Complementation of aprataxin deficiency by base excision repair enzymes

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ABSTRACT

Abortive ligation during base excision repair (BER) leads to blocked repair intermediates containing a 5′-adenylated-deoxyribose phosphate (5′-AMP-dRP) group. Aprataxin (APTX) is able to remove the AMP group allowing repair to proceed. Earlier results had indicated that purified DNA polymerase β (pol β) removes the entire 5′-AMP-dRP group through its lyase activity and flap endonuclease 1 (FEN1) excises the 5′-AMP-dRP group along with one or two nucleotides. Here, using cell extracts from APTX-deficient cell lines, human Ataxia with Oculomotor Apraxia Type 1 (AOA1) and DT40 chicken B cell, we found that pol β and FEN1 enzymatic activities were prominent and strong enough to complement APTX deficiency. In addition, pol β, APTX and FEN1 coordinate with each other in processing of the 5′-adenylated dRP-containing BER intermediate. Finally, other DNA polymerases and a repair factor with dRP lyase activity (pol λ, pol ι, pol θ and Ku70) were found to remove the 5′-adenylated-dRP group from the BER intermediate. However, the activities of these enzymes were weak compared with those of pol β and FEN1.

INTRODUCTION

Mammalian base excision repair (BER) is completed by two distinct subpathways, short patch or single-nucleotide (SN) and long patch (LP) BER, depending on how the 5′-deoxyribose phosphate (5′-dRP) group is excised (1). In SN BER, excision of the group is catalyzed by the lyase activity of DNA polymerase β (pol β) resulting a SN gapped intermediate with 3′-OH and 5′-phosphate groups at the margins of the gap (2). If the dRP group is modified, BER then switches to an alternate LP BER subpathway involving excision by flap endonuclease 1 (FEN1) and replacement of two to several nucleotides ahead of the site of base damage (3). The steps in both BER subpathways are processed sequentially with the product of each step acting as the substrate for the next step. Coordination between the BER en-

zymes may minimize release of the repair intermediates (4–6). DNA ligases can fail during the last ligation step of BER that results in covalent attachment of adenosine monophosphate (AMP) onto the 5′-phosphate terminus of the ligation substrate (7). One important example of this is the failed ligation reaction during the last step in BER, when the BER intermediate is passed to ligase prior to removal of the 5′-dRP group. This results in formation of a blocked BER intermediate containing a 5′-adenylated-deoxyribose phosphate (5′-AMP-dRP) group (8). Aprataxin (APTX), a member of the histidine triad (HIT) superfamily, removes the 5′-AMP moiety from this abortive ligation product, allowing further attempts at completing repair. Purified samples of the BER enzymes pol β and FEN1 were found to be capable of removing the entire 5′-AMP-dRP group from BER intermediate. Their enzymatic rates were similar to each other and comparable to that of APTX (8). The results suggest that these BER enzymes could process blocked BER intermediates in APTX-deficient cells.

Mutations in the *aptx* gene cause the autosomal recessive neurodegenerative disease known as Ataxia with Oculomotor Apraxia Type 1 (AOA1) (9). APTX is considered a BER factor interacting with several BER proteins, including XRCC1, PARP-1 and APE1 (10,11). However, the function of APTX in the context of cellular DNA repair has remained elusive. For example, some studies have revealed differences in the repair rates of normal and APTX-deficient cells, but a slower rate of repair in APTX-deficient cells was not confirmed by others (12–15). In the *Saccharomyces cerevisae* model system, deletion of the *aptx* (*hnt3*) gene fails to alter cell sensitivity to DNA lesion-inducing agents, such as methyl methanesulfonate (8,16), yet it is likely that abortive ligation occurs during BER. These results suggested the presence of alternate mechanisms for repairing the 5′-adenylated BER intermediates in the absence of APTX. The potential roles for the BER enzymes pol β and FEN1 was recently revealed in the study with purified enzymes (8), however, the question of whether the expression levels and activities of these enzymes are sufficient to perform a complementation role in APTX-deficient cells is unknown.

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In the present study, we evaluated BER enzymes for complementation of a deficiency in APTX activity for processing of the 5′-AMP and 5′-adenylayed-dRP-containing abortive ligation products. Pol β and FEN1 enzymatic activities were investigated in different cell types using whole cell extracts prepared from AOA1 human lymphocytes, APTX null DT40 cells and pol β null and pol λ-deficient mouse embryonic fibroblasts (MEFs). The results revealed that pol β and FEN1 activities in the extracts were strong enough to complement APTX deficiency. In addition, pol λ could function in the case of pol β deficiency with 5- to 10-fold slower 5′-AMP-dRP removal rate than that of pol β. Other purified enzymes with 5′-dRP lyase activity, such as pol θ, pol ι and Ku70 could remove 5′-AMP-dRP group, but had minimal activity.

MATERIALS AND METHODS

Materials

Oligodeoxyribonucleotides with a 6-carboxyfluorescein (FAM) label at the 3′-end were from the Midland Certified Reagent Co. Recombinant wild-type human pol β, human FEN1 and uracil-DNA glycosylase (UDG) with 84 amino acids deleted from the amino-terminus, and recombinant wild-type human pol λ and pol θ were purified as described (8,17,18). Recombinant human APTX and Ku70 were purchased from Fitzgerald and Enzymax, respectively. The 5′-DNA adenylation kit and ethylenediaminetetraacetic acid (EDTA) free protease inhibitor tablets were from Sigma-Aldrich and Roche Applied Science, respectively. Rabbit polyclonal anti-FEN1 (ab17993) and mouse monoclonal anti-pol β (ab3181) antibodies were from Abcam. The secondary antibodies, goat anti-mouse immunoglobulin G (IgG) (H+L)-horseradish peroxidase (HRP) conjugate and goat anti-rabbit IgG (H+L)-HRP conjugate, were from Bio-Rad. SuperSignal West Pico Chemiluminescent substrate and Restore Western Blot Stripping Buffer were from Pierce Biotechnology Inc. Protein A-sepharose CL-4B and protein G-agarose were from GE Heathcare and Sigma-Aldrich, respectively.

Cell lines and growth conditions

The human cell line derived from the peripheral blood of a Japanese AOA1 patient used in this study has a point mutation within the HIT domain of APTX involving substitution of proline for leucine at position 206 (L938:P206L/P206L). This mutation inactivates APTX, is associated with early onset of AOA1, and is the most frequent cause of AOA1 in Japan (19). Human AOA1 (L938) and the wild-type (C2ABR) cell lines were previously described (10). These cell lines were maintained at 37°C in RPMI 1640 medium from Gibco containing 10% fetal bovine serum (HyClone). The APTX null DT40 cell line has the *aptx* gene deletion from valine 78 onward. This results in lack of the catalytic HIT domain, a part of the N-terminal FHA domain, and the C-terminal zinc finger domain of APTX (20). Wild-type and APTX null DT40 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% chicken serum (Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 2 mM L-glutamine

(Gibco) in a 5% CO_2 incubator at 39.5°C. Pol $\beta^{-/-}$ and pol $\beta^{+/+}$ MEF cell lines were previously developed in our laboratory and described (21). Similarly, pol $\lambda^{-/-}$ and pol $\beta^{-/-}$ pol $\lambda^{-/-}$ MEF cell lines were developed in our laboratory and described (22).

Preparation of DNA substrates

DNA substrates used in this study were reported previously (8). The gapped and nicked DNA substrates with 5′-AMP group at the 3′-end of FAM-labeled oligonucleotides were prepared as described previously (8). Illustrations of the DNA substrates used in this study are provided in Scheme 1.

The enzymatic assays using purified proteins

The dRP lyase assays and single-turnover kinetic measurements with purified enzymes pol β, pol λ, pol θ, pol ι and Ku70 were performed as reported previously (8). For the reactions including pol β and FEN1, pol β and APTX, or APTX and FEN1 enzyme combinations, the protein mixture was preincubated at 37°C for 3 min, and the reaction was initiated by addition of UDG-pretreated DNA substrate (100 nM) to the mixture (10 μl final volume) that contained 50 mM Hepes, pH 7.5, 20 mM KCl, 0.5 mM EDTA and 2 mM DTT. The reaction mixture including FEN1 contained $MgCl_2$ at a final concentration of 10 mM. The incubation was continued at 37°C for 15 min and the reaction products were stabilized by addition of freshly prepared 1 M $NaBH_4$ to a final concentration of 100 mM. The reaction products were then mixed with gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol) and separated by electrophoresis in a 15% polyacrylamide gel as described previously (8).

Preparation of cell extracts

Cell extracts were prepared as described (23). Cell lines used in this study were AOA1 (L938) and wild-type (C2ABR) human cell lines, pol $\beta^{+/+}$, pol $\beta^{-/-}$, pol $\lambda^{-/-}$ and pol $\beta^{-/-}$ pol $\lambda^{-/-}$ MEFs and wild-type and APTX null chicken DT40 cell lines. Briefly, cell pellets were thawed by resuspension in buffer containing 10 mM Tris–HCl, pH 7.8, 200 mM KCl, 1 mM EDTA, 25 mM NaF, 20% glycerol, 0.1% NP-40, 1 mM DTT and protease inhibitors. The suspension was rotated for 1 h at 4°C and extracts were clarified by centrifugation in a microcentrifuge at full speed for 15 min at 4°C. The protein concentration of the extract was determined by the Bio-Rad assay.

Enzymatic assays in cell extracts

The dRP lyase, FEN1 excision and APTX deadenylation assays for reference reactions were performed as described previously (8). For reaction mixtures using cell extracts and the gapped 5′-AMP-dRP DNA substrate, the reaction was initiated by addition of cell extract (50 μg) and stopped at the indicated time points by the addition of freshly prepared NaBH4 to a final concentration of 100 mM. The incubation was then continued for 30 min on ice. For reaction mixtures
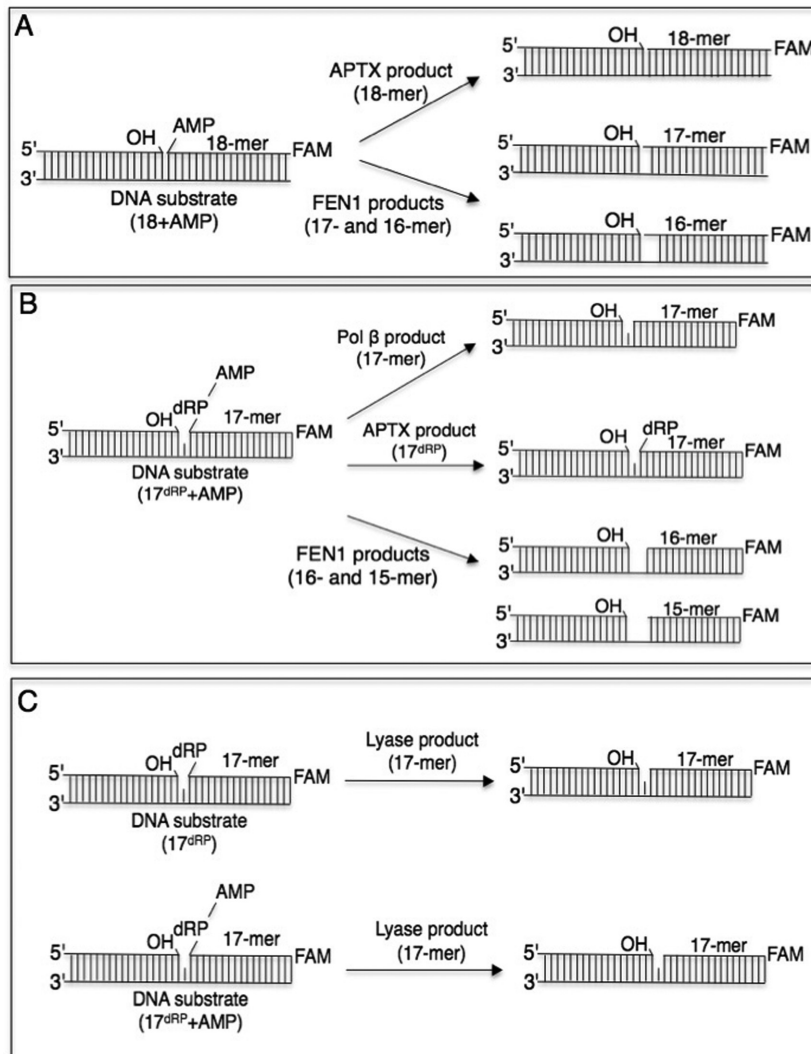
Scheme 1. Representations of DNA substrates and reaction products used in this study. (**A**) Illustration of the nicked 5′-AMP-containing DNA substrate (18+AMP) and the reaction products after 5′-AMP removal by APTX (18-mer) and one or two nucleotide excision by FEN1 (17- or 16-mer). (**B**) Illustration of the gapped 5′-AMP-dRP-containing DNA substrate (17^{dRP}+AMP) and the reaction products after 5′-AMP removal by APTX (17^{dRP}), 5′-AMP-dRP removal by pol β (17-mer), and one or two nucleotide excision by FEN1 (16- or 15-mer). (**C**) Illustration of the gapped 5′-dRP (17^{dRP}) and 5′-AMP-dRP (17^{dRP}+AMP)-containing DNA substrates and reaction products after 5′-dRP or 5′-AMP-dRP removal by dRP lyase activity (17-mer). In panels A, B and C, FAM indicates the presence of a fluorescent tag and the dRP represents a 5′-dRP group. The DNA substrates containing a dRP group were formed after UDG treatment of the double-stranded DNA substrates.

using cell extracts and the nicked 5′-AMP or the gapped 5′-AMP-THF DNA substrates, the reaction was initiated as above and stopped at the indicated time points by mixing with gel loading buffer. Reaction products were analyzed as described above.

Immunoblotting

Immunoblotting analysis was conducted as described (24). Cell extracts (50 μg) were loaded onto a 4–12% Bis–Tris NuPAGE gel, separated by electrophoresis in NuPAGE MES running buffer, and then proteins were transferred onto a nitrocellulose membrane at 25V overnight. After transfer, the membrane was blocked overnight in 5% non-fat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST). The membrane was first probed with

rabbit polyclonal antibody against FEN1 in 5% nonfat dry milk in TBST overnight at 4°C. After washing with TBST, the membrane was further incubated with secondary anti-rabbit IgG antibody at room temperature and visualized by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent substrate. The membrane was stripped for 10 min at 37°C and then 30 min at room temperature by incubation in Restore Western Blot Stripping Buffer. After washing with TBST three times, the membrane was blocked in 5% nonfat dry milk in TBST overnight at 4°C. The pol β antibody was similarly subjected to immunodetection.

Immunodepletion of FEN1

FEN1 protein was depleted by immunodepletion as reported (25). Briefly, protein A-sepharose and protein G-

agarose bead suspensions (100 μl each) were washed five times with phosphate-buffered saline (PBS) and resuspended in 200 μl PBS. FEN1 affinity-purified polyclonal antibody and pre-immune serum (20 μl each), respectively, were added to 100 μl protein A/G suspension and the mixture was incubated for 2 h at 4°C with gentle shaking. Anti-FEN1/sepharose or pre-immune/sepharose suspensions were washed five times with 0.5 ml PBS and resuspended in 200 μl PBS. Cell extracts from wild-type and APTX null DT40 cell lines (200 μg for each cell extract) were mixed with 100 μl anti-FEN1/sepharose or pre-immune/sepharose suspensions and incubated for 2 h at 4°C with gentle shaking. The mixture was then centrifuged and the supernatant fraction was collected and then directly used in both enzymatic activity assays and immunoblotting as described above.

RESULTS

Processing of the 5′-AMP-containing abortive ligation product by cell extracts

APTX is known to remove 5′-AMP from the abortive ligation product adenylated at the 5′-phosphate. FEN1 also removes 5′-AMP together with one or two nucleotides via its endonuclease activity. In contrast, pol β does not function on this adenylated 5′-phosphate-containing abortive ligation product (8).

Here, we first confirmed a deficiency in 5′-AMP removal using cell extracts from APTX-deficient AOA1 and APTX null DT40 cell lines. In reference reactions using purified enzymes, products of APTX and FEN1 activities, as described above, were evaluated for comparison (Figure 1A and D). Analysis of 5′-AMP removal in cell extract from the AOA1 cell line revealed a deficiency, as expected, but a detectable level of activity was observed (Figure 1C). Similar results were obtained using extract from APTX null DT40 cells (Figure 1F). In cell extracts from wild-type cell lines (Figure 1B and E), FEN1 excision of 5′-AMP plus one or two nucleotides, as well as strong APTX removal of the 5′-AMP group, were observed. In contrast, FEN1 excision products were predominant in extracts from APTX-deficient AOA1 (Figure 1C) and APTX null DT40 (Figure 1F) cell lines. These results demonstrated a capacity of FEN1 to complement APTX deficiency (Supplementary Figure S1A and B). Finally, we confirmed that FEN1 expression level was unaffected by APTX deficiency in these cell lines (Supplementary Figure S2A and B).

Processing of the 5′-adenylated-dRP-containing BER intermediate by cell extracts

It is known that the 5′-adenylated-dRP-containing BER intermediate in SN gapped DNA can be processed by purified APTX, pol β or FEN1 (8). APTX removes only the 5′-AMP moiety, pol β removes the entire 5′-adenylated-dRP group via its lyase activity and FEN1 removes the 5′-adenylated-dRP group along with one or two nucleotides. In the present study, we investigated processing of the 5′-adenylated-dRP-containing BER intermediate in whole cell extracts from APTX-deficient AOA1 and APTX null DT40 cell lines.

In reference reactions using purified enzymes, products of pol β, APTX and FEN1 activities, as described above, were evaluated for comparison (Figure 2A and D). Using cell extracts from wild-type cell lines, products similar to those in the reference reactions were observed (Figure 2B and E). Using extracts from APTX-deficient AOA1 and APTX null DT40 cell lines, results indicating 5′-AMP-dRP removal by pol β were seen together with a weak residual APTX-like removal of 5′-AMP (Figure 2C and F). FEN1 removal of 5′-AMP-dRP plus one nucleotide was observed along with a weak band corresponding to 5′-AMP-dRP plus two nucleotides in cell extracts from the AOA1 cell line (Figure 2C), but this two nucleotide FEN1 excision product was much stronger in extract from the APTX null DT40 cell line (Figure 2F). These results confirm that pol β and FEN1 provide alternative reactions for removing the blocking 5′-AMP-dRP group and their activities in the cell extracts were strong enough to complement the deficiency in APTX activity (Supplementary Figure S1C and D). Finally, we confirmed that pol β expression level was also unaffected by the APTX deficiency in these cell lines (Supplementary Figure S2A and B).

Next, in control experiments, enzymatic activities in cell extracts from DT40 cell lines were investigated using a DNA substrate with the abasic site analog tetrahydrofuran (THF). THF fails to act as a substrate for the pol β lyase reaction because it lacks a C1′ carbon with aldehydic character required in the lyase reaction (2). With the gapped 5′-AMP-dRP substrate, results similar to those described above (Figure 2D–F) were obtained in reference reactions (Supplementary Figure S3A) and those using cell extracts from wild-type and APTX null DT40 cell lines (Supplementary Figure S3B). With the gapped 5′-AMP-THF substrate, products for APTX 5′-AMP removal and FEN1 excision were observed in reference reactions (Supplementary Figure S3C) and using cell extract from the wild-type DT40 cell line (Supplementary Figure S3D, lines 19–23). In cell extracts from the APTX null DT40 cell line, FEN1 products were observed plus a clear band of residual APTX-like removal of 5′-AMP (Supplementary Figure S3D, lines 24–28). These results confirmed roles of pol β lyase and FEN1 activities in processing of the 5′-adenylated BER intermediates in cell extracts.

Fate of the 5′-adenylated-dRP-containing BER intermediate by cell extracts from pol β null cells

In order to further understand the fate of abortive ligation products involving the abasic site BER intermediate, we next investigated 5′-AMP-dRP removal using cell extracts from isogenic pol β$^{+/+}$ and pol β$^{-/-}$ MEFs. Similar results to those described above (Figure 2A and D) were obtained in reference reactions (Figure 3A) and using cell extract from pol β$^{+/+}$ MEFs (Figure 3C) as described above (Figure 2B and E). However, as expected, we failed to observe the normal level of pol β lyase product using cell extracts from pol β$^{-/-}$ MEFs. Note that the lyase product was not completely absent in cell extract from pol β$^{-/-}$ MEFs (Figure 3B). FEN1 excision activity was similar in cell extracts from both of these cell lines (Figure 3B and C). We also confirmed that FEN1 protein expression level in these
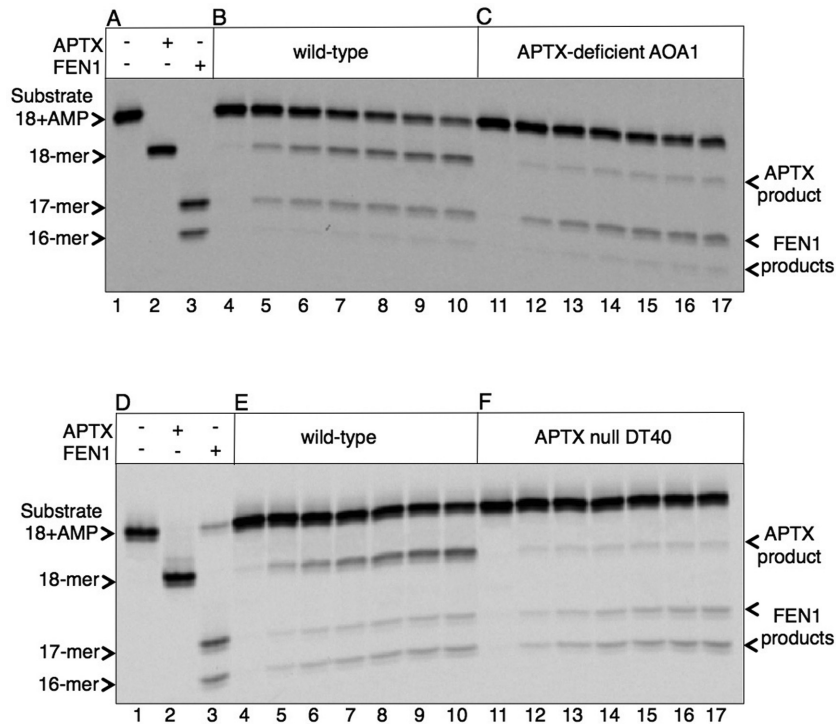
Figure 1. Processing of the 5′-AMP-containing abortive ligation product by cell extracts. (**A, D**) Lane 1 is a minus enzyme control, lanes 2 and 3 are reference reaction products of purified enzymes APTX and FEN1, respectively. (**B, C**) Reaction products observed in cell extracts from wild-type (lanes 4–10) and APTX-deficient AOA1 (lanes 11–17) human cell lines. (**E, F**) Reaction products observed in cell extracts from wild-type (lanes 4–10) and APTX null (lanes 11–17) DT40 cell lines. Lanes 4–10 and 11–17 correspond to time points 1, 5, 10, 15, 20, 25, 30 min, respectively. Illustrations of the nicked 5′-AMP-containing DNA substrate and reaction products are shown in Scheme 1A. The quantifications for relative contribution of APTX and FEN1 activities in cell extracts were provided in Supplementary Figure S1A and B.
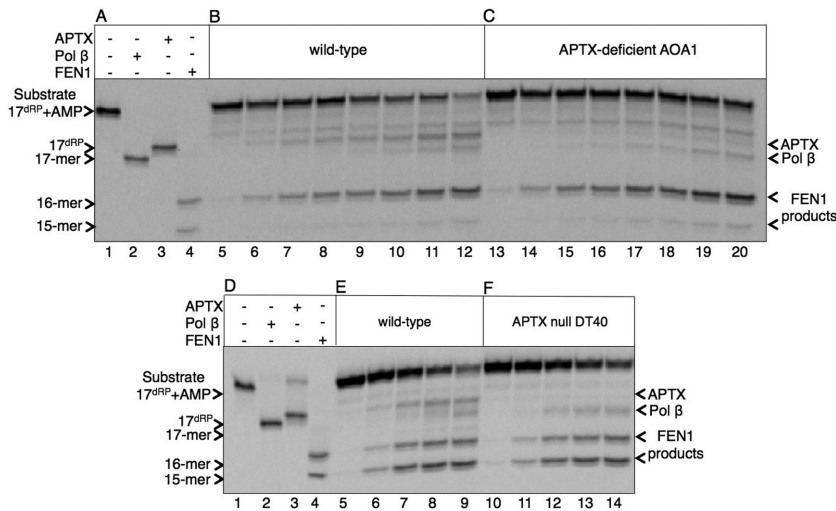

Figure 2. Processing of the 5′-adenylated-dRP-containing BER intermediate by cell extracts. (**A, D**) Lane 1 is a minus enzyme control, lanes 2, 3 and 4 are reference reaction products of purified enzymes pol β, APTX and FEN1, respectively. (**B, C, E, F**) Reaction products observed in cell extracts from wild-type human (lanes 5–12, panel B), and DT40 (lanes 5–9, panel E) cell lines and from APTX-deficient AOA1 (lanes 13–20, panel C), and APTX null DT40 (lanes 10–14, panel F) cell lines. Lanes 5–12 and 13–20 correspond to time points 1, 5, 8, 10, 15, 20, 25, 30 min, respectively. Lanes 5–9 and 10–14 correspond to time points 1, 5, 10, 15, 30 min, respectively. Illustrations of the gapped 5′-AMP-dRP-containing DNA substrate and reaction products are shown in Scheme 1B. The quantifications for relative contribution of APTX, pol β and FEN1 activities in cell extracts were provided in Supplementary Figure S1C and D.
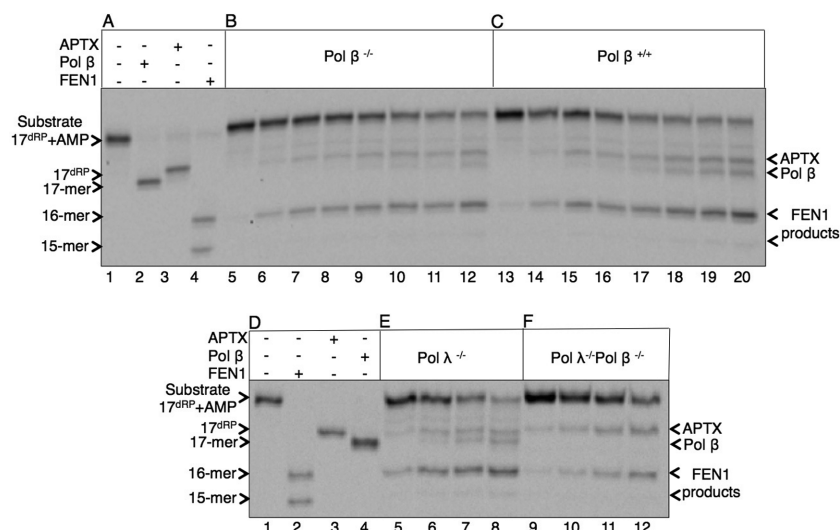
Figure 3. Processing of the 5′-adenylated-dRP-containing BER intermediate by cell extracts from MEFs. (**A**) Lane 1 is a minus enzyme control, and lanes 2, 3 and 4 are reference reaction products of purified enzymes pol β, APTX and FEN1, respectively. (**B**, **C**) Reaction products observed in cell extracts from pol β$^{-/-}$ (lanes 5–12) and pol β$^{+/+}$ (lanes 13–20) MEFs. (**D**) Lane 1 is a minus enzyme control, and lanes 2, 3 and 4 are reference reaction products of purified enzymes FEN1, APTX and pol β, respectively. (**E**, **F**) Reaction products observed in cell extracts from pol λ$^{-/-}$ (lanes 5–8) and pol λ$^{-/-}$ pol β$^{-/-}$ (lanes 9–12) MEFs. Lanes 5–12 and 13–20 correspond to time points 1, 5, 8, 10, 15, 20, 25, 30 min, respectively. Lanes 5–8 and 9–12 correspond to time points 5, 10, 20, 30 min, respectively. Illustrations of the gapped 5′-AMP-dRP-containing DNA substrate and reaction products are shown in Scheme 1B.

wild-type and pol β$^{-/-}$ MEFs was unaffected by the pol β deficiency (Supplementary Figure S2C).

The weak 'residual' lyase activity observed in cell extracts from pol β null cells could be due to several other enzymes with known lyase activity. To test this possibility, we investigated 5′-adenylated-dRP removal using cell extracts from pol λ$^{-/-}$ and pol λ$^{-/-}$ pol β$^{-/-}$ double knockout MEFs. We obtained similar results in reference reactions (Figure 3D) to those described above (Figure 3A). The 'residual' lyase activity was not observed in cell extracts from the double knockout cell line (Figure 3F), but the expected level of pol β activity and FEN1 activity was observed in cell extract from pol λ$^{-/-}$ MEFs (Figure 3E). These results indicated the 'residual' lyase activity in the absence of pol β was due to pol λ.

Fate of the 5′-adenylated-dRP-containing BER intermediate by cell extracts from FEN1-immunodepleted cells

The results described so far revealed robust activity of FEN1 in processing of the 5′-adenylated-dRP-containing BER intermediate. In some cases, FEN1 activity was stronger than pol β dRP lyase activity (c.f., Figures 2 and 3). To further confirm the pol β lyase effect, FEN1 was depleted in cell extracts from DT40 cell lines by immunodepletion using an affinity-purified FEN1 antibody and pre-immunue IgG as a control. With a gapped 5′-AMP-dRP substrate, the usual products to those described above (Figure 2D) were observed in reference reactions (Figure 4A). Using cell extracts from the wild-type and APTX null DT40 cells treated with pre-immunue IgG (Figure 4B), similar products were obtained as described above (Figure 2E and F). Using cell extracts from FEN1-immunodepleted wild-type DT40 cells, strong pol β and APTX products were observed (Figure 4C, lanes 15–19), whereas pol β lyase was

the predominant activity able to remove the entire 5′-AMP-dRP group in the case of both APTX and FEN1 deficiency (Figure 4C, lanes 20–24). Finally, we confirmed that FEN1 immunodepletion did not affect the pol β level in these cell lines (Supplementary Figure S2D).

We also investigated APTX and FEN1 activities in these cell extracts using the nicked 5′-AMP-containing DNA substrate (Supplementary Figure S4). Similar results in reference reactions were obtained (Supplementary Figure S4A) to those described above (Figure 1D). Using cell extracts from wild-type and APTX null DT40 cells treated with pre-immune IgG (Supplementary Figure S4B), similar APTX and FEN1 products were obtained as described above (Figure 1E and F). Using cell extracts from FEN1-immunodepleted wild-type DT40 cells (Supplementary Figure S4C, lines 14–18), no reduction in 5′-AMP removal was observed, but a reduction in FEN1 products was observed, as expected. Using cell extracts from FEN1-immunodepleted APTX null DT40 cells (Supplementary Figure S4C, lines 19–23), only weak deadenylation activity was observed.

Coordination between FEN1, APTX and pol β for processing of 5′-adenylated-dRP-containing BER intermediate

We investigated the effect of pol β and APTX on FEN1 activity, as well as the effect of APTX on the pol β lyase activity against the 5′-adenylated-dRP-containing BER intermediate.

We first ascertained the effect of the pol β on FEN1 excision activity against the gapped 5′-AMP-dRP- and the gapped 5′-AMP-THF-containing DNA substrates (Figure 5). Using the gapped 5′-AMP-dRP substrate, FEN1 activity was slighty increased as a function of adding pol β, but this analysis was complicated by the pol β lyase ac-
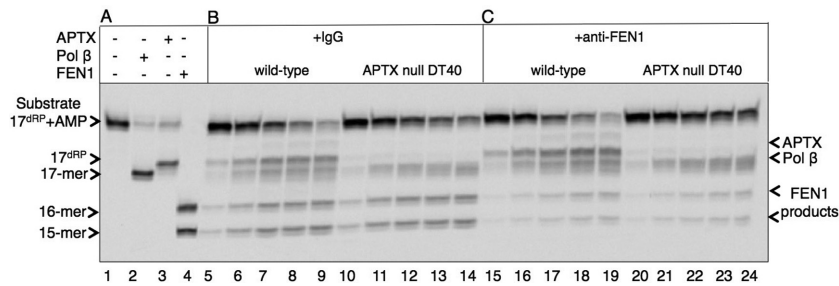
Figure 4. Effect of FEN1 immunodepletion on processing of the 5′-adenylated-dRP-containing BER intermediate by cell extracts. (**A**) Lane 1 is a minus enzyme control, and lanes 2, 3 and 4 are reference reaction products of purified enzymes pol β, APTX and FEN1, respectively. (**B**) Reaction products observed in cell extracts from wild-type (lanes 5–9) and APTX null (lanes 10–14) DT40 cells treated with pre-immunue IgG. (**C**) Reaction products observed in cell extracts from FEN1-immunodepleted wild-type (lanes 15–19) and APTX null (lanes 20–24) DT40 cells. Lanes 5–9, 10–14, 15–19 and 20–24 correspond to time points 1, 5, 10, 15, 30 min. Illustrations of the gapped 5′-AMP-dRP-containing DNA substrate and reaction products are shown in Scheme 1B.
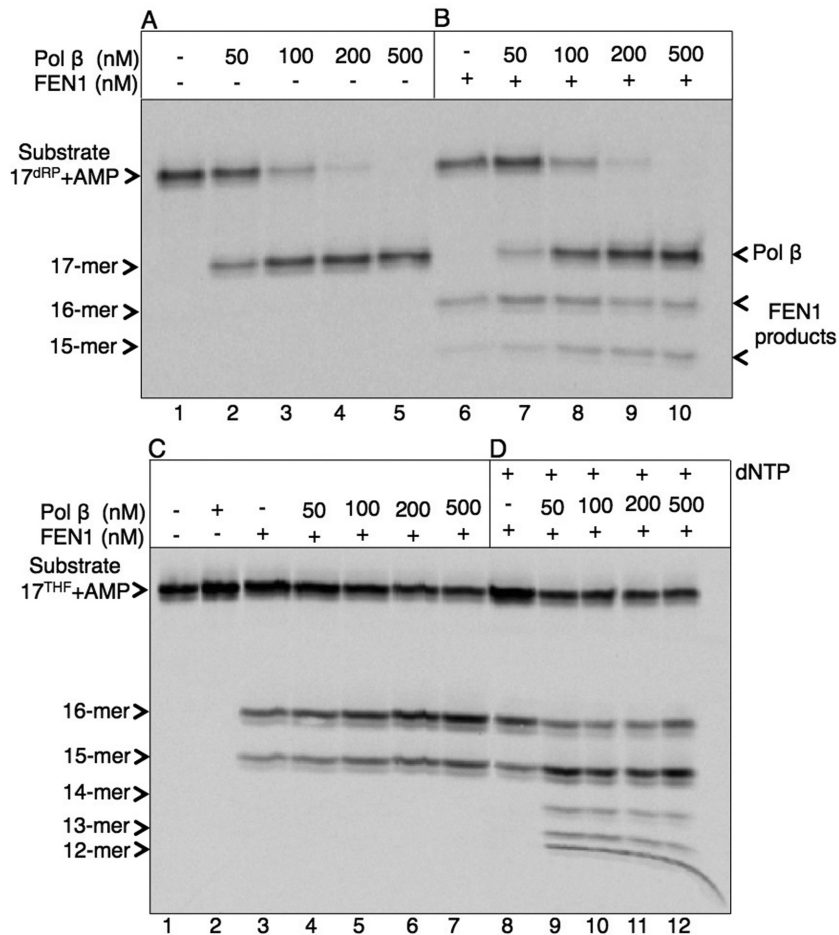

Figure 5. Effect of pol β on FEN1 excision activity. (**A**, **B**) Lane 1 is a minus enzyme control for the 5′-AMP-dRP-containing DNA substrate. Lanes 2–5 are reaction products for 5′-AMP-dRP removal by pol β alone and lane 6 is the FEN1 excision product in the absence of pol β. Lanes 7–10 are reaction products in the presence of both pol β and FEN1. (**C**) Lane 1 is a minus enzyme control for the 5′-AMP-THF-containing DNA substrate. Pol β does not function on this substrate (lane 2). Lane 3 is the FEN1 excision product in the absence of pol β. Lanes 4–7 are reaction products in the presence of both pol β and FEN1. (**D**) Pol β and FEN1 coordination in processing of the 5′-AMP-THF-containing DNA substrate in the presence of dNTPs (5 μM). Lane 8 is the FEN1 excision product in the absence of pol β and lanes 9–12 are FEN1 excision products in the presence of both pol β and FEN1. The reaction mixtures including both proteins contained 50–500 nM pol β and 100 nM FEN1.
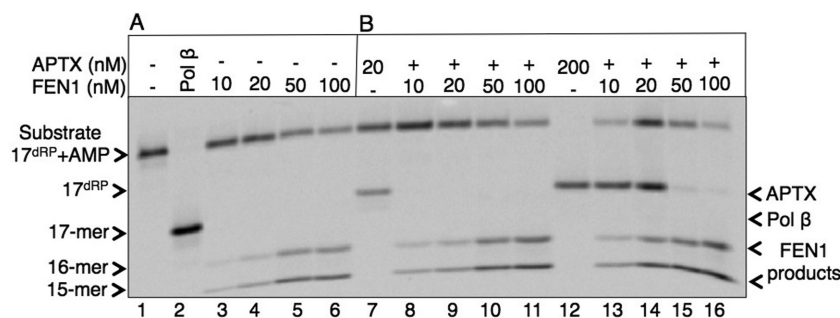
Figure 6. Effect of APTX on FEN1 excision activity. (**A**) Lane 1 is a minus enzyme control for the 5′-AMP-dRP-containing DNA substrate and lane 2 is the pol β 5′-AMP-dRP removal product. Lanes 3–6 are FEN1 excision products in the presence of FEN1 alone. (**B**) Lanes 7 and 12 are 5′-AMP removal products in the presence of APTX alone. Lanes 8–11 and 13–16 are reaction products in the presence of both APTX (20 nM) plus FEN1 (10–100 nM) and APTX (200 nM) plus FEN1 (10–100 nM), respectively.

tivity (Figure 5A and B). Using the gapped 5′-AMP-THF-containing substrate that is refractory to pol β lyase activity (2), pol β stimulation of FEN1 incision was observed, consistent with a functional interaction between these enzymes in resolving of the 5′-adenylated BER intermediates (Figure 5C). This experiment was repeated in the presence of all four deoxynucleoside triphosphates (dNTPs). The results revealed stronger two nucleotide excision products plus additional FEN1 excision products, consistent with strand-displacement synthesis by the polymerase (Figure 5D). FEN1 and pol β are known to have a functional partnership in LP BER (26). These results reinforced the idea that pol β and FEN1 also coordinate in processing of the 5′-adenylated-dRP during LP BER.

We next examined the effect of APTX on FEN1 activity against the gapped 5′-AMP-dRP-containing DNA substrate. In control reactions, FEN1 excision products indicating two nucleotide excision along with 5′-AMP-dRP removal were observed (Figure 6A). In the reactions including both FEN1 and APTX, FEN1 excision products were predominant as a function of adding FEN1 for both a limited and higher amount of APTX (Figure 6B). In both cases, FEN1 excision activity was stimulated by APTX (Supplementary Figure S5A). Overall, the results indicate that the robust activity of FEN1 observed in the cell extracts (Figures 2 and 3) in resolving the 5′-adenylated-dRP-containing BER intermediates could be due to stimulation of FEN1 excision activity by other BER enzymes such as pol β and APTX.

Finally, we evaluated the possibility of coordination between APTX and pol β. With the gapped 5′-AMP-dRP-containing DNA substrate, 5′-AMP removal products were observed as a function of adding APTX in the control reactions (Figure 7A). In the reactions including both pol β and APTX, pol β lyase removal was predominant indicating 5′-AMP-dRP removal along with minor 5′-AMP removal products (Figure 7B). Quantification of the results showed APTX mediated ~2-fold stimulation in pol β lyase activity (Supplementary Figure S5B). For the gapped 5′-AMP-THF-containing DNA substrate on which pol β cannot function, only APTX 5′-AMP removal products were obtained in the reactions including both pol β and APTX (Figure 7D), when compared to the reactions with the 5′-

Table 1. Catalytic rate constants of 5′-AMP-dRP removal by enzymes with dRP lyase activity used in this study

Enzyme	k_{obs} (per min)
Pol β	3
Pol λ	0.5
Pol θ	0.05
Pol ι	0.03
Ku70	0.01

All of the kinetic measurements were repeated in triplicate and the numbers represent the mean values of three seperate experimental values. Plots of product formation are provided in Supplementary Figure S6.

adenylated-natural abasic site substrate (Figure 7C) as described above (Figure 7B).

Enzymology of enzymes for processing of the 5′-adenylated-dRP-containing BER intermediate

We investigated enzymes with known dRP lyase activity using purified pol λ (17), pol θ (18), pol ι (27) and Ku70 (28) for a role in removal of the 5′-adenylated-dRP-containing BER intermediate. The results showed that pol λ activity was lower than that of pol β for 5′-dRP (Figure 8A) and 5′-AMP-dRP removal (Figure 8B). The activities of other enzymes, i.e. pol θ, pol ι and Ku70, were much weaker for both 5′-dRP (Figure 8C) and 5′-AMP-dRP (Figure 8D) removal than pol β and pol λ. Based on single-turnover kinetic measurements, the rate comparison of these enzymes for 5′-AMP-dRP removal showed that pol λ has a potential as a back-up BER enzyme, while the other enzymes did not seem strong enough to provide significant complementation activity in a case of APTX deficiency (Supplementary Figure S6A and B, Table 1).

DISCUSSION

The present study examined alternative mechanisms to complement a deficiency in APTX activity in mammalian and chicken BER. In a previous report on findings with purified enzymes (8), we suggested a model for the fate of the blocked 5′-adenylated-dRP-containing BER intermediate in a cell deficient in APTX. In this model, SN BER involving pol β and LP BER involving FEN1 could impact removal of the adenylated BER intermediates, especially in
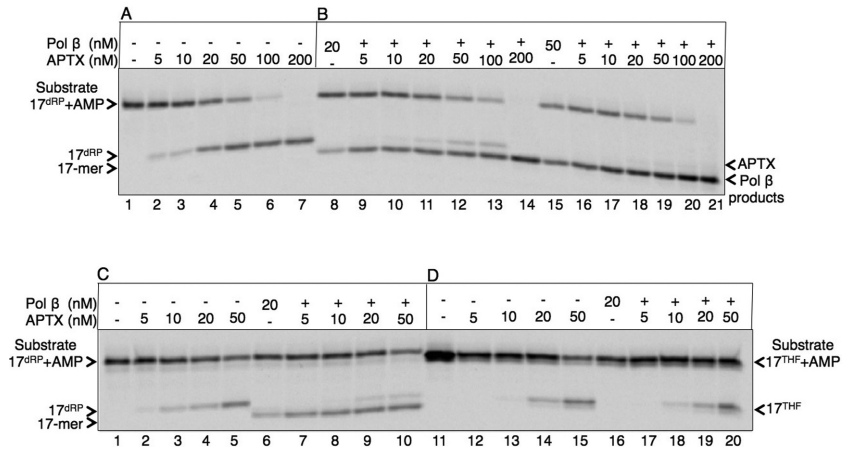
Figure 7. Effect of APTX on pol β lyase activity. (**A**, **B**) Lane 1 is a minus enzyme control for the 5′-AMP-dRP-containing DNA substrate. Lanes 2–7 are 5′-AMP removal products in the presence of APTX alone. Lanes 8 and 15 are 5′-AMP-dRP removal products in the presence of pol β alone. Lanes 9–14 and 16–21 are reaction products in the presence of both pol β (20 nM) plus APTX (5–200 nM) and pol β (50 nM) plus APTX (5–200 nM), respectively. (**C**, **D**) Lanes 1 and 11 are minus enzyme controls for the 5′-AMP-dRP- and 5′-AMP-THF-containing DNA substrates, respectively. Lanes 2–10 are reaction products for processing of the 5′-AMP-dRP substrate as described in panels A and B. Lanes 12–15 are 5′-AMP removal products in the presence of APTX alone. Pol β does not function on this substrate (lane 16). Lanes 17–20 are reaction products in the presence of both pol β (20 nM) and APTX (5–50 nM).
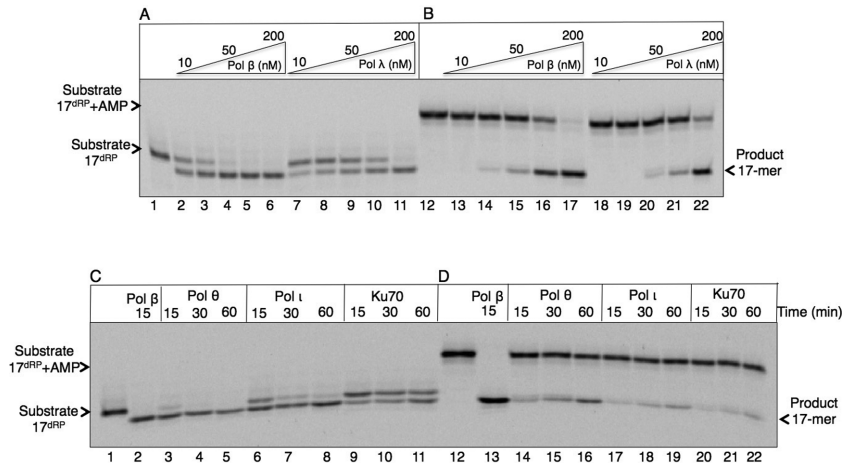

Figure 8. Processing of the 5′-dRP and 5′-adenylated-dRP-containing BER intermediates by enzymes with dRP lyase activity. (**A**, **B**) Lanes 1 and 12 are minus enzyme controls for 5′-dRP and 5′-AMP-dRP-containing DNA substrates, respectively. Lanes 2–6 and 13–17 are pol β lyase reaction products for 5′-dRP and 5′-AMP-dRP removal, respectively. Lanes 7–11 and 18–22 are pol λ lyase reaction products for 5′-dRP and 5′-AMP-dRP removal, respectively. The lyase reactions contained pol β or pol λ at indicated concentrations and 100 nM DNA substrate, and were incubated for 15 min. (**C**, **D**) Lanes 1 and 12 are minus enzyme controls for 5′-dRP and 5′-AMP-dRP-containing DNA substrates, respectively. Lanes 2 and 13 are pol β control reaction products for 5′-dRP and 5′-AMP-dRP removal, respectively. Lanes 3–5, 6–8 and 9–11 are pol θ, pol ι and Ku70 lyase reaction products, respectively, for 5′-dRP removal. Lanes 14–16, 17–19 and 20–22 are pol θ, pol ι and Ku70 lyase reaction products, respectively, for 5′-AMP-dRP removal. The lyase reactions contained 50 and 500 nM pol β for 5′-dRP and 5′-AMP-dRP-containing DNA substrates, respectively. For both substrates, the lyase reaction included 500 nM pol θ, pol ι or Ku70 and 100 nM DNA substrate, with incubation for the indicated time points. Illustrations of the gapped 5′-dRP and 5′-dRP-AMP-containing DNA substrates and reaction products are shown in Scheme 1C.

the absence of APTX activity. In this study, using whole cell extracts from APTX-deficient cell lines, we demonstrated that the activities of pol β and FEN1 were strong enough to enable these enzymes to substitute for APTX activity.

A robust FEN1 excision effect in the repair of the 5′-adenylate blocks at either the 5′-dRP group or the 5′-phosphate group was observed. The ratio of one nucleotide to two nucleotide FEN1 excision products were different in the cell extracts studied here. This could reflect a difference in specificity between the mammalian and chicken enzymes, but the explanation for this observation is a topic for future study. In addition, we also observed a stimulation of FEN1

excision activity by pol β and APTX. It is known from other studies that functional cooperation between BER proteins is an important feature of LP BER (4–6,26). Therefore, the findings here provide another example of a functional interaction among BER proteins. This effect may allow the cell to avoid making the 5′-blocked repair intermediates available for cell death signaling.

The present study also examined the potential role of several other enzymes with dRP lyase activity, i.e. pol λ, pol θ, pol ι and Ku70 in processing of the 5′-adenylated-dRP-containing BER intermediates. The findings revealed the potential for back-up lyase removal of the 5′-adenylated-

dRP by pol λ, whereas the other enzymes tested did not seem to have strong enough activity to play a significant back-up role, except in specialized cases. Consistent with this idea was the finding that extract from the double knockout pol β$^{-/-}$ pol λ$^{-/-}$ cell line did not have residual lyase activity, indicating that the main source of the weak residual activity in the absence of pol β was pol λ. However, the pol λ lyase activity against the 5′-adenylated BER intermediate was much lower than that of pol β.

The relative contributions of SN and LP BER subpathways in resolving of blocking 5′-adenylates in different APTX-deficient cell types have not yet been adressed, and more studies will be needed to understand the molecular mechanisms that play roles in disease phenotype expression associated with APTX deficiency. Other processes, including transcriptional defects (10), have been also reported to contribute to the neurodegenerative phenotype of AOA1 patients and it is unclear how this could relate to the current findings. Nevertheless, the results are consistent with the hypothesis that pol β and FEN1 could influence disease phenotypes associated with APTX deficiency.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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