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RESEARCH ARTICLE

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The overexpression of TcAP1 endonuclease confers resistance to infective *Trypanosoma cruzi* trypomastigotes against oxidative DNA damage

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Abstract

Trypanosoma cruzi, the causative agent of Chagas' disease survives to DNA damage generated by ROS/RNS inside to their different hosts. In recent eukaryotes, oxidative DNA damage is repaired mainly by the Base Excision Repair (BER) pathway, being essential the apurinic/apyrimidinic endonuclease activity. Using a pTREX-gfp vector, the nucleotide sequence that encodes *T. cruzi* AP endonuclease TcAP1 (orthologue of human APE1) and a putative TcAP1 dominant negative (TcAP1DN), were transfectedand expressed in *T. cruzi* epimastigotes. TcAP1-GFP and TcAP1DN-GFP were expressed in those modified epimastigotes and found in the parasite nucleus. The endonucleases were purified under native conditions and the AP endonuclease activity was evaluated. While TcAP1 presents the expected AP endonuclease activity TcAP1DN does not. Moreover, TcAP1DN partially inhibits in vitro TcAP1 enzymatic activity. Transfected epimastigotes expressing TcAP1-GFP and TcAP1DN-GFP were differentiated to infective trypomastigotes. The infective parasites maintained both proteins (TcAP1-GFP and TcAP1DN-GFP) in the nucleus. The overexpression of TcAP1-GFP in epimastigotes and trypomastigotes increases the viability of both parasite forms when exposed to oxidative stress while the expression of TcAP1DN-GFP did not show any in vivo inhibitory effect, suggesting that endogenous TcAP1 constitutive expression overcomes the TcAP1DN inhibitory activity. Our results show that TcAP1 is important for trypomastigote survival under oxidative conditions similar to those found in infected mammalian cells, then increasing its permanence in the infected cells and the possibility of development of Chagas disease.

KEYWORDS
DNA base excision repair, oxidative DNA damage, *T. cruzi* trypomastigote survival

1 | INTRODUCTION

Chagas disease, caused by *Trypanosoma cruzi*, a parasitic protozoan, is one of the most important zoonotic diseases transmitted by vectors in the American continent. Nowadays there are 6-7 millions of infected people and more than 10 thousand annual deaths related with this pathology.[1,2] To date, there are no available vaccines to control this disease and the more efficient method of prevention remains being vector control.[2,3] An effective treatment to the chronic phase of the

illness is also lacking[4] making necessary the search of more efficient and safer drugs with new therapeutic targets.

T cruzi presents an indirect life cycle with three cellular forms: epimastigote (extracellular, replicative, and non-infective form), trypomastigote (non-replicative and infective form) and amastigote (intracellular and replicative form).[5–7] Along its life cycle, the parasite is exposed to oxidative damage generated by constant production of reactive oxygen and nitrogen species (ROS/RNS) by their hosts. While most of the parasites die some are able to resist this damage and establish a chronic infection.[8,9]

ROS/RNS react with lipids, proteins, and DNA. While lipids and proteins may be re-synthesized DNA damage have to be repaired[10–12] mainly by the DNA Base Excision Repair (BER) pathway.[13–16] BER is a highly conserved pathway in eukaryotes that begins with the recognition of the oxidized base by one of many DNA glycosylases, which remove the damaged base, generating an AP site that is recognized by an apurinic/apyrimidinic endonuclease (AP endonuclease). This enzyme cleaves the DNA strand resulting in the formation of a one-nucleotide gap flanked by 3′-hydroxyl and 5′-deoxyribosephosphate (5′ dRP) ends. Afterwards, DNA polymerases adds a new nucleotide and DNA ligases seal the nick in the DNA backbone.[7,17,18]

Different studies have shown the importance of AP endonucleases activity in other trypanosomatids such as *Leishmania major* and *Trypanosoma brucei*.[19,20] In those protozoa, the overexpression of AP endonucleases confers resistance against genotoxic agents. In *T. cruzi*, the presence of two enzymes with AP endonuclease activity involved in the BER pathway has been described: TcAP1 and TcAP2.[14,21] The overexpression of TcAP1, but not of TcAP2, protects epimastigotes against acute and sustained oxidative stress.[13,14]

McNeill and Wilson[22] described a dominant negative form of the human apurinic/apyrimidinic endonuclease APE1 that lacks detectable nuclease activity and binds substrate DNA with higher affinity than the wild-type protein. This APE1 mutant form (APE1DN) possesses two amino acid substitutions at the active site (E96Q and D210N) and inhibits the trypanosome enzymes TcAP1 and TcAP2 activities in assays performed in vitro.[14] Interestingly, amino acids proper of the catalytic region of human APE1 are fully conserved in *T. cruzi* TcAP1 AP endonuclease. Taking these results into account, we have substituted the same amino acid described by McNeill and Wilson for the human APE1 in the active site of the *T. cruzi* TcAP1 to generate a putative negative dominant form of that AP endonuclease (TcAP1DN).

In order to overexpress the wild-type TcAP1 endonuclease and its putative negative dominant form (TcAP1DN) in *T. cruzi* epimastigotes the appropriate recombinant expression vectors were constructed. Those recombinant proteins were purified under native condition and their endonuclease activities were tested in vitro. TcAP1 but not TcAP1DN showed the expected AP endonuclease activity. On the other hand, TcAP1DN showed an inhibitory effect on the TcAP1 endonuclease activity.

Non-infective epimastigotes overexpressing TcAP1 and TcAP1DN were transformed to trypomastigotes, the infective form of the parasite. We show that the TcAP1 overexpression in non-infective epimastigotes is maintained in the nucleus of infective trypomastigotes after metacyclogenesis (differentiation) and protects both parasite cellular forms when chased with oxidative agents. Surprisingly, the inhibitory effect of TcAP1DN on the TcAP1 AP endonuclease activity observed in vitro, is not able to diminish parasite viability when exposed to oxidative stress.

2 | MATERIALS AND METHODS

2.1 | Parasite cultures

T. cruzi epimastigotes (Y strain) were cultivated at 28°C in LIT medium (Liver Infusion Tryptose: 5 g/L tryptose, 5 g/L liver extract, 3.97 g/L NaCl, 0.395 g/L KCl, 3.12 g/L HPO_4Na_2, 2 g/L glucose)[23] supplemented with 10% inactive fetal bovine serum (FBS, Biological Industries, Beit-Haemek, Israel), antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL, Biological Industries) and hemin (20 µg/L) (Sigma-Aldrich, Saint Louis, MO).

2.2 | Cell cultures

Vero cells (ATCC® CCL-81TM) were cultivated in RPMI 1640 medium (Biological Industries) supplemented with 5% active FBS and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL) (Biological Industries). Cell cultures were maintained at 37°C, 5% CO_2, and 95% relative humidity.

2.3 | Generation of TcAP1DN by TcAP1 site direct mutagenesis

A previously obtained pGEM-T-easy-*tcap1* plasmid was used to create a double mutant TcAP1 protein (TcAP1DN) by site-directed mutagenesis using the QuikChange® Lightning Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer indications. TcAP1DN possesses two amino acid substitutions at the AP endonuclease active site (residues Glu260Gln and Asp411Asn). Site-directed mutagenesis was performed by overlapping PCR[22] to sequentially introduce two codon changes. The following primers were used: E260Q-Primer: 5′-GCGTTGTGTCTGCAG**CAA**ACGAAGCTGAACCCG-3′); D411N-Primer: 5′-GTTTCATCTGGGCAGGC**AAC**CTGAATGTCGCCG-3′). Codon change is shown underlined. Mutated pGEM-T-easy-*tcap1dn* plasmid was confirmed by automatic DNA sequencing (not shown).

2.4 | *tcap1* and *tcap1dn* pTREX expression vector construction

tcap1 and *tcap1dn* DNA coding sequence were amplified by PCR using Platinum Taq High Fidelity polymerase (Invitrogen, Sao Paulo, Brazil) and inserted in the pTREX-*his-gfp* vector to generate pTREX-*his-tcap1-gfp* and pTREX-*his-tcap1dn-gfp* constructs. With these expression vectors, fusion proteins with an 8-histidine tail and a GFP tag in the N-terminal and C-terminal regions, respectively, were produced. With the aim of separating the GFP domains from the remaining fusion proteins, a nucleotide coding sequence that generates a helix-forming peptide linker (AEAAAKEAAAKEAAAKA) was introduced in order to reduce the interference between the GFP and the AP endonuclease amino acid sequences.[24] The primers used were: sense 5′-GCTCTAGAATGCCGTCGGGACC-TAAGG-3′ and antisense 5′- CCCAAGCTTCCTGCG-CAGCCACATCTGC-3′ for TcAP1 and TcAP1DN; those primers present restriction sites for *Xba* I and *Hind* III enzymes in the sense and in the antisense sequences, respectively. TcAP1 and TcAP1DN DNA coding sequences were amplified using previously obtained pGEM-T-easy-*tcap1* and pGEM-T-easy-*tcap1dn* plasmids as templates. The correct insertion of each sequence was confirmed by PCR, enzymatic digestion and automatic DNA sequencing (not shown).

2.5 | Transfection of pTREX-*his-tcap1-gfp* and pTREX-*his-tcap1dn-gfp* expression vectors in *cruzi* epimastigotes

Epimastigotes in the exponential phase of growth were electroporated with pTREX-*his-tcap1-gfp* or pTREX-*his-tcap1dn-gfp* constructs and with the empty pTREX-*his-gfp* plasmid 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 incubated with 50-100 μg of each plasmid. Electroporation was performed at 0.3 kV and 500 μF in two pulses separated by 1 min maintaining the parasites on ice. Immediately, transfected epimastigotes were transferred to 20% FBS LIT medium. After 24 h, 250 μg/mL of G418 antibiotic were added increasing the antibiotic concentration to 500 μg/mL at 72 h and the transfected parasites were maintained for 4 weeks. Transfection efficiency was assessed by fluorescence microscopy.

2.6 | Detection of recombinant tcAP1-GFP and tcAP1DN-GFP proteins in transfected epimastigotes

Recombinant TcAP1-GFP and TcAP1DN-GFP were detected in transfected epimastigotes homogenates by Western blot assays using an anti-GFP polyclonal antibody (Sigma-Aldrich). Briefly, parasite protein extracts were separated by electrophoresis in 10% acrylamide gels. Afterwards, proteins were transferred to a nitrocellulose membrane and incubated with 5% bovine serum albumin (BSA) in PBS 0.05% Tween-20 at 4°C overnight. The membrane was then incubated for 2 h with the anti-GFP polyclonal antibody (Sigma-Aldrich) in a 1:4000 v/v dilution in 1% BSA in PBS 0.05% Tween-20 at room temperature and subsequently with a secondary goat anti-rabbit antibody coupled to horseradish peroxidase (HRP, Jackson Immuno Research Laboratories, Inc, West Grove, PA). Results were visualized by chemiluminescence using the Supersignal West Pico kit (Thermo Scientific, Rockford, IL).

2.7 | TcAP1-GFP and TcAP1DN-GFP protein purification under native conditions

Recombinant TcAP1-GFP and TcAP1DN-GFP proteins were purified from transfected epimastigote cultures by affinity chromatography using HisPur Ni-Nta agarose resin (Thermo Scientific) in native conditions, following the manufacturer indications.

2.8 | Subcellular location of TcAP1-GFP and TcAP1DN-GFP fusion proteins in transfected epimastigotes and in differentiated infective trypomastigotes

Subcellular location of TcAP1-GFP and TcAP1DN-GFP proteins in transfected *T. cruzi* epimastigotes and trypomastigotes were assayed by immunodetection as described.[15] Briefly, following parasite fixation (70% ice methanol for 30 min), parasites were treated with blocking solution (1% BSA w/v, 0.1% saponine v/v, 3% calf serum v/v in PBS) for 2 h at 37°C and incubated overnight at 4°C with a monoclonal anti-GFP antibody (Thermo Scientific). Samples were then washed and further incubated with a secondary antibody conjugated to Alexa 488 fluorochrome (Molecular Probes, Eugene, OR). Nuclear and kinetoplastid DNA were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL for 5 min). Samples were evaluated by fluorescence microscopy using 430 ± 20 nm and 520 ± 20 nm filters for blue and green fluorescence, respectively. Photographs were processed computationally to determine the overlap of DAPI (pseudocolor red) and Alexa fluor 488 (green).

2.9 | AP endonuclease activity assays

To determinate TcAP1-GFP and TcAP1DN-GFP AP endonuclease activity, a 25 mer DNA oligonucleotide was designed (THF-oligo). This oligo contains an artificial AP site by dSpacer addition (similar to tetrahydrofuran structure) in the 13th nucleotide position (5′-CCGCTAGCGGGT-**dSpacer**-ACCGAGCTCGAAT-3′). THF-oligo was labeled with $P\gamma^{32}$ at

the 5′ end using *DNA 5 End Labeling System* (Promega, Madison, WI) kit and then hybridized with an unlabeled complementary oligonucleotide (3′-GGCGATCGCC-CA**C**TGGCTCGAGCTTA-5′).

To evaluate TcAP1-GFP and TcAP1DN-GFP AP endonuclease activities, 2 pg of labeled THF-oligo was incubated with TcAP1-GFP or TcAP1DN-GFP at increasing protein concentrations (0.1, 0.3, 1.0, 3.0, and 10 ng) in BER buffer solution (50 mM hepes KOH pH 7.8, 0.36% BSA w/v, 70 mM KCl, 5 mM $MgCl_2$, and 0.5 mM DTT) in a final volume of 25 µl for 30 min at 37°C. As a positive control the THF-oligo was incubated with 1U of Exonuclease III (*E. coli* AP endonuclease, New England Biolabs, Ipswich, MA). As a negative control the untreated labeled THF-oligo was used. To inactivate the enzymes all samples were heated at 75°C for 10 min after incubation. Afterwards, an equivalent volume of BER buffer (96% formamide v/v, 20 mM EDTA, 5 mM Tris pH 7,5, 0.05% xylene cyanol w/v, 0.05% bromophenol blue w/v) was added. Finally, all samples were heated at 95°C for 5 min and electrophoretically separated in formamide-acrilamide denaturing gels (20% acrilamide w/v) at 60 watts for 1 h. The radioactivity was detected using Personal Molecular Imager FX System (Biorad, Hercules, CA).

The capacity of TcAP1DN-GFP protein to inhibit the TcAP1-GFP AP endonuclease activity in in vitro assays was also determined. Briefly, 2 pg of labeled THF-oligo were pre-incubated with 10 ng of TcAP1DN-GFP purified protein for 15 min at 37°C in BER buffer. Afterwards, increasing concentrations of the recombinant TcAP1-GFP protein (0.1, 0.3, 1.0, 3.0, and 10 ng) were added and the samples were further incubated for 15 min at 37°C. The densitometric analysis of bands was performed using the Quantity One (Biorad) program version 4.6.3.

2.10 | *cruzi* epimastigotes in vitro cellular differentiation to trypomastigotes (metacyclogenesis)

To obtain infective trypomastigotes that expresses TcAP1-GFP or TcAP1DN-GFP proteins, transfected epimastigotes expressing those proteins were submitted to in vitro cellular differentiation (metacyclogenesis) using Triatomine Artificial Urine (TAU) 3AAG medium.[25,26] Briefly, 6×10^7 transfected epimastigotes per mL maintained in the logarithmic phase of growth in LIT medium were collected and centrifuged at 7.000*g* for 15 min at 10°C. Afterwards, the parasites were concentrated to 5×10^8/mL and exposed to nutritional stress in TAU medium (190 mM NaCl, 8 mM phosphate buffer pH 6.0, 17 mM KCl, 2 mM $MgCl_2$, 0.35 g/L $NaHCO_3$) for 2 h at 28°C. Finally, 1×10^9 parasites were added to 200 mL of TAU3AAG medium (10 mM proline, 50 mM L-glutamic acid, 2 mM L-aspartic acid, and 10 mM glucose), and incubated for 96 h at 28°C. Metacyclic trypomastigotes were purified from the TAU3AAG medium by DEAE-cellulose columns.[27]

2.11 | Obtainment of cell-derived trypomastigotes

To increase the number of infective trypomastigotes expressing TcAP1-GFP, TcAP1DN-GFP or GFP alone (control), metacyclic trypomastigotes were immediately used to infect culture Vero cells. After incubation for 24 h at 1:10 cell:parasite ratio at 37°C the infected Vero cell cultures were washed with sterile PBS 1X and supplemented with fresh RPMI 5% FBS. After 4 days, cell-derived trypomastigotes were collected from the supernatant for viability assays.

2.12 | Viability assays of transfected epimastigotes and trypomastigotes exposed to oxidative stress

A 2×10^7/mL epimastigotes or infective trypomastigotes expressing TcAP1-GFP or TcAP1DN-GFP were exposed to increasing H_2O_2 concentrations (200, 350, or 500 µM; Sigma-Aldrich) in LIT medium at 30°C (epimastigotes) or RPMI culture medium at 37°C (trypomastigotes). After 30 min of incubation, the parasites were washed once with PBS and incubated with fresh medium for 4 h at 30°C or 37°C to allow DNA repair.[13] Subsequently, parasites were resuspended in Grace's medium (epimastigote) or RPMI without red phenol medium (trypomastigote) and 100 µl of parasite aliquots were incubated with 10 µl AlamarBlue reagent (Invitrogen, Rockford, lL) in 96 well plates to evaluate their viability. The plates were further incubated for 4 h at 37°C and the fluorescence was measured at 560/590 excitation/emission (VarioskanTM Flash Multimode Reader, Thermo Scientific).

2.13 | Statistical analysis

Statistical analyses were performed using the commercially available software *Graph Pad Prism* version 5. For viability assays, all experiments were done in triplicate. Significant data differences were compared using Student's *T*-test.

3 | RESULTS

3.1 | Human APE1 and *cruzi* TcAP1 endonucleases share amino acids proper of the active site allowing the generation of TcAP1DN

Following McNeill and Wilson,[22] who described a dominant negative form of the human apurinic/apyrimidinic endonuclease, a plasmid expressing the mutant form of

trypanosome TcAP1 endonuclease (TcAP1DN) was constructed. Though human APE1 and *T. cruzi* TcAP1 endonucleases show a low identity percentage (~20%),[14] the residues proper of the active site are conserved (Figure 1, asterisks). Consequently, TcAP1DN mutant possesses the same two amino acid substitutions at the active site (E260Q and D411N) as those described by McNeill and Wilson[22] for the human counterpart (Figure 1, arrowheads).

3.2 | TcAP1-GFP and TcAP1DN-GFP are expressed in transfected epimastigotes and located in the parasite nucleus

Protein expression associated to GFP-labeled TcAP1 and TcAP1DN was detected in total protein homogenates of transfected epimastigotes by Western blot using an anti-GFP primary antibody. In epimastigotes transfected with an empty vector plasmid (Figure 2A, lane 1) a band close to 30 kDa corresponding to GFP expression is shown. Epimastigotes overexpressing either TcAP1-GFP (Figure 2A, lane 2) or TcAP1DN-GFP (Figure 2A, lane 3) show protein bands of approximately 75 kDa, corresponding to the expected mass of the recombinant proteins (TcAP1 or TcAP1DN GFP-tagged).

TcAP1-GFP and TcAP1DN-GFP subcellular localization was determined in transfected *T. cruzi* epimastigotes by immunofluorescence using an anti-GFP antibody. Both proteins were found as granules in the parasite nucleus (Figure 2B, arrows). Merge experiments demonstrate absence of those proteins in the *T. cruzi* kinetoplast (Figure 2B, arrowheads). Control epimastigotes transfected with empty vector show GFP as granules evenly distributed in the parasite cytoplasm (Figure 2B, GFP control).

3.3 | TcAP1, but not TcAP1DN, shows AP endonuclease activity

A 25 mer DNA oligonucleotide (THF-oligo) possessing an artificial AP site at position 13 (Figure 3A) that can be recognized and processed by AP endonucleases was synthesized. This THF-oligo was 5′-γP^{32} labeled and aligned with the non-labeled complementary strand. An AP endonuclease activity generates a radioactive labeled 12 mer fragment, while the absence of that enzymatic activity maintains the labeled 5′-γP^{32} 25 mer oligonucleotide (Figure 3A). The labeled products were separated by electrophoresis in denaturing 96% formamide-20% acrylamide gels and the resulting oligonucleotides were detected using a Personal Molecular Imager FX System (Biorad).

To perform the AP endonuclease in vitro activity assays, the recombinant TcAP1 and TcAP1DN proteins were obtained under native conditions. Figure 3B shows that TcAP1 recombinant protein presents AP endonuclease activity since it is able to efficiently process the substrate producing a 12 mer labeled product when 3.0 and 10 ng of the purified TcAP1 wild-type protein were used. Contrarily, the same concentrations of the purified TcAP1DN

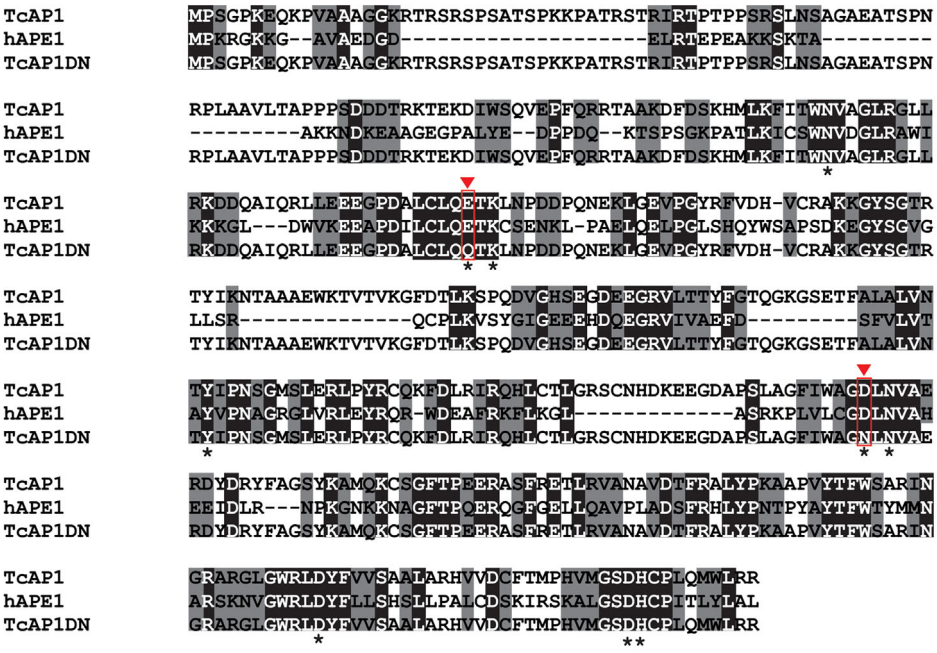

FIGURE 1 Multiple amino acid sequences alignment of the *T. cruzi* TcAP1, Homo sapiens APE1, and *T. cruzi* TcAP1DN proteins showing E260Q and D411N amino acid substitutions. Conserved residues are highlighted in black (identical) or gray (similar). Asterisks (*) indicate conserved AP endonuclease residues critical for AP binding and catalysis. Arrow-head (▼) indicates mutated residues in TcAP1DN

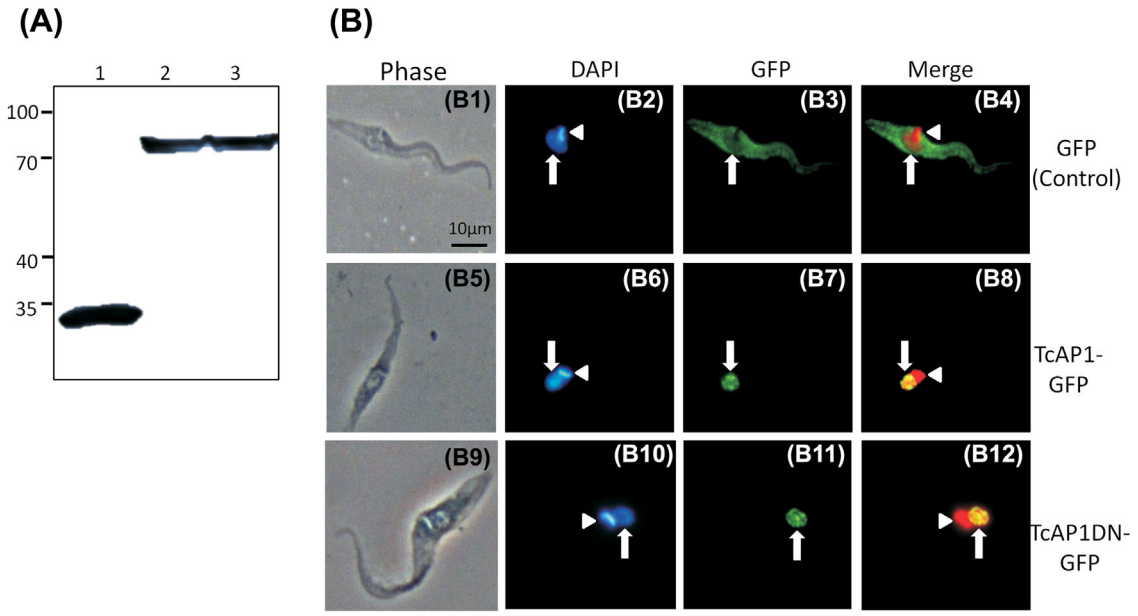

FIGURE 2 TcAP1 and TcAP1DN GFP fusion proteins are expressed in *T. cruzi* epimastigotes and located in parasite nuclei. A, TcAP1 and TcAP1DN GFP fusion proteins were detected by Western blot using an anti-GFP antibody Lane 1: GFP expression in epimastigotes transfected with pTREX-gfp empty plasmid (expected molecular mass: ~30 kDa); Lane 2: TcAP1-GFP expression in epimastigotes transfected with pTREX-*gfp-tcap1* plasmid (expected molecular mass: ~75 kDa); Lane 3: TcAP1DN-GFP expression in epimastigotes transfected with pTREX-*gfp-tcap1*dn plasmid (expected molecular mass: ~75 kDa). B, GFP from parasites transfected with an empty vector (B1-B4) and TcAP1-GFP (B5-B8) and TcAP1DN-GFP (B9-B12) proteins were detected in fixed transfected *T. cruzi* epimastigotes using an anti-GFP primary antibody and an anti-mouse secondary antibody conjugated to Alexa 488. Phase contrast (B1, B5, B9); DAPI (B2, B6, B10); GFP (B3, B7, B11); Merge (B4, B8, B12). For merge DAPI was used in red pseudo color. Arrows: nucleus; Arrowheads: kinetoplast. Bars 10 μm

recombinant protein are not able to process the labeled THF-oligo, indicating lack of AP endonuclease activity of that protein (Figure 3C). As a positive control, the labeled 25 mer oligonucleotide was treated with *E. coli* Exo III. This enzyme generates higher mobility bands (Figures 3B and 3C; C(+)) as a consequence of the exonuclease 3′-5′ activity of Exo III on the 12 mer labeled product resulting from the AP endonuclease activity.[28] As a negative control the labeled 25 mer oligonucleotide alone is shown (Figures 3B and 3C; C(−)).

3.4 | TcAP1DN acts as a TcAP1 partial inhibitor

In order to assay the possible inhibitory effect of the TcAP1DN on the TcAP1 AP endonuclease activity the labeled 25 mer substrate was pre-incubated with 10 ng of native recombinant TcAP1DN purified protein for 15 min at 37°C. Afterwards, increasing concentrations of the native recombinant TcAP1 protein (0.1, 0.3, 1.0, 3.0, and 10 ng) were added and the samples further incubated for 15 min at 37°C. Products were resolved by gel electrophoresis and the resulting bands were detected using Personal Molecular Imager FX System (Biorad). A partial inhibition of the TcAP1 AP endonuclease activity was observed which was evident when 3.0 ng (65%) and 10 ng (35%) of TcAP1 wild-type was assayed in the presence of 10 ng TcAP1DN (Figure 4) as compared with the enzymatic activity with 3.0 and 10 ng of TcAP1 on the same substrate (Figure 3B).

3.5 | TcAP1-GFP and TcAP1DN-GFP maintain their nuclear location in *cruzi* infective trypomastigotes obtained after epimastigote in vitro metacyclogenesis

In order to determine whether infective trypomastigotes maintain the TcAP1-GFP and TcAP1DN-GFP nuclear location, non-infective transfected epimastigotes were differentiated to trypomasitgotes by in vitro metacyclogenesis. Recombinant metacyclic trypomastigotes expressing TcAP1-GFP, TcAP1DN-GFP, or GFP alone (control) were immediately used to infect culture Vero cells in order to obtain cell-derived trypomastigotes. In those parasites, both recombinant proteins maintain their granulated nuclear location as detected by immunofluorescence using an anti-GFP antibody (Figure 5, arrows). Merge experiments demonstrated absence of those proteins in the *T. cruzi* kinetoplast (Figure 5, arroheads). Control trypomastigotes express GFP evenly distributed in the parasite cytoplasm (Figure 5, GFP control).

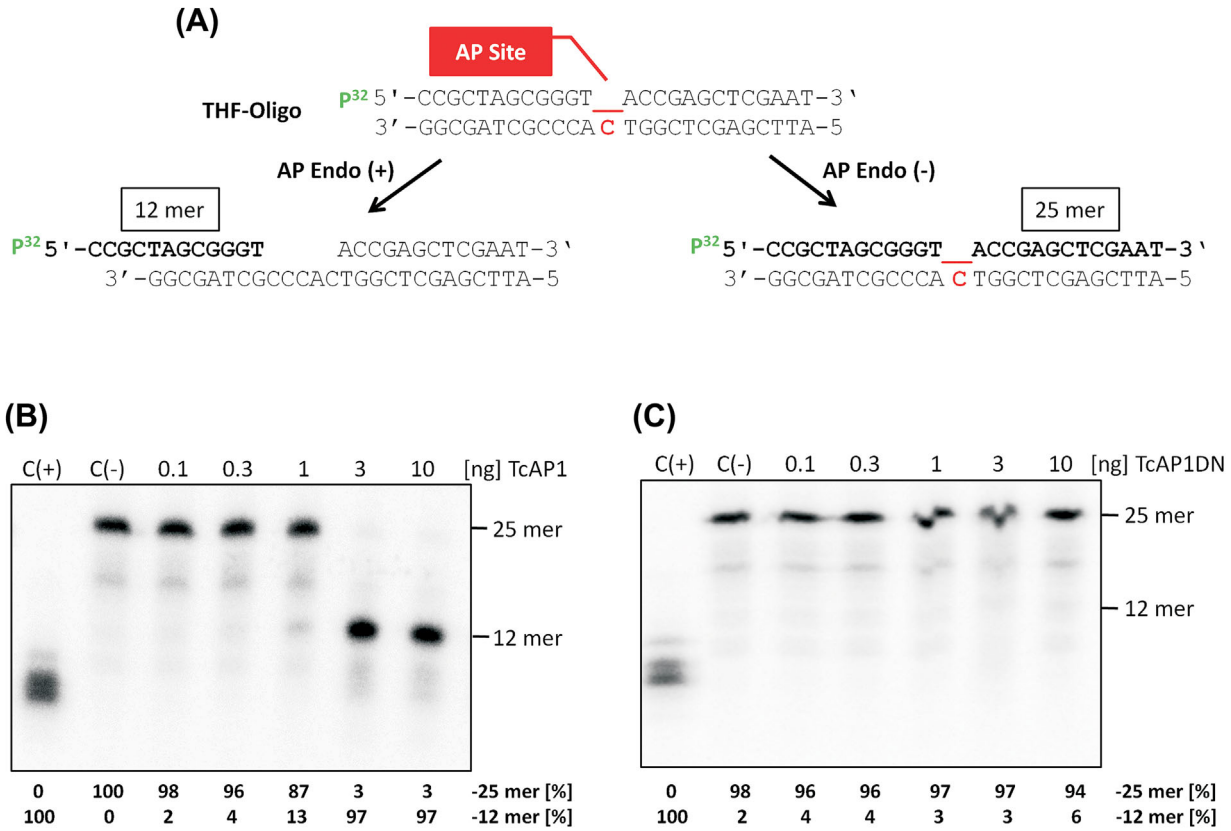

FIGURE 3 TcAP1, but not TcAP1DN, presents AP endonuclease activity. A, Schematic representation of the AP endonuclease activity detection using a 25 mer 5′ Pγ32 labeled THF-oligo substrate with an artificial AP site at position 13. The generation of a labeled 12 mer fragment indicates AP endonuclease activity. B, The THF-oligo substrate was incubated with 0.1, 0.3, 1, 3, and 10 ng of TcAP1 recombinant protein for 30 min at 37°C. A 12 mer fragment is observed as a result of TcAP1 activity when using 1, 3, and 10 ng of the enzyme. C, The THF-oligo substrate was incubated with 0.1, 0.3, 1, 3, and 10 ng of TcAP1DN recombinant protein for 30 min at 37°C. A 12 mer fragment is not observed at any protein concentration tested. In B and C: C(+): positive control, 25 mer oligonucleotide treated with Exo III; C(−): negative control, 25 mer oligonucleotide without enzyme. Band densitometric analysis was performed using the Quantity One (Bio Rad) version 4.6.3 program and expressed as percentage

3.6 | TcAP1 overexpression improves epimastigote and trypomastigote survival when exposed to acute oxidative stress while TcAP1DN does not present an in vivo effect

Viability of recombinant epimastigotes and trypomastigotes overexpressing either TcAP1-GFP or TcAP1DN-GFP proteins, were evaluated under acute oxidative stress using increasing H_2O_2 concentrations. The overexpression of TcAP1 wild-type protein in epimastigotes (Figure 6A) and trypomastigotes (Figure 6B) increases the viability of both parasite forms when compared to control parasites. Contrary to our expectations, the expression of TcAP1DN in epimastigotes (Figure 6C) and trypomastigotes (Figure 6D) do not show differences in their viability when compared to their control parasites with the exception of expressing TcAP1DN trypomastigotes that show an increase in their viability when submitted to 200 μM of H_2O_2 (Figure 6D) in three different experiments in triplicate.

4 | DISCUSSION

DNA repair includes diverse and well-developed mechanisms devoted to eliminate different injuries produced in the genetic integrity of eukaryotic cells.[29,30] Considering that cells have been exposed to DNA damage from their origins, mechanisms of DNA repair should have appeared early in evolution. This may explain why many of these repair mechanisms are highly conserved in prokaryotes and eukaryotes. DNA Base Excision Repair (BER) is an evolutionarily conserved process for maintaining nuclear and mitochondrial genomic integrity by eliminating damaged oxidized or alkylated bases that are generated endogenously or induced by genotoxicants, predominantly oxidative agents.[31,32]

Generally, each organism expresses one or two AP endonucleases belonging to the BER pathway, orthologous to *E. coli* exonuclease III (Xth) or *to E. coli* endonuclease IV (Nfo).[33] In *Homo sapiens* the major DNA repair AP

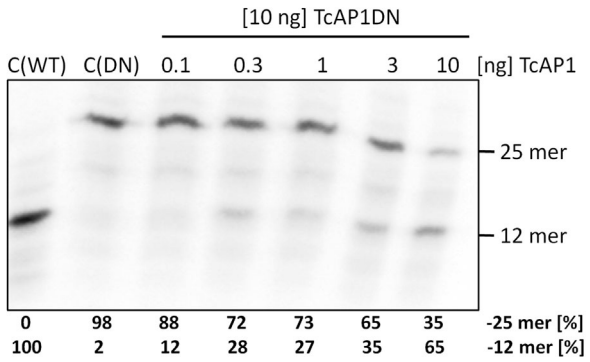

FIGURE 4 TcAP1DN partially inhibits the TcAP1 AP endonuclease activity. TcAP1DN purified protein (10 ng) was pre-incubated with the labeled THF-oligo at 37°C for 15 min. Afterwards, wild-type TcAP1 purified protein was added to the reaction mixture at increasing concentrations (0.1, 0.3, 1, 3, and 10 ng) and incubated at 37°C for an extra 15 min. TcAP1DN partial inhibition of the TcAP1 AP endonuclease activity is evident when higher concentrations of the wild-type protein were used

endonuclease is APE1 (HAP1, APEX, or REF1), an *E. coli* exonuclease III homolog that is essential for genome maintenance and cell survival.[34,35] APE1 promotes survival of different cell types after oxidative stress stimulation.[36–38] Suppression of APE1 expression by means of interference RNA (RNAi) stops cell cycle and induces apoptosis in several cellular types.[39] Similarly, knock-out mice for APE1 do not progress after the blastocyst stage.[40] These results show that APE1 is essential in the repair of oxidative cytotoxic DNA damage. Contrarily, *E. coli* strains lacking Exo III grow normally, although these bacteria show a high sensitivity to ROS.[41]

In *T. brucei*, the TBAPE1 AP endonuclease protein (*T. cruzi* TcAP1 orthologous,) was also characterized.[20] The purified recombinant TBAPE1 enzyme exhibited AP endonuclease activity in in vitro assays. This AP endonuclease is not essential for the parasite (TBAPE1-knock out bloodstream cellular forms are viable) but results in a significant increase in abasic DNA damage rendering cells hypersensitive to oxidative agents. On the other hand, overexpression of LmAP endonuclease in *L.* major, increases parasite resistance to hydrogen peroxide and methotrexate.[42]

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 methoxyamine (Mx),[13] an AP endonuclease activity inhibitor.[43,44] Those results indicate that the BER pathway is present and active in both parasite cellular forms. Several enzymes of the BER pathway (Uracil-DNA glycosylase, PARP, and DNA glycosylase TcOgg1) have been studied in *T. cruzi*.[45–47] In our laboratory DNA glycosylase NTH1, *T. cruzi* TcAP1 and TcAP2 AP endonucleases (*Homo sapiens* APE1 and APE2 orthologous, respectively), and TcFEN1 endonucleases have also been recently characterized.[14–16]

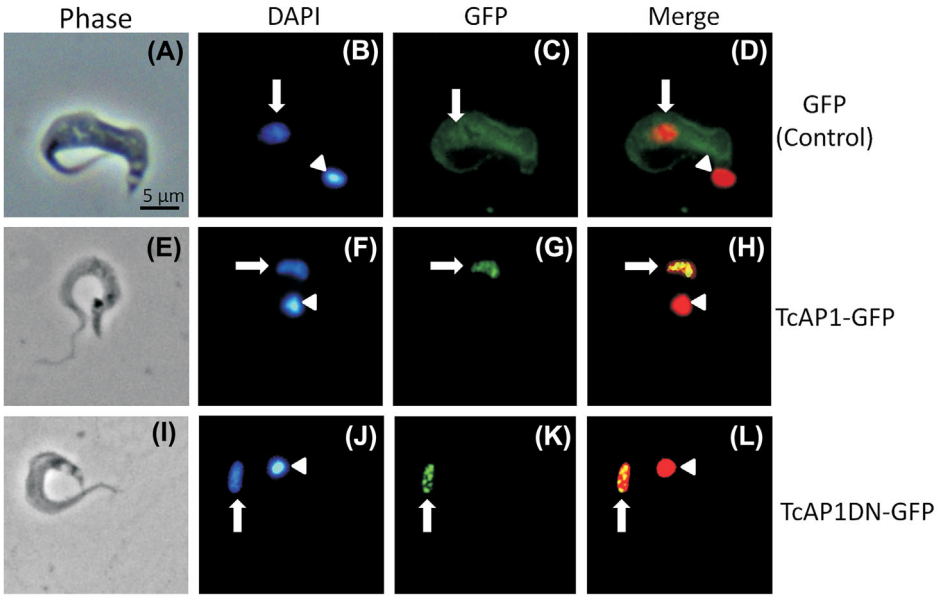

FIGURE 5 TcAP1 and TcAP1DN GFP fusion proteins remain in the nucleus of *T. cruzi* after epimastigote differentiation to infective trypomastigotes. GFP (A-D), TcAP1-GFP (E-H) and TcAP1DN-GFP (I-L) proteins were detected in fixed recombinant *T. cruzi* trypomastigotes using an anti-GFP primary antibody and an anti-mouse secondary antibody conjugated to Alexa 488. Phase contrast (A, E, I); DAPI (B, F, J); GFP (C, G, K); Merge (D, H, I). For merge DAPI was used in red pseudo color. Arrows: nucleus; Arrowheads: kinetoplast. Bars 5 µm

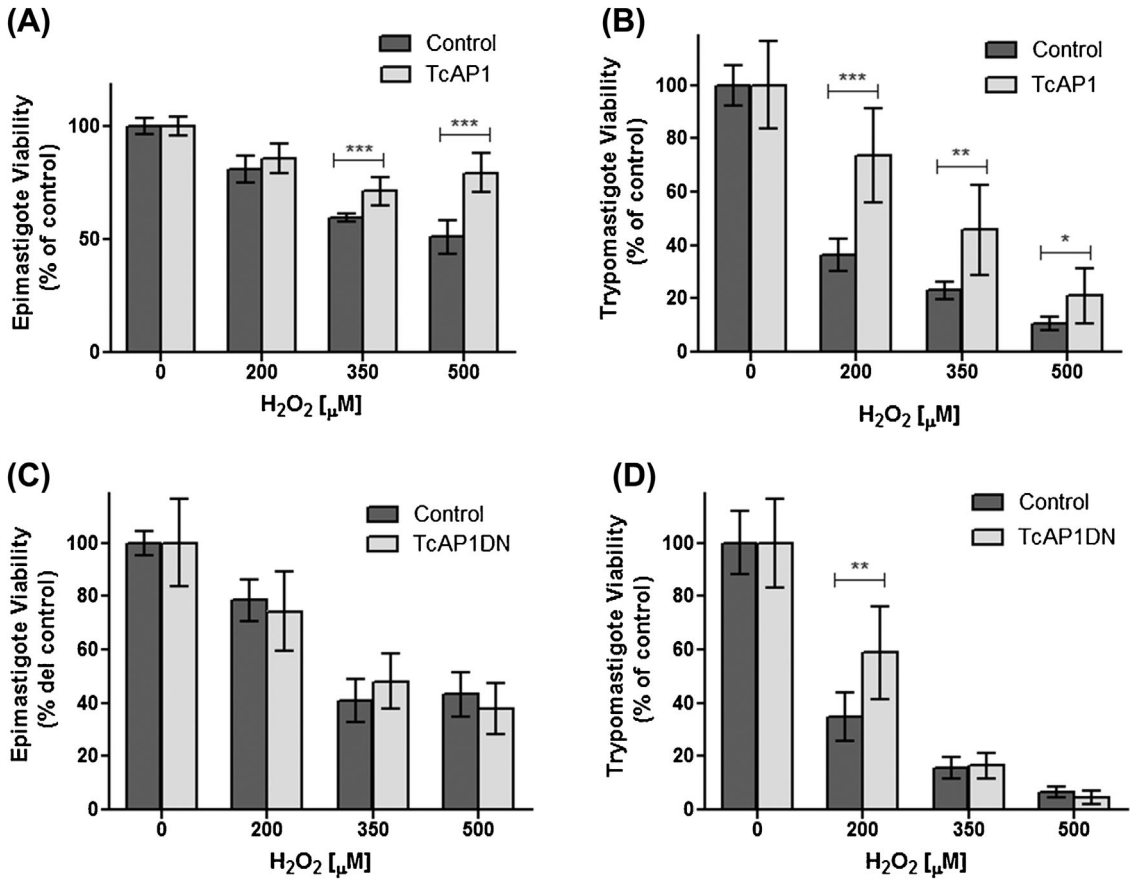

FIGURE 6 Overexpression of TcAP1 AP endonuclease, but not TcAP1DN, protects parasites against acute oxidative DNA damage. Epimastigotes (A) and trypomastigotes (B) overexpressing TcAP1 as well as epimastigotes (C) and trypomastigotes (D) expressing TcAP1DN were treated with increasing concentrations of H2O2 (200, 350, and 500 μM) for 30 min at 37°C. Viability was evaluated by the AlamarBlue assay and data were statistically analyzed using Student's *T*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

TcAP1 and TcAP2 AP endonucleases are constitutively expressed in the three cellular forms of the parasite (epimastigote, trypomastigote, and amastigote) and are localized in the nucleus but not in the kinetoplast or cytoplasm. Interestingly the overexpression of *T. cruzi* TcAP1 but not of TcAP2 increases epimastigotes survival when submitted to oxidative stress, suggesting that TcAP1 is the major AP endonuclease in this parasite.[14] Additionally, TcAP1 possesses a stronger in vitro AP endonuclease activity than TcAP2 as evidenced by the lower enzyme concentration required to detect its enzymatic activity. On the other hand Mx, a compound that impairs DNA repair through indirect inhibition of APE1 activity, also inhibits the recombinant TcAP1 and TcAP2 activities in vitro and increases the cytotoxic effect induced by oxidative agents in *T. cruzi* epimastigote and trypomastigote cellular forms.[13,14]

In this report, we show that TcAP1 endonuclease maintains its expression and cellular location after in vitro differentiation of non-infective epimastigotes to infective trypomastigotes cellular forms. The purified recombinant TcAP1 enzyme shows DNA endonuclease activity in a THF-oligo substrate as it was previously described by us using a different AP substrate.[14] Considering that RNAi approaches are not feasible to be applied in *T. cruzi*[48,49] and in order to determine the participation of the intrinsic TcAP1 endonuclease activity in parasite survival to oxidative stress an experimental approach based in the expression of a TcAP1 putative negative dominant was used. This purified recombinant TcAP1DN does not present AP endonuclease activity and, while showing a partial inhibitory effect on the wild-type TcAP1 enzyme activity when assayed in vitro, it does not decrease the parasite viability when expressed in epimastigotes and trypomastigotes exposed to oxidative agents. The absence of an inhibitory in vivo effect suggests that endogenous TcAP1 overcomes the TcAP1DN inhibitory activity. This is consistent with our previous results showing that TcAP1 is active and present as a constitutive enzyme in the three cellular forms of the parasite.[14]

As shown before[14] overexpression of TcAP1 increases epimastigote parasite viability when exposed to H_2O_2 (Figure 6A). Interestingly, trypomastigote infective forms overexpressing TcAP1 also shows protection of parasite

viability when exposed to the all tested H_2O_2 concentrations (Figure 6B). Considering that trypomastigotes are the parasite cellular forms that are exposed to oxidative burst in the parasitophorous vacuole inside the macrophages and other mammalian infected cells,[8] our results suggest that the BER pathway is of upmost importance for the parasite protection when inside the infected cells. Thus, TcAP1 may be considered as a possible target for the inhibition of *T. cruzi* DNA repair after mammal cells infection, leading to parasite loss of viability.

In summary TcAP1 is important for infective trypomastigote survival under oxidative conditions which are similar to those found in infected mammalian cells then increasing its permanence in the infected cells and, as a consequence, the possibility of development of Chagas disease.

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