Requirement for human AP endonuclease 1 for repair of 3′-blocking damage at DNA single-strand breaks induced by reactive oxygen species

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The major mammalian apurinic/apyrimidinic (AP) endonuclease (APE1) plays a central role in the DNA base excision repair pathway (BER) in two distinct ways. As an AP endonuclease, it initiates repair of AP sites in DNA produced either spontaneously or after removal of uracil and alkylated bases in DNA by monofunctional DNA glycosylases. Alternatively, by acting as a 3′-phosphoesterase, it initiates repair of DNA strand breaks with 3′-blocking damage, which are produced either directly by reactive oxygen species (ROS) or indirectly through the AP lyase reaction of damage-specific DNA glycosylases. The endonuclease activity of APE1, however, is much more efficient than its DNA 3′-phosphoesterase activity. Using whole extracts from human HeLa and lymphoblastoid TK6 cells, we have investigated whether these two activities differentially affect BER efficiency. The repair of ROS-induced DNA strand breaks was significantly stimulated by supplementing the reaction with purified APE1. This enhancement was linearly dependent on the amount of APE1 added, while addition of other BER enzymes, such as DNA ligase I and FEN1, had no effect. Moreover, depletion of endogenous APE1 from the extract significantly reduced the repair activity, suggesting that APE1 is essential for repairing such DNA damage and is limiting in extracts of human cells. In contrast, when uracil-containing DNA was used as the substrate, the efficiency of repair was not affected by exogenous APE1, presumably because the AP endonuclease activity was not limiting. These results indicate that the cellular level of APE1 may differentially affect repair efficiency for DNA strand breaks but not for uracil and AP sites in DNA.

Abbreviations: AP, abasic (apurinic/apyrimidinic); APE, AP endonuclease; BER, base excision repair; β-pol, DNA polymerase β; 3′P, 3′-phosphate; 3′PG, 3′-phosphoglycolate; ROS, reactive oxygen species; UDG, uracil-DNA glycosylase; WCE, whole cell extract.

Introduction

Reactive oxygen species (ROS), which are generated either endogenously as respiratory by-products or exogenously by various environmental factors, including ionizing radiation, induce DNA damage that have been implicated in the etiology of many pathological conditions and in aging (1–4). Such DNA lesions include various abnormal base adducts, such as thymine glycol, 8-oxoguanine, abasic (AP) sites and DNA strand breaks (2). Many of these lesions are either toxic or mutagenic and thus may cause either cellular death or neoplastic transformation. Genomic integrity after the ROS reaction is restored primarily via the DNA base excision repair (BER) pathway, which consists of a series of reactions involving multiple repair proteins (5–7). In the case of oxidized base lesions, repair in mammalian cells is initiated by DNA glycosylases, including the endonuclease III homolog (NTH1) and 8-oxoguanine DNA glycosylases (OGG1 and OGG2), that have associated AP lyase activity and generate strand breaks via β-elimination (7–9). These abnormal 3′-end structures (3′-blocking damage) prevent DNA polymerases from carrying out repair synthesis (10–12). Thus it is likely that oxidative base adducts form repair intermediates with 3′-blocking termini in vivo.

The 3′-blocking ends are also direct products of ROS, which attack and fragment the deoxyribose residues in DNA (2,13). The ROS reaction thus produces a mixture of DNA strand breaks with various 3′-end structures, including 3′-phosphoglycolate (3′PG) and 3′-phosphate (3′P) termini (2,11,14–17). Because all 3′-blocking groups arising as direct and indirect products of ROS prevent repair synthesis (11,15,17), it is crucial for cells to remove these blocking ends before DNA repair synthesis can be initiated. Removal of these 3′-blocking groups is carried out by AP endonuclease (APE) (13,18). In addition to endonuclease activity for repair of the AP sites, the major mammalian APE (APE1), like all APEs, possesses DNA 3′-phosphoesterase activity (3′-end cleaning activity) (19).

In a multi-component reaction pathway like BER, one particular factor may be limiting and thus critically affect the overall repair efficiency. It was suggested that DNA polymerase β (β-pol), which carries out DNA synthesis (20,21), is the rate limiting factor in repair of uracil in DNA (21,22). However, in contrast to the oxidized base-specific DNA glycosylases, repair of uracil is initiated by uracil-DNA glycosylase (UDG), an enzyme without AP lyase activity that produces AP sites after exciting uracil (7,23). Thus there is a clear dichotomy in the requirement for APE1 activity for uracil repair versus repair of oxidized bases and ROS-induced strand breaks, i.e. AP endonuclease activity for the former and 3′-phosphoesterase activity for the latter. Suh et al. compared the two types of human APE1 activities in kinetics studies and found that the AP endonuclease activity was ~100-fold more efficient than 3′-DNA phosphoesterase activity (19). We therefore hypothesized that unlike uracil repair, the level of APE1 in the cell might be limiting for repair of DNA strand breaks induced by ROS. This hypothesis is supported by our previous observation that APE1 activation after ROS generation was accompanied by
adaptive resistance of the cells to ROS (24). In this report we show, using extracts of human cells, that APE1 in such extracts was rate limiting in repair of 3’-blocking damage but not of uracil. These results suggest that the level of APE1 can be critical during repair of oxidative DNA lesions.

Materials and methods

Preparation of whole cell extract

All procedures for preparing cell extracts were performed at 0°C. HeLa S cells were grown in suspension culture containing S-MEM (Gibco Life Technologies) supplemented with 10% bovine serum (Hyclone) at 37°C in a 5% CO₂ incubator. Human TK6 lymphoblastoid cells were also grown in suspension in RPMI 1640 (Gibco Life Technologies) supplemented with 10% bovine serum. All cells were grown to mid log phase (~5×10⁶ cells/ml) before harvesting. The ‘whole cell extract’ (WCE) was prepared according to Manley et al. (25). Typically 5×10⁶ cells yielded ~20 mg of protein in WCE.

Depletion of APE1 protein from WCE using immunoadfinity chromatography

Anti-APE1 antisera was raised in rabbits by injecting purified full-length human APE1 (26,27). The antisera (~5 ml) was conjugated to an immunoadfinity matrix (IgG-Orientation Kit; Pierce) and then HeLa WCE (1 ml) was incubated with the matrix for 3 h at 4°C with gentle rocking, which was then poured into a column and the extract subsequently eluted from the matrix by gravity. As a control, an aliquot (50 µl) of the same batch of WCE was identically incubated without the matrix. Depletion of APE1 from the matrix-treated extract was confirmed by western blot analysis.

DNA substrates

Form I plasmid DNA was treated with various agents in order to induce single-strand breaks in 30–50% of the molecules, corresponding to a single break per molecule in 70–85% of the form II population based on a Poisson distribution. A 3 kb plasmid DNA, pBluescript SK(-) (Stratagene), was used to generate damage, except for DNAse I treatment, for which a 7 kb plasmid DNA was used. Form I DNA was treated with pancreatic DNAse to generate single-strand nicks containing 3’-OH/5’-phosphate. Bleomycin/Fe²⁺ was used to generate single-strand breaks containing 3’-PG (28). The treated DNA contained a small amount of linearized DNA, indicating the presence of double-strand breaks, presumably due to attack by bleomycin on both strands (2). The bleomycin-treated form II DNA contained no detectable AP sites (2), because treatment with APE1 did not increase the fraction of linear molecules (data not shown). Thus most AP sites were probably cleaved during preparation to form linear molecules due to the instability of intact AP sites. To generate H₂O₂-induced DNA single-strand breaks with various 3’-blocking ends, H₂O₂ (0.27 mg/ml) was treated with 0.1 mM H₂O₂, and 0.2 mM CuSO₄ for 10 min at 37°C. DNA strand breaks with 3’-α,β-unsaturated aldehyde were generated by treating plasmid DNA in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA with 0.04% OsO₄ at 70°C for 8 min, followed by removal of OsO₄ via dialysis and then treatment with human NTH1 in 50 mM NaCl and 1 mM dithiothreitol (9). In all cases, form II DNA was separated from the form I DNA and purified by two rounds of ultracentrifugation in CsCl/ethidium bromide. Plasmid DNA containing uracil was produced by treatment with sodium bisulfite (29).

DNA repair assay

Repair of form II DNA into form I DNA with WCE was carried out in an assay as described by Satoh and Lindahl (30). The reaction mixtures were incubated for 30 min at 30°C unless indicated otherwise. The amounts of WCE are described in the figure legends. Form I and form II plasmid DNA were separated by agarose electrophoresis. The relative band intensity was quantified by fluorescence of ethidium stained bands using an Eagle Eye II (Stratagene) and Storm system (Molecular Dynamics) with correction for ethidium binding difference between forms I and II, except in Figure 5, where repair was assayed by incorporation of [α-³²P]dCTP (30). In experiments involving radiolabeled substrates, the radioactivity in the DNA bands was quantified by PhosphorImager analysis (Molecular Dynamics).

DNA repair enzymes

Human APE1 and NTH1 were purified as reported earlier (9,31). DNA pol β was a generous gift of Dr S.H.Wilson (20), Purification of FEN1 was as described elsewhere (32). Human DNA ligase I was purified earlier (33) and its activity confirmed using DNA nicked by bovine DNAse I (data not shown).

Other enzymes and reagents

T4 polynucleotide kinase was purchased from Pharmacia Biotech and T4 DNA ligase was from New England Biolabs. ATP and dNTPs (dATP, dGTP, dCTP and dTTP) were obtained from Pharmacia Biotech. Other chemicals were purchased from Sigma and Fisher Scientific Co.

Results

Role of APE1 in repair of oxidative DNA damage

The in vitro repair system using human cell-free extracts has been extensively used to study DNA repair functions in the eukaryotes (30,34). Our aim was to examine BER efficiency using various damaged DNA substrates generated by genotoxic agents. The sites of damage were randomly distributed in the plasmid DNA. The substrates had the following different structures of the 3’-termini at the break sites: 3’-OH produced by cleavage with DNAse I; 3’PG produced by bleomycin (2); 3’-trans-α,β-unsaturated aldehyde (3’-phosphosugar) generated by the combined DNA glycosylase and AP lyase activities of human NTH1 on OsO₄-treated DNA (9); a mixture of 3’-blocking ends generated by H₂O₂, including 3’P and 3’PG (11,17). Thus these substrates, except that with 3’-OH nicks, represent the spectrum of modified 3’-termini at single-strand breaks generated in DNA directly or indirectly due to ROS reaction. As expected, DNA ligase alone was active with the DNA containing 3’-OH nicks but did not seal any of the other strand breaks (Figure 1). We then carried out complete repair reactions with HeLa WCE observed after incubation of various types of form II DNA with HeLa WCE (Figure 2). The conversion of form II DNA to form I was completely dependent on DNA repair synthesis, because the reaction occurred only in the presence of dNTPs (Figure 2B). Therefore, generation of form I DNA resulted from a genuine BER reaction. However, the repair occurred with different efficiencies depending on the 3’-termini. While nicks with 3’-OH was repaired relatively efficiently, repair of strand breaks with abnormal 3’-ends was inefficient [lane 2 of each set in Figure 2; note that the DNAse I-treated plasmid DNA was larger (7 kb) than the other substrates (3 kb)]. Because the repair reactions were performed under identical conditions in all cases, the differences in repair efficiency were due to the type of damage, i.e. the 3’-end structure at the DNA strand breaks. We tested the possibility that the amount of APE1, because of its weak 3’-end cleaning activity (19), was limiting in repair of these 3’-blocking damages. Addition of human APE1 significantly enhanced repair of the 3’-blocking ends, but did not affect repair of 3’-OH nicks (Figure 2, lanes 3 and 4 of each set compared with lane 2 of each). Enhancement of repair of ROS-induced strand breaks was also observed with the addition of Escherichia coli Xth (35) instead of APE1 (data not shown). This last result indicates that the APE1-mediated enhancement of repair was indeed due to the repair activity of APE1 and not because of the unrelated redox activation function of APE1, which is absent in the E.coli enzyme (36,37).

Using bleomycin-treated DNA, we quantitatively examined the repair kinetics. The extent of repair increased as a function of the amount of cell extract up to 100 µg (Figure 3A). However, there was a steep, initial linear range up to 5 µg, after which the slope of repair declined (Figure 3A). This decrease may be due to the inhibitory effect of unrelated factors such as non-specific DNA-binding proteins present in WCE. Because it was not our goal to investigate this inhibitory effect, all experiments on kinetics were carried out in the initial linear range (2.5 µg WCE). Under this condition, the extent of repair was linearly dependent on time up to 90 min (Figure 3B), which again validated our assay condition of...
Fig. 1. Form II plasmid DNA with strand breaks, induced by treatment with DNase I, bleomycin (bleo), OsO4 and NTH1 and H2O2, were incubated with (+) or without (−) T4 DNA ligase (T4lig) for 5 h at 16°C. ctl, undamaged 3 kb plasmid DNA. pBluescript SK(−) plasmid DNA (3 kb) (Stratagene) was used except for that treated with DNase I (pIZ266, 7 kb) (unpublished). Arrowheads at the sides indicate forms I and II. M, size marker.

Fig. 2. APE1-dependent enhancement of DNA single-strand break repair. (A) Form II plasmid DNA was incubated with HeLa WCE. In the third and fourth lanes in each set human recombinant APE1 was added at 200 (+) and 400 ng (++)+, respectively. The amount of WCE in each assay was 2.5 µg for DNA treated with DNase I or bleomycin and 15 µg for H2O2-treated and 5 µg for OsO4+NTH-treated DNA. (B) Role of dNTPs. (Left) DNAse I-treated DNA was incubated with 2.5 (+) or 5 µg (++) of WCE in the absence of dNTPs. (Right) Damage-containing DNA was incubated with (+) or without (−) 5 µg of WCE in the absence of dNTPs.

Fig. 3. Quantitation of repair of 3′PG-containing DNA. (A) Amount of repaired product (form I) was measured as a percentage of total DNA after 30 min incubation with different amounts of WCE. (B) Kinetics of repair after incubation with 2.5 µg WCE. (C) Stimulation of repair dependent on APE1. DNA was incubated with 2.5 µg of WCE and various amounts of purified APE1 (0, 1.5, 3, 6, 15 and 30 ng) and the amount of repaired form I DNA was quantified. Relative repair efficiency denotes fold increase in repair product compared with that of WCE alone. (D) (Top) HeLa WCE, immunodepleted of APE1, was used for repair of 3′PG-containing DNA. Lane 1, no WCE; lane 2, 2.5 µg of mock-treated WCE; lane 3, 2.5 µg of depleted WCE; lane 4, depleted WCE (2.5 µg) plus 50 ng of APE1. (Bottom) Western blot of mock-treated (M), depleted (D) and eluent (E) using an anti-APE1 antibody. The arrow indicates the APE1 band.

Repair efficiency and concluded that APE1 was not important in the BER pathway (38). Although Winters et al. showed a neutralizing effect of the antibody with purified enzymes (38), it was possible that this effect was diminished in the crude extract (38). We believe that, in order to validate their conclusion, it would be necessary to physically remove APE1 from the reaction mixture, instead of preincubating with the antibody. Therefore, we therefore depleted WCE of APE1 using an immunoaffinity column conjugated with anti-APE1 antibody, which was monitored by western blot analysis (Figure 3D, bottom). This depleted WCE substantially inhibited the repair reaction (Figure 3D, top). To exclude the possibility that this immunodepletion procedure could have removed other repair enzymes in addition to APE1 or that the repair activity was simply inactivated during the procedure, the depleted WCE was supplemented with purified recombinant APE1, which resulted in complete restoration of repair (Figure 3D). Therefore, contrary to the earlier observation (38), our results clearly indicate that APE1 is essential and limiting for removal of 3′-blocking damage in the repair pathway for ROS-induced DNA strand breaks.

Lack of repair enhancement by other BER enzymes
To test whether the enhancement was unique to APE1 or similar enhancement could be observed by addition of other BER proteins as well, we tested the effect of FEN1 and DNA ligase I (32,39) on repair of ROS-induced DNA strand breaks.
Involvement of these enzymes in the BER pathway was established earlier (39). As shown in Figure 4A, addition of exogenous FEN1 failed to enhance overall repair (lanes 6–8 compared with lane 2), while in the same experiment APE1 addition caused a robust increase (lanes 3–5). A similar absence of repair enhancement were observed after addition of DNA ligase I (lanes 3 and 4 compared with lane 2 in Figure 4B) and even after supplementation with APE1 the ligase could not stimulate the reaction further (lanes 6 and 7 compared with lane 5). Addition of β-pol to the reaction did not enhance the activity either (Figure 4C). Therefore, the enhancement of BER was proportional to the level of APE1 alone.

Uracil repair is not affected by APE1 addition

Repair of uracil, a common mutagenic lesion formed in DNA by deamination of cytosine, has been extensively investigated and is used as the paradigm for the BER pathway (21,40,41). In vitro repair studies have shown that after removal of uracil by UDG, the DNA strand is cleaved at the resulting AP site by APE1, which generates a 5′-deoxyribose phosphate residue. As mentioned earlier, this group is then removed by the dRPase activity of β-pol or FEN1 (42–44). Subsequent filling of the nucleotide gap by β-pol and sealing of the nick by DNA ligase I or a complex of DNA ligase III and XRCC1 complete the repair process (21,41,45). To test whether APE1 could also enhance this repair process, we monitored repair of uracil by incorporation of [α-32P]dCMP in uracil-containing plasmid DNA present as both forms I and II (Figure 5). Incorporation of 32P was undetectable in undamaged control DNA under our experimental conditions (data not shown). In contrast to the results on repair of 3′-blocking damage, addition of exogenous APE1 did not enhance repair of uracil-containing DNA (Figure 5, lanes 2–4). These results indicate that the AP endonuclease activity was not limiting for repair of uracil in DNA.

We then tested whether β-pol could be limiting in repair of uracil. Addition of purified β-pol to the reaction mixture showed increased nucleotide incorporation, indicating enhanced repair of uracil in the substrate DNA (Figure 5, lanes 5–7). In contrast, addition of APE1 to the reaction stimulated by β-pol did not further affect the extent of repair (Figure 4, lane 6). These results thus confirm the earlier studies that suggested that β-pol was limiting in repair of uracil-containing DNA (21,22).

Discussion

We have described a correlation between the APE1 level and overall repair efficiency for ROS-induced DNA strand breaks in human cell-free extracts. Such a repair process did not only require APE1, but was also enhanced by addition of exogenous APE1. This enhancement of repair was specific for ROS-induced DNA strand breaks, i.e. those with 3′-blocking damage, and was not observed during repair of uracil in DNA. Instead, β-pol and not APE1 stimulated repair of uracil, consistent with the earlier studies (20). These two distinct effects of APE1, namely a rate limiting role of APE1 in repair of DNA strand breaks but not of uracil, may be due to its bipartite functions. After removal of uracil by UDG, repair of AP sites requires the endonuclease activity of APE1 while repair of the DNA strand breaks requires its DNA 3′-phosphoesterase activity (19). All human glycosylases responsible for repairing ROS-induced base adducts have intrinsic AP lyase activity, including NTH1, which removes thymine glycol in DNA (9), and OGG1 and the recently identified human OGG2, which repair the highly mutagenic 8-oxoguanine in DNA (8). It is thus possible that repair of ROS-induced base adducts needs APE1 for 3′-phosphoesterase activity and a change in the APE1 level affects the overall repair efficiency. In contrast, as we observed, a change in APE1 level would not have a significant effect on repair of AP sites generated by monofunctional DNA glycosylases such as UDG (Figure 5). The conclusion is consistent with the earlier observation that a β-pol nullizygous cell line was highly sensitive to an alkylating

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**Fig. 4.** Effect of supplementation with other repair enzymes on the repair reaction. (A) 3′PG-containing DNA was incubated with no (lane 1) or 2.5 µg of WCE (lane 2) and with APE1 (lane 3, 20 ng; lane 4, 40 ng; lane 5, 200 ng) or FEN1 (lane 6, 20 ng; lane 7, 40 ng; lane 8, 200 ng). (B) 3′PG-containing DNA was incubated with no (lane 1) or 2.5 µg of WCE (lane 2) and with human DNA ligase I (lanes 4 and 5, 20 ng; lanes 3 and 6, 200 ng) or APE1 (lanes 5–7, 50 ng). (C) 3′PG-containing DNA was incubated with no (lane 1) or 2.5 µg of WCE (lane 2) and with 20 ng of APE1 (lane 3) or 20 ng of β-pol (lane 4).

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**Fig. 5.** Effect of APE1 and β-pol on uracil repair. (Top) Plasmid DNA containing uracil was incubated with no (lane 1) or 2.5 µg of WCE in the presence of 6.25 mCi [α-32P]dCTP (lane 2) and supplemented with APE1 (lanes 3, 6 and 7, 20 ng; lane 4, 40 ng) or β-pol (lanes 5 and 6, 20 ng; lane 7, 40 ng). (Bottom) Incorporation of radioactivity in the same DNA bands as analyzed in a PhosphorImager.
agent but showed a normal phenotype to hydrogen peroxide, a ROS generator (46).

The distinct roles of APE1 have profound implications for the in vivo regulation of APE1 and its effect on cellular recovery after DNA damage (24). This regulation is complex because of activation of APE1 by ROS as well as its potential autoregulation (24,26,47). Further experiments, particularly in vivo studies on transgenic mice/conditional APE1 knockout and DNA ligase I in a multiprotein base excision repair complex from the in vivo grants ES08457, CA53791, AG10514, ES06676 and GM47251. species and its correlation with their adaptive response to genotoxicity at UTMB for preparation of plasmid DNA. The excellent secretarial help of 24. Ramana, C.V., Boldogh, I., Izumi, T. and Mitra, S. (1998) Activation of incisive comments. Ms Julie Lock provided expert technical assistance. We truncated mouse

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