Role of DNA Polymerase β in the Excision Step of Long Patch Mammalian Base Excision Repair

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The two base excision repair (BER) subpathways in mammalian cells are characterized by the number of nucleotides synthesized into the excision patch. They are the “single-nucleotide” BER pathway and the “long patch” (several nucleotides incorporated) BER pathway. Both of these subpathways involve excision of a damaged base and/or nearby nucleotides and DNA synthesis to fill the excision gap. Whereas DNA polymerase β (pol β) is known to participate in the single-nucleotide BER pathway, the identity of polymerases involved in long patch BER has remained unclear. By analyzing products of long patch excision generated during BER of a uracil-containing DNA substrate in mammalian cell extracts we find that long patch excision depends on pol β. We show that the excision of the characteristic 5'-deoxyribose phosphate containing oligonucleotide (dRP-oligo) is deficient in extracts from pol β null cells and is rescued by addition of purified pol β. Also, pol β-neutralizing antibody inhibits release of the dRP-oligo in wild-type cell extracts, and the addition of pol β after inhibition with antibody completely restores the excision reaction. The results indicate that pol β plays an essential role in long patch BER by conducting strand displacement synthesis and controlling the size of the excised flap.

Base excision repair of uracil in DNA is initiated by uracil-DNA glycosylase, which removes uracil by cleavage of the base-sugar glycosidic bond (1). The AP site resulting from this DNA glycosylase activity is then processed by AP endonuclease, DNA polymerase β (pol β), and DNA ligase I (2, 3), resulting in the replacement of one nucleotide. Processing of the AP site can also be accomplished by a different subpathway of BER, resulting in a longer DNA repair patch of several nucleotides (4, 5). A satisfactory understanding of the enzymes participating in this “long patch” BER and the mechanisms involved has not been achieved. It was shown that long patch BER reconstituted with partially purified components depended on PCNA (4) and that long patch BER in cell extract is sensitive to PCNA antibody (5). It was suggested that DNA polymerases δ or ε are involved in the gap-filling step during long patch BER (4, 5) because these enzymes are known to be stimulated by PCNA and these polymerases are proficient in reconstituted long patch BER systems (6, 7). Alternatively, if the role of PCNA in long patch BER is limited to the stimulation of FEN1 cleavage of a flap DNA substrate (8–10), other polymerases may also be considered as participants in long patch BER. There are observations suggesting that pol β can operate during long patch BER. Both DNA polymerases β and δ can function in long patch BER reconstituted with purified proteins (7), and it was demonstrated that cell extract-mediated long patch BER is inhibited by antibody to pol β (7). Also, pol β null cell extract does not repair a reduced abasic site in a linear DNA substrate that is repaired through long patch BER in pol β-containing wild-type cell extract (11). Further, the absolute amount of long patch BER activity (substrate repaired per mg of extract protein) in the pol β null cell extract is less than that in the isogenic wild-type cell extract. Taken together, these results suggest that pol β, in addition to its role in single-nucleotide patch repair, could play an important but yet unknown role in long patch BER. In light of this background information, we directly addressed the role of pol β in the excision process of long patch BER performed by mammalian cell extracts.

MATERIALS AND METHODS

Cells and Extracts—Normal human lymphoid cell line AG9387 was obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Cells were grown in medium recommended by the supplier. The DNA pol β-knockout mouse fibroblasts and the isogenic wild type cell lines were grown as described (12). Whole cell extracts were prepared from 3–5 g of cells by the method of Manley et al. (13) and dialyzed overnight against buffer containing 25 mM Hepes-KOH, pH 7.9, 2 mM dithiothreitol, 12 mM MgCl₂, 0.1 mM EDTA, 17% glycerol, and 0.1 M KCl. Extracts were aliquoted and stored at –80 °C.

Proteins and Antibodies—Human DNA pol β was purified as described (14). Polyclonal antibodies against human pol β were raised in rabbit and were affinity-purified on a pol β-Sepharose column.

Construction of Closed Circular M13 DNA Containing a Single Uracil Residue—Double-stranded closed circular DNA containing single uracil (U-DNA) was constructed as described (15) by priming single-stranded M13 DNA with the 5'-labelled oligonucleotide "pUCGCCC-GATCACAAGCTTATTCGAGTACCG for internally labeled uracil-containing substrate and "pUCGCCCGATCAAGCTTATTCGAGTACCG for the control DNA substrate.

Excision Assay—Standard 10-μl reactions contained 50–100 ng of internally labeled U-DNA, 45 mM Hepes-KOH (pH 7.8), 70 mM KCl, 7.5 mM MgCl₂, 1 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 3.4% glycerol, 50 μM each of dGTP, dATP, dTTP, dCTP, 1 μg of 30-mer single-stranded oligonucleotide (as a carrier), and 20 μg of a cell extract protein. DNA repair synthesis reactions were carried out at 32 °C for the indicated times. After the reaction, excision products were stabilized by the addition of 0.5 mM NaBH₄ to a final concentration of 0.1 M and incubated for 30 s on ice. Then 12 μl of formamide-dye solution was added, and the reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel.

RESULTS

Excision Products Generated by Human Cell Extract—To assess the role of pol β in long patch BER of uracil-DNA

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The abbreviations used are: AP sites, apurinic/apyrimidinic sites; abasic sites; BER, base excision repair; PCNA, proliferating cell nuclear antigen; FEN1, flap endonuclease; pol β and pol δ, DNA polymerases β and δ; dRP, 5’-deoxyribose phosphate; dRP-oligo, 5’-deoxyribose phosphate oligonucleotide.

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substrate DNA with a regular C:G base pair at the same position was degraded (Fig. 1, lane 2).

Because oligonucleotides containing 5'-dRP may migrate differently in polyacrylamide gels than the oligonucleotide markers used, we engineered a marker with the same nucleotide sequence as the expected excision product and with the dRP residue at the 5'-end. First, we constructed an oligonucleotide duplex with a uracil residue in one strand 4 bases upstream of the 3'-end and with a labeled phosphate group next to it on the 5'-side. This oligonucleotide duplex was then treated with bacterial uracil-DNA glycosylase and endonuclease IV. The combined action of these enzymes releases the 5'-deoxyribose phosphate-containing oligonucleotide dRpCpGpG. This product was stabilized by reduction with NaBH4 and subjected to high resolution electrophoresis. The dRpCpGpG molecule almost co-migrated with the 3-mer marker (Fig. 1c). We conclude that during long patch BER of the U-DNA the dRP residue can be excised with at least three nucleotides located immediately 3' to the damaged base.

Excision of dRP-oligo Depends on pol β—The use of pol β-knockout mouse embryonic fibroblasts with a homozygous deletion in the pol β gene allowed us to test directly whether pol β is involved in excision steps of long patch BER. We found that wild-type mouse cell extract released dRP-oligo as a major excision product in long patch BER. This product was strongly reduced in the pol β null cell extract, and the size distribution of excision products was different (Fig. 2). To confirm that the observed reduction in dRP-oligo release was due to the absence of pol β, the purified enzyme was added to cell extract prepared from pol β null cells (Fig. 3). As little as 2 ng of pol β could reconstitute dRP-oligo release to the level observed in wild-type cell extract (Fig. 3, compare lanes 4 and 6). The addition of 4 ng of pol β further stimulated dRP-oligo excision (Fig. 3, compare lanes 5 and 6).

Additional evidence for a role of pol β in excision was obtained by using antibody specific to pol β. The addition of antibody blocked the excision of dRP-oligo and resulted in an excision product pattern characteristic for the pol β null cell extract (Fig. 4, lanes 2–4). This excision deficiency in the presence of antibody was then corrected by addition of pol β to the antibody-containing reaction (Fig. 4, lane 5), indicating that the effect of antibody was highly specific and limited to blockade of pol β function. This antibody to pol β is known to inhibit the enzyme’s DNA polymerase activity (16). Based on these experiments, we conclude that DNA repair synthesis per-
formed by pol β is required for dRP-oligo excision in long patch BER.

**DISCUSSION**

**DNA Polymerases in Base Excision Repair**—Previous studies had shown that pol β is the major DNA polymerase for the single-nucleotide BER pathway (12) whereas pol δε are thought to be involved in PCNA-dependent long patch BER (4, 5). Recent findings, however, suggest that pol β and pol δ can substitute for each other in long patch BER reconstituted with purified proteins (7). The substitution of different polymerases in BER was also confirmed by the competence of pol β null cell extracts in the in vitro repair of both natural and reduced abasic sites in closed circular DNA (11, 17). Thus, the biochemical proficiency of pol β and other polymerases in both subpathways for base excision repair has been documented. Yet, the question remained as to the DNA polymerase of choice for the long patch BER subpathway and its precise role(s) in the sequential mechanism. In this report we present data demonstrating that pol β is the major DNA polymerase involved in long patch BER in mammalian cells. Several experimental approaches used in this study support this conclusion. First, the excision step of long patch BER is dependent upon the pol β status of the cell extract: long patch excision is reduced in pol β-deficient cells but can be reconstituted by the addition of purified pol β. Second, pol β-neutralizing antibody inhibits long patch BER excision and especially release of the dRP-oligo product. These results were unexpected because pol β has not been proposed to participate in the long patch BER reaction. The striking homogeneity of the excision product size (i.e. the dRP-oligo) indicates that the proteins involved in excision may predetermine the length of the excised oligonucleotide, and pol β is a good candidate for this function. It was shown earlier that when a suitable substrate is provided, FEN1 is able to release the AP-site 5'-sugar phosphate as part of an oligonucleotide but does not favor any particular flap size (18). However, as we have demonstrated here in the presence of pol β, the excision is almost strictly limited to release of the dRP-oligo, and in pol β-deficient cell extract this particular excision product was significantly reduced.

The Role of pol β in Coordination of BER—It appears that different steps of the BER reaction are coordinated and directed by multiple interactions between the participating proteins (3). For example, after removal of the T base, from the G-T mismatch, G-T-DNA glycosylase remains bound to the AP site and must be displaced by AP endonuclease (19). Further, AP endonuclease was shown to interact with pol β, and this interaction stimulates the pol β lyase repair reaction (20). After this step in the repair process pol β appears to play a central role in determining the subpathway: removal of 5'-sugar phosphate by pol β (21, 22) and interaction with DNA ligase I (23) results in single-nucleotide BER. Alternatively, as we demonstrate here, a pol β-dependent excision reaction may switch repair to the long patch BER subpathway. In conclusion, whereas multiple pathways function in BER, pol β-dependent DNA repair operates in mammalian cells for both single-nucleotide BER and long patch BER.

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