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Noncatalytic, N-terminal Domains of DNA Polymerase Lambda Affect Its Cellular Localization and DNA Damage Response

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Abstract

Specialized DNA polymerases, such as DNA polymerase lambda (Pol λ), are important players in DNA damage tolerance and repair pathways. Knowing how DNA polymerases are regulated and recruited to sites of DNA damage is imperative to understanding these pathways. Recent work has suggested that Pol λ plays a role in several distinct DNA damage tolerance and repair pathways. In this paper, we report previously unknown roles of the N-terminal domains of human Pol λ for modulating its involvement in DNA damage tolerance and repair. By using Western blot analysis, fluorescence microscopy, and cell survival assays, we found that the BRCA1 C-terminal (BRCT) and proline/serine-rich (PSR) domains of Pol λ affect its cellular localization and DNA damage responses. The nuclear localization signal (NLS) of Pol λ was necessary to overcome the impediment of its nuclear localization caused by its BRCT and PSR domains. Induction of DNA damage resulted in recruitment of Pol λ to chromatin, which was controlled by its BRCT and PSR domains. In addition, the presence of both domains was required for Pol λ -mediated tolerance of oxidative DNA damage but not DNA methylation damage. These findings suggest that the N-terminal domains of Pol λ are important for regulating its responses to DNA damage.

Graphical Abstract

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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Western blots, nuclear localization data, and chromatin association data collected with mouse Pol λ proteins in MEF $\lambda^{-/-}/\beta^{-/-}$ and with human Pol λ proteins in Huh7; cell survival data collected with MEF $\lambda^{-/-}/\beta^{-/-}$ expressing Pol λ proteins in the absence of DNA damaging treatment (PDF)

INTRODUCTION

The proliferation of life is dependent upon high-fidelity replication of genetic material. However, the maintenance of genetic material is constantly challenged by exogenous and endogenous agents, which damage DNA via chemical modifications such as alkylation, oxidation, and formation of complex DNA base adducts. DNA damage can perturb replication and alter the coding potential of bases, as demonstrated by the notable oxidative lesion 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), which can base pair with adenosine causing G → T transversions during replication.^{1–4} Several strategies are employed by cells to counteract the deleterious effects of DNA damage. One pathway, which serves to replace damaged bases, is base excision repair (BER). During BER, gap-filling DNA synthesis is performed by an X-family DNA polymerase. The X-family of DNA polymerases includes DNA polymerases beta (Pol β), lambda (Pol λ), mu (Pol μ), and terminal deoxynucleotidyl transferase (TdT). It is widely accepted that Pol β is the primary BER polymerase, while the other X-family DNA polymerases are involved in DNA end-joining repair and tissue-specific recombination.^{5–10} However, recent work has challenged the stringency of this notion. For example, Pol λ plays a backup role to Pol β in BER and directly interacts with other BER proteins.^{11–14}

Another DNA repair pathway is nonhomologous end-joining (NHEJ), which serves to repair double-stranded DNA breaks. Repair of DNA by NHEJ often requires gap-filling by a DNA polymerase to make DNA ends suitable for ligation. Pol λ has been implicated in NHEJ as it interacts with other proteins involved in NHEJ and can perform gap-filling in substrates that mimic NHEJ intermediates.^{15–19} Furthermore, Pol λ may also function in DNA damage tolerance as it is capable of efficiently performing translesion synthesis (TLS) on DNA substrates that contain various different lesions.^{20–25} However, the manner in which a specific DNA polymerase, such as Pol λ , is recruited for each of these DNA damage repair and tolerance pathways is not well established.

The domain structure of Pol λ may indicate how Pol λ is distinguished from other DNA polymerases and recruited to specific types of DNA damage in cells. While all X-family DNA polymerases possess the Pol β -like domain consisting of the typical finger, palm, and thumb polymerase subdomains, Pol λ also contains noncatalytic N-terminal domains (Figure 1). These domains include a putative nuclear localization signal (NLS), a BRCA1 C-terminal (BRCT) domain, and a proline/serine-rich (PSR) domain. The putative NLS of

Pol λ resembles the canonical type of lysine- and arginine-rich NLS recognized by Importin α , although it has not been directly characterized.^{5,26,27} BRCT domains have been implicated in protein-protein and protein-DNA interactions.^{28–32} The BRCT domain is highly conserved among Pol λ proteins in different organisms. However, the Pol λ BRCT domain has no significant sequence homology to the BRCT domains of other DNA repair proteins such as Pol μ , replication factor C (RFC), DNA ligase IV (LigIV), and X-ray repair cross-complementing protein 4 (XRCC4), suggesting unique roles for the Pol λ BRCT domain in its cellular functions. The Pol λ BRCT domain interacts with XRCC4-LigIV complex and is required for Pol λ to perform alignment-based gap-filling in vitro and for Pol λ to protect cells from oxidative DNA damage.^{12,17,19} Relative to the Pol λ BRCT domain, less is known about the role of its PSR domain. By using structural prediction and sequence analysis, it has been proposed that the Pol λ PSR domain is targeted for post-translation modifications that may regulate Pol λ during DNA repair.⁵ This prediction is supported with evidence that the PSR domain of Pol λ can be phosphorylated by cyclin-dependent kinase 2 in vitro and that Pol λ is phosphorylated in human cells in a cell-cycle dependent manner.³³ Furthermore, studies have indicated that the Pol λ N-terminal domains directly affect its polymerase activity.^{24,34} Together, these observations suggest a model wherein the involvement of Pol λ in some types of DNA damage tolerance and repair is mediated by its BRCT and PSR domains. However, there are limited cellular data to support this model.

To investigate the cellular roles of the N-terminal domains of Pol λ , we examined the contribution of each of these domains to (i) Pol λ localization, (ii) recruitment of Pol λ to sites of DNA damage, and (iii) the ability of Pol λ to function in DNA damage tolerance and repair pathways in cells. We found that the Pol λ BRCT and PSR domains counteracted the function of its NLS and impeded its nuclear accumulation. In response to DNA damage, Pol λ associated with chromatin and this association was affected by its BRCT, PSR, and Pol β -like domains. Furthermore, the ability of Pol λ to rescue cells after DNA damage was impacted differently by its BRCT, PSR, and Pol β -like domains. We concluded that the involvement of Pol λ in DNA damage tolerance and repair is mediated through its BRCT and PSR domains.

EXPERIMENTAL PROCEDURES

Molecular Cloning.

For localization studies, the open reading frame (ORF) for human and mouse Pol λ and Pol β was cloned into pCDNA3.1 mammalian expression plasmid (Life Technologies) using 5' and 3' *Hind*III and *Xba*I restriction sites, respectively. For fluorescence microscopy studies, the ORF for enhanced green fluorescent protein (GFP) was cloned upstream of the polymerase ORF between *Nhe*I and *Hind*III restriction sites. A construct containing only GFP was generated by cloning the GFP ORF into pCDNA3.1 between *Hind*III and *Xba*I restriction sites. For Western blot analysis, a FLAG tag with a start codon (MDYKDDDDK) was cloned upstream of the polymerase ORF between *Nhe*I and *Hind*III restriction sites. To generate deletion mutants (Figure 1), the 5' and 3' segments of the Pol λ ORF flanking the deleted segment were PCR amplified to insert a *Bam*HI cut site in place of the deleted

segment These DNA fragments were then ligated into pCDNA3.1 between *Hind*III and *Xba*I restriction sites. The deletion mutants were GFP-and FLAG-tagged in the same way as full-length Pol λ as describee above. The sequences of all constructs were confirmed by DNA sequencing. The expression of these constructs in cells was confirmed by Western blot (Figures 2 and S1).

Mammalian Cell Culture and Treatments.

Human hepatocarcinoma 7 (Huh7) cells and mouse embryonic fibroblasts with homozygous Pol λ and Pol β knockout (MEF $\lambda^{-/-}/\beta^{-/-}$) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), minimum essential medium nonessential amino acids, 100 μ g/mL streptomycin sulfate, and 100 units/mL penicillin G sodium. Cells were allowed to reach 90% confluency before being plated for assays. For fluorescence microscopy and Western blotting, cells were dissociated using 0.25% trypsin-EDTA, counted, and plated at the indicated cell number. Transfections were performed 24 h after plating using Lipofectamine 2000 according to the manufacturer's instructions. After transfection, cells were incubated for 24 h before samples were prepared for Western blotting and fluorescence microscopy. All culture medium supplies were purchased from Life Technologies with the exception of FBS, which was purchased from Thermo Scientific.

Western Blotting.

Cells were seeded (250 000 cells per well) in six-well dishes. After 24 h, the cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, the cells were washed and dissociated into cold PBS using a cell scraper. The cells were pelleted at $1000 \times g$ for 2 min at 4 °C in a benchtop centrifuge. The pellets were resuspended in 100 μ L of SDS-PAGE Sample Buffer (62.5 mM Tris, 1% SDS, 10% (w/v) glycerol, 0.005% bromophenol blue, 10% β ME, pH 6.8), heated to 95 °C for 5 min, and then mixed vigorously for 1 min. The proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The blots were developed using Amersham ECL Western blotting Detection Kit (GE Healthcare) according the manufacturer's instructions. The primary antibodies used were rabbit anti-FLAG monoclonal antibody (Sigma F2555), rabbit anti-GAPDH monoclonal antibody (Cell Signaling 14C10), and rabbit anti-Histone H3 polyclonal antibody (Cell Signaling 9715). All antibodies solutions for Western blotting were prepared by 1000-fold dilution in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6). In the case where soluble proteins were removed, the cell pellets were resuspended in hypotonic lysis buffer (10 mM Tris, 2.5 mM MgCl₂, 0.5% Nonidet P40 substitute, 1 mM phenylmethanesulfonyl fluoride, pH 7.4), incubated on ice for 8 min, and then pelleted for 30 s at $16\,000 \times g$. The supernatant liquid was removed, and the remaining chromatin pellet was resuspended in SDS-PAGE sample buffer as described above. In experiments where methylmethanesulfonate (MMS) or hydrogen peroxide (H₂O₂) treatments were performed, cells were treated with MMS or H₂O₂ in growth medium (or mock treated with fresh growth medium) for 1 h prior to the removal of soluble proteins.

Nuclear Localization Assay.

Cells were seeded on 18 mm glass coverslips in 12-well dishes at 60 000 cells per well. After 24 h, the cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, cells were fixed by treatment with 4% formaldehyde in PBS at room temperature for 10 min. The slides were washed with PBS before being mounted into VectaShield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) stain (Vector Laboratories). Images of the DAPI and GFP channels were collected for 30 randomly chosen cells with an Olympus IX81 inverted epifluorescence microscope at room temperature through a UPLANFLN 40X Oil-immersion 0.75 numerical aperture objective using MetaMorph Premier Version 7.7.0.0 software. Images were collected as tiff file format without further processing. Localization was quantified using ImageJ software. Nuclear boundaries for each image were determined using the DAPI channel. Pixel density was then measured in the GFP channel for the nucleus and the whole cell. Background pixel density was determined from the mean pixel density of a region lacking cells and then subtracted from the nuclear and whole cell pixel densities. Nuclear localization was then quantified as percent by dividing the corrected nuclear pixel density by the corrected whole cell pixel density and multiplying the fraction by 100%. Statistical validation was performed using two-way ANOVA at a confidence interval of 99.9% followed by Tukey's multiple comparisons test with GraphPad Prism version 7.00 (GraphPad Software). Representative images after colorization using Olympus Fluoview Ver.2.0b software are shown (Figures 3A, S2, S3, and S4).

Chromatin Association Assay.

The following protocol was modified from a previous report.³⁵ Cells were seeded on 18 mm glass coverslips in 12-well dishes at 60 000 cells per well. After 24 h, the cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, the cells were washed with cold PBS and treated with hypotonic lysis buffer for 8 min on ice. The cells were then washed with cold PBS and immediately fixed using 4% formaldehyde in PBS at room temperature for 10 min. The coverslips were then washed with PBS and mounted in VectaShield Mounting Medium with DAPI stain (Vector Laboratories). The cells were observed using an Olympus FV 1000 Filter Confocal microscope. Images were collected using Olympus Fluoview Ver.2.0b software at room temperature through a UPLFLN 40X Oil-immersion 1.30 numerical aperture objective. Images were converted directly to tiff file format without further processing. Representative images after colorization using Olympus Fluoview Ver.2.0b software are shown (Figures 5A, S5, S6, and S7).

Cell Survival Assay.

MEF $\lambda^{-/-}/\beta^{-/-}$ cells were seeded in 96-well plates at 4000 cells per well. After 24 h, the cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, cells were treated with varying concentrations of MMS or H₂O₂ in 100 μ L of growth medium for 1 h. The cells were washed with PBS and cultured in fresh medium. After 48 h, the cells were washed with PBS and assayed using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). A_{490} and A_{650} were

collected using a Flexstation 3 microplate reader (Molecular Dynamics). Cell survival was calculated and expressed as a percentage using eq 1, where the $(A_{490} - A_{650})$ of cells that were not treated with DNA damaging agent was assigned as “100% Survival”. Each data point represents the mean of three independent biological replicates:

$$\text{Survival (\%)} = 100 \times [(A_{490} - A_{650}) / (100\% \text{ Survival})] \quad (1)$$

RESULTS

Model System Selection and Preparation and Pol λ Mammalian Expression Constructs.

To study the cellular roles of the N-terminal domains of Pol λ , we generated deletion mutant constructs lacking one or more of the NLS, BRCT domain, PSR domain, or Pol β -like domain (Figure 1). To this end, the full-length or deletion mutant Pol λ ORF from human or mouse was cloned into mammalian expression plasmid pCDNA3.1 with N-terminal GFP or FLAG tags to allow for detection by fluorescence microscopy or Western blot, respectively. Human hepatocellular carcinoma cells (Huh7) were chosen as the primary model system in this study for several reasons. Foremost, we sought to investigate cellular localization and DNA damage response of human Pol λ , and thus we required a human cell line. Furthermore, our techniques relied on transient expression, Western blot, and fluorescence microscopy; thus, we required a cell line that is amenable to efficient transfection with large nuclei to facilitate imaging. Lastly, the chromatin association assay used here involved a mild-detergent wash to remove proteins not tightly associated with chromatin prior to imaging, and thus, we required a strongly adherent cell line that would resist detachment during the wash. Huh7 satisfies all of these requirements. In addition to the above studies, we also sought to investigate the roles of the N-terminal domains of Pol λ in DNA damage tolerance and repair using cell survival assays, and thus, we required a cell line lacking endogenous Pol λ for these studies. It has been shown previously that cells lacking Pol λ exhibit only a marginal sensitivity to DNA damaging agents.^{11–14} However, a double knockout of Pol λ and Pol β significantly enhances DNA damaging agent sensitivity compared to an individual knockout of either Pol λ or Pol β .^{11,13,14} Therefore, we chose to conduct our cell survival assays in homozygous Pol λ and Pol β knockout mouse embryonic fibroblasts (MEF $\lambda^{-/-}/\beta^{-/-}$), a generous gift from Dr. Samuel H. Wilson at the National Institute of Environmental Health Sciences. For these studies, we used the mouse Pol λ ORF to prepare mammalian expression constructs containing full-length Pol λ and Pol λ deletion mutants. To demonstrate the generality of our results and to justify usage of mouse cells for the survival assays, we also conducted localization assays and chromatin association experiments in MEF $\lambda^{-/-}/\beta^{-/-}$. Results of localization assays and chromatin association experiments in MEF $\lambda^{-/-}/\beta^{-/-}$ are presented as Supporting Information. As noted herein, very similar cellular localization and chromatin association were observed between human Pol λ in Huh7 and mouse Pol λ in MEF $\lambda^{-/-}/\beta^{-/-}$, thus substantiating the generality of our results for mammalian Pol λ and strengthening our conclusions.

Validation of Pol λ Expression Constructs.

Western blot of whole cell extracts prepared from cells transiently expressing FLAG-tagged constructs for 24 h revealed single bands at the expected sizes for the constructs (Figures 2A and S1). While the relative levels of full-length human Pol λ and most of the human Pol λ deletion mutants were similar in Huh7, human Pol λ (NLS) was nearly 10-fold less abundant possibly owing to ubiquitin-mediated, cytosolic degradation of Pol λ as previously shown.^{36–38} Notably, the relative protein level of mouse Pol λ in MEF $\lambda^{-/-}/\beta^{-/-}$ differed from that of human Pol λ in Huh7. This may be due to differences between the rates of cellular growth and protein degradation of these two cell lines. To further validate proper expression, epifluorescence microscopy was performed on cells transiently expressing the GFP-tagged Pol λ constructs to visualize localization. Full-length Pol λ and Pol λ deletion mutants possessing an NLS (i.e., Pol λ (BRCT), Pol λ (PSR)) were located primarily in nucleus (Figures 3A and S3). Conversely, the Pol λ deletion mutants lacking an NLS (i.e., Pol λ (NLS), Pol λ (NLS-BRCT), and Pol λ (N)) were located throughout the cells (Figures 3A and S2A). Notably, we observed differences in the nuclear localization of the deletion mutants lacking an NLS. This prompted further investigation into the impact of the N-terminal domains on nuclear localization of Pol λ . Importantly, transient expression of the constructs studied here did not result in notable abnormalities in growth kinetics or cellular morphology (data not shown).

Determination of the Impact of the Pol λ N-Terminal Domains on Its Nuclear Localization.

Pol λ possesses a putative NLS on its N terminus.^{5,26,27} Besides the NLS, the other N-terminal domains of Pol λ may affect its nuclear localization. To investigate this possibility, we sought to determine the contributions of the BRCT and PSR domains to nuclear localization of Pol λ . GFP-tagged Pol λ and sequential Pol λ deletion mutants (Figure 1; Pol λ (NLS), Pol λ (NLS- BRCT), and Pol λ (N)) were transiently expressed in cells, and fluorescence microscopy was used to quantify their cellular localization. As controls, nuclear localization of Pol β (which possesses a characterized NLS) and GFP alone (which does not possess any specific localization signal motifs) were quantified.³⁹ As expected, Pol β was $78 \pm 5\%$ localized within the nucleus, whereas GFP alone was only $24 \pm 6\%$ localized within the nucleus (Figure 3). Importantly, the nuclear localization of Pol β determined via our method is practically equivalent to the nuclear localization of Pol β reported previously using immunofluorescence staining of untagged, endogenous Pol β with a Pol β -specific primary antibody ($75 \pm 8\%$), thus validating our method.³⁹

Consistent with the presence of a canonical NLS, full-length Pol λ was $92 \pm 3\%$ localized within the nucleus, while Pol λ (NLS) was only $29 \pm 4\%$ localized within the nucleus (Figure 3). Interestingly, Pol λ (NLS-BRCT) was $45 \pm 6\%$ localized within the nucleus, a significant improvement compared to the NLS deletion alone (Figure 3). Moreover, Pol λ (N), the Pol β -like domain of Pol λ , showed further improvement of nuclear accumulation to $54 \pm 5\%$ (Figure 3). To further substantiate our results and validate using mouse cells for other studies as noted above, we also quantified localization of GFP-tagged mouse Pol λ and mouse Pol λ sequential deletion mutants in MEF $\lambda^{-/-}/\beta^{-/-}$ (Figure S2). Nuclear localization was very similar for mouse Pol λ proteins in MEF $\lambda^{-/-}/\beta^{-/-}$. However, the increase in nuclear localization upon truncation of the Pol λ N-terminus was less

pronounced for mouse Pol λ proteins in MEF $\lambda^{-/-}/\beta^{-/-}$ (Figure S2). This might have been due to the morphological differences between Huh7 and MEF cells. The nuclei of MEF cells encapsulate a larger fraction of the cellular volume than the nuclei of Huh7, which may have skewed comparison of nuclear localization between the two cell lines. Nonetheless, the increasing trend in nuclear localization upon truncation of the Pol λ N-terminus was observed in both Huh7 and MEF $\lambda^{-/-}/\beta^{-/-}$. In summary, these results suggest that, without an NLS, both the BRCT and PSR domains reduce the nuclear accumulation of Pol λ . Importantly, the sequential deletion mutants did not accumulate in the nucleus to the same extent as full-length Pol λ , thereby complicating comparative functional studies of these mutants in cell-based assays. To facilitate further studies of the Pol λ N-terminal domains, internal deletion mutants lacking only the BRCT or PSR domain were generated (Figure 1; Pol λ (BRCT) and Pol λ (PSR)). Because of the presence of the NLS, the internal deletion mutants localized to the nucleus to the same extent as full-length Pol λ (Figures S3 and S4).

Analysis of the Involvement of the Pol λ N-Terminal Domains in its DNA Damage-Induced Chromatin Association.

Some proteins involved in DNA damage tolerance and repair associate tightly with chromatin in response to DNA damage.^{35,40,41} Therefore, to explore the DNA damage response of Pol λ , we used fluorescence microscopy and Western blot to detect Pol λ tightly associated with chromatin in cells treated with DNA damaging agents. These assays utilized a hypotonic lysis buffer containing a nonionic detergent to remove proteins that were not tightly associated with insoluble structures, such as chromatin.^{35,40,41} Consistent with previous studies, treatment of cells with either MMS or H₂O₂ resulted in recruitment of full-length Pol λ to chromatin in a dose-dependent manner (Figures 4 and S5).^{12,37} Interestingly, nearly 80% of the transiently expressed, GFP-tagged Pol λ was associated with chromatin after 1 h treatment with 10 mM MMS, despite competition with endogenous Pol λ in the Huh7 cells (Figure 4C). However, this may be an indirect effect caused by cellular toxicity at high concentrations of MMS. Significant physiological effects indicative of such toxicity were observed at 10 mM MMS, as made apparent by smaller, irregularly shaped nuclei (Figure 4A, 10 mM MMS). Therefore, further chromatin association experiments were conducted with 1 mM MMS treatment, which induced chromatin-association of approximately 20% of the transiently expressed, GFP-tagged Pol λ and did not result in apparent toxic effects (Figure 4A).

We next tested the Pol λ internal deletion mutants for chromatin association (Figure 5). Pol λ (BRCT) did not associate with chromatin in response to treatment with either reagent, which suggested that the BRCT domain is required for chromatin association of Pol λ (Figures 5, S6, and S7). Unexpectedly, chromatin association of Pol λ (PSR) occurred independent of DNA damage (Figures 5, S6, and S7). The relative amount of chromatin-associated Pol λ (PSR) and Pol λ (N) after treatment with MMS was similar to that of full-length Pol λ as determined by quantitation of Western blots (Figure 5C). Intriguingly, in the absence of both the BRCT and PSR domains, Pol λ still exhibited DNA damage-induced chromatin association (Figures 5, S6, and S7), which implied that the N-terminal domains play a regulatory role rather than a direct role in mediating interactions required for the DNA damage response of Pol λ . We also performed chromatin association experiments Pol λ

proteins in MEF $\lambda^{-/-}/\beta^{-/-}$. Importantly, the results of these studies in MEF $\lambda^{-/-}/\beta^{-/-}$ were consistent with our studies on human Pol λ proteins in Huh7, thus providing a qualitative confirmation of our results and validating usage of mouse cells for cytotoxicity studies as noted above. In summary, the results from our chromatin association assays suggest that, in the presence of the BRCT domain, the PSR domain is required to prevent chromatin-association in the absence of extensive DNA damage. Similarly, in the presence of the PSR domain, the BRCT domain is required for DNA damage-induced chromatin association to occur.

Determination of the Importance of the Pol λ BRCT and PSR Domains in DNA Damage Tolerance and Repair.

To determine whether the BRCT and PSR domains of Pol λ influence its participation in cellular DNA damage tolerance and repair, we performed cell survival assays on MEF $\lambda^{-/-}/\beta^{-/-}$ cells transiently expressing FLAG-tagged Pol λ or the Pol λ internal deletion mutants (Figure 1). In brief, cells were treated with MMS or H₂O₂ and then allowed to recover for 2 days before being queried for survival. MEF $\lambda^{-/-}/\beta^{-/-}$ are hypersensitive to MMS and H₂O₂ and have been used previously to show that Pol λ plays a backup role to Pol β in BER.^{11–13} We first performed a control experiment to determine whether transient expression of full-length Pol λ or the Pol λ internal deletion mutants negatively affected survival of cells in the absence of DNA damaging treatment. We found that survival of cells expressing Pol λ internal deletion mutants in the absence of DNA damaging treatments was indistinguishable from that of cells expressing full-length Pol λ or an empty vector (Figure S8). However, transient expression of wild-type Pol λ resulted in a moderate improvement in survival compared to cells expressing an unmodified pCDNA3.1 plasmid (empty) after treatment with either MMS (Figure 6) or H₂O₂ (Figure 7). Expression of Pol λ (N) also improved survival of cells after MMS treatment (Figure 6) but failed to improve survival of cells after H₂O₂ treatment (Figure 7), which indicated that the N-terminal domains are dispensable for Pol λ to participate in tolerance and repair of MMS-induced DNA damage but are required for Pol λ to participate in tolerance and repair of H₂O₂-induced DNA damage. Interestingly, we found that transient expression of Pol λ (BRCT) or Pol λ (PSR) negatively impacted survival of cells after treatment with MMS compared with cells transfected with an empty plasmid (Figure 6). In contrast, Pol λ (BRCT) and Pol λ (PSR) did not cause decreased survival compared with cells transfected with an empty plasmid after treatment with H₂O₂ (Figure 7). Importantly, there were not significant differences in survival of cells transiently expressing wild-type Pol λ , the deletion mutants of Pol λ , or an unmodified plasmid in the absence of DNA damaging treatment (Figure S8).

DISCUSSION

Cells utilize many strategies for tolerating or repairing DNA damage. Among them are NHEJ, BER, and TLS, each of which requires the action of a specialized DNA polymerase. To date, 16 DNA polymerases have been identified in human cells. Most of these DNA polymerases are restricted to a particular cellular function. For example, DNA polymerase η (Pol η) functions primarily for TLS, while Pol β functions for BER.^{7–10,42,43} In contrast, the functions of Pol λ appear to be less restricted as it can participate in NHEJ, BER, and

TLS.^{11–25} It has been proposed that the unique N-terminal domains of Pol λ may be involved in regulating its participation in these responses to DNA damage. Considering that the primary BER polymerase, Pol β possesses only the catalytic domains (Figure 1), it is unclear whether the N-terminal domains of Pol λ contribute to its involvement in BER. While recent studies have attributed these domains to some protein–protein interactions, their cellular roles remain to be determined.^{1–17} Here, we demonstrated that the N-terminal domains of Pol λ strongly affected its cellular localization as well as its involvement in DNA damage response and repair.

BRCT and PSR Domains of Pol λ Affect Its NLS-Independent Nuclear Accumulation.

The NLS is a powerful signal for targeting proteins to the nucleus. On the basis of sequence analysis, however, some proteins that function in the nucleus do not possess an NLS, such as DNA polymerase epsilon subunit 4, proliferating cell nuclear antigen (PCNA), as well as replication factor C subunits 2 and 5. Proteins that lack an NLS are still able to shuttle between the cytosol and the nucleus through (i) unassisted diffusion through the nuclear pore complex in the case of proteins smaller than approximately 65 kDa; (ii) being captured during reformation of the nucleus in telophase in the case of many eukaryotic cell types; and (iii) cotransport through direct binding to a different proteins that possess an NLS.^{44–46} Our localization studies demonstrate that the putative NLS of Pol λ was required for efficient accumulation in the nucleus. Interestingly, our results suggest that the NLS of Pol β is weaker than the putative NLS of Pol λ as demonstrated by the significantly greater nuclear accumulation of full-length Pol λ compared to Pol β (Figure 3). As Pol β lacks the N-terminal BRCT and PSR domains present in Pol λ , it is possible that Pol λ evolved to possess a stronger NLS to counteract the negative impact of the BRCT and PSR domains for Pol λ nuclear import (Figures 3 and S2).

Interestingly, Pol λ (AN), which lacks the putative NLS, demonstrated significant nuclear accumulation (Figures 3 and S2). One explanation for this phenomenon is that Pol λ may also possess an unidentified NLS within its Pol β -like domain. However, this putative, weaker NLS may be insufficient to counteract the inhibitory effects of the BRCT and PSR domains. Alternatively, Pol λ (N) may associate with other nuclear-targeting proteins and be cotransported into the nucleus. Association to these other nuclear-targeting proteins may be limited by the BRCT and PSR domains. Therefore, deletion of the BRCT and PSR domains could have improved nuclear accumulation of Pol λ (N). This possibility is supported by previous studies that have demonstrated interactions between Pol λ and other DNA repair proteins, including alkyladenine-DNA glycosylase, 8-oxoG-DNA glycosylase, and XRCC4-LigIV complex, each of which possess an NLS based on sequence analysis.^{11,17} Another alternative explanation is that the smaller size of the deletion mutants was responsible for the observed improvement of nuclear accumulation upon deletion of the N-terminal domains. However, even the smallest mutant we studied, GFP-Pol λ -(N), is approximately 65 kDa, which is over the size-exclusion limit of 40–60 kDa for unassisted diffusion into the nucleus.^{44–46} Furthermore, GFP (27 kDa) is far smaller than this size limit but exhibited significantly lower nuclear accumulation than the larger GFP-Pol λ -(N) (Figures 3 and S2). These observations suggest that size was not the primary determining factor for nuclear accumulation of the Pol λ mutants that lack an NLS studied here.

Pol λ Associates with Chromatin in Response to DNA Damage, and This Association Is Regulated by Its BRCT and PSR Domains.

PCNA and Pol η are known to participate in DNA damage tolerance and exhibit relocalization from the detergent-soluble fraction of the nucleus to the insoluble chromatin fraction in response to DNA damage.^{35,40} Previous reports have suggested Pol λ exhibits a similar DNA damage response and associates with chromatin after cells are treated with H₂O₂.³⁷ Our results further add to the known DNA damage responses of Pol λ by demonstrating chromatin association in response to MMS treatment (Figures 4 and 5). Different types of DNA damage are induced by these DNA damaging agents; MMS primarily methylates individual bases, while H₂O₂ causes both oxidation of individual bases and strand-breakage.^{47,48} Methylated and oxidized DNA bases are tolerated by TLS to prevent cell death and repaired by BER, while strand-breaks require end-joining pathways such as NHEJ.

Previous studies have demonstrated that the BRCT domain is responsible for direct interaction between Pol λ and the NHEJ protein complexes XRCC4-LigIV and Ku, while the Pol β -like domain is responsible for direct interaction of Pol λ with the TLS factor PCNA and several BER pathway enzymes.^{12,17,18,49,50} Therefore, the chromatin-associated Pol λ foci observed here (Figures 4, 5, and S5–S7) may have corresponded to a culmination of TLS, NHEJ, and BER complexes containing Pol λ . However, despite its requirement for interactions with NHEJ proteins, the Pol λ N terminus was not absolutely required for chromatin association to occur (Figures 5, S6, and S7).^{17,18} Furthermore, previous studies have shown that DNA damage from MMS is primarily repaired by BER pathway.⁴⁷ Taken together, this evidence suggests that chromatin-associated Pol λ in our assays was present within BER or TLS complexes rather than NHEJ complexes.

Strikingly, despite the full N terminus being dispensable for chromatin association, the BRCT domain became absolutely required for chromatin association when the PSR domain was present (Figures 5, S6, and S7). This suggests that the PSR domain negatively impacted association of Pol λ to BER or TLS complexes. Such negative regulation may allow cells to limit recruitment of full-length Pol λ to BER or TLS complexes when DNA damage is not overly abundant and thus may be responsible for limiting Pol λ to play a back-up role in BER.^{11,14} In support of this notion, Pol λ lacking only the PSR domain exhibited constitutive chromatin-association in the absence of exogenously induced DNA damage (Figures 5, S6, and S7) suggesting that the BRCT domain caused unnecessary recruitment to chromatin. While we have not fully defined the mechanism of chromatin recruitment of Pol λ , our results lay the groundwork for further investigation into the cellular roles of the Pol λ N-terminal domains and hint to an interesting role for the previously ill-characterized PSR domain in negatively regulating association of Pol λ to BER or TLS complexes.

BRCT and PSR Domains of Pol λ Are Required for Oxidative DNA Damage Tolerance but Have Different Effects on DNA Methylation Damage Tolerance.

Previous in vitro mechanistic studies have shown that the N-terminal domains of Pol λ are not required for catalytic activity, but they influence polymerase fidelity with damaged and undamaged DNA templates.^{24,25,34,51–54} We sought to further characterize the role of the N-

terminal domains in conferring tolerance to DNA damage. As expected based on previous studies, wild-type Pol λ was able to confer tolerance against MMS and H₂O₂ treatments (Figures 6 and 7).^{11–14} Intriguingly, expression of only the Pol β -like domain of Pol λ protected cells from MMS-induced cytotoxicity (Figure 6) but not from H₂O₂ (Figure 7). This observation was unexpected as Pol λ (N) associated with chromatin after H₂O₂ treatment (Figure S7). However, chromatin association of Pol λ (N) in response to H₂O₂ treatment was likely due to the formation of oxidized bases that are bypassed by TLS and repaired by BER. Pol λ (N) could participate in TLS or BER due to its similarity to Pol β and the ability to interact with PCNA and DNA glycosylases.^{11,12,49,50} The lack of improvement in survival of cells expressing Pol λ (N) and challenged with H₂O₂ (Figure 7) could have been due to the formation of DNA strand-breaks. This type of DNA damage is generally considered to be more cytotoxic than oxidative base lesions and is repaired through recombination and end-joining pathways in which Pol λ (N) is unlikely to participate as it lacks the BRCT domain required for Pol λ to participate in NHEJ in vitro.^{16,17,19}

Interestingly, we found that expression of Pol λ mutants lacking only its BRCT domain or its PSR domain was detrimental to the survival of cells challenged by MMS (Figure 6). This observation suggests a complicated interplay between the Pol λ BRCT and PSR domains during tolerance and/or repair of DNA methylation damage. It is possible that, without the BRCT domain, Pol λ was unable to be recruited to chromatin, as suggested from the loss in chromatin association of Pol λ (BRCT) (Figures 5, S5, and S6), but still could interact with some DNA damage tolerance and repair proteins such as PCNA and DNA glycosylases.^{11,12,49,50} Therefore, Pol λ (BRCT) may have formed inactive complexes with TLS/BER proteins and prevented them from functioning for damage tolerance and repair pathways, resulting in a negative impact on cell survival after challenged with extensive DNA damage. Similarly, the constitutive chromatin association of Pol λ -(PSR) (Figure 5) may have recruited such proteins to undamaged DNA, also preventing repair proteins from being successfully recruited to DNA damage. In contrast to cells treated with MMS, cells expressing Pol λ (BRCT) and Pol λ (PSR) and treated with H₂O₂ did not exhibit a loss in survival compared with the control cells expressing empty plasmid (Figure 7). As described above, single base lesions induced by the alkylating agent MMS and perhaps other types of DNA damaging chemicals are primarily repaired through BER, while DNA strand breaks generated by the oxidizing agents such as H₂O₂ are repaired through end-joining pathways. This implies that Pol λ (BRCT) and Pol λ (PSR) mutants have a negative impact on BER or TLS rather than the aforementioned end-joining pathways.

In summary, our results suggest that the noncatalytic domains of Pol λ regulate its cellular behavior. Previous studies have implied that Pol λ participates in BER, NHEJ, and TLS.^{15,19–21,49} Therefore, these proposed regulatory domains may be required for modulating the availability of Pol λ for binding to the specific substrates or other proteins of these pathways. Our results also support a model wherein the BRCT and PSR domains of Pol λ competitively regulate its association to DNA repair complexes. In this model, the Pol λ PSR domain prevents its BRCT and Pol β -like domains from associating with cellular DNA or DNA repair proteins in the absence of DNA damage. Upon induction of DNA damage, the negative regulation from the Pol λ PSR domain is relieved, thereby allowing the Pol λ BRCT

and Pol β -like domains to associate with DNA and DNA repair protein complexes. In agreement with this model, the Pol λ PSR domain is required to prevent chromatin association of Pol λ in the absence of DNA damage (Figures 5, S6, and S7). In the absence of both its BRCT and PSR domains, the proposed regulators, the Pol β -like domain of Pol λ , may behave similarly to Pol β as observed in previous studies.⁵⁵ Future work will identify the exact repair factors with which Pol λ exhibits DNA damage-induced association and determine the importance of the N-terminal domains for these specific interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
BER	base excision repair (BER)
BRCT	BRCA1 C-terminal
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
GFP	enhanced green fluorescent protein
H₂O₂	hydrogen peroxide
Huh7	Human hepatocarcinoma 7
LigIV	DNA ligase IV
MEF $\lambda^{-/-}/\beta^{-/-}$	mouse embryonic fibroblasts with homozygous Pol λ and Pol β knockout
MMS	methylmethane-sulfonate
NHEJ	nonhomologous end-joining
NLS	nuclear localization signal
ORF	open reading frame
PCNA	proliferating cell nuclear antigen

Polβ	DNA polymerases beta
Polη	DNA polymerase eta
Polλ	DNA polymerase lambda
Polλ(BRCT)	Pol λ lacking the BRCT domain
Polλ (N)	Pol λ lacking the NLS BRCT domain and PSR domain
Polλ(NLS)	Pol λ lacking the NLS
Polλ(NLS-BRCT)	Pol λ lacking the NLS and BRCT domain
Polλ-(PSR)	Pol λ lacking the PSR domain
Polμ	DNA polymerase mu
PSR	proline/serine-rich
RFC	replication factor C
TdT	terminal deoxynucleotidyl transferase
TLS	translesion synthesis
XRCC4	X-ray repair cross-complementing protein 4

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Figure 1.

Domain structures of Pol β , Pol λ , and the Pol λ deletion mutants. The domain structures of full-length human Pol β and Pol λ are illustrated. Numbers below each domain interface indicate amino acid residue numbers of the wild-type, human proteins. The Pol λ putative nuclear localization signal (NLS) is indicated along with the BRCA1 C-terminal (BRCT), proline-serine rich (PSR), and Pol β -like domains of full-length Pol λ and the Pol λ deletion mutants. The name of each Pol λ deletion mutant is given on the left of its domain structure.

Figure 2.

Western blot of Huh7 cells transiently expressing FLAG-tagged Polλ and the Polλ deletion mutants. (A) Cells were transfected with plasmids encoding FLAG-tagged constructs indicated above each lane. Whole-cell lysates were analyzed by Western blot with the indicated antibodies. Detection of GAPDH was used as a loading control. (B) Western blot bands for Polλ proteins were quantified by densitometry and normalized to the GAPDH loading control for each lane. Band intensities were then normalized to wild-type Polλ for comparison.

Figure 3.

Localization of GFP, Polβ, Polλ, and the sequential N-terminal deletion mutants of Polλ in Huh7 cells. (A) Cells were transfected with plasmids encoding GFP-tagged constructs indicated above each image. The cells were then fixed, mounted, and imaged by epifluorescence microscopy. For each image, the GFP channel (GFP), the DAPI channel (DAPI), and a merge of the GFP and DAPI channels (GFP+DAPI) are shown. The white scale bars (bottom-right corner) represent 30 μm. (B) Localization was quantified using ImageJ software to analyze images of 30 randomly chosen cells for each construct. The mean percent nuclear localization of each condition is plotted and displayed as a numerical value above each bar. The error bars represent the SD of each data set. One-way ANOVA followed by Tukey's multiple comparison tests was used to test for significance in differences of localization between constructs. An asterisk denotes that the indicated data sets are not statistically different from each other at a confidence interval of 99.9% based upon Tukey's post hoc test. All other pairwise combinations of data sets were found to be significantly different from each other.

Figure 4.

Dose response of MMS-induced chromatin association of Pol λ in Huh7. (A) Cells were plated and transfected with plasmid encoding GFP-tagged Pol λ . The cells were treated with different concentrations of MMS as indicated above each image. After 1 h of treatment, the cells were washed with a hypotonic solution containing nonionic detergent to remove soluble proteins. The cells were then fixed, mounted, and imaged by confocal fluorescence microscopy. Cells not washed or treated with MMS are in the leftmost column. For each image, the GFP channel (GFP) and a merge of the GFP and DAPI channels (GFP+DAPI) are shown. The white scale bars (bottom-right corner) represent 30 μ m. (B) Cells were plated and transfected with a plasmid encoding FLAG-tagged Pol λ . The cells were then treated with different concentrations of MMS indicated above each lane. After 1 h of MMS treatment, the cells were isolated and treated with a hypotonic solution containing nonionic detergent to remove the soluble cytosolic and nuclear proteins. The resulting chromatin fractions were isolated and analyzed by Western blot with the indicated antibodies. Detection of Histone H3 was used as a loading control. Blotting for GAPDH in the chromatin fractions was used to validate removal of soluble, nonchromatin-bound proteins. (C) Western blot bands corresponding to Pol λ were quantified by densitometry and normalized to the Histone H3 loading control for each lane. Band intensities were then normalized to Pol λ from untreated, unwashed cells for comparison.

Figure 5.

Chromatin association of the Pol λ deletion mutants in Huh7 in response to MMS treatment. (A) Cells were plated and transfected with plasmids encoding GFP-tagged constructs as indicated to the left of each set of images. The cells were then treated with 1 mM MMS or mock treated without MMS for 1 h. The cells were washed with a hypotonic solution containing nonionic detergent to remove soluble proteins. The cells were then fixed, mounted, and imaged by confocal fluorescence microscopy. For each construct, images of cells without MMS treatment (No MMS) and with MMS treatment (1 mM MMS) are

shown. For each image, the GFP channel (GFP) and a merge of the GFP and DAPI channels (GFP+DAPI) are shown. The white scale bars (bottom-right corner) represent 30 μm . (B) Cells were plated and transfected with plasmids encoding FLAG-tagged constructs as indicated above each lane. The cells were then treated with 1 mM MMS or mock treated without MMS for 1 h. The cells were then isolated and washed with a hypotonic solution containing nonionic detergent to remove the soluble cytosolic and nuclear proteins. The resulting chromatin fractions were analyzed by Western blot with the indicated antibodies. Blots of chromatin fractions without MMS treatment (No MMS) and with MMS treatment (1 mM MMS) are shown. Detection of Histone H3 was used as a loading control. (C) Western blot bands for Pol λ proteins were quantified by densitometry and normalized to the Histone H3 loading control for each lane. Band intensities were then normalized to the band corresponding to Pol λ after MMS treatment for comparison.

Figure 6.

Cell survival assay with Polλ and the Polλ deletion mutants in MEF $\lambda^{-/-}/\beta^{-/-}$ treated with MMS. Cells were plated and transfected with plasmids encoding FLAG-tagged constructs indicated in the key (top-right corner). The cells were treated with the indicated [MMS] for 1 h. The medium was then replaced with fresh medium, and the cells were allowed to recover for 48 h before being assayed for cell survival. The mean percent survival is plotted for each condition. The error bars represent SD of each data set from triplicate biological replicates. Data from cell transfected with an empty pCDNA3.1 plasmid (empty) are plotted as a black solid line with circles. Wild-type Polλ is a blue solid line with circles. Polλ (ΔN) is a green solid line with circles. Polλ(ΔBRCT) is a scarlet broken line with circles. Polλ(ΔPSR) is a gray broken line with circles.

Figure 7.

Cell survival assay with Polλ and deletion mutants in MEF $\lambda^{-/-}/\beta^{-/-}$ treated with H₂O₂. Cells were plated and transfected with plasmids encoding FLAG-tagged constructs indicated in the key (top- right corner). The cells were treated with the indicated [H₂O₂] for 1 h. The medium was then replaced with fresh medium, and the cells were allowed to recover for 48 h before being queried for cell survival. The mean percent survival is plotted for each condition. The error bars represent SD of each data set from triplicate biological replicates. Data from cell transfected with an empty pCDNA3.1 plasmid (empty) are plotted as a black solid line with circles. Wild-type Polλ is a blue solid line with circles. Polλ(ΔN) is a green solid line with circles. Polλ(ΔBRCT) is a scarlet broken line with circles. Polλ(ΔPSR) is a gray broken line with circles.