The Apoptogenic Toxin AIP56 Is a Metalloprotease A-B Toxin that Cleaves NF-κB P65

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Abstract

AIP56 (apoptosis-inducing protein of 56 kDa) is a major virulence factor of Photobacterium damselae piscicida (Phdp), a Gram-negative pathogen that causes septicemic infections, which are among the most threatening diseases in mariculture. The toxin triggers apoptosis of host macrophages and neutrophils through a process that, in vivo, culminates with secondary necrosis of the apoptotic cells contributing to the necrotic lesions observed in the diseased animals. Here, we show that AIP56 is a NF-κB p65-cleaving zinc-metalloprotease whose catalytic activity is required for the apoptogenic effect. Most of the bacterial effectors known to target NF-κB are type III secreted effectors. In contrast, we demonstrate that AIP56 is an A-B toxin capable of acting at distance, without requiring contact of the bacteria with the target cell. We also show that the N-terminal domain cleaves NF-κB at the Cys29-Glu60 peptide bond and that the C-terminal domain is involved in binding and internalization into the cytosol.

Introduction

The NF-κB family of transcription factors is evolutionarily conserved and comprises five members (NF-κB 1 (p50), NF-κB 2 (p52), RelA (p65), RelB and cRel), which form different combinations of homo- or heterodimers [1]. Under normal physiological conditions, NF-κB complexes remain inactive in the cytosol through association with the IkB proteins that mask the nuclear localization domains on NF-κB subunits. A variety of stimuli, including bacterial and viral products and cytokines, acting via cellular receptors such as Toll-like receptors (TLRs), Interleukin-1 receptor (IL-1R) and TNF receptors (TNFRs), trigger a signalling cascade that leads to phosphorylation and degradation of the inhibitory IkB proteins with rapid activation and transport of the NF-κB complexes to the nucleus, resulting in the up-regulation of inflammatory and anti-apoptotic genes [2].

NF-κB activation is considered to be the central initiating event of host responses to microbial pathogen invasion [2]. Therefore, it is not surprising that successful microbial pathogens have evolved complex strategies to interfere with NF-κB signalling. A number of pathogenic bacteria were recently found to interfere with this pathway by targeting different intermediates of the NF-κB activation cascade [2–4].

Photobacterium damselae piscicida (Phdp) is a Gram-negative bacterium that infects several warm water fish species worldwide and is recognized as one of the most threatening pathogens in mariculture [5–8]. In acute infections, a rapid septicemia develops and is recognized as one of the most threatening pathogens in mariculture [5–8]. This leads to the impairment of host immune defences and to the release of the cytotoxic contents of the phagocytes, contributing to the formation of the necrotic lesions observed in the diseased animals.

We have previously shown that phagocyte apoptosis observed in Phdp infections results from the activity of AIP56, a plasmid-encoded exotoxin secreted by virulent strains, and that passive...
immunization with anti-AIP56 rabbit serum protects against Phdp infection [17,21]. These results implicated AIP56 as a key virulence factor of Phdp. However, the molecular target(s) of the toxin remained unidentified and nothing was known about its structure-function relationship.

AIP56 is synthesized as a precursor protein with a cleavable N-terminal signal peptide that is removed during secretion, originating a 497-amino acid mature toxin with the conserved HEIVH zinc-binding motif within its N-terminal region [21], similarly to tetanus neurotoxin [22]. The N-terminal region of AIP56 is homologous to NleC [21,23], a type III secreted effector present in several enteric pathogenic bacteria, while the C-terminal region is highly similar to an uncharacterized hypothetical protein of Acrythosiphon pisum bacteriophage APSE-2 [24] and to the C-terminal portion of a hypothetical protein of the monarch butterfly Danaus plexippus (Figure S1). This suggested that AIP56 is a two domain protein, belonging to the group of A-B type toxins that includes diphtheria and tetanus toxins [23,25,26].

Recently, it was shown that NleC inhibits NF-κB activation and represses NF-κB-dependent transcription by cleaving NF-κB p65 within its N-terminal region [27–31].

Here, we show that AIP56 is a zinc-metalloprotease that cleaves NF-κB p65 and that its enzymatic and apoptogenic activities are correlated. In contrast to NleC, which is delivered into the host cell’s cytosol through a type III secretion system, AIP56 is an A-B type exotoxin with an N-terminal domain responsible for the proteolytic activity and a C-terminal domain involved in binding and internalisation into target cells.

### Results

The metalloprotease signature of AIP56 is essential for its apoptogenic activity

In order to clarify the role played by the zinc metalloprotease activity of AIP56, a mutant (AIP56AAIVAA) containing a disrupted putative zinc-binding motif was produced. The oligomerization state and secondary structure content of the toxin were undisturbed by the introduced mutations (Figure S2) and atomic absorption spectroscopy did not detect zinc in AIP56AAIVAA, while in AIP56 equimolar amounts of zinc (0.93±0.04 mol zinc/mol protein) were present. When tested ex vivo, AIP56AAIVAA failed to induce apoptosis of sea bass phagocytes, whilst a large number of cells with apoptotic morphology were observed after treatment with AIP56 (Figure 1A). These results indicate that an intact metalloprotease domain is essential for the apoptogenic activity of AIP56.

It is worth noting that the AIP56 concentrations used in the present work are biologically relevant, since they are similar to those detected in the plasma of infected fish (Figure S3).

AIP56 is a zinc dependent metalloprotease that cleaves NF-κB p65 at the Cys39-Glu40 peptide bond

When incubated with sea bass cell lysates, AIP56 cleaved p65 with the appearance of a lower MW fragment (Figure 1B). Proteolysis of p65 did not occur in cell lysates incubated with AIP56AAIVAA or with AIP56 in the presence of the metalloprotease inhibitor 1,10-phenanthroline (Figure 1B). The p65 fragment was recognised by an antibody specific for a peptide located at the C-terminal region of p65 indicating that the AIP56-mediated p65 cleavage occurred within the N-terminal region, where the Rel-homology domain is located. To map the cleavage site, recombinant sea bass p65Rel domain (sbp65Rel) was incubated with the toxin. SDS-PAGE analysis showed that AIP56 cleaved recombinant sbp65Rel in vitro (Figure 1C), and N-terminal sequencing of the cleaved fragment revealed that the cleavage occurred at the Cys39-Glu40 peptide bond, similar to what was described for NleC [27]. Experiments using in vitro synthesised 35S-labeled sea bass p65Rel domain (sbp65Rel) and three sbp65Rel mutants (sbp65RelC39A, sbp65RelE40A and sbp65CE39-40AA) showed that mutation of the evolutionarily conserved Cys39 had no effect on p65 cleavage by either AIP56 or NleC (Figure S4). However, mutation of the following Glu40 inhibited cleavage and double mutation of Cys39 and Glu40 completely abolished p65 proteolysis by AIP56 and NleC (Figure S4).

To determine if cellular intoxication by AIP56 involves cleavage of NF-κB p65, sea bass peritoneal leukocytes were incubated with wild type toxin or with AIP56AAIVAA mutant and p65 proteolysis assessed by Western bloting. Wild type AIP56 caused NF-κB p65 depletion, whilst AIP56AAIVAA was inactive (Figure 1D). It has been reported that caspase-3 can cleave p65 [32,33]. To investigate whether caspases are involved in AIP56-dependent cleavage of p65, cells were incubated with the toxin in the presence or absence of the pan-caspase inhibitor ZVAD-FMK (Figure 1E), previously shown to block AIP56-induced apoptosis [19]. In these experiments, ZVAD-FMK was effective in protecting cells from AIP56-induced apoptosis (data not shown), but did not affect NF-κB p65 cleavage (Figure 1E), indicating that AIP56-mediated p65 depletion is a caspase-independent event. Taken together, the above results demonstrate that the metalloprotease activity of AIP56 is responsible for the cleavage of NF-κB p65 at the Cys39-Glu40 peptide bond.

AIP56 has two domains

The primary structure of AIP56 suggests that this toxin comprises two functional domains and could be an A-B toxin with its two moieties linked by a single disulphide bond (Figure S1) [23]. Therefore, in order to define domain boundaries within the toxin, limited proteolysis experiments were performed. SDS-PAGE analysis of AIP56 digested with chymotrypsin, trypsin or proteinase K revealed that the toxin is highly resistant to trypsin digestion, whereas chymotrypsin and proteinase K cleaved AIP56 into two major fragments with approximately 32 and 24 kDa (Figure 2A). These two fragments were only detected upon treatment with the reducing agent DTT, suggesting that they are linked by a disulphide bridge (Figure 2B). N-terminal Edman
Figure 1. AIP56 is a zinc-metalloprotease that cleaves NF-κB p65 at the Cys39-Glu40 peptide bond. (A) Disruption of the zinc-metalloprotease signature abolishes AIP56 apoptogenic activity. Sea bass peritoneal leukocytes collected from 5 animals were incubated with AIP56 or AIP56AAAVAA, for 4 h at 22°C. Mock-treated cells were used as controls. Images shown are representative cytospin preparations stained with Antonow’s for labelling neutrophils (brown) followed by Hemacolor. Note the presence of several apoptotic cells (arrowheads) in the sample incubated with AIP56 and their absence in cells incubated with AIP56AAAVAA and in mock-treated cells. The percentage of apoptotic phagocytes, determined by morphological analysis, is depicted in the dot plot. (B) Incubation of cell lysates with AIP56, AIP561-285C262S and nicked AIP56 resulted in p65 cleavage. Lysates were incubated for 2 h at 22°C with 1 μM of the indicated proteins in the presence or absence of the metalloprotease inhibitor 1,10-phenanthroline (O-phe) and p65 cleavage assessed by Western blotting. (C) AIP56 cleaves NF-κB p65 at the Cys39-Glu40 peptide bond. Recombinant sea bass p65Rel (7.5 μM) was incubated in the presence or absence of 1 μM of AIP56 for 3 h at 22°C and analysed by SDS-PAGE. Edman degradation of the cleaved sbp65Rel (cl-sbp65Rel) identified the sequence E40GRSA44 showing that cleavage occurred after the conserved C39. (D) Incubation of leukocytes with AIP56, but not with AIP56AAAVAA, AIP561-285C262S, AIP561-491C485, nicked AIP56 (AIP56nic) or reconstituted AIP56 (AIP56rcit) leads to p65 depletion. Leukocytes were incubated with 10 μg/ml of the indicated proteins for 2 h at 22°C and p65 cleavage was assessed.
sequencing revealed that chymotrypsin cleavage occurred between Phe^{285} and Phe^{286}, in the amino-acid stretch flanked by the two unique cysteine residues (Cys^{262} and Cys^{298}) of AIP56 (Figure 2C). Altogether, these results indicate that AIP56 is composed of two domains linked by a disulphide bridge.

The N-terminal domain is responsible for the catalytic activity and the C-terminal domain is implicated in binding to target cells

To better understand the function of the two AIP56 domains, constructs corresponding to the N- and C-terminal portions of the toxin (AIP56^{1-285} and AIP56^{286-497}, respectively) were designed, taking into account the boundary defined by the chymotrypsin cleavage site (Figure 2C). Purification of these two recombinant proteins using the experimental conditions used for the full-length toxin revealed that they display a major propensity to oxidize leading to the formation of DTT-sensitive dimers (Figure S5A), a phenomenon that could have a functional impact and complicate subsequent analyses. Therefore, versions of the constructs with the single cysteine replaced by serines (AIP56^{1-285}C262S and AIP56^{286-497}C298S, respectively) were produced. The mutants are indistinguishable from the non-mutant proteins, as assessed by CD (Figure S5B and S5C) with the N-terminal domain composed mainly of α-helices, whereas β-sheet is the predominant secondary structure of the C-terminal moiety (Figure 2D). Furthermore, the weighted sum of the CD spectra of the N- and C-terminal domains reproduces the spectrum of the entire protein (Figure 2D), indicating conservation of the native structure.

To test the catalytic activities of the AIP56 N- and C-terminal domains, AIP56^{1-253}C262S and AIP56^{286-497}C298S were incubated with fish leukocyte lysates (Figure 1B) or with in vitro translated ^35S-labeled αb55Rel (Figure S5D). The C-terminal construct did not display catalytic activity, whereas the N-terminal domain cleaved p65, similarly to the full-length toxin. However, neither changes in cellular p65 levels (Figure 1D) nor apoptosis (Figure 3A) were observed in sea bass leukocytes incubated with the N- or C-terminal truncate or with a mixture of both. This indicates that the two AIP56 domains are non-toxic and suggests that they need to be part of the same molecule to elicit a biological effect.

The cytosolic location of NF-κB p65 could mean that the lack of toxicity of the N-terminal domain was related to its inability to enter the cells and reach its target. Hence, a strategy to deliver the N-terminal domain into the cell cytosol was designed. Chimeric proteins consisting of the N-terminal portion of Bacillus anthracis LF fused to the AIP56 protease domain (LF^{11-263}AIP56^{1-261}) or to the C-terminal domain (LF^{11-263}AIP56^{269-495}) were produced. Intoxication assays were performed in the presence of PA, the receptor-binding subunit for LF [34]. In cells incubated with LF^{11-263}AIP56^{1-261}, the p65 levels were significantly reduced, confirming that LF^{11-263}AIP56^{1-261} was successfully delivered into the cell cytosol, while no changes in p65 levels were observed in cells incubated with LF^{11-262}AIP56^{269-495} (Figure 3B). Accordingly, LF^{11-263}AIP56^{299-495} did not display apoptogenic activity, while incubation with LF^{11-263}AIP56^{261} resulted in an increased number of cells with apoptotic morphology (Figure 3B), similar to what was observed in cells incubated with AIP56. Thus, delivery of the AIP56 N-terminal domain into the cytosol reproduces the toxic effect of the full length toxin, confirming that this domain is responsible for the toxin’s catalytic and apoptogenic activities. These results also suggest that the C-terminal domain of AIP56 is involved/required for entrance of the toxin into cells. To investigate this possibility, AIP56^{286-497}AAIVAA, AIP56^{1-285}AAIVAA, or AIP56^{286-497}C262S were used in competition experiments with AIP56. Both p65 cleavage and apoptosis were monitored in these experiments. AIP56^{286-497}AAIVAA and AIP56^{286-497}C298S inhibited AIP56-mediated p65 degradation, whereas no effect could be observed when AIP56^{1-285}AAIVAA was used as competitor (Figure S3, right panel). These results indicate that the C-terminal domain mediates binding of the toxin to the cell surface and entry into the cells.

Translocation of AIP56 requires integrity of the Cys^{262}, Cys^{298} linker but the disulphide bridge is not an absolute requirement for toxicity

Results obtained in experiments using N- and C-terminal truncates of AIP56 suggested that the two domains must be part of the same molecule to display toxicity. In order to investigate if the two domains of the toxin bound by a disulphide bridge are able to intoxicate cells, we nicked the toxin with chymotrypsin. Nicking of the toxin and integrity of the disulphide bridge linking the two fragments were confirmed by reducing and non-reducing SDS-PAGE (Figure S6A). Surprisingly, no changes in p65 cellular levels (Figure 1D) and no apoptosis (Figure 4A) were observed upon incubation of sea bass peritoneal cells with nicked toxin. Similar results were obtained using a reconstituted version of the toxin (Figure 1D and 4A) consisting of disulfide-bound AIP56^{1-285}/AIP56^{286-497} along with trace amounts of AIP56^{1-285} and of AIP56^{286-497} homodimers and monomers (Figure S6A). Although nicking abolished cellular toxicity, it did not induce major structural changes (Figure S6B) and only a 1°C decrease in Tm (39.0 ± 0.1°C for AIP56 and 38.2 ± 0.2°C for nicked AIP56; mean ± SD of 16 measurements in four independent experiments) was measured by DSC. More importantly, nicked AIP56 retained both proteolytic activity against p65 in vitro (Figure 1B and 4B) and cell binding ability, as indicated by the partial inhibition of the AIP56-mediated p65 cleavage and apoptosis in competition experiments (Figure 4C). These results suggest that the integrity of the linker region between the two cysteine residues is needed for toxin internalization, in contrast to what is known for the diphtheria, tetanus and botulinum toxins, where nicking of the inter-cysteine loop is required for toxicity [26,35].

In tetanus and botulinum neurotoxin type A, it has been shown that the disulphide bridge is essential for neurotoxicity [36,37]. We found that disruption of the disulphide bridge linking Cys^{262} and Cys^{298} of AIP56 by alkylation (Figure S6D) did not affect the catalytic activity of the toxin in vitro (Figure 4B), but partially compromised its toxicity (Figure 4D), suggesting that in AIP56 the disulphide bridge plays a role in the intoxication process but is not an absolute requirement for toxicity.

Discussion

In this study, we report the structural and functional characterization of AIP56 as an A-B type bacterial exotoxin that cleaves by Western blotting. (E) AIP56-mediated p65 cleavage is caspase-independent. Leukocytes were incubated with 2 μg/ml AIP56 in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK for 2 h at 22°C, and p65 cleavage was assessed by Western blotting. Numbers to the left of the panels refer to the position and mass of the molecular weight markers, in kDa.

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Considering the anti-apoptotic functions of NF-κB, and in particular, of its p65 subunit [39–40], AIP56-mediated depletion of NF-κB p65 likely explains the disseminated phagocyte apoptosis observed in Phdp infections that contributes to subvert the host immune response and determines the outcome of the infection [17,19,21]. This adds to a general theme of host-pathogen interaction that has recently emerged, consisting in the induction of apoptosis of the host immune cells to the pathogen advantage [41–43].

Here we demonstrate that, similar to the anthrax lethal factor and to the clostridial neurotoxins [44,45], AIP56 is a zinc-endopeptidase but with a catalytic activity towards NF-κB p65. Furthermore, we show that AIP56 is organized into two distinct domains linked by a single disulphide bond. The N-terminal domain of AIP56 harbours the catalytic activity of the toxin and cleaves NF-κB p65 at the Cys39-Glu40 peptide bond, within the p65 N-terminal Rel homology domain, where several key residues of p65 known to be involved in DNA interaction are located [46–48]. In the last decade, several reports revealed that Cys38 of human p65 (Cys39 in sea bass p65) interacts with the phosphate backbone of NF-κB binding sites [46], that its oxidation and nitrosylation inhibit DNA binding [49] and that it is targeted by several inhibitors of NF-κB with anti-inflammatory and/or anticancer properties [50–60]. More recently, it was shown that hydrogen sulphide-linked sulfhydration of Cys38 of human p65 plays a key role in regulating the anti-apoptotic actions of NF-κB [40]. Therefore, cleavage of sea bass p65 by AIP56 disrupts a segment crucial for DNA interaction. Considering that the proteolytic activity of AIP56 towards p65 is similar to the one previously described for NleC (both proteases cleave p65 at the same peptide bond), and based on the observation that p65 cleavage by NleC compromises NF-κB dependent transcription.

**Figure 2.** AIP56 is composed of two domains linked by a disulphide bridge. (A) Limited proteolysis of AIP56 with chymotrypsin and proteinase K produces two major fragments. AIP56 (0.6 mg/ml) was incubated with 0.25, 1.25, 6.25 or 25 μg/ml of chymotrypsin, trypsin and proteinase K for 30 min on ice and digests analysed by reducing SDS-PAGE. The proteases (marked as *) and undigested AIP56 (marked as **) were loaded as controls. (B) The two AIP56 digestion fragments are linked by a disulphide bridge. AIP56 was incubated with or without 25 μg/ml chymotrypsin (Chym) for 30 min on ice and digests analysed under reducing (+DTT) or non-reducing (−DTT) SDS-PAGE. Numbers to the left and right of the panels refer to the position and mass of the molecular weight markers, in kDa. (C) Schematic representation of AIP56. (D) Far-UV CD spectra of AIP56 (thick solid line), AIP561–285 (thin solid line), AIP56286–497 (thin dashed line) and the weighted sum of AIP561–285 and AIP56286–497 spectra (thick dashed line).

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Cys298) is involved in translocating the toxin into the host cell. Pseudomonas [79]. Alternatively, after endocytosis, unprocessed AIP56 may be intoxicating the cells, similarly to what was described for other toxins. A-B toxins may result from a very small amount of processed in the bacterial culture supernatant or in the serum of infected fish [21]. Furthermore, despite several attempts, we were unable to detect proteolytic processing of AIP56 upon its interaction with host cells. If AIP56 needs to be processed in order to exert its effect, the lack of detection of processed toxin may result from a very small amount of processed not detectable in our experiments) being sufficient to intoxicate the cells, similarly to what was described for other toxins. Alternatively, after endocytosis, unprocessed AIP56 may be translocated into the cytosol as described for Pseudomonas exotoxin A [80,81] or may localize in an endosome membrane (e.g. endosomal membrane) with the catalytic domain facing the cytosolic compartment where it can interact with and cleave p65. Studies aiming at discriminating between these hypotheses will be developed in the future.

It is now recognised that horizontal transfer of entire genes or portion of genes plays a key role in generating diversity in pathogens by allowing them to acquire novel phenotypic characteristics. Indeed, there are several examples of bacterial genes with a mosaic structure, composed of diverse segments with different origins [82–87]. The structure of AIP56 suggests that the toxin has a chimeric structure, having an N-terminal catalytic domain highly identical to the type III effector NleC and a C-terminal domain homologous to a hypothetical protein of the bacteriophage APS-2. The actual transfer events that gave rise to such a chimeric protein toxin remain to be disclosed.

The AIP56 catalytic domain and NleC have the same NF-κB p65 cleavage activity. However, NleC requires a type III secretion machinery for activity, while AIP56 has an intrinsic ability to reach the cytosol, due to the presence of the additional C-terminal domain that functions as a “delivery module”. This difference may have relevant implications when considering the use of both pathogen-derived molecules as therapeutic agents in situations associated with uncontrolled activation of NF-κB such as inflammatory diseases and cancer.

**Materials and Methods**

Additional details can be found in Supporting Information.

**Ethics statement**

This study was carried out in accordance with European and Portuguese legislation for the use of animals for scientific purposes (Directive 86/609/EEC; Decreto-Lei 129/92; Portaria 1005/92). The work was approved by Direcção Geral de Veterinária, the Portuguese authority for animal protection (ref. 004933, 2011-02-22).

**Fish**

Sea bass (Dicentrarchus labrax), were kept in a recirculating, ozone-treated salt-water (25–30‰) system at 20±1°C, and fed at a ratio of 2% body weight per day. Fish were euthanized with 2-phenoxethanol (Pantac; >5 ml/10 L).

**Production and purification of recombinant proteins**

DNA coding sequences were cloned into Ncol/Xhol restriction sites of pET-28a (+) (Novagen) as described in Supporting Information. Mutants were generated by site directed mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Stratagene) following manufacturer’s instructions. Recombinant His-tagged proteins were expressed in E. coli BL21(DE3) cells. AIP56, AIP561-285, AIP56286-497, AIP56286-497C298S, NleC, LF1-264, AIP561-261 and LF1-264AIP56299-497 were purified from the soluble fraction of induced bacteria by metal-affinity chromatography. After this step, AIP56, AIP561-285, AIP56286-497 were subjected to anion exchange chromatography, whereas AIP56286-497C298S and NleC were subjected to size exclusion chromatography. AIP56AAVa1 and AIP561-285C298S were purified from inclusion bodies by metal-affinity chromatography under denaturing conditions, refolded by dialysis against sea bass PBS (sbPBS; phosphate buffer saline with osmotic strength adjusted to 322 mOsm) with 10% (v/v) glycerol and purified by size exclusion chromatography. For reconstitution of AIP56, AIP561-285 and AIP56286-497 were mixed in equimolar amounts in 8 M urea, 1 mM DTT and refolded by extensive dialysis against sbPBS. Nicked AIP56 was obtained by limited proteolysis with 25 μg/ml chymotrypsin, as described below, followed by metal-affinity chromatography purification. To prepare alkylated toxin, 65 μM AIP56 in 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM DTT was incubated with 5 mM iodoacetamide (Sigma) for 30 min at RT and dialysed against 20 mM Tris-HCl pH 8.0, 200 mM NaCl.
Figure 3. AIP56 N-terminal domain plays the catalytic role and the C-terminal domain is involved in binding and entry into cells. (A) AIP56[1–285]C262S and AIP56[286–497]C298S lack apoptogenic activity. Leukocytes collected from 5 animals were incubated with AIP56, AIP56[1–285]C262S, AIP56[286–497]C298S, or a mixture of AIP56[1–285]C262S and AIP56[286–497]C298S (50 μg/ml each) for 4 h at 22 °C. The percentage of apoptotic phagocytes was determined by morphological analysis of cytospin preparations stained with Hemacolor. (B) Delivery of AIP56 N-terminal domain into the cell’s cytosol using B. anthracis LF/PA system reproduces the activity of full length AIP56. Leukocytes from 4 animals were incubated for 4 h at 22 °C with 20 nM LF[11–263] and AIP56[1–261] or LF[11–263] and AIP56[299–497] in the presence of 10 nM PA. Cells incubated with 2 μg/ml (35 nM) AIP56 were used as positive control. Cleavage of NF-κB p65 was detected by Western blotting and the occurrence of apoptosis by morphological analysis of cytospin preparations stained with Hemacolor. Note the presence of several apoptotic cells in the samples incubated with PA+LF[11–263] and AIP56[1–261]. (C) AIP56 C-terminal domain is involved in toxin binding and entry into the target cells. AIP56 AAIVAA and AIP56[286–497]C298S, but not AIP56[1–285]C262S, inhibit AIP56-associated p65 cleavage and apoptogenic activity. Leukocytes collected from 7 fish were incubated with AIP56[1–285]C262S, AIP56[286–497]C298S, or AIP56 AAIVAA at final concentrations of 0.35, 1.75 or 3.5 μM for 15 min on ice, followed by further 15 min incubation on ice with 8.75 nM (0.5 μg/ml) AIP56 in the presence of the competitors. The competitor:AIP56 molar ratios are indicated. Cells incubated with AIP56 in the absence of competitors or with 3.5 μM of each competitor alone were used as controls. Cells were washed, transferred to 22 °C and incubated for 4 h prior to determination of the percentage of apoptotic cells by morphological analysis of cytospin preparations stained with Hemacolor. Left panel presents the box plot of percentage of apoptotic cells (the middle bar corresponds to the median and the lower and upper side of the boxes, the first and third quartiles; circles signal extreme observations). The inhibitory effect of the highest dose of each competitor upon AIP56-mediated cleavage of p65 was assessed by Western blotting.

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Figure 4. AIP56 toxicity requires integrity of the linker but the disulfide bridge is dispensable for intoxication. (A) Nicked and reconstituted AIP56 are not apoptogenic. Leukocytes collected from 3 animals were incubated with nicked or reconstituted AIP56 (AIP56nic and AIP56rct, respectively) for 4 h at 22 °C and the percentage of apoptotic cells determined by morphological analysis of cytospin preparations stained with Hemacolor. Cells treated with AIP56 and mock-treated cells were used as positive and negative controls, respectively. (B) Nicked and alkylated AIP56 (AIP56alk) display proteolytic activity in vitro in the same dose range as AIP56. 35S-labeled sbp65Rel was incubated for 2 h at 22 °C with wild type, nicked or alkylated AIP56 and cleavage assessed by autoradiography. (C) Nicked AIP56 competes with intact AIP56 and inhibits its toxicity. Leukocytes collected from 3 animals were incubated with 3.5 μM nicked AIP56 (AIP56nic) for 15 minutes on ice followed by further 15 min incubation on ice with 8.75 nM of AIP56. Mock-treated cells, cells incubated with 8.75 nM AIP56, or cells incubated with 3.5 μM of AIP56AAIVAA, AIP561–285 or AIP56286–497 before incubation with 8.75 nM of AIP56 were used as controls. Cells were washed, transferred to 22 °C and incubated for 4 h. The percentage of apoptotic cells was determined by morphological analysis of cytospin preparations stained with Hemacolor and the p65 cleavage was assessed by Western blotting. (D) Disruption of the disulphide bridge linking Cys262 and Cys298 partially compromises AIP56 toxicity. Leukocytes collected from 5 animals were incubated with AIP56 or AIP56alk for 4 h at 22 °C and the percentage of apoptotic cells determined by morphological analysis of cytospin preparations stained with Hemacolor. Left panel presents the box plot of percentage of apoptotic cells (the middle
Sea bass NF-κB p65 REL homology domain (sbp65Rel) was purified from the soluble fraction of induced bacteria by metal-affinity chromatography. Untagged 35S-labeled sbp65Rel and sbp65Rel mutants (sbp65RelC39A, sbp65RelE40A, and sbp65C39E40AA) were produced using the TNT T7 Quick Coupled transcription/Translation kit (Promega), in the presence of Redivue™ L-[35S] methionine (specific activity of 1000 Ci/mmol).

Limited proteolysis
AIP56 at 0.6 mg/ml in 10 mM Tris-HCl pH 8.0, 200 mM NaCl was incubated with 0.25 to 25 μg/ml trypsin, chymotrypsin or protease K (molar ratios of protease:AIP56 of approximately 1:10 to 1:1000) for 30 min on ice. Proteases were inactivated by addition of PMSF to a final concentration of 250 μM. Digests were analysed by reducing and non-reducing SDS-PAGE. The two major chymotrypsin digestion fragments were subjected to N-terminal sequencing.

Circular dichroism spectroscopy (CD)
Far UV CD spectra were acquired on an Olis DSM 20 circular dichroism spectropolarimeter controlled by the Globalworks software. The spectrum is the average of three scans collected at 20°C with a 0.2 mm path length cuvette and with an integration time of 4 seconds. Proteins were dissolved in 10 mM Tris-HCl, 50 mM NaCl, pH 8.0 and concentrations were determined by absorbance measurements. Analysis of the protein secondary structure was performed using the Globalworks software algorithm.

Cells
Sea bass peritoneal leukocytes were obtained as previously described [19] and used at a density of 2×10⁶ cells/ml. The peritoneal population of cells consists of approximately 70% macrophages and 20% neutrophils with the presence of small numbers of eosinophilic granular cells, lymphocytes and erythro-macrophages and 20% neutrophils with the presence of small populations of cells consists of approximately 70% macrophages and 20% neutrophils. The preparations were centrifuged at 1200×g for 5 min, washed twice then resuspended in PBS to a concentration of 5×10⁶ cells/ml. The cells were pre-treated for 30 min at 22°C with 25 μM of the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK). In experiments using LF chimeric proteins, inhibitors N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) and 1,10-phenanthroline (Sigma) were used at 5 mM.

Ex vivo. peritoneal leukocytes were treated for 2 or 4 h as described above in the section “Apoptotic activity assays”, collected by centrifugation, washed, resuspended in sbPBS and lyzed by addition of SDS-PAGE sample buffer. Cleavage of p65 was evaluated by Western blotting, as described above.

Apoptogenic activity assays
Cells were incubated for 4 h at 22°C with AIP56 or AIP56 related proteins at indicated concentrations. Where indicated, the cells were pre-treated for 30 min at 22°C with 25 μM of the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK). In experiments using LF chimeric proteins, inhibitors N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) and 1,10-phenanthroline (Sigma) were used at 5 mM.

Statistical analysis
Statistical analysis was performed using a randomized block design, where fish are treated as blocks and the concentration of the treatments/competitors as a factor. The data, percentage of apoptotic cells, have been transformed using the arcsine transformation. Post-hoc comparisons were performed using the Tukey's Honest Significant Difference test. Significance was defined for p<0.05.

Supporting Information
Figure S1. Schematic diagram of the primary structure of AIP56 and AIP56-related proteins. Grey: signal peptides (experimentally determined for AIP56 [21] and predicted for the remaining proteins using SignalP at http://www.cbs.dtu.dk/services/SignalP/ [91,92]; Yellow: regions with high identity to NeC and AIP56 N-terminal catalytic domain; Green: regions with high identity to AIP56 linker polypeptide; Orange: regions with high identity to APSE-2 and AIP56 C-terminal domain; Red: zinc-metalloprotease signature HEXXH; White: regions with low identity to AIP56 domain, NeC or APSE-2. Conserved zinc-metalloprotease signature HEXXH, cysteine residues, and other signalling amino acids are represented at their relative positions. AIP56-related proteins were retrieved by Blast analysis of the AIP56 protein sequence against the non-redundant protein sequences database (updated from [23]).

Figure S2. Disruption of the zinc-metalloprotease signature does not induce major structural changes in AIP56. Native-PAGE (A)
and size exclusion chromatography (B) of AIP56 and AIP56\textsuperscript{AAIVAA} showing that disruption of the zinc-binding motif did not affect the monodispersity/stokes radius of the protein. In Native-PAGE, BSA electrophoretic mobility is shown for reference purposes. (C) Far-UV CD spectra of wild-type AIP56 (thick line) and AIP56\textsuperscript{AAIVAA} (thin line) showing that the secondary structure content of the toxin was also unaffected by the introduced mutations.

(TIF)

**Figure S3** The AIP56 concentration in the plasma of infected fish is in the same range as those used in the present work. (A) The presence of AIP56 in plastras (5 μl aliquots) from sea bass infected with a lethal dose of \textit{Pldp} strain PP3 was determined by Western blotting. Different concentrations of recombinant AIP56 (5 μl) were loaded as standards. Numbers at the left refer to the position and mass (in kDa) of the molecular weight markers. (B) Concentrations of AIP56 in the plasmas analysed in (A), determined by densitometry, using a recombinant AIP56 standard curve.

(TIF)

**Figure S4** The AIP56 cleavage-determining residues are evolutionarily conserved. (A) Mutations in Cys\textsuperscript{39} and Glu\textsuperscript{40} of sbp65Rel inhibit proteolytic activity by AIP56 and NleC. \textsuperscript{38}S-labeled sbp65Rel, sbp65RelC39A, sbp65RelE40A or sbp65CE39-40AA were incubated for 2 h at 22°C with 100 nM of the indicated proteins and cleavage assessed by autoradiography. (B) Alignment of the p65 N-terminal region from different species (NCBI accession numbers: \textit{Homo sapiens}, AAA36408; \textit{Mus musculus}, NP\_033071; \textit{Gallus gallus}, NP\_990460; \textit{Xenopus laevis}, AAH70711; \textit{Monodelphis domestica}, XP\_001379653; \textit{Danio rerio}, AAO26404). The residues mutated in (A) are shadowed grey.

(TIF)

**Figure S5** Structural and functional analysis of recombinant AIP56 N- and C-terminal domains. (A) Reducing and non-reducing SDS-PAGE of purified AIP56\textsuperscript{1–285} and AIP56\textsuperscript{286–497}. Numbers at the left refer to the position and mass (in kDa) of the molecular weight markers. (B) Far-UV CD spectra of AIP56\textsuperscript{1–285} (thick solid line) and AIP56\textsuperscript{286–497} (thin line) showing that the secondary structure content of the toxin is in the same range as those used in the present work. (C) Far-UV CD spectra of AIP56 1–285 and AIP56 286–497. AIP56 N- and C-terminal domains. (D) Reducing and non-reducing SDS-PAGE of AIP56 and alkylated AIP56 (AIP56alk). Numbers on the left of the panels indicate the mass of the molecular weight markers, in kDa.

(TIF)

**Materials and Methods S1** Additional details about the constructs used in this study, protein production and purification, protein quantification, PAGE and Western blotting, analysis of the zinc content, analytical size exclusion chromatography, circular dichroism spectroscopy (CD), differential scanning fluorimetry (DSF), determination of AIP56 concentration in the plasma of infected fish, are provided as Supporting Materials and Methods.

(DOCX)

**Table S1** Primers used in this study.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: DSS AdV JFA PJBP SMR. Performed the experiments: DSS LMGP FFdS ARM RMB TQF. Analyzed the data: DSS AdV NMSdS JEA PJBP SMR. Contributed reagents/materials/analysis tools: IZ CM. Wrote the paper: DSS AdV NMSdS.
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