Identification of a residue critical for the excision of 3′-blocking ends in apurinic/apyrimidinic endonucleases of the Xth family

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ABSTRACT

DNA single-strand breaks containing 3′-blocking groups are generated from attack of the sugar backbone by reactive oxygen species or after base excision by DNA glycosylase/apurinic/apyrimidinic (AP) lyases. In human cells, APE1 excises sugar fragments that block the 3′-ends thus facilitating DNA repair synthesis. In *Leishmania major*, the causal agent of leishmaniasis, the APE1 homolog is the class II AP endonuclease LMAP. Expression of LMAP but not of APE1 reverts the hypersensitivity of a *xth nfo* repair-deficient *Escherichia coli* strain to the oxidative compound hydrogen peroxide (H_2O_2). To identify the residues specifically involved in the repair of oxidative DNA damage, we generated random mutations in the *ape1* gene and selected those variants that conferred protection against H_2O_2. Among the resistant clones, we isolated a mutant in the nuclease domain of APE1 (D70A) with an increased capacity to remove 3′-blocking ends *in vitro*. D70 of APE1 aligns with A138 of LMAP and mutation of the latter to aspartate significantly reduces its 3′-phosphodiesterase activity. Kinetic analysis shows a novel role of residue D70 in the excision rate of 3′-blocking ends. The functional and structural differences between the parasite and human enzymes probably reflect a divergent molecular evolution of their DNA repair responses to oxidative damage.

INTRODUCTION

Oxidative DNA damage is induced by ionizing radiation (1), by reactive oxygen species produced by the aerobic metabolism (2,3) and by the action of some antitumoral agents such as neocarzinostatin and bleomycin (4). The attack of DNA by free radicals gives rise to oxidative base damage, abasic sites and single-strand breaks containing modified 3′-ends, such as 3′-phosphoglycolate (3′-PG) and 3′-phosphate (3′-P) (5–7). Damaged 3′-ends may also arise as intermediates during base excision repair (BER), resulting from the cleavage of the apurinic/apyrimidinic (AP) site by bifunctional DNA glycosylases. For instance, human 8-oxoguanine-DNA glycosylase (OGG1) and the endonuclease III (NTH) have intrinsic associated β-lyase activity that cleaves the phosphodiester backbone 3′ to the abasic site through a β-elimination reaction to produce a 3′-α, β-unsaturated aldehyde (3′-dRP) (8–10). In contrast, endonuclease VIII-like (NEIL) glycosylases incise the abasic site by β,δ-elimination generating a 3′-P terminus at the DNA strand break (11). These 3′-blocking damages must be removed prior to DNA repair synthesis. The current model for excision of 3′-damaged ends is initiated by a 3′-phosphodiesterase or 3′-phosphatase activity that cleaves the phosphodiester bond at 5′ position of the 3′-blocking group. The product is a single nucleotide gap with 3′-hydroxyl (3′-OH) and 5′-phosphate termini. The repair is then accomplished by the incorporation of one nucleotide by a DNA polymerase (DNA repair synthesis) and the covalent sealing of the strand break by a DNA ligase (DNA sealing) (12).

Cells have different ways to handle 3′-blocking ends. *Escherichia coli* possesses two class II abasic (AP) endonucleases capable of repairing such strand breaks by excision of the 3′-blocking groups: exonuclease III and endonuclease IV, encoded by *xthA* and *nfo* genes (13–15). *xthA* and *xthA nfo* strains are highly sensitive to hydrogen peroxide (H_2O_2) (16), oxidative agent that induces strand breaks with 3′-blocking damages as primary lethal lesion (17). In human cells, the exonuclease III homolog is AP endonuclease 1, APE1. *In vitro*, the human enzyme can

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catalyze the hydrolysis of 3′-PG residues (18–22) and also the removal of the 3′-dRP generated after β-elimination (23–25) although its 3′-phosphodiesterase function has been reported less efficient than its incision activity on AP sites (19,20,22). In fact, the expression of human AP endonuclease can replace bacterial exonuclease III at repairing alkylation-induced AP sites, but not at processing the strand breaks generated by oxidative agents (16,26,27). In spite of this, APE1 has been shown to provide the major phosphoglycolate removal activity in human cell extracts (28) although other sources of such activity cannot be ruled out (18,29–33). APE1 also has DNA 3′-phosphatase activity *in vitro*, but polynucleotide kinase phosphatase (PNKP) has been reported as the primary 3′-phosphatase activity responsible for the excision of 3′-P lesions in mammalian cells (25). In addition, APE1 has 3′ to 5′ exonuclease activity (34), which could be physiologically relevant in the removal of mismatched or damaged nucleotides incorporated during the synthesis step of BER (35,36). Finally, APE1 can also incise DNA at the 5′-position of oxidized pyrimidine bases such as 5,6-dihydro-thymine or 5,6-dihydro-2′-deoxyuracil (DHU), thus initiating a repair process known as nucleotide incision repair (NIR) (37).

Human cells possess a second AP endonuclease of the exonuclease III family, APE2, which is endowed with strong 3′ to 5′ exonuclease and 3′-phosphodiesterase activities but a very low AP endonuclease activity (32). The presence of these biochemical activities in the purified protein suggests an important role in the processing of 3′-blocking ends and in the proofreading of 3′-mismatched nucleotides.

Kinetoplastid parasites such as *Leishmania* are exposed to a highly oxidative environment within the host macrophage. Our previous studies suggest that *L. major*'s AP endonuclease LMAP must play an important role in parasite survival mediating the repair of apurinic/apyrimidinic sites and 3′-blocked termini induced by oxidative stress since in addition to its function as an AP endonuclease, LMAP has an equally efficient 3′-phosphodiesterase and a significant 3′-phosphatase activity (38). In contrast to human APE1, over-expression of LMAP in *E. coli* protects against both methyl methanesulphonate (MMS) and H_2O_2 indicating that LMAP efficiently repairs the DNA lesions caused by these two DNA damaging agents (38,39). Therefore, we were interested in identifying the specific residues involved in the recognition and/or cleavage of 3′-blocking ends at single strand breaks by these two AP endonucleases. Since LMAP has no obvious structural particularities that may account for such different enzymatic behavior, we sought to isolate mutants with altered repair capacity of this type of lesions. In the present study, we report the isolation of a mutant in the nuclease domain of APE1 that protects *xth nfo E. coli* strain against H_2O_2-induced DNA damage and has an increased 3′-phosphodiesterase and 3′-phosphatase activities. The mutation consists in a single base change, leading to the single amino acid change D70A. The opposed mutation at the corresponding position in LMAP (A138D) was then introduced by site-directed mutagenesis and the complementation phenotypes and enzymatic properties of these mutants were determined. The study

reveals a novel role for D70 of APE1 in the repair of damaged 3′-ends and discusses its possible implication in the process of catalysis.

MATERIALS AND METHODS

Expression plasmids

The construction of expression vectors pKK-ape1 and pKK-LMAP has been previously described (38). The pKK-LMAP^{A138D} mutant plasmid was constructed by using the QuickChange mutagenesis kit (Stratagene) with the primers 5′-cat tac gtg gaa cgt cga cgg cct acg cgg g-3′ and 5′-ccc gcg tag gcc gtc gac gtt cca cgt aat g-3′ on the plasmid template pKK-LMAP. For the convenience of subsequent identification, these primers also contain a silent change that results in a novel SalI restriction enzyme site (underlined). Plasmids pKK223-3, pKK-LMAP, pKK-ape1 and pKK-LMAP^{A138D} were transformed into BW528 strain (*nfo-1::Kan Δ* (*xth-pncA*)) for subsequent complementation studies.

For protein expression purposes, APE1^{D70A} was partially digested with NdeI and HindIII from pKK-APE1^{D70A} and a band corresponding to full-length APE1^{D70A} was cloned into pET28a, previously digested with NdeI and HindIII. pET28-LMAP^{A138D} was generated by using QuickChange mutagenesis kit (Stratagene) with primers 5′-cat tac gtg gaa cgt cga cgg cct acg cgg g-3′ and 5′-ccc gcg tag gcc gtc gac gtt cca cgt aat g-3′ on the plasmid template pET28a-LMAP.

To produce pX63-LMAP^{A138D}, LMAP-A138D was amplified by PCR from pET28-LMAP^{A138D} with primers 5′-gca gat cta tgg cct cga agc-3′ and 5′-gca gat ctc atg ggt gtc gca-3′. The PCR product was digested with BglII and cloned into BglII-digested pX63NEO. A clone with the correct orientation was chosen after digestion with EcoRI and DNA sequencing.

Isolation of a mutant *ape1* and lethality assay in *E. coli*

A random library of *ape1* mutants was generated by transforming the mutator strain XL1-Red (Stratagene, La Jolla, CA, USA) with the prokaryotic *ape1* expression plasmid, pKK-ape1, according to manufacturer's protocol. Cells were transformed with 40 ng of pKK-ape1, plated on Luria-Bertani (LB) agar with ampicillin (Amp) and incubated overnight at 37°C. Next day, approximately 200 colonies were scraped and washed off the agar with LB and transferred to 10 ml LB + Amp and grown for 24 h. Subsequently, this culture was diluted 1:10 000 and grown for an additional 24 h before plasmid purification.

An overnight culture of strain BW528 harboring the mutagenized plasmid library was diluted 100-fold in LB + Amp and incubated at 37°C until it reached an optical density of 0.8 at 600 nm. The culture was then spun down, washed and resuspended in phosphate-buffered saline (PBS). Cells suspension was exposed to 1 mM H_2O_2 for 30 min at 37°C, diluted 100-fold and incubated in liquid LB + Amp at 37°C overnight. Next day, cells were exposed to 5 mM H_2O_2 following standard lethality assay conditions and plated on LB + Amp. Plasmid DNA

was isolated from viable colonies that arose on the selective media and was used to re-transform BW528, to confirm the H_2O_2-resistant phenotype. Additionally, *ape1* gene from individual clones was cutoff with EcoRI and HindIII, cloned into pKK223-3 and the plasmid used to transform BW528 to check for complementation following H_2O_2 treatment. The nucleotide sequence of the entire *ape1* gene of the mutagenized pKK-*ape1* plasmids was determined and one such mutant with a missense mutation in *ape1* resulting in the substitution of aspartate for alanine at residue 70 was chosen for further study.

Survival curves were obtained by exposing approximately 10^8 cells from exponentially growing cultures to a range of drug concentrations for 30 min at 37°C in LB medium. Serial dilutions were made in PBS prior to plating on LB agar with ampicillin. Colonies were counted after incubation at 37°C for 24 h.

Substrates, enzymes and antibodies

The 21-mer oligonucleotides containing a tetrahydrofuranyl residue (THF) (5′-cct gcc ctg [THF]gc agc tgt ggg) and an abasic site (AP) (5′-cct gcc ctg [AP]gc agc tgt ggg) were from Trevigen (Gaithersburg, MD, USA). The oligonucleotides harboring a 3′-PG residue (5′-cct gcc ctg-3′[PG]), a 5′-phosphate (5′[P]-gc agc tgt ggg-3′) and the oligonucleotide containing a DHU residue (5′-cct gcc ctg [DHU]gc agc tgt ggg) were synthesized by Eurogentec (Liège, Belgium). The rest of oligonucleotides were synthesized at the Analytical Services of IPBLN (Granada, Spain), including the exonuclease substrates 5′-cctgccctgT and 5′-cctgccctgC. Oligonucleotides were labeled at the 5′-end using [γ-^{32}P]ATP (3000 Ci/mmol; GE Healthcare) and T4 polynucleotide kinase (Promega). The ^{32}P-labeled oligonucleotides were purified using MicroSpin G25 columns (GE Healthcare) and annealed to 1.5-fold molar excess of the complementary strand by incubating at 95°C for 2 min, followed by slow cooling to room temperature. THF was annealed to a complementary oligonucleotide containing A opposite the lesion (CompA); 3′-PG was annealed to CompA and 5′-P to produce a strand break; DHU was annealed to a complementary oligonucleotide with G opposite the base lesion CompG; C and T exonuclease substrates were annealed to CompG and 5′-P. For the preparation of a nicked DNA with a 3′-dRP residue or a 3′-P, the AP site-containing duplex was treated with an excess of *E. coli* endonuclease III (Trevigen) or Fpg (kindly provided by Dr Boiteux), respectively for 1 h at 37°C. The reaction mixture was treated with proteinase K for 30 min at 37°C and extracted with a phenol–chloroform mixture and the DNA was precipitated from the aqueous phase by ethanol precipitation. After drying, the DNA was resuspended in H_2O and re-hybridized.

Histidine-tagged AP endonucleases were purified on Ni^{2+}-charged HiTrap Chelating HP columns (Amersham Biosciences) as previously described (38). The eluates were concentrated and applied to a Superdex-75 gel filtration column (Amersham Biosciences). The His-APE1 and His-LMAP fusion proteins were eluted in phosphate buffer (50 mM sodium phosphate, pH 7.0, 150 mM NaCl and 10 mM 2-mercaptoethanol), aliquoted and stored in 20% glycerol at −80°C. Protein concentration was determined using NanoDrop ND-1000 Spectrophotometer.

Polyclonal rabbit antiserum raised against the recombinant LMAP was generated as previously described (39). The antiserum was purified by chromatography in a protein A-sepharose column (Sigma) according to the manufacturer's instructions. Rabbit anti-human APE1 antiserum was purchased from Alpha Diagnostic (San Antonio, TX, USA). An antibody directed towards *L. major* mevalonate kinase was used as protein loading control (our laboratory stocks).

Enzyme assays and kinetic analysis

In a standard reaction (10 μl final volume), the corresponding ^{32}P-labeled duplex DNA was incubated in reaction buffer [20 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, 1% bovine serum albumin (BSA)] with increasing amounts of AP endonuclease or protein from whole cell extract as indicated in the figures. The NIR activity was assayed in a specific buffer previously described with minor variations (20 mM HEPES–KOH pH 6.8, 25 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT and 1% BSA) (37). Reactions were carried out at 37°C for 10 min and stopped by adding 5 μl of formamide dye, followed by heating for 5 min at 95°C before loading in the gels. When any of these parameters was changed it is indicated in the figure legend. The products of the reactions were resolved by denaturing 20% polyacrylamide gel electrophoresis (PAGE) (19:1 acrylamide:bisacrylamide). Gels were scanned and band intensities quantified using a Storm PhosphorImager and ImageQuant 5.2 software (Molecular Dynamics).

Kinetic analysis was performed using the enzymatic assays described above with variations. Briefly, initial rates of 3′-PG excision were determined by incubating various enzyme concentrations of APE1 (1–5 nM), LMAP (0.1–1 nM), LMAP^{A138D} (1–5 nM) or APE1^{D70A} (0.1–1 nM) with increasing concentrations (31–2000 nM) of the 5′-^{32}P-labeled 3′-PG-containing duplex for 150 s at 37°C under standard reaction conditions. AP site incision initial rates were determined by incubating APE1 (0.1–0.5 nM), LMAP (0.1–0.5 nM), LMAP^{A138D} (1–5 nM) or APE1^{D70A} (1–5 nM) with increasing concentrations (31–500 nM) of the 5′-^{32}P-labeled THF-containing duplex for 150 s at 37°C. In all cases, at least five independent data points from three independent experiments were used. By determining the initial rates (V) at each substrate concentration [S], a plot of substrate concentration against reaction rate was produced. Kinetic parameters were estimated by utilizing the software SigmaPlot 2002 for Windows version 8.0. The K_m and k_{cat} values were determined from nonlinear hyperbolic fits and used to calculate the efficiency of 3′-PG excision and AP site incision (k_{cat}/K_m).

Parasites and transfection

Clone P2 of *L. major* 252 line was a gift from Dr Stephen M. Beverley (Washington University). Promastigote forms were grown at 28°C in M199 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated

fetal bovine serum. Parasites were maintained by sequential passages *in vitro*. Transfection of *L. major* was performed essentially as described (40). Logarithmic-phase promastigotes were transfected by electroporation with 50 μg of plasmid DNA. Clones were obtained by plating on M199 semisolid medium supplements containing 0.032 mg/ml of geneticine (G418, Gibco, Invitrogen). All experiments using transfected cells were carried out in the presence of 0.032 mg/ml G418.

Viability of *Leishmania* upon exposure to H_2O_2 and methyl methanesulphonate

Mid log-phase transfected cells at $10^7 \, ml^{-1}$ in M199 medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen) containing 0.032 mg/ml of geneticine (G418, Gibco Invitrogen) were exposed to increasing concentrations of H_2O_2 (Merck) or MMS (Merck) for 1 h at 28°C. After exposure to the genotoxic agent, cells were centrifuged, washed and resuspended in the same volume of PBS buffer. Cells were then inoculated into 96-well flat bottomed microtiter plates (Nunc, Denmark) at a density of 10^6 cells/100 μl. Wells without cells served as control. Twenty microlitres of Resazurin (1.1 mg/ml, Sigma) were added to each well and incubated in the dark for 1 h at 28°C. After the incubation period, the reaction was stopped by adding 50 μl of 3% Sodium Dodecyl Sulfate (SDS). Cell viability was estimated by measuring the fluorescence at 570 nm excitation wavelength and 590 nm emission wavelength in a SpectraMax® GEMINI EM microplate reader (Molecular Devices, Sunnyvale, CA, USA). Experiments for each compound were performed three times, in duplicate. Standard curves were obtained after serial dilution of *Leishmania* cells in 100 μl of PBS and measurement of the fluorescence after 1 h of incubation with 20 μl of Resazurin.

RESULTS

Genetic selection of a mutant APE1 protein that confers resistance to H_2O_2 in *E. coli*

Having previously shown that APE1 and LMAP differ in their ability to protect *E. coli* against oxidative agents such as H_2O_2 (38), we were interested in gaining insight into the structural determinants that might account for these different properties. Since the respective catalytic domains contain no readily identifiable motifs that could be easily targeted for mutagenesis, a strategy was devised based on the genetic selection of *ape1* mutants that had gained the ability to restore the resistance to oxidative damage of the *E. coli* strain BW528 (*xth nfo*), which is deficient in the major AP endonucleases, exonuclease III and endonuclease IV (41). It can be hypothesized that one such mutant would have an enhanced capability to process oxidative damage by excising the 3′-blocking groups generated at DNA single-strand breaks. To identify such mutants, the *E. coli* expression plasmid expressing full-length *ape1* was randomly mutagenized and selected for colonies that survive after serial treatments with H_2O_2. Several clones were isolated and sequenced. One carrying

a single base mutation ($A_{209} \rightarrow C$) that leads to a D70A substitution in APE1 was chosen for further study. Only another base substitution was identified in other clones consisting in the transition mutation $A_{209} \rightarrow G$ that generates a related amino acid change (D70G).

The primary sequence alignment of APE1 with other AP endonucleases from different species shows that D70 is located in a highly conserved motif (Figure 1A). This residue appears to be restricted to mammalian enzymes since other eukaryotic and prokaryotic sequences have alanine or asparagine at the same position. An exception is human APE2 that, together with yeast APN2, presents an asparagine instead of an aspartate. Interestingly, in the AP endonuclease LMAP from *L. major*, D70 is substituted by an alanine at residue 138. We therefore set out to make the mutant A138D of LMAP in order to confirm the role of this amino acid in oxidative DNA damage processing. Thus, APE1^{D70A} and LMAP^{A138D} were expressed in the BW528 strain to carry out lethality assays with H_2O_2 and MMS. Figure 1B shows the western blot analysis of the level of expression of the four proteins in BW528 at the conditions used in the lethality assays. In agreement with the selection strategy used in the isolation of the APE1^{D70A} mutant that was specifically aimed at identifying the residues involved in H_2O_2-induced damage repair, indeed the expression of APE1^{D70A} mutant significantly increased the survival of the repair-deficient strain treated with H_2O_2. In contrast, cells expressing either APE1 or LMAP^{A138D} were equally sensitive to the oxidative agent and exhibited a behavior similar to the *xth nfo* deficient background (Figure 1C).

To evaluate the effect of the mutations on the repair of cellular AP sites, the resistance of the different strains to the killing effect of the alkylating agent MMS was determined (Figure 1D). The sensitivity of BW528 expressing either APE1 or APE1^{D70A} was similar as previously shown for the mutant APE1^{D70N} (42). Likewise, the expression of LMAP and LMAP^{A138D} reverted the sensitivity of strain BW528 to MMS. These results suggest that, at least in *E. coli*, residues D70 and A138 have a major role in the removal of DNA 3′-blocking lesions generated by oxidative attack while their participation in the processing of AP sites is less critical.

Effect of mutations D70A and A138D on the removal of 3′-blocking termini

To establish the effect of mutations D70A and A138D on the enzyme activities, we first compared the 3′-phosphodiesterase activity of wild-type and mutant enzymes in a standard cleavage assay using nicked DNA with a 3′-PG residue as substrate (Figure 2A). There are disagreeing reports about the electrophoretic behaviour of a PG-containing oligonucleotide respect to the corresponding 3′-OH product. In our hands, the electrophoretic mobility of the 9-mer product was higher than the corresponding 9-mer with the phosphoglycolate group, an observation shared by others (28). Upon incubation at 37°C, 5 nM of purified APE1^{D70A} or LMAP proteins removed most of the 3′-PG residues in 10 min while the same enzyme amounts of APE1 or the LMAP^{A138D}
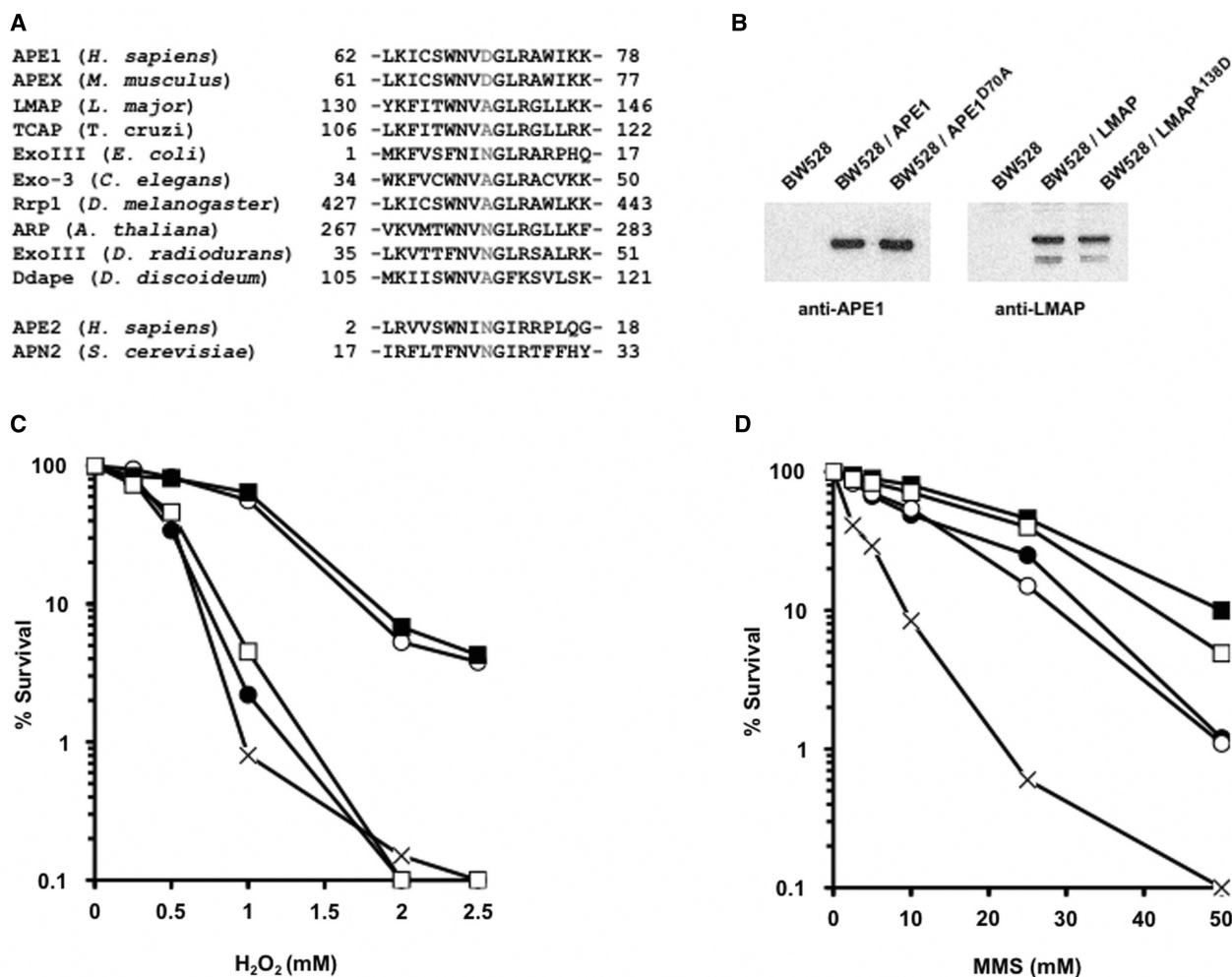
A

APE1 (*H. sapiens*)	62	-LKICSWNVDGLRAWIKK-	78
APEX (*M. musculus*)	61	-LKICSWNVDGLRAWIKK-	77
LMAP (*L. major*)	130	-YKFITWNVAGLRGLLKK-	146
TCAP (*T. cruzi*)	106	-LKFITWNVAGLRGLLRK-	122
ExoIII (*E. coli*)	1	-MKFVSFNINGLRARPHQ-	17
Exo-3 (*C. elegans*)	34	-WKFVCWNVAGLRACVKK-	50
Rrp1 (*D. melanogaster*)	427	-LKICSWNVAGLRAWLKK-	443
ARP (*A. thaliana*)	267	-VKVMTWNVNGLRGLLKF-	283
ExoIII (*D. radiodurans*)	35	-LKVTTFNVNGLRSALRK-	51
Ddape (*D. discoideum*)	105	-MKIISWNVAGFKSVLSK-	121
APE2 (*H. sapiens*)	2	-LRVVSWNINGIRRPLQG-	18
APN2 (*S. cerevisiae*)	17	-IRFLTFNVNGIRTFFHY-	33

B

C

D

Figure 1. Identification of an amino acid residue in the AP endonucleases APE1 and LMAP critical for the resistance to H_2O_2 in *E. coli*. (**A**) Sequence alignment of the APE1 amino acid motif that includes D70 with other AP endonucleases of the exonuclease III family. (**B**) AP endonuclease protein levels in 2 µg of protein cell extracts from each strain were determined by western blotting. (**C** and **D**) Sensitivity assay of *xth nfo E. coli* strains following treatment with DNA damaging agents. *Escherichia coli* strains: BW528/pKK223-3 (cross); BW528/pKK223-LMAP (filled square); BW528/pKK223-LMAP^{A138D} (open square); BW528/pKK223-APE1 (filled circle); and BW528/pKK223-APE1^{D70A} (open circle). A total of 10^8 cells from exponentially growing cultures were exposed to a range of (**C**) H_2O_2 or (**D**) MMS concentrations for 30 min at 37°C in LB medium. All points are the mean of at least three independent experiments.

mutant converted only around 60% and 50%, respectively, of the substrate into the corresponding 3′-OH form. In a time course experiment, the 3′-PG excision activity was analyzed over time intervals during 15 min (Figure 2B). For all four reactions, the product formation was linear over the time points monitored. In agreement with the previous assay, the specific activity was significantly increased in the case of the D70A mutant enzyme, whereas the mutation A138D produced a decrease in 3′-PG excision activity.

In order to quantify the extent by which the mutations affect the catalytic activity of APE1 and LMAP enzymes, the changes in steady-state kinetic parameters were determined for the excision of 3′-PG from the nicked DNA duplex substrate used in standard assays described earlier. The kinetics of excision was determined as a function of 3′-PG substrate concentration under steady-state conditions (Figure 2C and D). An estimation of the k_{cat}/K_m

ratio revealed that the APE1^{D70A} mutant was 7-fold more efficient as a 3′-phosphodiesterase than APE1 and in the same manner, LMAP catalyzes the excision of the 3′-PG moiety at a rate over 5-fold higher than the LMAP^{A138D} mutant. In addition, the data indicate that LMAP removes 3′-PG ends approximately 6-fold more efficiently than wild-type APE1. The differences in catalytic activity arise primarily from evident changes in k_{cat} values, while the K_m for the 3′-PG substrate remains unaltered.

Similar differences were also found for the 3′-phosphodiesterase activity measured on a 3′-dRP substrate (38). Damaged 3′-ends consisting in a nicked DNA with a 3′-dRP residue can arise in the course of BER repair by the action of bifunctional DNA glycosylases such as NTH and OGG1, which are endowed with AP lyase activity and catalyze a β-elimination reaction. Figure 3A shows that the enzymes lacking the aspartate at position 70
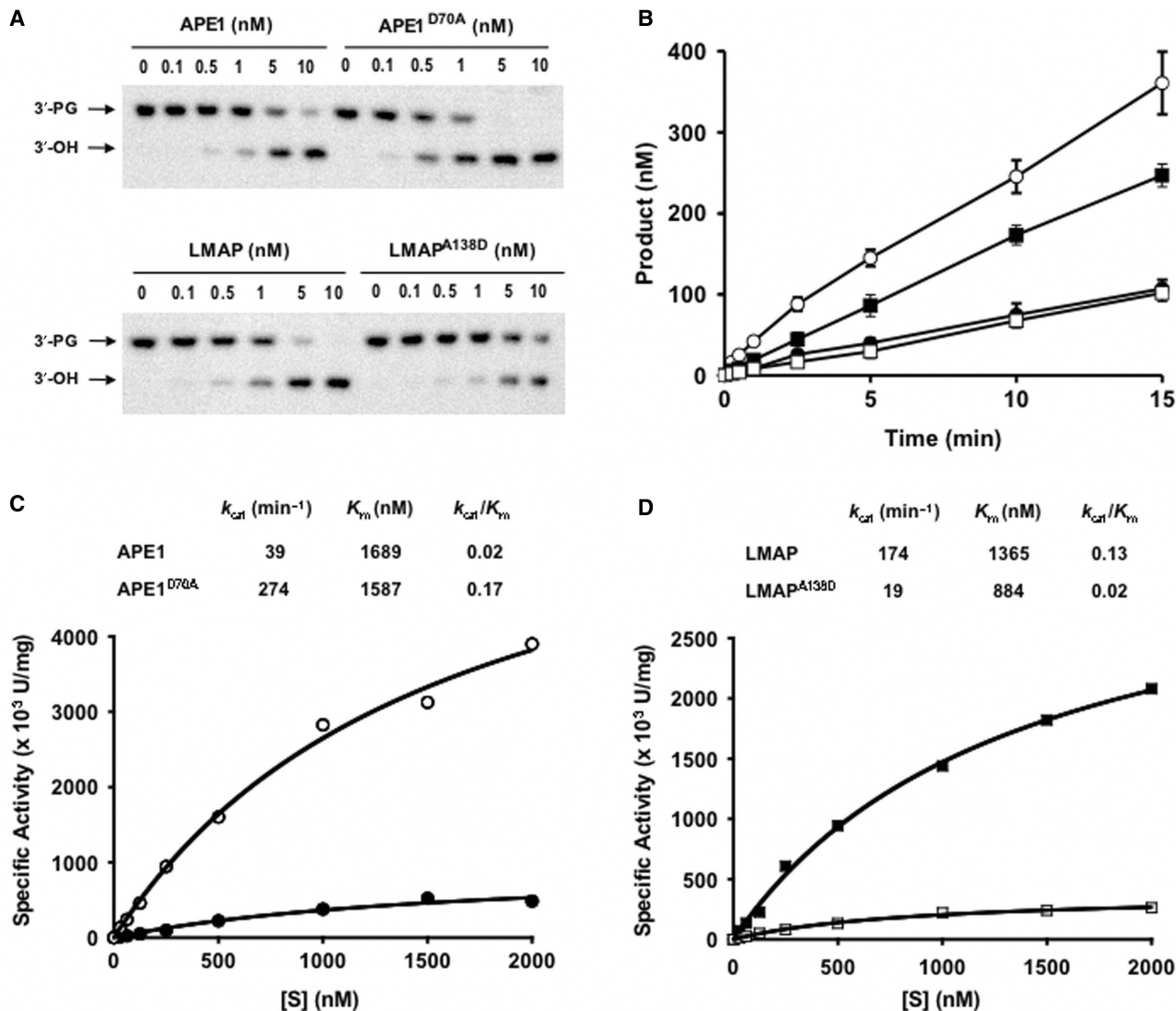
Figure 2. Mutations D70A in APE1 and A138D in LMAP affect the excision of 3′-PG. (**A**) The 3′-phosphodiesterase activity of APE1, APE1[D70A] (upper gel) LMAP and LMAP[A138D] (lower gel) was tested on 21-mer DNA duplex containing a nick with a 3′-PG residue and labeled at the 5′ of the lesion-carrying strand. Increasing amounts of the AP endonucleases (0–10 nM) were incubated for 10 min at 37°C with the oligonucleotide substrate (500 nM) under standard reaction conditions. The products of the reactions were separated by denaturing 20% PAGE. Positions in the gel of the 3′-PG substrate and the 3′-OH product of the reaction are indicated with arrows. (**B**) Time course assay. Excision of phosphoglycolate was monitored over a 15-min time course using 500 nM of 3′-PG substrate and 1 nM of enzyme at 37°C in standard buffer. The reactions were performed and terminated as described in the Materials and Methods section. The amount of product was quantified and plotted for time points between 0 min and 15 min. Experiments were performed in triplicate. (**C** and **D**) Kinetic parameters for the 3′-phosphodiesterase activity of (C) APE1 (filled circle), APE1[D70A] (open circle), (D) LMAP (filled square) and LMAP[A138D] (open square) enzymes. The k_{cat} and K_m values represent the average from three independent determinations. Standard deviations did not exceed 20% for any of the values calculated.

(APE1[D70A]) or 138 (LMAP) have a higher 3′-phospho-diesterase activity also on this type of lesion. Relatively low amounts (0.5 nM) of APE1[D70A] or LMAP catalyze the excision of all of the 3′-dRP substrate used in the assay. For APE1 and LMAP[A138D], the complete excision of the fragmented 3′-phosphoribose residues requires an enzyme concentration five times higher.

When the repair of a modified base is initiated by a NEIL glycosylase, a possible DNA product is a gap with a 3′-P. This toxic intermediate can also be processed by an AP endonuclease, although the 3′-phosphatase activity of APE1 is relatively inefficient (25). We therefore

investigated whether the mutations in APE1 and LMAP also altered their 3′-phosphatase activity. To this end, the purified proteins were incubated with a ^{32}P-labeled 21-mer oligonucleotide duplex carrying a central single-strand break with a 3′-P end and the activity was examined by the substrate conversion into a moiety with lower electrophoretic mobility that corresponds to the 3′-OH product (Figure 3B). The activity assay showed that mutation D70A in APE1 stimulates the excision of the 3′-P from single-strand breaks. The mutation A138D in LMAP had the opposite effect and reduced the 3′-phosphatase activity. Taken together, these results indicate that APE1[D70A]
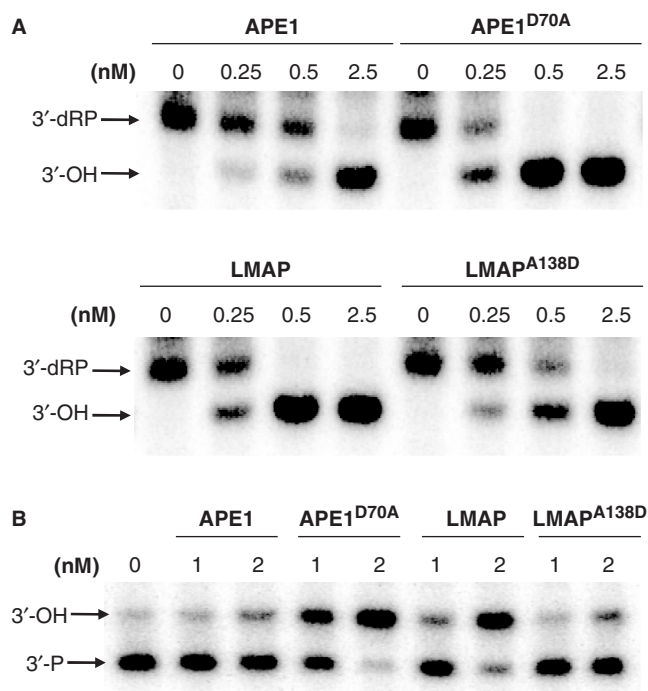
A

B

Figure 3. Effect of the amino acid substitutions on the removal of different 3'-blocking groups. (**A**) The 3'-phosphodiesterase activity of APE1, APE1[D70A] (upper gel), LMAP and LMAP[A138D] (lower gel) was tested on 21-mer DNA duplex containing a nick with a 3'-dRP residue and labeled at the 5' of the lesion-carrying strand. Increasing amounts of the AP endonucleases (0–2.5 nM) were incubated for 10 min at 37°C with the oligonucleotide substrate (5 nM) under standard reaction conditions. (**B**) The 3'-phosphatase activity was tested on a ^{32}P-labeled 21-mer DNA duplex containing a nick with a 3'-P residue produced as described in Materials and Methods section. Two concentrations of enzyme (1 nM and 2 nM) were incubated 10 min at 37°C with the double-stranded oligonucleotide substrate (5 nM) under standard reaction conditions. The products of the reactions were separated by denaturing 20% PAGE. Positions in the gel of the substrates and the 3'-OH product of the reaction are indicated with arrows.

mutant has an increased catalytic capacity to process different types of 3'-damaged ends. Conversely, the mutation A138D impairs the ability of LMAP to excise 3'-blocking termini.

Study of the 3' to 5' exonuclease and nucleotide incision activities of wild-type and mutant AP endonucleases

Oxidized nucleotides such as 8-oxodGTP can be misincorporated during the BER process, giving rise to 3'-blocking ends that inhibit the subsequent DNA ligation step. APE1 is the major exonuclease activity in human cells that excises 3'-8oxoG (36) and removes mismatched nucleotides within a single-strand break. We were therefore interested in finding out how the mutations generated affected the exonuclease function and to this end we determined the excision of dTMP from a T/G mismatch and dCMP from a C/G correct base pair (Figure 4A). The change of aspartate for alanine in APE1 substantially enhanced the removal of the mismatched nucleotide but also increased, although to a lesser extent, the excision of correctly paired nucleotide. On the other hand, mutation A138D in LMAP

decreased the excision of both dTMP and dCMP substrates. It is therefore probable that APE1's exonuclease also participates in the repair of lethal DNA damages generated by misincorporation of oxidized nucleotides to the DNA.

In addition to the exonuclease function, human APE1 is endowed with another enzymatic activity potentially relevant for the protection against oxidative damage. The human enzyme has been reported to initiate the repair of oxidized bases through the incision of the DNA strand at 5' of the damaged base (37). The NIR activity of APE1 provides an alternative and complementary repair pathway to BER. The NIR activity was examined by monitoring the incision of a DHU-containing oligonucleotide substrate (21-mer) under reaction conditions previously described by Gros *et al.* (Figure 4B). In comparison to other activities tested, the incision activity was not significantly modified in the presence or absence of an aspartate in human and leishmanial AP endonucleases suggesting that repair of oxidative base damage induced within DNA has a minor influence in the protection conferred by APE1[D70A] or LMAP. The experiments were carried out in a specific NIR reaction buffer that differs from the AP endonuclease/3'-phosphodiesterase buffer essentially in pH and magnesium concentration. When the reactions were performed using this second buffer, less incision product (9-mer) was detected (data not shown).

Mutations D70A and A138D decrease the AP incision activity

Mutagenesis studies have shown that APE1 mutants at residue D70 display lower AP endonuclease-specific activity than the wild-type enzyme (43,44). To determine the effect of the A138D mutation on the incision of AP sites by LMAP, initial reaction rates were measured by incubating the enzymes with increasing concentrations of 21-nt duplex DNA substrate containing a THF residue. In agreement with the above-mentioned studies, we found that wild-type APE1 cleaves AP sites more efficiently than D70A mutant with a k_{cat} value for the incision of an AP site aproximately 10-fold higher (202 versus 17 min^{-1}) (Figure 5A). No gain of incision activity was observed mutating LMAP to LMAP[A138D] as might have been expected (Figure 5B). Instead, the mutation produced a 3-fold decrease in the k_{cat} value with apparently no effect on the K_m, indicating that D138 in LMAP cannot exert the same function as D70 in APE1. Even though the human and parasite AP endonucleases have structurally similar active sites, subtle differences must exist that affect the processing of AP sites by the two enzymes.

Effect of Mg^{2+} concentration on the enzymatic activities of APE1 and LMAP

Structural and mutagenesis data have led to postulate the participation of the carboxylate group of D70 in the coordination of one metal ion during the catalytical process that leads to AP site cleavage. However, the function of D70 appears to be restricted to mammalian AP endonucleases since this amino acid residue is absent
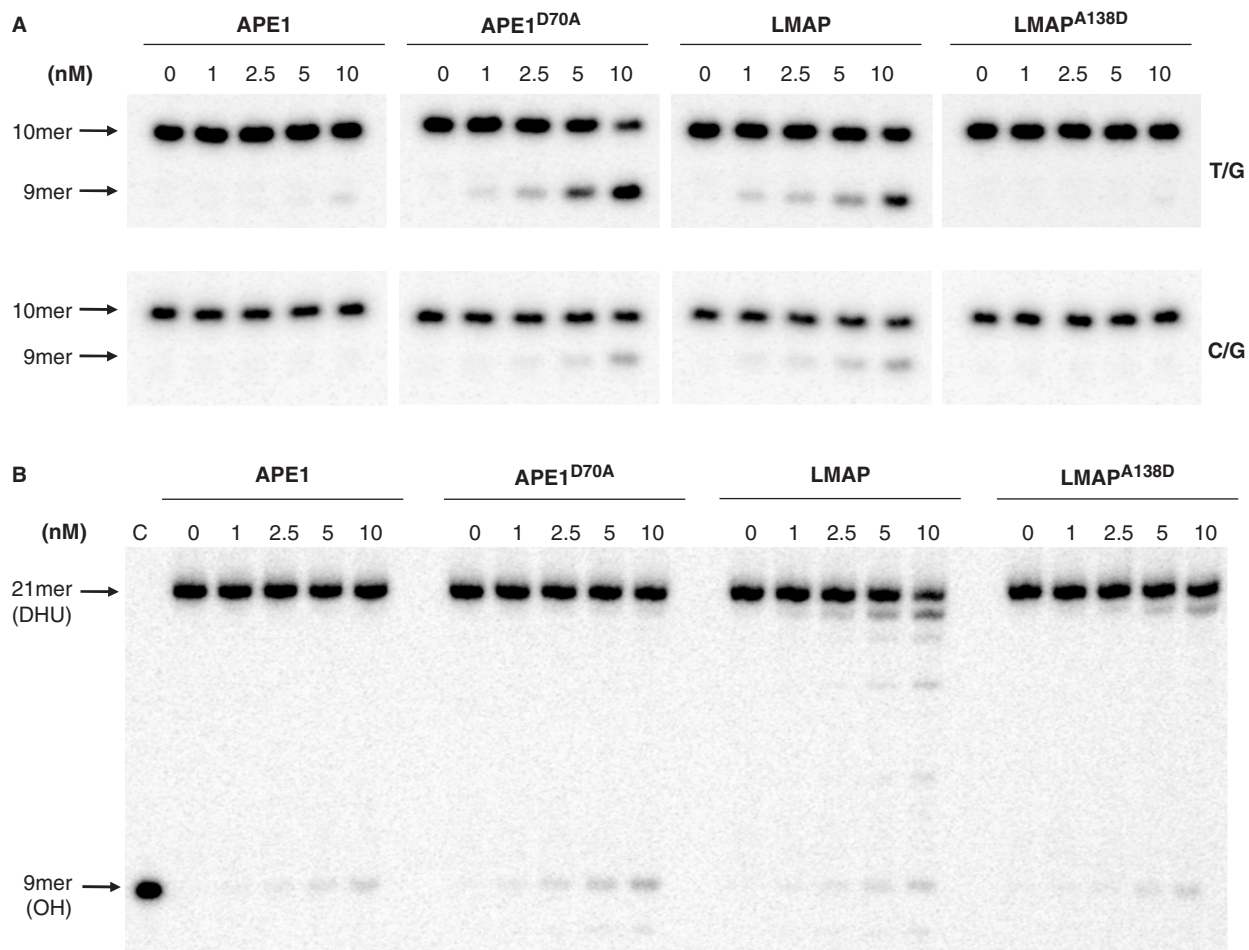
Figure 4. Exonuclease and NIR activities. (**A**) The 3′ to 5′ exonuclease activity of APE1, APE1[D70A], LMAP and LMAP[A138D] was tested on a ^{32}P-labeled 21-mer DNA duplex containing a nick with a 3′-T/G mismatch (T/G) (upper gels) or a 3′-C/G correct pair (C/G) and a phosphate group at 5′. Increasing amounts of the AP endonucleases (0–10 nM) were incubated for 10 min at 37°C with the oligonucleotide substrate (5 nM) under standard reaction conditions. (**B**) The NIR activity was tested on a ^{32}P-labeled 21-mer DNA duplex containing a DHU residue. The enzymes (0–10 nM) were incubated for 10 min at 37°C with the double-stranded oligonucleotide substrate (5 nM) under specific NIR reaction conditions described in Materials and Methods section. The products of the reactions were separated by denaturing 20% PAGE. A 9-mer oligonucleotide was loaded as migration control (C) for the NIR product. Positions in the gel of the substrates and the 3′-OH product of the reaction are indicated with arrows.

in the catalytic sites of protozoan and prokaryotic enzymes. To investigate the role of D70 in metal ion coordination, we compared the activities of APE1, LMAP and their respective mutant forms over a range of Mg^{2+} concentrations. Irrespectively of the concentration tested, APE1[D70A] and LMAP showed higher 3′-phosphodiesterase activity than APE1 and LMAP[A138D] (Figure 6A and B, left). In agreement with previous studies, APE1 (and LMAP[A138D]) reached a maximum of 3′-PG excision activity at Mg^{2+} concentrations around 2.5 mM, the excision being severely inhibited (below 25%) at 10 mM and higher ion concentrations (Figure 6A and B, right) (45,46). In contrast, APE1[D70A] and LMAP exhibited a different pattern of inhibition by magnesium. Both enzymes reached maximum activity at higher metal concentrations and were more refractory to Mg^{2+} inhibition, thus still retaining about 50% of activity at 20 mM (Figure 6A and B). These results confirm that amino acid changes D70A in APE1 and

A138D in LMAP have opposing effects on the 3′-phosphodiesterase activity and show that the residue D70 has an important role in magnesium-mediated inhibition. Thus, when a certain metal concentration is reached, D70 may bind to Mg^{2+} which results in inhibition of the enzyme activity.

To investigate whether the D70A mutation also altered Mg^{2+} inhibition using a different substrate, the AP endonuclease activity was measured on a THF-containing oligonucleotide at a variety of magnesium concentrations. Under the conditions assayed, APE1 exhibited the highest activity at 5 mM of metal while the specific activity of APE1[D70A] did not reach a maximum until 40 mM of Mg^{2+} was added to the reaction (Figure 6C). Inhibition was not evidenced until 80 mM was reached (data not shown). For the parasite enzymes LMAP and LMAP[A138D], the activity peaks were also obtained at different magnesium concentrations (10 and 2.5 mM, respectively) (Figure 6D). For both enzymes containing
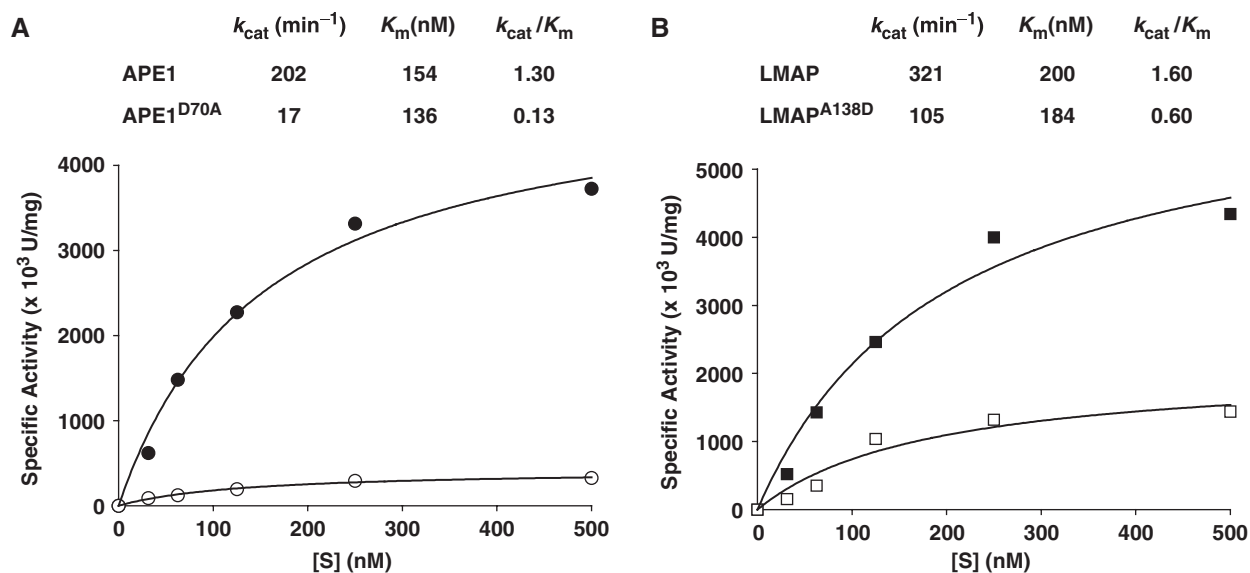
A

	k_{cat} (min^{-1})	K_m(nM)	k_{cat}/K_m
APE1	202	154	1.30
APE1^{D70A}	17	136	0.13

B

	k_{cat} (min^{-1})	K_m(nM)	k_{cat}/K_m
LMAP	321	200	1.60
LMAP^{A138D}	105	184	0.60

Figure 5. Michaelis–Menten plots for the calculation of the K_m and V_{max} values of the AP endonuclease activity. (**A**) APE1 (filled circle), APE1^{D70A} (open circle); (**B**) LMAP (filled square), LMAP^{A138D} (open square). The k_{cat} and K_m values represent the average from three independent determinations. Standard deviations did not exceed 20% for any of the values calculated.
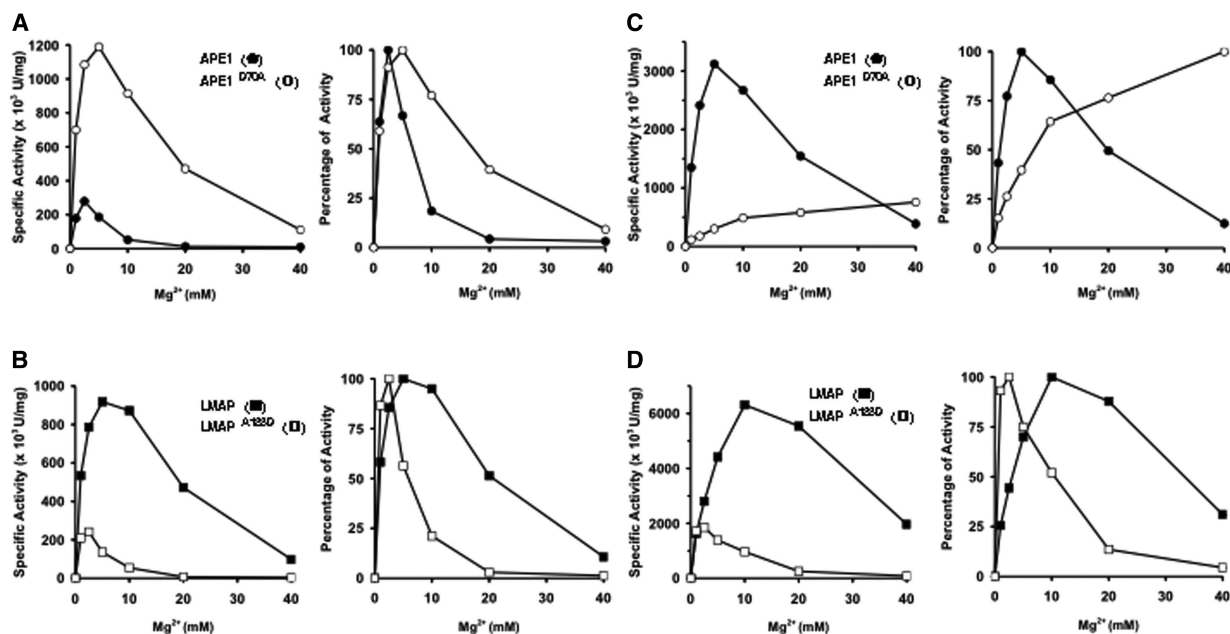
Figure 6. Comparison of the activities of wild-type and mutant AP endonucleases at different magnesium concentrations. (**A** and **B**) The 3′-phosphodiesterase and (**C** and **D**) AP endonuclease activity assays were carried out in standard buffer with increasing Mg^{2+} concentrations (1–40 mM) by incubating the enzymes with 500 nM of the 3′-PG or THF substrate, respectively. Specific activities (left) and relative activities (right) are represented for each of the enzymes. Relative activities were calculated assigning a value of 100% to the maximal activity reached for an enzyme at a given Mg^{2+} concentration. To monitor the excision of phosphoglycolate and AP site incision in the absence of magnesium in the reaction mixture, a reaction with 20 mM EDTA was included. Enzymes are represented with the following symbols: APE1 (filled circle), APE1^{D70A} (open circle), LMAP (filled square) and LMAP^{A138D} (open square). Units are defined as picomoles of reaction product generated per minute at 37°C.

aspartate, the AP endonuclease activity was inhibited by lower metal concentrations. However, it should be noted that at optimal metal concentrations, both mutant enzymes have lower AP endonuclease activities than the respective wild-type proteins.

Over-expression of LMAP^{A138D} sensitizes *Leishmania* cells to oxidative agents

The biochemical analysis of the mutants described in this work evidences subtle but significant differences between the parasite and human AP endonucleases that could
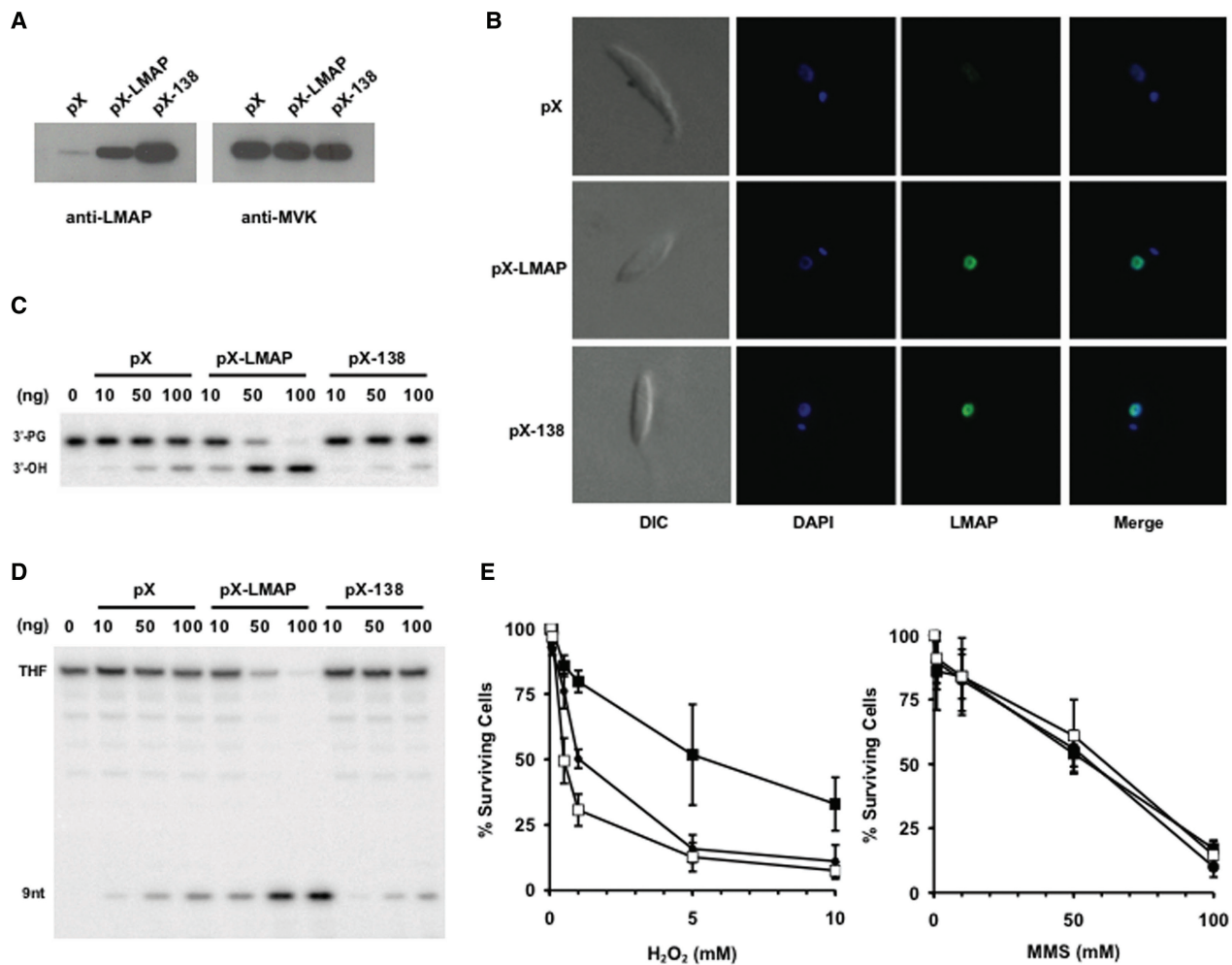
Figure 7. Sensitivity of *Leishmania* cells to H_2O_2 and MMS-induced DNA damages. (**A**) Western blot showing LMAP protein levels in whole cell extracts from 2.5×10^6 parasites harboring vector pX63-Neo (cell line pX), expressing wild-type LMAP (cell line pX-LMAP) or LMAP[A138D] (cell line pX-138). As control of charge, level of mevalonate kinase protein was measured in the three cell lines. (**B**) Immunofluorescence analysis was performed to establish the intracellular localization of LMAP in *L. major* cell lines pX, pX-LMAP and pX138. Nuclear and kinetoplast DNA were stained with DAPI and LMAP and LMAP[A138D] were detected with anti-LmAP and FITC conjugated anti-rabbit secondary antibody. Images were collected with a Zeiss Axiophot microscope. (**C**) The 3'-phosphodiesterase and (**D**) AP endonuclease activities of whole cell extracts from cell lines pX, pX-LMAP and pX-138 were tested on 21-mer DNA duplex containing a nick with a 3'-PG residue or a THF residue, respectively, and labeled at the 5' of the lesion-carrying strand. Increasing amounts of protein (0–100 ng) were incubated for 30 min at 37°C with the oligonucleotide substrate (2000 nM) under standard reaction conditions. The products of the reactions were separated by denaturing 20% PAGE. (**E**) A total of 10^7 cells/ml from mid-log phase *Leishmania* cultures were exposed to increasing concentrations of H_2O_2 (left) or MMS (right) concentrations for 1 h at 28°C in M199 medium supplemented with 10% fetal bovine serum. After exposure to the genotoxic agent, cells were centrifuged, washed, resuspended in the same volume of PBS buffer and incubated with resazurin for 1 h at 28°C. Fluorescence was measured at 570 nm excitation wavelength and 590 nm emission wavelength. Experiments for each compound were performed three times, in duplicate.

affect the catalytic process during oxidative DNA-damage repair. To further examine the role of residue A138 of the LMAP enzyme in parasite survival under genotoxic stress, the mutant enzyme LMAP[A138D] was over-expressed in *L. major* cells. A cell line over-expressing LMAP[A138D] (pX-138) was generated by transfection with the expression vector pX63Neo-LMAP[A138D] and selection of clones resistant to G418 at 32 µg/ml. Cell lines over-expressing LMAP wild-type (pX-LMAP) and a control cell line (pX) harboring the empty expression vector have been obtained and described elsewhere (47). The level of expression of the selected clones was determined by western blot using a purified antibody directed against pure recombinant

LMAP (Figure 7A). Protein quantity standardization was carried out using an antibody directed against *L. major* mevalonate kinase (MVK). Cell lines pX-LMAP and pX-138 expressed 9- and 16-fold higher levels of LMAP, respectively, than cell line pX. It was also checked that growth rate was not affected by the protein over-expression.

The cellular localization of the expressed proteins was determined by immunofluorescence microscopy using the purified antibody mentioned above. As shown in Figure 7B, LMAP and the mutant LMAP form were both clearly located in the nucleus. Constitutive endogenous expression levels of LMAP are significantly lower than

those obtained in over-expressing parasites. In mammalian cells, different forms of APE1 have been localized in the nucleus and mitochondria (48,49). Even in cells that over-express the protein, no LMAP appeared to be associated with the mitochondrion or kinetoplast. However, we cannot rule out the possibility that a minor fraction is present in this organelle.

To further characterize the constructed cell lines, whole cell extracts were assayed for cleavage activity using a DNA substrate harboring a nick with a 3′-PG end or an AP site analog (Figure 7C and D, respectively). The expression of the LMAP wild-type protein in the selected clone was associated with at least a 5- and 8-fold increase in the 3′-PG excision and AP site-cleavage activity levels, respectively. In contrast, the over-expression of LMAP^{A138D} in cell line pX-138 reduced the level of 3′-phosphodiesterase around 5-fold respect to the endogenous activity of cell line pX (according to the product formed at 100 ng of protein extract) while surprisingly, the level of AP site incision remained similar. It should be taken into consideration that repair activities of recombinant proteins may not necessarily reflect effective cellular repair since factors that modulate incision activities may be absent in the *in vitro* assay.

In an earlier study it was demonstrated that parasites overproducing LMAP have an enhanced resistance to H$_2$O$_2$ (47). Here, we compared the viability of cell lines overproducing either wild-type LMAP or LMAP^{A138D} in the presence of the DNA damaging agents H$_2$O$_2$ and methyl methanesulphonate. Figure 7E (left) shows the effect on cell survival after 1-h exposure to increasing concentrations of H$_2$O$_2$. Following treatment with 0.5 mM H$_2$O$_2$, the number of viable cells expressing LMAP^{A138D} is reduced to 50% while the proportion of surviving cells transfected with the empty vector or over-expressing LMAP remains 76% and 86%, respectively. Indeed, exposure to 1 mM H$_2$O$_2$ has a minor effect on the survival of cell-line pX-LMAP (80%) but produces a considerable impact on cell line pX (50%) and even more pronounced on cell line pX-138 (30%). Therefore, while parasites expressing LMAP were protected against H$_2$O$_2$, cells over-expressing LMAP^{A138D} became even more sensitive to killing by this oxidative agent than cells transfected with the empty vector. It can be concluded that residue A138 plays an important role in the repair of oxidative DNA damage by LMAP and its substitution by an aspartate has an impact on the viability of cells exposed to oxidative agents. In contrast, the expression of the LMAP or LMAP^{A138D} proteins did not have a significant effect on survival after treatment with MMS, which alkylates mainly purine bases in DNA and produces AP sites (50) (Figure 7E, right). Similar results have been obtained expressing the *Ape1* cDNA in CHO cells (51), suggesting that the constitutive levels of AP-endonuclease activity in both organisms are sufficient to repair the AP sites generated by alkylating agents. Thus, the lethal effect observed after MMS treatment is probably due to the block of DNA replication by the alkylation-induced DNA adducts such as N^3-methyl adenine.

DISCUSSION

Human APE1 protein has been subjected to an exhaustive search for residues that are critical for cleavage of DNA at AP sites (43,45,46,52–61). Active site residues involved in hydrolysis and metal coordination or AP site binding have been identified by site-directed mutagenesis on highly conserved amino acids and by the analysis of the crystal structure of APE1 bound to substrate abasic DNA (56). Besides its AP endonuclease activity, APE1 can remove some types of 3′-blocking lesions at DNA single-strand breaks *in vitro*, on account of its 3′-phosphodiesterase, 3′ to 5′ exonuclease and 3′-phosphatase activities. However, even if the different nuclease activities of the APE1 protein appear to share a common active site, the human enzyme carries out these other types of chemical reactions at a lower rate than AP site cleavage (19,25,38,62). It results obvious that some unnoticed structural elements of the enzyme must modulate substrate specificity, although to date, the catalytical determinants for the hydrolysis of 3′-end damaged termini have not been fully understood. More intriguingly, other AP endonucleases belonging to the same family, such as exonuclease III and the AP endonuclease from *L. major* LMAP, share with APE1 the overall 3D structure and most of the catalytic groups in their active sites and can catalyze the removal of damaged nucleotides and phosphate groups from 3′-ends more efficiently than APE1 (38,63,64). All the biochemical reactions mentioned, including AP site cleavage, entail the hydrolysis of a P–O bond but the substrates differ in size, charge and/or structure. It is therefore of interest to understand how similar catalytic sites can account for different substrate specificities.

As shown by protein engineering and *in vitro* evolution experiments, single amino acid substitutions can drastically change substrate specificity and increase the activity of any enzyme. In this regard, there are several examples of how mutations can alter the activity and reaction specificity of DNA repair enzymes (65). We have taken advantage from such functional plasticity and using a complementation assay in a repair-deficient *E. coli* strain, have selected a mutant in the nuclease domain of the human AP Endonuclease 1 (APE1^{D70A}) that confers resistance to H$_2$O$_2$-induced DNA damage. D70 is a poorly conserved residue located in the middle of a highly conserved region comprising amino acids 62–78 of the human protein (Figure 1A). *In vitro*, the purified APE1^{D70A} protein shows a higher capacity to process different types of 3′-blocking ends including 3′-PG, 3′-dRP and 3′-P groups. It also has an enhanced exonuclease activity on mismatched 3′-nucleotides within single-strand breaks. A comparative analysis with APE2 has allowed for the identification of F266 and W280 as two residues critical for 3′ to 5′ DNA exonuclease activity and APE1 mutants with increased exonuclease activity have been generated (66). However, these residues do not appear to be responsible for the increased phosphodiesterase and exonuclease activities of *Leishmania* AP endonuclease which exhibits the same residues as APE1.

Our results suggest that aspartate 70 modulates the substrate specificity of the enzyme. In agreement, the change of alanine by an aspartate at the equivalent position in LMAP clearly decreases the excision of 3′-blocking ends by the parasitic enzyme. A kinetic analysis shows that the replacement of aspartate by an alanine in APE1 enhances the rate of cleavage of 3′-PG termini without affecting the K_m, which indicates that catalysis or a step following catalysis is accelerated by the amino acid change. In other words, the aspartate residue limits somehow the catalytic turnover of APE1 and LMAP^{A138D} for 3′-damage excision. It is worth noting that this negative effect is probably due to the charge and not the size of the aspartate side chain since the catalytically efficient exonuclease III and human APE2 have asparagine at the same position. On the contrary, D138 in LMAP and D70 in APE1 must play different roles during the AP site incision reaction since both D70A and A138D mutations reduce the rate of AP site cleavage. It is probable that the parasitic enzyme possesses adaptive second site features that substitute the function carried out by D70 in human APE1 and confer the maximal AP endonuclease activity.

Several structural and biochemical studies have suggested that amino acid D70 contributes to the coordination of the cationic metal at the active site of APE1 (44,46,54). However, the aspartate is substituted by asparagine or alanine in other AP endonucleases (i.e. exoIII and LMAP) and its replacement by mutagenesis does not have a dramatic effect on the incision activity of APE1 (43; this study). When the specific activities of wild-type and mutant enzymes were determined over a range of metal concentrations, the activity versus magnesium concentration curves showed two different effects of the metal ion as previously described. Low magnesium concentrations stimulate catalysis, as this ion is required for the cleavage reaction, while high metal concentrations result inhibitory for the activity (52). Furthermore, lower magnesium concentrations were required for inhibition of wild-type APE1 than for the mutant D70A. The opposite effect was observed with LMAP and mutant A138D indicating that metal ion-mediated inhibition is more pronounced on those enzymes with aspartate. The inhibition of APE1 activity by high Mg^{2+} concentrations has been hypothesized to be due to an oversaturation of the enzyme catalytic site when the two proposed existent metal sites are occupied simultaneously (67). In this scenario, it can be hypothesized that at high metal concentrations, aspartate 70 attracts Mg^{2+} ions to the active site that interfere with the catalytic process. A close examination of the APE1-DNA crystal structure suggests an alternate possibility. The situation of D70 makes possible an interaction with the DNA strand complementary to the AP site DNA. Such interaction might distort the DNA and contribute to the positioning of the substrate in the active site. At inhibiting metal concentrations, the 'extra' metal ions recruited by D70 would perturb the interaction and hence, the substrate alignment at the catalytic site. The partial loss of AP incision activity of the D70A mutant might be due at least in part, to a poor substrate orientation in the active site when the aspartate is absent. It is tempting to speculate that the interaction between D70 and the DNA strand opposite to the AP site (when D70 is not bound to Mg^{2+}) favors cleavage of AP sites by exerting an influence on the DNA conformation as described above, but somehow impedes the correct positioning of the more 'bendable' bonds that bear phosphoglycolates, phosphates or mismatched nucleotides. We are aware that the available data are not sufficient to understand the role of residue D70 during AP site or 3′-end cleavage and further structural and biochemical experimentation will be required to fully establish its function.

Regardless of the possible catalytic mechanism, our work reveals significant differences in the processing of 3′-blocking DNA damages between kinetoplastid parasites and humans. The presence of alanine or aspartate in the AP endonucleases from these two organisms provides an exceptional perspective on the molecular evolution of their DNA repair responses to oxidative damage. It is reasonable to think that genetic changes took place probably according to the amount and different types of DNA damage they were exposed to. In *Leishmania* cells, the efficient functioning of its oxidative damaged DNA repair pathways is likely critical for survival during the oxidative burst that takes place in the mammalian host. In support of this, we have shown that over-expression of LMAP protects the parasite against high levels of oxidative stress induced by H_2O_2. Although DNA single-strand breaks are probably the primary lethal lesion induced by H_2O_2, AP site and exonuclease repair activities of APE1 can possibly contribute to this protection. In contrast, expression of mutant LMAP^{A138D} protein sensitizes the cells treated with this oxidative agent. An excess of mutant enzyme in the cell may block access to the 3′-damaged strand breaks of the endogenous LMAP thus slowing down repair and increasing cell death. Taken together, our results provide novel structural and functional information that might be exploited in the rational design of selective inhibitors to treat leishmaniasis.

DNA single-strand breaks are also frequent lesions in human DNA and their repair of vital importance for the cell. *In vivo*, the catalytical deficit of APE1 as 3′-phosphodiesterase might be compensated by the action of other factors that activate or complement APE1 function. For instance, the XRCC1 protein, absent in *Leishmania*, has been shown to act as a scaffold and modulator of different BER activities. XRCC1 physically interacts with APE1 and stimulates, at least *in vitro*, its AP endonuclease and 3′-phosphodiesterase activities (68). In addition, recent studies show that human cells possess other enzymes with strong 3′-phosphodiesterase activities *in vitro* like APE2, a second class II AP endonuclease, and aprataxin, the gene product defective in the neurological disorder AOA1 (ataxia oculomotor apraxia-1). These enzymes might assist APE1 in the repair of 3′-PG lesions (32,33). Thus, human cells have evolved to possess a variety of DNA repair factors capable to initiate the processing of DNA single-strand breaks and maintain genome integrity. Why one of these major factors, APE1, has not been fully optimized during evolution in terms of reaction rate is an important question that should be answered in order to understand its function.

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