REV3 and REV1 Play Major Roles in Recombination-independent Repair of DNA Interstrand Cross-links Mediated by Monoubiquitinated Proliferating Cell Nuclear Antigen (PCNA) * ‡

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DNA interstrand cross-links (ICLs) are the most cytotoxic lesions to eukaryotic genome and are repaired by both homologous recombination-dependent and -independent mechanisms. To better understand the role of lesion bypass polymerases in ICL repair, we investigated recombination-independent repair of ICLs in REV3 and REV1 deletion mutants constructed in avian DT40 cells and mouse embryonic fibroblast cells. Our results showed that Rev3 plays a major role in recombination-independent ICL repair, which may account for the extreme sensitivity of Rev3 mutants to cross-linking agents. This result raised the possibility that the NER gap synthesis, when encountering an adducted base present in the ICL repair intermediate, can lead to recruitment of Rev3, analogous to the recruitment of polymerase η during replicative synthesis. Indeed, the monoubiquitination-defective Proliferating Cell Nuclear Antigen (PCNA) mutant exhibits impaired recombination-independent ICL repair as well as drastically reduced mutation rate, indicating that the PCNA switch is utilized to enable lesion bypass during DNA repair synthesis. Analyses of a REV1 deletion mutant also revealed a significant reduction in recombination-independent ICL repair, suggesting that Rev1 cooperates with Rev3 in recombination-independent ICL repair. Moreover, deletion of REV3 or REV1 significantly altered the spectrum of mutations resulting from ICL repair, further confirming their involvement in mutagenic repair of ICLs.

Bifunctional alkylation agents generate DNA interstrand cross-links (ICLs), which prevent strand separation required for essential DNA functions such as replication, transcription, and recombination. Because an ICL compromises both strands of the double helix, recombination with an undamaged homologous sequence is required for error-free repair. This has been shown in both prokaryotic and eukaryotic systems (1, 2). However, ICL repair also occurs in a recombination-independent fashion. Our investigations in mammalian cells have suggested an NER- and translesion synthesis-based error-prone mechanism of ICL repair in which the gap created by the NER dual incisions is resynthesized through participation of lesion bypass DNA polymerases (3, 4). This error-prone mechanism may account for the mutagenic impact of ICLs. A similar mechanism has been demonstrated in budding yeast, suggesting that recombination-independent ICL repair may be a highly conserved mechanism in eukaryotes (5, 6).

The budding yeast mutants REV3 and REV1 are characterized by their marked reduction in UV-induced mutability (7) and profound sensitivity to ICLs during G1 or stationary phases. REV3 encodes the catalytic subunit of DNA polymerase ζ (Polζ), a member of the B-type lesion bypass polymerase family (8). Mouse embryonic fibroblasts (MEFs) derived from REV3+/− embryos and an avian REV3+/− mutant exhibited severe sensitivity to DNA cross-linking agents (2, 9–11), suggesting an important role for Polζ in the repair of DNA ICLs. REV1 encodes a deoxycytidyltransferase (12, 13), which has been proposed to act sequentially with Polζ to bypass DNA lesions (14). In addition, the C terminus of Rev1 interacts with a number of other translesion synthesis (TLS) polymerases, including Polλ (15, 16). This C-terminal region is essential for effective DNA damage tolerance, suggesting that it may have an important role in coordinating TLS (22).

Although it is clearly established that loss of Rev3 and Rev1 leads to profound cross-link sensitivity, it is not clear whether Rev3 functions in recombination-dependent or -independent or other yet-to-be-defined repair pathways (22). To elucidate the mechanism of lesion bypass processes during recombination-independent ICL repair, we analyzed a series of mammalian and avian mutants defective in the TLS process. In this report, we show that Polζ is likely the predominant lesion bypass polymerase in recombination-independent ICL repair, with its involvement facilitated by the monoubiquitination of PCNA. Deletion of REV1 also resulted in a major defect in recombination-independent ICL repair and reduced mutation formation, suggesting that Rev3 and Rev1 acts as one functional module during ICL repair.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture Conditions—REV3 mutant and wild-type MEF cells were maintained in minimal essential medium supplemented with 10% fetal calf serum. DT40 wild-type cells, DT40 REV3−/− cells, and REV1−/− cells were all cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 3% chicken serum. PCNA−/− DT40 cells complemented with human PCNA or human PCNA with the K164R substitution were similarly maintained. Western blot was performed using an anti-PCNA monoclonal antibody (PC10, Abcam) to verify the expression of wild-type and mutant human PCNA in DT40 cells with endogenous PCNA deletion.

Preparation of DNA Substrate with Defined ICLs—Preparation of site-specific psoralen- or mitomycin C (MMC)-cross-linked plasmid substrates has been described previously (3, 4). Specific to this study, the 18-mer oligonucleotide for generating psoralen-cross-linked plasmids were 5′-ggggCTAGCgggggga-3′, and 5′-ccccGTACGccccca-3′ (Uppercase letters indicate an Nhel restriction site containing the single psoralen target site Tpa). The MMC-cross-linked plasmids were prepared with the 19-mer oligonucleotides 5′-tagataCTGAATagat-3′ and 5′-tagataCTGAATagat-3′, which contains only one single CpG motif (embedded in a BspD1 recognition site (uppercase)) for the formation of a single MMC-cross-link. Denaturing PAGE analysis of the purified cross-linked oligonucleotide and cross-linked plasmid substrates used in this study is shown in supplemental Fig. S1.

Luciferase Reporter Reactivation Assay—This assay was performed essentially as described previously (3, 4). For DT40 cells, the cross-linked reporter substrates were introduced by electroporation (Amaxa Nucleofector). The FuGene 6 reagent (Roche Diagnostics) was used for transient transfections into MEF cells. A β-galactosidase reporter plasmid was included in each sample as an internal control. Each experiment was repeated at least three times and the standard deviation is provided for each data point in the form of error bars. The linear range of luciferase activity was individually determined for each cell line.

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2 The abbreviations used are: ICL, interstrand cross-link; TLS, translesion synthesis, MEF, mouse embryonic fibroblast, MMC, mitomycin C, NER, nucleotide excision repair; Pol, polymerase; PCNA, proliferating cell nuclear antigen; CMV, cytomegalovirus.
Mutation Analysis—For identification of ICL-induced mutations, psoralen- or MMC-cross-linked plasmid DNA substrates were transfected into 

RESULTS

To study ICL repair in vivo, we generated plasmid reporter substrates with a site-specific ICL placed between the CMV promoter and the initiation ATG of a luciferase reporter (4). Therefore, removal of ICLs can be monitored by expression of the luciferase gene, as the presence of the ICL constitutes an absolute block to luciferase gene transcription. Since undamaged homologous sequences were not supplied in the assay, this system measures primarily absolute block to luciferase expression as the presence of the ICL constitutes an absolute block to luciferase gene transcription. Since undamaged homologous sequences were not supplied in the assay, this system measures primarily absolute block to luciferase expression as the presence of the ICL constitutes an absolute block to luciferase expression resulting from ICL removal (25.9 and 33.4% repair efficiency, respectively) (Fig. 1A). Similarly, when psoralen- or MMC-cross-linked pCMV-Luc DNA substrates were transfected into the wild-type DT40 cell line, the ICL repair efficiency were 48 and 28%, respectively (Fig. 1B). Together, these results indicate that the recombination-independent ICL repair mechanism, first identified in human cells (3, 4), operates in both mouse and avian cells. When two independent REV3−/− MEF cell lines were tested, we found that the ICL repair efficiency was drastically decreased (1.9 and 3.0%, respectively) (Fig. 1A). This result suggested that REV3 is a critical component in recombination-independent ICL repair. To further validate this result, we analyzed a REV3−/− DT40 mutant constructed by replacement targeting. Again, REV3 deletion substantially decreased the level of recombination-independent ICL repair with both psoralen- and MMC-cross-linked plasmid substrates (Fig. 1B). Taken together, these results strongly suggest that polymerase ζ plays a major role in recombination-independent ICL repair. Next, we asked whether Rev1 is also involved in the recombination-independent ICL repair. We examined a REV1−/− DT40 deletion mutant with both psoralen- and MMC-cross-linked luciferase substrates. As shown in Fig. 1C, loss of Rev1 clearly decreased repair efficiency, suggesting that Rev1 also has an important role in recombination-independent ICL repair.

Eukaryotic mutants lacking REV3 display severe cross-link sensitivity. Interestingly, however, cells appear to maintain an extremely low level of Rev3. To date, the Rev3 protein remains undetectable by immunological means. A restricted Rev3 level can serve as a rate-limiting step for recombination-independent ICL repair. Hence, heterozygous loss of REV3 may have the potential to give rise to a haploinsufficiency phenotype. In addressing this question, we tested two REV3+−/− MEF cell lines (Fig. 1D). ICL repair efficiency in two of the REV3+−/− clones was ~50% of that in the REV3 wild-type cells, indeed suggesting a haploinsufficiency of the REV3+−/− cells in recombination-independent ICL repair. This result is consistent with the notion that the level of Polζ might be one of the limiting factors for restricting the contribution of recombination-independent repair in overall ICL removal.

ICL repair utilizing the recombination-independent mechanism depends on the dual incisions introduced by the NER excinucleases to create a gapped intermediate, which leads to the uncoupling of the double helix (Fig. 3). Normally, gaps derived from monoadduct lesions would be resynthesized by Polδ
or Polε. However, a gap intermediate derived from an ICL would have the excised oligonucleotide attached to the single-stranded region by the remaining cross-linking moiety. Because neither Polδ nor Polε possesses effective lesion bypass activity (17), gap repair synthesis would most likely be stalled by the remaining lesion. Presumably, Rev3 and/or Rev1 are required to extend repair synthesis over the remaining cross-linked nucleotide residue. However, a mechanistic link allowing Rev3 to interface with stalled NER repair synthesis is missing. We reasoned that the recruitment of Rev3 could be mediated by PCNA ubiquitination as has been shown during replicative lesion bypass as stalled replication forks (18, 19). To address this hypothesis, we constructed two DT40 cell lines with deletion of the endogenous PCNA gene but complemented by wild-type human PCNA and mutant human PCNA K164R. Parental DT40 cells was used as a control.

**FIGURE 2.** ICL repair in DT40 cells with endogenous PCNA deletion and complemented by wild-type human PCNA and mutant human PCNA K164R. Parental DT40 cells was used as a control.

ACCELERATED PUBLICATION: Polɛ-mediated Lesion Bypass in DNA Cross-link Repair

**TABLE 1**

| Cell lines and genotype | No. of clones analyzed | No. of mutations identified | Mutation frequency |
|-------------------------|------------------------|----------------------------|-------------------|
| MEF REV3+/+            | 80                     | 7                          | 8.75%             |
| MEF REV3-/-            | 56                     | 0                          | 0%                |
| DT40 REV3+/+           | 102                    | 6                          | 5.88%             |
| DT40 REV3-/-           | 68                     | 0                          | 0%                |
| DT40 Rev1+/+           | 86                     | 1                          | 1.16%             |
| DT40 hPCNA             | 79                     | 5                          | 6.33%             |
| DT40 hPCNA K164R       | 73                     | 0                          | 0%                |

The mutation rate and the nature of mutations from the DT40-hPCNA cells closely resembles that of the parental wild type DT40 cells (Table 1 and data not shown), despite a noticeable difference in their ICL repair efficiency (19.1% versus 29.7%) (Fig. 2A), presumably due to the ectopic expression of PCNA from an exogenous promoter. However, no mutated PCMV-LUC plasmid was recovered from 73 colonies derived from cells expressing PCNA-K164R. This result further strengthened the notion that monoubiquitination of PCNA at lysine 164 is important for the error-prone ICL repair. Collectively, these results offer additional support for an ICL repair model in which Rev3 and Rev1 act in concert in bypassing processed ICL repair intermediate, similar to the sequential mode of action found in the yeast system (14), and PCNA monoubiquitination enables recruitment of the TLS components.

**DISCUSSION**

Our group and others have recently demonstrated the error-prone recombination-independent ICL repair in both yeast and mammals. While the principle steps of this pathway are established, the lesion bypass components and how they engage in the lesion bypass step were not clear. The results we described provide firm answers to these questions. First, we show that Rev3 and Rev1 are the principle lesion-bypass components of this pathway. Second, initiation of the lesion bypass is controlled by monoubiquitination of PCNA at lysine 164. The latter conclusion also broadened the PCNA switch model of TLS from replicative synthesis to repair synthesis bypass.

Our result showed that cells expressing monoubiquitination-defective PCNA exhibited a markedly reduced ICL repair capacity. Monoubiquitination of PCNA at lysine 164 is induced by a wide-array of DNA damaging reagent, including cross-linking agents (18). However, its role in facilitating lesion bypass has been shown only in replicative DNA synthesis when replication forks are stalled by monoaduct lesions (19). Our present study suggests that ICL repair synthesis also relies on PCNA monoubiquitination. Given that NER polymers δ and ε both depend on PCNA and RFC for their function in NER (20), the repair synthesis machinery thus partially resembles the DNA replicative elongation complex. Combined together, it appears that the presence of stalled DNA polymerase along with RFC and monoubiquitinated PCNA may be sufficient to trigger a switching to lesion bypass polymerases. This notion is further supported by the finding that DNA template, RFC, and a properly loaded PCNA clamp is sufficient to initiate PCNA monoubiquitination (21). Our previous study with a human mutant (XP30RO) suggested that Polγ has a role in recombination-independent ICL repair (3). However, when comparing the XPV and the Rev3 mutants (3.3-fold versus 10-fold reduction in repair efficiency), it is quite clear that Rev3 loss resulted in a more severe deficit in ICL repair, suggesting that Polγ is the principle lesion bypass polymerase, while Polη may play a secondary role in mouse and avian cells.

Rev1 and Rev3 perform lesion bypass in a cooperative manner (14).
Although we have shown that PCNA monoubiquitination is critical in ICL repair, it remains to be determined whether a direct interaction between PCNA and Rev3 is required to allow its recruitment. The unavailability of a Rev3 antibody precludes the possibility of examining such an interaction in mammalian cells by immunological means. However, recent work suggests that Rev1 may be able to allocate the Rev1/Rev3 module to the stalled repair site. Rev1 was found to interact directly with PCNA (22) and to co-localize with PCNA upon DNA damage (23). Monoubiquitinated PCNA was found to directly stimulate Rev1-dependent single base addition, a step that precedes the Rev3-dependent extension (21). More recently, the Y-family DNA polymerases were found to possess ubiquitin-binding motifs (24). In Rev1, this motif localizes precisely at the region required for Rev1 interaction with PCNA (22, 24). Hence, Rev1 may function as a key mediator for monoubiquitinated PCNA to promote Rev1/Rev3-dependent TLS during ICL repair. Collectively, our results help to further complete a model (Fig. 3) of recombination-independent ICL repair in mammalian cells by revealing the critical role of Rev3/Rev1 in this process and by showing that monoubiquitinated PCNA is required for the switch to the key step of lesion bypass during repair synthesis. Further enhancing this assertion, the ICL-induced mutagenic spectrum shifted correspondingly in cells bearing Rev3, Rev1, and PCNA mutations. While this paper was at the final stage of preparation, similar findings have been reported in budding yeast system (25), indicating that the recombination-independent ICL repair is a well conserved mechanism in eukaryotes.

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