DNA Polymerase β-mediated Long Patch Base Excision Repair

POLY(ADP-RIbose) POLYMERASE-1 STIMULATES STRAND DISPLACEMENT DNA SYNTHESIS*

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Recently, photoaffinity labeling experiments with mouse cell extracts suggested that PARP-1 functions as a surveillance protein for a stalled BER intermediate. To further understand the role of PARP-1 in BER, we examined the DNA synthesis and flap excision steps in long patch BER using a reconstituted system containing a 34-base pair BER substrate and five purified human enzymes: uracil-DNA glycosylase, apurinic/apyrimidinic endonuclease, DNA polymerase β, flap endonuclease-1 (FEN-1), and PARP-1. PARP-1 stimulates strand displacement DNA synthesis by DNA polymerase β in this system; this stimulation is dependent on the presence of FEN-1. PARP-1 and FEN-1, therefore, cooperate to activate long patch BER. The results are discussed in the context of a model for BER sub-pathway choice, illustrating a dual role for PARP-1 as a surveillance protein for a stalled BER intermediate and an activating factor for long patch BER DNA synthesis.

Base excision repair (BER)1 is a major DNA repair pathway protecting mammalian cells against single-base DNA damage by methylating and oxidizing agents among other genotox- cants. Biochemical and genetic studies indicate that BER is mediated through two sub-pathways differentiated by repair patch size and the enzymes involved (1–5). These sub-pathways are designated “single nucleotide” BER and “long patch” (2–15-nucleotide repair patch). In both sub-pathways, repair is initiated by removal of a damaged base by a DNA glycosylase and repair then proceeds through a number of sequential enzymatic steps involving excision gap formation, repair patch synthesis, and DNA ligation (6–10). Several lines of evidence suggest that some of the sequential steps may be coordinated such that the product of one reaction can be handed off to the next enzyme in the series (11). In keeping with this idea, photoaffinity cross-linking studies of BER intermediates in crude cell extracts revealed that poly(ADP-ribose) polymerase-1 (PARP-1) is a DNA nick surveillance protein that binds only weakly to BER intermediates when single nucleotide BER proceeds normally to completion. In contrast, when single nucleotide BER is stalled by a block in the excision step, PARP-1 binds strongly to the BER intermediate, along with apurinic/apyrimidinic endonuclease (APE), DNA polymerase β (β-pol), and flap endonuclease-1 (FEN-1) (12). The significance of this recognition specificity by PARP-1 was investigated here.

PARP-1 is a relatively abundant nuclear protein that is highly conserved and found in all eukaryotes, except yeast. Our recent cross-linking studies pointed to a PARP-1 role in BER (12), and biochemical and genetic studies had already implicated PARP-1 in BER (13–17). The results of Dantzer et al. (13) suggested involvement of PARP-1 in both single nucleotide BER and long patch BER sub-pathways; extract from PARP-1 knockout cells had a modest defect in single nucleotide BER and a dramatic defect in long patch BER. However, the molecular mechanism of this role in the BER sub-pathways remains unclear, as pointed out recently by Poirier and co-workers (17).

To gain a better understanding of the role of PARP-1 in BER, we reconstituted a portion of the long patch BER reaction using a uracil-containing oligonucleotide substrate and the following purified human enzymes: uracil-DNA glycosylase (UDG), APE, β-pol, FEN-1, and PARP-1. Our results show that PARP-1, along with FEN-1, stimulates β-pol strand displacement DNA synthesis of the long patch BER excision patch.

EXPERIMENTAL PROCEDURES

Materials—Synthetic oligodeoxyribonucleotides were obtained from Oligos Etc. Inc. (Willoville, OR). [γ-32P]dATP, [γ-32P]ddATP, and [α-32P]dCTP (3000 Ci/mmol) were purchased from Amersham Pharmaceuticals. Terminal deoxynucleotidyltransferase and polynucleotide kinase were from Promega (Madison, WI). Human APE, UDG, FEN-1, and PARP-1 were purified as described previously (5, 12).

β-Pol Preparations—Recombinant wild-type human β-pol and mutants K35A, K68A, K72A, and K35A/K68A/K72A were overexpressed and purified to homogeneity as described previously (18). The samples were evaluated by circular dichroism (CD) spectroscopy (Applied Photophysics PiStar-180 spectrometer (Applied Photophysics, Surrey, United Kingdom) at the Macromolecular Interactions Facility (University of North Carolina)).

DNA Substrates and 3′-End Labeling—Preparation of DNA substrates and 3′-end labeling were essentially as described previously (5). The sequence of the uracil (U) or tetrahydrofuran (F)-containing DNA substrate was as follows: 5′-CTGCCGTGATGCGCU/FGTACGGATC-3′.

UDG Treatment of DNA Substrate—Prior to assembly of the repair reaction, the uracil-containing DNA substrate (1 μM) was pre-treated with 100 nM UDG in 60 mM Hepes, pH 7.5, 20 mM KCl, and 2 mM diethiothreitol (DTT). The reaction mixture was incubated at 37 °C for 20 min.

Reconstitution of Repair Reaction—The repair reaction was assembled on ice in a reaction mixture (10 μl final volume) that contained 50 mM Hepes, pH 7.5, 20 mM KCl, 2 mM DTT, 10 mM MgCl2, 4 mM ATP, 20 μM each of dATP, dGTP, dTTP, and 2.5 μM [α-32P]dCTP (specific activity 1 × 106 cpm/μmol) and 100 nM DNA substrate. The reaction was initiated by adding APE (10 nM), wild-type β-pol or mutants of β-pol, FEN-1, PARP-1, or NAD+ (1 mM), as indicated in the figure legends (Fig. 1). Note that all reaction mixtures contained UDG to a final concentration of 10 nM. Incubation was at 37 °C for 25 min. DNA products were measured as described previously (19).
additions are indicated. Photographs of autoradiograms are shown illustrating the incorporation of [32P]dCMP into DNA.

reaction mixture was supplemented with NAD to study the stimulatory effect of PARP-1/FEN-1 was blocked when the gap was filled by PARP-1 (with or without FEN-1), and PARP-1, NAD, APE, and β-pol, as indicated. c, a 34-base pair duplex DNA containing a uracil residue (lanes 1 and 2), tetrahydrofuran (F) (lanes 3 and 4) at position 16, one-nucleotide-gapped DNA with 5'-phosphate (lanes 5 and 6), or with 3'- and 5'-hydroxyl groups at the margins of the gap (lanes 7 and 8) was pretreated with UDGS as in a. The pretreated DNA (100 nM) was then incubated with (+) or without (−) FEN-1 and/or PARP-1, APE, and β-pol, as indicated. Incubation was for 25 min at 37 °C. A schematic of each DNA is shown on top of the photograph.

**FEN-1 Excision Activity**—The DNA substrate was treated with UDG as above. The reaction mixture (10 µl final volume) was assembled on ice and contained 50 mM Hepes, pH 7.5, 20 mM KCl, 2 mM DTT, 10 mM MgCl₂, 4 mM ATP, 20 µM each of dATP, dGTP, dTTP, and dCTP, and 50 nM 32P-labeled DNA. The reaction was initiated by adding FEN-1 (20 nM), PARP-1 (50 nM), and ω-pol (2 nM), as indicated in the figure legends (Fig. 5). Note that all reaction mixtures contained APE to a final concentration of 10 nM. Incubation was at 37 °C.

**dRP Lyase Activity Assay** —Preparation of the dRP substrate and the dRP lyase assay was as described previously (19).

**RESULTS**

**PARP-1 and FEN-1 Are Strand Displacement Synthesis Factors in Long Patch BER**—We reconstituted portions of long patch BER in vitro using a 34-base pair oligonucleotide sub- strate containing a lesion (uracil) at position 16 (i.e. U-DNA in Fig. 1a) and five purified human enzymes: UDG, APE, β-pol, FEN-1, and PARP-1. The DNA products, in this system without PARP-1 and FEN-1, are described under **Materials and Methods**. The reaction conditions and product analyses are described under “Experimental Procedures.” The positions of single nucleotide (SN) BER products, long patch (LP) BER products, and dNMP adducts are indicated. Photographs of autoradiograms are shown illustrating the incorporation of [3H]dCMP into DNA. a, a 34-base pair duplex DNA containing a uracil residue at position 16 (U-DNA) was pretreated with UDG (10 nM) at 37 °C for 20 min. b, the resulting AP-DNA (100 nM) was then incubated with (+) and/or without (−) FEN-1, PARP-1, NAD, APE, and β-pol, as indicated. c, a 34-base pair duplex DNA containing a uracil residue (lanes 1 and 2), tetrahydrofuran (F) (lanes 3 and 4) at position 16, one-nucleotide-gapped DNA with 5'-phosphate (lanes 5 and 6), or with 3'- and 5'-hydroxyl groups at the margins of the gap (lanes 7 and 8) was pretreated with UDGS as in a. The pretreated DNA (100 nM) was then incubated with (+) or without (−) FEN-1 and/or PARP-1, APE, and β-pol, as indicated. Incubation was for 25 min at 37 °C. A schematic of each DNA is shown on top of the photograph.

**Role of PARP-1 in Long Patch BER**

**Characteristics of Long Patch BER Strand Displacement Synthesis by β-pol**—To further examine β-pol strand displacement DNA synthesis in BER, we first conducted quantitative measurements of the effect of PARP-1 and FEN-1 on the rate of one-nucleotide gap filling by β-pol, using a one-nucleotide gapped substrate (data not shown). The steady-state rate of one-nucleotide gap filling was found to be identical in the presence and absence of PARP-1/FEN-1 (data not shown), indicating that intrinsic DNA polymerase activity on this BER substrate was not altered by PARP-1/FEN-1. Next, we examined the influence of DNA substrate structure on product formation by β-pol (Fig. 1c). β-Pol alone in these experiments added primarily one nucleotide, with substrates corresponding to the one-nucleotide-gapped BER intermediate (Fig. 1c, lanes 1, 3, 5, and 7); little strand displacement DNA synthesis was observed, except in the case of the tetrahydrofuran-containing AP site DNA (Fig. 1c, lane 3) where modest strand displacement synthesis occurred along with abundant one-nucleotide gap filling. In contrast, when PARP-1 and FEN-1 were present, the β-pol products were longer, representing strand displacement DNA synthesis (Fig. 1c, lanes 2, 4, and 6). The stimulatory effect of PARP-1/FEN-1 was strong in each case, except with the DNA substrate lacking a 5'-hydroxyl group in the one-nucleotide gap (Fig. 1c, lanes 7 and 8), where no stimulation of strand displacement synthesis was observed. Control experiments showed that PARP-1 and FEN-1 either alone or in combination did not have DNA synthesis activity (data not shown).

**Role of the 5'-Deoxyribose Phosphate in β-pol Strand Displacement Synthesis**—In light of the results above indicating modest strand displacement synthesis by β-pol alone with the 5'-tetrahydrofuran phosphoglycerate substrate, the 5'-phosphate group is required for highest efficiency gap-filling DNA synthesis, but the 5'-phosphate has only a modest effect on the equilibrium constant (Kₐ) for DNA binding (20). An explanation for these properties has been suggested by X-ray crystallography (21). The 5'-phosphate group binding specificity is directed by dRP lyase active site residues in the amino-terminal 8-kDa domain of β-pol (19). Thus, the crystal structure of a complex with a one-nucleotide-gapped substrate indicated that the 5'-phosphate is bound by three lysine residues, Lys-35, Lys-68, and Lys-72 (21), and the functional significance of these residues...
was confirmed by site-directed mutagenesis (19). The K35A mutant was found to have lower 5'-phosphate recognition activity than the wild-type 8-kDa domain, when studied with a longer gapped DNA molecule (five nucleotides). A triple mutant (K35A/K68A/K72A) in the isolated 8-kDa domain was dRP lyase-deficient and also failed to discriminate between phosphorilated and unphosphorilated forms of gapped DNA substrates (19). Using site-directed mutagenesis and peptide chemistry, Lys-72 was identified as the Schiff base nucleophile (20).

Based upon this information, we entertained several ideas as to how PARP-1/FEN-1 might stimulate strand displacement synthesis by β-pol after one-nucleotide gap filling: (i) PARP-1/FEN-1 may compete for binding to the 5'-dRP moiety at the gap margin, and as a result the β-pol could be altered in its contacts with the downstream oligonucleotide relieving a constraint to strand displacement synthesis; (ii) PARP-1/FEN-1 may inhibit the dRP lyase, and persistence of the 5'-dRP-containing intermediate may influence conformational features that provoke strand displacement DNA synthesis; and (iii) interactions among β-pol, PARP-1, and FEN-1 may influence the DNA so as to relieve a constraint of β-pol strand displacement synthesis. To explore these ideas, we prepared site-directed mutants in intact β-pol of residues in the dRP lyase active site. Lys-35, Lys-68, Lys-72 were changed to alanine either singly or in combination with each other. These mutant β-pol were purified, characterized for folding properties by CD analysis (Fig. 2c), and finally examined for dRP lyase activity (Fig. 2b) and strand displacement DNA synthesis (Fig. 2d). The CD spectra of these alanine mutants were similar to that of the wild-type enzyme, as illustrated in Fig. 2c. First, alanine substitution at Lys-35 had no effect on dRP lyase activity, and near wild-type strand displacement DNA synthesis was observed in the presence of PARP-1/FEN-1 (data not shown). In contrast, the K72A mutant and the K35A/K68A/K72A triple mutant, which have lost most of the dRP lyase activity (Fig. 2b, lanes 3 and 4), showed strand displacement synthesis without addition of PARP-1/FEN-1 (Fig. 2d, lanes 2, 4, and 6). These results indicate that the presence of the 5'-dRP group can trigger strand displacement DNA synthesis by β-pol. Next, we examined whether these two β-pol mutants could respond to PARP-1/FEN-1 stimulation of strand displacement synthesis. The results showed that strand displacement synthesis could be stimulated by the presence of PARP-1/FEN-1 (Fig. 2d, compare lanes 3 and 5 with lanes 4 and 6, respectively). Since, these mutants responded to PARP-1/FEN-1 stimulation of strand displacement synthesis (Fig. 2d, compare lane 2 with lanes 4 and 6), the presence of the 5'-dRP group does not seem to fully explain the PARP-1/FEN-1 effect. Nevertheless, since similar results were obtained with the persistent 5'-dRP group or with PARP-1/FEN-1, strand displacement synthesis in these two cases may be mediated through a common molecular mechanism. The results indicated that (i) the 5'-phosphate and the 5'-dRP is required for β-pol strand displacement synthesis and (ii) the persistence of the dRP group can switch β-pol from one-nucleotide gap filling to strand displacement DNA synthesis.

**Effect of PARP-1 on dRP Lyase and Flap Excision in Long Patch BER**—To further examine strand displacement DNA synthesis by β-pol and to pinpoint step(s) in the BER reaction sequence that may be influenced by PARP-1/FEN-1, we examined both the dRP excision activity of β-pol and the flap excision activity of FEN-1 under reaction conditions similar to those used in the reconstituted BER reaction shown in Fig. 1. The results showed that the dRP lyase activity of β-pol was not influenced by FEN-1 or PARP-1, or when PARP-1 and FEN-1 were added together (data not shown).

Next, the influence of PARP-1 and β-pol on FEN-1 excision activity was examined using a four-nucleotide overhang-containing DNA substrate. With FEN-1 alone, there was an increase in flap excision with time, corresponding to a rate of 10 nM concentration of the substrate consumed per min (Fig. 3, b and c). Addition of either PARP-1 or β-pol to the reaction mixture stimulated the excision activity of FEN-1 by 7- and 4-fold, respectively. When PARP-1 and β-pol were added together, the excision activity of FEN-1 was ~10-fold higher than with FEN-1 alone (Fig. 3c). Both β-pol and PARP-1 alone failed to show any FEN-1-like activity (Fig. 3b, lanes 1 and 10). Taken together, these results suggest a functional interaction between PARP-1, β-pol, and FEN-1 in two of the key steps of long
patch BER: strand displacement synthesis to produce the repair patch and 5'-flap cleavage to release the flap.

**DISCUSSION**

The *in Vitro System for Long Patch BER*—To gain a better understanding of the involvement of PARP-1 in BER, we have now used two approaches. First, we applied a new photocross-linking technique to probe for crude extract proteins capable of binding to BER intermediates (12). We found that PARP-1 was cross-linked abundantly to a BER intermediate with a reduced sugar in the AP site (12). These results suggested to us that PARP-1 may be involved in triggering long patch BER by recognizing a stalled single nucleotide BER intermediate. Second, in the present study, we reconstituted a partial long patch BER reaction system and delineated a step where PARP-1 appears to be involved in long patch BER. PARP-1 stimulates strand displacement DNA synthesis of β-pol acting on the stalled single nucleotide BER intermediate substrate, but this occurred only in the presence of FEN-1, indicating a functional interaction between these two enzymes for strand displacement synthesis. The stimulation of strand displacement synthesis by β-pol was reversed by the FPARP-1 substrate NAD⁺.

Sub-pathways in BER—It is clear that β-pol is involved in both single nucleotide BER and long patch BER and that both processes can occur simultaneously in the mouse fibroblast cell extract. Yet, if the 5'-dRP group in the gap is not processed, single nucleotide BER will be blocked, and β-pol in cooperation with PARP-1/FEN-1 will conduct strand displacement synthesis. The dRP group appears to be critical in facilitating strand displacement synthesis by β-pol. Therefore, the contribution of each BER sub-pathway will depend on processing of 5'-dRP group and the abundance of PARP-1/FEN-1. This notion is supported by our observation that the dRP lyase minus mutants of β-pol or the reduced sugar-containing AP site-DNA substrate could support strand displacement synthesis especially in the presence of PARP-1/FEN-1. The dRP group may serve as a sensor for recruitment of PARP-1 onto the BER intermediate, and PARP-1 may activate long patch BER by recruiting other long patch BER proteins. Finally, the role of PARP-1 in long patch BER also appears to be strongly regulated by the PARP substrate NAD⁺, since addition of NAD⁺ to the *in vitro* long patch BER system used here completely blocked the PARP-1 effect on strand displacement DNA synthesis. It has long been proposed (e.g., Refs. 23, 24) that fluctuating NAD⁺ and ATP levels are associated with PARP-1 activation as a function of cellular DNA damage. Fluctuation of the NAD⁺ level could be an important regulating factor in BER sub-pathway choice, and further experiments will be required to gain insight into the possible regulatory role.

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