Nucleic Acids Research, 1999, Vol. 27, No. 3 771–777

Apurinic/apyrimidinic endonuclease genes from the Trypanosomatidae *Leishmania major* and *Trypanosoma cruzi* confer resistance to oxidizing agents in DNA repair-deficient *Escherichia coli*

Juana Pérez[+], Cláribel Gallego, Víctor Bernier-Villamor, Ana Camacho, Dolores González-Pacanowska and Luis M. Ruiz-Pérez*

Instituto de Parasitología y Biomedicina 'López-Neyra', Consejo Superior de Investigaciones Científicas, C/ Ventanilla 11, 18001-Granada, Spain

Received October 1, 1998; Revised and Accepted December 4, 1998

DDBJ/EMBL/GenBank accession nos U92486, U92487

ABSTRACT

Apurinic/apyrimidinic (AP) sites in DNA are considered to be highly mutagenic and must be corrected to preserve genetic integrity. We have isolated cDNAs from the Trypanosomatidae *Leishmania major* and *Trypanosoma cruzi* capable of complementing the deficiency of exonuclease III and dUTPase in the *Escherichia coli* mutant BW286. This double mutant is non-viable at 37°C due to an accumulation of non-repaired sites following excision of uracil from DNA. The genes were expressed as β-galactosidase–AP endonuclease fusion proteins and as such are active in repair of AP sites in *E.coli*. The *Trypanosoma* and *Leishmania* sequences have unique N-termini containing sequences that correspond to probable nuclear transport signals, while the C-terminal domains exhibit pronounced similarity to exonuclease III. The *L.major* gene was overexpressed as a histidine-tagged protein and recombinant enzyme exhibited endonuclease activity on AP DNA *in vitro*. Furthermore, expression of the enzymes in AP endonuclease-deficient *E.coli* mutants conferred significant resistance to killing by methylmethane sulphonate and peroxides. This study constitutes one of the first descriptions of DNA repair enzymes in these pathogenic organisms where oxidative stress is an important mechanism of both drug-mediated and intracellular killing.

INTRODUCTION

Active oxygen species such as the superoxide radical, hydroxyl radical and H_2O_2 can directly or indirectly damage cellular DNA. These species are produced during normal aerobic metabolism (1) or from exposure to exogenous agents (2). DNA damage includes modified bases, abasic (AP) sites and strand breaks with deoxyribose fragments at their termini. Non-oxidized AP sites are also formed by spontaneous hydrolysis (3) and by alkylating agents, for example methylmethane sulphonate (MMS), as secondary products when damaged bases hydrolyze from DNA spontaneously or with the assistance of DNA N-glycosylases (4–6).

AP sites are both cytotoxic and mutagenic and must be corrected to restore genetic integrity. The major enzymes initiating this DNA excision repair process are AP endonucleases, the main class of which (class II AP endonucleases) hydrolyze the phosphodiester bond immediately 5′ of the abasic site (7). Many AP endonucleases also present 3′-repair diesterase activity that selectively removes fragments of deoxyribose from 3′-termini of DNA strand breaks produced by free radical attack (8–10).

Escherichia coli contains two major class II AP endonucleases, exonuclease III that accounts for 95% of the total AP endonuclease activity in extracts of untreated wild-type *E.coli* (11) and endonuclease IV which is inducible by superoxide generating agents (12). *Escherichia coli* mutants lacking either of these activities showed increased sensitivity to killing by the oxidants H_2O_2 and bleomycin and to the alkylating agent MMS (13; reviewed in 8), indicating the physiological importance of these enzymes.

The high sensitivity of parasites to the reagent H_2O_2 and organic peroxides (14) and to both drug-generated and phagocyte-derived oxygen reactive species (15,16) is well documented. Likewise, the resistance capacity to H_2O_2 of *Leishmania donovani* has been directly related to the virulence of the promastigote forms of that strain (17). However, while parasites, and in particular protozoa, are highly sensitive to oxidative damage which is the main mechanism of intracellular amastigote killing, little is known regarding the enzymatic mechanisms involved in DNA repair or how the inappropriate oxidized bases are excised from the parasite genome.

Here we describe the isolation of cDNA clones from the Trypanosomatidae *Leishmania major* and *Trypanosoma cruzi* encoding DNA repair enzymes with marked sequence similarity to exonuclease III. The cDNAs were obtained by screening an expression library for complementation of deficiency of exonuclease III and dUTPase in *E.coli*. The purified recombinant protein showed endonuclease activity on AP DNA. Expression of

*To whom correspondence should be addressed. Tel: +34 958 203802; Fax: +34 958 203323; Email: lmruiz@ipb.csic.es

[+]Present address: Departamento de Microbiología, Facultad de Farmacia, Campus Cartuja, Universidad de Granada, 18071 Granada, Spain

L.major and *T.cruzi* AP endonucleases, under the control of different promoters, conferred resistance to oxidant and alkylating agents in *E.coli* mutants lacking either exonuclease III or endonuclease IV. Thus, AP endonucleases from Trypanosomatidae can function in bacteria exerting the proposed functions for exonuclease III in *E.coli* regarding repair of AP sites generated by peroxides and alkylating agents.

MATERIALS AND METHODS

Strains and growth media

The bacterial strains used in this study derive from *E.coli* K-12 and were provided by Dr Bernard Weiss (Johns Hopkins University School of Medicine, Baltimore, MD): AB1157 wild-type, BW286 [*dut-1*, Δ(*xth-pncA*)] (18) and BW528 [*nfo-1*::Kan Δ(*xth-pncA*)] (13) were grown in standard LB or on LB agar. For the BW286 strain (18), LB was supplemented with 250 µg/ml thymidine. The *T.cruzi* Y strain and *L.major* 252 strain were grown in LIT and M199 medium (Gibco) respectively, both of them supplemented with 10% fetal bovine serum (Gibco).

Trypanosoma cruzi and *Leishmania major* cDNA libraries

The cells used for construction of cDNA libraries were epimastigotes from the Y strain of *T.cruzi* and promastigotes derived from the 252 strain of *L.major*. Log phase, 24×10^6 parasites/ml for *T.cruzi* and 17×10^6 parasites/ml for *L.major*, were used. Isolation of poly(A)$^+$ mRNA, library construction and complementation screening were carried out as described (19). Sequencing was performed using the dideoxy Sanger terminator method in an Applied Biosystem 373 DNA sequencer. Appropriate primers were synthesized at the Analytical Services of the Instituto de Parasitología y Biomedicina, Granada. General methods for DNA manipulation were as described (20).

Leishmania major genomic DNA cloning

A genomic *L.major* library was constructed in λEMBL3 (Stratagene) and packed with GigapackR III Gold Packaging Extract (Stratagene). Approximately 6×10^5 p.f.u. were replica plated onto nitrocellulose and screened. An *Eco*RI fragment (1079 bp) from the partial cDNA of *L.major* was used as a probe for the screening. Five positive clones were isolated and one was selected for digestion mapping and analysis. After digestion with *Sal*I, a 4.1 kb fragment was cloned in pBluescript KS(–) that contained, along with 3.6 kb of the 5′ upstream genomic region, 514 bp of the 5′ coding sequence of the *L.major* AP endonuclease gene. The genomic sequence was fully determined by the analysis of another 2.1 kb *Sal*I fragment containing the remaining 827 bp of the 3′ region of the AP endonuclease gene together with 1273 bp of the 3′-UTR and agreed with the one obtained by cDNA sequencing.

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank databases under accession nos U92486 and U92487 for the *tcap* and *lmap* sequences respectively.

RT–PCR analysis

For mapping the splice acceptor site, cDNA was synthesized using as primer an oligonucleotide complementary to positions +516 to +535 of the *tcap* gene. The resulting cDNA was used as template in a first PCR using as primers the same oligonucleotide and a 23mer complementary to the 3′-end of the splice leader sequence (23-SL) of *T.cruzi*. The reaction products were diluted and used as template in a second PCR reaction using as primers 23-SL and an oligonucleotide complementary to positions +258 to +277. Products were separated by gel electrophoresis and hybridized with *tcap*.

Construction of plasmids for expression in DNA repair-deficient *E.coli*

For expression and complementation studies in *E.coli*, the entire coding sequence of the *T.cruzi* gene (*tcap*) was amplified using the PCR technique and cloned in pKK223.3 (Pharmacia). Oligonucleotide primers for amplification of the AP endonuclease coding region, ATG-AP (TATGCCGTCGGGACCTAAG) and TGA-AP (ACGCGGATCCAAGCTTATCACCTGCGCAG) were synthesized. ATG-AP was designed so that blunt ends generated by the PCR reaction were ligated into the *Sma*I site of the vector. TGA-AP contained a *Hin*dIII restriction site. The *tcap* gene was inserted downstream of the *tac* promoter of pKK223.3 to give plasmid pKtcap. Double strand DNA sequencing was performed to confirm the correct sequence after amplification.

Complementation studies

Plasmids pBKlmap, pBKtcap and pKtcap were used to transform by electroporation the different *E.coli* expression hosts AB1157, BW286 and BW528. Bacteria were grown in LB containing 50 mg/l ampicillin and/or 50 mg/l kanamycin. pKK223.3 and a pBK-CMV derivative containing 1330 bp of a non-coding sequence were used as controls (pBKdv). The gradient plate assays were prepared basically according to Cunningham *et al.* (13). To 50 ml of molten (50°C) LB agar, agents were added. The medium was poured into 12 × 12 cm square Petri dishes elevated at one edge. After the agar hardened, the plate was leveled and overlaid with another 50 ml of molten LB agar without agents. Fresh stationary phase bacterial cultures were diluted to an OD_{595} of 0.5. An aliquot of 40 µl of culture was diluted further in 2 ml of molten soft agar to 42°C and transferred to a microscope slide. Gradient plates were inoculated by dipping the edge of a warm microscope slide in the suspension and then touching the surface of the plate. Incubation was for 48 h at 28°C for BW286 cells and 24 h at 37°C for AB1157 and BW528. To visualize bacterial growth, plates were stained with an acridine orange solution and photographed under UV light. The average of at least three experiments using three independent transformants for each plasmid are shown in the figures. Standard deviations did not exceed 5% the average value.

The agents assayed were the alkylating agent MMS (Merck) and the chemical oxidants H_2O_2 and tBO_2H (t-butylhydroperoxide). The concentration of the agents distributed from bottom to top were: 0–12 mM MMS, 0–28 µM tBO_2H and 0–2 mM H_2O_2 for constructs using pBK-CMV and 0–6 mM MMS, 0–14 µM tBO_2H and 0–1 mM H_2O_2 for constructs performed using pKK223.3.

Expression and purification of recombinant protein

The *lmap* gene coding sequence was isolated by PCR and cloned into the *Nde*I and *Hin*dIII sites of pET28a (Novagen). The resulting plasmid, pETlmap, was sequenced in order to confirm that the correct reading frame was used, with the polyhistidine tail

placed in the N-terminal position. This construct was used to transform the *E.coli* expression host BL21(DE3). The production of polyhistidine–LMAP fusion proteins was induced in mid logarithmic *E.coli* cultures grown in LB containing 50 μg/ml kanamycin by 0.4 mM isopropyl-1-thio-β-D-galactoside. After 6 h of growth at 37°C, bacteria were pelleted by centrifugation, resuspended in 4 ml of 5 mM imidazole, 50 mM NaCl, 20 mM Tris–HCl, pH 7.9, buffer and sonicated. The soluble supernatant was applied to a nickel-chelated agarose affinity column and protein was purified as described by the manufacturer (Novagen).

Enzyme assays

An 800 bp fragment of a non-coding sequence from *L.major* was used as template for PCR amplification using dUTP instead of dTTP. This DNA was named U-DNA. Approximately 8 μg of GeneClean (Promega)-purified U-DNA was used as substrate for *E.coli* uracil-DNA glycosylase (1 U) (Boehringer Mannheim). Following phenol/chloroform extraction, the AP site-containing DNA was precipitated with cold ethanol. Aliquots of this abasic DNA were then incubated for 60 min at 37°C with purified recombinant polyhistidine–LMAP (1 μg) in buffer A (30 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 50 mM KCl, pH 7.4) and *E.coli* exonuclease III (0.02 U) in buffer B (30 mM HEPES, 200 mM NaCl, 5 mM $MgCl_2$, 50 mM KCl, pH 7.4) which inhibits 3′→5′ exonuclease activity. U-DNA was also directly incubated under the same conditions with purified polyhistidine–LMAP or exonuclease III for use as negative controls. Similar reactions were performed using as substrate a DNA amplified by PCR with a normal mixture of dNTPs (T-DNA) and therefore with no potential AP sites. Samples were electrophoresed on 1.5% agarose gels, Southern blotted, hybridized using the initial 800 bp DNA as probe and analyzed by autoradiography.

Immunological techniques

Anti-LMAP antibodies were obtained by injection of rabbits with 300 μg of purified recombinant protein in 1 ml of complete Freund's adjuvant (Bacto). Antisera used in this study were collected 7 weeks after the initial injection. For immunoblotting, protein samples were transferred from SDS–PAGE gels to nitrocellulose membranes. Bound antibodies were detected using anti-rabbit antibodies coupled to alkaline phosphatase. The stains were visualized with *p*-nitrophenyl phosphate in diethanolamine buffer.

RESULTS

Isolation of the *Leishmania major* AP endonuclease gene

A *L.major* ZAP Express cDNA expression library was screened for complementation of dUTPase and exonuclease III deficiency in *E.coli*. The bacterial strain used for screening (BW286) contains both the *dut-1* (dUTPase) and Δ(*xth-pncA*)90 (exonuclease III) alleles which in combination are lethal on rich medium at 37°C (18). Of the plasmids isolated, one has been shown to encode a protein with dUTPase activity (20). Four of the remaining clones (which appeared to contain overlapping inserts) were selected and the complete nucleotide sequence of both strands of the longest cDNA insert was determined. The sequence established (pBKlmap) contained an open reading frame but sequences corresponding to the spliced leader (a 39 nt sequence added by *trans*-splicing) were not found at the 5′-end. The sequence terminated with a 16 bp poly(A) tail at 997 bp from the stop codon. Codon usage studies and the absence of sequences characteristic of the 5′-untranslated region (UTR) of other *Leishmania* genes suggested that the coding sequence was truncated at the 5′-end. This was also the case of the remaining positive clones. Moreover, this observation raised the possibility that the gene was expressed as a fusion protein in BW286 cells and, as such, was capable of repair of AP sites. Indeed, when SDS gels of BW286 cells transformed with pBKlmap were analyzed, a band of 50 kDa was observed in transformed cells whose apparent molecular weight agreed with that of a protein resulting from the fusion of the predicted coding sequence to β-galactosidase (Fig. 1A, lane 3). Further screening of a genomic library resulted in isolation of clones containing the complete AP endonuclease sequence for *L.major*. Of the 1341 bp identified as the structural sequence, the first 94 were absent in the cDNA sequence. Thus, the fusion protein complementing AP deficiency in BW286 cells lacked ~32 amino acids of the N-terminus. The ATG considered as initiation codon was selected on the basis of codon usage studies as well as homology analysis and comparison with the sequence isolated after screening the *T.cruzi* cDNA library as described below. The sequence predicts a protein of 48.6 kDa and a pI of 8.8 and its C-terminal region shows pronounced similarity with the major human/*E.coli* exonuclease III family of AP endonucleases (Fig. 2).

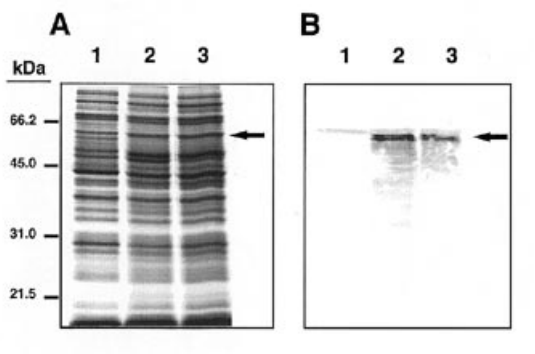

Figure 1. SDS–PAGE electrophoresis (**A**) and western blot analysis (**B**) of crude extracts from DNA repair-deficient *E.coli* (BW286 strain) expressing *T.cruzi* (pBKtcap) and *L.major* (pBKlmap) AP endonucleases fused to β-galactosidase. Lane 1, BW286/pBKdv; lane 2, BW286/pBKtcap; lane 3, BW286/pBKlmap. Samples in (B) were made to react with LMAP antibodies and detection was performed as described in Materials and Methods.

Isolation of the *Trypanosoma cruzi* AP endonuclease gene

A similar strategy to the one used in the case of *L.major* was used for isolation of the *T.cruzi* AP endonuclease gene. Of the positive clones selected, one of them hybridized with the *L.major* AP endonuclease probe. Plasmid DNA was isolated and the insert fully sequenced. An ORF was identified that encoded a protein comprising 405 amino acids with a calculated M_r of 45.2 kDa and a pI of 10.1. The cDNA sequence starts with 4 bp identical to the 3′-end of the splice leader sequence of *T.cruzi* and terminates with a 22 bp poly(A) tail. When SDS–polyacrylamide gels of BW286 cells transformed with pBKtcap were examined, a band of 49 kDa was observed that was not present in control cells transformed with pBKdv (Fig. 1A, lane 2). Hence, in the screening procedure

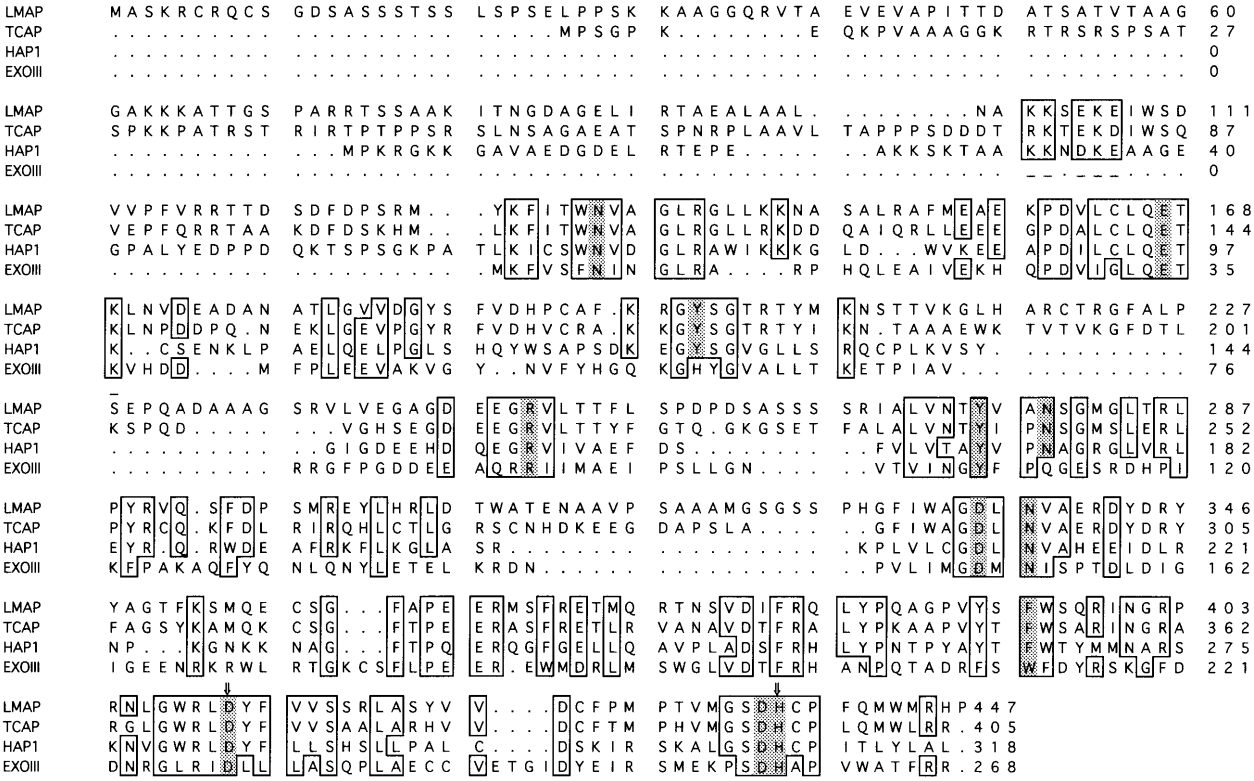

Figure 2. Alignment of the LMAP and TCAP predicted amino acid sequence with human AP endonuclease HAP1 (21) and *E.coli* exonuclease III (EXO III) (22). The protein sequences were aligned using the Pileup program from the UWGCG sequence analysis software. The main residues involved in HAP1 and EXO III DNA interaction, metal binding and catalytic mechanism are shaded. Arrows point to the active site amino acids responsible for the activation of a molecule of water and the consequent nucleophilic attack on the phosphodiester bond (27,28). The probable nuclear localization signals are underlined.

tcap was apparently also expressed as a fusion protein although in this case, the β-galactosidase domain was fused to a non-truncated version of *T.cruzi* AP endonuclease and a linker region of three amino acids corresponding to translation of the 5′-UTR region was also introduced. RT–PCR was performed to fully establish the position of the splice acceptor site for *tcap* which was located 4 bp upstream of the translation initiation site previously established. Thus, the initiation codon is the first ATG identified 3′ downstream of the splice acceptor site.

The deduced amino acid sequences of the *lmap* and *tcap* genes were compared with other reported class II AP endonucleases to analyze structural relationships. Figure 2 shows an alignment with exonuclease III from *E.coli* and the human HAP1 gene.

Complementation studies

We have used the *E.coli* BW286 strain for complementation screening of the cDNA libraries. The *dut* mutants, which are deficient in dUTPase, incorporate large amounts of uracil into their DNA. The uracil is removed by uracil-DNA glycosylase, thereby generating apyrimidinic sites. Exonuclease III (*xth*) initiates the repair of AP sites in *E.coli*, so that the double mutant is non-viable at 37°C due to the accumulation of such unrepaired sites. The viability of BW286 was completely restored to wild-type by expression of TCAP and LMAP cDNAs as fusion proteins (pBKtcap and pBKlmap) (Fig. 1A) showing that the protozoan endonucleases are able to repair damage generated as a result of uracil misincorporation. Complementation of the temperature-dependent phenotype was even attained in the case of pBKlmap where the AP protein was a truncated version lacking 32 amino acids of the N-terminus and fused to β-galactosidase.

Furthermore, both *T.cruzi* and *L.major* AP endonucleases overproduced as fusion proteins conferred significant resistance in BW286 cells to the mutagenic agents tBO_2H, H_2O_2 and MMS (Fig. 3, lanes 5 and 6). These experiments were performed at 28°C since control BW286 cells transformed with the pBK-CMV derivative (pBKdv) (Fig. 3, lane 4) are unable to grow at 37°C.

Expression of the entire coding sequence of *tcap* in pKK223.3 (pKtcap) as an individual protein was also analyzed for its capacity to confer resistance. Thus, pKtcap was able to restore the viability at 37°C of BW286 cells (with a reversion efficiency of 73%, versus 0.3% using pKK223.3 as control) indicating that the protein was expressed to significant levels under control of the *tac* promoter. However, attempts to quantify levels of TCAP by western blot in BW286 or BW528 cells were unsuccessful suggesting that they are extremely low or it is unstable. The consequences of TCAP overexpression on the sensitivity of BW286 cells grown at 28°C to tBO_2H, H_2O_2 and MMS are shown in Figure 4. *Trypanosoma cruzi* AP endonuclease re-established resistance to MMS and peroxides to levels similar to wild-type cells. When studies were performed at 37°C (Fig. 5), pKtcap not only restored the viability of BW286 cells, but also conferred resistance to the alkylating and oxidizing agents assayed (Fig. 5A–C, lane 6) even though damage in cells at this

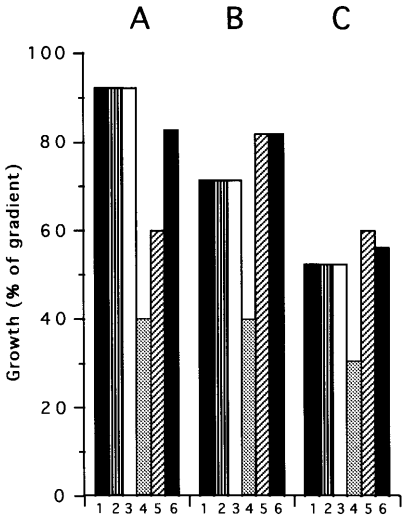

Figure 3. Complementation of the repair-deficient BW286 strain by pBKlmap and pBKtcap (Materials and Methods). Each bar represents the relative survival of cells in the presence of a linear gradient of the DNA damaging agents. The agar contained from the bottom to the top: (**A**) 0–28 µM tBO_2H; (**B**) 0–2 mM H_2O_2; (**C**) 0–12 mM MMS. Bars 1–6 in each panel represent the following strain/plasmid: 1, AB1157/pBKdv; 2, AB1157/pBKtcap; 3, AB1157/pBKlmap; 4, BW286/pBKdv; 5, BW286/pBKtcap; 6, BW286/pBKlmap. These experiments were carried out at 28°C.

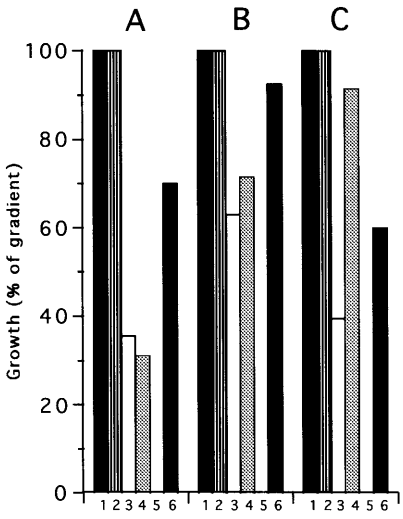

Figure 5. Resistance to oxidant and alkylating agents conferred by expression of TCAP in *xth⁻nfo⁻* (BW528) and *dut⁻xth⁻* (BW286) mutant strains. Bars 1–6 in each panel represent: 1, AB1157/pKK 223.3; 2, AB1157/pKtcap; 3, BW528/pKK223.3; 4, BW528/pKtcap; 5, BW286/pKK223.3; 6, BW286/pKtcap. (**A**) 0–14 µM tBO_2H; (**B**) 0–1 mM H_2O_2; (**C**) 0–6 mM MMS. The complementation of the temperature-sensitive mutation of BW286 by *tcap* can be observed in lanes 5 (no growth) and 6. Petri dishes were incubated at 37°C.

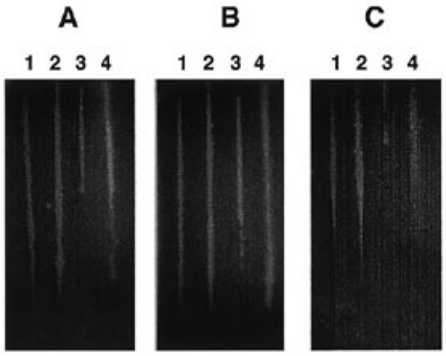

Figure 4. Resistance conferred by expression of TCAP in BW286 cells. (**A**) tBO_2H; (**B**) H_2O_2; (**C**) MMS. Lane 1, AB1157/pKK 223.3; lane 2, AB1157/pKtcap; lane 3, BW286/pKK 223.3; lane 4, BW286/pKtcap. The length of cell growth along the gradient is a measure of the strain resistance to the different agents. Petri dishes were incubated at 28°C.

temperature is extensive due to complete absence of dUTPase activity which leads to massive uracil misincorporation.

The ability of TCAP to restore resistance to MMS and peroxides was further tested in the *xth-nfo* double mutant BW528 lacking both exonuclease III and endonuclease IV activity. No effect on the sensitivity to tBO_2H (Fig. 5A, lanes 3 and 4) and only marginal effects on the sensitivity to H_2O_2 (Fig. 5B, lanes 3 and 4) were found in this strain yet resistance to MMS (Fig. 5C, lanes 3 and 4) was similar to levels exhibited by AB1157 cells.

Purification of recombinant LMAP and antibody specificity

Overexpression of *lmap* as a his-tagged protein using the expression host *E.coli* BL21(DE3) is illustrated in Figure 6. *Leishmania major* AP endonuclease constituted ~20% of soluble cell protein obtained following sonication of BL21(DE3)/pETlmap cells. The affinity purification procedure based on polyhistidine–LMAP fusion protein production resulted in a homogeneous preparation judged by SDS–PAGE and Coomassie blue staining (Fig. 6A, lane 2). Antibodies raised against purified LMAP were examined by immunoblotting. Purified antiserum reacted specifically with the purified fractions of M_r 48 600, the size of the purified polypeptide (Fig. 6B). Immunoblots also revealed some additional cross-reactive polypeptides of lower M_r in cells transformed with pBKtcap and pBKlmap (Fig. 1B, lanes 1 and 2) suggesting possible protein degradation.

AP endonuclease activity assays

A defective DNA was prepared by using dUTP instead of dTTP in the amplification by PCR. AP sites were created after incubation with *E.coli* uracil-DNA glycosylase. The resulting DNAs are stable (Fig. 7A, lanes 1 and 2) and are not altered by incubation with either purified recombinant LMAP or *E.coli* exonuclease III (Fig. 7A, lanes 3 and 4). When the abasic DNA was further incubated with purified recombinant polyhistidine–LMAP or *E.coli* exonuclease III, DNA degradation was observed (Fig. 7A, lanes 5 and 6) due to hydrolysis of the AP sites. DNA containing dTTP remained unaltered after incubation with either enzyme (Fig. 7B).

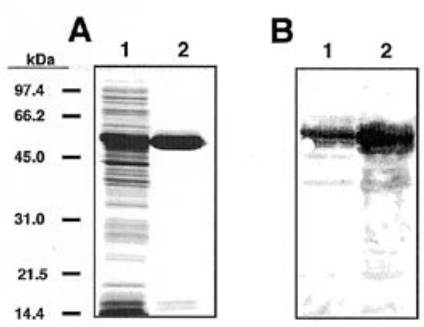

Figure 6. Purification of recombinant polyhistidine–LMAP. (**A**) SDS–PAGE. (**B**) Western blot of soluble extract from *E.coli* BL21(DE3)/pETlmap induced with IPTG and expressing polyhistidine–LMAP (1) and affinity-purified enzyme (2).

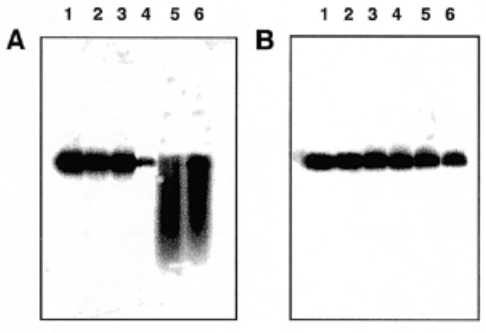

Figure 7. AP endonuclease assay. (**A**) DNA was amplified by PCR using dUTP in place of dTTP (U-DNA). (**B**) DNA was amplified with dTTP (T-DNA) and used as control. Lane 1, U-DNA (A) and T-DNA (B); lane 2, DNA treated with 1 µg of LMAP; lane 3, DNA treated with 0.02 U of *E.coli* exonuclease III; lane 4, DNA treated with 1 U of *E.coli* uracil-DNA glycosylase; lane 5, DNA treated with *E.coli* uracil-DNA glycosylase and then with 1 µg of LMAP; lane 6, DNA treated with *E.coli* uracil-DNA glycosylase and then with *E.coli* exonuclease III.

DISCUSSION

We have isolated *L.major* and *T.cruzi* cDNAs, encoding cellular enzymes that attack AP sites in DNA. The predicted LMAP and TCAP polypeptides present, as described for other AP endonucleases, two well-differentiated domains. The protozoan C-terminal domains (considered as 319 amino acids for *L.major* and 301 amino acids for *T.cruzi*) are highly conserved between each other (60% identity) and more distantly related to prokaryotic AP endonucleases than to eukaryotic counterparts. Hence, there is a 38% identity of the *L.major* and 41% of the *T.cruzi* sequences with the HAP1 endonuclease of human cells (21,26), 37 and 39% of the *L.major* and *T.cruzi* endonucleases, respectively, with the Rrp1 *Drosophila melanogaster* homologous region (23), 42% identity of both protozoan sequences with AP endonuclease from *Dictyostelium discoideum* (24) and 30% identity of both sequences with exonuclease III of *E.coli* (22).

Invariant amino acids involved in catalysis or substrate binding are especially conserved (Fig. 2). Mol *et al.* (25) and Gorman *et al.* (26) proposed that the mechanism for the hydrolytic cleavage of the P–O3′ bond in the abasic site is a nucleophilic attack of the hydroxyl group for *E.coli* exonuclease III and human HAP1. The residues involved in this attack and in stabilization of the active site are entirely conserved in the protozoan deduced sequence. Amino acids believed to be important in the pocket which recognizes the AP site, those that bind to the scissile phosphate or residues that could participate in metal binding are all present in the *T.cruzi* and *L.major* AP endonucleases.

Likewise, similar to the *D.melanogaster* Rrp1 (23) and Arp from *Arabidopsis thaliana* (27) the protozoan enzymes have an extended and highly charged N-terminal domain. These N-termini, 128 residues in *L.major* and 104 in *T.cruzi*, share 37% identity and exhibit low similarities with the N-terminal domains of other eukaryotic AP endonucleases or with any other sequences in the available DNA databases. In addition, the lengths of the N-termini are intermediate between human HAP1 and *D.melanogaster* Rrp1 and contain probable nuclear localization signals KKSEK (amino acids 102–106, *L.major*) and RKTEK (amino acids 78–82, *T.cruzi*) which are closely related to consensus targeting sequences and are consistent with the nuclear localization of AP endonucleases (28).

Database searching revealed also that amino acids 27–105 in *L.major* and 1–84 in *T.cruzi* exhibit 22% identity with histone H1 of *E.coli*. It has been reported that the *D.melanogaster* Rrp1 AP endonuclease protein efficiently renatures homologous single-stranded DNA *in vitro*, a property that is associated with its unique N-terminal region. Likewise, a ssDNA renaturation activity for histone H1 has also been described (23). The existence of DNA binding or renaturing activity associated with the N-terminus of the protozoan enzymes and the possible role of these properties in endonuclease activity is yet to be established.

The non-viability of BW286 *E.coli* mutants at 37°C is due to the incapability of endonuclease IV (which represents only ~5% of total AP endonuclease activity) to repair the high level of AP sites (21). The fact that the *L.major* and *T.cruzi* proteins were able to restore complete viability at restrictive temperature and that the recombinant polyhistidine fusion protein was able to degrade a PCR-amplified DNA using dUTP in place of dTTP suggests that the protozoan enzymes are effective AP endonucleases with a capacity for AP site repair equivalent to that of exonuclease III.

We tested further the role of these enzymes in the resistance to different mutagenic agents: the alkylating agent MMS and the oxidative agents H_2O_2 and tBO_2H. MMS alkylates mainly purine nitrogens in DNA and generates AP sites via spontaneous and enzymatic hydrolysis of glycosidic bonds. In contrast, a much wider spectrum of lesions is generated by peroxides, including DNA strand breaks with 3′ fragmented deoxyribose groups (reviewed in 8,29). The expression of the protozoan proteins conferred resistance not only to MMS but also to oxidative agents in BW286 indicating that TCAP and LMAP proteins are active in the repair of AP sites and may participate in the repair of other oxygen radical-generated DNA lesions.

The expression of *tcap* in BW528 cells, that lack both exonuclease III and its backup enzyme endonuclease IV, gave only a slight increase in resistance to H_2O_2 and did not confer resistance to tBO_2H. Similar results were obtained when the expression of human HAP1 in *E.coli nfo* mutants was analyzed (28). Cunningham *et al.* (13) have demonstrated a requirement for endonuclease IV, but not exonuclease III for resistance to tBO_2H. Apparently, endonuclease IV has an important role in damage induced by tBO_2H and in its absence, only partial resistance is obtained. In contrast, lesions caused by H_2O_2 and

MMS are repaired effectively by exonuclease III and to a lesser extent by Apn1, a yeast endonuclease homologous to endonuclease IV (30).

In summary, we have identified protozoan enzymes with AP endonuclease activity capable of conferring resistance to alkylating and oxidizing agents in *E.coli* hypersensitive mutants. This work constitutes the first report on enzymes involved in base excision repair in parasitic protozoa and provides information concerning the possible biological role of trypanosomatid AP endonucleases in DNA damage. Further studies will determine the contribution of the activity of TCAP and LMAP to base excision repair in general, as well as the importance of this pathway in defense against intracellular oxidative stress.

ACKNOWLEDGEMENTS

We are grateful to Dr Bernard Weiss for the *E.coli* mutant strains BW286 and BW528. These studies were supported by grants from the Spanish Programa Nacional de Investigación (BIO97-0659) and the Plan Andaluz de Investigación (CVI.199). J.P. was supported by a postdoctoral fellowship from the Spanish Ministerio de Educación y Ciencia. C.G. and V.B. are Predoctoral Fellows of the Ramón Areces Foundation. A.C. is a Predoctoral Fellow of the Plan Andaluz de Investigación.

REFERENCES

1 Halliwell,B. (1987) *FASEB J.*, **1**, 358–364.
2 Hutchinson,F. (1985) *Prog. Nucleic Acid Res.*, **32**, 115–154.
3 Lindahl,T., Sedgwick,B., Sekiguchi,M. and Nakabeppu,Y. (1988) *Annu. Rev. Biochem.*, **57**, 133–158.
4 Lindahl,T. and Nyberg,B. (1972) *Biochemistry*, **11**, 3610–3618.
5 Wallace,S. (1988) *Environ. Mol. Mutagen.*, **12**, 431–477.
6 Sancar,A.and Sancar,G.B. (1988) *Annu. Rev. Biochem.*, **57**, 29–67.
7 Warner,H.R., Demple,B., Deutsch,W.A., Kane,C.M. and Linn,S. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 4602–4606.
8 Demple,B. and Harrison,L. (1994) *Annu. Rev. Biochem.*, **63**, 915–948.
9 Johnson,A.W. and Demple,B. (1988) *J. Biol. Chem.*, **263**, 18009–18016.
10 Johnson,A.W. and Demple,B. (1988) *J. Biol. Chem.*, **263**, 18917–18022.
11 Yajko,D.M. and Weiss,B. (1975) *Proc. Natl Acad. Sci. USA*, **72**, 688–692.
12 Chan,E. and Weiss,B. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 3189–3193.
13 Cunningham,R.P., Saporito,S.M., Spitzer,S.G. and Weiss,B. (1986) *J. Bacteriol.*, **168**, 1120–112.
14 Docampo,R. and Moreno,S.N.J. (1984) *Free-Radical Intermediates in the Antiparasitic Action of Drugs and Phagocytic Cells*. In Pryor,W.A. (ed.), *Free Radicals in Biology.* Academic Press, New York. NY. pp. 243–288.
15 Docampo,R. and Moreno,S.N.J. (1984) *Rev. Infect. Dis.*, **6**, 223–238.
16 Docampo,R., (1990) *Chem. Interact.*, **73**, 1–27.
17 Goyal,N., Roy,U.and Rastogi,A.K. (1996) *Free Radical Biol. Med.*, **21**, 638–689.
18 Taylor,A.F. and Weiss,B. (1982) *J. Bacteriol.*, **151**, 351–357.
19 Camacho,A., Arrebola,R., Peña-Díaz,J., Ruiz-Pérez,L.M. and González-Pacanowska,D. (1997) *Biochem. J.*, **325**, 441–447.
20 Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1995) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York, NY.
21 Robson,C.N. and Hickson,I.D. (1991) *Nucleic Acids Res.*, **20**, 5519–5523.
22 Saporito,S., Smith-White,B.J. and Cunnigham,R.P. (1988) *J. Bacteriol.*, **170**, 4542–4547.
23 Sanders,M., Lowenhaupt,K. and Rich,A. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6780–6784.
24 Freeland,T.M., Guyer,R.B., Ling,A.Z. and Deering,R.A. (1996) *Nucleic Acids Res.*, **24**, 1950–1953.
25 Mol,C.D., Kuo,C.F., Thayer,M.M., Cunningham,R.P. and Tainer,J.A. (1995) *Nature*, **374**, 381–386.
26 Gorman,M.A., Morera,S., Rothwell,D.G., La Fortelle,E., Mol,C.D., Tainer,J.A., Hickson,I.D. and Freemont,P.S. (1997) *EMBO J.*, **16**, 6548–6558.
27 Babiychuk,E., Kushnir,S., Montagu,M.V. and Inze,D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 3299–3303.
28 Demple,B., Herman,T. and Chen,D.S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 11450–11454.
29 Lindahl,T. (1982) *Annu. Rev. Biochem.*, **51**, 61–87.
30 Ramotar,D., Popoff,S.C. and Demple,B. (1991) *Mol. Microbiol.*, **5**, 149–155.