The major surface-metalloprotease of the parasitic protozoan, *Leishmania*, protects against antimicrobial peptide-induced apoptotic killing

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Summary

Human infection by the vector-borne protozoan *Leishmania* is responsible for substantial worldwide morbidity and mortality. The surface-metalloprotease (leishmanolysin) of *Leishmania* is a virulence factor which contributes to a variety of functions including evasion of complement-mediated parasite-killing and host intramacrophage survival. We tested the hypothesis that leishmanolysin serves to protect parasites from the cytolytic effects of various antimicrobial peptides (AMPs) which are important components of the innate immune system. We found that members of the α- and θ-defensins, magainins and cathelicidins had substantially higher leishmanicidal activity against leishmanolysin-knock out mutants of *L. major*. Using the magainin analogue, pexiganan, as a model peptide we show that AMP evasion is due to rapid and extensive peptide degradation by wild-type parasites. Pexiganan-treatment of knock out mutants induced disruption of surface-membrane permeability and expression of features of apoptosis including smaller cell size, loss of mitochondrial membrane potential, exposure of surface phosphatidylin serine as well as induction of caspase 3/7 activity. These results demonstrate leishmanolysin as a virulence factor preventing AMP-mediated apoptotic killing. This study serves as a platform for the dissection of the AMP-mediated death pathways of *Leishmania* and demonstrates the potential that AMP evasion plays during host infection by this parasite.

Introduction

Human infection with the vector-borne protozoan *Leishmania* causes substantial worldwide morbidity and mortality. Mammalian infection is initiated by deposition of motile promastigotes during feeding by infected sandflies. Thereafter parasites encounter a range of antimicrobial factors prior to binding and engulfment by host macrophages (Chang et al., 2003) wherein they differentiate into and replicate as intracellular amastigotes within phagolysosomes. Multiple surface molecules are important for *Leishmania* virulence including a family of surface-localized, zinc-dependent metalloproteases (leishmanolysin, gp63 or MSP) (Chang and Chang, 1986; Etges et al., 1986; Wilson et al., 1993) which are the only ecto-proteases of all pathogenic *Leishmania* and serve as ligands for binding macrophage complement and fibronectin receptors (Russell and Wilhelm, 1986; McGwire and Chang, 1994; Brittingham et al., 1999). Leishmanolysin degrades a wide range of proteins, including complement which serves to protect parasites from lysis as well as generates an additional epitope (C3bi) for binding to complement type 3 receptors (Brittingham et al., 1995). Leishmanolysin-knock out mutants of *L. major* have increased complement sensitivity and lead to smaller lesion size than wild-type parasites in mouse models of infection (Joshi et al., 2002). In addition, the protease also protects parasites from intraphagolysosomal degradation in macrophages presumably by inactivation of hydrolytic enzymes (Chaudhuri et al., 1989; McGwire and Chang, 1994).

Antimicrobial peptides (AMPs) are structurally diverse cationic proteins with intrinsic antimicrobial activity and are components of the innate immune systems of a wide variety of unicellular and multicellular organisms (Ganz and Lehrer, 1999). The AMPs of mammals and amphib-
ians are produced by variety of cell types and predomi-
nate within the skin, gastrointestinal and respiratory tracts
where they are the first line of defence against microbial
invasion. AMPs have potent antimicrobial activity
against prokaryotic and eukaryotic pathogens as well as
viruses. Work by others has shown that diverse AMPs
have activity against Leishmania. These include amphib-
ian derived peptides magainin (Guerrero et al., 2004),
temporin (Mangoni et al., 2005), dermaseptin (Gaidukov
et al., 2003) and most recently bombinin (Mangoni et al.,
2006), which kill Leishmania by disruption of cell surface
membranes resulting in osmotic lysis of parasites. Other
peptides such as the cecropin-melittin hybrids, have also
been shown to kill Leishmania by the same mechanism
(Diaz-Achirica et al., 1998).

Leishmania can undergo apoptotic cell death when
exposed to various agents known to induce apoptosis in
mammalian cells. Staurosporine or camptothecin treat-
ment of Leishmania induce cell size changes, breakdown
of mitochondrial membrane potential, leakage of cyto-
chrome c and activation of caspase 3-like activity. These
ultimately result in the cleavage of intranuclear proteins
such as poly-ADP ribose and induction of DNA laddering
(Arnoult et al., 2002; Sen et al., 2004a,b). Mitefosine and
hydrogen peroxide treatment of Leishmania donovani
induces many of these same features (Das et al., 2001;
Paris et al., 2004). Nitric oxide induces Leishmania ama-
zonensis to undergo caspase-independent apoptosis
(Holzmuller et al., 2002) also characterized by DNA
degradation. Indeed, genes for ancestral caspases
(metacaspases) have been found in the genomes of
L. major and L. donovani and the related American and
African trypanosomes (El-Sayed et al., 2005). Whether
the proteins expressed from these genes, or other pro-
teases, are responsible for the caspase-like activities
induced in apoptotic Leishmania remains to be
determined.

We have previously characterized the activities of
AMPs against the trypanosomatid protozoa (McGwire
et al., 2003). Here we report that leishmanolysin pro-
teolytically inactivates multiple AMPs and prevents AMP-
induced apoptosis. To our knowledge, this work is the first
demonstration of AMP-mediated induction of apoptosis in
Leishmania and underscores the importance of leish-
manolysin as a virulence factor in AMP resistance and
protection from peptide-induced apoptotic death.

Results

Activities of diverse AMPs against Leishmania

The leishmanicidal activities of mammalian defensins (α,
β and δ) (Wilson et al., 1999; Jia et al., 2001; Tran et al.,
2002), various ovine and porcine cathelicidins (Brogden
et al., 2001; Lehrer and Ganz, 2002) and pexiganan (Ge
et al., 1999), an analogue of magainin, which is found on
the skin of the African clawed frog were compared using a
high throughqut MTT survival assay described previously
(McGwire et al., 2003). We have found that cathelicidins
had relatively higher activity against wild-type promastig-
otes of L. amazonensis (shown in Fig. 1A) and L. major
(in Fig. 1C) than did those of the other classes. AMP
activity was tested both by adding peptides directly into
parasite cultures as well as to cells washed in phosphate-
buffered saline (PBS). Parasite killing by the cathelicidins
was approximately 50% greater when tested in PBS (not
shown).

Resistance to AMP-mediated killing is dependent on
presence of leishmanolysin

We tested the hypothesis that leishmanolysin renders
Leishmania relatively resistant to killing by certain AMPs.
For this we initially compared AMP killing of wild type and
leishmanolysin-deficient L. amazonensis (McGwire and
Chang, 1994; 1996) and found the latter to be more effi-
ciently killed by the α- defensins, cryptdin-4 (Fig. 1B) and
cryptdin-1 (see Supplementary material) and the cyclized
defensin, Theta-II (Fig. 1B). In contrast we did not observe
differences in the killing of these lines with the cathelicidin,
Protegrin-1 (Lehrer and Ganz, 2002) or human
β-defensin-3 (Jia et al., 1999) (Fig. 1B). Similar results
were seen using other cathelicidins (SMAP and OV-1)
(Gennaro and Zanetti, 2000) (Sawai et al., 2002) and
human B-defensin-2 (not shown). Because the
leishmanolysin-deficient variant of L. amazonensis
expresses approximately 10% of the surface-localized
leishmanolysin as the wild type we used a leishmanolysin
knock-out line (KO) of L. major, completely devoid of
leishmanolysin expression (Joshi et al., 2002), for further
analysis. We compared AMP-killing of these lines with that
of an episomally complemented KO line expressing the
gene for leishmanolysin (KO + 63). We found dramatic
differences in the killing of the leishmanolysin-expressing
and KO lines with both cathelicidins and pexiganan
(Fig. 1C). In the case of pexiganan the killing of the KO
line was particularly striking, being ~50% greater than that
of wild type or KO + 63 lines. Differences were noted in
the differential killing of wild type and leishmanolysin-
deficient lines of the two Leishmania species with Theta-II
and Protegrin-1 (compare Fig. 1B and C) which probably
represents the inherent susceptibility of different Leishmania to different AMPs. Dose-dependent analysis of pexiganan-mediated killing against the L. major parasite lines is shown in Fig. 1D. Increasing concentrations of peptide, up to 50 μM, killed the KO much more efficiently than the wild-type line. The KO + 63 line was also protected from pexiganan killing but this protective effect was gradually lost as the concentration of peptide was increased and was equal to that of the KO line using 100 μM peptide.

Antimicrobial peptide activity can be potentiated by leishmanolysin inhibition

We suspected that the protective effect of leishmanolysin against AMPs was due to their proteolytic degradation. We tested the whether parasite killing could be potentiated by coincubation of AMPs with a leishmanolysin inhibitor. For this we used a non-hydrolysable mutant version of the MARCKs-related protein, a known substrate for leishmanolysin (Corradin et al., 2002). Both wild-type L. major and L. amazonensis were preincubated for 30 min with increasing amounts of inhibitor and then incubated with lytic doses of pexiganan or PG-1 under our standard conditions (Fig. 2A). Incubation of cell lines with the protease inhibitor alone did not affect their viability (not shown). We observed an increase in the AMP killing as the concentration of inhibitor was increased. At 50 pM inhibitor the killing of the wild-type L. major was the same as that of the KO line. Although PG-1 is more potent than pexiganan at killing wild-type L. major, leishmanolysin inhibition further potentiated PG-1 killing exceeding that of the KO line. These results strongly suggested that the protection in AMP-killing of wild-type cells was due to peptide degradation by leishmanolysin.

Antimicrobial peptides are proteolytically degraded leading to their inactivation

In order to confirm that the protection of wild-type L. major was due to proteolytic inactivation of AMPs, we tested the capacity of leishmanolysin to degrade both pexiganan (Fig. 2B) and cryptdin (see Supplementary material, Fig. S1). Incubation of pexiganan with glutaraldehyde-fixed wild type and KO + 63 lines led to degradation of the peptide whereas incubation with the KO line did not (upper right portion of Fig. 2B). Peptide degradation directly correlated with leishmanolysin proteolytic activity (Fig. 2B, right lower panel). In each case the peptides were degraded only in the presence of zinc, an essential cofactor for leishmanolysin activity. In order to test the kinetics of pexiganan degradation, replicate reactions using both the wild type and KO lines were performed and processed for SDS-PAGE analysis at increasing times of incubation from 15 to 120 min (Fig. 2C). This analysis showed that only incubation of the peptide with the wild-type cells resulted in complete and rapid degradation of the peptide by 15 min. We detected no appreciable loss of
peptide by incubation with the KO line further indicating that cells without leishmanolysin cannot degrade peptide. In order to characterize pexiganan degradation by leishmanolysin, we analysed degradation products by mass spectroscopy (Table 1) and determined that pexiganan was cleaved at 14–15 residues producing at least 13 different peptide fragments consistent with the previous characterization of leishmanolysin as a broad-spectrum endoprotease.

**Pexiganan-mediated cell death of leishmanolysin-deficient Leishmania occurs by apoptosis**

Because most AMPs, like pexiganan, are highly cationic and interact with and disrupt cell membranes, we measured the permeability of the surface membranes of cells upon disruption of their external membranes and interacts with DNA causing a measurable increase in fluorescence. Wild type, KO and KO + 63 lines were exposed to increasing amounts of pexiganan for 90 min in the presence of SYTOX. Cells incubated with 0.5% Triton-X 100 served as controls to demonstrate complete permeability. The KO line showed rapid and sustained influx of SYTOX at pexiganan amounts above 12.5 μM whereas wild type and KO + 63 lines were relatively impermeable to SYTOX at the same concentrations (Fig. 3). All cells lines treated with detergent had a rapid and sustained influx of SYTOX, occurring within the 1 min of incubation, whereas those treated with PG-1 had a slow influx of dye being maximal at 90 min.

In order to detect whether pexiganan induced large alterations in surface membranes, we analysed pexiganan-treated cell lines by scanning and transmission electron microscopy (EM). Scanning EM of all untreated
lines did not show gross differences in the surface membranes (leftmost panels in Fig. 4). Pexiganan treatment of the KO line caused ~70–80% of the cells to become rounded losing their typical elongated shape consistent with the decrease in their viability. Despite this, closer analysis of the surface of the treated KO line by scanning EM (seen in the middle set of EM panels of Fig. 4) did not reveal gross disruption of the external membrane. In contrast, transmission EM analysis of the pexiganan-treated KO line revealed complete decompartmentalization of the internal membranes not seen in the wild type or KO + 63 lines (rightmost EM panels in Fig. 4). Flow cytometric analysis of all treated and untreated cell lines showed that only the pexiganan-treated KO line had a significant decrease in cell size (Fig. 4, rightmost panels). This was apparent in cells treated with as little as 6.25 μM of pexiganan and increased at higher concentrations (see Supplementary material, Fig. S2). We did not observe a decrease in cell size of the wild-type line at even the highest concentration of pexiganan. We noted a slight decrease in the size of a minor proportion of cells of the KO + 63 line causing a decrease in their viability. Despite this, closer analysis of the surface of the untreated KO line revealed complete decompartmentalization of the internal membranes not seen in the wild type or KO + 63 lines (rightmost EM panels in Fig. 4). Flow cytometric analysis of all treated and untreated cell lines showed that only the pexiganan-treated KO line had a significant decrease in cell size (Fig. 4, rightmost panels). This was apparent in cells treated with as little as 6.25 μM of pexiganan and increased at higher concentrations (see Supplementary material, Fig. S2). We did not observe a decrease in cell size of the wild-type line at even the highest concentration of pexiganan. We noted a slight decrease in the size of a minor proportion of cells of the KO + 63 line at the highest concentration of pexiganan, which correlated with the decrease in its viability at this high concentration of pexiganan (Fig. 1D).

We measured the effect of pexiganan on the mitochondrial membrane potential of the cell lines using rhodamine 123 (Divo et al., 1993; Guerrero et al., 2004). Flow cytometric analysis of rhodamine 123 staining of pexiganan-treated KO cells demonstrated a dose-dependent breakdown of potential indicated by a decrease in fluorescence (Fig. 5A, and Supplementary material, Fig. S3). In contrast, the mitochondrial membrane potential of peptide-treated wild type and KO + 63 cells remained intact under the same conditions. A minor proportion of cells of the KO + 63 line showed a decrease in mitochondrial membrane potential at the highest level of pexiganan (see Supplementary material) consistent with both the cell size and viability data of this line with high-dose pexiganan. All cell lines treated with the mitochondrial poison FCCP, had a decrease in fluorescence (as indicated in Fig. 5A). Interestingly there was a marked decrease in fluorescence in pexiganan-treated KO-cells than those treated with FCCP suggesting that the peptide is more potent than FCCP in causing mitochondrial dysfunction.

The changes in cell size and mitochondrial membrane potential of pexiganan-treated KO cells are similar to features described previously in apoptotic Leishmania (Arnoux et al., 2002). This suggested to us that pexiganan induces features of apoptosis in KO cells. To confirm this we tested whether peptide-treated cell lines stained with annexin-V and propidium iodide. Untreated and pexiganan-treated cell lines were stained with both reagents and analysed by flow cytometry (Fig. 5B). Untreated lines remained largely unstained by both dyes. Peptide-treatment of the wild type and KO + 63 lines resulted in the production of a minor population of dual fluorescent cells. In stark contrast, pexiganan treatment of the KO line produced a dramatic increase (from 0.2% to 76%) in the number of dual staining cells. This highly suggested that the KO line undergoes apoptotic cell death due to pexiganan exposure. This effect is abolished by the rapid degradation of the peptide at the surface of leishmanolysin-expressing cells.

### Table 1. Pexiganan cleavage products generated by leishmanolysin degradation.

<table>
<thead>
<tr>
<th>Molecular mass</th>
<th>Pexiganan cleavage sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1072.01</td>
<td>K-P-G-K-A-P-V-K-I-L</td>
</tr>
</tbody>
</table>

*a.* Cleavage sites in parentheses cannot be definitively assigned because the identical masses of peptides precluded their absolute identification.

**Pexiganan selectively induces caspase 3/7 activity in leishmanolysin-deficient Leishmania**

Induction of apoptosis of Leishmania coincides with the activation of caspase-like cysteine proteases (Das *et al.*, 2002).
2001; Arnoult et al., 2002). To test whether pexiganan treatment of the *L. major* lines induced such activity we measured the cleavage of the caspase 3/7-specific substrate Z-DEVD-R110 of cell lysates prepared before and after 2 h incubation with 12.5 μM of pexiganan (Fig. 6). Pexiganan-treated KO cells had significantly more caspase activity than untreated controls. In contrast, leishmanolysin-expressing cells showed minimal increases in activity. As a test of the specificity of caspase activation, cells were preincubated with the cysteine protease inhibitors E-64 and BAF (Fig. 6) and then assessed for caspase 3/7 activity after peptide treatment. Preincubation of the KO line with either inhibitor prior to incubation with pexiganan resulted in a substantial decrease in caspase 3/7 activity. The reduction in caspase activity was more using BAF than with E-64 at the same concentration. The slight increase in caspase activity of the leishmanolysin-expressing lines was also diminished in the presence of the inhibitors. Lastly, we measured whether pexiganan treatment of the KO line led to the degradation of DNA as seen using other compounds known to induce apoptosis in *Leishmania*. Agarose gel analysis of total DNA purified from untreated and peptide-treated cell lines revealed that DNA degradation occurred only in the KO line treated with pexiganan (Fig. 6B). Preincubation of the KO line with E-64 partially protected the KO line from pexiganan-induced DNA degradation whereas BAF had no effect.

**Discussion**

Here we demonstrate that leishmanolysin serves to effectively protect parasites from killing by AMPs. This effect was found for a wide range of peptides including...
members of the β-defensins, cathelicidins and magainins and suggests that the evasion of AMPs by Leishmania may be important to parasite virulence. Like Leishmania, the evasion of AMP-mediated cytolysis has also been shown for other microbes. Staphylococcus and Salmonella can resist AMP killing via changes in the components of their cell walls leading to decreased association with peptides (Gunn, 2001) and Salmonella expresses a protease which can lead to AMP degradation (Guina et al., 2000). In addition, Staphylococcus can resist peptide killing by liberation of an exoprotein, staphylokinase, which can bind to and inactivate defensins (Jin et al., 2004). Staphylococcus aureus and Escherichia coli express several classes of proteases which impart resistance to lactoferrin B, a cationic peptide derived from the N-terminal portion of lactoferrin (Ulvatne et al., 2002). Both Proteus mirabilis and Staphylococcus express metalloproteases that degrade the human cathelicidin LL-37 leading to its inactivation (Belas et al., 2004; Sieprawska-Lupa et al., 2004). Cathelicidins appear to be more potent than pexiganan or α-defensins against wild-type Leishmania suggesting that cathelicidins may be less susceptible to leishmanolysin degradation and/or may interact more efficiently with the surface membrane. These may be related to differences in charge or secondary structure rather than overall peptide length because pexiganan and the cathelicidins tested are similar in size but differ in their ability to kill wild-type parasites. Alternatively, cathelicidin degradation products may be nearly as efficient at killing parasites as intact peptide. It is not entirely clear why cathelicidins have reduced activity to Leishmania in culture medium when compared with PBS. This may be due to degradation by released leishmanolysin (McGwire et al., 2002) or due to a reduction in the interaction of peptides with the parasite membrane due to the presence of serum components in the medium. The increase in pexiganan- and PG-1-mediated parasite killing in the presence of leishmanolysin inhibitor suggests, however, that these intact peptides are more potent at killing than their degradation products. Interestingly, we noted that PG-1 had higher levels of killing of wild-type cells at high concentrations of protease inhibitor than that of the KO line suggesting that the protease-inhibitor and peptide may work synergistically to kill the wild-type parasites. The reduction in survival of the wild-type parasites in the presence of both the protease inhibitor and AMP to the level seen in the KO line discounts the possibility that leishmanolysin, in the absence of its proteolytic activity, serves simply as a protective shield against AMP attack. The degradation of the pexiganan was remarkably rapid.

Fig. 4. Electron microscopic and flow cytometric analyses of pexiganan-treated Leishmania lines. Wild type, KO and KO + 63 lines were treated with pexiganan (12.5 μM, 2 h at 25°C) and analysed for membrane disruption with scanning (left and middle panels) and transmission (right) electron microscopy. The KO line had extensive disruption of the internal membranes when treated with peptide despite a relatively intact external membrane. Peptide treatment did not lead to disruption of the internal or external membrane systems of the lines expressing leishmanolysin. Pexiganan-treated lines were analysed by flow cytometry (rightmost panels) to determine changes in the relative size or granularity of cells. The solid arrow denotes cell populations of normal cell size and granularity seen in all cell types. White arrows denote cell populations, in the treated KO line, which show a downward shift in forward scatter indicative of smaller cell size consistent with apoptosis. The results shown are a representative of three experiments which revealed similar results.

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Fig. 5. Breakdown of mitochondrial membrane potential and annexin-V and propidium iodide staining of leishmanolysin-deficient Leishmania treated with pexiganan.
A. Pexiganan-treated cell lines were analysed for alterations in mitochondrial membrane potential using flow cytometry to measure increasing fluorescence after incubation with rhodamine 123. This dye selectively fluoresces in cells with functional mitochondria. The Wt and KO + 63 lines showed a high accumulation of the dye intracellularly whereas the KO had diminished accumulation of the dye under the same conditions indicative of a breakdown of the mitochondrial membrane potential which was similar to cells treated with 7.5 µM of the protonophore carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP, as indicated).
B. Untreated and pexiganan-treated lines (as indicated) were stained with both annexin-V-FITC (measured in the horizontal axis) and propidium iodide (PI) (in the vertical axis) followed by flow cytometric analysis. Dually stained cells appear in the upper right quadrant and are seen predominantly in the pexiganan-treated KO line. The majority of control cells of each line incubated in buffer alone did not stain. Indicated in the upper right quadrant is the percentage of the total cell population that stain with both PI and annexin-V. Shown are representative experiments of at least three experiments with gave similar results.

and extensive, occurring within 15 min at 12 sites in this 22 amino acid peptide to generate at least 13 different peptides. The finding that leishmanolysin has preference for cleavage of peptides on either side of lysine, or possibly other positively charged residues, suggests that certain leishmanolysin isoforms may have specifically evolved to have substrate specificity for inactivation of cationic peptides such as AMPs. Interestingly, the complemented KO mutant is more susceptible to killing at higher amounts of pexiganan than wild-type L. major. The
complemented KO line expresses one of two isoforms expressed by the wild-type line. We hypothesize that the lack of expression of the additional isoform in the complemented line leads to its increased susceptibility to high concentrations of pexiganan.

Surface membrane disruption of *Leishmania* is an important mode of killing by various AMPs. Both unmodified and N-terminal lipidated cecropin A-mellitin hybrid peptides (Diaz-Achirica et al., 1998), temporins (isolated from the amphibian *Rana Temporaria*) (Mangoni et al., 2005) and magainin-2 (a member of the magainin family of peptides expressed in the skin of *Xenopus Laevis*) (Guerrero et al., 2004) affect surface membranes of *L. donovani* leading to a decrease in intracellular ATP. This ATP loss appears not to be a result of a mitochondrial toxicity. Pexiganan-treatment of the KO cells did not result in gross changes in surface membranes visualized by SEM but SYTOX permeability was dramatically increased and cells stained with propidium iodide. The change in surface membrane permeability is consistent with the surface active nature of cationic AMPs and we hypothesize that this provides an essential trigger of the downstream events of cell death. While AMP-induced cell death may occur by several different pathways simultaneously our data argue strongly that the process involves apoptosis. Pexiganan treatment also led to a concomitant decrease in cell size and exposure of surface membrane phosphatidyl serine, which are features of apoptotic *Leishmania* (Arnoult et al., 2002; Sen et al., 2004b). In addition, the internal membranes and mitochondria potential of this line was dramatically affected by pexiganan. Although this is consistent with previous findings that magainins can disrupt mitochondrial membranes and uncouple respiration (Westerhoff et al., 1989) we do not yet know whether pexiganan treatment of the KO line affects their level of intracellular ATP. Overall these results strongly suggest that pexiganan induces apoptotic death of leishmanolysin KO mutants and that the changes in surface membrane permeability may be, in part, important in this phenomenon.

The peptide may act at the surface membrane triggering an intracellular signalling pathway leading to mitochondrial membrane disruption and activation of premetacaspases. Alternatively, pexiganan could transit through the surface membrane and directly act on cytosolic components or the mitochondrial membrane. TEM analysis showed drastic effects of pexiganan on the intracellular membranes of a proportion of KO cells and this was supported by flow cytometric analysis showing two populations with distinct differences in both cell size and rhodamine fluorescence. Although these changes were dose-dependent the presence of two populations suggests that cell death was not a synchronous process or that some cells may be relatively resistant to the effects of pexiganan. Pexiganan induces the activation of caspase 3/7-like protease activity which is likely the result of leakage of cytochrome c from disrupted mitochondria. Whether the apoptotic-like cell death reported for here is a caspase-dependent or -independent event is unclear. While preincubation of the KO line with the caspase inhibitor BAF greatly diminished pexiganan-induced caspase 3/7 activation, we do not yet know whether it inhibits the apoptotic phenotype. Caspase-like proteases of *Leishmania* can act on several intracellular targets to mediate DNA fragmentation (Das et al., 2001; Arnoult et al., 2002), but DNA fragmentation can also occur during non-caspase-
mediated apoptosis (Holzmuller et al., 2002). Interestingly, BAF did not prevent pexiganan-induced DNA degradation of the KO line whereas the broad spectrum cysteine protease inhibitor, E-64 did, suggesting that DNA degradation by pexiganan may be independent of caspase 3/7 activation and may be the result of the activity of the abundant cysteine proteases expressed by Leishmania (Mottram et al., 2004). The degraded DNA did not have the typical ladder-like pattern typically seen in apoptotic higher eukaryotic cells. This has been seen in Leishmania undergoing apoptosis as a result of serum deprivation and heat shock and has been hypothesized to be due to the massive decompartmentalization of cysteine proteases from parasite lysosomes (Zangger et al., 2002). Indeed, our data suggest that non-caspase cysteine proteases are responsible for the DNA fragmentation in AMP-induced apoptosis.

The work presented here demonstrates that leishmanolysin functions to protect parasites from killing by AMPs and ascribes an important new function to this virulence determinant. Leishmanolysin may be important for evasion of AMPs during the early stage of mammalian infection, serving to allow more parasites to survive extracellularly prior to establishing intracellular residence in macrophages. The concentration of AMPs used in our in vitro assays is within that expected to be present normally in the skin (Liu et al., 2002). Further the concentration of AMPs at sites of inflammation is thought to be even higher. For purposes of sensitivity the parasite numbers used in our assays are 10^2- to 10^3-fold higher than would be expected to be found inoculated by the bite of a single sandfly or present within an infected macrophage (10^1-10^4 parasites). Thus on a per cell basis the local concentration of AMPs in vivo would be within the range we have used. The degradation products of AMPs encountered by cells during infection may serve other roles, such as enhancing macrophage migration to inflammatory sites, alteration of their phagocytosis or lead to their activation or deactivation. Whether AMPs are important for the control of intracellular Leishmania within infected macrophages akin to that found in during Salmonella infection is not known (Rosenberger et al., 2004). Sandflies express their own AMPs which are induced by Leishmania infection. Other Leishmania virulence factors, such as lipophosphoglycan, are known to modulate the expression of sandfly AMP and may also aid in the survival of parasites in this milieu (Boulanger et al., 2004). The inactivation of these AMPs by leishmanolysin may be important for parasite survival in the sandfly midgut.

Our results have implications in the design of AMPs as potential antileishmanial compounds. Modification of AMPs, such as pexiganan, to decrease their propensity for leishmanolysin degradation, increase their membrane affinity and leishmanicidal activity is also worthy of systematic study. This is not only relevant to Leishmania but also to related parasites, the South American and African trypanosomes, both of which express a homologues of leishmanolysin (Grandgenett et al., 2000; LaCount et al., 2003).

**Experimental procedures**

**Parasites**

Cells used in this study are: (i) Leishmania major (NHOM/SN/74/Seidman), its leishmanolysin KO derivative (termed KO, in this article) and the back leishmanolysin transfected line (termed KO + 63 in this article) (Joshi et al., 2002); and (ii) wild type and leishmanolysin-deficient variants of L. amazonensis (McGwire and Chang, 1994; 1996). Strains were routinely cultivated as insect forms in M199 containing 10% HIFBS.

**Antimicrobial peptides**

Cryptdin-1 and -4 were produced and purified as described elsewhere (Wilson et al., 1999; Ayabe et al., 2000) and were the kind gifts of Drs C. A. Wilson (Washington University, St. Louis, MO) and D. P. Satchell (University of California at Irvine). Human β-defensins were purified from an insect cell/baculovirus expression system, as described elsewhere (Jia et al., 1999). A non-AMP of 32 aa (KLHMAPEFSDQVRRKAKIGERRSDGDIVSRDL), based on the sequence of Trypanosoma cruzi mitochondrial hsp40 (Klein et al., 1995), was used as a control. Peptides based on the sequence of the antimicrobial C-terminal regions of the cathelicidins (SMAP 29, PG-1) (Broden et al., 2001; Lehrer and Ganz, 2002), SMAP 28 (Gennaro and Zanetti, 2000), ovispirin (an 18-aa peptide resembling the N-terminus of SMAP 29) (Sawai et al., 2002), and pexiganan (Ge et al., 1999) were synthesized on an Applied Biosystems model 433 A synthesizer as described previously (McGwire et al., 2003).

**Peptide degradation assays and structural analysis of peptide degradation products**

The purification of leishmanolysin from L. amazonensis was performed as described previously (McGwire et al., 2002). For degradation assays, 10 μg of peptide were incubated in 100 μl of PBS at 37°C and indicated times containing 10^-2 glutaraldehyde-fixed parasites or -1 μg of purified leishmanolysin. After incubation cells were removed from reactions by microcentrifugation and peptide products were analysed by SDS-PAGE on 15% gels followed by Coomassie-Blue staining. Zinc chelation was performed by preincubation of cells or protease in 25 mM of 1, 10-phenanthroline for 1 h prior to addition to the peptide substrate. For analysis of degradation products capillary-liquid chromatography tandem mass spectrometry (Nano-LC MS/MS) was performed on a Micromass hybrid quadrupole time-of-flight Q-TOF (TM) II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal nanospray source (New Objective, Woburn, MA) operated in positive ion
mode. Mass spectra was acquired using MassLynx 4.0. Sequence information from the MS/MS data was processed using the Mascot Distiller software and database searches was performed using the MASCOT (Matrix Science, Boston, MA) programs.

Parasite survival assay

A standard parasite survival assay was used as described previously for *Leishmania* (McGwire et al., 2003). Routinely 10^7 parasites were incubated in 100 μl of MTT reagent followed by treatment with 10% SDS for 6–8 h followed by reading in spectrophotometer at 570 nm. Treated parasites were compared with parasites incubated in the same conditions either in buffer alone or in the presence of a non-AMP peptide at the same concentration. Cells were incubated for 2 h at the indicated concentration of peptide prior to analysis with the MTT assay. Cells were preincubated in a leishmanolysin peptidomimetic inhibitor (Corradin et al., 2002) at the indicated concentration 30 min prior to addition of AMP.

SYTOX Green Assay for cell membrane permeability

A total of 10^7 parasites were washed thrice in PBS then incubated in the dark with 1 μM SYTOX Green (Promega) in PBS for 15 min as described (Chicharro et al., 2001). Fluorescence was measured every 5 min after peptide addition for up to 2 h. Control for maximum fluorescence was shown by addition of 0.5% Triton X-100. Fluorescence was measured in a microplate reader with excitation and emission wavelengths of 485 and 520 nm respectively.

Caspase 3/7 activity assay

Caspase 3/7 protease activity was measured using the Apo-1 Homogenous Caspase 3/7 Activity Assay kit (Promega, Madison, WI). The assay was carried out according to the manufacturer’s instructions with the following minor modifications. A total of 10^7 parasites were incubated in 100 μl of PBS with or without pexiganan for 2 h followed by addition of kit reagents. At the completion of the reactions the increase in fluorescence, indicative of the cleavage of the Z-DEVD-R110 substrate, was read fluorimetrically for 2 h at excitation and emission wavelengths of 485 and 530 nm respectively. Protease inhibitors E-64 (Sigma, St Louis, MO) and 1-3-Boc-aspartyl-fluoromethyl ketone (BAF) (Calbiochem, San Diego, CA) were added in control reactions at 100 μM for 30 min prior to addition of 6.25–12.5 μM pexiganan.

DNA fragmentation analysis

Total DNA was extracted from parasites using 2.5 mM TRIS HCL, pH 7.4, 1.5 M LiCl and 0.1% Triton X-100 and precipitated with ethanol and then resuspended in deionized water followed by fractionation in 1% agarose gels in 1 × TRIS Borate buffer and visualized by staining with Ethidium Bromide as described previously (McGwire and Chang, 1996).

Flow cytometric analysis

Flow cytometry analysis was performed using a FACS Calibur flow cytometer and CellQuestPro software (Becton Dickinson, Mount View, CA, USA). Cells were prepared as indicated above and stained with annexin V-FITC and propidium iodide using the Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer’s instructions. For measurement of mitochondrial membrane potential changes cells were treated as indicated above and loaded for 5 min at 25°C with 0.3 μg ml^(-1) of rhodamine 123 as described previously (Diaz-Achirica et al., 1998). Cells were washed thrice in PBS prior to flow cytometric analysis using 488 and 525 nm excitation and emission wavelengths respectively. Cells incubated under the same conditions with 7.5 μM with the mitochondrial poison, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), were used as a positive control.

Electron microscopy

Cells were lightly centrifuged and then gently resuspended in fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) overnight at 4°C. Fixed cells were processed and visualized as described previously (McGwire et al., 2003).

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References


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**Supplemental material**

The following supplementary material is available for this article online:

**Fig. S1.** Metalloprotease mediated protection is via proteolysis of the AMP, cryptdin-1.

A. Differential killing of wild type (black) and a leishmanolysin-deficient variant of *L. amazonensis* (white) (McGwire and Chang, 1994) by matrilysin-activated recombinant cryptdin-1 (Wilson et al., 1999).
B. Cryptdin-1 were analysed by SDS-PAGE after incubation with increasing numbers of fixed *L. amazonensis* (upper portion of panel B) or purified leishmanolysin (gp63)(lower portion of panel B). Lower portion of panel A shows gelatin zymography of indicated *L. major* lines. The degradation of peptides were dependent on increasing cell number (shown in the upper right portion of panel B) and on the presence of Zn (as indicated) a known cofactor for leishmanolysin activity.

**Fig. S2.** Dose dependence of pexiganan-induced changes in the cell size of *Leishmania* lines. Wild type, KO and KO + 63 *L. major* lines were treated with increasing amounts of pexiganan (as indicated for 2 h at 25°C) and analysed by flow cytometry for shifts in forward- and side-scatter to determine changes in the relative size or granularity of cells. The solid arrow denotes cell populations of normal cell size and granularity seen in all cell types. White arrows denote cell populations, in the treated KO line, which show a downward shift in forward scatter indicative of smaller cell size consistent with apoptosis.

**Fig. S3.** Dose dependence of pexiganan-induced breakdown in mitochondrial membrane potential of *Leishmania* lines. Indicated cell lines were treated with increasing amounts of pexiganan (from 6.25 to 25 μM, as indicated), stained with rhodamine 123 and then analysed for alterations in mitochondrial membrane potential using flow cytometry to measure increasing fluorescence. The KO line showed a progressive decrease in the accumulation of the dye intracellularly with when treated with increasing amounts of peptide. This was in contrast to the lack of changes in fluorescence of leishmanolysin-expressing lines treated with the same concentrations of peptide. All cell lines treated with 7.5 mM of the protonophore carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP, as indicated) demonstrated a breakdown in mitochondrial breakdown. The results shown are a representative of three experiments which revealed similar results.

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