THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 8, pp. 4658–4664, February 22, 2008

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

Received for publication, November 12, 2007, and in revised form, December 20, 2007 Published, JBC Papers in Press, December 26, 2007, DOI 10.1074/jbc.M709275200

Caixia Guo[‡], Tie-Shan Tang[§], Marzena Bienko[¶1], Ivan Dikic[¶], and Errol C. Friedberg[‡2]

From the [‡]Laboratory of Molecular Pathology, Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9072, the [§]Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, and the [¶]Institute for Biochemistry II, Goethe University Medical School, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany

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-dihydrodiol epoxide or UVB radiation.

Translesion DNA synthesis (TLS)[3] 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 vertibrates, 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 3′ → 5′ proofreading exonuclease activity and replicate undamaged DNA *in vitro* with low fidelity and weak processivity (4). Members of this family in mammalian cells include Polκ, Polι, and Polη, all of which can extend primers for varying distances past various types of template damage (4). A fourth member of the Y-family, REV1 protein, is able to catalyze the incorporation of only one or two dCMP moieties, regardless of the template base composition (5). Polκ, Polι, and Polη have been shown to interact with REV1 protein via a highly conserved C-terminal domain in REV1 (6–8). These polymerases also interact with PCNA (9, 10), and recent observations suggest that PCNA plays a key role in promoting the access of specialized polymerases to arrested replication forks (11–15).

Disruption of the *PolK* gene in mouse and chicken cells results in significant sensitivity to killing by benzo[*a*]pyrene-dihydrodiol epoxide (BPDE) and UV radiation (16–18). Polκ-deficient mouse embryonic stem and fibroblast cells also show moderate sensitivity to methyl methanesulfonate (19). Consistent with these results, primer extension assays have shown that human Polκ can support TLS across sites of base loss, acetylaminofluorene-G adducts, benzo[*a*]pyrene-G adducts, and thymine glycol (4). However, the enzyme does not support primer extension past thymine-thymine (T<>T) dimers or [6,4]pyrimidine-pyrimidone photoproducts (4). Similar to DNA polymerase Polξ, Polκ is also efficient in extending sites of replicative bypass by other specialized polymerases during TLS, at least *in vitro* (12). Furthermore, overexpression of Polκ in mammalian cells promotes pleiotropic genetic alterations and tumorigenesis (20, 21).

The relaxed fidelity of Polκ renders it error prone when copying undamaged DNA. Hence, access of the enzyme to sites of undamaged DNA must be tightly regulated to avoid mutational catastrophes. It has been reported that Polκ accumulates in microscopically discrete nuclear foci in UV radiation- or BPDE-treated cells (22–24). In addition, the C-terminal 97 amino acids of Polκ, which include a C2HC zinc finger, a bipartite nuclear localization signal, and a putative PCNA binding site, are important for the localization of Polκ in nuclear foci (22). However, the underlying mechanism of TLS by Polκ and other specialized DNA polymerases remains unclear.

To further our understanding of the role of Polκ in TLS and in spontaneous and DNA damage-associated mutagenesis, we searched for proteins that interact with mouse Polκ by screening a mouse testis cDNA library using the yeast two-hybrid system (6). We report here that ubiquitin binds strongly to Polκ bait protein. We examined the interaction of ubiquitin with Polκ and show that this interaction requires two novel zinc fingers, called ubiquitin-binding motifs (UBZs) (25), resident in

* This study was supported in part by Grants ES11344 from NIEHS, National Institutes of Health (to E. C. F.), and DI 931/1-1 from the Deutsche Forschungsgemeinschaft (to I. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Ernst Schering Foundation.

[2] To whom correspondence should be addressed. Tel.: 214-648-4020; Fax: 214-648-4067; E-mail: friedberg.errol@pathology.swmed.edu.

[3] The abbreviations used are: TLS, translesion DNA synthesis; UBZ, ubiquitin-binding zinc finger motif; Ub, ubiquitin; CHX, cycloheximide; GST, glutathione *S*-transferase; PCNA, proliferating cell nuclear antigen; BPDE, benzo-[*a*]pyrene-dihydrodiol epoxide; mPolκ, mouse Polκ; hPolκ, human Polκ; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; MEF, mouse embryonic fibroblast.

the C-terminal half of Polκ. We also demonstrate that Polκ binds monoubiquitinated PCNA more robustly than nonubiquitinated PCNA. The UBZs are required for Polκ to form nuclear foci after UV radiation. We measured the half-life of endogenous Polκ as 5.4 h and show that mutational disruption of the UBZs does not alter the half-life of Polκ protein. Finally, we examined levels of Polκ 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 m*PolK* 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 to produce GST fusion proteins as reported (10, 26). A series of mutant m*PolK* 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).

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 and HEK293T (human embryonic kidney) cells were transfected with the indicated constructs using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. Cells were harvested for further analysis 48 h after transfection. The SV40-transformed human fibroblast MRC5 was kindly provided by Dr. Alan R. Lehmann, University of Sussex. MRC5 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection and UV irradiation were carried out as described previously (10).

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 as described (17). Treatments using genotoxic agents were as follows. 1), BPDE (NCI National Institutes of Health carcinogen repository) was dissolved in dimethyl sulfoxide. 1 mM BPDE was added to exponentially growing cells and incubated for 1 h, and the cells were washed twice with phosphate-buffered saline and incubated with fresh medium. 2), UVB radiation at 25 J/m^2 was performed in a UV cross-linker (UV Stratalinker 2400, Stratagene). Nuclear extracts were harvested as described previously (27) at different time points.

Protein Half-life Determination—COS7 cells were transfected with the wild-type and UBZs mutant HA-Polκ constructs. Twenty-four h later, the transfected cells were aliquoted into 8–10 35-mm dishes to continue to culture for ~16 h. The half-life of Polκ was determined by treating cells with 25 μg/ml cycloheximide (CHX) (Sigma) for 0–7 h to inhibit protein synthesis and then preparing cell lysates to determine Polκ levels by Western blotting. For endogenous Polκ, wild-type MEFs were treated with 25 μg/ml CHX for 0–9 h. The content of Polκ and β-actin bands was quantified by Photoshop histogram. To calculate the half-life of Polκ protein, the content of the Polκ Western blot bands at each time point was normalized to the control (0 h) Polκ content and also refers to the β-actin content. The normalized data from several independent experiments were averaged together and semi-log plots were generated in Origin 4.1. Linear regression was performed, and the half-life was calculated from the fitted line equation.

Antibodies—Rabbit polyclonal anti-HA and mouse monoclonal anti-HA and anti-Myc were purchased from Covance. Anti-FLAG M2 agarose affinity gel and anti-FLAG M2 monoclonal antibodies were purchased from Sigma. Hamster polyclonal antiserum against mouse Polκ was made by our laboratory (27). Rabbit polyclonal antiserum against mouse Polκ was generated with a 14-amino acid peptide (CNYLKIDTPRQEANE) containing an N-terminal cysteine residue conjugated with keyhole limpet hemocyanin as described (28). Anti-PCNA antibodies were purchased from Santa Cruz Biotechnology.

Lysate Preparation, Co-immunoprecipitation, and Western Blotting—COS7 cells were transfected with pCMV-HA-mPolκ and pCMV5-FLAG-Ub. Harvested cell lysates were immunoprecipitated with anti-FLAG antibodies. HEK293T cells were transfected with pCMV-Myc-mPolκ 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-mPolκ, and 40 h later they were UV-irradiated (25 J/m^2). 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).

GST Pulldown Assay—GST fusion proteins were expressed and purified on glutathione-agarose (Sigma) as described (6). Purified mPolκ was pulled down by GST-Ub as described previously (26). For interaction between truncated/mutant mPolκ and GST-PCNA or GST-Ub constructs, transfected COS7/MRC5 cells were lysed with HEPES buffer and incubated with equal amounts of GST fusion proteins as described previously (10, 26). Samples were separated by SDS-PAGE and detected by immunoblotting with polyclonal antibodies against mPolκ or with monoclonal antibodies against Myc (9E10), HA (16B12), or PCNA.

Immunofluorescence Microscopy—MRC5 cells were transfected using a panel of mutated/truncated EGFP-mPolκ and EGFP-hPolκ constructs and cultured for ~40 h. They were then UV-irradiated and processed for immunofluorescence as described previously (10). Images were acquired using a Nikon Eclipse TE2000-U confocal laser scanning microscope and processed using Adobe Photoshop 7.0. A minimum of 200 nuclei were analyzed for each construct and treatment.

RESULTS

Mouse Polκ Interacts with Ubiquitin via Two Ubiquitin-binding Zinc Finger Domains and Undergoes Ubiquitination in Vivo—By screening a mouse testis cDNA library using mouse Polκ as bait, we identified both ubiquitin B and REV1 as inter-

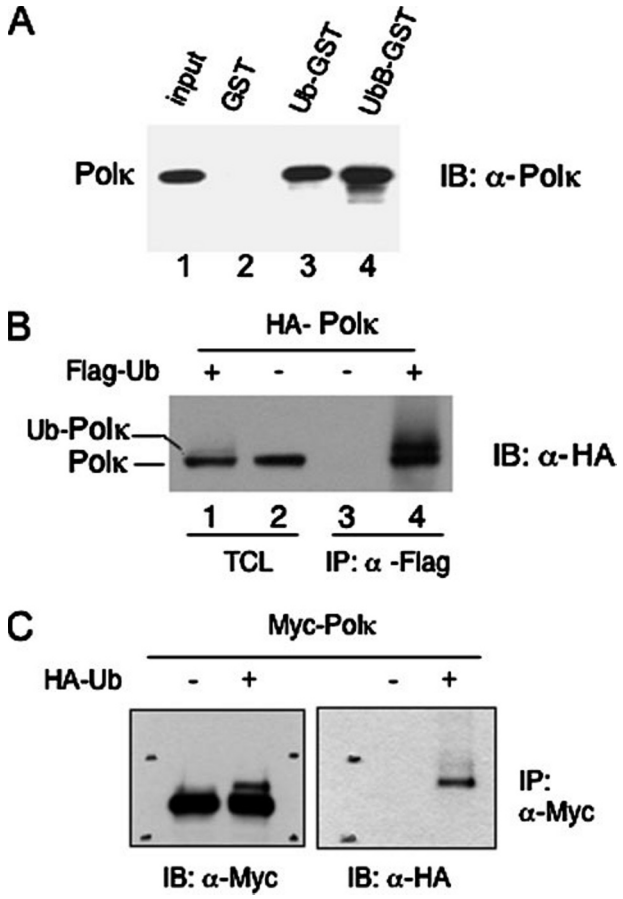

FIGURE 1. **Interaction between Polκ and ubiquitin.** *A*, shown is GST-ubiquitin pulldown of purified mouse Polκ protein as indicated. *Lane 1*, input containing one-tenth of the Polκ used in the experiments; *lane 2*, GST + Polκ; *lane 3*, GST-Ub + Polκ; *lane 4*, GST-UbB + Polκ. Bound proteins were detected by immunoblot (*IB*) analysis with α-Polκ antibody. *B*, anti-FLAG M2 agarose affinity gel was incubated with the COS7 cell lysates expressing HA-Polκ 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-Polκ constructs. The lysates were immunoprecipitated (*IP*) with α-Myc antibody and subjected to immunoblotting with α-Myc and α-HA as indicated.

acting moieties. Because ubiquitin B is a polypeptide containing four tandem ubiquitin moieties, we anticipated that Polκ would also bind to monoubiquitin. This was confirmed by GST pulldown using purified Polκ and GST-ubiquitin (Fig. 1*A*). We observed that mouse Polκ protein, in addition to binding ubiquitin, undergoes monoubiquitination *in vivo*. HA-Polκ and FLAG-ubiquitin were expressed in COS7 cells, and cell lysates were immunoprecipitated with anti-FLAG antibodies. Not surprisingly, HA-Polκ protein was detected in the immunoprecipitate, reflecting its interaction with ubiquitin (Fig. 1*B*). However, an additional slower migrating band was reproducibly observed, suggesting the presence of monoubiquitinated HA-Polκ protein (Fig. 1*B*). This was directly confirmed by immunoprecipitation of Polκ from cells cotransfected with Myc-Polκ and HA-Ub followed by immunoblotting with anti-HA antibodies (Fig. 1*C*).

Sequence analysis revealed that the duplicated C2HC zinc cluster domains in mouse Polκ (29) are in fact novel ubiquitin-binding domains called UBZs (25). The mouse UBZs (each ~30 amino acids in length) are located between amino acid residues 608 and 800 (Fig. 2*A*). To determine whether the UBZs in Polκ are required for binding ubiquitin, we incubated a fragment of Polκ bearing just the two UBZs (UBZ1 + UBZ2) with GST-ubiquitin and confirmed the interaction (Fig. 2*B*). To further document the requirement of the UBZs in Polκ for its interaction with ubiquitin, we generated a series of mutant constructs that deleted the N-terminal UBZ (UBZ1) (Polκ-UBZ1Δ), the C-terminal UBZ (UBZ2) (Polκ-UBZ2Δ), or both (Polκ-UBZΔ). Additionally, we generated constructs in which the amino acids Asp-642 and/or Asp-784 were mutated to Ala (D642A in UBZ1*, D784A in UBZ2*, D642A and D784A in UBZ*). Deletion of either UBZ significantly impaired binding to GST-ubiquitin (Fig. 2*C*), and deletion of both UBZs completely eliminated the interaction (Fig. 2*C*). Similar results were obtained when selected amino acids in the Polκ UBZs were mutated to alanine (Fig. 2*D*). Mutational inactivation of the UBZs in Polκ also impaired its monoubiquitination (Fig. 2*E*), and their deletion completely abolished monoubiquitination of Polκ (Fig. 2*E*). Collectively, these results suggest that the Polk UBZs are required for interaction between Polκ and ubiquitin and for monoubiquitination of the polymerase.

The UBZs Are Required for Enhanced Association between Polκ 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 Polη, Polι, and REV1 protein (10, 11, 25, 30). To determine whether an enhanced association also exists between Polκ and monoubiquitinated PCNA, we examined their interaction by GST pulldown experiments (26). Consistent with results shown previously (31), the interaction of purified Polκ with PCNA-Ub was more robust than with native PCNA (Fig. 3*A*). To determine whether the enhanced interaction is mediated via the UBZ domains, we incubated cell lysates expressing wild-type Polκ or those carrying mutations in the UBZ domains with GST-PCNA fusion proteins. As shown in Fig. 3*B*, the enhanced association with GST-PCNA-Ub was not observed with UBZ mutant preparations. To further support the result, HA-Polκ 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, Polκ was significantly increased after UVC treatment (Fig. 3*C*). Interestingly, the level of UBZ-deleted Polκ in chromatin fractions was significantly reduced after UVC treatment (Fig. 3*C*). 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 Polκ was significantly increased after UVC treatment (Fig. 3*D*).

In summary, the results of the experiments reported thus far indicate that mouse Polκ can interact with ubiquitin *in vitro*, an interaction that requires functional UBZs, and that Polκ can itself undergo monoubiquitination. A robust association between monoubiquitinated PCNA and Polκ also requires functional UBZs.

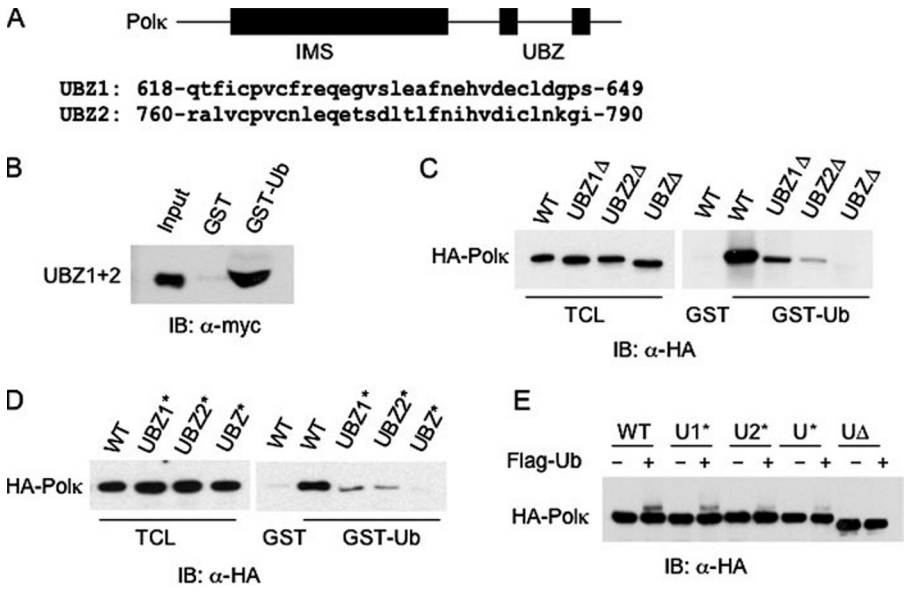

FIGURE 2. **The UBZs in Polκ are required for association between Polκ protein and ubiquitin.** *A*, shown is a schematic representation of mouse Polκ protein domains and the amino acid sequence of the UBZs. IMS, ImpB/MucB/SamB. *B*, COS7 cell lysates expressing Myc-Polκ-UBZ1 and -UBZ2 were pulled down with GST or GST-Ub fusion proteins as indicated. Bound proteins were detected by immunoblot (*IB*) analysis with α-Myc antibody. Input contains 2% of the lysates used in the experiment. *C* and *D*, shown are COS7 cell lysates expressing full-length Polκ and Polκ with the UBZs deleted (*C*) or Polκ with the UBZs mutated (*D*). HA-tagged Polκ proteins were pulled down with GST or GST-Ub fusion proteins as indicated. Bound proteins were detected by immunoblot analysis with α-HA. The total cell lysates (*TCL*) *lanes* contain 2% of the lysates used in the experiments. *WT*, wild type. *E*, COS7 cell lysates were transfected with (+) or without (−) FLAG-Ub together with HA-Polκ constructs. The cell lysates were separated and detected by immunoblot analysis with α-HA antibody.

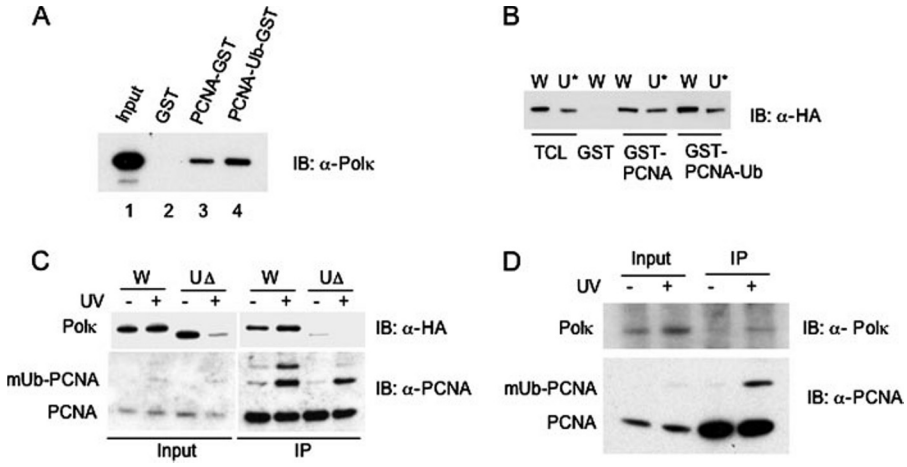

FIGURE 3. **The UBZs in Polκ protein mediate increased association between Polκ and monoubiquitinated PCNA.** *A*, shown is GST pulldown of purified mouse Polκ protein as indicated. *Lane 1*, input containing one-tenth of the Polκ used in the experiments; *lane 2*, GST + Polκ; *lane 3*, GST-PCNA + Polκ; and *lane 4*, GST-PCNA-Ub + Polκ. Bound proteins were detected by immunoblot (*IB*) analysis with α-Polκ antibody. *B*, lysates of MRC cells expressing wild-type (*W*) and UBZ* (*U**) Polκ proteins tagged with HA epitopes at their N termini were incubated with GST fusion proteins as indicated. Bound proteins were detected by immunoblotting with α-HA. The total cell lysates (*TCL*) *lanes* contain 2% of the lysates used in the experiments. *C*, MRC cells were transfected with wild-type and UBZ-deleted Polκ and were UV-irradiated (25 J/m^2) 40 h later. They were then incubated for 7 h prior to Triton extraction and cross-linking. Equal amounts of Triton-insoluble proteins were solubilized and immunoprecipitated with α-PCNA, and the retained proteins were analyzed by Western blotting with α-HA (*top*) or α-PCNA (*bottom*). *Input lanes* contain 4% of the lysates used in the experiments. *D*, MEFs were UV-irradiated (25 J/m^2) and then incubated for 7 h prior to Triton extraction and cross-linking. Immunoprecipitation with α-PCNA was performed as in *C*. The retained proteins were analyzed by Western blotting with α-Polκ (*top*) or α-PCNA (*bottom*). *Input lanes* contain 4% of the lysates used in the experiments. Data are representative of three independent experiments.

Polκ UBZs Are Required for Association of Polκ 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 Polκ constructs into fibroblasts. We observed strict nuclear localization of EGFP-mouse Polκ protein, regardless of the presence or absence of the UBZs (Fig. 4*A*). As reported previously for human Polκ (22), in ~4% of cells transfected with wild-type EGFP-mouse Polκ the protein was concentrated in nuclear foci (Fig. 4*A*). When cells transfected with EGFP-mouse Polκ were exposed to UV radiation and incubated for 8–16 h, the fraction of cells with discrete nuclear foci increased to ~55.3% (Fig. 4*B*). Interestingly, the number of cells with mouse Polκ foci was higher than that observed when cells were transfected with human Polk and exposed to UV radiation (Fig. 4*B*). This observation was confirmed using a different EGFP-human Polκ construct (22). Furthermore, foci were not detected (with or without UV radiation exposure) in cells transfected with EGFP-mouse Polκ lacking the UBZs (Fig. 4*A*). Similar results were obtained with EGFP-mouse Polκ carrying mutations in the UBZs (Fig. 4*B*). Hence, the UBZ domains are required for association of Polκ with replication factories in cells exposed to UV radiation.

Mutation of the Polκ UBZs Does Not Alter the Half-life of the Protein—Given that Polκ is intrinsically error prone, regulation of Polκ levels is presumably important for maintenance of genetic integrity. To investigate the stability of Polκ *in vivo*, MEFs were treated with CHX for various lengths of time. Endogenous Polκ was degraded slowly with a half-life of 5.4 h (Fig. 5*A*). To determine whether the UBZ domains affect the half-life of the protein, COS7 cells were transfected with wild-type and UBZ mutant Polκ 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 Polκ, we observed essentially similar half-lives between wild-type and UBZ mutant Polκ (Fig. 5, *B* and *C*).

Levels of Polκ Expression Are Increased in Cells Exposed to BPDE or UVB Radiation—Cells from two groups of independently generated Polκ knock-out mice are abnormally sensitive

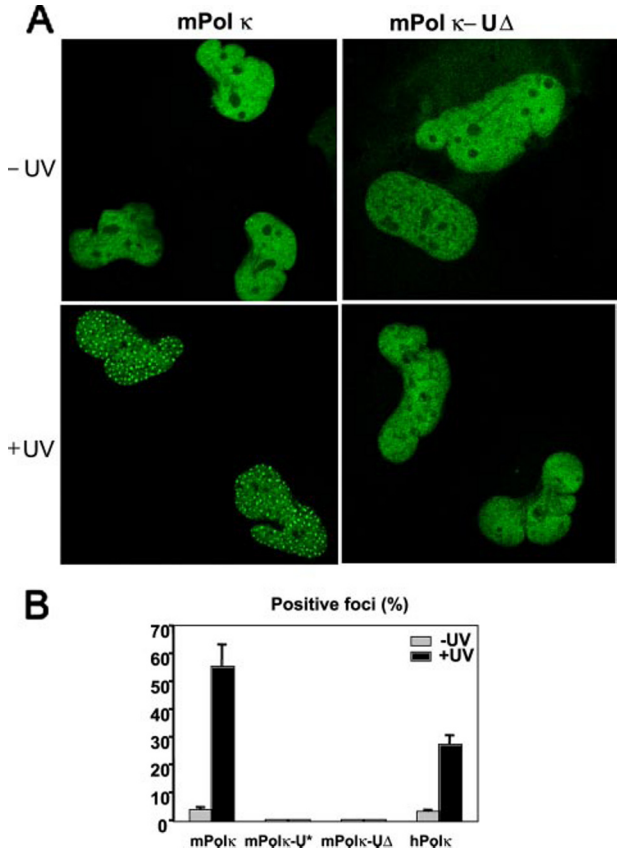

FIGURE 4. **The UBZs are required for association of Polκ with replication factories in cells exposed to UV radiation.** *A*, MRC5 cells were transfected with plasmids encoding full-length or deletion mutants of EGFP-Polκ as indicated. Forty h after transfection, cells were UV-irradiated (10 J/m^2) (*bottom panel*) and incubated for 8 h before fixation with paraformaldehyde. The distribution of Polκ 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 Polκ focus formation after UV radiation. MRC5 cells were transfected with a panel of EGFP-Polκ mutants and incubated for 40 h. Cells were irradiated with 10 J/m^2 UVC and further incubated for 8 h. The proportion of EGFP-Polκ-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.

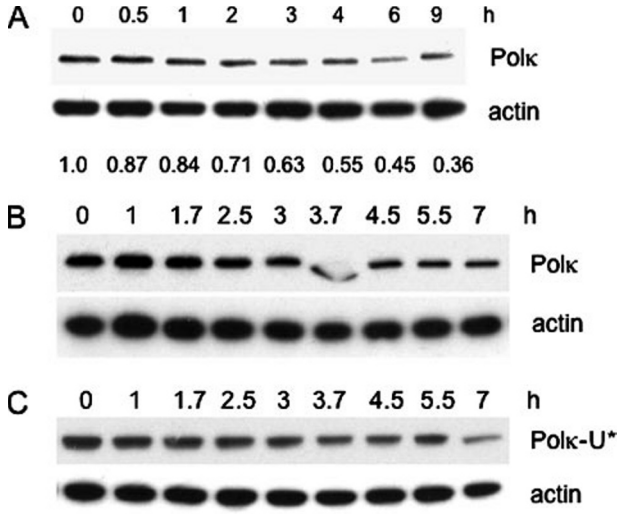

FIGURE 5. **The UBZ domains do not affect the stability of Polκ.** *A*, MEFs were treated with CHX (25 μg/ml) for the indicated times, and the whole cell extracts were immunoblotted with α-Polκ and α-actin antibodies. *B* and *C*, COS7 transfected with wild-type (*B*) or UBZ mutant (*C*) HA-Polκ was treated with CHX (25 μg/ml) for the indicated times, and the whole cell extracts were immunoblotted with α-HA and α-actin antibodies.

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 Polκ after UVB and BPDE treatments. Examination of MEFs exposed to UVB radiation at different times revealed a progressive increase in the amount of Polκ 24–48 h after UVB exposure (Fig. 6*A*). Similarly, increased steady-state levels of Polκ were observed 8–30 h after exposure of MEFs to 1 mM BPDE for 1 h (Fig. 6*B*). 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-Polκ antibodies, and bound endogenous Polκ was detected with hamster anti-Polκ antibodies. Consistent with the results shown in Fig. 6*B*, increased levels of Polκ were observed 8–24 h after BPDE treatment (Fig. 6*C*).

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 Polκ during TLS in mammalian cells, we searched for interacting partners and identified ubiquitin. Hence, like the other Y-family polymerases, Polη, Polι, and Rev1 (25, 26, 30), Polκ binds ubiquitin. Although the precise biological function of this interaction remains to be determined, this binding likely reflects an interaction of Polκ with monoubiquitinated PCNA (31). The present study demonstrates that recently identified ubiquitin-binding motifs in Polκ (UBZs) are required for its interaction with PCNA, suggesting specific molecular events associated with the Polκ/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 Polι (25), the UBZs in Polκ 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 Polκ protein, suggesting that the basal level of Polκ foci may represent a response to spontaneous DNA damage. Surprisingly, the number of cells with visible mouse Polκ foci (~55.3%) is significantly greater

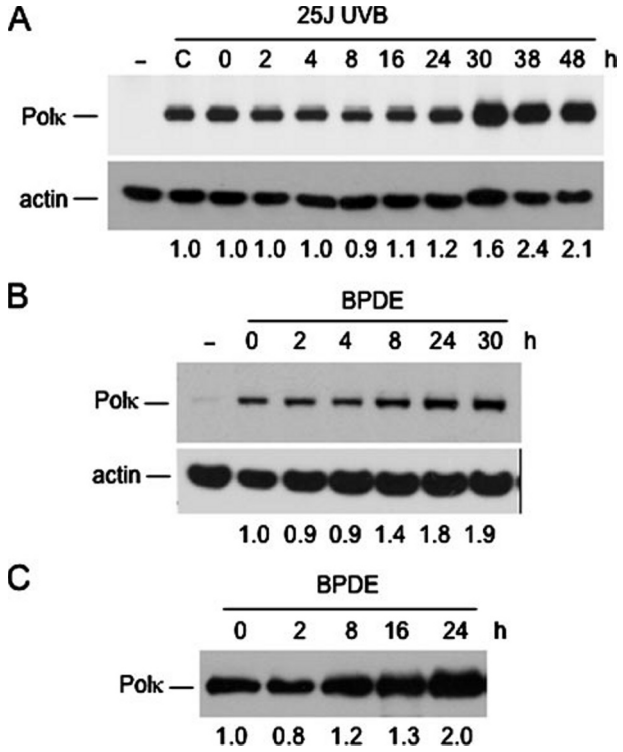

FIGURE 6. Time course of nuclear steady-state levels of Polκ with UVB (25 J/m^2) (*A*) or BPDE (1 mM) (*B*) treatments as determined by Western blotting. No signal was detected when a Polκ mutant was used. β-Actin served as a loading control (*bottom panel*). The intensity of Polκ and β-actin bands was quantified by Photoshop histogram. The intensity of the Polκ Western blot bands at each time point was normalized to that of the control (0 h) Polκ content and compared with the β-actin content. The normalized data from several independent experiments were averaged and are indicated under each *lane*. *Lane C* is an untreated sample. *C*, shown is the time course of cellular levels of Polκ with BPDE treatments as determined by immunoprecipitation. Whole cell lysates were harvested at times after exposure of mouse embryo fibroblasts to 1 mM BPDE for 1 h. Equal amounts of whole cell lysate were immunoprecipitated with rabbit anti-Polκ antibodies, and bound endogenous Polκ was detected with hamster anti-Polκ antibodies. The intensity of Polκ bands at each time point was quantified by Photoshop histogram and normalized to the control (0 h) Polκ content.

than that observed with human Polκ (~25%) upon exposure to UV radiation (22).

The present studies also demonstrate that like other Y-family polymerases, mouse Polκ protein can be monoubiquitinated and that the UBZs in the protein are required for this modification. The biological significance of monoubiquitination of Y-family polymerases is not understood. However, this post-translational modification may contribute to regulation of Y-family polymerases in or out of replication factories (25, 33).

We reported previously the presence of multiple *PolK* transcripts in mouse testis (27). Many of the putative Polκ protein isoforms thus identified lack UBZ domains. It is thus of considerable interest to determine whether the putative Polκ isoforms are indeed expressed *in vivo* and what novel biological functions they may have.

In addition to protein-protein interactions, Polκ activity may be regulated by its cellular levels. Polκ is apparently a relatively stable protein *in vivo*, and mutation of the UBZs does not alter the half-life of the protein. Consistent with this observation, the majority of Polκ *in vivo* is not monoubiquitinated, and polyubiquitinated Polκ is apparently absent.

Polκ-deficient mouse and chicken cells manifest sensitivity to killing by BPDE (16), suggesting a specific requirement for Polκ to bypass this planar polycyclic lesion in DNA. Conceivably, adducts in DNA with similar planer 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 Polκ protein after such treatments. The biological significance of these expression patterns remains to be established.

Acknowledgments—We thank Dr. Alan R. Lehmann for MRC5 cells and EGFP-hPolκ plasmid, Dr. Valerie Gerlach for preparing EGFP-C1-hPolκ construct and rabbit anti-Polκ, Drs. Tom Gillete and Lisa McDaniel for helpful discussion, and J. Nicole Kosarek for critical reading of the manuscript.

REFERENCES

1. Friedberg, E. C. (2005) *Nat. Rev. Mol. Cell Biol.* **6,** 943–953
2. Friedberg, E. C., Lehmann, A. R., and Fuchs, R. P. (2005) *Mol. Cell* **18,** 499–505
3. Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) *Mol. Cell* **8,** 7–8
4. Bebenek, K., and Kunkel, T. A. (2004) *Adv. Protein Chem.* **69,** 137–165
5. Lawrence, C. W. (2004) *Adv. Protein Chem.* **69,** 167–203
6. Guo, C., Fischhaber, P. L., Luk-Paszyc, M. J., Masuda, Y., Zhou, J., Kamiya, K., Kisker, C., and Friedberg, E. C. (2003) *EMBO J.* **22,** 6621–6630
7. Ohashi, E., Murakumo, Y., Kanjo, N., Akagi, J., Masutani, C., Hanaoka, F., and Ohmori, H. (2004) *Genes Cells* **9,** 523–531
8. Tissier, A., Kannouche, P., Reck, M. P., Lehmann, A. R., Fuchs, R. P., and Cordonnier, A. (2004) *DNA Repair* **3,** 1503–1514
9. Lehmann, A. R., Niimi, A., Ogi, T., Brown, S., Sabbioneda, S., Wing, J. F., Kannouche, P. L., and Green, C. M. (2007) *DNA Repair* **6,** 891–899
10. Guo, C., Sonoda, E., Tang, T. S., Parker, J. L., Bielen, A. B., Takeda, S., Ulrich, H. D., and Friedberg, E. C. (2006) *Mol. Cell* **23,** 265–271
11. Kannouche, P. L., Wing, J., and Lehmann, A. R. (2004) *Mol. Cell* **14,** 491–500
12. Prakash, S., Johnson, R. E., and Prakash, L. (2005) *Annu. Rev. Biochem.* **74,** 317–353
13. Ulrich, H. D. (2004) *Cell Cycle* **3,** 15–18
14. Stelter, P., and Ulrich, H. D. (2003) *Nature* **425,** 188–191
15. Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H., and Yamaizumi, M. (2004) *EMBO J.* **23,** 3886–3896
16. Ogi, T., Shinkai, Y., Tanaka, K., and Ohmori, H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15548–15553
17. Schenten, D., Gerlach, V. L., Guo, C., Velasco-Miguel, S., Hladik, C. L., White, C. L., Friedberg, E. C., Rajewsky, K., and Esposito, G. (2002) *Eur. J. Immunol.* **32,** 3152–3160
18. Okada, T., Sonoda, E., Yamashita, Y. M., Koyoshi, S., Tateishi, S., Yamaizumi, M., Takata, M., Ogawa, O., and Takeda, S. (2002) *J. Biol. Chem.* **277,** 48690–48695
19. Takenaka, K., Ogi, T., Okada, T., Sonoda, E., Guo, C., Friedberg, E. C., and Takeda, S. (2006) *J. Biol. Chem.* **281,** 2000–2004

20. Bavoux, C., Hoffmann, J. S., and Cazaux, C. (2005) *Biochimie* (*Paris*) **87,** 637–646
21. Bavoux, C., Leopoldino, A. M., Bergoglio, V., O-Wang, J., Ogi, T., Bieth, A., Judde, J. G., Pena, S. D., Poupon, M. F., Helleday, T., Tagawa, M., Machado, C., Hoffmann, J. S., and Cazaux, C. (2005) *Cancer Res.* **65,** 325–330
22. Ogi, T., Kannouche, P., and Lehmann, A. R. (2005) *J. Cell Sci.* **118,** 129–136
23. Bergoglio, V., Bavoux, C., Verbiest, V., Hoffmann, J. S., and Cazaux, C. (2002) *J. Cell Sci.* **115,** 4413–4418
24. Bi, X., Slater, D. M., Ohmori, H., and Vaziri, C. (2005) *J. Biol. Chem.* **280,** 22343–22355
25. Bienko, M., Green, C. M., Crosetto, N., Rudolf, F., Zapart, G., Coull, B., Kannouche, P., Wider, G., Peter, M., Lehmann, A. R., Hofmann, K., and Dikic, I. (2005) *Science* **310,** 1821–1824
26. Guo, C., Tang, T. S., Bienko, M., Parker, J. L., Bielen, A. B., Sonoda, E., Takeda, S., Ulrich, H. D., Dikic, I., and Friedberg, E. C. (2006) *Mol. Cell. Biol.* **26,** 8892–8900
27. Guo, C., Gao, T., Confer, N., Velasco-Miguel, S., and Friedberg, E. C. (2005) *DNA Repair* **4,** 397–402
28. Velasco-Miguel, S., Richardson, J. A., Gerlach, V. L., Lai, W. C., Gao, T., Russell, L. D., Hladik, C. L., White, C. L., and Friedberg, E. C. (2003) *DNA Repair* **2,** 91–106
29. Gerlach, V. L., Aravind, L., Gotway, G., Schultz, R. A., Koonin, E. V., and Friedberg, E. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11922–11927
30. Plosky, B. S., Vidal, A. E., Fernandez de Henestrosa, A. R., McLenigan, M. P., McDonald, J. P., Mead, S., and Woodgate, R. (2006) *EMBO J.* **25,** 2847–2855
31. Bi, X., Barkley, L. R., Slater, D. M., Tateishi, S., Yamaizumi, M., Ohmori, H., and Vaziri, C. (2006) *Mol. Cell. Biol.* **26,** 3527–3540
32. Moldovan, G. L., Pfander, B., and Jentsch, S. (2007) *Cell* **129,** 665–679
33. Huang, T. T., and D'Andrea, A. D. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 323–334
34. Ogi, T., Mimura, J., Hikida, M., Fujimoto, H., Fujii-Kuriyama, Y., and Ohmori, H. (2001) *Genes Cells* **6,** 943–953