Conservation of the pro-apoptotic nuclease activity of endonuclease G in unicellular trypanosomatid parasites

Sreenivas Gannavaram, Chetan Vedvyas and Alain Debrabant*

Laboratory of Bacterial, Parasitic and Unconventional Agents, Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda MD 20892, USA

*Author for correspondence (e-mail: alain.debrabant@fda.hhs.gov)

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Summary

Endonuclease G is a mitochondrial protein implicated in DNA fragmentation during apoptosis in cell types ranging from fungi to mammals. Features of programmed cell death have been reported in a number of single-celled organisms, including the human trypanosomatid parasites Leishmania and Trypanosoma. However, the protozoan cell death pathways and the effector molecules involved in such processes remain to be identified. In this report, we describe the pro-apoptotic function of endonuclease G in trypanosomatid parasites. Similar to metazoans, trypanosome endoG showed intrinsic nuclease activity, is localized in mitochondria and is released from this organelle when cell death is triggered. Overexpression of endoG strongly promoted apoptotic cell death under oxidant or differentiation-related stress in Leishmania and, conversely, loss of endoG expression conferred robust resistance to oxidant-induced cell death in T. brucei. These data demonstrate the conservation of the pro-apoptotic endonuclease activity of endoG in these evolutionarily ancient eukaryotic organisms. Furthermore, nuclear DNA degradation by endoG upon release from mitochondria might represent a caspase-independent cell death mechanism in trypanosomatid parasites as genes encoding caspase-like proteins have not been identified in their genomes.

Key words: Trypanosomatid parasites, Programmed cell death, DNA fragmentation, Apoptotic Nucleases, Endonuclease G

Introduction

Unicellular trypanosomatid protozoan parasites cause a wide variety of human diseases, ranging from localized self-healing cutaneous lesions primarily caused by Leishmania major to fatal visceral infections caused by Leishmania donovani (Murray et al., 2005) or human African trypanosomiasis, also known as sleeping sickness caused by Trypanosoma brucei (Brun and Balmer, 2006), or Chagas disease caused by Trypanosoma cruzi (Moncayo and Ortiz Yanine, 2006). The toxicity of the currently available drugs to treat these infections, together with the increasing incidence of drug resistance and lack of effective vaccines, has led to the emergence of these parasitic diseases as serious public health problems (http://www.who.int/tdr). This has evoked recent interest in the investigation of programmed cell death (PCD) pathways in these organisms to discover novel parasite control strategies.

It is often argued that the PCD pathways emerged along with multicellular organisms (Vaux et al., 1994), and the existence and role of such a counter-intuitive programme in single-celled organisms such as human trypanosomatid parasites remains controversial and poorly understood (Welburn et al., 1997; Ameisen, 2002; Duszenko et al., 2006). However, some of the features of apoptosis similar to those seen in multicellular organisms have been observed in trypanosomatid. These include loss of mitochondrial transmembrane potential, release of cytochrome c, condensation of nuclear chromatin, fragmentation of genomic DNA and surface binding of annexin-V in response to a variety of stress signals and parasiticidal drugs (Ameisen et al., 1995; Welburn et al., 1996; Debrabant et al., 2003; Duszenko et al., 2006). Other biochemical evidence for the protease and nuclease activities that correlate with this cell death phenotype suggested the existence of an intrinsic cell death pathway in unicellular organisms, including Leishmania and Trypanosoma (James and Green, 2004).

In multicellular organisms, apoptosis involves activation of caspases that orchestrate a downstream cascade of initiators and effectors that result in the morphological changes commonly described as the apoptotic phenotype (Green and Kroemer, 2004). Although several studies reported a caspase-like activity when Leishmania parasites were treated with anti-leishmanial drugs (Lee et al., 2002), hydrogen peroxide (Das et al., 2001) or inhibitors of protein kinase C (Arnoult et al., 2002; Sen et al., 2007), the parasite proteins with caspase-like activity have not been identified. Furthermore, the genomes of Leishmania and other trypanosomatid parasites do not contain orthologs of metazoan caspases. However, metacaspases, proteins with structural similarity to caspases, have been described in Leishmania and Trypanosoma (Helms et al., 2006; Gonzalez et al., 2007; Lee et al., 2007). The catalytic activity of Leishmania metacaspase is distinct from caspase activity, suggesting that the metacaspase is unlikely to be accountable for the caspase-like activity in Leishmania (Gonzalez et al., 2007; Lee et al., 2007).

Although DNA fragmentation is commonly observed in trypanosomatid PCD, the processes and mechanisms involved in
the fragmentation have never been investigated. Moreover, trypanosomatid parasites, just like Caenorhabditis elegans, do not contain orthologs of caspase-activated DNase (CAD), one of the best-characterized nucleases in mammalian apoptosis. Studies of apoptotic DNA fragmentation in C. elegans involving functional genetic screens have identified several additional nucleases apart from NUC1/DNaseII, the primary apoptotic nuclease in the worm (Wu et al., 2000), indicating the existence of multiple DNA degradation pathways (Parrish and Xue, 2003). Some of these nucleases are conserved from yeast to mammals, with well-defined physiological functions, including RNA processing, DNA replication and DNA damage and repair (Parrish and Xue, 2003; Samejima and Earnshaw, 2005). However, the specific role played by the majority of these cell-death-related nucleases with regard to functional sites and stages in DNA fragmentation remains to be determined.

Other than CAD in mammals (Enari et al., 1998) and NUC1/DNaseII in C. elegans (Wu et al., 2000), endoG is the most well-characterized cell death nuclease in C. elegans and mammals (Parrish et al., 2001; Li et al., 2001; Van Loo et al., 2001). Initial studies have shown that endoG is activated during cytotoxic death to induce chromosomal DNA fragmentation (Li et al., 2001; Parrish et al., 2001). EndoG is localized in the mitochondria and translocates to the nucleus during apoptosis (Li et al., 2001; Van Loo et al., 2001). Therefore, endoG belongs to the mitochondrial-resident proteins including cytochrome c, Smac/DIABLO and apoptosis-inducing factor acting as cell death effectors that are released in response to apoptotic stimuli (Wang, 2001).

To identify potential nucleases involved in the nucleosomal DNA degradation observed during trypanosomatid PCD, we performed a comprehensive homology search based on the currently well-characterized apoptotic nucleases of metazoans. We have identified putative genes encoding endoG in the genomes of Leishmania and Trypanosoma species. To investigate the endonuclease activity and possible role of the putative endoG in the PCD of trypanosomatid parasites, we have examined Leishmania parasites that overexpress endoG and, conversely, performed RNAi-based knockdown of endoG in T. brucei and investigated their response to hydrogen peroxide, a known trigger of PCD in these organisms. In this report, we describe the activity, intracellular localization and involvement of endoG in parasite PCD.

Results
Identification of genes encoding putative endoG proteins in the genomes of Leishmania and Trypanosoma
Evidence for DNA degradation, implying activation of nuclease(s), upon triggering of PCD has been demonstrated in Leishmania and Trypanosoma (Murphy and Welburn, 1997; Ridgley et al., 1999; Das et al., 2001; Lee et al., 2002; Zanger et al., 2002). We identified genes encoding putative endonuclease G proteins in the genome databases of Leishmania major (LmjF10.0610) and Trypanosoma brucei (Tb927.8.4040, Tb927.8.4090). The predicted open reading frames of trypanosomatid endoG encode a protein that is nearly twice as big as its mammalian ortholog. A comparison of trypanosomatid endoG amino acid sequences with the human, mouse and nematode endoG molecules showed several blocks of conserved amino acid residues in their predicted nuclease domains (Fig. 1). Overall, primary amino acid sequence comparison revealed that Leishmania and Trypanosoma endoG proteins display ~30% identity with human endoG. A comparison of the predicted nuclease domains of Leishmania and Trypanosoma
endoG revealed 48% identity between their sequences. Furthermore, trypanosome endoG shares structural features characteristic of a family of DNA/RNA nonspecific nucleases, of which endoG is a member. The DRGH motif shared between members of this family of nucleases is found in Leishmania and Trypanosoma endoG, although the aspartic acid is substituted by serine (Fig. 1). Of particular importance, the catalytically important histidine and asparagine residues essential for binding to divalent metal ions (Schafer et al., 2004) are also conserved in trypanosome endoG (Fig. 1). In addition, comparison of endoG sequence of L. major with L. infantum, the causative agent of the visceral form of leishmaniasis, showed complete identity. No significant homology between trypanosome endoG and its mammalian homolog was found in the N-terminal 50 amino acid residues, known to harbor mitochondrial targeting signals. However, use of MITOPROT, a mitochondrial targeting sequence prediction tool (Claros and Vincens, 1996) (http://ihg.gsf.de/ihg/mitoprot.html) enabled prediction of a mitochondrial targeting signal in the amino terminus of trypanosome endoG. Together, the sequence homology, conservation of catalytically important amino acid residues and probable mitochondrial localization indicated that trypanosome genomes contain genes encoding endoG.

Trypanosome endoG has nuclease cleavage activity
To explore the enzymatic properties of trypanosomatid endoG, wild-type T. brucei endoG and a mutant with point mutations H220A and E261A were expressed in Escherichia coli as histidine-tagged proteins (rTbEG and rTbEGm, respectively). These point mutations have been shown individually to abolish the nuclease activity in bovine endoG (Schafer et al., 2004). Polyclonal antibodies raised against TbEG protein reacted with rTbEG in western blots and specifically with an ~65 kDa protein in T. brucei lysates (Fig. 2A). Incubation of genomic DNA with rTbEG revealed the nuclease cleavage activity of this protein (Fig. 2B). The rTbEGm protein did not show DNA degradation in the nuclease assay, indicating that histidine and glutamic acid residues at positions 220 and 261 are required for the enzymatic activity of TbEG (Fig. 2B). Mammalian endoG has been shown to cleave preferentially double-stranded DNA, single-stranded DNA and RNA (Schafer et al., 2004). When rTbEG was incubated with total RNA, degradation of RNA was observed (Fig. 2C), whereas rTbEGm did not show RNase activity (Fig. 2C). Furthermore, to assess whether rTbEG was able to create nicks in fixed nuclei, procyclic T. brucei cells were fixed, permeabilized and treated with rTbEG and rTbEGm. The results showed that ~38% of cells treated with rTbEG were positive by the terminal deoxynucleotidyltransferase enzyme-mediated dUTP end labeling (TUNEL) assay, whereas only background levels of TUNEL-positive cells occurred in mutant controls (Fig. 2D). This indicated that rTbEG can cleave chromatin substrates in addition to genomic DNA and RNA.

To verify whether Leishmania endoG also had nuclease activity, we first attempted to express this protein in E. coli. However, the Leishmania endoG expression was toxic to E. coli. Therefore, wild-type Leishmania endoG was overexpressed in Leishmania using the expression plasmid pKSNeo (Zhang et al., 1996) and the resulting hemagglutinin (HA)-tagged endoG protein was assessed for nuclease activity. To confirm the specificity of the nuclease activity, a mutant form of Leishmania endoG was generated with point mutations S206G, H209A and E249A. The ectopic expression of wild-type and mutant endoG was analyzed by western blot in the Leishmania lysates from promastigotes and amastigotes, the two developmental stages of the parasite adapted for axenic growth in vitro (Debrabant et al., 2004). The results showed that the antibodies against HA reacted with an ~60 kDa protein (Fig. 3A) in lysates of both promastigotes and axenic amastigotes expressing endoG (Fig. 3A, lanes 2 and 5) and mutant endoG (Fig. 3A, lanes 3 and 6) but not in control cells transfected with the expression plasmid alone (Fig. 3A, lanes 1 and 4). The apparent molecular mass of the HA-tagged Leishmania endoG is higher than its predicted size. Similarly, in the case of T. brucei, endoG had an apparent molecular mass larger than its predicted mass of 56 kDa. Such an aberrant mobility assessed by SDS-PAGE has been reported previously for human endoG (Widlak et al., 2001) and might be a result of an intrinsic characteristic of endoG proteins. The nuclease assays were performed with ectopically expressed HA-tagged endoG that was immunoprecipitated using antibodies against HA from Leishmania lysates. To confirm that the immunoprecipitated material contained comparable amounts of HA-tagged proteins, equal volumes of the anti-HA pulldown material from LdEG, LdEGm and control KS lysates were resolved by SDS-PAGE and the resultant immunoblots were probed with biotinylated antibodies against rTbEG. The results showed that the Leishmania

Fig. 2. Nuclease activity of recombinant T. brucei endoG protein (α-rTbEG). (A) Polyclonal antibodies raised against full-length T. brucei endoG protein reacted with procyclic T. brucei lysates (L) and the TbEG recombinant protein (R). Pre-immune serum was used as a control (NRS). (B) A nuclease cleavage assay was performed with wild-type (rTbEG) or mutant TbEG (H220A and E261A, rTbEGm) proteins using genomic DNA (gDNA). Treatment with aurintricarboxylic acid (ATA) inhibited DNA degradation, confirming the specificity of TbEG for nucleic acid substrates. (C) Nuclease assay as in B, using total RNA as a substrate. (D) TUNEL assay of p-formaldehyde-fixed T. brucei procyclic cells incubated with rTbEG (light black line) or rTbEGm protein (dark black line) analyzed by flow cytometry. Untreated T. brucei cells (gray shaded peak) served as a control.
endoG is recognized by the antibodies against rTbEG and that similar amounts of LdEG and LdEGm proteins are immunoprecipitated under these conditions (Fig. 3B). A DNA degradation assay with the precipitated material, followed by analysis with a Bioanalyzer, revealed specific degradation of an ~10 kb plasmid substrate in LdEG samples (Fig. 3C). No major substrate degradation was noticeable with LdKS and only partial degradation was observed with LdEGm (Fig. 3C). By comparison, the substrate was completely degraded after incubation with the rTbEG control. These results showed that the endoG proteins from both Leishmania and T. brucei have an intrinsic nuclease activity and, similar to their mammalian orthologs, the conserved amino acid residues S206, H209 and E249 in Leishmania and H220 and E261 in T. brucei are required for the nuclease activity.

Mitochondrial localization of endoG in L. donovani and T. brucei
To determine the cellular localization of native endoG in Trypanosoma and Leishmania, antibodies against rTbEG were used in immunofluorescence studies. Immunofluorescence assay with these antibodies revealed that endogenous TbEG is localized in mitochondria in T. brucei, as indicated by its colocalization with Mitotracker Red, a mitochondrial marker (Fig. 4A, top panel). Immunofluorescence assay with antibodies against endoG revealed that endogenous Leishmania endoG is also localized in mitochondria (Fig. 4B, top panel). Only background reactivity was obtained with the pre-immune serum in these experiments.
Trypanosomatid endonuclease G (Fig. 4A,B, bottom panels). IFA with antibodies against HA showed that ectopically expressed *Leishmania* endoG was properly targeted to the mitochondria as it colocalized with Mitotracker Red (Fig. 4C, top panel). Similarly, the mutant *Leishmania* endoG was also targeted to the mitochondria (not shown).

### Overexpression of endoG in *Leishmania* induces parasite cell death and increases sensitivity to H$_2$O$_2$

To determine whether the putative *Leishmania* endoG was involved in PCD in this organism, parasites overexpressing either endoG or mutant endoG were analyzed. Overexpression of endoG or endoG mutant proteins in *Leishmania* did not affect the growth of the promastigote stage of *Leishmania* in vitro (data not shown). However, when the parasites were switched to axenic amastigote growth conditions (Debrabant et al., 2004), a significant reduction in the number of viable cells could be detected in *L. donovani* overexpressing endoG (LdEG) parasite cultures over time (Fig. 5A). By contrast, axenic amastigote parasites overexpressing mutant endoG (LdEGm) did not show a significant difference in comparison with controls for up to five days in culture; however, these cells showed a small but significant decrease in viable cells in the late stationary phase (Fig. 5A). TUNEL labeling revealed increased nicking in the genomic DNA of LdEG cells (Fig. 5B) and an increasingly higher proportion (~40%) of these cells were TUNEL positive over time in culture (Fig. 5C) compared with KS controls. The LdEGm cells also showed a moderate increase (~15%) in the number of TUNEL-positive cells when they reached the stationary phase, probably reflecting the residual nuclease activity of the mutant enzyme. These results indicate that overexpression of endoG in *Leishmania* results in degradation of genomic DNA, and the consequent cell death is dependent on its nuclease activity.

To investigate whether the overexpression of endoG alters the sensitivity of these cells to known triggers of PCD in *Leishmania*, wild-type and transfected axenic amastigotes were treated with 2 mM H$_2$O$_2$ for 60 minutes and subjected to TUNEL labeling. A 2.5-fold increase in TUNEL-positive cells was found in LdEG (51%) in comparison with wild-type or LdKS cells (~20%, Fig. 5D). This difference in the percentage of TUNEL-positive cells between LdKS and LdEG cells was statistically significant (*P*<0.05) (Fig. 5E). By contrast, LdEGm cells showed no significant increase (*P*>0.05) in TUNEL positivity in comparison with that of LdKS cells (Fig. 5E).
In order to examine the effects of overexpression of endoG on the differentiation and survival of intracellular amastigotes, human macrophages derived from elutriated monocytes were infected with *Leishmania major* promastigotes overexpressing either wild-type or mutant endoG. No difference in the number of amastigotes per hundred macrophages was found after 6 or 24 hours of infection between control cells transfected with KS and EG or EGm plasmid constructs, indicating that overexpression of endoG did not alter the infectivity of the parasite. Staining of the infected macrophages at the defined time-points revealed that overexpression of endoG results in elimination of ~99% of *Leishmania* amastigotes by the macrophages within 96 hours of infection, which is significantly higher than that shown by KS control transfectants (*P*<0.001, Fig. 6A). This reduction in amastigote number in LmEG was also significant compared with LmEGm expressors (*P*<0.001). Staining of infected macrophages at 6 hours post infection showed TUNEL-positive *Leishmania* nuclei in LmEG cells and not in LmEGm or LmKS controls, suggesting that overexpression of endoG mediates DNA degradation in LmEG cells, as was observed with axenic amastigotes (Fig. 6B). TUNEL-positive intracellular amastigotes were also observed in macrophages infected with either wild-type or transfectant parasites and treated with lipopolysaccharide (LPS) and human IFN-γ for 48 hours (Fig. 6C), suggesting that intracellular amastigotes are killed through PCD in activated macrophages. Taken together, these results show that *Leishmania* overexpressing endoG spontaneously undergo cell death either in vitro or in infected macrophages and that these cells are more sensitive to H2O2-induced PCD, suggesting a possible role of endoG in *Leishmania* PCD.

Translocation of endoG during parasite cell death

Having established the nuclease activity, cellular localization and increased propensity to undergo PCD as a result of overexpression, we next tested whether trypanosome endoG is released from the mitochondria along with another cell death protein, cytochrome c, upon treatment with cell death stimuli, as evidenced in studies using mammalian endoG (Li et al., 2001). To this end, we performed cell fractionation of axenic amastigotes, which were either treated or not treated with H2O2, under conditions identical to those used in the previous experiments to trigger parasite PCD. The cytoplasmic and mitochondrial fractions from these extracts were resolved by SDS-PAGE and immunoblotted with antibodies against HA. This revealed that, under normal physiological conditions, *Leishmania* endoG is associated with mitochondria, which is in agreement with the immunofluorescence results above (Fig. 4), and that, following induction of cell death with H2O2, endoG is released from mitochondria and detected in the cytoplasmic fractions (Fig. 7, antibody against HA). Re-probing of the blots with antibody against rTbEG showed that endogenous endoG is also released from mitochondria in control LdKS cells following treatment with H2O2 (Fig. 7, antibody against rTbEG). This redistribution of endoG was also observed in wild-type *L. donovani* axenic amastigotes treated with H2O2 (data not shown). Furthermore, re-probing these blots with antibodies against *L. donovani* cytochrome c showed a similar release of cytochrome c from mitochondria after H2O2 treatment (Fig. 7). To confirm the purity of the subcellular fractions, these blots were also probed for the mitochondria-associated protein heat-shock protein 70.
(Hsp70), using antibodies against *T. brucei* Hsp70 (a kind gift of P. Englund, Johns Hopkins University, MD) (Law et al., 2007) and for a cytoplasmic protein, adenine phosphoribosyltransferase (LdAPRT) using antibodies against *L. donovani* APRT (a kind gift of B. Ullman, Oregon Health and Science University, OR) (Zarella-Boitz et al., 2004). The results showed the presence of LdAPRT only in the cytoplasmic fractions (Fig. 7, antibody against LdAPRT) and retention of Hsp70 in the mitochondrial fractions after H$_2$O$_2$ treatment (Fig. 7, antibody against TbHsp70). As expected, immunoblotting with the antibody against TbHsp70 resulted in a single band in *L. donovani* lysates (Fig. 7B) and colocalized with Mitotracker Red in immunofluorescence studies (data not shown). Taken together, these results show that the release of endoG from the mitochondria in response to oxidant stress is a controlled process and is not due to a nonspecific release of mitochondrial components in the cytoplasm. Furthermore, these results are in agreement with the previously reported release of mammalian endoG from mitochondria during apoptosis and further support the role of endoG in trypanosomatid PCD.

RNAi of endoG in *T. brucei* results in increased resistance to cell death induced by H$_2$O$_2$

In order to assess further the role of endoG in trypanosomatid PCD, we investigated the effect of depletion of endoG on parasite growth and sensitivity to H$_2$O$_2$ in *T. brucei* using an RNAi approach. Gene database searches revealed that *T. brucei* has two copies of endoG that are 99% identical at the nucleotide and protein level. A tetracycline-inducible RNAi vector was constructed and transfected into *T. brucei*, following a technique described previously (Wirtz et al., 1999). Synthesis of dsRNA was induced by adding tetracycline to the cell culture. Cell growth was not inhibited even after nine days, indicating that endoG is not essential for procyclic *T. brucei* growth in vitro (Fig. 8A). A northern blot indicated that induction of RNAi resulted in a nearly complete loss of mRNA encoding endoG three days after induction (Fig. 8A, inset). *T. brucei* cells deficient in endoG were then treated with
H$_2$O$_2$ to examine whether the deficiency in endoG impaired nicking of genomic DNA. TUNEL labeling followed by analysis by fluorescence-activated cell sorting (FACS) revealed that 43% of non-RNAi induced cells were TUNEL positive after treatment with H$_2$O$_2$, whereas, in RNAi induced cells, only 13% of cells were TUNEL positive (Fig. 8B). The difference in the percentage of TUNEL-positive cells between control and H$_2$O$_2$-treated cells was statistically significant ($P<0.05$; Fig. 8C). These results show that parasites with decreased levels of endoG are more resistant to PCD induced by H$_2$O$_2$ and therefore lend further support for a role for endoG in trypanosome PCD.

**Discussion**

The molecular apparatus that regulates PCD in multicellular organisms has been widely investigated. Several unicellular organisms, including the protozoan human parasites *Leishmania* and *Trypanosoma*, display features of apoptotic cell death; however, molecular evidence for the existence of a regulated cell death program still needs to be demonstrated conclusively. By comparison, several orthologs of important mammalian apoptotic proteins have been discovered in fungi, underlining the conservation of proapoptotic and mitochondrial apoptotic pathways in single-celled organisms (Buttner et al., 2006; Cheng et al., 2006).

In trypanosome parasites, the triggering of caspase-like activity and/or nuclease activity have been implicated in cell death either in cultures in stationary phase (Lee et al., 2002; Nguewa et al., 2004) or cells treated with H$_2$O$_2$ (Ridgley et al., 1999; Das et al., 2001), camptothecin, mitofosine, prostaglandin-D2 and a range of other inducers (reviewed in Duszenko et al., 2006). Despite the caspase-like activity reported from trypanosomatids undergoing cell death, orthologs of caspases, caspase-dependent DNase or other cell-death-related nucleases have not been identified.

Endonuclease G was identified as a mitochondrial nuclease that induces caspase-independent DNA fragmentation in mouse liver tissues (Li et al., 2001) and in *C. elegans* (Parrish et al., 2001). However, because of discrepancies in the mouse endoG knockouts in the initial studies, the role of endoG in mammalian apoptosis remains less well established (Irvine et al., 2005; David et al., 2006). By contrast, a recent report in budding yeast employing clonogenic assays confirmed endoG as a cell death nuclease in yeast (Buttner et al., 2007). Our results are in line with the studies in yeast and support the argument that, in trypanosomatid parasites, endoG has an apoptotic nuclease function.

Mammalian and nematode endoG are synthesized as precursor proteins with an N-terminal mitochondrial targeting signal that is removed upon import into mitochondria. By contrast, we could not detect any such processing with *Leishmania* and *T. brucei* endoG for up to 60 minutes of chase following a 10 minute [$^{35}$S]-methionine metabolic labeling of parasites (data not shown). Trypanosomend oG protein is nearly twice as big in comparison with its mammalian ortholog and has very limited homology outside of the putative nuclease domain. Interestingly, the aspartic acid in the conserved DRGH motif, found in DNA/RNA nonspecific nucleases, is substituted by a serine in trypanosome endoG. Such substitutions in the DRGH motifs (D to N/E) are known in other organisms (Schafer et al., 2004). Site-directed mutagenesis studies in bovine endoG resulted in a nearly 50% reduction in the DNA cleavage activity when the aspartic acid was substituted with alanine (Schafer et al., 2004). The nuclease activity from the purified trypanosome endoG demonstrated that the presence of the serine residue instead of aspartic acid in the DRGH motif is compatible with the nuclease activity. The biological significance, if any, of such a substitution in trypanosomatids would be interesting to explore.

Overexpression of endoG in *Leishmania* affected the growth of the amastigote stage of this parasite both in vitro in axenic cultures and ex vivo inside macrophages. When the infected macrophages were stimulated with LPS and human IFN-γ under conditions that trigger amastigote killing through an oxidative burst (Carrera et al., 1996; Gantt et al., 2001), DNA degradation was evident in wild-type or transfected parasites, indicating that triggering of PCD is not limited to cells that overexpress endoG but also occurs in parasites with baseline level of endogenous endoG. Furthermore, LdEG cells showed heightened sensitivity to cell death induced by H$_2$O$_2$. The cell death mediated by H$_2$O$_2$ was mainly apoptotic-like at low cell densities as LdEG cells were predominantly TUNEL positive but negative in propidium iodide staining. The overexpression of endoG in the promastigote stage of *Leishmania* did not result in spontaneous cell death or increased sensitivity to H$_2$O$_2$ under the conditions used for the axenic amastigotes. The reasons for the amastigote stage-specific effects of reduced survival and heightened sensitivity to cell death are not immediately obvious. It is known that mitochondrial energy metabolism and Kreb’s cycle activities are developmentally regulated in the life cycle of trypanosomatids (El Fakhry et al., 2002). Thus, it is probable that the mitochondria in amastigotes are metabolically more active as they rely on glucose and fatty acids for generating ATP. As metabolism of fatty acids takes place in mitochondria, it is conceivable that the perturbations in mitochondria due to accumulation of endoG could result in increased susceptibility to PCD in amastigotes.

When the nuclease activity of the endoG was abolished by point mutations, the overexpression of such mutant endoG did not result in increased sensitivity to H$_2$O$_2$, demonstrating that the nuclease activity of endoG is necessary for its apoptotic function and is not an artifact of overexpression. In addition, the effects of overexpression of endoG in *Leishmania* amastigotes are consistent with the results of overexpression studies in *C. elegans* (Parrish et al., 2001), HeLa cells (Schafer et al., 2004) or yeast (Buttner et al., 2007), resulting in increased sensitivity to triggers of cell death. Conversely, when endoG-depleted *T. brucei* cells were exposed to H$_2$O$_2$, there was significantly less DNA damage in RNAi-induced cells in comparison with non-induced controls. This is also in complete agreement with the RNAi experiments performed in *C. elegans* that led to impaired DNA degradation and delayed cell death (Parrish et al., 2001). Taken together, these data suggest that overexpression of endoG predisposes *Leishmania* cells to undergo cell death, and depletion of endoG confers resistance to the DNA degradation associated with cell death in *T. brucei*, strongly supporting a role for endoG in trypanosomatid PCD.

Investigations into the intrinsic nuclease activity of trypanosomatid endoG revealed that bacterially expressed *T. brucei* endoG can cleave naked DNA and RNA substrates. Furthermore, the TbEG-induced DNA damage in p-formaldehyde-fixed *T. brucei* cells indicated that TbEG can induce DNA damage in chromatin substrates, as does the human endoG (Widlak et al., 2001). Similarly, we demonstrated nuclease activity of immunoprecipitated LdEG from *Leishmania* lysates. The biochemical characteristics of mammalian endoG in addition to RNAi-based functional genetic screens in *C. elegans* implied that endoG is a part of a nuclease cascade that acts in a hierarchical
sequence during apoptosis (Widlak et al., 2001; Parrish and Xue, 2003). Currently, it is not known whether additional nucleases are also involved in trypanosomatid DNA degradation during PCD conditions. The endoG described in this report is the first pro-apoptotic nuclease in trypanosomatid parasites. The genomes of trypanosomatid parasites encode many nucleases that might possess cell death functions. It will be of interest to delineate the functions of additional nucleases in trypanosomatid PCD.

Similar to metazoan endoG, trypanosome endoG showed mitochondrial localization in immunofluorescence studies. Cell fractionation following the H2O2 treatment revealed that endoG is released from mitochondria into the cytosol under these conditions. In HeLa cells, release of endoG from mitochondria requires caspase activity (Arnould et al., 2003). By contrast, the activities of endoG and metacaspase do not converge and function in independent cell death pathways in yeast (Buttner et al., 2007). Future studies are required to understand what factors trigger the release of endoG from mitochondria in trypanosomes.

Several suggestions have been made regarding the purpose of the existence of an apoptosis-like mechanism in trypanosomatid parasites, ranging from an altruistic response, especially under conditions of limiting nutrient availability, to a contribution to the virulence of these parasites (Welburn et al., 1997; Debrabant et al., 2003; James and Green, 2004; Wanderley et al., 2006; van Zandbergen et al., 2006). However, the essential question of how the apoptotic cell death programme fits into the biology of trypanosomatid parasites, or more generally any single-celled organism, remains a matter of intense investigation. The identification of trypanosomatid nucleases such as endoG and defining their role in parasite PCD would provide significant insights into the apoptotic processes in these protozoan parasites and their complex interactions with the human hosts.

In conclusion, our study represents the first report of the identification and characterization of a parasite-encoded endonuclease involved in PCD in trypanosomatid parasites. The observation that endoG represents a mitochondrion-originating cell death component that does not require activation by caspsases makes it a significant step in the molecular characterization of this pathway in trypanosomatid parasites. Leishmania endoG-null mutants might help define the role of endoG in PCD and also help address fundamental questions such as the biological relevance of PCD in these organisms. An understanding of cell death effectors in evolutionarily ancient organisms could illuminate the evolutionary origins of cell death processes. Such components of PCD in trypanosomatid parasites could be exploited to develop novel anti-parasitic drugs.

**Materials and Methods**

**Plasmids and parasite cultures**

*Leishmania donovani* promastigotes (strain 1S, clone 2D, WHO designation: MHOM/S/76/1S-CL2D) were grown in M199 medium containing 10% heat-inactivated fetal bovine serum. Promastigotes were transfected by electroporation and selected for growth in medium containing Geneticin (G418) up to 100 μg/ml, as described previously (Selvapandiyan et al., 2001). These drug-resistant cells were used in all subsequent experiments. *L. major* clone V1 (MHOM/IL/80/Friedlin) promastigotes were grown at 26°C in medium in 199 supplemented (M199/S) with 20% fetal calf serum. The *Leishmania* expression plasmid pKSNeo (Zhang et al., 1996) was used to express either the full-length or the mutant forms of *L. major* endoG in transfected *Leishmania* parasites. For this purpose, the full-length gene encoding *L. major* endoG was amplified with oligos based on the *L. major* putative endoG sequence. The point mutations S206G, H209A and E249A were introduced by PCR and selected for growth in medium containing Geneticin (G418) up to 100 μg/ml. The recombinant proteins were expressed from E. coli and purified in native conditions through Ni-agarose column chromatography according to the manufacturer’s protocol (Qiagen). The *Leishmania* gene encoding cytomegotype c was amplified by PCR using *L. major* genomic DNA and primers based on the *L. major* gene encoding putative cytomegotype c (LmjF16.1310) and ligated into pCR T7/CT-Topo. The plasmid encoded histidine-tagged *L. major* cytomegotype c protein was expressed in E. coli and purified as above. The recombinant purified *T. brucei* endoG and *L. major* cytomegotype c proteins were used to generate polyclonal antibodies in rabbits according to the manufacturer’s protocol (Spring Valley Laboratories, Woodbine, MD).

**RNAi of endoG in *T. brucei***

*Trypanosoma brucei* strain 29-13, which harbors integrated genes for T7 RNA polymerase and the tetracycline repressor (Wirtz et al., 1999), were cultured at 27°C in SDM-79 medium containing 10% fetal bovine serum. Based on the nucleotide sequence of putative endonuclease G in *T. brucei*, oligos were designed to amplify a 476 bp DNA fragment (918-1393bp of the putative TBeG open reading frame) that had no significant sequence identity with the rest of the *T. brucei* genome sequences. This fragment was subcloned into HindIII-Xhol sites of the pZJM vector (Wang et al., 2000), the resultant recombinant plasmid was linearized with NotI and the DNA transfected into the procytic form of *T. brucei*. Transfectants were selected in the presence of 2.5 μg/ml phloxycin (Sigma) and a clonal cell line obtained by limiting dilution. Total RNA (10 μg/lane) prepared from day five after tetracycline induction of RNAi was resolved on a formaldehyde gel and blotted onto a nitrocellulose membrane. To verify the reduction of mRNA encoding endoG upon induction of RNAi, a 402 bp DNA fragment (1-402 bp of the putative TBeG open reading frame) was used as a probe in a northern blot.

**RTUNEL assay**

*L. donovani* axenic amastigotes or procyclic *T. brucei* (1.5×10⁷ cells) were pelleted by centrifugation, resuspended in growth medium containing 2 mM H2O2 and incubated for 60 minutes at 37°C and 5 % CO2 for *Leishmania* and 27°C for *T. brucei* along with the untreated controls. The cells were washed twice in cold PBS and fixed by incubation in 2% p-formaldehyde for 45 minutes, followed by permeabilization with 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate for 2 minutes on ice. The permeabilized cells were stained with the TUNEL reaction mixture (In situ Cell Death Detection Kit Fluorescein, Roche) for 60 minutes at 37°C and analyzed using a FACS Calibur flow cytometer (Becton Dickinson). FL1-H fluorescence was recorded on 10,000 events and analyzed using FlowJo software. Data are expressed as a percentage of the TUNEL-positive cells.

**Nuclease cleavage assay**

One microgram of *Leishmania* genomic DNA was incubated for 30 minutes at 37°C with recombinant wild-type or mutant *T. brucei* endoG (200 ng purified protein) in assay buffer consisting of 10 mM KCl, 3 mM MgCl2, 0.5 mM diithreitol, 20 mM HEPES, pH 7.5. The cleavage products were resolved on a 1.2% agarose gel and stained with ethidium bromide. In cleavage assays with RNA, 5 μg of *Leishmania* total RNA was used under assay conditions identical to those described above. The cleavage products were resolved on formaldehyde gels and visualized by ethidium bromide staining. *Leishmania* lysates were prepared in lysis buffer containing 25 mM HEPES pH 7.5, 5 mM MgCl2, 150 mM NaCl, 1% Triton-X-100, 1 mM diithreitol. The cell suspension was incubated on ice for 30 minutes and the resulting lysate centrifuged at 16,000 g for 30 minutes. HA-tagged endoG proteins were immunoprecipitated with rabbit polyclonal antibodies against HA (Sigma) overnight and the immune complexes were precipitated with protein–A–sepharose beads (Roche). The beads were washed sequentially with lysis buffer, wash buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 M KCl, 1% Triton-X100) and resuspended in assay buffer, as described above.

**Immunofluorescence assay**

Immunofluorescence was performed essentially as described previously (Selvapandiyan et al., 2001) except that the cells were preincubated with Mitotracker Green FM (Molecular Probes) to label the mitochondria. Rat antibodies against HA (Roche) were used to detect ectopically expressed endoG in *Leishmania* axenic amastigotes, and rabbit antibodies against rTBeG were used to determine the cellular localization of endogenous endoG in procytic *T. brucei* and *Leishmania* promastigotes.

**Cell fractionation**

Cell fractionation was performed following a published protocol (Schnittler et al., 2000). Briefly, control and H2O2-treated (2 mM, 60 minutes) *Leishmania* cells were washed three times in 15 mM MES buffer (20 mM MOPS, pH 7.0, 250 mM sucrose, 3 mM EDTA). The cell pellet was resuspended in 0.2 ml MES buffer.
containing 1 mg/ml digitonin and protease inhibitor cocktail (Roche Applied Science). The suspension was incubated at room temperature for 5 minutes and centrifuged at 10,000 g for 5 minutes. The resulting supernatant was collected as a cytosolic fraction, and the heavy membrane pellet enriched for mitochondria was resuspended in phosphate buffer (20 mM sodium phosphate, pH 7.0, 3 mM EDTA).

Macrophase infection
Human elutriated monocytes were resuspended at 1.8 × 10⁶ cells/ml in RPMI medium containing 10% FBS macrophage colony-stimulating factor (20 ng/ml, ProSpec, Israel), plated in 0.5 ml on eight-chamber Lab-Tek tissue-culture slides (Miles Laboratories) and incubated for 9 days for differentiation into macrophages. The differentiated macrophages were infected with L. major using MDP-CDN (Nature, 2001a, 413-50).

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