Polκ protein is a eukaryotic member of the DinB/Polκ branch of the Y-family DNA polymerases, which are involved in the tolerance of DNA damage by replicative bypass. Despite universal conservation through evolution, the precise role(s) of Polκ in this process has remained unknown. Here we report that mouse Polκ can physically interact with ubiquitin by yeast two-hybrid screening, glutathione S-transferase pulldown, and immunoprecipitation methods. The association of Polκ with ubiquitin requires the ubiquitin-binding motifs located at the C terminus of Polκ. In addition, Polκ binds with monoubiquitinated proliferating cell nuclear antigen (PCNA) more robustly than with non-ubiquitinated PCNA. The ubiquitin-binding motifs mediate the enhanced association between monoubiquitinated PCNA and Polκ. The ubiquitin-binding motifs are also required for Polκ to form nuclear foci after UV radiation. However, the ubiquitin-binding motifs do not affect Polκ half-life. Finally, we have examined levels of Polκ expression following the exposure of mouse cells to benzo[a]pyrene-dihydriodiol epoxide or UVB radiation.

Translesion DNA synthesis (TLS) is one of several biochemical mechanisms by which cells can tolerate DNA damage that arrests semiconservative DNA synthesis (1, 2). This process requires the action of specialized DNA polymerases present in bacteria (such as Escherichia coli), lower eukaryotes, and vertebrates. Lower eukaryotes, particularly vertebrates, contain multiple such enzymes, suggesting the ability to bypass many types of DNA damage.

Several specialized DNA polymerases are members of a novel polymerase family, the Y-family (3). These enzymes are devoid of DNA polymerase Polκ, which include a C2HC zinc finger, a bipartite binding zinc finger motif; Ub, ubiquitin; CHX, cycloheximide; GST, glutathione S-transferase; PCNA, proliferating cell nuclear antigen; BPDE, benzo[a]pyrene-dihydriodiol epoxide; mPolκ, mouse Polκ; hPolκ, human Polκ; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; MEF, mouse embryonic fibroblast.

Requirements for the Interaction of Mouse Polκ with Ubiquitin and Its Biological Significance*

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the C-terminal half of Polk. We also demonstrate that Polk binds monoubiquitinated PCNA more robustly than nonubiquitinated PCNA. The UBZs are required for Polk to form nuclear foci after UV radiation. We measured the half-life of endogenous Polk as 5.4 h and show that mutational disruption of the UBZs does not alter the half-life of Polk protein. Finally, we examined levels of Polk expression following exposure of mouse cells to BPDE or UVB radiation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—For yeast two-hybrid screening, pGBT9/mouse Polk plasmid was cloned as described (6). For binding assays, full-length mouse Polk cDNA was cloned in pCMV-Myc or pCMV-HA (Clontech) to generate Myc or HA fusion proteins. For confocal study, mouse Polk cDNA with the first ATG codon deleted was PCR-amplified and cloned in the SalI site of pEGFP-C3 (Clontech) to generate an EGFP fusion protein. Human Polk cDNA was PCR-amplified and cloned in the BamHI site of pEGFP-C1 (Clontech). Isolated UBZs of mPolk were PCR-amplified and cloned into pCMV-Myc (Clontech) or pGEX4T-2 vectors (Amersham Biosciences). Ubiquitin, PCNA, and PCNA-ubiquitin chimera (25) were subcloned in pGEX4T-2 vectors to produce GST fusion proteins as reported (10, 26).

**Cell Culture and Treatments**—COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transient transfection experiments COS7 cell were transfected with pCMV-Myc-mPolk or pGEX4T-2 vectors to produce GST fusion proteins as reported (10, 26). A series of mutant mPolk constructs was generated using the QuikChange site-directed mutagenesis kit (Stratagene). Ubiquitin was cloned in pcDNA3-HA as described (25).

**Yeast Two-hybrid Assay**—The pGBT9/mouse Polk plasmid was used to screen a mouse testis cDNA library as described (6).

**Nuclear Protein Extraction and Western Blotting**—Wild-type MEFs were prepared and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection and UV irradiation were carried out as previously described (27).

**GST Pulldown Assay**—COS7 cells were transfected with pCMV-HA-mPolk and pCMV5-FLAG-Ub. Harvested cell lysates were immunoprecipitated with anti-FLAG antibodies. HEK293T cells were transfected with pCMV-Myc-mPolk and pcDNA3-HA-Ub. Harvested cell lysates were immunoprecipitated with anti-Myc antibodies. Immunoprecipitation and immunoblotting were performed as described (26). MRC cells were transfected with HA-mPolk, and 40 h later they were UV-irradiated (25 J/m²). They were then incubated for 7 h prior to Triton extraction and cross-linking. Triton-insoluble proteins were solubilized and immunoprecipitated with anti-PCNA as described (10).
**Polk Interacts with Ubiquitin**

**Figure 1. Interaction between Polk and ubiquitin.**

A. Shown is GST-ubiquitin pulldown of purified mouse Polk protein as indicated. Lane 1, input containing one tenth of the Polk used in the experiments; lane 2, GST + Polk; lane 3, GST-UB + Polk; lane 4, GST-UBB + Polk. Bound proteins were detected by immunoblot (IB) analysis with α-Polk antibody. B. Anti-FLAG M2 agarose affinity gel was incubated with the COS7 cell lysates expressing HA-Polk and FLAG-Ub or FLAG (control) as indicated. The total cell lysates (TCL) lanes (lanes 1 and 2) contain 2% of the lysates used in the experiments. Lanes 3 and 4, immunoprecipitation of lysates with α-FLAG. Bound proteins were detected by immunoblot analysis with α-HA antibody. C. HEK293T cells were transfected with (+) or without (−) HA-Ub together with Myc-Polk constructs. The lysates were immunoprecipitated (IP) with α-Myc antibody and subjected to immunoblotting with α-Myc and α-HA as indicated.

The UBZs Are Required for Enhanced Association between Polk and Monoubiquitinated PCNA—Recent studies have demonstrated that monoubiquitination of PCNA in cells exposed to UV radiation promotes a more robust interaction of this accessory replication protein with Polk, Polε, and Rev1 protein (10, 11, 25, 30). To determine whether an enhanced association also exists between Polk and monoubiquitinated PCNA, we examined their interaction by GST pulldown experiments (26). Consistent with results shown previously (31), the interaction of purified Polk with PCNA-Ub was more robust than with native PCNA (Fig. 3A). To determine whether the enhanced interaction is mediated via the UBZ domains, we incubated cell lysates expressing wild-type Polk or those carrying mutations in the UBZ domains with GST-PCNA fusion proteins. As shown in Fig. 3B, the enhanced association with GST-PCNA-Ub was not observed with UBZ mutant preparations. To further support the result, HA-Polk and its UBZ deletion derivatives were expressed in cells exposed to UV radiation to generate monoubiquitinated PCNA. The chromatin fraction was then isolated and immunoprecipitated with PCNA antibodies. Consistent with the results shown above, the amount of precipitated wild-type, but not UBZ-deleted, Polk was significantly increased after UVC treatment (Fig. 3C). Interestingly, the level of UBZ-deleted Polk in chromatin fractions was significantly reduced after UVC treatment (Fig. 3C). We isolated the chromatin fraction from wild-type cells after UVC treatment and immunoprecipitated it with anti-PCNA antibodies. Consistent with the results shown above, the amount of precipitated endogenous Polk was significantly increased after UVC treatment (Fig. 3D).

In summary, the results of the experiments reported thus far indicate that mouse Polk can interact with ubiquitin in vitro, an interaction that requires functional UBZs, and that Polk can itself undergo monoubiquitination. A robust association between monoubiquitinated PCNA and Polk also requires functional UBZs.
PolkUBZs Are Required for Association of Polk with Replication Factories in Cells Exposed to UV Radiation—To validate the results described above in living cells, we transfected wild-type and UBZ-deleted EGFP-mouse Polk constructs into fibroblasts. We observed strict nuclear localization of EGFP-mouse Polk protein, regardless of the presence or absence of the UBZs (Fig. 4A). As reported previously for human Polk (22), in ∼4% of cells transfected with wild-type EGFP-mouse Polk the protein was concentrated in nuclear foci (Fig. 4A). When cells transfected with EGFP-mouse Polk were exposed to UV radiation and incubated for 8–16 h, the fraction of cells with discrete nuclear foci increased to ∼55.3% (Fig. 4B). Interestingly, the number of cells with mouse Polk foci was higher than that observed when cells were transfected with human Polk and exposed to UV radiation (Fig. 4B). This observation was confirmed using a different EGFP-human Polk construct (22). Furthermore, foci were not detected (with or without UV radiation exposure) in cells transfected with EGFP-mouse Polk lacking the UBZs (Fig. 4A). Similar results were obtained with EGFP-mouse Polk carrying mutations in the UBZs (Fig. 4B). Hence, the UBZ domains are required for association of Polk with replication factories in cells exposed to UV radiation.

Mutation of the Polk UBZs Does Not Alter the Half-life of the Protein—Given that Polk is intrinsically error prone, regulation of Polk levels is presumably important for maintenance of genetic integrity. To investigate the stability of Polk in vivo, MEFs were treated with CHX for various lengths of time. Endogenous Polk was degraded slowly with a half-life of 5.4 h (Fig. 5A). To determine whether the UBZ domains affect the half-life of the protein, COS7 cells were transfected with wild-type and UBZ mutant Polk and were treated with CHX for various lengths of time. Although the turnover rate of these exogenous proteins (∼3.7–4.2 h) was relatively faster than that of endogenous Polk, we observed essentially similar half-lives between wild-type and UBZ mutant Polk (Fig. 5B and C). Levels of Polk Expression Are Increased in Cells Exposed to BPDE or UVR Radiation—Cells from two groups of independently generated Polk knock-out mice are abnormally sensitive
to BPDE and less so to UV radiation exposure (16, 17). To elucidate the underlying mechanism of this sensitivity, we examined the levels of nuclear Polk after UVB and BPDE treatments. Examination of MEFs exposed to UVB radiation at different times revealed a progressive increase in the amount of Polk 24–48 h after UVB exposure (Fig. 6A). Similarly, increased steady-state levels of Polk were observed 8–30 h after exposure of MEFs to 1 mM BPDE for 1 h (Fig. 6B). To further support this conclusion, whole cell lysates were harvested at different times after exposure of MEFs to 1 mM BPDE for 1 h. Equal amounts of whole cell lysate were immunoprecipitated with rabbit anti-Polk antibodies, and bound endogenous Polk was detected with hamster anti-Polk antibodies. Consistent with the results shown in Fig. 6B, increased levels of Polk were observed 8–24 h after BPDE treatment (Fig. 6C).

**DISCUSSION**

Persistent arrested DNA replication can threaten the viability of dividing cells. The observation that many eukaryotic cells, in particular those from higher eukaryotes, are endowed with multiple low fidelity specialized DNA polymerases that can catalyze DNA synthesis past sites of base damage in vitro has yielded important insights about DNA damage tolerance (2).

Regardless of the specific types of base damage in DNA handled by TLS, a question of considerable interest is how switching is effected at sites of arrested replication between high fidelity polymerases in the replicative machinery and one or more specialized enzymes that support TLS. Recent observations indicate that PCNA provides the central scaffold to which various TLS polymerases can bind to access the replicative ensemble stalled at a lesion and to execute their roles in lesion bypass (32, 33). However, it remains to be determined how a particular polymerase is selected to carry out TLS past a blocking lesion.

To further our understanding of the biological role of Polk during TLS in mammalian cells, we searched for interacting partners and identified ubiquitin. Hence, like the other Y-family polymerases, Polη, Polk, and Rev1 (25, 26, 30), Polk binds ubiquitin. Although the precise biological function of this interaction remains to be determined, this binding likely reflects an interaction of Polk with monoubiquitinated PCNA (31). The present study demonstrates that recently identified ubiquitin-binding motifs in Polk (UBZs) are required for its interaction with PCNA, suggesting specific molecular events associated with the Polk/PCNA interaction, especially in cells exposed to DNA-damaging agents such as UV radiation. Our studies represent the first demonstration of this phenomenon in living cells. Similar to the UBZs in Polη and the ubiquitin-binding motif in Polk (25), the UBZs in Polk are critical for the accumulation of the protein in replication foci when cells suffer DNA damage. Unlike the ubiquitin-binding motifs in Rev1 (26), deletion/mutation of the UBZs completely abolished the basal level of focus formation by wild-type Polk protein, suggesting that the basal level of Polk foci may represent a response to spontaneous DNA damage. Surprisingly, the number of cells with visible mouse Polk foci (~55.3%) is significantly greater.

**FIGURE 4.** The UBZs are required for association of Polk with replication factories in cells exposed to UV radiation. A, MRC5 cells were transfected with plasmids encoding full-length or deletion mutants of EGFP-Polk as indicated. Forty h after transfection, cells were UV-irradiated (10 J/m²) (bottom panel) and incubated for 8 h before fixation with paraformaldehyde. The distribution of Polk or deletion mutants (as indicated) was observed directly by autofluorescence of EGFP. The images show unirradiated (top) or UV-irradiated (bottom) transfected cells. B, shown is Polk focus formation after UV radiation. MRC5 cells were transfected with a panel of EGFP-Polk mutants and incubated for 40 h. Cells were irradiated with 10 J/m² UVC and further incubated for 8 h. The proportion of EGFP-Polk-expressing cells in which the protein was localized in nuclear foci was determined. All experiments were carried out in triplicate. Error bars indicate the standard deviation.

**FIGURE 5.** The UBZ domains do not affect the stability of Polk. A, MEFs were treated with CHX (25 µg/ml) for the indicated times, and the whole cell extracts were immunoblotted with α-Polk and α-actin antibodies. B and C, COS7 transfected with wild-type (β) or UBZ mutant (C) HA-Pokk was treated with CHX (25 µg/ml) for the indicated times, and the whole cell extracts were immunoblotted with α-HA and α-actin antibodies.
Polk-deficient mouse and chicken cells manifest sensitivity to killing by BPDE (16), suggesting a specific requirement for Polk to bypass this planar polycyclic lesion in DNA. Conceivably, adducts in DNA with similar planar polycyclic structures generated by cholesterol and cholesterol derivatives, such as steroid hormones and estrogen, generate the same requirement. Consistent with this notion, Polk mRNA is highly expressed in the adrenal cortex early during mouse embryonic development (28).

We and other laboratories reported previously that the mouse PolK gene is transcriptionally up-regulated following exposure to UVB and BPDE treatments (28, 34), suggesting that exposure to these DNA-damaging agents promotes up-regulation of the gene. Consistent with this interpretation, the present studies demonstrate a progressive increase in steady-state levels of Polk protein after such treatments. The biological significance of these expression patterns remains to be established.

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