Apoptosis-like programmed cell death induces antisense ribosomal RNA (rRNA) fragmentation and rRNA degradation in Leishmania

PK Padmanabhan1,2, M Samant1,2, S Cloutier1,2, MJ Simard3 and B Papadopoulou*1,2

Few natural antisense (as) RNAs have been reported as yet in the unicellular protozoan Leishmania. Here, we describe that Leishmania produces natural asRNAs complementary to all ribosomal RNA (rRNA) species. Interestingly, we show that drug-induced apoptosis-like programmed cell death triggers fragmentation of asRNA complementary to the large subunit gamma (LSU-γ) rRNA, one of the six 28S rRNA processed fragments in Leishmania. Heat and oxidative stress also induce fragmentation of asrRNA, but to a lesser extent. Extensive asrRNA cleavage correlates with rRNA breakdown and translation inhibition. Indeed, overexpression of asLSU-γ RNA accelerates rRNA degradation upon induction of apoptosis. In addition, we provide mechanistic insight into the regulation of apoptosis-induced asrRNA fragmentation by a 67 kDa ATP-dependent RNA helicase of the DEAD-box subfamily. This helicase binds both sense (s)LSU-γ and asLSU-γ rRNAs, and appears to have a key role in protecting rRNA from degradation by preventing asrRNA cleavage and thus cell death. Remarkably, the asrRNA fragmentation process operates not only in trypanosomatid protozoa but also in mammals. Our findings uncover a novel mechanism of regulation involving asrRNA fragmentation and rRNA breakdown, that is triggered by apoptosis and conditions of reduced translation under stress, and seems to be evolutionarily conserved.

Cell Death and Differentiation (2012) 19, 1972–1982; doi:10.1038/cdd.2012.85; published online 6 July 2012

Antisense RNAs (asRNAs) are common in prokaryotes and can mediate a plethora of regulatory processes, including transcription interference, RNA processing, RNA stability or ribosome binding.1 In eukaryotes, natural ascripts have established roles in epigenetic silencing, genomic imprinting, alternative splicing, messenger RNA (mRNA) editing, rRNA nuclear transport, stability and translation, as well as in the formation of endogenous siRNAs.2 The endogenous RNA interference (RNAi) pathway in eukaryotes is one example where asRNA production has a major role in the formation of sRNA–asRNA duplexes recognized by the RNAi machinery.3 It is becoming clear that asRNAs have an important role in regulating gene expression, but their production and function remain poorly understood.

Leishmania spp. causes a broad range of human diseases known as leishmaniasis. These parasites alternate between two major developmental forms, promastigotes in the insect vector and amastigotes in the phagolysosome of mammalian macrophages. In the absence of typical RNA pol II promoter sequences, polycistronic transcription units in Leishmania are processed to generate mature rRNAs though coupled 5’-trans-splicing and 3’-cleavage/polyadenylation reactions.4 Natural asRNAs complementary to the L. major Friedlin chromosome 1,5 or to specific protein-coding transcripts5,7 and to non-coding developmentally regulated RNAs6 have been described in Leishmania. The existence of ascripts is puzzling in the absence of any RNAi machinery in Leishmania6 and their putative role in regulating gene expression is still unknown.

Apoptosis, one of the programmed cell death mechanisms, is believed to operate in multicellular organisms to control various physiological processes, but also in response to various stress-induced mechanisms.10 However, there is now increasing evidence that apoptosis-like programmed cell death (ALPCD) occurs in unicellular organisms11,12 including Leishmania.13–15 Cell death in Leishmania may be helpful in controlling parasite’s density in response to limited resources and/or for ensuring propagation only of the cells that are fit to transmit the disease. Interestingly, it has been shown that apoptotic Leishmania in the virulent inoculum enhance the transmission and development of the disease.16 Dying Leishmania show typical hallmarks of apoptosis despite the absence of homologs to mammalian key regulatory or effector molecules like caspases.13,14,17 Various stress conditions, such as nitric oxide,18 reactive oxygen species,19 heat stress,20 starvation,21 and anti-Leishmania drugs

Received 16.3.12; revised 15.5.12; accepted 15.5.12. Edited by RA Knight; published online 06.7.12
(e.g., miltefosine (MF) or antimonials)\textsuperscript{22–24} have been reported to induce morphological and biochemical features of ALPCD in \textit{Leishmania}.

In this study, we show that induction of ALPCD triggers fragmentation of asRNA complementary to ribosomal RNA (rRNA) in \textit{Leishmania}. asRNA cleavage is correlated with rRNA degradation and inhibition of general translation. A 67 kDa DEAD-box ATP-dependent RNA helicase is implicated in this process by preventing asrRNA cleavage, and hence protecting rRNA from degradation and \textit{Leishmania} from cell death. Our findings uncover a novel regulatory pathway that seems to be conserved in unicellular protozoa but also in higher eukaryotes.

**Results**

Natural asRNA complementary to the rRNA is produced in \textit{Leishmania}. To identify small non-coding RNAs in \textit{Leishmania}, we cloned and sequenced small RNAs (<200 nt) and found that some of these sequences were opposite to the large subunit gamma (LSU-\textgreek{g}) rRNA (data not shown). A unique feature of \textit{Leishmania} and related parasites is that the large 28S rRNA subunit is processed to yield six stable RNA fragments, which include the LSU-\textgreek{g} (1840 nt), LSU-\textgreek{b} (1570 nt), LSU-\textgreek{y} (213 nt), LSU-\textgreek{d} (180 nt), LSU-\textgreek{c} (70 nt) and LSU-\textgreek{z} (140 nt).\textsuperscript{25} Although asRNAs against few mRNAs\textsuperscript{5,7} and non-coding RNAs\textsuperscript{8} have been detected in \textit{Leishmania}, the presence of asrRNA species has not been investigated yet in protozoan parasites.

To further search for asRNA in \textit{Leishmania}, we used strand-specific reverse transcribed-polymerase chain reaction (RT-PCR) and northern blot hybridization with srRNA-specific riboprobes for detecting the 28S, 18S and 5.8S rRNA species. These studies revealed the presence of asRNA complementary to all rRNA species in both developmental forms of \textit{Leishmania} (Figures 1a and b and Supplementary Figure S1). The present study focused on LSU-\textgreek{y} rRNA, one of the six 28S rRNA processed fragments. Strand-specific RT-PCR analysis (Supplementary Table 1) revealed a LSU-\textgreek{y} rRNA PCR product of the expected size (Figure 1a). The asLSU-\textgreek{y} rRNA/sLSU-\textgreek{y} rRNA ratio was estimated by quantitative real-time PCR to be 1/300 (data not shown). asLSU-\textgreek{y} rRNA was also detected in both life stages by northern blot hybridization using the 173 nt single-stranded (ss) DNA probe (Figure 1b). Interestingly, the length of asLSU-\textgreek{y} rRNA was similar to that of the sLSU-\textgreek{y} rRNA (213 nt) (Figures 1b and c). A higher molecular weight band of ~3 kb was also detected by hybridization corresponding most likely to the precursor asLSU-\textgreek{y} RNA (Figures 1b and c), suggesting that asrRNA is processed.

![Figure 1](image)
asRNA complementary to the *Leishmania* LSU-γ rRNA is enriched in translating ribosomes. To assess whether asLSU-γ RNA is associated with the ribosome, total RNA was sedimengd and fractionated in a sucrose gradient to separate the ribosomal subunits from monosomes and polysomes (Figure 1d, upper panel). RNA extracted from each fraction was resolved on a 10% denaturing acrylamide gel and hybridized with the 173 nt ss-DNA probe to detect asLSU-γ RNA. Northern blot hybridization revealed a ~200 nt product corresponding to the mature asLSU-γ RNA enriched in the 60S subunit (F4), the 80S monosome (F5–6) and polysome (F7–12) fractions (Figure 1d, lower panel). Smaller asLSU-γ-hybridizing fragments ranging from ~40–150 nt were also detected in 80S and polysome fractions (Figure 1d, F5–F12, lower panel), suggesting that part of the asLSU-γ RNA is further cleaved. Hybridization with a 5'-end-labeled oligonucleotide probe recognized the mature sLSU-γ rRNA transcript (213 nt) and smaller RNA species, which may correspond to degradation products (Figure 1e). sLSU-γ rRNA and asLSU-γ rRNA fragments were enriched in the same sucrose gradient fractions (Figure 1e, F5–F6 and F7–F12). Sequence of sLSU-γ rRNA-derived fragments confirmed their size from ~35–150 nt (Supplementary Figure S2), corroborating the hybridization data in Figure 1e. Neither sLSU-γ- nor asLSU-γ-derived fragments were detected in the 60S fraction (data not shown), which suggests that fragmentation of sLSU-γ and asLSU-γ rRNAs occurs in the context of the assembling ribosome.

Sequence analysis of several clones corresponding to either the mature sLSU-γ and asLSU-γ rRNAs, or their derived RNA products allowed us to map the 5' and 3' ends of these RNAs. Using 5'-random amplification of cDNA ends (RACE) we found that the 5'-end of the mature asLSU-γ RNA was complementary to the 3'-end of the sLSU-γ rRNA (Supplementary Figures S2 and S3). Mapping of the 3'-end of the mature asLSU-γ RNA showed complementarity to the first nucleotide of the sLSU-γ rRNA (Supplementary Figure S2). Thus, both ends of the asLSU-γ RNA are complementary to those of the sLSU-γ RNA (Supplementary Figure S2B), indicating that mature sLSU-γ and asLSU-γ rRNAs have the same length (213 nt). Internal cleavages of asLSU-γ RNA generated one nucleotide overhang at their 5'-end with respect to the 3'-end of the corresponding sLSU-γ rRNA fragments (58 (as)/57 (s) and 151 (as)/150 (s)) (Supplementary Figure S2A–S2B), suggesting that these RNA molecules may interact before cleavage.

Exposure of *Leishmania* to heat and oxidative stress leading to reduced levels of general translation induce fragmentation of the asLSU-γ RNA. *Leishmania* promastigote to amastigote differentiation is mainly triggered by temperature increase (from 25 to 37 °C) and drop in pH. We investigated whether differentiation signals could modulate asLSU-γ RNA fragmentation. RNA samples isolated either from *L. infantum* promastigotes subjected to heat-stress or from axenic amastigotes treated with H$_2$O$_2$ to induce oxidative stress were enriched for small RNAs (~200 nt) and analyzed by primer extension using a primer corresponding to nucleotides 101–118 of LSU-γ rRNA (Supplementary Table 1). Both heat and H$_2$O$_2$ stresses triggered a marked increase in asLSU-γ RNA fragmentation over the control (Figures 2a and b). Heat stress significantly reduced global translation as illustrated by polysome-profiling analysis.
Supplementary Figure S4A, upper panel) and induced more fragmentation of both asLSU-\(\gamma\) and sLSU-\(\gamma\) rRNAs in comparison with unstressed cells (Supplementary Figures S4A-S4B, bottom panels). Although the fragmentation pattern between heat-stressed and unstressed cells was generally similar, some bands were different, suggesting distinct cleavage events (Supplementary Figure S4A-S4B). Upon heat stress, asLSU-\(\gamma\) and sLSU-\(\gamma\) rRNA fragments were shifted mainly to the monosomes (Supplementary Figure S4A-S4B, F5-7), whereas under physiological conditions these were also detected in heavy polysomes (Supplementary Figure S4A-S4B, F12). Furthermore, \textit{L. infantum} axenic amastigotes shown recently to undergo a reduced translation\(^{27}\) exhibited more asLSU-\(\gamma\) RNA fragmentation than promastigotes (Figure 2c).

**asLSU-\(\gamma\) rRNA fragmentation is dramatically induced upon ALPCD.** Exposure of \textit{Leishmania} to \(\text{H}_2\text{O}_2\)\(^{19}\) or to antileishmanial drugs such as trivalent antimony (SbIII) or MF was shown to induce ALPCD.\(^{23,24}\) \textit{Leishmania} axenic amastigotes were treated with various drug concentrations for 24 h, and primer extension analysis was carried out on total \textit{Leishmania} RNA. Parasites treated with SbIII or MF demonstrated a dramatic increase in the asLSU-\(\gamma\) RNA cleavage pattern (Figure 3a) in a concentration-dependent manner (Figure 3b). In contrast, other cytotoxic drugs that do not trigger apoptosis in \textit{Leishmania} (e.g., hygromycin-B, paramomycin sulphate and neomycin/Geneticin (G418)) (M Ouellette, personal communication) failed to induce the asrRNA fragmentation process (Figure 3a), even at much higher concentrations (Supplementary Figure S5). Northern blot hybridization revealed that MF-induced asLSU-\(\gamma\) RNA fragmentation (Figure 3b) correlates with reduced levels of both the precursor and mature asLSU-\(\gamma\) RNAs (Figure 3c, upper panel) and with an increased accumulation of smaller RNA products (Figure 3c, bottom panel; prolonged exposure), further supporting that asLSU-\(\gamma\) RNA fragmentation is the result of cleavage reactions.

**asrRNA fragmentation upon induction of apoptosis is correlated with extensive rRNA degradation and translation inhibition.** Apoptosis in yeast\(^{28}\) and in mammals\(^{29}\) triggers rRNA degradation. To test whether induction of asrRNA cleavage during apoptosis has an effect on the degradation of srRNA, we evaluated LSU-\(\gamma\) rRNA expression levels in parasites treated with MF. Interestingly, northern blot hybridization revealed that accumulation of the mature sLSU-\(\gamma\) RNA is inversely correlated with increasing MF concentrations (Figure 4a, upper panel) and asLSU-\(\gamma\) RNA cleavage (Figure 3b). Primer extension analysis also revealed a decrease of LSU-\(\gamma\) rRNA in axenic amastigotes treated with the apoptosis-inducing agents MF and SbIII, but not with other cytotoxic drugs (Figure 4b), hence corroborating the northern blot data (Figure 4a, upper panel). Thus, degradation of the sLSU-\(\gamma\) RNA (Figure 4a, upper panel) seems to be related to increased asLSU-\(\gamma\) RNA cleavage (Figure 3b). Consistent with these data, northern blot hybridization on RNA from MF-treated promastigotes showed an increased accumulation of asLSU-\(\gamma\) RNA fragments in the 80S- and polysome-enriched fractions (Figure 4c). MF treatment not only resulted in LSU-\(\gamma\) rRNA degradation but also in the breakdown of all rRNA species.
Antisense rRNA fragmentation and apoptosis
Padmanabhan et al.

Figure 4  Fragmentation of asLSU-γ RNA upon induction of apoptosis is correlated with extensive degradation of the sLSU-γ LSU-γ rRNA in L. infantum axenic amastigotes. (a, upper panel) Northern blot analysis of RNA samples extracted from L. infantum amastigotes treated with various concentrations of MF (0–20 μM) for 24 h. (a, bottom panel) Ethidium bromide (EB)-stained RNA gel is shown here. (b) Primer extension analysis of L. infantum axenic amastigotes treated for 24 h with either G418 (25 μg/ml) or paramomycin sulphate (150 μg/ml) or hygromycin-B (80 μg/ml) or MF (20 μM) and/or Sb III (25 μM). A primer complementary to nucleotides 96–213 of sLSU-γ rRNA was used for the analysis. Arrows indicate the degradation pattern of sLSU-γ rRNA in MF- and SbIII-treated samples. (c) Lysates from Leishmania promastigotes treated with MF (25 μM) were fractionated by a 15–45% sucrose gradient. RNA samples were isolated from the respective fractions as indicated in Figure 1d, resolved on a 10% acrylamide gel and hybridized with the 173 nt ss-DNA probe to detect asLSU-γ RNA. The asLSU-γ RNA fragments enriched in the 80S and polysome fractions are indicated.

(Figure 4a, bottom panel). rRNA degradation following MF treatment had a dramatic effect on global translation as illustrated by polysome-profiling analysis (Supplementary Figure S6).

Next, we investigated whether induction of apoptosis could trigger asrRNA fragmentation and rRNA degradation also in macrophage-derived amastigotes. Primer extension analysis to detect asLSU-γ and sLSU-γ cleavage products was carried out on RNA extracted from L. infantum infected THP-1 macrophages treated with MF. Similarly to axenic amastigotes (Figures 3a and 4b), an increased fragmentation pattern of asLSU-γ rRNA was observed in MF-treated macrophages already at 24 h post infection, which was correlated with enhanced degradation of the sLSU-γ rRNA (Figures 5a and b). asLSU-γ fragmentation and sLSU-γ rRNA degradation were further enhanced at 48 and 72 h post infection (Figures 5a and b).

To further investigate the link between asrRNA fragmentation and rRNA degradation, we generated parasite strains overexpressing either the sLSU-γ or the asLSU-γ rRNA (Figure 6a). Overexpression of the asLSU-γ rRNA was confirmed by quantitative RT-PCR (qRT-PCR) (Figure 6b). All rRNA species were degraded more rapidly in MF-treated parasites overexpressing the asLSU1.2-RNA (Figure 6c, upper panel). Moreover, primer extension analysis revealed more degradation of the sLSU-γ rRNA in the asLSU1.2-overexpressing Leishmania in comparison with the sLSU1.2-overexpressing parasites (Figure 6d). Furthermore, northern blot showed a reduction in the mature asLSU-γ RNA levels in MF-treated asLSU-γ RNA overexpressing parasites (Figure 6c, lower panel) in line with increased asLSU-γ RNA fragmentation under those conditions (Figures 3a and b). Additionally, primer extension kinetics showed that LSU-γ rRNA degradation appears generally at the same time interval than asLSU-γ RNA fragmentation (Supplementary Figure S7).

An ATP-dependent DEAD-box RNA helicase of 67 kDa interacts with both sLSU-γ and asLSU-γ rRNAs to prevent asrRNA fragmentation. To investigate the mechanism of rRNA degradation induced upon apoptosis, we searched for Leishmania protein factors binding either to the sLSU-γ or asLSU-γ rRNA using a modified UV-crosslinking method (Materials and Methods). Two major bands of ~67 kDa and ~30 kDa were detected on 15% SDS-PAGE with both the sLSU-γ and asLSU-γ RNA templates (Figure 7a). These bands were gel-excised and analyzed by mass spectrometry analysis (MS/MS) identifying several proteins potentially interacting with the sLSU-γ and asLSU-γ rRNAs (Supplementary Figure S8 and data not shown). We concentrated on LinJ.32.0410 encoding an ATP-dependent RNA helicase of 67 kDa (HEL67) belonging to a conserved subfamily of DEAD-box helicases. The other proteins, including the 30 kDa UV-crosslinked protein will be the subject of a future study. The Leishmania HEL67 homolog shares 53% and 38% amino-acid sequence identity with the Drosophila Belle and VASA proteins, respectively, and 51% identity with the S. cerevisiae Ded1p protein (Supplementary Figure S9).
To investigate whether HEL67 protein has a role in asrRNA fragmentation, we generated a L. infantum null HEL67 (\(C^0/C^0\)) mutant by replacing the HEL67 gene by two different selection marker genes (Figure 7b, left panel). Gene replacement was confirmed by PCR (data not shown) and by Southern blot hybridization (Figure 7b, right panel). The lack of the 3.1 kb wild-type Lin HEL67 band and the HYG- and NEO-hybridizing bands of 2.3 kb and 2.1 kb, respectively, confirmed HEL67 gene inactivation. We also generated an add-back mutant (Lin HEL67 (\(C^0/C^0\)/\(C^0\))) expressing the HEL67 gene into Lin HEL67 (\(C^0/C^0\)/\(C^0\)) background (Figure 7b, left panel). In contrast to wild-type cells, Lin HEL67 (\(C^0/C^0\)/\(C^0\)) promastigotes exposed to elevated temperature (37°C) and acidic pH combined stress demonstrated a marked induction of asrRNA fragmentation (Figure 7c, left panel). Remarkably, asrRNA fragmentation in Lin HEL67 (\(C^0/C^0\)/\(C^0\)) was as high as in MF-treated parasites undergoing apoptosis (Figure 7c, left panel and 3a, b). This phenotype was rescued in Lin HEL67 (\(C^0/C^0\)/\(C^0\))REV (Figure 7c, left panel). The level of the mature asrRNA decreased significantly in Lin HEL67 (\(C^0/C^0\)/\(C^0\)) as estimated by qRT-PCR in comparison with the wild-type or to the add-back mutant (Figure 7c, right panel). Moreover, induction of asrRNA fragmentation correlates with a rapid rRNA breakdown in Lin HEL67 (\(C^0/C^0\)/\(C^0\)) (Figure 7d). These findings support a key role of Lin HEL67 in preventing asrRNA cleavage and rRNA degradation, hence protecting the parasite from apoptosis.

**Discussion**

Here, we report that ALPCD triggers fragmentation of asrRNA complementary to the LSU-\(\gamma\) rRNA in Leishmania that is correlated with rRNA breakdown. Additionally, we provide mechanistic insight into the regulation of this process by an ATP-dependent RNA helicase of the DEAD-box subfamily.
that interacts with both sLSU-γ and asLSU-γ rRNAs and protects rRNA from degradation by preventing asrRNA cleavage and cell death.

Although few asRNAs against mRNAs\textsuperscript{6,7} and non-coding RNAs\textsuperscript{8} have been reported previously in \textit{Leishmania}, asRNA complementary to rRNA species has not been investigated yet in protozoan parasites. Recently, in plants, it was shown that overaccumulation of the chloroplast asRNA AS5 is correlated with decreased abundance and inefficient 5S rRNA maturation\textsuperscript{30}. Here, we report that \textit{Leishmania} produces natural asRNA complementary to all rRNA species and that this asRNA is associated with the 80S and polyribosomes. We further show that part of the mature asLSU-γ RNA is cleaved into smaller RNA products (~40–150 nt) and this cleavage is markedly induced upon heat or oxidative stress, conditions where general translation is shown to be significantly reduced (Supplementary Figure S4 and Cloutier \textit{et al.}\textsuperscript{27}). srRNA is also fragmented within \textit{Leishmania} translating ribosomes under physiological conditions similarly to bacteria which could degrade rRNA in misassembled ribosome subunits\textsuperscript{31}, especially under stress conditions. In \textit{Leishmania}, heat stress leads to increased cleavage of the sLSU-γ RNA within monosomes, suggesting that most of stress-induced rRNA degradation occurs in assembled ribosomes. Our observations that the mature asLSU-γ RNA is fully complementary to the srRNA, that sLSU-γ and asLSU-γ RNA-derived fragments overlap to a large extent and that internal cleavages generate one nucleotide overhang might indicate an interaction between srRNAs and asrRNAs before their fragmentation, possibly through endoribonucleolytic activity.

An original finding in this study is that induction of ALPCD triggers a dramatic increase in asLSU-γ RNA fragmentation, which is correlated with an extensive breakdown of RNA and translation inhibition. Only conditions or drugs known to trigger
apoptosis in Leishmania could induce asrRNA fragmentation and rRNA degradation. Previous studies in yeast have reported that apoptosis induces specific rRNA degradation and translational arrest. Cleavage of monosome- and polysome-associated 28S rRNA has also been reported in human leukemia cells and in lymphoid cells undergoing apoptosis. A recent report demonstrated that asoligonucleotide-mediated stabilization of endogenous ribozyme-like non-coding rRNAs induced massive cell death via apoptotic and nonapoptotic mechanisms in lung cancer cells. However, asrRNA cleavage has not been reported, yet, as a consequence of apoptosis. We did not observe any significant accumulation of the precursor or mature asLSU-γ RNAs during stress (data not shown), indicating that asrRNA cleavage is central to this regulation. We show that asLSU-γ RNA fragmentation upon induction of apoptosis may directly
link to rRNA breakdown. Indeed, ectopic overexpression of asrRNA and rRNA fragments and accelerates rRNA degradation upon MF-triggered apoptosis. The fact that rRNA breakdown appears generally at the same time intervals as the asrRNA cleavage further favours the possibility that these two events are interrelated.

Another important finding here is the identification of HEL67 that seems to have a protective role in preventing fragmentation of asrRNA and rRNA degradation through its interaction with sLSU-γ and asLSU-γ rRNAs. The Leishmania HEL67 belongs to the superfamily 2-RNA helicases that are ubiquitously involved in various stages of RNA processing and RNP remodeling by promoting ATP-dependent conformational changes and structural transitions. HEL67 shares high sequence identity to the Drosophila Belle and VASA DEAD-box proteins, and to the yeast Ded1p. Belle promotes mitotic chromosome segregation in Drosophila via the endo-siRNA pathway. The germ-line-specific VASA have diverse roles in regulating mRNA translation, germline differentiation, and piwi-interacting RNA-mediated transposon silencing. The Ded1/DDX3 protein functions both as a repressor and an activator of translation. A Leishmania mutant deficient in HEL67 exhibits a marked increase in asLSU-γ RNA fragmentation when exposed to temperature and acidic pH stress, which is correlated with rapid degradation of the srRNA. Remarkably, the asLSU-γ RNA fragmentation pattern seen in Leishmania HEL67 mutant is comparable with that induced by MF in parasites undergoing apoptosis. Interestingly, an apoptosis-related phenotype has been reported for a null mutant of the mouse VASA homolog. Increased fragmentation of the asLSU-γ RNA in LinHEL67 is associated with reduced accumulation of the mature asLSU-γ rRNA, further indicating that HEL67 somehow prevents cleavage of the asrRNA. We propose that accumulation of asrRNA cleavage products may serve as a signal for accelerating rRNA breakdown under conditions of severe stress or apoptosis where global translation is markedly reduced. It remains to be seen, however, how asrRNA cleavage contributes to the degradation of srRNA. Our data support the possibility that under physiological conditions, HEL67 binds to both srRNA and asrRNAs within the context of their RNA-protein complexes.
Antisense rRNA fragmentation and apoptosis
Padmanabhan et al

translating ribosomes, hence preventing extensive cleavage of the asrRNA and protecting sRNA from degradation. Under conditions of severe stress or apoptosis, translation of HEL67 could be diminished and/or conformational changes within the ribosome could alter HEL67-rRNA interactions, hence allowing RNases to attack the non-translating ribosomes and to initiate rRNA breakdown.

In summary, here we describe for the first time that ALPCD and to a lesser extent stress trigger a regulated process involving fragmentation of asrRNA that is linked to rRNA degradation and translational arrest. This novel mechanism of asrRNA fragmentation seems to be conserved through evolution and it may represent a novel hallmark of apoptosis.

Materials and Methods

Parasite strains and cell culture. Leishmania infantum MHOM/MA/ITMAP-263, the parental strain for all the parasite lines employed in this study, was cultured as promastigotes in SDM-79 medium (pH 7.3) supplemented with 5% CO2 for 4 days as described. 40 Parasites were subjected to H2O2 (0–100 µM) or different drug treatments, including MF (Cayman Chemical, Ann Arbor, MI, USA) (0–40 µM), Stilb (Sigma) (0–100 µM), G418 (Sigma) (0–150 µg/ml), paramomycin sulphate (Sigma) (0–750 µg/ml) and hygromycin-B (Sigma) (80 µg/ml). T. brucei procaryotic cells were grown in SDM-79 medium until they reached logarithmic phase. The THP-1 human acute monocytic leukemia cell line was infected with L. infantum as previously described 40 but without adding PMA. THP-1 cells were seeded (25 × 103/ml) in a 75 ml flask and allowed to grow for 72 h.

DNA constructs and transfections. The vector pSPBT1YNEO1.2 was constructed as follows. The YNEO fragment where Y is a 92 bp polypyrimidine stretch, NEO the neomycin phosphotransferase gene for resistance to G418 and x the intergenic region of the L. enriettii alpha-tubulin gene was amplified from vector pSPSYNEOxLUC, and inserted into NotI of pSPBT1. 41 The 1.2 kb fragment harboring the last part of LSU-x (309 bp), the full-length LSU-y (213 bp) and the first part of LSU-y (414 bp) in sense (sLSU1.2) or antisense (asLSU1.2) orientation was amplified by PCR using specific primers (Supplementary Table 1). The amplified PCR products were cloned into pGEM-T easy vector (Promega) and sequenced. 41 The 1.2 kb fragment was inserted into the HindIII site of vector pSPBT1YNEOx. To inactivate the HEL67 gene of L. infantum (Lin32.0410) (TriTrypDB, http://tritrypdb.org), HEL67 5' and 3'-flanking regions were amplified from genomic DNA using a PCR fusion-based strategy and fused to the NEO and hygromycin phosphotransferase (HYGO) genes. For overexpressing the HEL67 gene, the HEL67 ORF was amplified by PCR and cloned into the XbaI and HindIII sites of vector pSP72S2EOX expressing the zeomycin (ZEO) marker. All primer sequences are described in Supplementary Table 1. Purified plasmid DNA (10–20 µg), Qiagen Plasmid Mini Prep Kit, Toronto, Ontario, Canada) was transfected into Leishmania by electroporation as described. 41 Stable transfectants were selected and cultured with either 0.025 mg/ml G418 (Sigma) or 0.080 mg/ml Hygromycin-B (Sigma) or 1 mg/ml zeomycin (Sigma).

DNA and RNA analysis and hybridizations. Genomic DNA of L. infantum was extracted using DNAzol (Life Technologies Inc., Toronto, Ontario, Canada) following the manufacturer's instructions. Total RNA was extracted from L. infantum after lysis with Trizol (Invitrogen) and analyzed on 1.2% agarose gels. PCR was carried out using the manufacturer’s instructions. For strand-specific (SS) RT-PCR, we used forward primers for 28S, 18S, 5.8S, LSU-x, LSU-y and LSU-J rRNAs (Supplementary Table 1). The resulting cDNA was treated with RNase H (Invitrogen) and PCR was carried out with the same forward and gene-specific reverse primers. The GAPDH gene was used for normalization of the qSS-RT-PCR data. Similarly, the RNA from T. brucei and the THP-1 cell line was used for SS-RT-PCR with specific primers described in Supplementary Table 1. Primers (Supplementary Table 1) were labeled with [γ-32P]ATP following the polyadenylate kinase protocol (PNK; New England Biolabs). Total RNA was isolated from drug-treated and untreated L. infantum promastigote and amastigote and T. brucei procaryotic samples and used for RT-reactions with labeled forward primers to detect asLSU-y rRNA cleavage products, and reverse primers to detect sLSU-y cleavage products. RNA from H2O2-treated and untreated THP-1 cells was isolated and used for primer extension with a forward primer corresponding to LSU-4685-4686 of the 28S rRNA (Supplementary Table 1). The resulting radiolabeled cDNA was resolved on 10% Urea acrylamide gel (Sequagel, National Diagnostics, Atlanta, GA, USA) and visualized by autoradiography. A 40×174 DNA/HindIII dephosphorylated DNA marker (Promega, Madison, WI, USA) was labeled with [γ-32P]ATP and PNK (New England Biolabs) according to the manufacturer’s recommendations and used as a size marker.

5' and 3'-end mapping of asrRNA and sRNA products. asLSU-y rRNA cleavage products were cloned by 5’-RACE. L. infantum RNA population of ~200 nt was enriched with the Ambion mirVana miRNA Isolation Kit and ligated with 5’-RACE adapter as per the manufacturer’s instructions (First choice RLM-RACE kit; Ambion). Treatments with CIP to remove the 5'-phosphate group and tobacco acid pyrophosphatase to remove the cap structure of mRNAs were omitted. The 5’-RACE adapter-RNA product was converted to cDNA using random hexamer and then PCR-amplified with Taq DNA polymerase (Qiagen) using adapter-specific primer (Ambion) and forward primer (P1) corresponding to nucleotides 1-18 of LSU-y for antisense and adapter-specific primer and reverse primer (P2) for sRNA (Supplementary Table 1). Nested PCR fragments with 5’-inner primer (provided with the kit) and gene-specific forward primer (P1) (Supplementary Table 1) for asLSU-y RNA and reverse primer (P2) and 5’-inner primer for sLSU-y RNA were used. To map the 3’-end of asLSU-y RNA, ~2 µg of total RNA was extended using poly-A polymerase (NEB) in the presence of ATP. The 3'-polyadenylated RNA was converted into cDNA using an oligo-dT primer. The cDNA was used as a template for PCR with oligo-dT and a reverse primer (P2) complementary to nucleotides 96-213 of sLSU-y RNA (Supplementary Table 1). The amplified PCR products were cloned into pcDNA3-T easy vector (Promega) and sequenced.

Polysem-profiling analysis. Approximately 3 × 106 L. infantum were treated with 100 µg/ml cycloheximide (Sigma) for 10 min, washed with phosphate-buffered saline, lysed with a Dounce homogenizer and 40 A260 nm units of the lysate supernatant was layered on top of a 15–45% linear sucrose gradient (10 ml) in gradient buffer (50 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 3 U/ml RNAGuard (Amersham)) as described. 41 Total RNA was isolated from each fraction following three volumes of ethanol precipitation with 1/10 volume of sodium acetate (3M, pH-5.2) and 50 µg of glycogen. The RNA was resolved on a 10% Urea acrylamide gel and analyzed by northern blot hybridization to detect sLSU-y and asLSU-y rRNAs.

In vitro UV-crosslinking studies. UV-crosslinking of in vitro-transcribed radiolabeled sLSU-y and asLSU-y transcripts and total Leishmania protein lysates (2 mg/ml) was performed as described previously 42 with some modifications. The mixture was transferred to a microplate and UV-irradiated using a Stratagene UV crosslinker (Agilent Technologies, Santa-Cla, CA, USA) (3 × 102 μJ, 254 nm bulbs) on ice for 15 min. The protein-crosslinked RNA was isolated by Trizol, digested with RNase A, 1 mg/ml (Invitrogen), RNase T1 10 U and RNase V1 (Ambion) and resolved on 15% SDS-PAGE gel. The radioactive bands were cut from the gel, left out for 10 weeks for radioactivity decay and then sent for MS/MS analysis.
Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. We thank Drs Marc Ouellette and Michaela Müller for critical reading of the manuscript. PP was a recipient of a postdoctoral fellowship from the Canadian Institutes of Health Research (CIHR) Strategic Training Program STP-53924 and the Centre for Host–Parasite Interactions (CHPI). MJS is a CIHR New Investigator supported by CIHR. This study was supported by the CIHR operating grant MCP-12182 awarded to BP. BP is a member of a CIHR Group on Host–Pathogen Interactions and of the Centre for Host–Parasite Interactions ‘Programme Regroupements Straté’giques’ of the Fonds de Québec pour la Recherche sur la Nature et les Technologies.

1. Thomason MK, Storz G. Bacterial antiseNSE rRNA: how many are there, and what are they doing? Annu Rev Genet 2010; 44: 167–188.
2. Faghihi MA, Wahlestedt C. Regulatory roles of natural antisense transcripts. Nat Rev Mol Cell Biol 2009; 10: 637–643.
3. Ketting RF. The many faces of RNAi. Cell 2003; 116: 205–219.
4. Robinson KA, Beverley SM. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. Mol Biochem Parasitol 2003; 128: 217–228.
5. Debrabant A, Lee N, Bertholet S, Duncan R, Nakhasi HL. Programmed cell death in Leishmania major Friedlin chromosome 1 initiates in both directions within a single region. Mol Cell Biol 2003; 11: 1291–1299.
6. Kapler GM, Beverley SM. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. Mol Biochem Parasitol 2003; 128: 217–228.
7. Dumas C, Chov C, Muller M, Papadopoulou B. A novel class of developmentally regulated noncoding RNAs in Leishmania. Eukaryot Cell 2006; 5: 2033–2046.
8. Smirnov D, Duszenko M, Ruiz AJ, Scoulica E, Bastien P, Fasel N. Programmed cell death in Leishmania amazonensis amastigotes. J Cell Sci 2002; 115: 341–353.
9. Carmona-Gutierrez D, Jungwirth H, Eisenberg T, Madeo F. Cell cycle control of cell death pathways in trypanosomatids gives insights into protozoan mechanisms of cell death. Trends Parasitol 2007; 23: 376–383.
10. Debrabant A, Lee N, Bertholet S, Duncan R, Nakhasi HL. Programmed cell death in trypanosomatids and other unicellular organisms. Int J Parasitol 2003; 33: 257–267.
11. Smirnov D, Duszenko M, Ruiz AJ, Scoulica E, Bastien P, Fasel N et al. Targeting essential pathways in trypanosomatids gives insights into protozoan mechanisms of cell death. Parasit Vectors 2010; 3: 107.
12. Debrabant A, Lee N, Bertholet S, Duncan R, Nakhasi HL. Programmed cell death in trypanosomatids and other unicellular organisms. Int J Parasitol 2003; 33: 257–267.
13. van Zandbergen G, Bollinger A, Wenzel A, Staat K, Myler PJ. Transcriptional regulation of the Leishmania major Friedlin chromosome 1 initiates in both directions within a single region. Mol Cell Biol 2003; 11: 1291–1299.
14. Kapler GM, Beverley SM. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. Mol Biochem Parasitol 2003; 128: 217–228.
15. Dumas C, Chov C, Muller M, Papadopoulou B. A novel class of developmentally regulated noncoding RNAs in Leishmania. Eukaryot Cell 2006; 5: 2033–2046.
16. Smirnov D, Duszenko M, Ruiz AJ, Scoulica E, Bastien P, Fasel N. Programmed cell death in Leishmania amazonensis amastigotes. J Cell Sci 2002; 115: 341–353.
17. Gonzalez LJ, Desponts C, Schaft C, Mottram JC, Fasel N. Leishmania major metacaspase can replace yeast metacaspase in programmed cell death and has arginine-specific cysteine peptidase activity. Int J Parasitol 2007; 37: 161–172.
18. Ketting RF. The many faces of RNAi. Cell 2003; 116: 205–219.
19. Cloutier S, Laverdiere M, Chou MN, Boilard N, Papadopoulou B. Translational control through eIF2alpha phosphorylation during the Leishmania differentiation process. PLoS ONE 2012; 7: e35985.
20. Claverie T, Alzate JF, Alvarez-Barrientos A, Gonzalez VM, Jimenez-Ruiz A. Heat-induced transcriptional mapping of the amplified region encoding the 18S rRNA maturation in vitro. FEBS Lett 2010; 583: 1291–1299.
21. Zangger H, Mottram JC, Fasel N. Cell death in Leishmania induced by stress and differentiation: programmed cell death or necrosis? Cell Death Differ 2002; 9: 1126–1139.
22. Vergnes B, Gourbal B, Girard I, Sundar S, Drummelsmith J, Ouellette M. A proteomics screen implicates HSP83 and a small kinetoheadin calpain-related protein in drug resistance in Leishmania donovani clinical field isolates by modulating drug-induced programmed cell death. Mol Cell Proteomics 2007; 6: 88–101.
23. Paris C, Loiseau PM, Bories C, Beaud J, Millettoine induces apoptosis-like death in Leishmania donovani promastigotes. Antimicrob Agents Chemother 2004; 48: 852–859.
24. Sereno D, Holzmüller P, Mangt I, Cuny G, Ouassai A, Lemesre J-L. Antimonal-mediated DNA fragmentation in Leishmania infantum amastigotes. Antimicrob Agents Chemother 2001; 45: 2094–2095.
25. White TC, Rudenko G, Borst P. Three small RNAs within the 16 kb trypanosome rRNA transcription unit are analogous to domain VII of other eukaryotic 28S rRNAs. Nucleic Acids Res 1986; 14: 9471–9489.
26. Zilberstein D, Shapira M. The role of pH and temperature in the development of Leishmania parasites. Annu Rev Microbiol 1994; 48: 449–470.
27. Clouter S, Laverdiere M, Chou MN, Boilard N, Csapoda B. Programmed cell death in Leishmania major Friedlin chromosome 1 initiates in both directions within a single region. Mol Cell Biol 2003; 11: 1291–1299.
28. Kapler GM, Beverley SM. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. Mol Biochem Parasitol 2003; 128: 217–228.
29. Danial NN, Korsmeyer SJ. Cell death: critical control points. Nat Rev Mol Cell Biol 2009; 10: 637–643.
30. Shemarova IV. Signaling mechanisms of apoptosis-like programmed cell death in yeast. Nucleic Acids Res 2008; 36: 2874–2888.
31. Shemarova IV. Signaling mechanisms of apoptosis-like programmed cell death in yeast. Nucleic Acids Res 2008; 36: 2874–2888.
32. King KL, Jewell CM, Bottert CD, Ciddowick JS. 28S ribosome degradation in lymphoid cell apoptosis: evidence for caspase and Bcl-2-dependent and -independent pathways. Cell Death Differ 2000; 7: 994–1001.
33. Shawcross DE, Hotto AM, Bollenbach TJ, Stern DB. Overacumulation of the chloroplastis rRNA ASS is correlated with decreased abundance of 5S rRNA in vivo and inefficient 5S rRNA maturation in vitro. RNA 2011; 17: 230–243.
34. Deutscher MP. Maturation and degradation of ribosomal RNA in bacteria. Prog Mol Biol Transl Sci 2009; 85: 269–391.
35. Houge G, Dickelander S, Boe R, Lanotte M. Selective cleavage of 28S rRNA variable regions V3 and V13 in myeloid leukemia cell apoptosis. FEBS Lett 1993; 315: 16–20.
36. Boulanger BM, Mulcahy JT, Soll J. Programmed cell death in protozoa. Annu Rev Genet 1993; 27: 9471–9489.
37. Boulanger BM, Mulcahy JT, Soll J. Programmed cell death in protozoa. Annu Rev Genet 1993; 27: 9471–9489.
38. Boulanger BM, Mulcahy JT, Soll J. Programmed cell death in protozoa. Annu Rev Genet 1993; 27: 9471–9489.
39. Boulanger BM, Mulcahy JT, Soll J. Programmed cell death in protozoa. Annu Rev Genet 1993; 27: 9471–9489.
40. Boulanger BM, Mulcahy JT, Soll J. Programmed cell death in protozoa. Annu Rev Genet 1993; 27: 9471–9489.