Involvement of Vertebrate Polκ in Translesion DNA Synthesis across DNA Monoalkylation Damage

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DNA lesions that escape excision repair pathways can cause arrested DNA replication. This replication block can be processed by translesion DNA synthesis (TLS), which is carried out by a number of specialized DNA polymerases. A sequential lesion bypass model has been proposed; one of the lesion-specific polymerases inserts nucleotide(s) opposite the damaged template, followed by extension from the inserted nucleotide by the same or another polymerase. Polζ and Polκ have been proposed as candidates for executing the extension step in eukaryotic cells. We previously disrupted separately Rev3, the catalytic subunit of Polζ, and Polκ in chicken B lymphocyte DT40 cells. We found that each cell line showed significant UV sensitivity, implying that both contribute to UV radiation damage repair. In the present studies we generated REV3−/−POLK−/− double knock-out cells to determine whether they participate in the same or different pathways. The double mutant was viable and proliferated with the same kinetics as parental REV3−/− cells. The cells showed the same sensitivity as REV3−/− cells to UV, ionizing radiation, and chemical cross-linking agents. In contrast, they were more sensitive than REV3−/− cells to mono-functional alkylating agents, even though POLK−/− cells barely exhibited increased sensitivity to those. Moreover Polκ-deficient mouse embryonic stem and fibroblast cells, both of which have previously been shown to be sensitive to UV radiation, also showed moderate sensitivity to methyl methanesulfonate, a mono-functional alkylating agent. These data imply that Polκ has a function in TLS past alkylated base adducts as well as UV radiation DNA damage in vertebrates.

Chromosomal DNA in living organisms is continually exposed to a variety of genotoxic agents from exogenous and endogenous sources. Unrepaired DNA damage can lead to replication fork arrest and the formation of gaps and breaks in sister chromatids. Such DNA lesions are processed by two major postreplication repair pathways: homologous recombination repair and translesion DNA synthesis (TLS)1 (1). While homologous recombination repair promotes DNA synthesis by facilitating recombination between damaged sister chromatids with the other intact ones, TLS functions by synthesizing DNA past lesions using a number of specialized DNA polymerases (2–4).

A number of TLS polymerases have been identified in yeasts and mammals. Polη, Polζ, and Polκ are conserved between species, while Polκ is lacking in budding yeast. Biochemical studies have suggested that lesion bypass is effected by two sequential nucleotide incorporation events (4). The first step is insertion of nucleotides opposite the damaged template nucleotide(s), and the second step is extension from the inserted nucleotide(s). While mammalian and yeast Polη efficiently promotes replication through major UV-induced DNA lesions such as cyclobutane pyrimidine dimer both as an inserter and as an extender (5–7), Polζ appears to act more efficiently at extending from the inserted nucleotide(s).

Yeast and mammalian Polζ is comprised of the Rev3 catalytic subunit and the Rev7 subunit (8–10). Rev1, a member of the Y family of DNA polymerases, has limited deoxyctydyltransferase activity (8, 11) and can physically interact with Rev7 as well as with other Y family polymerases, such as Polκ, Pol, and Polη (12–14). We previously characterized REV3−/− cells from the chicken B lymphocyte DT40 cell line, and observed that Rev3 is involved in the maintenance of chromosomal DNA as well as in the tolerance of various types of DNA damage (15). We also observed that Rev1, Rev3, and Rev7 may act as a functional unit in cellular tolerance of a variety of genotoxic stresses, as do the orthologous yeast proteins (16).

Polκ is a member of the Y family of DNA polymerases together with Polη, Polζ, and Rev1 (17). Purified human Polκ can bypass adducts of N2-acetylanilinothiourea and benzeneurea, and templates containing 8-oxoguanine, abasic sites, and 1,N4-ethenedeoxyadenosine (18–22). In addition, Polκ efficiently extends various nucleotides incorporated opposite O6-methyl guanine and 8-oxoguanine (23). Therefore, it has been suggested that the role of Polκ in TLS is as an extender, but at present in vivo evidence in support of this suggestion is lacking.

In this study, we generated REV3−/−POLK−/− double knock-out cells and analyzed their sensitivity to various DNA-damaging agents. The double mutant cells displayed the same sensitivity to UV radiation, ionizing radiation (IR), and cross-linking agents as did parental REV3−/− cells. However, they showed increased sensitivity to monofunctional alkylating agents. Additionally, Polκ-deficient mouse embryonic stem (ES) cells and embryonic fibroblasts (MEF) showed a moderate increase in sensitivity to killing following exposure to the monofunctional alkylating agents methyl methanesulfonate (MMS). Collectively, these observations suggest that Polκ might have a conserved in vivo function in TLS past monoalkylated bases in vertebrates.

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2 The abbreviations used are: TLS, translesion DNA synthesis; Pol, polymerase; ES, embryonic stem; MEF, mouse embryonic fibroblast; MMS, methyl methanesulfonate; IR, ionizing radiation; MMC, mitomycin C; MNG, N-methyl-N-nitro-N-nitrosoguanidine; DMEM, Dulbecco’s modified Eagle’s medium; PP, photoproduct.
**MATERIALS AND METHODS**

**Cell Culture and Gene Targeting**—The conditions for cell culture, selection, and DNA transfections of DT40 cells have been described previously. The REV3<sup>−/−</sup> POLK<sup>−/−</sup> double knock-out cells were produced from REV3<sup>−/−</sup> cells (15) by disrupting the single POLK gene in the sex chromosome in DT40 cells with a chicken POLK disruption construct containing a puromycin-resistant selection marker cassette (24). REV1<sup>−/−</sup> POLK<sup>−/−</sup> double knock-out cells were generated from REV1<sup>−/−</sup> cells (25) with a POLK disruption construct containing a histidinol-D-resistant selection marker cassette (24). To express chicken POLK, a full-length chicken POLK was inserted into the expression vector p176 (26), linearized with PvuI, and transfected into the cells. Generation of Polk-targeted mouse ES cells and MEFs have been described previously (27, 28).

**Analyses of Chromosome Aberrations and Sister Chromatid Exchange Events**—Measurement of chromosome aberrations and sister chromatid exchanges was performed as described previously (24).

**Colony Formation Assay**—Colony formation assay of DT40 cells with media containing methyleneblue was performed as previously described for measuring sensitivities to UV, IR, cisplatin (cis-platinum (II) diaminodichloride), and MMS (24). For exposure of cells to mitomycin C (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Nacalai Tesque, Kyoto, Japan), cells were treated at 39.5 °C in 1 ml of complete medium containing MMC or MNNG for 1 h. Exposure of cells to ethyl methanesulfonate (Nacalai Tesque) was performed in medium containing no serum for 1 h, followed by addition of serum to recover the cells that are bound to the bottom of the dishes. The sensitivity of Polk-targeted mouse ES cells to MMS was determined by measuring the colony-forming efficiency of cells treated over a range of doses as described (27). Wild-type TT2 and Polk-targeted ES cells were grown on feeder plates supplied with ES medium; ES medium contains 15% fetal calf serum and 1,000 units/ml Leukemia inhibitory factor in DMEM. Before MMS treatment, cells were harvested, replated on feeder plates, and allowed to adhere to plates for 6 h. After 16-h MMS treatment in ES medium, cells were washed with phosphate-buffered saline once, and cultured for 10 days in ES medium, then fixed, stained, and counted. For exposure of wild-type and Polk-deficient MEFs to MMS, harvested cells were plated in DMEM supplemented with 15% fetal calf serum and allowed to adhere for 2 h. MMS treatment was performed in DMEM for 16 h. Viable colonies were counted after 2 weeks culture.

**RESULTS**

**Generation of REV3<sup>−/−</sup> POLK<sup>−/−</sup> Double Knock-out Cells**—DT40 chicken cells possess a single POLK gene in the sex chromosome (24). To produce REV3<sup>−/−</sup> POLK<sup>−/−</sup> double knock-out cells, the single POLK gene in a REV3<sup>−/−</sup> DT40 cell line was disrupted with a gene targeting construct designed to delete all the DNA polymerase motifs of POLK. Targeting events were verified by the appearance of a smaller band and the disappearance of the band that appears in wild-type cells (Fig. 1A, lanes 9 and 10). We isolated two independent REV3<sup>−/−</sup> POLK<sup>−/−</sup> clones and measured their kinetics of proliferation. The growth rate of REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells was indistinguishable from that of REV3<sup>−/−</sup> cells, which proliferates with significantly slower kinetics than wild-type and POLK<sup>−/−</sup> cells (Fig. 1B). Likewise, the level of spontaneous chromosome aberrations for REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells was similar to that of REV3<sup>−/−</sup> cells (Table 1). In our previous studies, POLK-disrupted cells exhibited a significant increase in the level of spontaneous sister chromatid exchanges (3.5 ± 1.5 for POLK<sup>−/−</sup> cells versus 2.1 ± 1.6 for wild-type cells, means ± S.E.) (24). In the REV3<sup>−/−</sup> background, POLK disruption did not increase spontaneous sister chromatid exchanges (5.0 ± 2.5 for REV3<sup>−/−</sup> cells versus 5.1 ± 2.6 for REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells). This
observation suggests that although both Rev3 and Polk play important roles in maintaining the integrity of chromosomal DNA during the cell cycle, Polk cannot fulfill this function in the absence of Rev3.

REV3<sup>−/−</sup> POLK<sup>−/−</sup> and REV3<sup>−/−</sup> Cells Have the Same Sensitivity to UV Radiation, IR, and Cross-linking Agents—To analyze the DNA repair capacity of REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells, we examined viability of cells after various genotoxic treatments using colony formation assays. POLK<sup>−/−</sup> cells are sensitive to UV radiation, but we did not observe a significant increase in the sensitivity of REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells to this agent (Fig. 2A). Disruption of POLK in wild-type or in REV3<sup>−/−</sup> cells did not increase sensitivity to IR exposure and cross-linking agents (Fig. 2, B–D). These observations suggest that Polk and Rev3 interact in the same pathway in response to UV radiation-induced adducts and that Polk does not function in TLS across IR-induced base damage or adducts generated by cross-linking agents.

Elevated Sensitivity of REV3<sup>−/−</sup> POLK<sup>−/−</sup> Cells to Monoalkylating Agents—We previously showed that POLK<sup>−/−</sup> DT40 cells do not display notably increased sensitivity to monoalkylating agents (24). However, in the present studies we observed that disruption of POLK significantly sensitized REV3<sup>−/−</sup> cells to these agents (Fig. 3, A–C). To confirm that the elevated sensitivities were caused by the disruption of the POLK gene in REV3<sup>−/−</sup> cells, REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells were reconstituted with chicken POLK cDNA. Expression of the chicken POLK gene restored the tolerance of REV3<sup>−/−</sup> POLK<sup>−/−</sup> cells efficiently (Fig. 3, A–C). These results indicate that Polk functions in TLS past monoalkylated adducts.

To determine whether Polk has a role in TLS past monoalkylated adducts in mammalian cells, we examined Polk-deficient mouse ES cell lines. Wild-type, Polk<sup>+/+</sup>, and two independent Polk<sup>−/−</sup> cells were exposed to MMS. Polk<sup>−/−</sup> cells exhibited the same sensitivity as wild-type cells. However, Polk<sup>−/−</sup> cells showed moderately increased sensitivity to killing by MMS (Fig. 4A). Increased MMS sensitivity was observed also in Polk-deficient MEF cells (Fig. 4B). Thus, Polk may have a role in bypassing DNA lesions induced by mono-functional alkylating agents. Furthermore, the data suggest complex functional interactions between Polk and Rev3, since the two genes are epistatic for UV radiation sensitivity, whereas Polk can act as a back up for Rev3 in cellular tolerance to MMS. This complex interaction led us to examine functional interactions between Polk and Rev1 by generating REV1<sup>−/−</sup>- POLK<sup>−/−</sup> double mutant cells.

Phenotypic Comparison between REV1 and REV3 Disruption in a POLK<sup>−/−</sup> Background—It has been reported that the Rev1 protein binds to both Rev7 and Polk, which implies that these genes may have functional relationships (12–14). Despite this biochemical result, we recently reported that REV1<sup>−/−</sup>, REV3<sup>−/−</sup>, and REV1<sup>−/−</sup>- REV3<sup>−/−</sup> DT40 cells and triple mutant cells show the same sensitivity to MMS, IR, UV radiation, and cross-linking agents (16). We therefore concluded that Rev1 may act cooperatively with Rev3 but perhaps not with other DNA polymerases. To determine whether or not Rev1 and Polk function inde-
In conclusion, we took advantage of the high targeting efficiency of DT40 and generated REV3−/−POLK− and REV1−/−POLK− double knock-out cells. By analyzing the mutant cells, we have identified an in vitro function of Polκ in bypassing DNA lesions produced by monofunctional alkylating agents. Relationships with other TLS polymerases, such as Polη encoded by RAD30/XPF gene, will be analyzed in the future.

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Vertebrate Polκ across Monoalkylation Damage

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