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Expression, Functionality, and Localization of Apurinic/Apyrimidinic Endonucleases in Replicative and Non-Replicative Forms of *Trypanosoma cruzi*

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ABSTRACT

Trypanosoma cruzi is the etiological agent of Chagas disease. The parasite has to overcome oxidative damage by ROS/RNS all along its life cycle to survive and to establish a chronic infection. We propose that *T. cruzi* is able to survive, among other mechanisms of detoxification, by repair of its damaged DNA through activation of the DNA base excision repair (BER) pathway. BER is highly conserved in eukaryotes with apurinic/apirimidinic endonucleases (APEs) playing a fundamental role. Previous results showed that *T. cruzi* exposed to hydrogen peroxide and peroxinitrite significantly decreases its viability when co-incubated with methoxyamine, an AP endonuclease inhibitor. In this work the localization, expression and functionality of two *T. cruzi* APEs (TcAP1, *Homo sapiens* APE1 orthologous and TcAP2, orthologous to *Homo sapiens* APE2 and to *Schizosaccaromyces pombe* Apn2p) were determined. These enzymes are present and active in the two replicative parasite forms (epimastigotes and amastigotes) as well as in the non-replicative, infective trypomastigotes. TcAP1 and TcAP2 are located in the nucleus of epimastigotes and their expression is constitutive. Epimastigote AP endonucleases as well as recombinant TcAP1 and TcAP2 are inhibited by methoxyamine. Overexpression of TcAP1 increases epimastigotes viability when they are exposed to acute ROS/RNS attack. This protective effect is more evident when parasites are submitted to persistent ROS/RNS exposition, mimicking nature conditions. Our results confirm that the BER pathway is involved in *T. cruzi* resistance to DNA oxidative damage and points to the participation of DNA AP endonucleases in parasite survival. J. Cell. Biochem. 115: 397–409, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: *T. cruzi*; DNA DAMAGE/REPAIR; BER PATHWAY

Chagas disease is a neglected chronic illness with a high social impact that currently affects 8–10 million people in endemic countries of Latin America [Rassi et al., 2012]. This lifelong infection, also known as American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi* [Chagas, 1909]. Chagas disease is currently a major cause of morbidity and mortality in Latin America, enormously burdening economic resources and dramatically affecting patients' social and labor situations [Munoz-Saravia et al., 2010]. Recently, the appearance of *T. cruzi* infected vectors in the USA [Sarkar et al., 2010] and the identification of more than 300,000 persons carrying the parasite in this country [Bern and Montgomery, 2009] together with the globalization of this disease through immigration [Schmunis and Yadon, 2010] have converted this illness in a worldwide problem.

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Vectorial transmission of Chagas′ disease is produced by infected triatomine insects that upon feeding on mammal blood, deposits feces with infective parasites (metacyclic trypomastigotes) that are ingested in parasitophorous vacuoles in host cells. In the cytoplasm, trypomastigotes differentiate to round amastigotes that undergo 8–9 cycles of multiplication before transforming back to blood trypomastigotes that escape to circulation. Upon infection of target tissues trypomastigotes change back to intracellular amastigotes (amastigote nests) that maintain *T. cruzi* infection for life. Eventually, blood trypomastigotes may be ingested by a triatomine and transformed to epimastigotes in the vector′s midgut. After multiplication, epimastigotes move to the insect hindgut where they differentiate into infective metacyclic trypomastigotes [Tyler and Engman, 2001; De Souza, 2002].

In its life cycle, *T. cruzi* must overcome diverse oxygen and nitrogen reactive species [Graca-Souza et al., 2006; Gupta et al., 2009b; Piacenza et al., 2009]. Though it was clearly determined that modification of lipids and proteins resulting from the oxidative species assault in mammalian and insect parasite hosts are corrected through metabolism [Hernandez et al., 2006; Dhiman et al., 2008, 2009; Ferrer-Sueta and Radi, 2009; Cardoso et al., 2011], it was only recently shown that peroxynitrite (NOO^-) and hydrogen peroxide (H_2O_2) damage *T. cruzi* DNA and that the parasite is able to repair this damage [Cabrera et al., 2011].

Base excision repair (BER) is an evolutionarily conserved process for maintaining nuclear and mitochondrial genomic integrity by eliminating oxidized DNA bases [Mandavilli et al., 2002; Hegde et al., 2008]. Initiation of BER occurs by recognition of a damaged DNA base by a DNA glycosylase, which hydrolyses the N-glycosidic bond, creating an AP site. In a second step, an AP endonuclease cleaves the phosphodiester backbone 5′ to the AP site generating a DNA single-strand break with a 3′-hydroxyl and a 5′-deoxyribose phosphate terminus. AP site repair is then completed by a DNA polymerase and a DNA ligase [Barzilay and Hickson, 1995; Robertson et al., 2009; Kim and Wilson, 2012].

Generally, each organism possesses one or two AP endonucleases, orthologous to *E. coli* exonuclease III (Xth) or to *E. coli* endonuclease IV (Nfo) [Zharkov, 2008]. The major mammalian AP endonuclease, APE1, and the lately discovered APE2, are both Xth homologues [Demple et al., 1991; Seki et al., 1991; Hadi and Wilson, 2000].

To date, the presence of an AP endonuclease gene (TcAP1) has been described in *T. cruzi*; expression of this gene confers resistance to oxidant agents when probed in hypersensitive *E. coli*, deficient in exo III enzyme [Perez et al., 1999]. However, until now there have been no studies on its expression or activity in the parasite or in its possible protective effect to DNA damage. The viability of *T. cruzi* epimastigotes and trypomastigotes diminishes in the presence of H_2O_2 and NOO^-. This effect is significantly increased in parasites subsequently incubated with the drug methoxyamine (Mx) [Cabrera et al., 2011], an AP endonuclease activity inhibitor [Fortini et al., 1990; Yan et al., 2007]. Those results suggest that both parasite cellular forms express active AP endonucleases.

In this work the localization, expression and functionality of two *T. cruzi* APEs (TcAP1, *Homo sapiens* APE1 orthologous and TcAP2, *Homo sapiens* APE2 and *Schizosaccaromyces pombe* Apn2p orthologous) were determined. Our results indicate that these enzymes are present in the two replicative forms of the parasite (epimastigotes and amastigotes) as well as in the non replicative, infective trypomastigotes. TcAP1 and TcAP2 expression is not increased in the presence of H_2O_2 or NOO^-. Both enzymes are localized in the nucleus but not in the kinetoplast or cytoplasm of epimastigotes. Recombinant TcAP1 and TcAP2 AP endonuclease activities are inhibited with Mx or with a human APE1 negative dominant form. Interestingly overexpression of TcAP1 but not of TcAP2 increases survival of parasites when submitted to acute and sustained oxidative stress. These results suggest that the BER pathway and particularly TcAP1 play an important role in *T. cruzi* oxidative DNA damage resistance and could participate in the parasite persistence both in the insect vector and in the mammalian host cells.

MATERIALS AND METHODS

PARASITE CULTURE

T. cruzi epimastigotes (Dm28c strain) were cultivated at 28°C in a monophasic liquid medium [Diamond, 1968] (0.106 M NaCl, 23 mM KH_2PO_4, 12.5 g/L tryptose, 12.5 g/L tryptone, 12.5 g/L yeast extract, pH 7.2) supplemented with 10% bovine fetal serum, 7.5 mM hemine and antibiotics (75 U/ml penicillin and 75 mg/ml streptomycin). Trypomastigotes and amastigotes were obtained from infected RAW 264.7 cells cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C with 5% CO_2.

TcAP1 AND TcAP2 IDENTIFICATION

TcAP1 and TcAP2 endonucleases were identified by Western blot assays in epimastigotes, amastigotes, and trypomastigotes protein extracts using specific rabbit anti-TcAP1 and anti-TcAP2 purified antibodies prepared against immunogenic peptides present in the deduced amino acid sequences from each enzyme (Ac-TEKDIWSQ-VEPFQRRTAC-amide for TcAP1 and CAHFDGISLAPRPGKLEER-amide for TcAP2). Briefly, protein extracts were separated by electrophoresis in 8% acrylamide gels. Afterwards, proteins were transferred to nitrocellulose membranes and incubated with 5% bovine serum albumin (BSA) in PBS 0.05% Tween-20 at 4°C overnight. Subsequently, membranes were incubated 2 h with anti-TcAP1 or anti-TcAP2 antibody in a 1:2,000 (v/v) dilution in PBS 0.05% Tween-20 at room temperature. Membranes were incubated with a secondary anti-rabbit antibody coupled to horseradish peroxidase (HRP) and results were visualized by chemiluminescence.

The evaluation of expression levels of TcAP1 and TcAP2 in epimastigotes and trypomastigotes subjected to oxidative stress was measured using the same protocol described above. Briefly, 12×10^6 parasites were exposed to H_2O_2 and NOO^- (200 or 500 μM) for 0, 15, 30, 60, or 240 min at 28°C. Otherwise, 12×10^6 parasites were exposed to increasing concentrations of H_2O_2 (2, 5, 100, 200, 500, and 1,000 μM) for 30 min at 28°C. TcAP1 and TcAP2 identification was assessed by Western blot assay using the specific antibodies. Identification of α-tubulin with a specific mouse monoclonal antibody (Sigma, cat no. T-5168) was used as load control.

TcAP1, TcAP2, AND APE1-DN PLASMID CONSTRUCTIONS

TcAP1, TcAP2 and human apurynic/apyrimidinic endonuclease 1 negative dominant form (APE1-DN) DNA coding sequences were

amplified by PCR and inserted in the pTREX-his-GFP vector [Vazquez and Levin, 1999]. Fusion proteins with GFP and eight histidine residues in the C-terminal and in N-terminal regions respectively were produced. The primers used were: sense 5′-GCTCTAGAATGCCGTCGGGACCTAAGG-3′ and antisense 5′-CCCAAGCTTCCTGCGCAGCCACATCTGC-3′ for TcAP1; sense 5′-GCTCTAGAATGTTTATCATTAGTTGGAATGTG-3′ and antisense 5′-CCCAAGCTTGGAAATAACATCGGTAATTTCAAT-3′ for TcAP2; and sense 5′-GCTCTAGAATGCCGAAGCGTCGGAAAAAG-3′ and antisense 5′-CCCAAGCTTCAGTGCTAGGTATAGGGTGATA-3′ for APE1-DN. All those primers present restriction sites for *Xba*I and *Hind*III enzymes in the sense and in the antisense sequences, respectively. TcAP1 and TcAP2 DNA sequences were amplified using total *T. cruzi* DNA as template. The nucleotide sequence for APE1-DN was obtained by site directed mutagenesis from the human APE1 coding sequence previously inserted in a pGEM-T easy vector (Promega). The mutations inserted were E96Q and D210N, as described [McNeill and Wilson, 2007]. This mutated plasmid was used as substrate for PCR reaction to amplify the APE1-DN sequence. The correct insertion of each gene was confirmed by PCR, enzymatic digestion and automatic DNA sequencing (not shown).

TRANSFECTION AND OVEREXPRESSION OF TcAP1, TcAP2, AND APE1-DN IN *T. CRUZI* EPIMASTIGOTES

Epimastigotes in the exponential phase of growth were electroporated with the pTREX-his-tcap1-gfp, pTREX-his-tcap2-gfp, pTREX-his-ape1dn-gfp constructs and with the pTREX-his-gfp as a control vector. Briefly, 1×10^8 parasites/ml were washed in sterile PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM CaCl, 10 mM K_2HPO_4, 25 mM Hepes, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.6). Afterwards 4×10^7 parasites were separately incubated with 50–100 μg of each plasmid. The electroporation was performed at 0.3 kV and 500 μF in two pulses separated by 10 s maintaining the parasites on ice. Immediately, transfected epimastigotes were transferred to 20% FBS Diamond's medium; after 24 h, 250 μg/ml of G418 antibiotic were added increasing the antibiotic concentration to 500 μg/ml at 48 h. To evaluate transfection efficiency the parasites were observed in the epi-fluorescence microscope using a 520 ± 20 filter (green fluorescence). Considering the low percentage of transfection observed (around 10%), fluorescent transfected epimastigotes were sorted by FACS (FACSAria II), reaching 97% efficiency. These parasites showed fluorescence for more than 3 months, demonstrating transfection stability.

TcAP1, TcAP2, AND APE1-DN RECOMBINANT PROTEINS OBTENTION

Recombinant TcAP1 and APE1-DN proteins were generated in transfected pTREX-his-tcap1-gfp and pTREX-his-ape1dn-gfp *T. cruzi* epimastigotes as described above. On the other hand, recombinant TcAP2 protein was generated using the pLEXSY expression system (Jena Bioscience) in *Leishmania tarentolae*, a eukaryote phylogenetically close to *T. cruzi*. The pLEXSY plasmid generates a fusion protein with six histidine residues in the N-terminal region. The three recombinant proteins were purified by affinity chromatography using a HisPur Ni-NTA resin (Thermo Scientific) in native conditions.

AP ENDONUCLEASE ACTIVITY ASSAY

To determine the AP endonuclease activity a 24 mer synthetic DNA oligonucleotide with a uracil at position 8 (5′-CCGCTAGUGGGTACCGAGCTCGAAT-3′) was labeled at the 5′ end with $P\gamma^{32}$ using the DNA 5′ End Labeling System kit (Promega). This oligo was then hybridized with an unlabeled complementary oligonucleotide and incubated with 1 U of an *E. coli* uracil-DNA-glycosilase (New England Biolabs) to generate an AP site (oligo AP). Afterwards, epimastigote, trypomastigote, or amastigote protein supernatants (20,000*g*, 10 min) or TcAP1 or TcAP2 recombinant proteins were incubated with 2 pg of the oligo AP in BER buffer solution (50 mM Hepes KOH pH7.8, 0.36% p/v BSA, 70 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT) for 2 h at 30°C in the presence or absence of ATP. To inactivate the enzymes, samples were heated at 75°C for 10 min and one volume of formamide loading buffer (96% v/v formamide, 20 mM EDTA, 5 mM Tris pH 7.5, xylene cyanol 0.05% p/v, bromophenol blue 0.05% p/v) was added. After heating at 95°C for 5 min the samples were electrophoretically separated in denaturing 96% formamide-20% acrylamide gels. Labeled oligos were detected using a phosphorimager device (BioRad). As a negative control the untreated oligo AP was used. As positive control, the oligo AP was incubated with 1U Exonuclease III (*E. coli* AP endonuclease, New England Biolabs) in BER buffer. An AP endonuclease activity generates a radioactive labeled 7 mer fragment. To determine whether Mx, a known AP endonuclease inhibitor [Fortini et al., 1990; Yan et al., 2007] inhibits the AP endonuclease activity, parasite protein supernatants or recombinant TcAP1 or TcAP2 proteins were incubated at different times with oligo AP previously treated or not with 10 mM Mx for 15 min at 30°C. Additionally, inhibition of the AP endonuclease activity by an APE1 human endonuclease negative dominant (APE1-DN) [McNeill and Wilson, 2007] was assayed. For this purpose, recombinant TcAP1 and TcAP2 proteins were incubated with increasing concentrations of APE1-DN in presence of the oligo AP for 2 h at 30°C.

EXPRESSION AND SUBCELLULAR LOCATION OF TcAP1 AND TcAP2 GFP FUSION PROTEINS IN TRANSFECTED EPIMASTIGOTES

TcAP1 and TcAP2 GFP fusion proteins were detected in transfected epimastigotes by Western blot assays using anti-TcAP1, anti-TcAP2, and anti-GFP antibodies.

Subcellular location of TcAP1-GFP and TcAP2-GFP was assayed by immunodetection of GFP on transfected parasites smears fixed on 70% ice methanol for 30 min. Following fixation, samples were treated with blocking solution (BSA 1% p/v, saponine 0.1% v/v, calf serum 3% v/v in PBS) for 2 h at 37°C previous incubation overnight at 4°C with a monoclonal anti-GFP antibody (Thermo Scientific). Samples were then washed and incubated with a secondary antibody conjugated to Alexa 488 fluorochrome (Molecular Probes). Nuclear and kinetoplastid DNA were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Samples were evaluated by fluorescence microscopy observation using a 430 ± 20 nm filter and a 520 ± 20 nm for blue and green fluorescence, respectively. Photographs were processed computationally to determine the overlap of DAPI (pseudocolor red) and Alexa fluor 488 (green).

TRANSFECTED EPIMASTIGOTES VIABILITY ASSAY

Viability of *T. cruzi* epimastigotes overexpressing TcAP1 or TcAP2 and exposed to H_2O_2 or NOO^- was evaluated by the MTT [3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [Muelas-Serrano et al., 2000]. For this purpose, 12×10^6 parasites/ml were incubated with different concentrations of H_2O_2 (200, 350, or 500 μM; Sigma) or NOO^- (200, 500, or 1,000 μM; Cayman) in Diamond's culture medium at 28°C. After 30 min incubation, the parasites were washed twice with PBS and incubated with fresh medium for 4 h. Subsequently, 100 μl of parasites in 96 wells plates were incubated with 10 μl of 5 mg/ml MTT reagent plus 0.22 mg/ml phenazine metosulfate. After additional incubation for 4 h, the generated water insoluble formazan dye was dissolved in 100 μl of 10% w/v SDS/0.01M HCl. The plates were further incubated 30 min at 37°C and optical density (OD) in each well was determined using a microplate reader (Multiskan FC, Thermo Scientific) at 570 nm.

To generate sustained oxidative conditions, 12×10^6 parasites were incubated for 2 or 24 h in the presence of 5 mM glucose plus 10, 25, 50, and 100 mU glucose oxidase from *Aspergillus niger* (Sigma) at 28°C. These conditions generate a 45–65 μM H_2O_2 concentration as measured by the Amplex Red Hydrogen/Peroxide/Peroxidase kit (Invitrogen). Parasites were then washed and their viability was evaluated by MTT assay as described above.

STATISTICAL ANALYSIS

Statistical analyses were performed using the GraphPad Prism 5.0 program. All experiments were done in triplicate and results correspond to means ± SEM from at least three independent experiments. Significant data differences were analyzed applying the two-way ANOVA with Bonferroni posttest.

RESULTS

TcAP1 AND TcAP2 PRESENCE AND EXPRESSION IN *T. CRUZI*

In the *T. cruzi* genome there are orthologous sequences (TcAP1, described in the Y strain [Perez et al., 1999], and TcAP2, described for the CL Brener strain, GenBank accession No. 71654547) corresponding to APE1 and APE2 *Homo sapiens* DNA endonucleases. Figure 1 shows a multiple amino acid sequences alignment of the catalytic region of *T. cruzi* Dm28c TcAP1 (GenBank accession No. KC987037) and TcAP2 (GenBank accession No. KC987038) endonucleases with *Homo sapiens* APE1 and APE2 and with *Schizosaccharomyces pombe* Apn2p. Clearly, amino acids proper of the catalytic region of both *T. cruzi* AP endonucleases are conserved. On the other hand a hydrophobic pocket that has been described for human APE1 [Hadi et al., 2002] as required for AP sites recognition is conserved in TcAP1 but not in TcAP2 (Fig. 1). Indeed none of the known DNA AP2 endonucleases present that hydrophobic pocket which may be a cause of their lower AP site affinity and activity as compared to DNA AP1 endonuclease enzymes [Hadi et al., 2002]. However, amino acids found in this region for TcAP2 are conserved with Apn2p, the most important DNA AP endonuclease in *S. pombe* for DNA repair oxidative damage [Ribar et al., 2004]. Therefore, additionally to amino acid sequences conservation, TcAP1 and TcAP2 present features that are expected for canonic AP endonucleases and which are essential for their activity.

The presence of TcAP1 and TcAP2 in *T. cruzi* was detected by Western blotting using specific antibodies prepared against immunogenic peptides present in the deduced amino acid sequences from both enzymes (Fig. 2A,B). Proteins recognized by those antibodies are evident in the epimastigote, amastigote and trypomastigote cellular forms of the parasite showing the expected molecular masses for TcAP1 and TcAP2. Thus, in epimastigote the anti TcAP1 antibody recognizes two proteins of approximately 49 and 56 kDa, respectively, while the trypomastigote and amastigote cellular forms show a single ~58 kDa protein (Fig. 2A). On the other hand, these three cellular forms of the parasite show a single protein recognized by the specific TcAP2 antibody with the expected ~68 kDa mass (Fig. 2B).

TcAP1 AND TcAP2 ARE NOT OVEREXPRESSED IN PARASITES EXPOSED TO H_2O_2 OR NOO

T. cruzi epimastigotes and trypomastigotes were treated with increasing H_2O_2 concentrations for 30 min. Afterwards parasites were homogenated and proteins were separated by SDS–PAGE. Finally, TcAP1 and TcAP2 were detected using specific antibodies. As shown in Figure 2C,D (epimastigotes) and Figure 2E,F (trypomastigotes) the levels of those enzymes did not show any evident change when chased with H_2O_2 at the experimental conditions used. Similar results were obtained when parasites were exposed to 200 or 500 μM H_2O_2 for 30 min to 24 h and when exposed to increasing concentrations of NOO^- for 30 min (not shown).

Those results suggest that TcAP1 and TcAP2 AP endonucleases are constitutively expressed. This is of particular importance considering that the human ortholog AP endonuclease APE1 presents a genotoxic inducible expression [Ramana et al., 1998].

T. CRUZI PRESENTS A DNA AP ENDONUCLEASE ACTIVITY THAT IS INHIBITED BY MX

A 24 mer synthetic oligonucleotide labeled with P^{32} at the 5′ position, containing an abasic site at position 8 and aligned with the non labeled complementary strand, was incubated with supernatants from *T. cruzi* epimastigote, trypomastigote and amastigote homogenates (20,000*g*, 10 min) for 2 h in the presence or absence of ATP. In the absence of ATP, only a 7 mer fragment is observed in all parasite cellular forms corresponding to an oligonucleotide cut by the AP endonuclease activity (Fig. 3A). In the presence of 5 mM ATP, two main fragments of 7 and 24 mer were observed, the last one corresponding to the fully repaired 24 mer oligonucleotide. In Figure 3B the inhibitory effect of Mx on *T. cruzi* AP endonuclease activity is shown. The same synthetic oligonucleotide containing an abasic site at position 8, used in 3A, was incubated with 10 mM Mx for 15 min at 30°C. Afterwards, a supernatant from epimastigote homogenates (20,000*g*, 10 min) was added and further incubated for 2 h. Clearly, without Mx the synthetic 24 mer oligonucleotide is fully cut by the *T. cruzi* AP endonucleases. On the contrary, when the 24 mer synthetic oligonucleotide with an abasic site at position 8 was previously incubated for 15 min with Mx, the endonuclease activity is almost fully inhibited.

Those results indicate that: (i) there is an AP endonuclease activity in *T. cruzi* epimastigotes, amastigotes and trypomastigotes; (ii) these three *T. cruzi* cellular forms are able to repair damaged abasic DNA; (iii) the *T. cruzi* AP endonuclease activity is inhibited by Mx; (iv) the

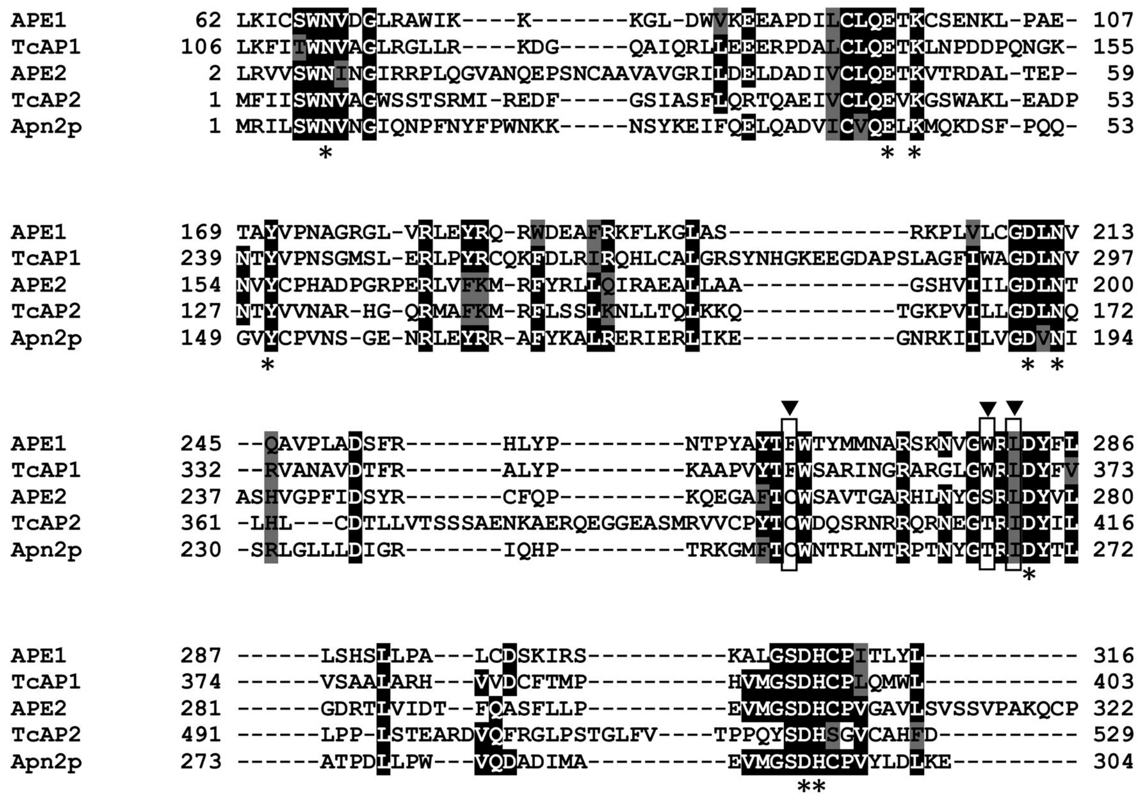

Fig. 1. Multiple amino acid sequences alignment of the *T. cruzi* TcAP1 and TcAP2 catalytic regions with *Homo sapiens* APE1 and APE2 and with *Schizosaccharomyces pombe* Apn2p. Conserved residues are highlighted in black (identical) or gray (similar). Asterisks (*) indicate AP endonuclease residues critical for AP binding and catalysis. Residues that influence the AP endonuclease ability of AP enzymes (hydrophobic pocket) are indicated by an arrow-head (▼).

fact that Mx inhibits *T. cruzi* AP endonucleases suggests the presence of a conserved mechanism of DNA BER repair from Kinetoplastids to mammals; (v) those results obtained in vitro validate our previous results obtained in vivo [Cabrera et al., 2011].

RECOMBINANT TcAP1 AND TcAP2 PROTEINS PRESENT AP ENDONUCLEASE ACTIVITIES THAT ARE INHIBITED BY MX AND BY A HUMAN APE1 NEGATIVE DOMINANT FORM

TcAP1 and APE1-DN recombinant proteins were obtained from *T. cruzi* epimastigotes transfected with the expression vectors pTREX-his-tcap1-gfp and pTREX-his-ape1dn-gfp, respectively. TcAP2 recombinant protein was obtained from *L. tarentolae* transfected with the expression vector pLEXSY-his-tcap2.

Purified recombinant TcAP1 or TcAP2 AP endonuclease enzymatic activities were determined incubating those proteins with a 24 mer synthetic oligonucleotide, containing an abasic site at position 8 for different times at 30°C.

As shown in Figure 4, the AP endonuclease activity of recombinants TcAP1 (A) and TcAP2 (B) increases as a function of incubation time, evidenced by a decrease in the intensity of the band corresponding to the oligonucleotide substrate (24 mer) and an increase in the intensity of the band corresponding to the enzymes product (7 mer). Additionally, the inhibitory effect of Mx on the activity of both AP endonucleases was assayed (Fig. 4C,D). Clearly, both recombinant enzymes are inhibited by this drug, as expected for DNA AP endonucleases.

Figure 4 shows the inhibitory effect of the human APE1 negative dominant form on the TcAP1 (4E) and on the TcAP2 (4F) AP endonuclease activities. APE1-DN possesses two amino acid substitutions at the active site residues Glu(96) (changed to Gln) and Asp(210) (changed to Asn). In vitro biochemical assays reveal that APE1-DN impedes wild-type AP endonuclease site incision function, presumably by binding AP-DNA and blocking normal lesion processing [McNeill and Wilson, 2007]. Clearly, both enzymes are inhibited by the DN form as evidenced by a decrease in the 7 mer product and an increase in the 24 mer non cleaved substrate, as a function of the dominant negative protein concentration.

Probably due to the presence of the hydrophobic pocket in its sequence, TcAP1 possesses a stronger AP endonuclease activity than TcAP2 as evidenced by the lower enzyme concentration required to detect its enzymatic activity (Fig. 4A,B) as well as by the higher APE1-DN concentration needed to inhibit that enzyme (Fig 4E,F).

TcAP1 AND TcAP2 GFP FUSION PROTEINS ARE EXPRESSED IN *T. CRUZI* EPIMASTIGOTES AND LOCATED THE PARASITE NUCLEI

TcAP1 and TcAP2 GFP fusion proteins overexpressed in transfected epimastigotes were detected using anti-TcAP1 (Fig. 5A), anti-TcAP2 (Fig. 5B), and anti-GFP (Fig. 5A,B) antibodies. Both fusion proteins

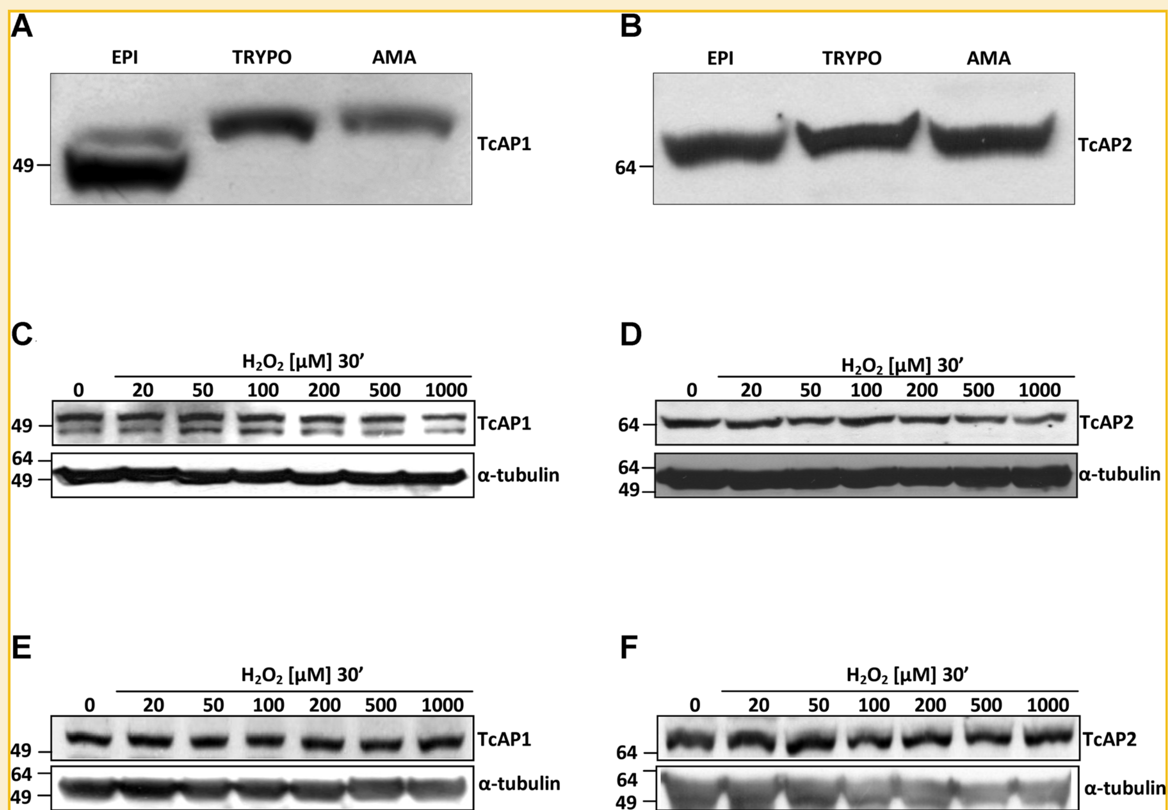

Fig. 2. TcAP1 and TcAP2 presence and their expression levels in *T. cruzi* cellular forms submitted to oxidative stress. Western blot detection of TcAP1 (A) and TcAP2 (B) in total protein homogenates from epimastigotes (EPI), trypomastigotes (TRYPO), and amastigotes (AMA), using specific anti-TcAP1 or anti-TcAP2 antibodies. Expression levels of *T. cruzi* AP endonucleases in epimastigotes (C,D) and trypomastigotes (E,F) treated with different initial H_2O_2 concentrations for 30 min. Enzymes were detected by Western blot using specific anti-TcAP1 (C,E) or anti-TcAP2 (D,F) antibodies. To normalize the levels of protein an anti-alpha tubulin antibody was used.

were clearly expressed (Fig. 5A,B, arrowheads). Using specific anti-peptide antibodies, both endogenous AP endonucleases were evidenced (Fig. 5A,B, asterisks). On the other hand GFP was detected in control epimastigotes transfected with empty vectors (Fig. 5A,B, white arrows). Densitometric analysis of TcAP1-GFP and TcAP2-GFP endonucleases showed 1.4- and 1.1-fold overexpression compared to the endogenous enzymes, respectively.

The human APE1 DNA repair enzyme was found in different subcellular locations (nucleus, cytoplasm and mitochondria), probably depending on the cell type analyzed and/or on their cellular physiological status [Kakolyris et al., 1998; Mitra et al., 2002; Jackson et al., 2005; Tell et al., 2005]. TcAP1 and TcAP2 subcellular localization was determined in *T. cruzi* epimastigotes expressing AP endonucleases fused to green fluorescent protein. Both TcAP endonucleases proteins were found only in the parasite nucleus (Fig. 5C). Thus, DAPI/TcAP1 and DAPI/TcAP2 merge experiments demonstrate absence of those enzymes in the *T. cruzi* kinetoplast (Fig. 5C). Control epimastigotes transfected with empty vectors show GFP evenly distributed in granules in the cytoplasm (Fig. 5C). TcAP1 and TcAP2 remain in the parasite nucleus even after incubation with 200 µM H_2O_2 for 30 min at 28°C (not shown).

All those results suggest that in *T. cruzi*: (i) Nuclear DNA damage may be repaired via AP endonucleases; (ii) Kinetoplast DNA do not repair their DNA via AP endonucleases; or (iii) An AP endonuclease other than TcAP1 and TcAP2 would be responsible for mitochondrial DNA AP endonuclease activity.

The fact that TcAP1 and TcAP2 are constitutively expressed (Fig. 2) and that both enzymes are found only in the parasite nucleus (Fig. 5C) may be related to the persistent exposure of *T. cruzi* to the oxidative species generated in their hosts as well as to the ancestral Trypanosomatids evolutionary position [Cavalier-Smith, 1993].

TcAP1 OVEREXPRESSION INCREASES PARASITE SURVIVAL WHEN SUBMITTED TO ACUTE AND SUSTAINED OXIDATIVE STRESS

Transfected epimastigotes overexpressing TcAP1 or TcAP2 endonucleases maintained in the exponential growth phase were treated for 30 min with increasing H_2O_2 or NOO^- concentrations. Afterwards the parasites were maintained 4 h in fresh supplemented medium to allow DNA repair [Cabrera et al., 2011] and their viability was measured by the MTT assay. Overexpression of TcAP1 enzyme induces a modest though clear increase in epimastigote viability when parasites are submitted to acute oxidative stress, either after H_2O_2 (Fig. 6A) or NOO^- (Fig. 6B). No such effect was evident for TcAP2 overexpression (Fig. 6C,D), except for 500 µM NOO^-.

On the other hand, sustained 45–65 µM H_2O_2 concentrations were obtained maintaining *T. cruzi* epimastigotes in 5 mM glucose plus

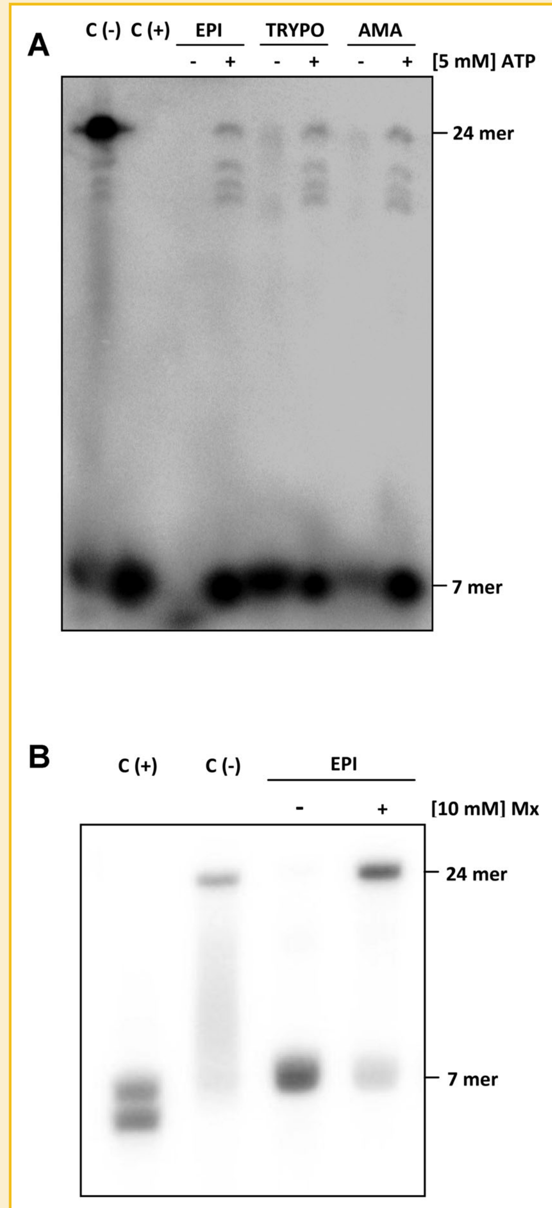

Fig. 3. AP endonuclease activity and DNA repair in *T. cruzi*. Inhibition by Mx. A: A 24 mer oligonucleotide containing an abasic site at position 8 was incubated with epimastigotes, trypomastigotes and amastigotes protein extracts for 120 min at 30°C, alone or in presence of 5 mM ATP. A 7 mer fragment is observed when protein extracts are incubated without ATP. Two main fragments of 7 and 24 mer are observed in the presence of ATP. B: A 24 mer oligonucleotide containing an abasic site at position 8 was incubated with epimastigotes protein extract for 60 min at 30°C, previously treated or non-treated for 2 h with 10 mM Mx. C(+): 24 mer oligonucleotide treated with *E. coli* UDG and Exo III; C(−): 24 mer oligonucleotide treated with *E. coli* UDG only.

10–100 mU glucose oxidase (GO) for 2 or 24 h. TcAP1 overexpression in epimastigotes submitted to those sustained H_2O_2 concentrations clearly increases parasite viability (Fig. 6E,F). As a whole, our results suggest that TcAP1 plays a role in the resistance of *T. cruzi* to oxidative DNA damage.

DISCUSSION

In mammals, cells from the immune system produce ROS/RNS as one of their mechanisms to control pathogen invasion [Valko et al., 2006; Ferrari et al., 2011]. Exogenously, cells receive different damaging signals that also lead to the generation of intracellular ROS [Herrling et al., 2003]. As a result, cells are equipped with different detoxifying and repairing mechanisms that are used to deal with damaging internal or external reactive species [Turrens, 2004; Valko et al., 2006].

T. cruzi, though does not present the glutation/glutation reductase system and catalase proper of recent eukaryotes, is well equipped with a battery of enzymes and molecules that efficiently control ROS/RNS [Krieger et al., 2000; Wilkinson et al., 2000, 2002a,b; Ariyanayagam and Fairlamb, 2001; Oza et al., 2002; Turrens, 2004; Piacenza et al., 2008]. This parasite is exposed to oxygen and nitrogen reactive species all along its life cycle. In the epimastigote extracellular replicative stage, the parasite is attacked by ROS produced during the blood digestion in the insect midgut [Graca-Souza et al., 2006]. On the other hand, opsonised infective non-replicative trypomastigotes interacting with macrophages in the mammal, recruit cytosolic subunits of the NADPH oxidase that associates with plasma membrane counterparts (included in a parasitophorous vacuole) resulting in the synthesis of large amounts of ROS/RNS directed against the internalized parasite [de Carvalho and de Souza, 1989; Piacenza et al., 2009]. Finally, amastigote intracellular replicative forms of the parasite mostly present in cardiac cells generate cardiac chronic inflammation which in turn provokes oxidative stress and mitochondrial dysfunction in cardiomyocytes [Munoz-Fernandez et al., 1992; Gupta et al., 2009a,b]; as a result ROS level increases resulting in an attack to these intracellular parasite forms.

As a consequence of this persistent oxidative attack, most parasites die; however, some survive establishing parasite persistence both in the insect and in mammals. In these parasites, damaged proteins and lipids are re-synthesized [Cardoso et al., 2011] while modified DNA should be repaired. Indeed, it was clearly established that H_2O_2 and NOO^- induces DNA damage in *T. cruzi* nucleus and kinetoplast and that this damage is repaired [Cabrera et al., 2011].

The main mechanism of oxidative DNA repair in eukaryotes is the base excision repair pathway [Slupphaug et al., 2003; Hegde et al., 2008; Robertson et al., 2009]. BER is an evolutionarily conserved process for maintaining nuclear and mitochondrial genomic integrity by eliminating oxidized, alkylated or mismatched bases that are generated endogenously or induced by ROS/RNS [Mandavilli et al., 2002; Hegde et al., 2008]. To date, the presence and activity of several enzymes of the BER pathway, such as UDGase (Uracil-DNA glycosylase), PARP and 8-oxoguanine DNA glycosylase have been studied in *T. cruzi* [Farez-Vidal et al., 2001; Pena-Diaz et al., 2004; Fernandez Villamil et al., 2008; Furtado et al., 2012]. Furthermore, the APE1 gene (TcAP1) has been described in this parasite; expression of this gene confers resistance to oxidant and alkylating agents when probed in hypersensitive *E. coli*, deficient in exonuclease III enzyme [Perez et al., 1999]. A sequence for another AP endonuclease (TcAP2) is also present in the *T. cruzi* genome (Fig. 1).

TcAP1 and TcAP2 were both expressed in the epimastigote, amastigote and trypomastigote cellular forms. TcAP1 shows two isoforms in epimastigotes (aprox. 49 and 56 kDa) while only one in

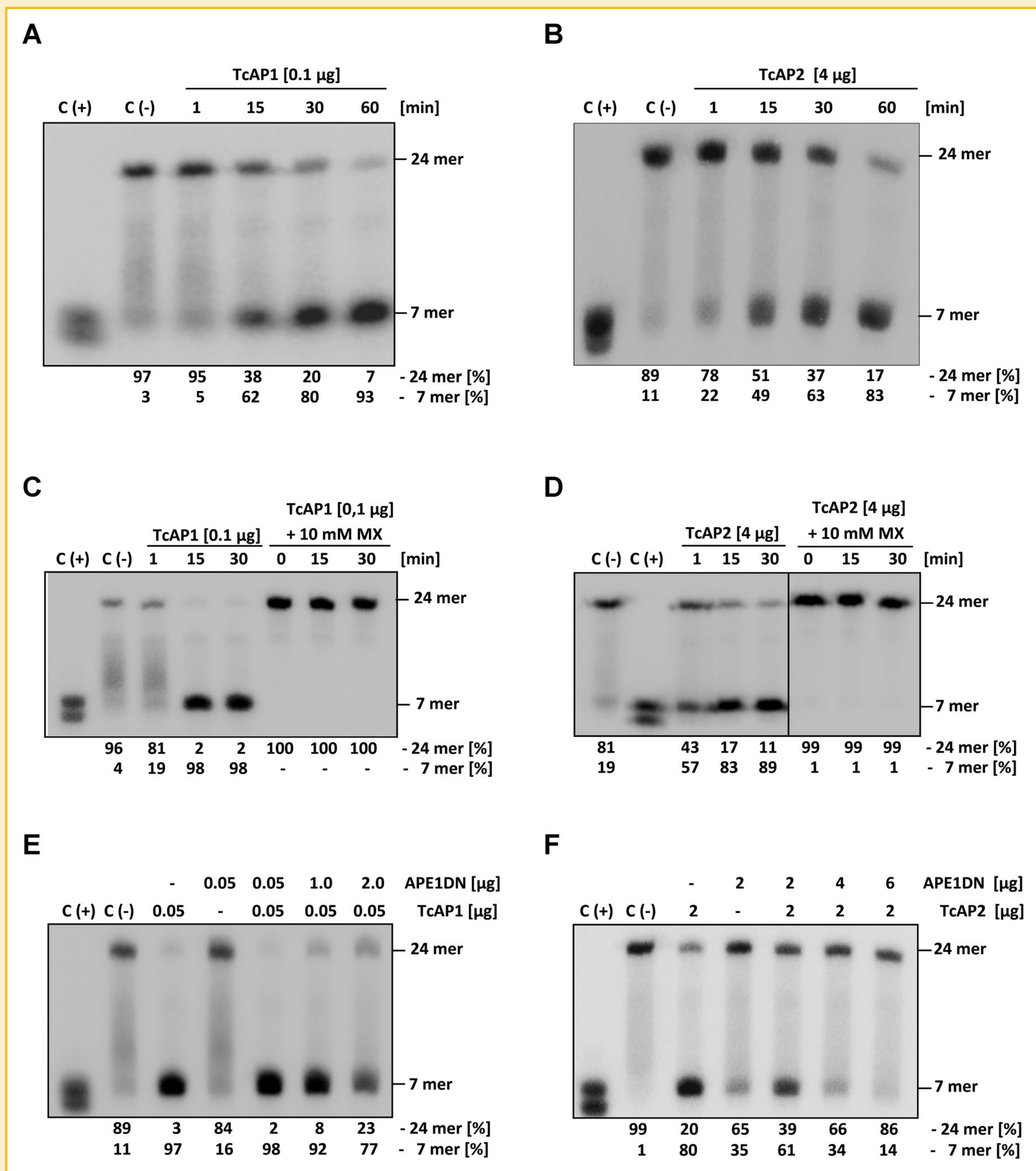

Fig. 4. Recombinant TcAP1 and TcAP2 AP endonuclease activities and their inhibition with Mx or with an APE1 negative dominant form. A 24 mer oligonucleotide containing an abasic site at position 8 was incubated with TcAP1 or TcAP2 recombinant proteins for 1, 15, 30, and 60 min at 30°C. A 7 mer fragment, corresponding to an oligonucleotide cut by the AP endonucleases, is observed as a result of TcAP1 (A) or TcAP2 (B) activities. Addition of 10 mM Mx (C,D) or of human APE1-DN recombinant protein (E,F) inhibits the activity of both enzymes. C(+): 24 mer oligonucleotide treated with *E. coli* UDG and Exo III; C(−): 24 mer oligonucleotide treated with *E. coli* UDG only. Band densitometric analysis was performed using the Quantity One (Bio Rad) version 4.6.3 program and expressed as percentage.

trypomastigotes and amastigotes (aprox. 58 kDa). Three isoforms of this endonuclease may be explained by the presence of several ATGs in the reading frame of the gene upstream of the initial ATG previously described for TcAP1 [Perez et al., 1999]. Indeed this mechanism of isoforms generation in different trypanosomatid stages was previously reported [Benabdellah et al., 2007; Rettig et al., 2011].

These authors propose that alternative trans-splicing is a mechanism that plays an important role in stage-regulated control of gene expression in trypanosomatids. Accordingly it was found that the *T. cruzi* LYT1 mRNA precursor coding for a 552 amino acid protein may be processed by alternative trans-splicing [Manning-Cela et al., 2002; Benabdellah et al., 2007]. Initiation of translation is forced to an in-

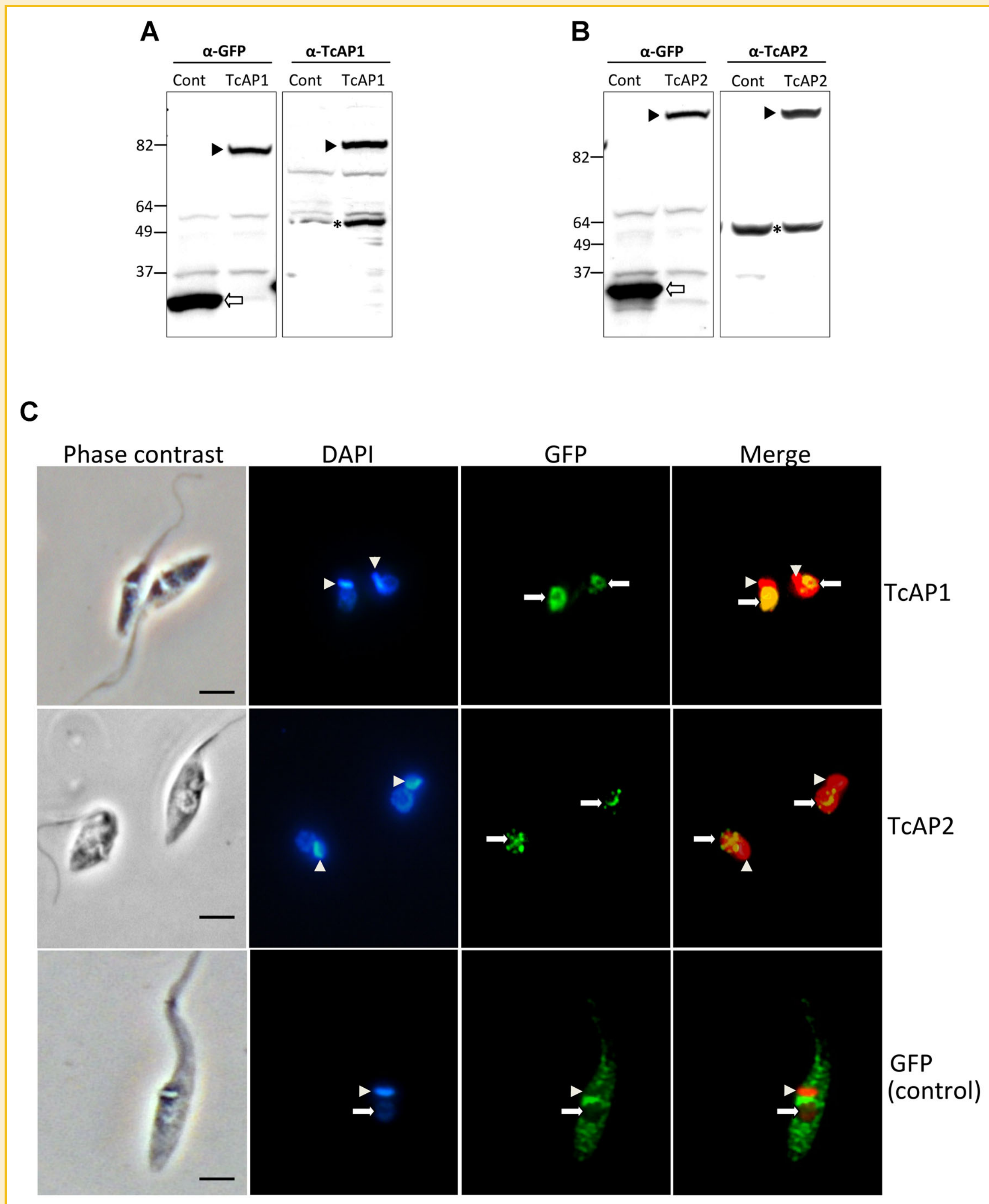

Fig. 5. TcAP1 and TcAP2 GFP fusion proteins are expressed in *T. cruzi* epimastigotes and located the parasite nuclei. TcAP1 and TcAP2 GFP fusion proteins were detected using anti-TcAP1 (A), anti-TcAP2 (B) and anti-GFP (A,B) antibodies. Arrowheads: TcAP1 (A) or TcAP2 GFP fusion proteins. Asterisks: Endogenous TcAP1 (A) and TcAP2 (B). White arrows: GFP control proteins. TcAP1, TcAP2, and control parasites (transfected with an empty vector) were detected in fixed *T. cruzi* epimastigotes (C) using an anti-GFP antibody and an anti-mouse secondary antibody conjugated to Alexa 488. For merge DAPI was used in red pseudo color. Arrows: nucleus; Arrowheads: kinetoplast. Bars 10 μm.

frame downstream AUG, and a shorter form of the protein is produced that lacks the first 28 amino acids, but it is otherwise identical to the larger form. While the longer mRNA is expressed mostly in the mammalian stages (trypomastigotes and amastigotes), the shorter one is most abundant in epimastigotes [Manning-Cela et al., 2002; Benabdellah et al., 2007]. In accordance with these results, studying the whole chromosome sequence display (using the TriTrypDB) we found several AG splice-acceptor sites 5′ to the annotated first one on the TcAP1 pre-mRNA molecule. Those additional AG could explain the presence of several TcAP1 isoforms. However, we do not discard

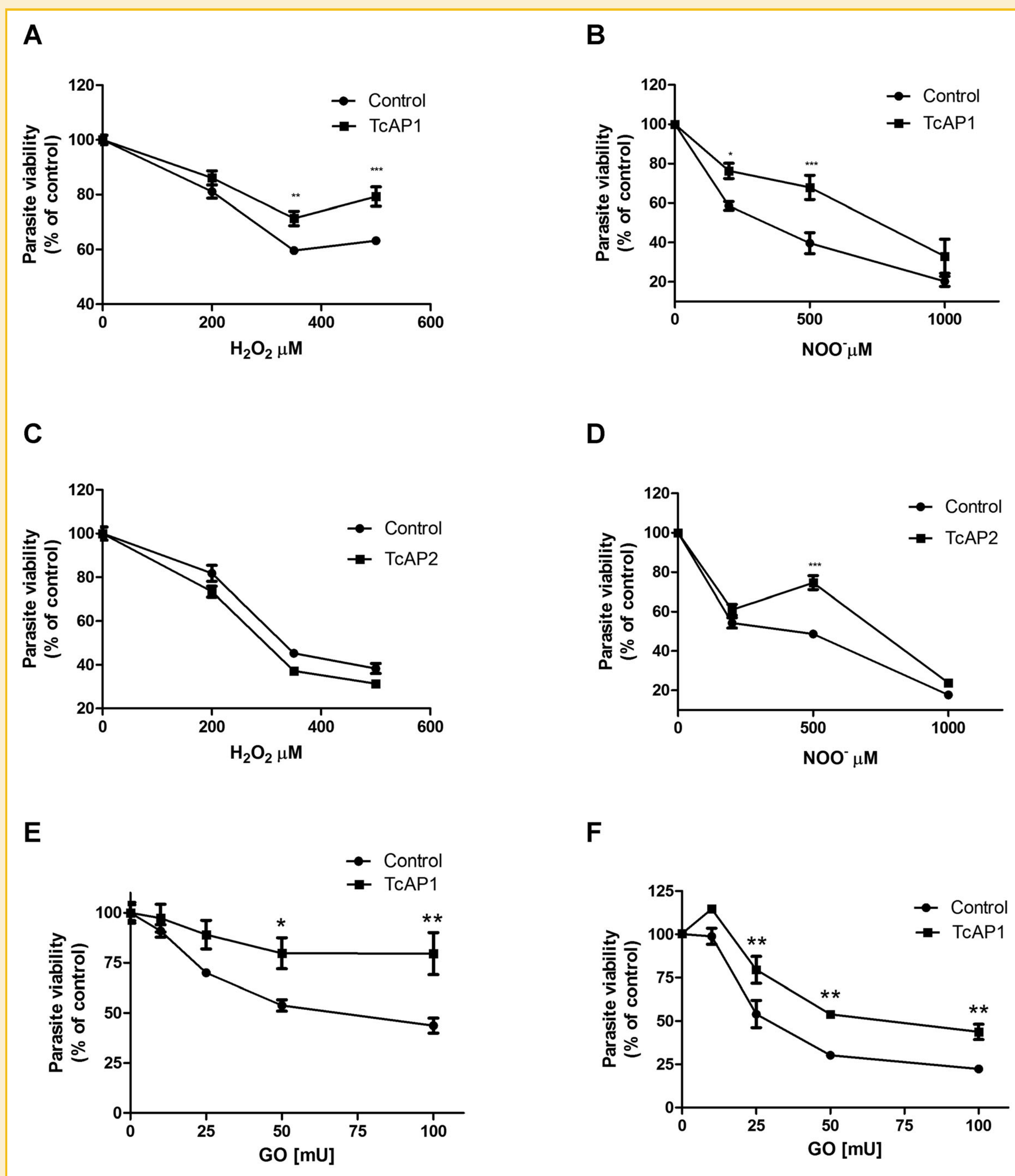

Fig. 6. Viability of TcAP1 and TcAP2 transfected epimastigotes submitted to acute and sustained oxidative stress. TcAP1 or TcAP2 overexpressing epimastigotes and their controls (transfected with empty vector) were treated for 30 min with different H_2O_2 (A,B) or NOO^- (C,D) initial concentrations. Afterwards, the parasites were washed and further incubated for 4 h in fresh medium. (E,F) show viability of TcAP1 transfected epimastigotes in the presence of sustained 45–65 μM H_2O_2 concentration for 2 (E) and 24 (F) h. Viability was determined by MTT assays. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.1$.

the presence of post translational modifications of the TcAP1 endonuclease that also may explain our results.

Contrarily to human APE1 [Wolff et al., 1991; Ramana et al., 1998; Fung et al., 2001; Silber et al., 2002], challenge of the parasite with oxidative agents does not induce a clear increase in the levels of TcAP1 and TcAP2 suggesting that both AP endonucleases are constitutively expressed. This is not surprising considering that all cellular forms of the parasite are continuously exposed to high levels of oxidative species. However, the level of expression of TcAP1 and TcAP2 endonucleases do not necessarily correlates with the activity of these enzymes.

Using in vitro assays applied in other eukaryotes [Hang et al., 1997; Castillo-Acosta et al., 2009] an AP endonuclease activity was found in epimastigote, amastigote and trypomastigote cellular forms

(Fig. 3); the AP oligonucleotide hydrolysis was inhibited by Mx which is consistent with a canonic AP endonuclease activity. Accordingly, two *T. cruzi* recombinant AP endonucleases presented AP endonuclease activity which was also inhibited by Mx (Fig. 4).

Human APE1, the major abasic endonuclease homologue to Exo III, displays a poor 3′ to 5′exonuclease activity while exhibiting a strong AP endonuclease and highly selective 3′phosphodiesterase activities [Seki et al., 1992; Wilson et al., 1995]. On the other hand human APE2, while showing a significant amino acid sequence similarity to the nuclease domains of APE1, exhibits a poor AP endonuclease activity. Those results suggest that differences in the amino acid sequences in the active sites lead to varying levels of endonuclease activity [Hadi et al., 2002].

Interestingly, deduced amino acid sequences for parasite TcAP1 and TcAP2 reveal that both present residues proper of the catalytic site expected for canonic AP endonucleases [Barzilay et al., 1995a,b]. In addition, TcAP1 shows a hydrophobic pocket that is needed for the recognition of apurynic/apyrimidinic sites in damaged DNA (Fig. 1). Similarly to human APE2, this pocket is absent in TcAP2; however, TcAP2 and *S. pombe* Apn2p share the same amino acids in that position. From these observations, it should be expected a strong AP endonuclease activity for TcAP1 while a weak one for TcAP2. Indeed we found that a low concentration of recombinant TcAP1 rapidly processes AP sites in a synthetic oligonucleotide while a higher concentration and an increasing time is required for TcAP2 (Fig. 4). As suggested for human APE2 [Burkovics et al., 2006] and *S. pombe* Apn2p [Sugimoto et al., 2005; Tanihigashi et al., 2006], TcAP2 may present a strong 3′-phosphodiesterase activity for removing 3′-phospho-α, β-unsaturated aldehyde ends induced by bifunctional DNA glycosylases. This proposal should be proved.

Consistent with the higher AP endonuclease activity shown by TcAP1 relative to TcAP2, a higher concentration of the human recombinant APE1-DN is needed to inhibit the recombinant TcAP1 enzyme. These results confirm that the presence of the hydrophobic pocket in TcAP1 amino acidic sequence is related to a higher activity of this AP endonuclease as compared with TcAP2 [Hadi et al., 2002].

In some mammalian cells the ortholog human APE1, when localized in the cytoplasm, is translocated to the nucleus as a consequence of oxidative stress. For this movement to take place, the first 20 amino acids of the amino terminal domain are essential [Jackson et al., 2005]. However other cell types show APE1 only in the nucleus [Kakolyris et al., 1998]. On the other hand, experimental removal of the first 33 amino acidic residues of the human APE1 amino terminal region favors its translocation to the mitochondria [Chattopadhyay et al., 2006]. Similarly, oxidative stress also induces relocation of APE1 to the mitochondria [Mitra et al., 2007]. On the other hand human APE2 is mainly located in the nucleus though a minor fraction was found in mitochondria [Tsuchimoto et al., 2001]. Though we have found that parasite TcAP1-GFP and TcAP2-GFP are located only in the epimastigote nucleus and that this location does not change upon exposition to ROS/RNS we cannot discard that endogenous TcAP1 and TcAP2 may have another subcellular location or that they suffer translocation upon exposition to oxidative agents.

Similarly, in the trypanosomatids *Leishmania major* [Perez et al., 1999; Castillo-Acosta et al., 2009] and *Trypanosoma brucei* [Charret et al., 2011] DNA AP endonucleases (LMAP and TBAPE1, respectively) are only present in the nucleus of those parasites. Taking all these results and ours into account, the nuclear location of DNA AP endonucleases may be a feature proper of Trypanosomatids.

Endogenous APE1 overexpression is observed in mammal cells exposed to genotoxic agents [Wolff et al., 1991; Ramana et al., 1998; Fung et al., 2001; Silber et al., 2002]. However, exogenous APE1 overexppression in C6 rat glyoma cells transfected with expression vectors carrying that endonuclease gene does not show an increase in survival after exposition to γ-radiation or alkylant agents [Herring et al., 1999]. Similar results were observed in CHO cells [Prieto-Alamo and Laval, 1999]. Parasite overexpressing TcAP1 exposed to H_2O_2 and NOO^- for short periods show a moderate increase in cell viability (Fig. 6A); this effect is not observed in transfected TcAP2 parasites except for 500 μM NOO^-. However, exposition of TcAP1 transfected parasites to sustained H_2O_2 concentrations reveals an important increase in cell viability, reflecting protection of epimastigotes against the oxidative assault. Accordingly, TBAPE1 depleted *T. brucei* cells show an increased susceptibility to nitric oxide (Charret et al.).

All these results are in agreement with the proposal that the BER pathway plays a key role in parasite survival and persistence in the different *T. cruzi* hosts. The possibility of searching for specific inhibitors of these enzymes, which could enhance the anti-parasitic effect generated by human host cells and/or improve the action of conventional anti chagasic drugs, is open.

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