

**Structural and functional characterization of AIP56,
an apoptogenic exotoxin of
Photobacterium damsela piscicida**

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an apoptogenic exotoxin of *Photobacterium damsela* piscicida.**

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LIST OF ABBREVIATIONS

ABIN1: A20-binding inhibitor of NF- κ B

ANTXR1: Tumour endothelial marker-8

ANTXR2: Capillary morphogenesis protein 2

APCs: Antigen presenting cells

BAFF: B-cell activating factor

BAFFR: BAFF receptor

BoNT: Botulinum neurotoxins

cAMP: Cyclic adenosine monophosphate

CD: Circular dichroism

CDCs: cholesterol dependent cytolysins

cGMP: Cyclic guanosine monophosphate

cIAP: Cellular inhibitor of apoptosis protein

ClyA: Cytolysin A

COPI: Coatamer protein complex I

CT: Cholera toxin

DAG: Diacylglycerol

DD: Dimerization domain

DLS: Dynamic light scattering

DSF: Differential scanning fluorimetry

DT: Diphtheria toxin

EF: Edema factor

EF-2: Elongation factor 2

ER: Endoplasmic reticulum

ERAD: ER-associated protein degradation

ERK: Extracellular-signal-regulated kinase

GTP: Guanosine-5'-triphosphate

IKK: I κ B kinase

IL: Interleukin

IL-1R: Interleukin-1 receptor

IP3: Inositol triphosphate

IκBs: Inhibitors of NF-κB

JNK: c-Jun N-terminal kinase

LF: Lethal factor

LPA: Lysophosphatidic acid

LT: Heat-labile enterotoxins

LTβ: Lymphotoxin β

LTβR: Lymphotoxin β receptor

MAPK: Mitogen activated protein kinase

MHC-II: Major Histocompatibility Complex class II

MYD88: Myeloid differentiation primary response gene 88

NF-κB: Nuclear factor-κB

NIK: NF-κB-inducing kinase

NLS: Nuclear localization signal

NOD: Nucleotide oligomerization domain

NTD: N-terminal domain

PA: Protective antigen

PAMPs: Pathogen-associated molecular patterns

PDB: Protein data bank

PDI: Protein disulphide isomerase

PE: *Pseudomonas* exotoxin A

PFTs: Pore-forming toxins

PhA: Phosphatidic acid

PIP2: Phosphatidylinositol 4,5-bisphosphate

PK: Protein kinase

PRRs: Pattern-recognition receptors

PT: Pertussis toxin

RHD: Rel homology domain

RIPs: Receptor interacting proteins

ROS: Reactive oxygen species

RSP3: Ribosomal protein S3

SAGs: Superantigen toxins

sbPBS: Sea bass phosphate saline buffer

SNARE: Soluble N-ethylmaleimide-sensitive factor activating protein receptor

STs: Heat-stable toxins

Stx: Shiga toxin

SUMO-1: Small Ubiquitin-like modifier protein 1

TAB: TAK-1 binding protein

TAD: Transactivation domain

TAK1: Transforming growth factor β -activated protein kinase 1

TCR: T-cell receptors

TeNT: Tetanus neurotoxin

TIRAP: Toll-interleukin 1 receptor domain-containing adaptor proteins

TlpA: TIR-like protein A

TLRs: Toll-like receptors

T_m: Melting temperature

TNF: Tumour necrosis factor

TNFRs: TNF receptors

TRAF: TNF-receptor associated factor

TRAIL: TNF-related apoptosis-inducing ligand

ABSTRACT

Bacterial exotoxins are amongst the principal virulence factors of pathogenic bacteria and usually contribute for the majority of symptoms and lesions caused by infection. The AIP56 toxin (apoptosis-inducing protein of 56 kDa) is a major virulence factor of *Photobacterium damsela piscicida* (*Phdp*), a Gram-negative bacterium causing a septicemic infection that is one of the most threatening diseases in mariculture. During infection, the toxin is secreted by *Phdp* and is systemically disseminated, triggering apoptosis of macrophages and neutrophils. The destruction of both professional phagocytes of the host allows bacterial evasion from phagocytosis, promoting bacterial septicemic spread. This process culminates in cell lysis by secondary necrosis with release of the phagocyte's cytotoxic contents, thus contributing to the necrotic lesions observed in the diseased animals.

Despite the toxic effects of AIP56 and their contribution to the pathology of *Phdp* infections are well known, knowledge about the toxin's structure-function relationships and the molecular determinants beyond its biologic activity was still missing.

The work herein presented shows that AIP56 is a zinc-metalloprotease that cleaves NF- κ B p65 at Cys³⁹, an evolutionarily conserved residue of the Rel homology domain, removing crucial residues involved in DNA interaction and compromising the NF- κ B activity. Cleavage was observed upon incubation of sea bass cell lysates or recombinant p65 Rel homology domain with AIP56 and was dependent on the metalloprotease signature of the toxin. Furthermore, the proteolytic activity towards NF- κ B p65 was found to be essential for toxicity, since mutations within the toxin's metalloprotease signature completely abolished its apoptogenic activity. Given the central role of the NF- κ B family of transcription factors in the host responses to microbial pathogen invasion (particularly regulating the expression of inflammatory and anti-apoptotic genes) it is not surprising that depletion of NF- κ B p65 is involved in the disseminated apoptosis observed in *Phdp* infections.

Bacterial toxins acting on intracellular targets enter cells through a multistep process involving specific binding to the cell surface, internalization via receptor-mediated endocytosis and translocation into the cytosol from an intracellular compartment. The structure of those toxins is adapted to their entry mechanisms and, to this end, these toxins are organized in a so-called AB-type structure consisting of a catalytic A subunit bound to a cell interacting B subunit(s) that mediate toxin binding to a cell receptor and delivery of the catalytic A subunit into the cytosol. Homology

analysis of AIP56 primary structure provided the first evidences of a two-domain/subunit structural organization. By performing limited proteolysis assays together with *in silico* secondary structure analysis we confirmed that AIP56 is organized in two structural protease-resistant domains held together by an unstructured flexible region containing two cysteine residues, involved in a disulphide bond. Furthermore, by using truncated versions of the toxin, designed based on the chymotrypsin cleavage site, we disclosed the role played by the two identified structural domains in AIP56's intoxication mechanism. The recombinant AIP56 N-terminal portion, containing the zinc-binding signature, exhibited proteolytic activity *in vitro* towards NF- κ B p65. In addition, delivery of the N-terminal region into the cell cytosol using the *Bacillus anthracis* PA/LF system reproduced the proteolytic and apoptogenic activities of the full length toxin, attributing to the N-terminal region the catalytic role. In contrast, the C-terminal portion of AIP56 exhibited no proteolytic activity *in vivo* or *in vitro* but, when used as a competitor, it efficiently prevented AIP56 toxic effects, demonstrating the involvement of the C-terminal region in toxin binding/entry into target cells.

Despite for AIP56 the boundaries of the A and B domains were not yet defined, its structural architecture adds the toxin to the family of the AB toxins alongside with diphtheria toxin, botulinum and tetanus neurotoxins and *Pseudomonas* exotoxin A. However, we found that AIP56 differs from those toxins in some structural features required for cell intoxication. AIP56 does not require proteolytic or any other kind of activation to display enzymatic activity *in vitro*. In addition, integrity of the region between the two cysteine residues (Cys²⁷⁸ and Cys³¹⁴), linking the two functional subunits, appears to be a requirement for toxicity since nicking of AIP56 at this region rendered the toxin inactive. These findings suggest that this region plays a role in the translocation of the toxin into the cell's cytosol and may be part of the yet unidentified translocation domain.

The catalytic region of AIP56 has homology with NleC, a type III secreted effector of enteropathogenic bacteria that has also been shown to target NF- κ B p65. Using CD spectroscopy and *in silico* secondary structure predictions we showed that these proteins are highly similar in terms of secondary structure. Still, even though AIP56 and NleC share structural and functional homology, AIP56 N-terminal region and NleC were found not to be cross compatible since substitution of AIP56's N-terminus by NleC resulted in a non-toxic chimera and the *Bacillus anthracis* PA/LF system also failed to deliver NleC into the cytoplasm of cells. Differential scanning fluorimetry analysis revealed that AIP56 catalytic region and NleC have different unfolding properties in response to pH, likely explaining their distinct behaviour.

The intrinsic abilities of AB toxins to enter target cells make these proteins attractive not only as tools in cell biology, to study cellular processes, but also as systems to deliver protein cargos inside targeted cells. This, associated to the extensive involvement of NF- κ B transcription factors in inflammation and cancer diseases establishes AIP56 has a promising therapeutic/biotechnological tool. Nevertheless, in depth knowledge on the toxin's structural features, intracellular pathways and molecular mechanisms of action are crucial before considering the use of AIP56 to these ends.

RESUMO

As exotoxinas bacterianas estão entre os principais factores de virulência das bactérias patogénicas e, frequentemente, são responsáveis pela maioria dos sintomas e lesões decorrentes da infecção.

A exotoxina AIP56 é um factor de virulência fundamental da *Photobacterium damsela piscicida* (*Phdp*), uma bactéria Gram-negativa causadora de uma septicemia que afecta várias espécies de peixes marinhos economicamente importantes. A toxina é secretada pela *Phdp* durante a infecção, dissemina-se sistemicamente e induz a apoptose de macrófagos e neutrófilos. A destruição simultânea dos dois tipos de fagócitos, induzida pela toxina, compromete a defesa imunológica do hospedeiro, promovendo a proliferação e distribuição sistémica da bactéria. Para além disso, a destruição dos macrófagos tem como consequência a eliminação ineficaz das células em apoptose, o que, por sua vez, resulta na lise dos fagócitos por necrose secundária, com libertação de moléculas citotóxicas que contribuem para a génese das lesões necróticas características da infeção pela *Phdp*.

Apesar dos efeitos tóxicos da AIP56 já serem conhecidos, e de estar bem documentada a sua participação na patologia associada às infecções pela *Phdp*, as relações entre a estrutura e a função da toxina e os determinantes moleculares que estão na base da sua actividade tóxica permanecem por identificar.

Trabalhos desenvolvidos no âmbito desta tese revelaram que a AIP56 é uma metaloprotease dependente de zinco que corta a subunidade p65 do NF- κ B no seu domínio Rel, mais concretamente na cisteína 39. A clivagem neste resíduo remove uma região do p65 essencial para a sua interacção com o DNA, desta forma comprometendo a sua atividade. O corte do p65 foi observado após incubação da AIP56 com extractos totais de leucócitos de robalo ou com o domínio Rel do p65 produzido *in vitro* e mostrou ser dependente da assinatura de metaloprotease presente na AIP56. Além do mais, a clivagem foi demonstrada ser essencial para a actividade tóxica da AIP56, uma vez que mutantes da toxina desprovidos de actividade catalítica revelaram-se não tóxicos. Tendo em conta a importância do NF- κ B na resposta do hospedeiro contra invasões microbianas, regulando a expressão de genes inflamatórios e anti-apoptóticos, é espectável que a depleção do NF- κ B esteja envolvida na apoptose disseminada observada durante as infeções pela *Phdp*.

As toxinas bacterianas que têm alvos intracelulares entram nas células por um processo que envolve a ligação específica a recetores celulares seguido de internalização e translocação para o citosol a partir de um compartimento organelar.

As estruturas dessas toxinas estão adaptadas aos seus mecanismos de entrada, possuindo uma organização do tipo AB, com uma subunidade A, que é responsável pela actividade enzimática, ligada a uma sub-unidade B, que leva a cabo a ligação e entrada da toxina nas células.

A existência de dois domínios na AIP56 foi inicialmente sugerida pelo resultado da análise comparativa da sua estrutura primária com a de proteínas semelhantes obtidas a partir de bases de dados. Durante o presente trabalho, através de ensaios de proteólise limitada e da análise *in silico* da estrutura secundária da AIP56, detectamos a existência de dois domínios unidos entre si por uma região desordenada e uma ponte dissulfureto. Usando versões truncadas da toxina, produzidas com base nos resultados da proteólise limitada, foi possível estabelecer o papel que os dois domínios estruturais da AIP56 desempenham no mecanismo de intoxicação. A porção amino-terminal recombinante, que contém a assinatura de metaloprotease, revelou ter actividade proteolítica contra o p65 do NF- κ B *in vitro* e quando introduzida no citosol das células alvo, usando o sistema LF/PA (*lethal factor/protective antigen*) do *Bacillus anthracis*, reproduziu as actividades proteolítica e apoptogénica da toxina. Estes resultados permitiram atribuir à porção amino-terminal da AIP56 a função catalítica. Pelo contrário, a porção carboxi-terminal da toxina não apresentou actividade catalítica *in vivo* nem *in vitro*. No entanto, quando usada como competidor, preveniu os efeitos tóxicos da AIP56, demonstrando que desempenha um papel importante na ligação e entrada da toxina nas células.

Apesar das fronteiras dos domínios A e B da AIP56 não estarem ainda bem definidas, os resultados obtidos permitiram incluir a AIP56 na família das toxinas do tipo AB, da qual fazem parte as toxinas da difteria, botulismo e tétano, e a exotoxina A da *Pseudomonas*, entre outras. No entanto, os nossos resultados também revelaram que, quando comparada com as outras toxinas do tipo AB, a AIP56 possui algumas diferenças no que respeita aos requisitos estruturais necessários para a intoxicação. Contrariamente a outras toxinas AB, a AIP56 não necessita de activação proteolítica ou qualquer outro tipo de activação para ser enzimaticamente activa *in vitro*. Não obstante, parece necessitar que a região de ligação entre as duas cisteínas (C262 a C298) esteja intacta, uma vez que uma incisão nesta região tornou a toxina inativa. Estas observações sugerem que a região de ligação entre as duas cisteínas participa na translocação da toxina para o citosol e poderá fazer parte do ainda não definido domínio de translocação.

A porção catalítica da AIP56 apresenta uma grande semelhança com o NleC, um efector de tipo III de bactérias entéricas patogénicas que também tem como alvo a subunidade p65 do NF- κ B. Resultados de dicroísmo circular e previsões *in silico*

revelaram que as duas proteínas são muito semelhantes em termos de estruturas secundárias. No entanto, apesar de a AIP56 e o NleC partilharem homologia estrutural e funcional, a região catalítica da AIP56 não é permutável pelo NleC, uma vez que a substituição da porção amino-terminal da AIP56 pelo NleC originou uma quimera sem toxicidade. Igualmente, não foi possível introduzir o NleC no citoplasma das células alvo usando o sistema LF/PA do *Bacillus anthracis* (que no entanto se mostrou eficaz na introdução do domínio catalítico da AIP56). De facto, resultados de fluorimetria diferencial de varrimento mostraram que a região catalítica da AIP56 e o NleC têm propriedades diferentes relativamente à sua desnaturação em resposta à acidificação, podendo estes resultados justificar as diferenças acima mencionadas.

A capacidade intrínseca das toxinas AB para entrarem nas células alvo torna-as atractivas não só como ferramentas para estudos de processos/mecanismos celulares mas também como mecanismos de transporte de diferentes cargas para o interior de células. Isto, associado envolvimento do NF- κ B em situações patológicas graves, incluindo doenças inflamatórias e cancerígenas, confere à AIP56 um enorme potencial como ferramenta para fins terapêuticos e/ou biotecnológicos. Todavia, a futura utilização da AIP56 nestes contextos, implica a detenção de um conhecimento profundo das características estruturais da toxina, das suas vias intracelulares e dos detalhes moleculares que estão na base da sua toxicidade.

CHAPTER I

INTRODUCTION

A. Bacterial exotoxins

1. Overview

The word toxin derives from the Greek τοξικον ("toxikon") that translates as "poison". The concept that pathogenic bacteria might induce harm and cause disease through the production of "poisons", is almost as old as the notion of pathogenic bacteria itself (Alouf, 2006). However, it was not until the 1880's that the term toxin was introduced; according to Carl Lamanna (Lamanna, 1990), first by E. Ray Lankester, in 1886, to name "poisons for animals produced by pathogenic bacteria" and two years later by Roux and Yersin (Roux & Yersin, 1888) to describe the "diphtherial poison" currently known as diphtheria toxin (Alouf, 2006). The discovery of this first bacterial protein toxin prompted outstanding discoveries not only in microbiology and infection but also in biological and medical sciences. The study of the biological effects of bacterial toxins at subcellular and molecular levels, revealed the high complexity and implications of these molecules in a variety of physiological and metabolic processes that ultimately lead to pathogenesis (Alouf, 2006).

Bacterial exotoxins are amongst the principal virulence factors of pathogenic bacteria and usually contribute for the majority of symptoms and lesions caused by infection (Balfanz et al, 1996; Menestrina et al, 1994). These toxins, typically of protein nature, act at a distance from the infectious site and can diffuse throughout the organism. Through diverse and sophisticated mechanisms, toxins manipulate host cell functions and attack crucial events in vital processes of living organisms in favour of microbial infection. This is often achieved by the recognition of specific cell surface receptors that, in addition to cell-specificity, provide anchoring to the cell surface, lower the diffusion space and consequently lead to an increase in toxin concentration. Upon binding to their cell receptor, these toxins can unleash their toxic program at the cell membrane level or can enter the cells and recognize and modify specific intracellular targets (Balfanz et al, 1996; Popoff, 2005).

2. Exotoxins acting at the plasma membrane

Three levels of action can be established among those toxins acting at the plasma membrane: (i) interference with signal transduction pathways, (ii) enzymatic activities towards membrane components and (iii) pore formation (Popoff, 2005).

2.1. Toxins interfering with signal transduction pathways

The Heat-stable toxins (STs) secreted by some enteropathogenic bacteria and the superantigen toxins (SAGs), secreted by many Gram-positive bacteria, such as

Staphylococcus aureus, *Clostridium perfringens* and *Streptococcus pyogenes*, and by the Gram-negative bacteria *Yersinia pseudotuberculosis* (Brosnahan & Schlievert, 2011; Muller-Alouf et al, 2001), are examples of bacterial toxins that act at the plasma membrane and interfere with signalling pathways through binding to a specific cell surface receptor (Popoff, 2005).

STs are produced by a variety of enteric pathogenic organisms, including diarrheagenic *Escherichia coli*, *Vibrio cholerae*, *Vibrio mimicus*, *Yersinia enterocolitica*, *Citrobacter freundii*, and *Klebsiella pneumonia* and are known to interfere with intestinal fluid homeostasis and induce diarrhea (Lin et al, 2010). These toxins are small peptides that fall into two families: STa (or ST-I) and STb (or ST-II) toxins, which differ on their physicochemical and biological characteristics (Lin et al, 2010). The STa members bind and activate guanylate cyclase C (de Sauvage et al, 1991; Schulz et al, 1990), a membrane protein receptor primarily expressed in intestinal mucosal cells involved in regulation of fluid and ion secretion, colon cell proliferation, and the gut immune system (Arshad & Visweswariah, 2012; Lucas et al, 2000). Activation of guanylate cyclase C by STa results in an increase in intracellular levels of cyclic guanosine monophosphate (cGMP) leading to the activation of protein kinase G (PKG) and protein kinase A (PKA), which in turn induce phosphorylation of chloride ion-channels on the apical membrane of intestinal cells causing water and chloride ions efflux resulting in watery diarrhea. In addition, cGMP-dependent PKA activation also leads to inhibition of the re-absorption of sodium by the Na⁺/H⁺-exchanger (Figure 1.1) (Hasegawa & Shimonishi, 2005; Weiglmeier et al, 2010).

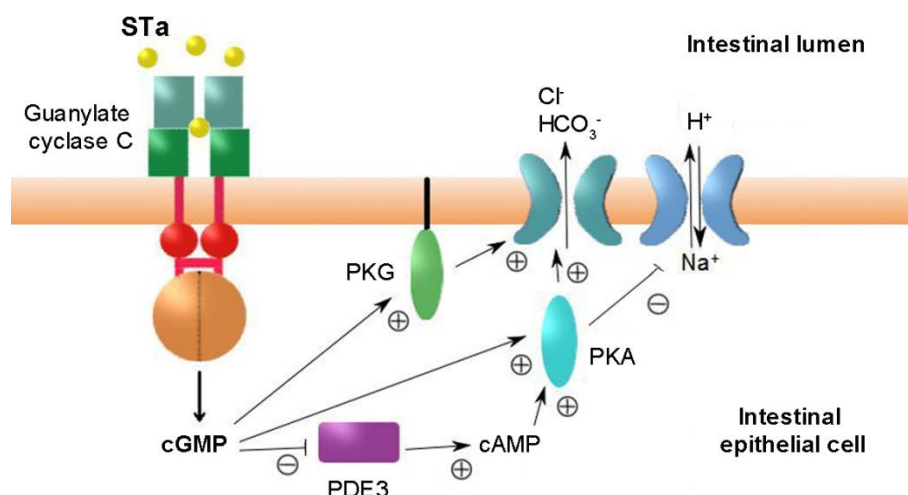


Figure 1.1. Heat-stable toxin a (STa) mechanism of action. Over-activation of guanylate cyclase C through binding of STa leads to an increase in intracellular levels of cyclic GMP (cGMP). This initiates a signalling cascade that ultimately results in secretion of electrolytes into the intestinal lumen. PDE3: cGMP-inhibitable phosphodiesterase 3; PKG: protein kinase G PKA: protein kinase A (Adapted from (Weiglmeier et al, 2010)).

SAGs are a particular class of bacterial toxins that share the ability to induce a powerful inflammatory response within the host (Muller-Alouf et al, 2001; Proft & Fraser, 2003). These enterotoxins hijack T-cell antigen recognition through binding to invariable regions of major histocompatibility complex class II (MHC-II), outside the antigen-binding groove, and to the $\nu\beta$ domain of T-cell receptors (TCR) at the surface of antigen presenting cells (APCs), causing a non-specific activation of the targeted-lymphocytes (Fraser, 2011; Li et al, 1999) (Figure 1.2). By crosslinking MHC-II and TCR, SAGs bridge T-cells and APCs, bringing co-stimulatory molecules closer to their ligands, which results in a more efficient interaction and signal transduction (Muller-Alouf et al, 2001; Proft & Fraser, 2003). As a consequence, both APC and T-cells are stimulated to release massive amounts of cytokines, overpowering the host regulatory network and causing tissue damage (Krakauer, 1999; Muller-Alouf et al, 2001; Proft & Fraser, 2003). Additionally, these toxins have also been described to bind to epithelial cells and equally stimulate the production of pro-inflammatory cytokines by these cells (Kushnaryov et al, 1984a; Kushnaryov et al, 1984b). This phenomenon, termed outside-in signalling, acts to recruit adaptive immune cells to the submucosa, initiating cytokine cascades that lead to toxic shock syndrome (Brosnahan & Schlievert, 2011). Established members of the SAGs family are several staphylococcal enterotoxins, which are among the leading causes of food poisoning, the toxic-shock syndrome toxin 1 from *Staphylococcus aureus*, which induces an acute and sometimes lethal vaginal and wound tissue infection, and the pyrogenic toxins of *Streptococcus pyogenes* involved in scarlet fever (Brosnahan & Schlievert, 2011; Muller-Alouf et al, 2001; Proft & Fraser, 2003).

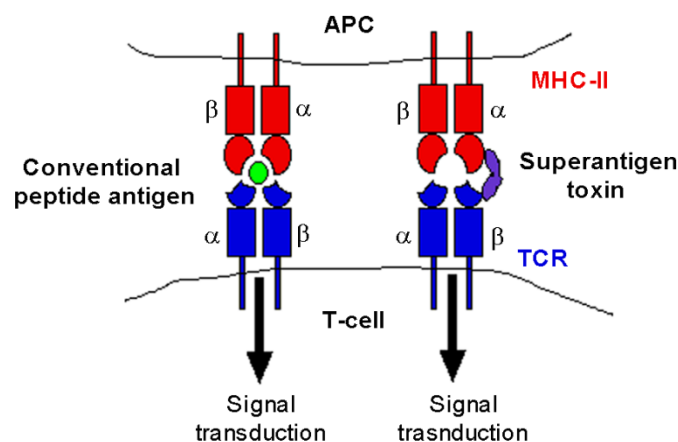


Figure 1.2. Superantigen toxins. Representation of T-cell activation by conventional peptide antigens or by superantigen toxins. TCR: T-cell receptor; APC: antigen presenting cell; MHC class II: major histocompatibility complex class II. (Adapted from <http://www.bio.davidson.edu/courses/immunology/students/spring2000/white/restricted/tss.html>)

2.2. Toxins acting by degrading membrane components

Some toxins act at the plasma membrane by displaying enzymatic activity towards membrane components. The phospholipids, being ubiquitous and key constituents of biological membranes are thus preferential targets and their hydrolysis, not only induces major changes in membrane fluidity and integrity (often resulting in cell damage), but also interferes with intracellular signalling pathways. The hydrolytic products of phospholipids often serve as second messengers in various intracellular signalling cascades involved in the regulation of important physiologic processes such as cell death and the immune response (Geny & Popoff, 2006b; Songer, 1997) (Figure 1.3).

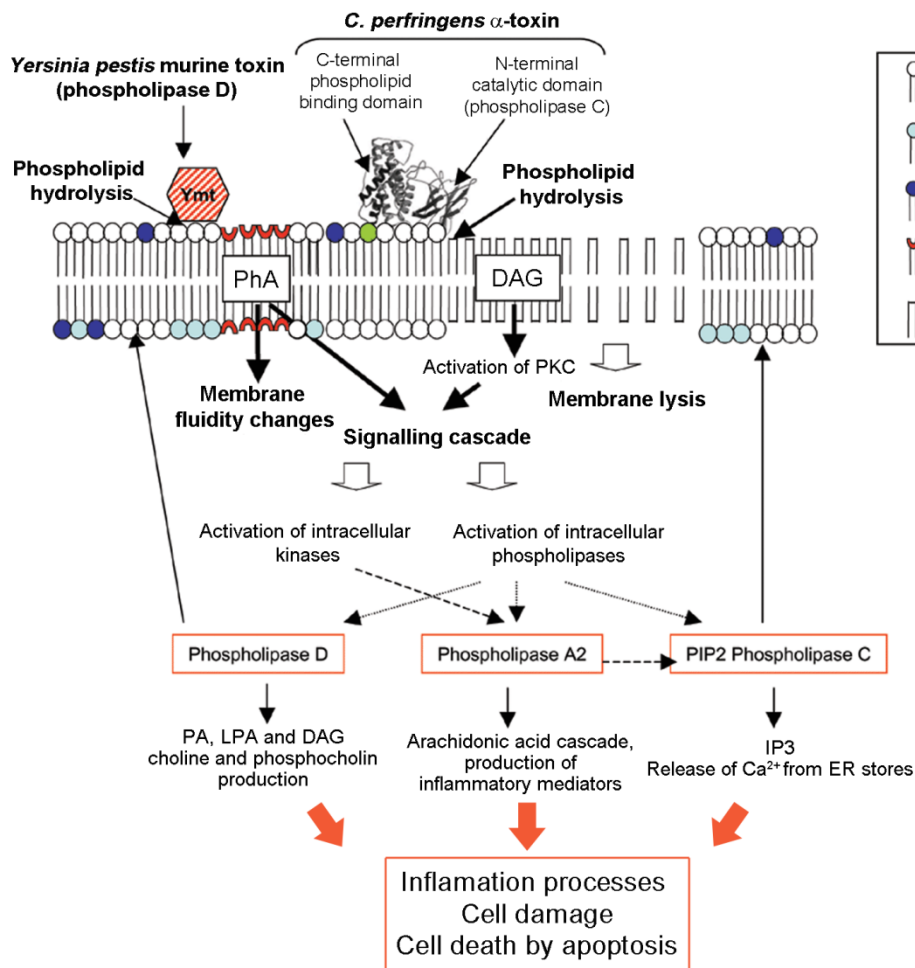


Figure 1.3. Cellular disturbances induced by bacterial toxins acting on membrane phospholipids. Toxins with phospholipase activity act by degrading membrane phospholipids. These toxins induce various changes in host cells including changes in membrane fluidity and membrane lysis. Phospholipid degradation products also act as second messengers in various intracellular kinases and phospholipases pathways involved in several physiological processes. PhA: phosphatidic acid; DAG: diacylglycerol; LPA: lysophosphatidic acid; IP3: inositol triphosphate; PKC: protein kinase C, ER: endoplasmic reticulum, PIP2: Phosphatidylinositol 4,5-bisphosphate. (Adapted from (Geny & Popoff, 2006b))

One example is the *Clostridium perfringens* α -toxin (Figure 1.3), which is a zinc-dependent phospholipase C with preferential activity towards phosphatidylcholine and sphingomyelin, that affects a variety of cells including platelets, leukocytes, fibroblasts and erythrocytes, causing hemolysis, dermonecrosis and lethality (Sakurai et al, 2004). In small amounts, the toxin causes limited hydrolysis of those phospholipids, generating diacylglycerol and ceramide. These molecules modulate the activity of intracellular protein kinases and endogenous phospholipases involved in several intracellular signalling cascades and in membrane phospholipid metabolism, ultimately resulting in hemolysis, contraction of blood vessels and superoxide production (Geny & Popoff, 2006b; Goñi et al, 2012; Sakurai et al, 2004). Moreover, at high concentrations, a more drastic effect is observed, which results in massive degradation of the lipids leading to membrane disruption (Sakurai et al, 2004). Another example is the *Yersinia pestis* murine toxin (Figure 1.3) with phospholipase D activity that hydrolyses the terminal phosphodiester bond of phospholipids (mainly phosphatidylethanolamine and, to a lesser extent, phosphatidylcholine and phosphatidylserine) generating phosphatidic acid (PhA) and an hydrophilic constituent (ethanolamine, serine or choline depending on the phospholipid hydrolysed) causing changes in membrane fluidity and also stimulating a variety of signal transduction pathways (Geny & Popoff, 2006b). Other bacterial phospholipases include phospholipase C from *Pseudomonas*, *Listeria* and various species of *Clostridium*, phospholipase A from *Helicobacter pylori* and phospholipase D from *Corynebacterium* (Songer, 1997; Titball, 1998).

2.3. Pore-forming toxins

The pore-forming toxins (PFTs) are the largest class of exotoxins and represent about 25% of all known bacterial toxins (Gonzalez et al, 2011; Iacovache et al, 2008). PFTs form pores in cell membranes and their action is often essential for the pathogenesis of their producing bacteria. Such pores are expectedly detrimental to the cell, allowing exodus of critical molecules, destroying the established membrane potential and ionic gradients, and contributing to osmotic swelling (Gonzalez et al, 2008). Notwithstanding, the events triggered by pore-formation in the membrane of a given target cell depend largely on toxin concentration, time of exposure and target cell type. At high concentrations PFTs induce numerous pores on the plasma membrane leading to cell necrosis. However, lower concentrations result in fewer pores, which may allow internalization and intracellular activity of PFT molecules (Geny & Popoff, 2006a).

One of the most fascinating aspects of these toxins is that, although they are secreted as fully soluble proteins, at their final stage they form a transmembrane pore/channel in the target membrane (Bischofberger et al, 2012). PFTs bind to the cell membrane, often through specific receptors, prior to pore-formation (Anderluh et al, 2003). The transition from membrane-bound to membrane-inserted state is often a multistep process, driven by structural rearrangements generating or exposing hydrophobic patches that spontaneously insert into the lipid bilayer to form a pore. According to the type of secondary structure of the membrane-spanning region, PFTs can be broadly classified as α -PFTs or β -PFTs (Iacovache et al, 2010).

The α -PFTs contain α -helical hydrophobic stretches that span the membrane (Gonzalez et al, 2008; Iacovache et al, 2010; Iacovache et al, 2008). The α -helical family includes the colicins produced by *Escherichia coli* (Cascales et al, 2007), the Cry toxins from *Bacillus thuringiensis* (Pardo-Lopez et al, 2012) and the cytolysin A (ClyA), also known as hemolysin E, from *Escherichia coli* and *Salmonella enterica* strains (Wallace et al, 2000). Whereas the structure of the soluble form of several α -PFTs has been determined (Iacovache et al, 2008), ClyA is the only α -PFT to which three-dimensional structures of both the soluble (Wallace et al, 2000) and pore structures are available (Mueller et al, 2009). Soluble ClyA is an elongated molecule composed exclusively of α -helices, with the exception of a short solvent exposed hydrophobic β -hairpin (Figure 1.4). Conversion of the soluble form of the toxin into its membrane inserted state requires oligomerization and major rearrangements in the toxin structure. Upon membrane encounter, the hydrophobic β -tongue converts into a hydrophobic α -helix, thought to spontaneously insert into the lipid bilayer, driving subsequent structural changes, oligomerization and pore formation (Iacovache et al, 2010; Mueller et al, 2009). The assembled ClyA pore forms a hollow cylinder composed of twelve toxin monomeric units with the structure of each protomer being substantially different from that in the soluble monomer (Figure 1.4) (Mueller et al, 2009). Nonetheless, it is important to note that cytolysin A is not a typical representative of the α -PFT, in particular because most other α -PFTs do not form well-defined stable oligomers (Iacovache et al, 2010). Indeed, this class of PFTs is a very heterogeneous group, gathering together toxins with very different structures, and the fact that most of these toxins do not form stable oligomeric complexes is a major drawback for structural studies. Therefore, the molecular details and the series of events that lead to the formation of a membrane inserted pore in many α -PFTs are yet unclear. For instance, the colicins contain a pore-forming domain that, in their soluble form, is composed by a bundle of α -helices sandwiching a hydrophobic helical hairpin (Hilsenbeck et al, 2004; Vetter et al, 1998; Wiener et al, 1997). It is known that pore

formation involves structural rearrangements within the toxin, driving the exposure of the hydrophobic helical hairpin and its insertion into the lipid membrane (Kienker et al, 1997; Song et al, 1991). However, a high-resolution structure of the pore formed by these toxins is still unavailable and although toxin multimerization is most likely required for the formation of a transmembrane open pore structure, definitive proof is still lacking (Iacovache et al, 2008).

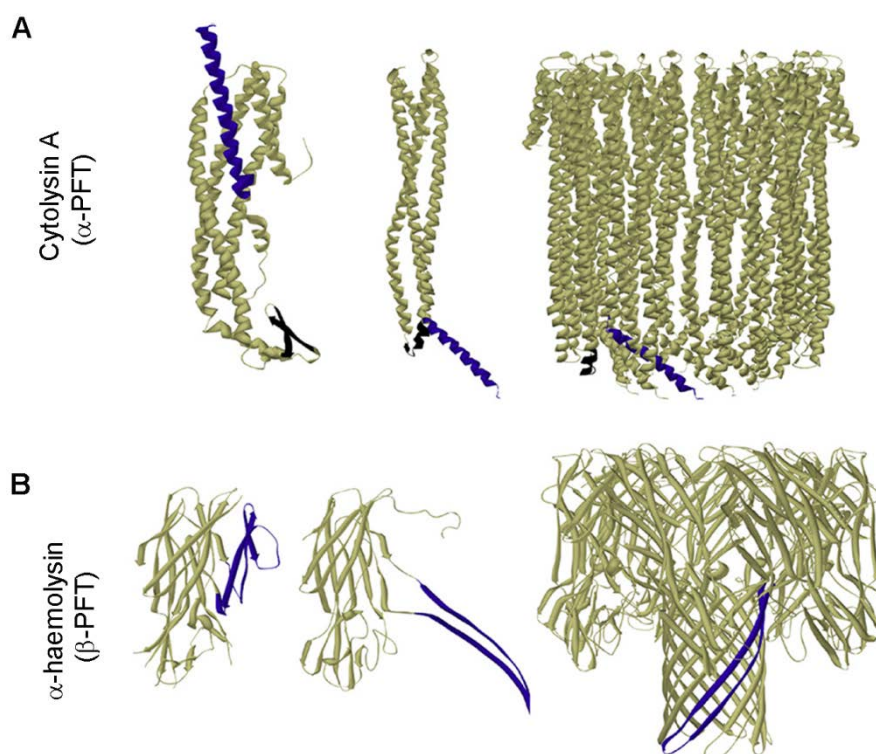


Figure 1.4. Three-dimensional structures of pore-forming toxins (PFTs). Structures of *Escherichia coli* cytolysin A and of *Staphylococcus aureus* α-haemolysin. **A)** Structure of soluble monomeric cytolysin A (left), structure of cytolysin A protomer (middle) and structure of cytolysin A dodecameric pore (right). The hydrophobic hairpin ("β-tong") is represented in black and the pore-forming domain in blue. **B)** Structure of soluble monomeric α-haemolysin (left), structure of α-haemolysin protomer (middle) and structure of α-haemolysin heptameric pore (right). The pore-forming domain is represented in blue. Note the conformational differences between the soluble and the protomeric toxin forms (Adapted from (Iacovache et al, 2010)).

The β-PTFs have so far been the largest and most studied class of PFTs and to which pore-formation is better understood. To this group of PFTs belong, among others, the *Staphylococcus aureus* α-hemolysin (Song et al, 1996), the aerolysin produced by various species of *Aeromonas* (Fivaz et al, 2001) and the cholesterol dependent cytolysins (CDCs) which include the *Streptococcus pyogenes* streptolysin O, the *Streptococcus pneumoniae* pneumolysin and the *Clostridium perfringens* perfringolysin O (Hotze & Tweten, 2012). The β-PFTs are characterized by containing pairs of amphipathic β-strands that, when combined

in oligomeric structures (a step that for certain toxins requires proteolytic activation) generate the hydrophobic surface required for membrane insertion to occur (Iacovache et al, 2010; Iacovache et al, 2008). Each protomer usually contributes with one or two amphipathic β -hairpins that together create an amphipathic β -barrel with a hydrophilic cavity and a hydrophobic outer surface (Iacovache et al, 2010; Iacovache et al, 2008). Exposure of the hydrophobic surface on the outside of the formed β -barrel is thought to provide the energy for membrane insertion, even though no evidence is currently available to support this hypothesis (Gonzalez et al, 2008). For most β -PFTs, membrane insertion is a concerted event preceded by toxin oligomerization into pre-pore structures (Gonzalez et al, 2008). (Iacovache et al, 2010; Iacovache et al, 2008). Yet, in the case of aerolysin, formation of the pre-pore-structure has never been observed and it appears that oligomerization, folding of the amphipathic β -barrel and membrane insertion are coupled events (Geny & Popoff, 2006a; Iacovache et al, 2006). Although for many toxins, the events driving the conversion of the pre-pore into membrane-inserted pores remain obscure, in general these events involve a second series of conformational changes (Thompson et al, 2011). The only high resolution structure of a β -PFT pore is of the *Staphylococcus aureus* α -haemolysin (Song et al, 1996). The pore has a mushroom structure and is composed by 7 toxin units with a transmembrane domain comprising a 14-strand antiparallel β -barrel (each protomer contributes with two β -strands) (Figure 1.4). Nevertheless, depending on the toxin, the number of monomers involved in pore formation can vary from 7 (as in the case of α -haemolysin, aerolysin (Iacovache et al, 2006) and staphylococcal α -toxin (Menestrina et al, 2003)) to up to 50 (in the case of CDCs (Hotze & Tweten, 2012)).

3. Exotoxins that enzymatically modify cytosolic targets

3.1. Overview

Many of the most potent bacterial toxins act inside host cells. The potency of intracellularly acting toxins is derived, in part, from the fact that most are enzymes that catalyse the covalent modification of specific cytosolic targets. Depending on their specific target and type of modification, these toxins can interfere with a variety of cellular processes that include inhibition of protein synthesis (e.g. diphtheria and shiga toxins), increase in second messenger cyclic adenosine monophosphate (cAMP) (e.g. cholera and adenylate cyclase toxins), disaggregation of the microfilament structure (e.g. *Clostridium difficile* toxins A and B) and inhibition of neurotransmitter release (tetanus and botulinum toxins) (Popoff, 2005) (Table 1.1).

To be successful, intracellularly acting toxins must access their substrates inside target cells. To enter the cells, these toxins bind to receptors at the cell surface and exploit the endocytic pathway to reach the cytosol from intracellular compartments (Montecucco & Papini, 1995; Sandvig et al, 2004). However, exceptions exist as is the case of *Bordetella pertussis* adenylate cyclase A that delivers its adenylate cyclase enzyme component into the cell cytoplasm directly from the plasma membrane in a process that does not depend on receptor-mediated endocytosis (Gordon et al, 1988; Osickova et al, 2010). Two conformers of this toxin, which exert different activities, are known. One is able to insert into the cell plasma membrane as a toxin translocation precursor that accomplishes delivery of the catalytic domain into the cytosol, while the other forms cation-selective pores that permeabilize the membrane for efflux of cytosolic potassium (Benz et al, 1994; Fiser et al, 2012; Osickova et al, 2010; Vojtova et al, 2006).

Apart from the above mentioned exception, cell entry/intoxication by toxins targeting cytosolic substrates is a four-step process involving (i) specific binding of the toxin to the cell surface, (ii) internalization, (iii) membrane translocation and, finally, (iv) modification of the cytosolic target (Montecucco & Papini, 1995; Montecucco et al, 1994). This elaborate mechanism of cell entry and action relies in a multi-domain architecture that has evolved and adapted to play specific roles in each of the above mentioned intoxication steps (Montecucco et al, 1994; Rossetto et al, 2000).

Table 1.1. Bacterial toxins that modify cytosolic targets.

Toxin	Bacteria	Mode of action	Intracellular Target	Effect	References
Diphtheria toxin	<i>Corynebacterium diphtheria</i>	ADP-ribosyltransferase	Elongation factor 2	Inhibition of protein synthesis	(Collier, 2001; Honjo et al, 1968)
<i>Pseudomonas</i> exotoxin A	<i>Pseudomonas aeruginosa</i>	ADP-ribosyltransferase	Elongation factor 2	Inhibition of protein synthesis	(Iglewski et al, 1977)
Cholera toxin	<i>Vibrio cholera</i>	ADP-ribosyltransferase	GTP-binding protein G of the adenylate cyclase complex	Increase of cAMP intracellular levels	(Cassel & Selinger, 1977; Spangler, 1992)
Heat labile enterotoxins	<i>Escherichia coli</i>	ADP-ribosyltransferase	GTP-binding protein G of the adenylate cyclase complex	Increase of cAMP intracellular levels	(Gill & Richardson, 1980; Spangler, 1992)
C3 and C3-like toxins	<i>Clostridium botulinum</i> <i>Clostridium limosum</i> <i>Staphylococcus aureus</i> <i>Bacillus cereus</i>	ADP-ribosyltransferase	Rho GTP-binding proteins	Actin cytoskeleton rearrangements	(Aktories & Hall, 1989; Fukui & Horiguchi, 2004; Rubin et al, 1988; Wilde et al, 2001)
Pertussis toxin	<i>Bordetella pertussis</i>	ADP-ribosyltransferase	Trimeric G proteins (α -subunit)	Inhibition of the G protein-coupled signalling pathways	(Locht et al, 2011; Okajima & Ui, 1984)
<i>Clostridium perfringens</i> Iota toxin	<i>Clostridium perfringens</i>	ADP-ribosyltransferase	Actin	Disruption of the cytoskeletal architecture	(Popoff & Bouvet, 2009; Sakurai et al, 2009; Schering et al, 1988; Vandekerckhove et al, 1987)
<i>Clostridium</i> spiroforme Iota-like toxin	<i>Clostridium spiroforme</i>	ADP-ribosyltransferase	Actin	Disruption of the cytoskeletal architecture	(Popoff & Boquet, 1988; Popoff et al, 1989; Simpson et al, 1989)
<i>Clostridium botulinum</i> C2 toxin	<i>Clostridium botulinum</i>	ADP-ribosyltransferase	Actin	Disruption of the cytoskeletal architecture	(Popoff & Bouvet, 2009; Vandekerckhove et al, 1988)
<i>Clostridium difficile</i> toxin A	<i>Clostridium difficile</i>	Glycosyltransferase	Rho GTP-binding proteins	Actin cytoskeleton rearrangements	(Aktories, 2007; Just et al, 1995b; Popoff & Bouvet, 2009)
<i>Clostridium difficile</i> toxin B	<i>Clostridium difficile</i>	Glycosyltransferase	Rho GTP-binding proteins	Actin cytoskeleton rearrangements	(Aktories, 2007; Just et al, 1995a; Popoff & Bouvet, 2009)
<i>Clostridium sordellii</i> lethal toxin	<i>Clostridium sordellii</i>	Glycosyltransferase	Rho-subfamily and Ras-subfamily of small GTPases	Actin cytoskeleton rearrangements	(Hofmann et al, 1996; Just et al, 1996; Popoff et al, 1996)

Table 1.1. Bacterial toxins that modify cytosolic targets. (cont)

Toxin	Bacteria	Mode of action	Intracellular Target	Effect	References
<i>Clostridium sordellii</i> hemorrhagic toxin	<i>Clostridium sordellii</i>	Glycosyltransferase	Rho-subfamily proteins (Rho, Rac and Cdc42)	Actin cytoskeleton rearrangements	(Genth et al, 1996)
<i>Clostridium novyi</i> α -toxin	<i>Clostridium novyi</i>	Glycosyltransferase	Ras family of small GTPases	Actin cytoskeleton rearrangements	(Selzer et al, 1996)
Cytotoxic necrotizing factors (CNF-1 and CNF-2)	<i>Escherichia coli</i>	Deaminase	Rho GTP-binding proteins (RhoA, Rac1, and Cdc42)	Actin cytoskeleton rearrangements	(Flatau et al, 1997; Lerm et al, 1999; Oswald et al, 1994; Schmidt et al, 1997)
Cytotoxic necrotizing factor	<i>Yersinia pseudotuberculosis</i>	Deaminase	RhoA	Actin cytoskeleton rearrangements	(Hoffmann et al, 2004)
<i>Bordetella</i> dermonecrotic toxin (DNT)	<i>Bordetella pertussis</i>	Deaminase	Rho GTP-binding proteins	Actin cytoskeleton rearrangements	(Fukui & Horiguchi, 2004)
<i>Pasteurella multocida</i> toxin (PMT)	<i>Pasteurella multocida</i>	Deaminidase	Heterotrimeric G-proteins	Activation of G-protein signalling pathways	(Orth et al, 2009)
Anthrax edema toxin	<i>Bacillus anthracis</i>	Calmodulin dependent adenylate cyclase	Catalysis the synthesis of cAMP from cellular ATP	Increase in intracellular cAMP levels	(Leppla, 1982; Tang & Guo, 2009)
<i>Bordetella pertussis</i> Adenylate cyclase A (CyaA)	<i>Bordetella pertussis</i>	Calmodulin adenylate cyclase	Catalysis the synthesis of cAMP from cellular ATP	Increase in intracellular cAMP levels	(Carbonetti, 2010; Wolff et al, 1980)
Shiga toxin and shiga-like toxins	<i>Shigella dysenteriae</i> <i>Enterohemorrhagic E. coli</i>	RNA N-glycosidase	28S ribosomal RNA	Inhibition of protein synthesis	(Endo et al, 1988; Sandvig, 2001)
Anthrax lethal toxin	<i>Bacillus anthracis</i>	Metalloprotease	MAPK kinases	Inhibition of MAPK signalling pathways	(Duesbery et al, 1998; Tonello & Montecucco, 2009; Vitale et al, 1998)
Tetanus toxin	<i>Clostridium tetani</i>	Metalloprotease	SNARE proteins	Inhibition of neurotransmitter release	(Schiavo et al, 1992a; Schiavo et al, 1994; Turton et al, 2002)
Botulinum toxins (type A to F)	<i>Clostridium botulinum</i>	Metalloprotease	SNARE proteins	Inhibition of neurotransmitter release	(Schiavo et al, 1992a; Schiavo et al, 1994; Schiavo et al, 1993a; Schiavo et al, 1993b; Turton et al, 2002)

3.2. The AB structure

In general terms, toxins that act intracellularly and enzymatically modify cytosolic targets are composed by two functional subunits: a catalytic moiety (also designated A subunit) that holds the toxin enzymatic activity and a binding moiety (also designated B subunit) involved in cellular uptake and delivery of the enzymatic component into the cytosol. Individually the two subunits are non-toxic and a concerted action of the two subunits is need for cell intoxication. Because of their architecture, these toxins are referred as AB toxins (Montecucco et al, 1994; Rossetto et al, 2000).

Toxins belonging to the AB toxin family can be synthesized and arranged in a multiplicity of ways (Figure 1.5). The different folding and assemblies of the A and B subunits to form a final toxin are closely related with the molecular processes of toxin biogenesis, and their structural features are adapted to the specific requirements of the intoxication pathway (Montecucco & Papini, 1995).

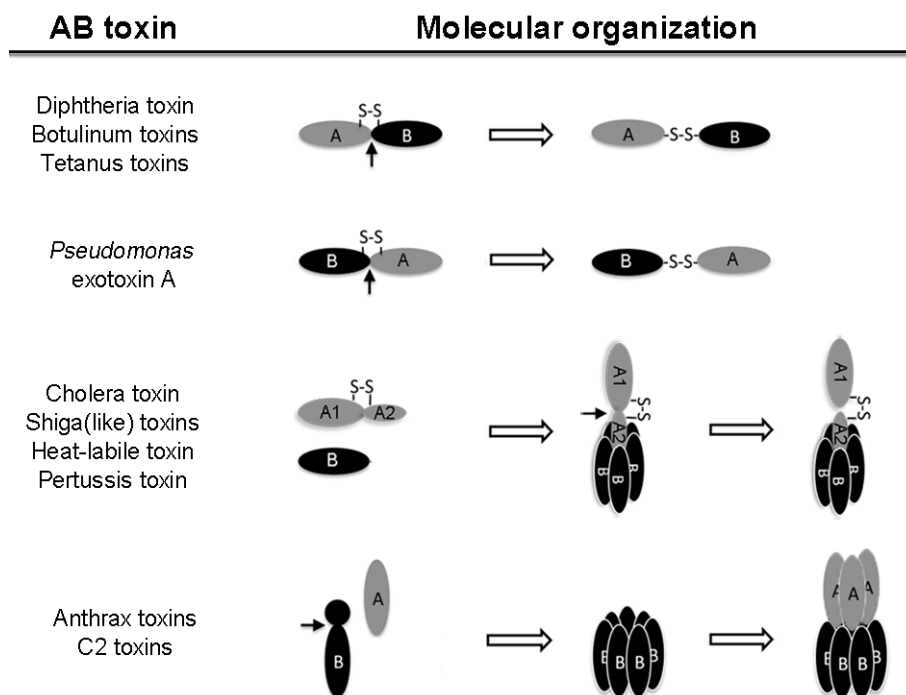


Figure 1.5. Molecular organizations of AB toxins. The A subunit is the enzymatically active portion of the toxin and the B moiety is the one responsible toxin binding and internalization. Protease cleavage sites are indicated by black arrows. (Adapted from (Jianjun, 2012))

3.2.1. AB toxins secreted as a single polypeptide chain

Toxins such as diphtheria toxin (DT) (Collier, 2001) and the clostridial neurotoxins (comprising the tetanus neurotoxin (TeNT) and the seven antigenically distinct botulinum neurotoxins (BoNT/A-G)) (Turton et al, 2002) are secreted by their producing bacteria as

single-chain polypeptides that upon secretion undergo proteolytic processing at a flexible and exposed region between two cysteine residues, yielding an A chain/subunit bound by a disulphide bridge to a B chain/subunit. Proteolytic processing of TeNT and BoNT results from the action of endogenous clostridial proteases (Ahnert-Hilger et al, 1990; DasGupta, 1971; Kriegelstein et al, 1991; Weller et al, 1989), whereas in the case of DT it can result from the action of bacterial proteases or occur at the cell surface mediated by a furin or furin-like protease (Gill & Dinius, 1971; Tsuneoka et al, 1993). The X-ray crystal structures of DT (Bell & Eisenberg, 1997; Choe et al, 1992) and intact BoNT/A (Lacy et al, 1998) and BoNT/B (Eswaramoorthy et al, 2004; Swaminathan & Eswaramoorthy, 2000) have been solved. However, in the case of TeNT, only the crystal structures of the isolated A- and B-chains (also called light and heavy chains, respectively) have been determined so far (Breidenbach & Brunger, 2005; Rao et al, 2005; Umland et al, 1997). These toxins share the same overall structure with three structural/functional domains: an N-terminal catalytic domain, which makes the A subunit, disulphide-bounded to a B subunit comprising a membrane translocation (T) domain and a receptor binding (R) domain (Collier, 2001; Turton et al, 2002) (Figure 1.6). Nonetheless, the spacial arrangement of the domains among these toxins differs. In BoNT, the three functional domains are arranged in a linear fashion, with the translocation domain flanked by the catalytic and binding domains, whereas in DT and the three domains are arranged in the shape of an “Y” with the T domain forming the lower segment and the T and R domains the upper angled segments of the “Y” (Figure 1.6).

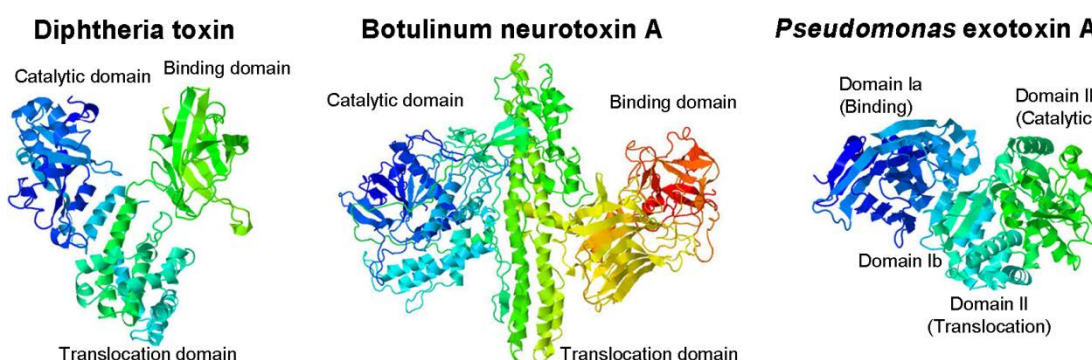


Figure 1.6. Three-dimensional structures of AB toxins secreted as a single polypeptide chain. Crystal structures of diphtheria toxin (PDB ID: 1SGK), botulinum neurotoxin A (PDB ID: 3BTA) and *Pseudomonas* exotoxin A (PDB ID: 1IKQ). PDB: Protein data bank.

The *Pseudomonas* exotoxin A (PE) is another example of an AB toxin secreted as a single polypeptide chain (Weldon & Pastan, 2011; Wolf & Elsasser-Beile, 2009). However, in contrast with the above mentioned toxins, PE proteolytic processing takes place upon toxin

internalization at an acidic compartment, presumably the endosomes, and is mediated by furin (Corboy & Draper, 1997; Fryling et al, 1992; Inocencio et al, 1994; Ogata et al, 1990; Ogata et al, 1992). PE is composed by three structural and functional domains (denominated I, II and III) (Allured et al, 1986; Hwang et al, 1987; Siegall et al, 1989; Wedekind et al, 2001). The X-ray crystal structures of PE revealed that the N-terminal domain I is divided into two non-sequential but structurally adjacent domains named Ia and Ib. Residues between Ia and Ib comprise domain II and domain III lies in the C-terminus (Allured et al, 1986; Wedekind et al, 2001) (Figure 1.6). Functionally, domain Ia is the receptor-binding domain and the major component of the B subunit, domain II contains the furin cleavage site and is involved in toxin translocation and intracellular trafficking, and domain III is the catalytically active domain and the primary constituent of the A subunit (Hwang et al, 1987; Siegall et al, 1989; Theuer et al, 1993; Wedekind et al, 2001); the functions of domain Ib are not yet clarified.

Equally secreted by their producing bacteria as single polypeptide chains are the clostridial glucosylating toxins (i.e. *Clostridium difficile* toxin A and B, the hemorrhagic and lethal toxins from *Clostridium sordelli* and the α -toxin from *Clostridium novyi*), also known as large clostridial toxins (Jank & Aktories, 2008; Just & Gerhard, 2004; Popoff & Bouvet, 2009). However, in contrast to DT, TeNT, BoNT, and PE, the proteolytic processing of the clostridial glucosylating toxins is the result of auto-catalytic activity (Aktories, 2007; Giesemann et al, 2008; Shen, 2010). In addition to the catalytic, translocation and binding domains, these toxins possess an autocatalytic/self-cutting protease domain (Aktories, 2007; Albesa-Jové et al, 2010; Giesemann et al, 2008; Shen, 2010) located in the N-terminal region preceded by the catalytic domain. The receptor binding domain is located in the C-terminus of the toxin and downstream to a hydrophobic region putatively involved in membrane translocation (Aktories, 2007; Albesa-Jové et al, 2010; Jank & Aktories, 2008). Thus, in these toxins, the AB model can be extended to an ABCD model where A is responsible for the catalytic activity, B for binding to target cells, C for auto-proteolysis, and D for delivering/translocation (Jank & Aktories, 2008).

3.2.2. AB₅ toxins

Toxins including cholera toxin (CT) (Bharati & Ganguly, 2011), shiga toxin (Stx) (Sandvig, 2001), heat-labile enterotoxins (LT) (Beddoe et al, 2010) and pertussis toxin (PT) (Locht et al, 2011) consist of an assembly of independently synthesized A and B subunits that associate by non-covalent bonds to form a final multimeric toxin. The toxin complexes consist of hetero-hexameric assemblies comprising a single A subunit linked to a pentamer of B-subunits (Figures 1.5), and thus the AB₅ designation (Beddoe et al, 2010; Merritt & Hol,

1995). The X-ray structures of several AB₅ members are known and reveal a remarkable degree of structural homology, despite the lack of sequence homology (Fraser et al, 2004; Stein et al, 1994; van den Akker et al, 1996; Zhang et al, 1995) (Figure 1.7).

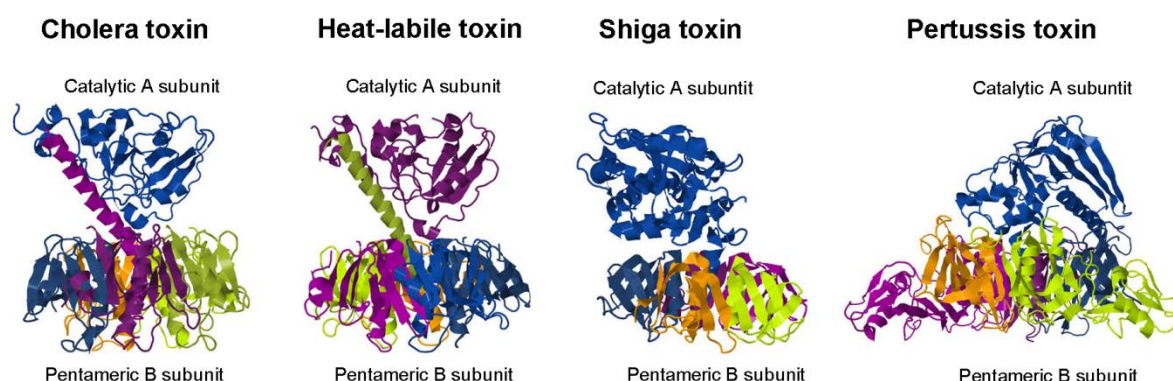


Figure 1.7. Three-dimensional structures of AB₅ toxins. Crystal structures of cholera toxin (PDB ID: 1XTC), heat-labile enterotoxin IIb (PDB ID: 1TII), shiga toxin (PDB ID: 1R4Q) and pertussis toxin (PDB ID: 1PRT). PDB: Protein data bank.

In AB₅ toxins, the B-pentamer is usually composed of identical B subunits (Beddoe et al, 2010; Merritt & Hol, 1995), with exception of PT where the B-pentamer is composed of four different subunits, named S2 to S5 (Locht et al, 2011). Assembly of the A and B subunits into AB₅ oligomers is a complex process that occurs in the periplasmic compartment of the bacterial cell envelope (Hirst et al, 1984; Mekalanos et al, 1983; Neill et al, 1983). The precise order of subunit interaction and the molecular determinants of the oligomerization process are not yet completely clarified. It is known that pentamerization of the B subunit(s) can be achieved *in vitro* in the presence or absence of the A subunit, indicating that the sequence of the B subunit contains all the necessary information for pentamer assembly (Lai et al, 1976; Loch et al, 2011; Olsnes et al, 1981; Sandvig, 2001; Spangler, 1992; Zrimi et al, 2010). However, A subunits are only able to interact with immature (not pentameric) B oligomers and oligomerization is faster in the presence of A subunits (Hardy et al, 1988; Streatfield et al, 1992), suggesting that binding of the A subunit must occur prior to complete B subunit pentamerization.

Upon secretion, AB₅ toxins undergo proteolytic activation within their A subunit at a trypsin- and/or furin-sensitive loop located at the C-terminal end yielding two disulphide-bridged domains (A1 and A2) (Booth et al, 1984; Garred et al, 1995; Grant et al, 1994; Krueger et al, 1991). The A1 domain comprises the catalytic function and is responsible for the modification of the intracellular target whereas the A2 domain consists of an α -helix that penetrates into the central pore of the pentameric B-subunit, thereby non-covalently

anchoring the A- and B-subunits together (Beddoe et al, 2010; Fraser et al, 2004; Sixma et al, 1993; Stein et al, 1994; Stein et al, 1992; Zhang et al, 1995). In the case of CT, the proteolytic processing of the A domain results from the activity of a *Vibrio cholerae* protease (Booth et al, 1984), whereas in the case of Stx (Garred et al, 1995), LT (Grant et al, 1994) and PT (Finck-Barbançon & Barbieri, 1996; Krueger et al, 1991) cleavage occurs at early stages of intracellular transport and is mediated by a target cell protease.

3.2.3. AB_{7/8} toxins

Anthrax toxins (lethal toxin and edema toxin), which derive from the combination of two independent A subunits (edema factor and lethal factor) with the same oligomeric B component, the protective antigen (PA), and *Clostridium botulinum* C2 toxin are the most prominent examples of the AB_{7/8} toxins (Barth & Aktories, 2011; Barth et al, 2004; Young & Collier, 2007). Similarly to AB₅, the AB_{7/8} toxins consist of a complex of independently synthesized A and B subunits that associate non-covalently to form a final multimeric toxin. The toxin complexes consist on a B heptameric or octameric oligomer, bound to multiple A subunits (Bann, 2012) (Figure 1.5).

In contrast with AB₅ toxins, formation of the AB_{7/8} toxin complexes occurs upon secretion of the A and B components by the producing bacteria. Assembly can take place at the extracellular medium (Ezzell et al, 2009; Kaiser et al, 2006; Panchal et al, 2005) or at the cell surface, upon binding of the B component to cell receptors (Barth et al, 2000; Beauregard et al, 2000; Ohishi & Miyake, 1985). Either way, toxin assembly requires proteolytic activation of the B component, as the result of the action of serum proteases (Ezzell & Abshire, 1992) or furin proteases located at the cell surface (Klimpel et al, 1992).

Proteolytic processing drives oligomerization of the B components into ring shaped structures called pre-pore (Figure 1.8). Pre-pore formation precedes the binding of the A components to form the functional toxin complex (Ascenzi et al, 2002; Barth et al, 2000; Beauregard et al, 2000; Mogridge et al, 2002). Until recently, only heptameric structures of pre-pore complexes, able to bind three A subunit molecules, were known (Barth et al, 2000; Lacy et al, 2004; Milne et al, 1994; Petosa et al, 1997; Santelli et al, 2004; Schleberger et al, 2006). However, in 2009, Kintzer and colleagues reported the existence of functional anthrax PA octameric pre-pore structures, capable of binding four A subunits (Kintzer et al, 2010; Kintzer et al, 2009) (Figure 1.8).

Anthrax toxins are the most extensively studied AB_{7/8} toxins and for which the molecular details of the assembly and intoxication are best understood (Bann, 2012; Puhar & Montecucco, 2007; Thoren & Krantz, 2011; Young & Collier, 2007). By contrast, structural

information regarding the C2 toxin is scarce and only a low resolution crystal structure is available (Schleberger et al, 2006). However, and even though the enzymatic components of C2 and anthrax toxins are structurally and functionally divergent, the binding components of those toxins share considerable sequence and structural homology (Barth et al, 2000; Popoff & Bouvet, 2009; Schleberger et al, 2006; Young & Collier, 2007). Both binding components consist of four functional domains: domain I is proteolytically cleaved during toxin activation and is involved in the interaction with the enzymatic components (domain I'); domain II is a flexible hydrophobic loop that inserts in the membrane playing a role in membrane translocation; domain III is involved in B subunit oligomerization; and domain IV is responsible for binding to the cell surface receptors (Lacy et al, 2004; Petosa et al, 1997; Schleberger et al, 2006) (Figure 1.8).

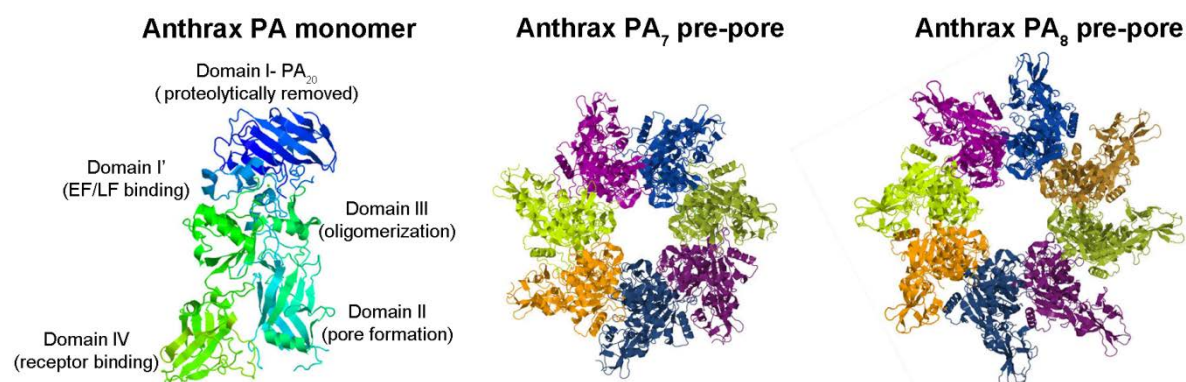


Figure 1.8. Three-dimensional structures of anthrax protective antigen (PA). Crystal structure of monomeric PA (PDB ID:1ACC) and axial views of X-ray crystal structures of heptameric (PDB ID:1TZO) and octameric (PDB ID: 3HVD) pre-pore structures. PDB: Protein data bank.

3.3. Mechanisms of intoxication

The first step of the entry of a bacterial protein toxin into the host cell is the binding to a specific receptor at the cell surface, which can be of proteic, lipidic or carbohydrate nature. Binding to the receptor not only determines cell target specificity, but also provides toxin anchoring to the cell membrane which is essential in the following intoxication steps (Lord et al, 1999; Montecucco et al, 1994). Host cells lacking the receptors for a particular toxin are generally resistant to intoxication, underscoring the importance of toxin-receptor complexes. Some receptor-bound toxins (e.g., DT and ST) are targeted for clathrin-dependent endocytosis (Murphy, 2011; Sandvig et al, 2010a; Sandvig et al, 2004), while other toxins, including CT and TeNT, enter cells by one of several alternative “clathrin-independent” endocytic pathways (Deinhardt et al, 2006; Torgersen et al, 2001).

The cytosol is the final destination of these toxins (or at least of the catalytic A subunit) and, when it comes to cytosolic entry these toxins fall into two main groups: (i) the “short-trip” toxins that reach the cytosol from the endosomal compartment, and the (ii) “long-trip” toxins that reach the cytosol from the endoplasmic reticulum (ER) (Figure 1.9) (Falnes & Sandvig, 2000; Montecucco & Papini, 1995; Montecucco et al, 1994; Sandvig et al, 2004). Either way, in order to reach the cytosol, toxins need to cross the membrane barrier and this is still the most obscure step of the entire intoxication process. Considering the hydrophilic nature of protein toxins and the hydrophobic nature of the membrane, it is clear that membrane translocation represents a major fundamental biologic problem for toxin activity and toxins have developed different strategies to overcome it (Falnes & Sandvig, 2000).

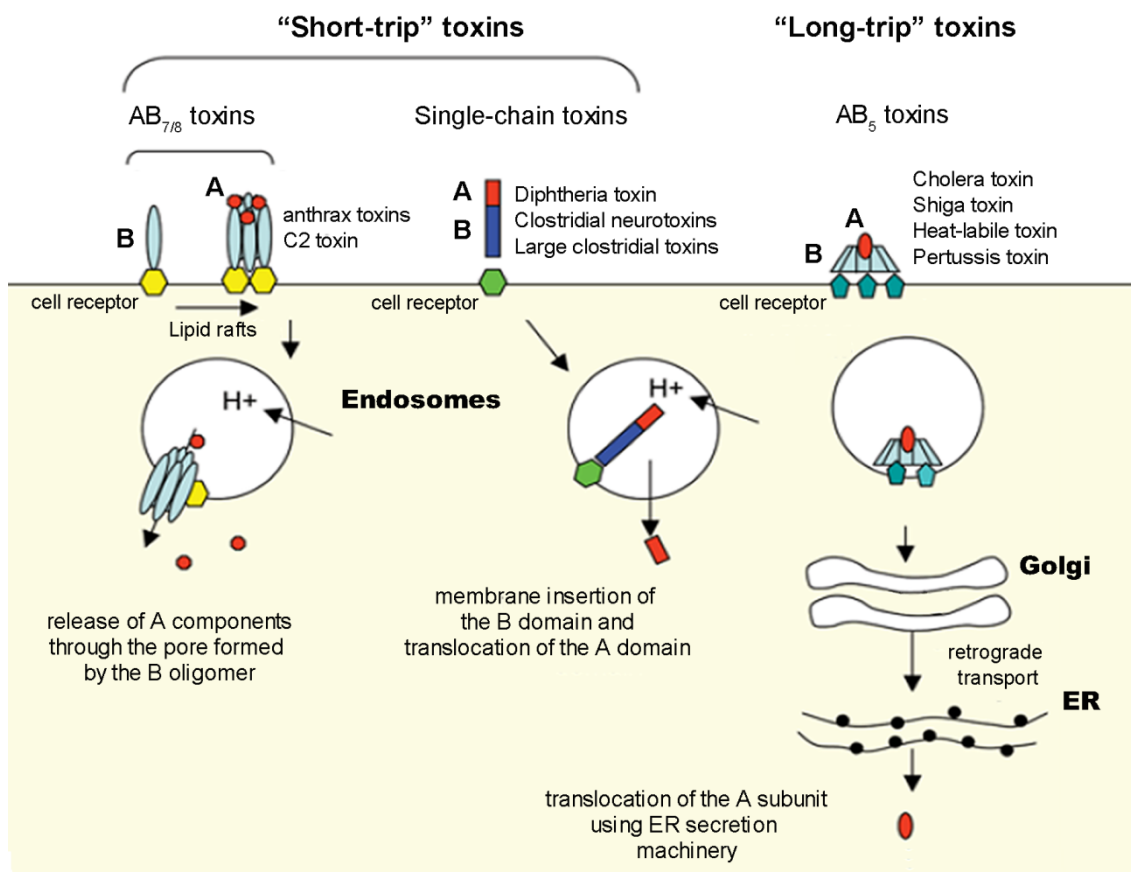


Figure 1.9. Intracellular pathways of AB toxins. Toxin B subunit(s) bind to cellular receptors and the AB toxin complex is internalized via receptor-mediated endocytosis. The “short-trip” toxins reach the endosomal compartment and exploit the endosomal acidification to translocate their catalytic A subunit to the cytosol. Under the low pH of the endosomal lumen the toxin undergoes conformational rearrangements that lead to the exposure of hydrophobic regions allowing membrane insertion of the toxin’s B subunit(s) and formation of ion-conducting channels across which partially unfolded A fragments translocate into the cytosol. The “long-trip” toxins follow a longer intracellular route and exploit the degradation pathways for misfolded proteins. These toxins travel retrogradely from the plasma membrane, via the trans-Golgi network (TGN) and the Golgi stack, to the endoplasmic reticulum (ER), from where the A subunit translocates to the cytosol using ER secretion machinery. (Adapted from (Geny & Popoff, 2006a))

3.3.1. “Short-trip” toxins

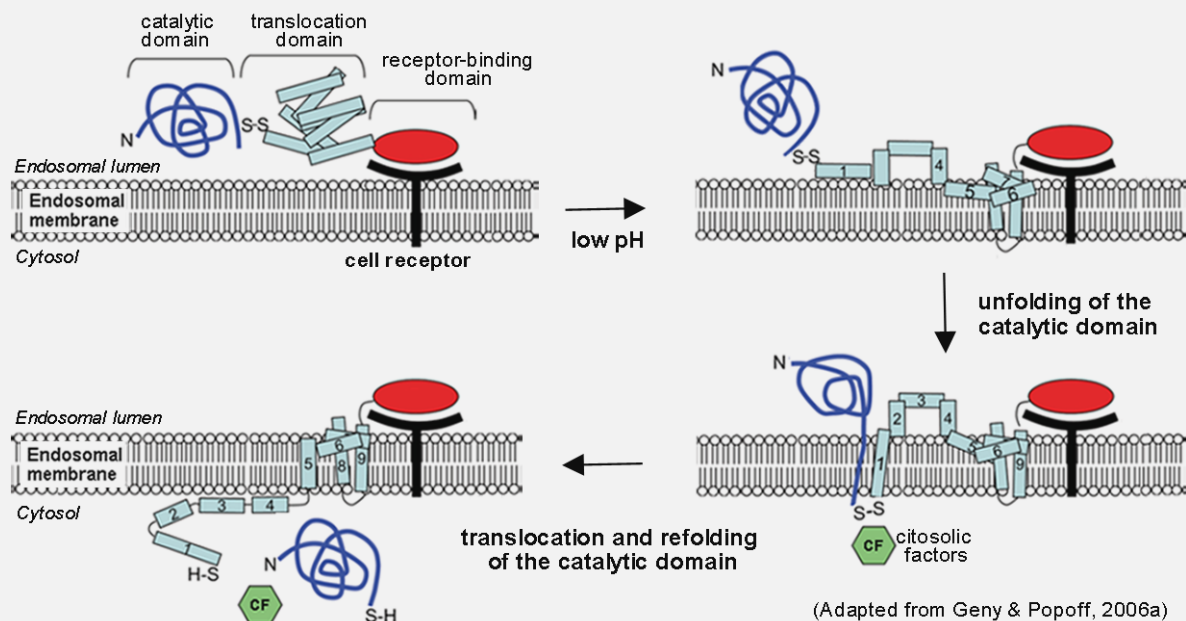
To transport their A fragments into the cytosol, several toxins including DT (see box1) (Murphy, 2011), anthrax toxins (see box2) (Young & Collier, 2007), the clostridial neurotoxins TeNT and BoNT (Turton et al, 2002), and the large clostridial toxins (Jank & Aktories, 2008; Pruitt & Lacy, 2012), exploit the acidification of the endocytic vesicles, as they traffic from the plasma into the cell, and translocate their A subunits into the cytosol from this acidic compartment (Figure 1.9).

Although the structural architecture of DT and clostridial neurotoxins differ greatly from anthrax toxins and the large clostridial toxins (discussed above in section 2.2.1), these toxins share molecular and structural features that meet the specific requirements to cross a lipid membrane. All these toxins share the property of forming ion channels in lipid bilayers at low pH (Bachmeyer et al, 2001; Barth et al, 2001; Blaustein, 2011; Giesemann et al, 2006; Hoch et al, 1985). Formation of these channels was observed not only in artificial membranes (Bachmeyer et al, 2001; Barth et al, 2001; Blaustein et al, 1989; Giesemann et al, 2006; Hoch et al, 1985; Koehler & Collier, 1991; Lang et al, 2008) but also in living cells (Bachmeyer et al, 2001; Barth et al, 2001; Eriksen et al, 1994; Giesemann et al, 2006; Lang et al, 2008; Li & Shi, 2006; Milne & Collier, 1993; Rainey et al, 2005; Sandvig & Olsnes, 1988) and in this sense, the B components of these toxins are also considered as pore-forming toxins (Bischofberger et al, 2012; Gonzalez et al, 2008; Reig & van der Goot, 2006). Channel formation involves conformational rearrangement of the toxin with insertion into the membrane of hydrophobic stretches within the B-subunit (T domain of DT and clostridial neurotoxins and domain II of anthrax PA) (Albesa-Jové et al, 2010; Bann, 2012; Barth et al, 2001; Blaustein et al, 1987; Chenal et al, 2009; Chenal et al, 2002; Galloux et al, 2008; Giesemann et al, 2006; Hoch et al, 1985; Montecucco et al, 1986; Qa'Dan et al, 2000; Shone et al, 1987; Silverman et al, 1994).

It is now currently accepted that the acid endosomal pH triggers conformational rearrangements within the toxin that enable: (i) the B subunit to insert into the membrane and form a transmembrane pore that serves as a tunnel for the A subunit to cross the membrane protected from the contact with lipids, and (ii) the A chain to unfold and translocate into the cytosol through the formed pore (Bann, 2012; Montal, 2010; Murphy, 2011; Pentelute et al, 2011; Rodnin et al, 2010; Thoren & Krantz, 2011; Turton et al, 2002; Wu et al, 2006). Under the neutral reducing environment of the cytosol, the A subunit refolds and the disulphide bond linking the A and B subunits is reduced, leaving behind the B subunit (Montal, 2010; Murphy, 2011; Thoren & Krantz, 2011; Turton et al, 2002). The molecular determinants involved in this last step of the intoxication process, particularly regarding the translocation

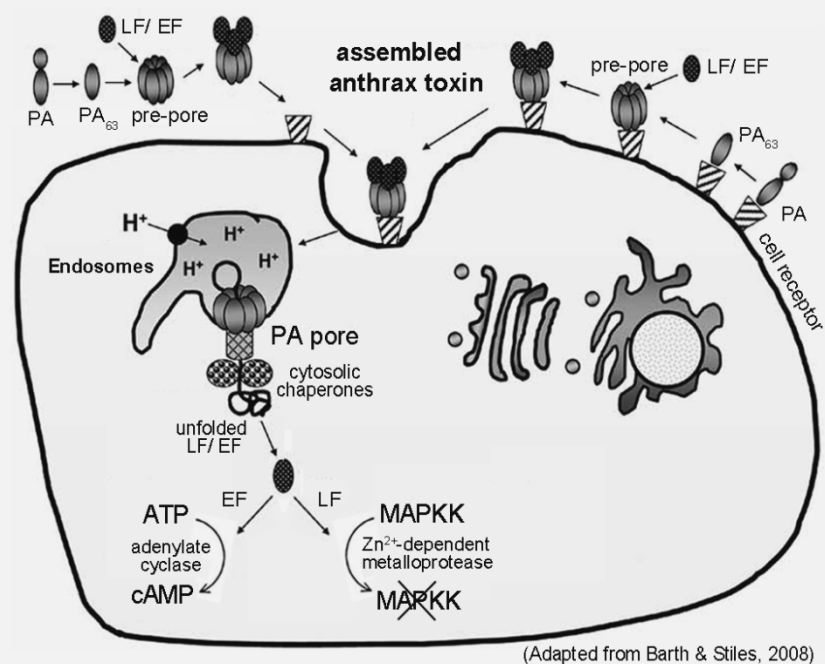
Box 1. Diphtheria toxin

Diphtheria toxin (DT) is one of the most extensively studied and well understood bacterial toxins (Collier, 2001). The toxin is secreted by *Corynebacterium diphtheria*, the causative agent of the diphtheria disease, an infectious disease that primarily affects the mucous membranes of the respiratory tract (respiratory diphtheria). Although not so commonly, diphtheria may also affect the skin (cutaneous diphtheria) and lining tissues in the ear, eye, and the genital areas (<http://www.cdc.gov/>). DT is composed by three domains: a catalytic domain, a binding domain which mediates the binding of the toxin to cell surface receptors and a translocation domain involved in the translocation of the catalytic domain into the cell cytosol (Collier, 2001). The toxin binds to the membrane anchored heparin binding epidermal growth factor-like precursor hb-EGF (Naglich et al, 1992). Receptor bound toxin is concentrated in clathrin coated pits and internalized into clathrin coated vesicles and then trafficked to the endosomal compartment (Moya et al, 1985; Murphy, 2011). Once in endosomes, acidification of the luminal pH triggers the dynamic unfolding of the toxin, with the exposure of hydrophobic regions, leading to toxin insertion into the endosomal membrane and formation of a cation-selective pore from across which unfolded catalytic domain translocates to the cytosol. Membrane insertion is mediated by the α -helical translocation domain comprising nine α -helices arranged in three helical layers (Collier, 2001; Geny & Popoff, 2006a; Murphy, 2011). It is widely recognized that the endosomal acidification is a key trigger event that induces conformational changes necessary for membrane insertion and pore formation (Collier, 2001; Geny & Popoff, 2006a; Murphy, 2011). Concerted protonation of key histidines located throughout the translocation domain and acidic amino acid residues within the loop between helices TH8-TH9, are thought to trigger conformational changes that drive membrane interaction and insertion of the transmembrane α -helices TH5-TH7 and TH8-TH9 (Kaul et al, 1996; Perier et al, 2007; Rodnin et al, 2010; Wang & London, 2009). However, membrane insertion and pore formation are not self-sufficient to conduct translocation of the catalytic domain across the endosomal membrane and some host cell factors (Murphy, 2011) along with ATP (Lemichiez et al, 1997) have been suggested to be involved in the process. Several studies have demonstrated the involvement of COPI (coatamer protein complex I) complex in facilitating the delivery of the catalytic domain into the cell cytosol (Lemichiez et al, 1997; Ratts et al, 2005; Trujillo et al, 2010). Additionally, the cytoplasmic thioredoxin reductase was also found to be essential for release of the DT catalytic domain into the cytosol and the cytosolic chaperone heat-shock protein 90 (Hsp90) likely to be involved in the refolding of the catalytic domain to generate a catalytically active ADP-ribosyltransferase (Ratts et al, 2003). Once at the cytosol, the catalytic domain of DT catalysis the transfer of the ADP-ribose moiety of NAD to the elongation factor 2 (EF-2) leading to the inhibition of protein synthesis (Honjo et al, 1968).



Box 2. Anthrax toxins

Anthrax toxins (lethal toxin and edema toxin) are secreted by *Bacillus anthracis*, the causative agent of anthrax disease. In its systemic form, anthrax infections are rapid-course deadly infections that result from inhalation or ingestion of *Bacillus anthracis* spores (Ascenzi et al, 2002). Lethal toxin and edema toxin derive from two independent enzymatically active subunits, the edema factor (EF) and lethal factor (LF), that associate with the common receptor-binding subunit, the protective antigen (PA) (Ascenzi et al, 2002; Young & Collier, 2007). Initial steps in the cellular intoxication involve assembly of the toxin complexes and binding of toxin to the



cellular receptor. Formation of the PA/LF or EF complexes can occur either at the cell surface, upon binding to the cell receptor, or at the extracellular medium (i.e. blood serum) (Bann, 2012; Kintzer et al, 2010). Either way, toxin assembly requires proteolytic activation of the PA resulting in the formation of a C-terminal 63 kDa fragment (PA₆₃) that oligomerizes into heptameric (Lacy et al, 2004; Milne et al, 1994) or octameric (Kintzer et al, 2010; Kintzer et al, 2009) ring-shaped structures (pre-pore) capable of binding 3 or 4 molecules, respectively, of LF or EF. Three anthrax cell receptors have been identified: the tumour endothelial marker-8 (ANTXR1) (Bradley et al, 2001), the capillary morphogenesis protein 2 (ANTXR2) (Scobie et al, 2003) and more recently, the integrin $\beta 1$ complexes (identified as a low-affinity PA receptor that was also found to potentiate ANTXR2-mediated endocytosis) (Martchenko et al, 2010). Nonetheless, a very recent study showed that ANTXR1 and ANTXR2 are the only two anthrax toxin receptors mediating lethality *in vivo* (Liu et al, 2012). Following lipid raft association, anthrax toxin-receptor complexes are internalized presumably via clathrin coated pits and then trafficked to early endosomes (Abrami et al, 2010; Abrami et al, 2003), where the acidic environment induces a series of conformational changes on the toxin complex that allow the pre-pore insertion into the membrane, forming a membrane spanning pore (Bann, 2012). There is only one model for the membrane active PA-pore (Nguyen, 2004), based on the mushroom shaped resolved structure of α -hemolysin (Song et al, 1996) (see Figure 1.4), according to which, the pore is an extended 14-stranded β -barrel structure (Nguyen, 2004) that acts as a protein-conducting translocase channel from where unfolded EF and LF translocate to the cytosol (Bann, 2012; Thoren & Krantz, 2011). The proton gradient (ΔpH) generated by the endosomal acidification is thought to be a major driving force promoting pore-formation and EF and LF unfolding and translocation (Krantz et al, 2006). Nevertheless, the binding of the translocating chains to the surface of the PA channel is proposed to assist the unfolding step (Thoren & Krantz, 2011; Thoren et al, 2009) and, auxiliary host cell factors such as the COPI complex (Tamayo et al, 2008) and the chaperones Hsp90 and cyclophilin A (Dmochewicz et al, 2011) have been proposed to assist the delivery of LF into the cytosol. LF is a zinc metalloprotease that specifically cleaves and inactivates mitogen activated protein (MAP) kinase kinases (Duesbery et al, 1998; Vitale et al, 1998) causing the impairment of the host immune responses (Turk, 2007). EF is a calcium- and calmodulin-dependent adenylate cyclase that catalysis the synthesis of cAMP from cellular ATP (Leppla, 1982) increasing cAMP intracellular levels, thus interfering with several intracellular signalling pathways and upsetting the water homeostasis causing edema (Turk, 2007).

of the catalytic A subunit across the pore, are not yet completely understood and differ among toxins. Nevertheless, evidences suggest that it is not exclusively toxin- and pH-dependent since ATP (Lemichiez et al, 1997) as well as several cytosolic proteins such as coatamer protein complex I (COPI) (Lemichiez et al, 1997; Ratts et al, 2005; Tamayo et al, 2008; Trujillo et al, 2010), chaperoning proteins (Dmochewitz et al, 2011; Haug et al, 2004; Haug et al, 2003; Kaiser et al, 2011; Ratts et al, 2003) thioredoxin reductase (Ratts et al, 2003) and cyclophilin A, a peptidyl-prolyl *cis/trans* isomerase (Kaiser et al, 2011; Kaiser et al, 2009), have been reported to be involved in the translocation process.

3.3.2. “Long-trip” toxins

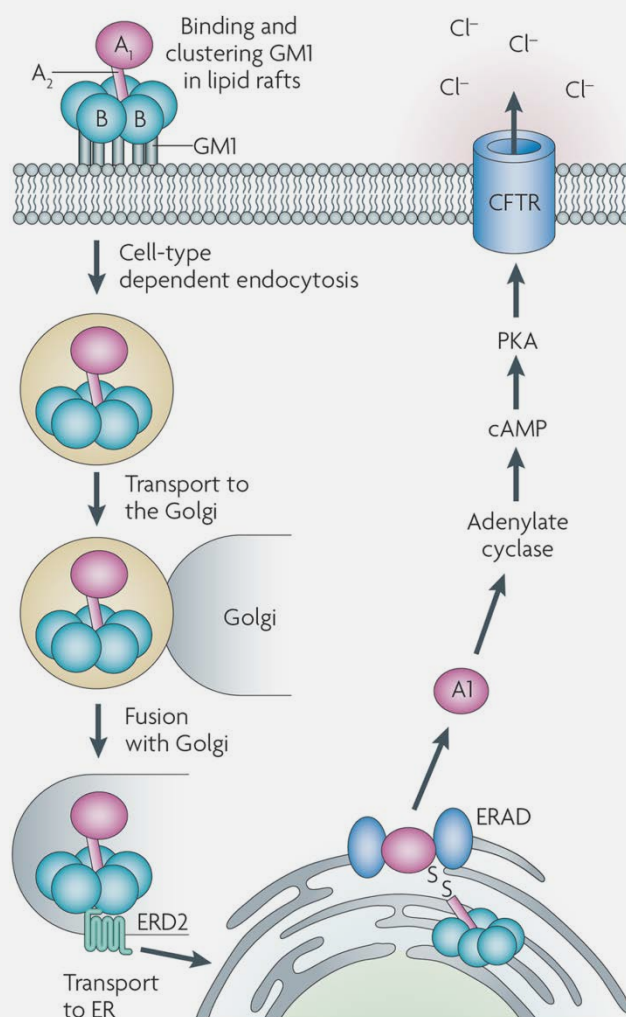
Members of the AB₅ toxin family such as CT (see box 3), Stx, LT and PT follow a different route and take a longer trip to access cytosolic substrates (Beddoe et al, 2010) (Figure 1.9). These toxins exploit one of several retrograde trafficking pathways and travel from the plasma membrane, via early endosomes and Golgi network, to the ER (Beddoe et al, 2010; Locht et al, 2011; Sandvig et al, 2010a; Spooner & Lord, 2012; Wernick et al, 2010). Once at the ER these toxins disguise themselves as misfolded proteins and use the machinery of the ER-associated protein degradation (ERAD) pathway (the process by which misfolded proteins are sorted and exported from the ER lumen to the cytosol for proteasomal degradation) to translocate to the cytosol (Beddoe et al, 2010; Locht et al, 2011; Sandvig et al, 2010a; Spooner & Lord, 2012; Wernick et al, 2010).

The pentameric B components of these AB₅ toxins are multivalent lectins that recognize the oligosaccharide domain of membrane glycolipids at the cell surface: Upon association with lipid rafts, the toxin-receptor complexes have the ability to move retrogradely and function as transport vehicles from the plasma membrane to the ER, via Golgi network (Cho et al, 2012; Lencer & Saslowsky, 2005). At the C-terminus of their A2 domain, CT and LT contain a KDEL or a KDEL-like (RDEL) sequence (known ER retention and retrieval signals that bind to KDEL-receptors (Munro & Pelham, 1987; Raykhel et al, 2007)), respectively, that emerge beyond the boundaries of the B-subunits and were shown to be important for toxicity of those toxins (Hazes & Read, 1997; Lencer et al, 1995). AB₅ toxins arrive the ER as fully folded stable molecules and are thus poor substrates for dislocation processes. The key to their toxicity hence lies on the ability to unfold upon ER arrival (Pande et al, 2007; Spooner & Lord, 2012). The molecular mechanisms involved in toxin unfolding and translocation are yet mostly obscure. Nevertheless, ER chaperones such as the redox-dependent chaperone, protein disulphide isomerase (PDI) were reported to be involved in the release of the A1 fragment (Taylor et al, 2011; Tsai et al, 2001) and the Sec61 translocon

and the derlin-1-Hrd1 complex to be involved in toxin translocation to the cytosol (Bernardi et al, 2008; Bernardi et al, 2010; Koopmann et al, 2000; Schmitz et al, 2000). Once at the cytosol, the A1 fragment must refold and escape the ultimate destination of the ER associated degradation (ERAD) pathway: the proteasomal degradation (Spooner & Lord, 2012; Wernick et al, 2010). The catalytic portions of these ER-trafficking toxins have an unusual low lysine contents which presumably helps them to escape ubiquitination and subsequent proteasomal degradation (Hazes & Read, 1997).

Box 3. Cholera toxin

Cholera toxin (CT) is secreted by the bacterium *Vibrio cholerae* the causative agent of cholera disease. CT is secreted in the intestinal lumen and acts on intestinal epithelial cells causing profuse, watery diarrhea and vomiting. CT is a hetero-hexameric protein complex composed of a catalytic subunit (A) bound to a pentameric ring shaped structure of identical (B) subunits responsible for the binding of the toxin to target cells (Wernick et al, 2010). CT binds to the ganglioside GM1 (monosialotetrahexosylganglioside) found in the plasma membrane microdomains (lipid rafts) (Fujinaga et al, 2003), and can cluster five GM1 molecules at once due to the ring-like pentameric structure of its binding domain (Merritt et al, 1994). Endocytosis of the toxin complex from the plasma membrane to early endosomes can occur by various mechanisms involving clathrin- and caveolin-dependent and independent mechanisms, and is followed by traffic of the toxin to early and recycling endosomes (Massol et al, 2004; Orlandi & Fishman, 1998; Torgersen et al, 2001). From the recycling endosomes, CT travels to the TNG, through an yet not completely understood process, but in general believed to be independent of the late endosomal pathway (Amessou et al, 2007; Ganley et al, 2008; Mallard et al, 2002), from where it is transported to the ER, apparently bypassing the Golgi-cisternae (Feng et al, 2004). Once at the ER, through the action of the chaperone protein disulphide isomerase (PDI), the catalytic domain unfolds and is released from the rest of the toxin (Tsai et al, 2001). The unfolded domain is then transferred across the ER membrane into the cytosol, presumably using the Sec61 channel (Schmitz et al, 2000), or the derlin-1-Hrd1 complex (Bernardi et al, 2008; Bernardi et al, 2010). Upon entry on the cytosol, the CT catalytic domain is refolded to a stable enzymatically active conformation and escapes proteasomal degradation, possibly with the help of cytosolic chaperones (Ampapathi et al, 2008; Pande et al, 2007; Wernick et al, 2010). At the cytosol, CT binds to NAD and catalyzes the ADP ribosylation of GTP-binding protein G of the adenylate cyclase complex (Cassel & Selinger, 1977; Spangler, 1992) leading to its super-activation and thus increasing intracellular cAMP concentrations interfering with several intracellular signalling pathways that lead to deregulation of intestinal fluid homeostasis (Viswanathan et al, 2009).



(From Viswanathan et al. 2009)

3.3.3. *Pseudomonas* exotoxin A: the double-route toxin

So far, the PE is the only example of a toxin reported to be able to translocate into the cytosol from the endosomal compartment and also to follow the retrograde pathway and translocate from the ER (Morlon-Guyot et al, 2009; Weldon & Pastan, 2011). Upon receptor-mediated endocytosis, the toxin reaches the endosomal compartment, where exposure to the low endosomal pH induces major conformational changes within the toxin (Farahbakhsh et al, 1987; Jiang & London, 1990; Mere et al, 2005). From this step two different scenarios have been described: i) the entire toxin inserts and translocates across the endosomal membrane in a process driven by ATP hydrolysis (Alami et al, 1998; Jiang & London, 1990; Mere et al, 2005; Morlon-Guyot et al, 2009; Taupiac et al, 1996) or ii) the toxin can be proteolytically processed (by furin) (Inocencio et al, 1994) and reduced (presumably by PDI or PDI like enzyme) (McKee & FitzGerald, 1999) generating a 37 kDa C-terminal fragment (lacking the receptor binding domain) which is retrogradely transported to the ER and translocated into the cytosol using ER machinery (Jackson et al, 1999; Ogata et al, 1990; Smith et al, 2006; Yoshida et al, 1991).

The relevance of each of the described pathways in PE toxicity is still controversial. Data from Morlon-Guyot and colleagues indicate that PE processing is dispensable for cell intoxication and that cytotoxicity results largely from the endosomal translocation of the entire toxin (Morlon-Guyot et al, 2009). However, several lines of evidence support the importance of the retro translocation from the ER for toxicity. At its C-terminus, PE contains a REDLK sequence that resembles the ER retrieval motif KDEL (Chaudhary et al, 1990). Deletion or the REDLK sequence significantly reduces PE toxicity (Chaudhary et al, 1990), and replacement of the REDLK sequence by KDEL results in an increased toxicity (Seetharam et al, 1991). In addition, the use of antibodies preventing the retrograde transport of the KDEL receptor from the Golgi complex to the ER resulted in reduced PE cytotoxicity (Jackson et al, 1999). Furthermore, treatment with drugs that disrupt the Golgi - ER transport protected cells from PE intoxication (Seetharam et al, 1991; Yoshida et al, 1991). Therefore, additional studies are needed in order to fully understand the intricate mechanisms of PE intoxication.

B. AIP56 toxin

1. AIP56: a key virulence factor of *Photobacterium damsela* *piscicida*

AIP56 (apoptosis inducing protein of 56 kDa) is an exotoxin secreted by the Gram-negative *Photobacterium damsela piscicida* (*Phdp*), the causative agent of fish pseudotuberculosis or photobacteriosis, a bacterial infection that seriously affects warm water marine fish species (Romalde, 2002; Romalde & Magarinos, 1997). Due to its wide host range, massive mortality, ubiquitous geographical distribution, widespread antibiotic resistance and lack of effective vaccines the disease is considered one of the most threatening infections in marine culture worldwide (Romalde, 2002; Toranzo et al, 2005).

Phdp infections are of a necrotizing character and are characterized by generalized septicaemia and bacteraemia with exuberant cytopathology with numerous foci of cell necrosis (do Vale et al, 2007a). Internally, infected fish exhibit haemorrhagic septicaemia and granulomatous lesions in the kidney, spleen and liver (Barnes et al, 2005; Magariños et al, 1996c; Thune et al, 1993). These granulomatous lesions appear as white tubercles, hence the designation of the diseases as pseudotuberculosis, and consist of accumulations of bacteria and apoptotic and necrotic cell debris (do Vale et al, 2007a; Magariños et al, 1996c; Romalde, 2002). In advanced phases of the disease, lesions in gut lamina propria were also observed, which are accompanied by extensive detachment of epithelial linings and shedding of isolated enterocytes that undergo anoikis (do Vale et al, 2007b).

Different studies have identified several factors/mechanisms participating in *Phdp* virulence (do Vale et al, 2007a; do Vale et al, 2003; do Vale et al, 2005; Magariños et al, 1996a; Magariños et al, 1994; Magariños et al, 1996b; Magariños et al, 1992; Noya et al, 1995). However, it is now well established that AIP56 is the main virulence factor of *Phdp* (do Vale et al, 2005), as supported by the fact that: (i) AIP56 is only secreted by *Phdp* virulent strains, (ii) passive immunization of sea bass with anti-AIP56 antibodies provides significant protection against *Phdp* challenge and, (iii) intraperitoneal injection of AIP56 reproduces the pathology of the disease and leads to a dose-dependent lethality (do Vale et al, 2007a; do Vale et al, 2005).

AIP56's virulence mechanism relies on the destruction of the host phagocytic cells (macrophages and neutrophils) (do Vale et al, 2005) through a process involving both the extrinsic and intrinsic apoptotic pathways, with activation of caspases -8, -9, and -3 and with the involvement of mitochondria (with loss of mitochondrial membrane potential, release of cytochrome c and over production of reactive oxygen species) (Costa-Ramos et al, 2011). This simultaneous targeting of both professional phagocytes is a powerful pathogenic

strategy that inevitably leads to a severe depletion in the number of these leukocytes, causing not only the impairment of phagocytosis (do Vale et al, 2007a; do Vale et al, 2007b; do Vale et al, 2003; do Vale et al, 2005) but also affecting cytokine production (Nascimento et al, 2007a; Nascimento et al, 2007b). The dramatic weakening of the host immune responses allows the rapid spread of the bacteria throughout the infected host, with massive extracellular multiplication occurring mainly in spleen and head kidney, hence contributing to the establishment of a septicaemic infection (do Vale et al, 2007a; do Vale et al, 2003). The bacterial spread with colonization of the organs triggers a local inflammatory response with the recruitment to the infectious *foci* of phagocytic cells that also undergo AIP56-induced apoptosis (do Vale et al, 2007a; do Vale et al, 2003; Silva, 2010).

Under normal conditions apoptotic cells are efficiently phagocytosed by scavenger cells, mainly macrophages, thus preventing necrotic cell death with autolysis (Maderna & Godson, 2003; Ren & Savill, 1998; Silva et al, 2008). However, in the context of *Phdp* infections, due to the depletion of macrophages, there is an inefficient clearing of the apoptosing cells, and the AIP56-induced phagocyte apoptosis culminates in secondary necrosis, with the release of their highly cytotoxic content (do Vale et al, 2007a). One of the most potent proteolytic and destructive enzymes is the neutrophil elastase, which was found in the blood of fish with terminal infection and whose levels correlate with the ones of active caspase-3 (do Vale et al, 2007a).

AIP56 toxin is the most abundant protein of mid-exponential phase virulent *Phdp* culture supernatants (do Vale et al, 2005) but, more importantly, during the course of infection it is abundantly secreted and systemically distributed, being detected in the plasma of the diseased animals as well as in areas with accumulation of apoptosing cells, namely in the spleen, head kidney and gut (do Vale et al, 2007a). Being an exotoxin, AIP56 exerts its cytotoxic activity at a distance without requiring direct contact between the pathogen and the target cells, which is decisive for the rapid course and high mortality of acute fish pasteurellosis. This AIP56-dependent pathogenicity mechanism is central in the etiopathogenesis of the disease and results in two effects that operate in concert against the host: evasion of the pathogen from a crucial defence mechanisms and the release of cytotoxic molecules causing tissue damage (do Vale et al, 2007a).

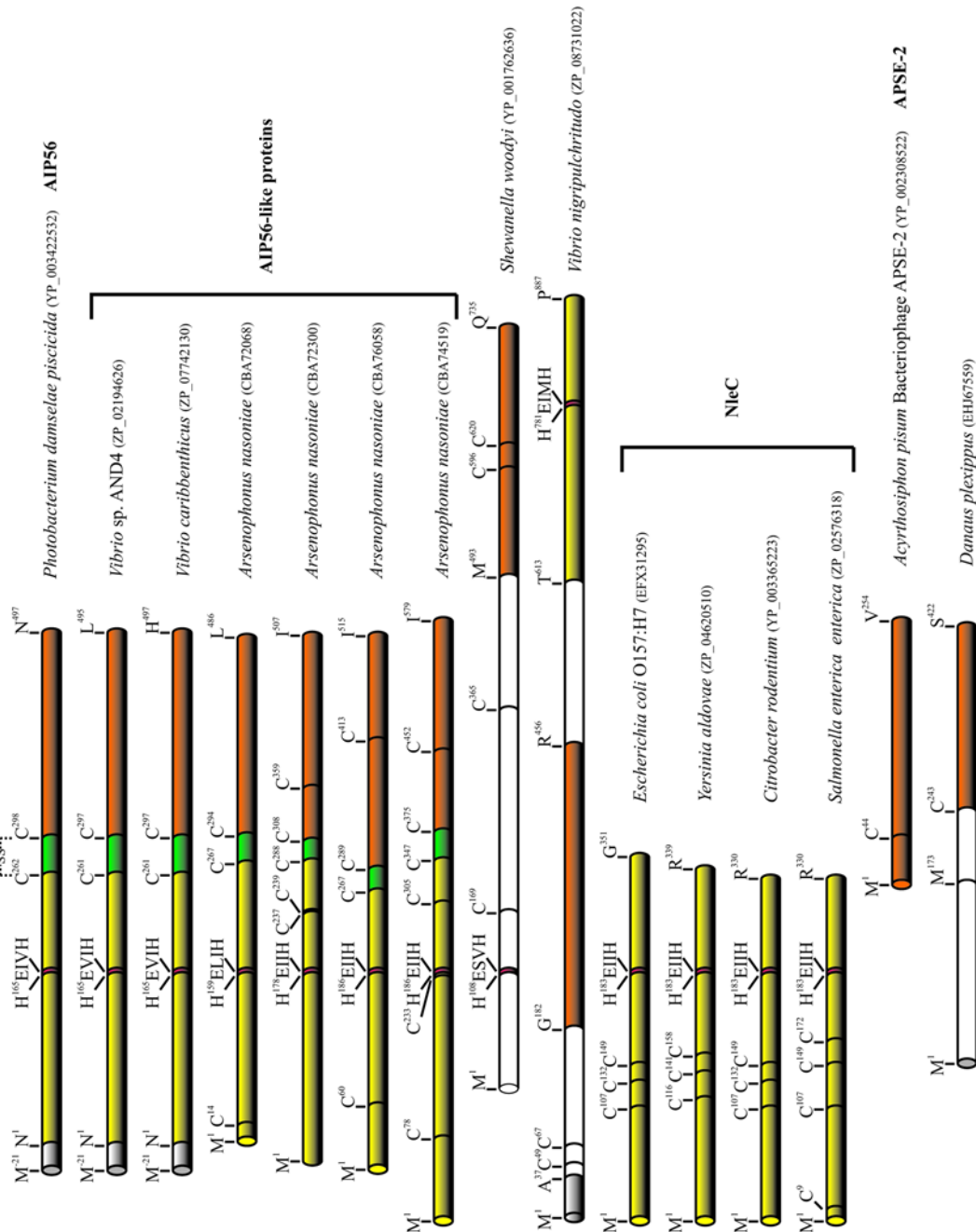
2. AIP56 primary structure and homologies

AIP56 is a plasmid-encoded exotoxin, synthesized as a precursor protein of 520 amino acids with an N-terminal cleavable signal peptide of 23 residues (Silva et al, 2010). When first characterized, (do Vale et al, 2005), the protein was annotated as a 513 amino acid precursor protein with a 16 amino acid residues cleavable signal peptide. However, an alternative GTG start codon exists 21 nucleotides upstream of the previously annotated ATG start codon (Silva et al, 2010). AIP56 has a conserved zinc metalloprotease signature (HEXXH) (Jongeneel et al, 1989), consisting of the amino acid sequence HEIVH, and only two cysteine residues (do Vale et al, 2005). Several AIP56 homologue proteins, from different organisms (Figure 1.10), were retrieved by Blast analysis of AIP56 against the non-redundant protein sequences database (Silva et al, 2010). The retrieved hits include full-length uncharacterized homologues of *Vibrio* species and *Arsenophonus nasoniae* (Wilkes et al, 2010), bacteria pathogenic to aquatic organisms and wasps, respectively. The N-terminal region (first 324 amino acids) of AIP56 also shares homology with NleC (do Vale et al, 2005; Silva et al, 2010), a type III secreted effector present in several enteric pathogenic bacteria, while the C-terminal region was found to be identical to an uncharacterized hypothetical protein of *Acrythosiphon pisum* bacteriophage APSE-2 (Degnan et al, 2009) and to the C-terminal of a recently annotated hypothetical protein of the monarch butterfly *Danaus plexippus*.

Recently, the AIP56 N-terminal homologue, NleC, was shown to be a zinc dependent metalloprotease with specific activity towards NF- κ B p65. This cleavage led to NF- κ B inactivation and consequent repression of NF- κ B-dependent gene transcription, particularly inhibiting the tumour necrosis factor (TNF)- α induced interleukin (IL)-8 expression and thus contributing to the dampening of the host inflammatory response during infection (Baruch et al, 2010; Muhlen et al, 2011; Pearson et al, 2011; Sham et al, 2011; Yen et al, 2010).

Although the pathological consequences of AIP56 have been well studied and documented, very little is known about its structure and how it exploits the host cellular machinery to reach its cellular targets and kill the cells.

Figure 1.10. Schematic diagram of the primary structure of AIP56 and AIP56-related proteins retrieved by Blast analysis of the AIP56 protein sequence against the non-redundant protein sequences database. Grey: signal peptides (experimentally determined for AIP56 (do Vale et al, 2005) and predicted for the remaining proteins using SignalP at <http://www.cbs.dtu.dk/services/SignalP/> (Bendtzen et al, 2004; Nielsen et al, 1997); Yellow: regions with high identity to NleC and AIP56 N-terminal catalytic domain; Green: regions with high identity with 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 domains, NleC or APSE-2. Conserved zinc-metalloprotease signature HEXXH, cysteine residues, and other signalled amino acids are represented at their relative positions. (Updated from (Silva et al, 2010))



C- Nuclear factor- κ B (NF- κ B)

1. Overview

The nuclear factor- κ B (NF- κ B) is a family of transcription factors consisting of homo- or heterodimers formed by association of different members of the Rel/NF- κ B family. The NF- κ B was first identified as a protein that binds to a specific decameric DNA sequence (ggg ACT TTTC C) within the intronic enhancer of the immunoglobulin kappa light chain in mature B and plasma cells (Sen & Baltimore, 1986). Presently, it is acknowledged that these proteins regulate the expression of hundreds of genes that are associated with diverse cellular processes, such as development, cellular growth and apoptosis, as well immune and inflammatory responses thereby playing a central role in the host response to infection by microbial pathogens (Gilmore & Wolenski, 2012; Le Negrata, 2012; Neish & Naumann, 2011; Rahman & McFadden, 2011; Vallabhapurapu & Karin, 2009).

Rel/NF- κ B proteins are evolutionarily conserved from the fruit fly *Drosophila melanogaster* to humans (Gilmore & Wolenski, 2012; Wang et al, 2006). More recently, Rel/NF- κ B homologues were also found in simple organisms such as sea anemones and corals (Meyer et al, 2009; Shinzato et al, 2011; Wolenski et al, 2011), sponges (Gauthier & Degnan, 2008) and in the horseshoe crab, *Carcinoscorpius rotundicauda*, considered to be a living fossil (Wang et al, 2006). Despite there is a paucity of information on the molecular components and regulation of the most primitive NF- κ B signalling pathways, their evolutionary conservation underlines the pivotal role of NF- κ B pathways in a multitude of important cellular processes (Gilmore & Wolenski, 2012).

2. The NF- κ B family of transcription factors

Transcriptionally active NF- κ B dimers are formed by combinatorial association of five subunits: NF- κ B 1 (p50), NF- κ B 2 (p52), RelA (p65), RelB and c-Rel (Figure 1.11). Fifteen possible combinations can arise from these five different proteins and this multiplicity contributes to transcriptional selectivity of the NF- κ B response (Oeckinghaus & Ghosh, 2009; Smale, 2012). All these dimers share the ability to bind a family of DNA-binding sites (9-11 base pairs in length) collectively known as κ B sites (Natoli et al, 2005). Each homo- or heterodimer may be activated in a cell type- and stimulus-specific manner and, while most dimer combinations are abundant in diverse cell types, others are rare and a few have not been detected directly. Nevertheless, it remains likely that the rare ones (such as c-Rel:RelB heterodimer) also exist in some cells under specific regulatory conditions (Ghosh et al, 2012;

Smale, 2012). The term NF- κ B commonly refers specifically to the p50/p65 heterodimer since it is the most abundant in most cells and was the first NF- κ B dimer to be identified (Gilmore, 1999).

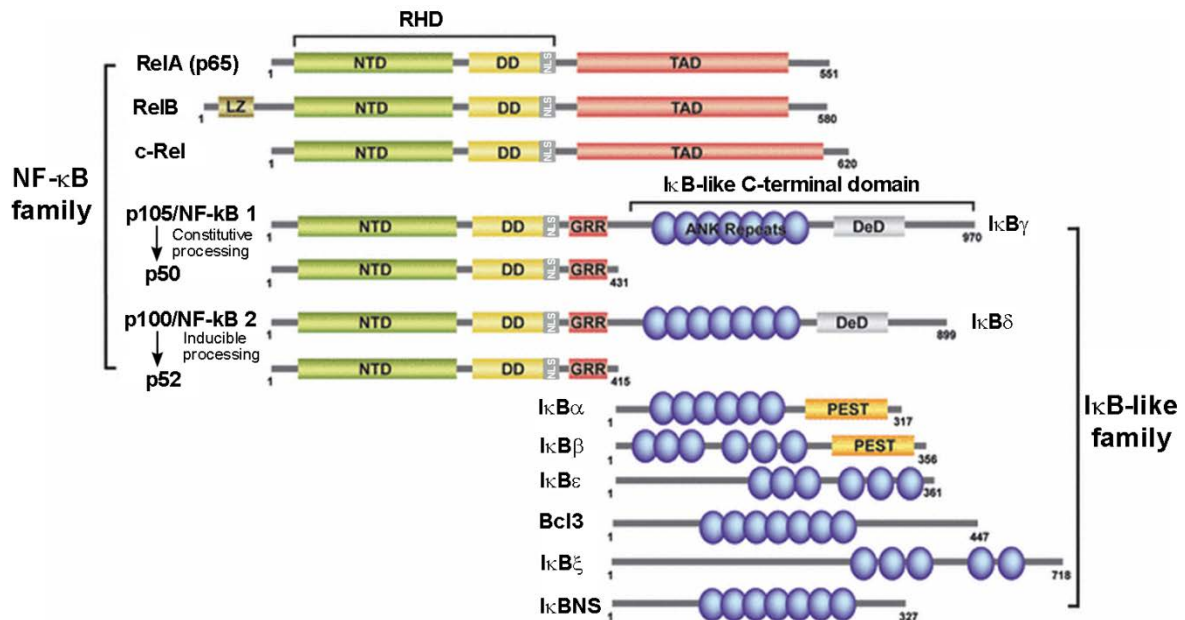


Figure 1.11. Members of the NF- κ B transcription factor family. The five protein members share a highly conserved Rel homology domain (RHD) at their N-terminal region that can be divided into three structural regions: the N-terminal domain (NTD) that contains sequences required for dimerization and nuclear localization, the dimerization domain (DD) that mediates the association of NF- κ B subunits to form combinatorial dimers, and the nuclear localization signal (NLS) (in grey). The RelA, RelB and c-Rel family members at C-terminal region contain the transcriptional activation domain (TAD). The p50 and p52 are synthesized as large precursors (p105 and p100, respectively) which undergo proteolytic processing. These proteins lack a C-terminal TAD and instead contain a glycine-rich region (GRR), remnant of their processing from p105 and p100 precursors. I κ B proteins are characterized by the presence of multiple ankyrin repeats and include the p105 and p100 proteins. (From (Ghosh et al, 2012))

The proteins in the NF- κ B family are related through the highly conserved N-terminal region, called Rel homology domain (RHD). This domain is roughly 300 residues in length and is responsible for the most critical NF- κ B functions (Ghosh et al, 2012). It can be divided into three structural regions: the N-terminal domain (NTD), the dimerization domain (DD) and the nuclear localization signal (NLS) (Figure 1.11). The DD alone mediates association of NF- κ B subunits to form dimers and together with the NTD perform the DNA binding function, which is further modulated by additional post-translational modifications, including phosphorylation and acetylation (Ghosh et al, 2012; Huang et al, 2010; Zheng et al, 2010). Based on sequences C-terminal to the RHD, the proteins of this family can be divided into two classes: (i) the p65, c-Rel and RelB, which contain a C-terminal transactivation domain

(TAD) essential for transcriptional activation, and (ii) the p50 and p52 proteins, which lack the TAD and instead contain a C-terminal glycine-rich region (Ghosh et al, 2012; Gilmore & Wolenski, 2012; Smale, 2012) (Figure 1.11). The TAD region mediates the interaction with various components of the transcriptional apparatus, including TATA-binding protein, transcription factor IIB, p300, and CBP transcriptional co-activators and is not conserved, at the amino acid level, between the different NF- κ B subunits (Chen & Greene, 2004). Thus, as a consequence of their lack of a C-terminal TAD, NF- κ B dimers composed only of p50 and/or p52 subunits fail to activate transcription (Ghosh et al, 2012). The p50 and p52 proteins are synthesized as large precursors, p105 and p100 which undergo processing with selective degradation of their C-terminal region containing ankyrin repeats (Figure 1.11). Processing is mediated by the ubiquitin/proteasome pathway and in the case of p100 processing is a postranslational and signal induced event whereas in the case of p105 processing is constitutive (Ghosh et al, 2012; Sun, 2012).

3. NF- κ B activation and gene expression

In quiescent cells, NF- κ B dimers are held captive in the cytoplasm via non-covalent interactions with a class of proteins called inhibitors of NF- κ B (I κ Bs) (Hinz et al, 2012) (Figure 1.11). These NF- κ B inhibitors are characterized by the presence of multiple ankyrin repeats which mediate the association between I κ B and NF- κ B dimers. As with the Rel/NF- κ B proteins, there are several I κ B proteins, with different affinities for individual Rel/NF- κ B complexes, which are regulated and expressed differently (Hinz et al, 2012). The I κ B proteins also include the NF- κ B precursor's p105 and p100, which contain at their C-terminal halves multiple ankyrin repeats that allow them to display an I κ B-like function (Figure 1.11). These ankyrin repeats interact with the DD and NLS regions of NF- κ B subunits, thus preventing their translocation to the nucleus (Ghosh et al, 2012; Hinz et al, 2012; Oeckinghaus & Ghosh, 2009). Activation of NF- κ B involves phosphorylation and degradation of I κ B or I κ B-like proteins, freeing NF- κ B dimers to migrate to the nucleus where they bind to κ B sites within the promoters/enhancers of target genes, and regulate transcription through the recruitment of co-activators and co-repressors (Gilmore & Wolenski, 2012; Hayden & Ghosh, 2008; Wang et al, 2006; Wolenski et al, 2011). Many different stimuli involved in several signalling cascades lead to activation of NF- κ B transcription factors. Nevertheless, in general terms, NF- κ B activation is controlled by two main signalling pathways: the canonical and the non-canonical pathway, which differ on the stimuli and on the activated NF- κ B dimers (Figure 1.12) (Oeckinghaus & Ghosh, 2009).

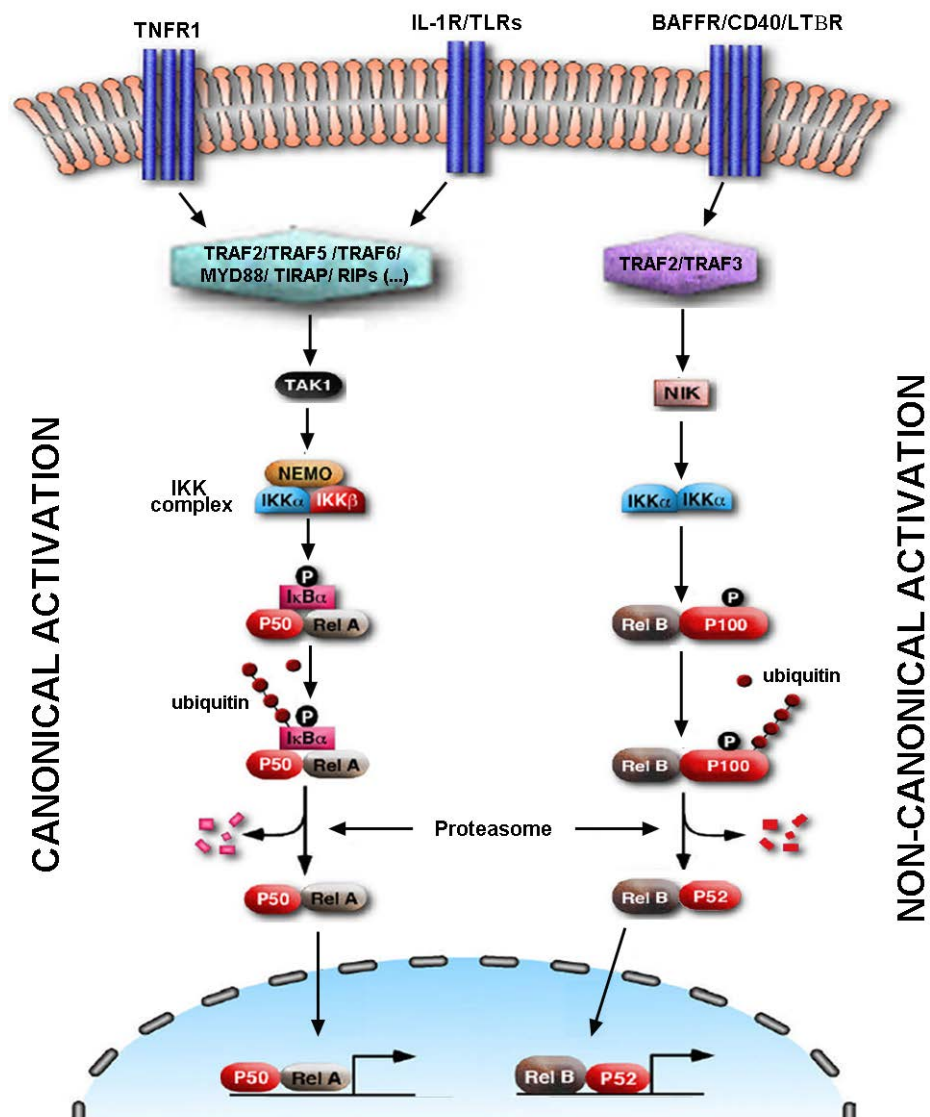


Figure 1.12. NF-κB activation pathways. Under resting conditions, NF-κB dimers are sequestered in the cytoplasm bound to inhibitory IκB proteins. The canonical NF-κB activation (left) is induced mainly by inflammatory stimuli including activation of TNF receptor-1 (TNFR1), IL-1 receptor (IL-1R) and Toll-like receptors (TLRs). Ligand binding to these receptors triggers signalling cascades involving several adaptor and signal transduction proteins that culminate in the activation of the protein kinase TAK-1 which in turn phosphorylates and activates the IκB kinase (IKK) complex (consisting of two catalytically active kinases, IKKα and IKKβ, and a regulatory subunit NEMO). Activated IKK phosphorylates IκB proteins bound to NF-κB dimers, mostly p65-containing heterodimers. Phosphorylated IκB proteins are targeted for ubiquitination and proteasomal degradation, freeing NF-κB dimers that translocate to the nucleus. In contrast, non-canonical NF-κB activation (right) leads to nuclear translocation of p100-containing dimers and is induced by a subset of TNF family cytokines, such as CD40 ligand, B-cell activating factor (BAFF) and lymphotoxin-β (LT-β) causing activation of the NF-κB-inducing kinase (NIK). NIK activation leads to phosphorylation and activation of the IKKα complexes, which in turn phosphorylate C-terminal residues in p100 leading to its ubiquitination and proteasomal processing into transcriptionally competent p52/RelB complexes that translocate to the nucleus and induce target gene expression. (BAFFR: B-cell activating factor receptor, LT-βR: lymphotoxin-β receptor, TRAF: TNFR-associated factor, MYD88: myeloid differentiation primary response protein 88; TIRAP: Toll-interleukin 1 receptor domain-containing adaptor protein; RIPs: receptor-interacting proteins) (Adapted from (Chen, 2005))

In the canonical pathway, the inducible degradation of I κ Bs, particularly I κ B α , leads to nuclear translocation of various NF- κ B complexes, comprising dimeric combinations of p65, c-Rel, RelB and p50, from which p50/p65 and c-Rel:p50 are the predominant activated dimers (Vallabhapurapu & Karin, 2009) (Figure 1.12). This pathway is considered to be the central initiating event of host responses to invasion by microbial pathogens and, typically, responds to (i) proinflammatory cytokines, which bind to cytokine receptors, such as IL-1 receptor (IL-1R) and TNF receptor-1 (TNFR1), or to (ii) pathogen-associated molecular patterns (PAMPs), including microbial and viral products, which bind to pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), RIG-I-like receptors, nucleotide oligomerization domain (NOD)-like receptors, and C-type lectin receptors (Le Negrte, 2012; Neish & Naumann, 2011; Vallabhapurapu & Karin, 2009). Ligand binding to these receptors, triggers distinct signalling cascades that use multiple adaptors (including TNFR-associated factors (TRAFs), myeloid differentiation primary response protein 88 (MYD88) and Toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP)) and intermediate transducing molecules (including IL-1 receptor-associated kinases (IRAK) and receptor-interacting proteins (RIPs)) converging in activation of the I κ B kinase (IKK) complex (Oeckinghaus & Ghosh, 2009). The IKK complex is composed by two highly homologous kinase subunits IKK α and IKK β and a regulatory subunit IKK γ (also named NEMO) (Israel, 2010) (Figure 1.12). Signal-induced activation of this complex, results in phosphorylation of I κ B α (bound to NF- κ B dimers) leading to its polyubiquitination subsequent proteasomal degradation, exposing NF- κ B nuclear localization domain and leading to nuclear translocation of the liberated NF- κ B dimers (Oeckinghaus & Ghosh, 2009). The IKK complex may also phosphorylate the TAD regions of p65 and c-Rel, while in the cytoplasm, thus also regulating their transcriptional activity. Once at the nucleus, NF- κ B dimers up-regulate the expression of a battery of genes, mostly related with the immune and inflammatory responses, including chemotactic chemokines (such as IL-8 and monocyte chemoattractant protein 1), immunoregulatory cytokines (such as TNF- α and IL-1) and neutrophil adhesion proteins (such as the Intracellular Adhesion Molecule). Furthermore, the secreted TNF- α and IL-1 β also start a feedback loop for a second phase of NF- κ B activation that continues the induction of robust immune responses (Le Negrte, 2012; Neish & Naumann, 2011; Vallabhapurapu & Karin, 2009).

The non-canonical NF- κ B activation pathway relies on the inducible processing of the ankyrin repeats of the p100 subunit through the activation of the NF- κ B-inducing kinase (NIK) (Razani et al, 2011; Sun, 2011; Sun, 2012) (Figure 1.12). NIK activation triggers IKK α phosphorylation and activation. The NIK-activated IKK α phosphorylates NF- κ B p100 within

its C-terminal region (containing the ankyrin repeats), leading to its polyubiquitination and subsequent proteasomal processing into the mature p52 form. As p100 preferentially interacts with RelB, the processing of p100 not only generates p52 but also causes p52/RelB nuclear translocation (Razani et al, 2011; Sun, 2011; Sun, 2012). Non-canonical NF- κ B activation is involved in the regulation of lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism (Razani et al, 2011; Sun, 2011; Sun, 2012). In contrast to the canonical activation, which responds to signals elicited by diverse stimuli, non-canonical NF- κ B activation, is restricted to specific members of the TNF cytokine family including B-cell activating factor (BAFF), CD40 ligand, lymphotoxin- β , TNFR2 ligands among others (Sun, 2011). A common feature on the non-canonical NF- κ B-stimulating receptors is the possession of a TRAF-binding motif, involved in the recruitment of TRAF members (particularly TRAF2 and TRAF3) to the receptor complex. This recruitment is essential to the activation of NIK and to the subsequent p100 processing (Sun, 2011).

The mechanisms involved in NF- κ B activation and in the regulation of its transcriptional activity are of remarkable complexity and are not yet completely understood. The intricacy of these mechanisms transcends the signalling cascades that lead to the release of NF- κ B proteins from their inhibitory I κ B proteins. Additional posttranslational modifications of NF- κ B subunits, including phosphorylation, acetylation, ubiquitination and methylation, also modulate target gene specificity and transcriptional activity, adding another layer to the regulation of NF- κ B activity (Chen & Greene, 2004; Huang et al, 2010; Perkins, 2006).

Deregulation of NF- κ B activity is a common scenario in many diseases. Uncontrolled NF- κ B activation is often associated with multiple inflammatory, autoimmune and cancer diseases (DiDonato et al, 2012; Rayet & Gelinas, 1999; Staudt, 2010). Furthermore, and given the central role of NF- κ B in coordinating host immune responses, many successful pathogens have acquired sophisticated mechanism to shut off NF- κ B signalling by developing subversive proteins or hijacking host signalling molecules (Le Negrate, 2012; Neish & Naumann, 2011; Rahman & McFadden, 2011).

CHAPTER II

PROJECT AIMS

AIP56 was identified as an exotoxin secreted by the fish bacterial pathogen *Photobacterium damsela* *piscicida* (*Phdp*). It acts by triggering apoptosis in macrophages and neutrophils, resulting in lysis of these phagocytes by post-apoptotic secondary necrosis. This AIP56-induced phagocyte cell death results not only in an impairment of host immune defenses, through the destruction of phagocytes, but also in the release of the cytotoxic contents of the phagocytes, leading to tissue damage and contributing to the formation of the necrotic lesions observed in the diseased animals.

Although *in vivo* studies showed that AIP56 is a key virulence factor of *Phdp*, many questions remain to be answered regarding the AIP56 molecular mechanism(s) of action. Analysis of the AIP56 sequence suggests the existence of two domains/subunits, as in many other bacterial toxins such as diphtheria and the clostridial neurotoxins, which have an enzymatic domain responsible for toxicity and a binding domain responsible for cell binding and internalization. Within its N-terminal region AIP56 also processes a zinc binding signature, typical of zinc-dependent metalloproteases, which would presumably be involved in the toxin's catalytic activity.

In this thesis, we aimed at extending the characterization of AIP56 by:

- 1. Analysing AIP56 catalytic activity and establishing its relevance for the toxin's biological activity
- 2. Identifying AIP56 molecular target(s)
- 3. Investigating the AIP56 domain structure and defining the role played by the AIP56 domains
- 4. Attempting to solve AIP56 crystal structure

CHAPTER III

MATERIAL AND METHODS

1. Recombinant proteins

The recombinant proteins used in this work are listed in table 3.1. Some were expressed in *E. coli* while others were synthesized *in vitro* as ^{35}S -labeled proteins using rabbit reticulocyte lysates.

Table 3.1. Recombinant proteins used in this study.

Name	Description	Expression system
AIP56	Wild type, full length AIP56 toxin	<i>E. coli</i>
AIP56^{AAIVAA}	AIP56 mutant in the zinc metalloprotease signature (His ¹⁶⁵ , Glu ¹⁶⁶ , His ¹⁶⁹ and His ¹⁷⁰ mutated to Ala)	<i>E. coli</i>
AIP56¹⁻²⁸⁵	AIP56 N-terminal portion (Asn ¹ -Phe ²⁸⁵)	<i>E. coli</i>
AIP56²⁸⁶⁻⁴⁹⁷	AIP56 C-terminal portion (Phe ²⁸⁶ -Asn ⁴⁹⁷)	<i>E. coli</i>
AIP56^{1-285C262S}	AIP56 ¹⁻²⁸⁵ cysteine mutant (Cys ²⁶² mutated to Ser)	<i>E. coli</i>
AIP56^{286-497C298S}	AIP56 ²⁸⁶⁻⁴⁹⁷ cysteine mutant (Cys ²⁹⁸ mutated to Ser)	<i>E. coli</i>
LF¹¹⁻²⁶³.AIP56¹⁻²⁶¹	Chimera consisting of the amino-terminal portion of anthrax lethal factor (Val ¹¹ -Arg ²⁶³) fused to AIP56 N-terminal region (Asn ¹ -Phe ²⁶¹)	<i>E. coli</i>
LF¹¹⁻²⁶³.AIP56²⁹⁹⁻⁴⁹⁷	Chimera consisting of the amino-terminal portion of anthrax lethal factor (Val ¹¹ -Arg ²⁶³) fused to AIP56 C-terminal region (Thr ²⁹⁹ -Asn ⁴⁹⁷)	<i>E. coli</i>
NleC	Type III secreted effector from enteropathogenic bacteria homologous to AIP56 N-terminal region	<i>E. coli</i>
NleC¹⁻²⁷⁰.AIP56²⁵¹⁻⁴⁹⁷	Chimera consisting of the first 270 amino acid residues (Met ¹ -Glu ²⁷⁰) of NleC fused to AIP56 C-terminal region (AIP56 ²⁵¹⁻⁴⁹⁷)	<i>E. coli</i>
LF¹¹⁻²⁶³.NleC	Chimera consisting to the amino- terminal portion of anthrax lethal factor (Val ¹¹ -Arg ²⁶³) fused to NleC	<i>E. coli</i>
LF¹¹⁻²⁶³.NleC^{Mut}	LF ¹¹⁻²⁶³ .NleC chimera cysteine mutant where Cys ¹⁰⁷ and Cys ¹⁴⁹ of NleC were mutated to His and Tyr, respectively	<i>E. coli</i>
sbp65Rel	Sea bass NF-κB p65 Rel homology domain (Met ¹ -Arg ¹⁸⁸)	<i>E. coli</i> / Rabbit reticulocyte
sbp65RelC39A	Sea bass NF-κB p65 Rel homology domain Cys ³⁹ mutant	Rabbit reticulocyte
sbp65RelE40A	Sea bass NF-κB p65 Rel homology domain Glu ⁴⁰ mutant	Rabbit reticulocyte
sbp65C39AE40A	Sea bass NF-κB p65 Rel homology domain Cys ³⁹ -Glu ⁴⁰ double mutant	Rabbit reticulocyte

1.1. Constructs

For *E. coli* protein expression, DNA coding sequences were cloned into the NcoI/XhoI restriction sites of pET-28a(+) vector (Novagen) in frame with a C-terminal 6xHis-tag. For production of ^{35}S -labeled proteins, untagged versions were engineered using a reverse

primer that introduces a stop codon before the 6xHis-tag of the pET-28a(+)vector. The primers used are listed in table 3.2.

Mutated versions of the proteins were obtained by site-directed mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer instructions.

Table 3.2. Primers used in this study. Restriction enzyme recognition sequences are underlined.

Primer designation	Nucleotide sequence 5'→3'
AIP56EHECFw1BstBI	CGCTTCGAAAGGCTGGGTACGATCAGTGACCGATATGAGGCTTCGCCTGACTTCGGCACCC TGACCTCTTTT
AIP56Fw4NcoI	GCGCCATGGACAACGATAAACCAGATGCAAGC
AIP56Fw6NcoI	GCGCCATGGTTCTCCCTAGCGCTAGCGCCG
AIP56Rv5XhoI	GCGCTCGAGATTAATGAATTGTGGCGCGTGGG
AIP56Rv7XhoI	GAGCTCGAGAAAGGCGCGCCCCGTTG
AIP56Rv9XhoI	GAGCTCGAGAAAAGAGGTCAGGGTGCCGAA
CtermAIP56Fw1SacI	GCGGAGCTCACTTTTGATGTACTAAATCGAAT
DLp65Fw1NcoI	CGCCCATGGAAGGTGTGTATGGATGGAGCCTG
DLp65Rv2XhoI	GCGCTCGAGTCACCTGTTGTCATAGATGGGCTGCG
DLp65Rv4XhoI	GCGCTCGAGCCTGTTGTCATAGATGGGCTGCG
EHECFw1NcoI	CATGCCATGGAAATTCCTCATTACAG
EHECRv1XhoI	CCGCTCGAGTTGCTGATTGTGTTTGTC
LFFw1NcoI	GCGCCATGGTAAAAGAGAAAGAGAAAAATAAAG
LFRv1SacI	GCGGAGCTCCCGTTGATCTTTAAGTTCTTC
NleCFw1SacI	GCGGAGCTCATGGAATTCCTCATTACA
NtermAIP56Fw1SacI	GCGGAGCTCAACAACGATAAACCAGATGCAAGC

AIP56

Recombinant AIP56 was expressed using the plasmid pETAIP56H⁺ (do Vale et al, 2005). This plasmid contains the AIP56 coding sequence including the 16-amino acid signal peptide that is removed from the protein upon secretion yielding a mature recombinant AIP56 (do Vale et al, 2005).

AIP56^{AAIVAA}

The plasmid (pETAIP56^{AAIVAA}) coding for a mutated version of AIP56, in which His¹⁶⁵, Glu¹⁶⁶, His¹⁶⁹, and His¹⁷⁰ of the zinc-binding signature were replaced by Ala, was obtained by site-directed mutagenesis of the pETAIP56H⁺ plasmid (do Vale et al, 2005). As AIP56^{AAIVAA} could not be produced as a soluble protein (see section 1.2 below) a construct coding for

mature AIP56^{AAIVAA} (lacking the signal peptide) was produced using pETAIP56^{AAIVAA} as template and the AIP56Fw4NcoI/AIP56Rv5XhoI primer combination.

AIP56¹⁻²⁸⁵, AIP56²⁸⁶⁻⁴⁹⁷, AIP56^{1-285C262S} and AIP56^{286-497C298S}

Plasmids coding for truncated versions of AIP56 were designed based on the chymotrypsin cleavage site (see section 2.6). pETAIP56H⁺ (do Vale et al, 2005) was subjected to PCR amplification using primers AIP56Fw4NcoI/AIP56Rv7XhoI for AIP56¹⁻²⁸⁵ and AIP56Fw6NcoI/AIP56Rv5XhoI for AIP56²⁸⁶⁻⁴⁹⁷. PCR fragments were cloned into pET-28a(+) in frame with a C-terminal His-tag, yielding pETAIP56¹⁻²⁸⁵ and pETAIP56²⁸⁶⁻⁴⁹⁷. Plasmids pETAIP56^{1-285C262S} and pETAIP56^{286-497C298S}, coding for mutated versions of AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷, in which Cys²⁶² or Cys²⁹⁸ were mutated to Ser (AIP56^{1-285C262S} or AIP56^{286-497C298S}), were obtained by site directed mutagenesis of pETAIP56¹⁻²⁸⁵ or pETAIP56²⁸⁶⁻⁴⁹⁷.

NleC

The NleC-coding sequence was amplified by PCR with primers EHECFw1NcoI and EHECRv1XhoI using total DNA (Sambrook & Russel, 2001) from *E. coli* O157:H7 strain 4462 as template. PCR products were cloned into pET-28a(+) in frame with a C-terminal 6xHis-tag yielding pETNleC.

NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷

For production of a chimeric protein consisting of NleC¹⁻²⁷⁰ fused to AIP56²⁵¹⁻⁴⁹⁷, the sequence coding for NleC¹⁻²⁷⁰ was amplified from pETNleC (see above) using the EHECFw1NcoI/ EHECRv1XhoI primer combination and the sequence coding for AIP56²⁵¹⁻⁴⁹⁷ was amplified from pETAIP56H⁺ (do Vale et al, 2005) using primers AIP56EHECFw1BstBI and AIP56Rv5XhoI. The PCR products were cloned into the pGEM-T-easy vector (Promega) and pGEM-T-easy carrying *nleC*¹⁻²⁷⁰ was digested with BstBI/XhoI to allow ligation of the *aip56*²⁵¹⁻⁴⁹⁷ fragment, excised from the pGEM-T-easy carrying *aip56*²⁵¹⁻⁴⁹⁷ by digestion with BstBI/XhoI. The resulting plasmid was digested with NcoI/XhoI and the excised fragment cloned into pET-28a(+), yielding pETNleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷.

LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷, LF¹¹⁻²⁶³·NleC and LF¹¹⁻²⁶³·NleC^{Mut}

Chimeric proteins consisting of the amino-terminal region of anthrax lethal factor (LF¹¹⁻²⁶³) fused to AIP56 N-terminal segment (AIP56¹⁻²⁶¹), AIP56 C-terminal portion (AIP56²⁹⁹⁻⁴⁹⁷) or NleC were constructed. The sequence encoding LF¹¹⁻²⁶³ was amplified with primers LFFw1NcoI/LFRv1SacI from plasmid pRSET A containing the LF gene (Zornetta et al, 2010), the regions encoding AIP56¹⁻²⁶¹ and AIP56²⁹⁹⁻⁴⁹⁷ were amplified from pETAIP56H⁺ (do Vale

et al, 2005) using primer combinations NtermAIP56Fw1SacI/AIP56Rv9XhoI and CtermAIP56Fw1SacI/AIP56Rv5XhoI, respectively, and the sequence coding for NleC¹⁻³³⁰ was amplified from pETNleC (see above) with primers NleCFw1Sac1/EHECRv1XhoI. PCR fragments were digested with SacI, and LF¹¹⁻²⁶³ ligated to AIP56¹⁻²⁶¹, AIP56²⁹⁹⁻⁴⁹⁷ or NleC. Ligations were subjected to PCR using the primer combination LFFw1NcoI/AIP56Rv9XhoI for LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LFFw1NcoI/AIP56Rv5XhoI for LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷, or LFFw1NcoI/EHECRv1XhoI for LF¹¹⁻²⁶³·NleC and PCR products were cloned into pET-28a(+). A mutated version of LF¹¹⁻²⁶³·NleC (LF¹¹⁻²⁶³·NleC^{Mut}), in which the NleC conserved Cys¹⁰⁷ and Cys¹⁴⁹ were replaced by His and Tyr, respectively, was produced by site-directed mutagenesis using the plasmid pETLF¹¹⁻²⁶³·NleC as template.

sbp65Rel, sbp65RelC39A, sbp65RelE40A and sbp65C39AE40A

The coding region (Met¹-Arg¹⁸⁸) of sea bass p65 REL domain (sbp65Rel) was amplified by PCR from cDNA obtained as described in (Pinto et al, 2012) using primers DLp65Fw1NcoI and DLp65Rv4XhoI. For production of ³⁵S-labeled sbp65Rel the primer DLp65Rv2XhoI, that introduces a stop codon before the XhoI restriction site, was used instead of DLp65Rv4XhoI, with the purpose of producing a protein without a 6xHis-tag. PCR products were cloned in pET-28a(+). Additional sbp65Rel mutants, where Cys³⁹ and/or Glu⁴⁰ were mutated to Ala (sbp65RelC39A, sbp65RelE40A, and sbp65C39AE40A, respectively) were produced.

1.2. Production of recombinant proteins

1.2.1. Cell-free expression of ³⁵S-labeled proteins

Untagged sbp65Rel and sbp65Rel mutants (sbp65RelC39A, sbp65RelE40A, and sbp65C39AE40A) were produced as ³⁵S-labeled proteins in rabbit reticulocyte lysates by *in vitro* translation using the TNT T7 Quick Coupled transcription/Translation kit (Promega), in the presence of RedivueTM L-[³⁵S] methionine (specific activity of N1000 Ci/mmol) following the manufacturer's instructions.

1.2.2. Protein production in bacteria

Before large-scale protein production, expression conditions and protein solubility were evaluated in small-scale cultures. Expression and solubility of the recombinant proteins was assessed by SDS-PAGE analysis. Different *E. coli* host strains and variable induction times and temperatures were tested to select the protocols resulting in higher yield of soluble

proteins. With exception of AIP56^{AAIVAA} and AIP56^{1-285C262S} that could only be obtained in the insoluble fraction as inclusion bodies, the remaining proteins were recovered soluble.

Recombinant His-tagged proteins were expressed in *E. coli* BL21(DE3) cells grown in Lysogenic broth (LB) (Bertani, 2004) supplemented with 50 µg/ml kanamycin. Protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and carried out overnight at 17 °C, except for AIP56^{AAIVAA}, AIP56^{1-285C262S}, NleC, LF¹¹⁻²⁶³·NleC, LF¹¹⁻²⁶³·NleC^{Mut} and sbp65Rel, which were expressed at 37 °C for 4 h. Starting cultures were grown overnight at 37 °C, used to inoculate fresh medium at 1:100 dilution and grown at 37 °C. For expressions at 17 °C, cultures were equilibrated to this temperature prior to induction. Protein expression was induced when bacterial cultures reached an OD₂₆₀ of approximately 0.6. Induced bacterial cells were collected by centrifugation and resuspended in 50 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 200 µg/ml lysozyme, 250 µg/ml phenylmethylsulfonyl fluoride (PMSF), in the proportion of 5 ml buffer per gram of cell pellet. Bacterial cells were lysed by freeze/thaw cycles followed by 20 min incubation on ice, in the presence of 10 µg/ml DNase I from bovine pancreas type IV (Sigma) and 10 mM MgCl₂, and sonication to ensure complete cell lysis. For AIP56 used in crystallization trials, induced bacterial cells were resuspended in 50mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 1% (v/v) Triton X-100, 100 µg/ml PMSF, and immediately lysed by sonication. Soluble and insoluble fractions were obtained by centrifugation of the total cell lysates at 16000 g for 15 min at 4 °C.

1.3. Protein purification

Recombinant proteins produced in *E. coli* were purified by chromatographic methods using commercially available chromatography columns and following the manufacturer's instructions. Affinity and ion-exchange chromatography was performed on a Biologic LP system (BioRad) at room temperature and size-exclusion chromatography was performed on a Biologic DuoFlow system (BioRad) at 4°C. Before loading onto chromatography columns, protein samples were centrifuged at 16000 g for 15 min at 4 °C. All chromatography buffers were filtered through 0.45 µm filters and degassed before use. Purified proteins were dialysed against the specified buffers and concentrated by ultrafiltration using Amicon Ultra-15 Centrifugal Filter Units (Millipore), divided into small aliquots, frozen in liquid nitrogen, and stored at -80 °C. The purification procedures (optimised for each protein) are described below.

1.3.1. Proteins purified from the soluble fraction of induced bacterial cells

AIP56

The protocol for AIP56 production was adapted from (do Vale et al, 2005) with some modifications to increase protein purity. The soluble fraction of induced bacterial cells, obtained as described in section 1.2.2, was supplemented with 20 mM imidazole and loaded onto HisTrap HP columns (GE Healthcare). Bound proteins were eluted with step-increasing concentrations of imidazole in 50 mM sodium phosphate buffer pH 7.4, 500 mM NaCl. Eluted fractions were collected into tubes containing 50 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, to lower protein and imidazole concentrations and minimize AIP56 precipitation. Fractions containing high ratio of AIP56/contaminant proteins, were pooled and dialysed overnight at 4 °C against 2 x 50 volumes of 20 mM Tris-HCl pH 8.0, 200 mM NaCl, using Spectra/Por 3 dialysis membrane (Spectrum Laboratories). Dialysed sample was subjected to anion-exchange chromatography using a Bio-Scale™ Unosphere Q cartridge (BioRad). Prior to loading into the column, the sample was diluted in 20 mM Tris-HCl to lower NaCl concentration to 66 mM. Protein elution was done using a linear NaCl gradient (66 mM to 1 M) in 20 mM Tris-HCl pH 8.0, at a flow rate of 2.5 ml/min for 60 min, and fractions containing AIP56 were pooled, dialysed against 10 mM Tris-HCl pH 8.0, 200 mM NaCl, and stored as described above.

To purify AIP56 for crystallization trials some modifications were introduced, resulting in significantly purer and more homogeneous protein preparations. AIP56 was isolated from the soluble fraction of induced bacterial cells by affinity chromatography as described above. Fractions containing high ratios of AIP56/contaminants were pooled, dialyzed against 20 mM Tris-HCl pH 8.0, 300 mM NaCl, using PD-10 desalting columns (GE Healthcare) and stored overnight on ice. The second purification step consisted in an anion-exchange chromatography using in a Bio-Scale™ High Q cartridge (BioRad). Before loading onto the column, the NaCl concentration was lowered to 100 mM by dilution in 20 mM Tris pH 8.0. Protein was eluted using a linear NaCl gradient (100 mM to 1 M) as described above. Eluted fractions containing highly pure AIP56 were pooled, dialyzed against 10 mM Tris-HCl pH 8.0, 200 mM NaCl, concentrated to 10 mg/ml, aliquoted and stored as described above (section 1.3).

AIP56¹⁻²⁸⁵, AIP56²⁸⁶⁻⁴⁹⁷ and AIP56^{286-497C298S}

Purification of AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷ was done as described above for AIP56. Purified AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷ were dialysed against 20 mM Tris-HCl pH 8.0, 200 mM NaCl and stored as described above (section 1.3).

AIP56^{286-497C298S} was purified by nickel-affinity chromatography as described for AIP56, except for the presence of 10% (v/v) glycerol in elution buffers. Selected elution fractions were pooled and subjected to size exclusion chromatography using a Sephacryl S100 HR column (GE Healthcare) equilibrated in 20 mM Tris-HCl pH 8.0, 200 mM NaCl. Protein was eluted in the same buffer, and fractions containing pure AIP56^{286-497C298S} were pooled, dialysed against 20 mM Tris-HCl pH 8.0, 200 mM NaCl and 10% (v/v) glycerol, and stored as described above (section 1.3).

NleC

NleC was purified by affinity chromatography as described for AIP56 but using 20 mM sodium phosphate buffer pH 8.0, 500 mM NaCl, with increasing concentrations of imidazole for protein elution. Fractions containing low amounts of contaminating proteins were pooled and subjected to size-exclusion chromatography (Sephacryl S100 HR column, GE Healthcare) in 20 mM Tris-HCl pH 8.0, 50 mM NaCl. Fractions containing NleC were pooled, dialysed against 20 mM Tris-HCl pH 8.0, 50 mM NaCl and stored as described above (section 1.3).

NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷

NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ was purified by nickel-affinity chromatography followed by anion-exchange chromatography as described for AIP56. Purified protein was dialysed against 20 mM Tris-HCl pH 8.0, 200 mM NaCl and stored as described above (section 1.3).

LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷, LF¹¹⁻²⁶³·NleC and LF¹¹⁻²⁶³·NleC^{Mut}

LF¹¹⁻²⁶³·NleC and LF¹¹⁻²⁶³·NleC^{Mut} were purified by nickel-affinity chromatography followed by anion-exchange chromatography as described for AIP56. LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ and LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷ were purified by nickel-affinity chromatography as described for AIP56. In the case of LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, to avoid the formation of protein aggregates, sonication of cell lysates was omitted and glycerol (5% (v/v) final concentration) was added to cell lysates and affinity chromatography buffers. Purified LF·AIP56 and LF·NleC chimeras were dialysed against 10 mM Tris-HCl pH 8.0, 200 mM NaCl and stored as described above (section 1.3).

sbp65Rel

His-tagged sbp65Rel was purified from the soluble fraction of induced bacterial cells by affinity chromatography using HIS-Select Nickel Affinity Gel (Sigma). The protein bound to the resin was eluted with increasing concentrations of imidazole in 50 mM sodium phosphate buffer pH 7.4, 300 mM NaCl. Fractions containing sbp65Rel were pooled, dialysed against

50 mM sodium phosphate buffer pH 7.4, 300 mM NaCl and stored as described above (section 1.3).

1.3.2. Proteins purified from the insoluble fraction of induced bacterial cells

AIP56^{AAIVAA} and AIP56^{1-285C262S} were obtained in the insoluble fraction of induced bacterial cells and therefore, were subjected to a refolding protocol. Refolding conditions were first optimised using AIP56 obtained as inclusion bodies from *E. coli* cells induced at 37 °C (do Vale et al, 2005), because its correct folding could be functionally assessed by testing its ability to induce apoptosis in sea bass peritoneal leukocytes. The established protocol was applied to AIP56^{AAIVAA} and AIP56^{1-285C262S}, and the biochemical and biophysical properties of the refolded proteins, determined by Native-PAGE, size-exclusion chromatography and circular dichroism spectroscopy (CD), compared to those of AIP56 or AIP56¹⁻²⁸⁵.

AIP56^{AAIVAA}

Inclusion bodies were extracted by washing the insoluble fraction of induced bacterial cells (obtained as described in section 1.2.2) with 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 1% (v/v) Triton X-100, 5% (v/v) glycerol, 0.5 mM EDTA followed by two washes in 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 5% (v/v) glycerol, 0.5 mM EDTA; centrifugations were performed at 10000 g for 15 min at 4 °C. Inclusion bodies were solubilized in 8 M urea, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 1 mM 2-mercaptoethanol, 5 mM imidazole and purified by zinc-affinity chromatography (HisTrap HP columns, GE Healthcare) under denaturing conditions. The protein was eluted with increasing concentrations of imidazole in 8 M urea, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 1 mM 2-mercaptoethanol. Fractions containing AIP56^{AAIVAA} were pooled, quantified by densitometric analysis of Coomassie Blue-stained gels (as described below in section 6.3) and concentration adjusted to 0.1 mg/ml. The protein was then subjected to refolding through dialysis against 3 x 50 volumes of sea bass phosphate saline buffer (sbPBS), 10% (v/v) glycerol, at 4 °C using Spectra/Por 3 dialysis membrane. The refolded protein was purified by size exclusion chromatography using a Sephacryl S200 HR column (GE Healthcare) in 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol and fractions containing pure AIP56^{AAIVAA} were pooled, dialysed against 10 mM Tris-HCl pH 8.0, 200 mM NaCl and stored as described above (section 1.3).

AIP56^{1-285C262S}

AIP56^{1-285C262S} was purified as described above for AIP56^{AAIVAA} except that size exclusion chromatography was performed in a Sephacryl S100 HR column (GE Healthcare) and the protein was stored in 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol.

1.4. Chemically and enzymatically modified AIP56

Alkylated AIP56

In order to disrupt the disulphide bridge linking Cys²⁶² and Cys²⁹⁸ by alkylation, AIP56 at 63 μ M 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, dialysed against 20 mM Tris-HCl pH 8.0, 200 mM NaCl and stored as described in section 1.3. Disruption of the disulphide bridge was confirmed by reducing and non-reducing SDS-PAGE analysis.

Nicked AIP56

In order to obtain nicked AIP56, purified recombinant AIP56 adjusted to 0.6 mg/ml in 10 mM Tris-HCl pH 8.0 with 200 mM NaCl was incubated with 0.05 mg/ml of chymotrypsin for 45 min on ice. The reaction was stopped by addition of 250 μ g/ml PMSF. Digestion products were applied to a HisTrap HP column (GE Healthcare) and the bound proteins eluted with step-increasing concentrations of imidazole in 50 mM sodium phosphate buffer pH 7.4, 500 mM NaCl. Elution fractions were analysed under reducing and non-reducing SDS-PAGE and the ones containing the two AIP56 digestion fragments linked by a disulphide bridge, pooled, dialysed against 10 mM Tris-HCl pH 8.0, 200 mM NaCl and stored as described in section 1.3.

1.5. Reconstitution of AIP56 from AIP56 truncates

Reconstituted AIP56 (AIP56rec) consists of AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷ linked by a disulphide bridge. Purified AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷ were mixed in equimolar amounts in 8 M urea, 1mM DTT, refolded by extensive dialysis against sbPBS and stored as described in section 1.3. Formation of the disulphide-bound heterodimer (AIP56¹⁻²⁸⁵/AIP56²⁸⁶⁻⁴⁹⁷) was confirmed by reducing and non-reducing SDS-PAGE analysis. Quantification of the heterodimer was performed by densitometric analysis of Coomassie Blue-stained gels (as described below section 6.3).

2. Biochemical, biophysical and structural characterization

2.1. Dynamic light scattering

Dynamic light scattering (DLS) was performed in *Zetasizer Nano ZS* (Malvern Instruments) at 20 °C in a 45 µL quartz cuvette (Hellma). Stability and homogeneity of purified AIP56 (1 mg/ml) in different buffers was evaluated by measuring scattering intensity (kilo counts per second) as a function of hydrodynamic diameter (nm) at fixed conditions (temperature and pH). 10 mM Tris-HCl pH 8.0 with NaCl concentrations ranging from 100 to 500 mM with or without glycerol (5 or 10% v/v) were tested. Data analysis was performed using the Dispersion Technology Software (DTS) 5.03 (Malvern Instruments). The polydispersity index (PI), which is representative of particle width distribution, was used as indicator of protein homogeneity and stability (Borgstahl, 2007).

2.2. Size exclusion chromatography

Protein samples at 0.25 mg/ml in 10 mM Tris-HCl pH 8.0, 200 mM NaCl were subjected to analytical size-exclusion chromatography in a Superose 12 10/300 column (GE Healthcare) using an ÄKTA Purifier FPLC system (GE Healthcare) at room temperature and a 0.5 ml/min flow rate. The column was pre-equilibrated with 10 mM Tris-HCl pH 8.0, 200 mM NaCl and protein elution was monitored by measuring the absorbance at 280 nm.

2.3. Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectra were acquired on an Olis DSM 20 circular dichroism spectropolarimeter continuously purged with nitrogen, equipped with a Quantum Northwest CD 150 Temperature-Controlled cuvette holder and controlled by the Globalworks software. Scans were collected between 190 and 260 nm in 1 nm intervals at 20 °C with a 0.2 mm path-length cuvette. Three scans with an integration time of 4 seconds were averaged for each measurement. Protein concentration was determined by absorbance measurements as described above. Proteins were dissolved in 10 mM Tris-HCl pH 8.0, 50 mM NaCl. The results are expressed in terms of mean residue ellipticity $[\Theta]_{MRW}$ in $\text{deg cm}^2 \text{dmol}^{-1}$, according to the equation $[\Theta]_{MRW} = \Theta_{obs} * Mw * 100 / (l * c * N)$ where Θ_{obs} is the observed ellipticity in mdeg, Mw is the protein molecular weight in g/mol, l is the cuvette path length in cm, c is the protein concentration in g/l and N is the number of residues of the protein. Analysis of the protein secondary structure was performed using the Globalworks software algorithm.

2.4. Analysis of zinc content

The zinc content of the proteins (0.1-0.2 mg/ml) was determined by atomic absorption spectroscopy with flame optimisation in an Atomic Absorption Spectrometer PU 9200X (Philips). All buffers were prepared with chemicals of the highest purity available and using Milli-Q grade water. All material was previously immersed for 24 h in 10% (v/v) nitric acid, washed with Milli-Q grade water and autoclaved in paper bags. Before metal determination, protein samples were extensively dialyzed against 10 mM Tris-HCl pH 8.0, 200 mM NaCl using Amicon Ultra-15 centrifugal filter devices (Millipore). Metal content was measured after standardization in the linear concentration range (0-1 ppm). Results are expressed as the mean $\pm$ SD of three independent measurements carried out in triplicate.

2.5. Differential scanning fluorimetry

The thermal stability of AIP56, AIP56^{nic}, LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·NleC, or LF¹¹⁻²⁶³·NleC^{Mut} at different pH was monitored by following SYPRO Orange (Invitrogen) fluorescence at 585 nm (excitation = 545nm) on an iQ5 Real Time PCR System (BioRad). Protein samples in 150mM phosphate/citrate buffer pH 7.0, 6.5, 6.0, 5.5 or 5.0 with 150 mM NaCl were mixed 1:1 (v/v) with dye solution in the same buffer in a final volume of 30 μ l in 96-well plates. AIP56 and nicked AIP56 were used at 5 μ M with 10x SYPRO Orange, LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ at 10 μ M with 20x SYPRO Orange, LF¹¹⁻²⁶³·NleC at 1 μ M with 40x SYPRO Orange and LF¹¹⁻²⁶³·NleC^{Mut} at 5 μ M with 40x SYPRO Orange. Controls included no protein and/or no dye. The melting curves were analysed using CFX Manager (BioRad) and the melting temperature (T_m) was calculated as the inflection point of the fluorescence curve of at least 7 measurements in 2 independent experiments.

2.6. Limited proteolysis

Recombinant proteins at 0.6 mg/ml in 10 mM Tris-HCl pH 8.0, 200 mM NaCl were incubated with trypsin, chymotrypsin or proteinase K at concentrations ranging from 0.25 to 25 μ g/ml for 30 min on ice. Proteases were inactivated by the addition of PMSF at a final concentration of 250 μ g/ml. Digests were analysed by SDS-PAGE under reducing and non-reducing conditions. The two major chymotrypsin digestion fragments were subjected to N-terminal sequencing.

3. Bioinformatic analysis

3.1. Biochemical parameters

Theoretical molecular weights and isoelectric points were calculated using the ProtParam tool (<http://web.expasy.org/protparam/>).

3.2. Secondary structure prediction

The secondary structures of AIP56 (NCBI accession number ABA00995) and NleC (NCBI accession number NP_286533) were predicted using the program PSIPRED via its web interface (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Jones, 1999; McGuffin et al, 2000) and confirmed using PredictProtein (<http://www.predictprotein.org/>) (Rost et al, 2004).

3.3. Protein sequence alignments and similarity analysis

Protein sequence alignments were performed using ClustalW2 multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) (Dunbrack, 2006). The percentages of similarity and identity were calculated with MatGAT (Campanella et al, 2003) using default parameters.

4. Activity assays

4.1. Fish and cells

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. Before collection of peritoneal leukocytes fish were euthanized with 2-phenoxyethanol (Panreac; >5 ml/10 L). 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 Direção Geral de Veterinária, the Portuguese authority for animal protection (ref. 004933, 2011-02-22). Sea bass peritoneal leukocytes were obtained as described (Costa-Ramos et al, 2011). Briefly, 12 to 20 days after stimulation with Incomplete Freund's Adjuvant, the peritoneal cells were collected in L-15 medium (Gibco) adjusted to 322 mOsm and supplemented with 10% (v/v) inactivated (60 °C, 40 min) fetal bovine serum (FBS, Gibco), 1% (w/v) penicillin/streptomycin (P/S, Gibco) and 20 U/ml heparin (Braun) (supplemented L-15). 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 erythrocytes (Costa-Ramos et al, 2011). Cells were used at a density of 2×10^6 cells/ml.

4.2. Apoptogenic activity assays

Cells were incubated for 4 h at 22 °C with the specified recombinant proteins at the indicated doses. Where indicated, the cells were pre-treated for 30 min at 22 °C with 25 µM of the pan-caspase inhibitor N-Benzylloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK). In experiments using LF chimeric proteins, cells were incubated with 20 nM of LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ (1.2 mg/ml) or LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷ (1.1 mg/ml) or 700 nM of LF¹¹⁻²⁶³·NleC or LF¹¹⁻²⁶³·NleC^{Mut} (48 mg/ml) with or without 10 nM of anthrax protective antigen (PA) and obtained as described (Tonello et al, 2004). Mock-treated cells were used as control. Apoptosis was assessed by analysis of cytospin preparations, using a set of morphological markers such as occurrence of cell rounding and shrinkage, nuclear fragmentation and/or chromatin condensation, cell blebbing and formation of apoptotic bodies (Hacker, 2000). Experiments were performed using cells collected from at least 3 individual fish. The percentage of apoptotic cells was determined in a blind fashion by scoring a minimum of 350 cells per slide.

4.3. NF-κB p65 cleavage assays

In vitro: NF-κB p65 cleavage was assessed using cell lysates, recombinant sbp65Rel produced in *E. coli* or translated *in vitro* as a ³⁵S-labeled protein. HeLa cells and sea bass peritoneal leukocytes were used to prepare cell lysates. Cells were incubated in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (v/v) Triton X-100 and 10% (v/v) glycerol for 30 min on ice, sonicated briefly and centrifuged. The supernatant of 2×10^6 cells (for sea bass cells) or (1×10^6 cells for HeLa cells) was incubated with 1 µM of the indicated proteins for 2 h at 22 °C and p65 cleavage evaluated by Western blotting as described in section 6.2. Recombinant sbp65Rel (7.5 µM in 50 mM sodium phosphate buffer pH 7.4, 300 mM NaCl) was incubated for 3 h at 22 °C with 1 µM AIP56 and p65 cleavage evaluated by SDS-PAGE. *In vitro* translated ³⁵S-labeled sbp65Rel and ³⁵S-labeled sbp65Rel mutants (sbp65RelC39A, sbp65RelE40A, and sbp65C39AE40A) were incubated with the indicated proteins in 10 mM Tris-HCl pH 8.0, 150 mM NaCl and 10% (v/v) glycerol for 2 h at 22 °C, and p65 cleavage assessed by autoradiography. When specified 1,10-phenanthroline (Sigma) was used at 5 mM.

Ex vivo: Peritoneal leukocytes were incubated for 2 or 4 h as described in section 4.2. After treatment, cells were collected by centrifugation, washed, resuspended in sbPBS and lysed by addition of SDS-PAGE sample buffer (50 mM Tris-HCl pH 8.0, 2% (w/v) SDS, 10% (v/v) glycerol, 0.017% (w/v) bromophenol blue, 2 mM EDTA pH 8.8, 10 mM DTT). Cleavage of p65 was evaluated by Western blotting, as described in section 6.2.

4.4. Competition assays

AIP56^{AAIVAA}, AIP56^{1-285C262S}, AIP56^{286-497C298S}, AIP56^{nic} and NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ were tested for their ability to inhibit AIP56's apoptogenic activity and AIP56-mediated p65 cleavage. Cells were pre-incubated for 15 min on ice with different concentrations (350 nM to 3.5 μ M) of AIP56^{AAIVAA}, AIP56^{1-285C262S}, AIP56^{286-497C298S}, AIP56^{nic} or NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ followed by incubation for further 15 min on ice with 8.75 nM (0.5 μ g/ml) of AIP56 in presence of the competitors. Unbound proteins were removed by washing with ice cold L-15 medium supplemented as described above and cells incubated at 22 °C for 4 h. AIP56's apoptogenic activity and AIP56-mediated p65 cleavage was assessed as described above (section 4.3).

5. AIP56 crystallization trials

5.1. Crystallization condition screening

AIP56 crystallization was screened using the vapor diffusion method at 22 °C in sitting drops. In order to identify potential crystallization conditions an extensive screening was performed using sparse-matrix crystallization kits from Hampton Research, namely IndexTM, Crystal ScreenTM, Crystal Screen 2TM, Crystal Screen CryoTM and PEG/Ion ScreenTM. Crystals were grown in 24 well plates (Cryschem plate, Hampton Research) containing 800 μ l of reservoir solution in each well and 4 μ l drops containing 2 μ l of AIP56 (10 mg/ml in 10 mM Tris-HCl pH 8.0, 200 mM NaCl) and 2 μ l of reservoir solution. Optimization of conditions that yielded microcrystals was performed by variation of pH, concentration of the precipitant agent and by testing different ratios of crystallization solution and protein within the drop. The effect of the presence of additives such as small molecules and detergents was also tested, for condition 0.2 M KSCN, 25% (w/v) PEG 2000, 0.1 M HEPES pH 7.9, 0.2% (v/v) glycerol using Additive ScreenTM and Detergent ScreensTM kits from Hampton Research.

5.2. Seeding

Streak-seeding (Stura & Wilson, 1991) was performed using microcrystals or small crystal plates grown as described above (section 5.1), using 0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 10% (w/v) PEG 6000, 0.1 M Tris-HCl pH 7.0 or 0.2 M KSCN, 25% (w/v) PEG 2000, 0.1 M HEPES pH 7.9 as reservoir solution. Seeds were transferred to sitting drops consisting of 2 μl of protein solution mixed with 2 μl of reservoir solution after overnight equilibration at 22 °C against 400 μl reservoir solution. Prior to being transferred to the new drop, crystals were washed in a clean drop of a solution with a lower precipitant concentration, to induce partial removal of the external layers of the crystals and generate a fresh crystallization surface. Crystallization solutions used in seeding experiments are summarized in table 3.3.

Table 3.3. List of conditions tested in seeding experiments.

Chemical composition of reservoir solution
0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 20% (w/v) PEG 2000, 0.1M Tris-HCl pH 7.0
0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 20% (w/v) PEG 2000, 0.1M Tris-HCl pH 7.9
0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 15% (w/v) PEG 8000, 0.1M Tris-HCl pH 7.0
0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 15% (w/v) PEG 8000, 0.1M Tris-HCl pH 7.9
0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 15%(w/v) PEG 8000, 0.1M Tris-HCl pH 7.0, 10% (v/v) glycerol
0.2 M $(\text{NH}_4)_2\text{HPO}_4$, 15% (w/v) PEG 8000, 0.1M Tris-HCl pH 7.0, 15% (v/v) glycerol
0.2 M KSCN, 20% (w/v) PEG 2000, 0.1 M HEPES pH 7.9
0.2 M KSCN, 25% (w/v) PEG 2000, 0.1 M HEPES pH 7.9
0.2 M KSCN, 15% (w/v) PEG 6000, 0.1 M HEPES pH 7.9
0.2 M KSCN, 20% (w/v) PEG 6000, 0.1 M HEPES pH 7.9
0.2 M KSCN, 10% (w/v) PEG 8000, 0.1 M HEPES pH 7.9
0.2 M KSCN, 15% (w/v) PEG 8000, 0.1 M HEPES pH 7.9
0.2 M KSCN, 20% (w/v) PEG 6000, 0.1 M HEPES pH 7.9, 0.2%(v/v) glycerol
0.2 M KSCN, 20% (w/v) PEG 6000, 0.1 M HEPES pH 7.9, 0.5% (v/v) glycerol
0.2 M KSCN, 20% (w/v) PEG 6000, 0.1 M HEPES pH 7.9, 1% (v/v) glycerol

5.3. *In situ* proteolysis crystallization

AIP56 (10 mg/ml) in 10 mM Tris-HCl pH 8.0, 200 mM NaCl was mixed with chymotrypsin at a 1:10000 (molar ratio) and used in crystallization trials with the sparse matrix crystallization kits PEG/Ion ScreenTM and Crystal Screen CryoTM from Hampton Research as described above (section 5.1).

5.4. Data collection

Glycerol was used as cryoprotective agent and concentrations that ensure cryo-protection were determined for each crystallization condition. Crystals were transferred from their mother liquor to 10 μ l drops of mother liquor containing increasing concentrations of glycerol (up to 20% v/v), mounted in loops (Molecular Dimensions), flash-cooled and stored in liquid nitrogen until data collection.

6. Miscellaneous

6.1. SDS-PAGE, Native-PAGE

SDS- and Native-PAGE were performed using the Laemmli discontinuous buffer system (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R-250 (BioRad). For SDS-PAGE analysis samples were boiled for 5 min in sample buffer (50 mM Tris-HCl pH 8.0, 2% (w/v) SDS, 10% (v/v) glycerol, 0.017% (w/v) bromophenol blue, 2 mM EDTA pH 8.8) with or without 100 mM DTT before loading. Proteins were resolved in 10-15% (w/v) polyacrylamide gels with 1% (w/v) SDS, prepared with acrylamide:bis-acrylamide (30:0.4) stock solution. Native-PAGE was performed in 10% (w/v) polyacrylamide gels prepared with acrylamide:bis-acrylamide (30:1) stock solution, but omitting SDS. Furthermore, samples were not subjected to thermal denaturation prior to loading and reducing agents and SDS were omitted from the loading buffer.

6.2. Western blotting

After SDS-PAGE proteins were transferred onto nitrocellulose membranes in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio Rad) with an applied current of 2 mA/cm² of membrane for 1h. Detection of AIP56 was done using an anti-AIP56 rabbit serum (do Vale et al, 2005). For detection of sea bass NF- κ p65, an anti-sea bass NF- κ B p65 rabbit serum (produced using the peptide SIFNSGNPARFVS located at the C-terminal region of sea bass p65 as antigen) was used and for detection of human NF- κ B p65 a commercially available α -p65 antibody (NF κ B p65 (C-20): sc-372, Santa Cruz Biotechnology) was used. Reactive bands were detected using anti-rabbit IgG alkaline phosphatase conjugate (Sigma) followed by BCIP/NBT development or using an anti-rabbit IgG horseradish peroxidase-linked secondary antibody (The binding site) followed by detection with SuperSignal West Dura Extended Duration Substrate (Pierce biotechnology). Blots shown are representative of at least three independent experiments.

6.3. Protein quantification

Unless otherwise specified, proteins were quantified by measuring absorbance at 280 nm and using the extinction coefficient calculated by the ProtParam tool (<http://www.expasy.org/tools/protparam.html>), using the Edelhoch method (Edelhoch, 1967), but with the extinction coefficients for Trp and Tyr determined by Pace et al (Pace et al, 1995). Alternatively, proteins were quantified by densitometric analysis of Coomassie Blue-stained gels with protein concentration inferred from BSA standards calibration curve.

6.4. Determination of AIP56 concentrations in the plasma of infected fish

Sea bass weighting 16.3 ± 2.4 g were infected intraperitoneally with a lethal dose (1.8×10^6 CFU/fish) of *Photobacterium damsela piscicida* (Phdp) strain PP3 (do Vale et al, 2007a). Growth conditions and inoculum preparation were carried out as described (do Vale et al, 2003; do Vale et al, 2005). Bacterial concentrations of the inoculum were confirmed by plating serial dilutions of the final bacterial suspensions onto TSA-1 plates and counting the number of CFU following incubation at 22 °C for 2 days. Plasmas were collected from moribund fish as previously described (do Vale et al, 2007a). Five-microliter aliquots of plasma or recombinant AIP56 standards (at 50, 25, 10, 2 and 1 µg/ml) were analysed by Western blotting. The concentrations of AIP56 in plasma were determined by densitometric analysis of the blot and inferred from AIP56 standards calibration curve.

6.5. 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 $p < 0.05$.

CHAPTER IV

RESULTS

1. Recombinant proteins

To conduct AIP56 structural and functional characterization studies, several recombinant proteins were engineered and produced. These are represented in Figure 4.1 and include AIP56, AIP56's N-terminal homologue, NleC, as well as several variants of these proteins and wild type and mutant versions of the Rel homology domain of sea bass NF- κ B p65 (the AIP56 putative target).

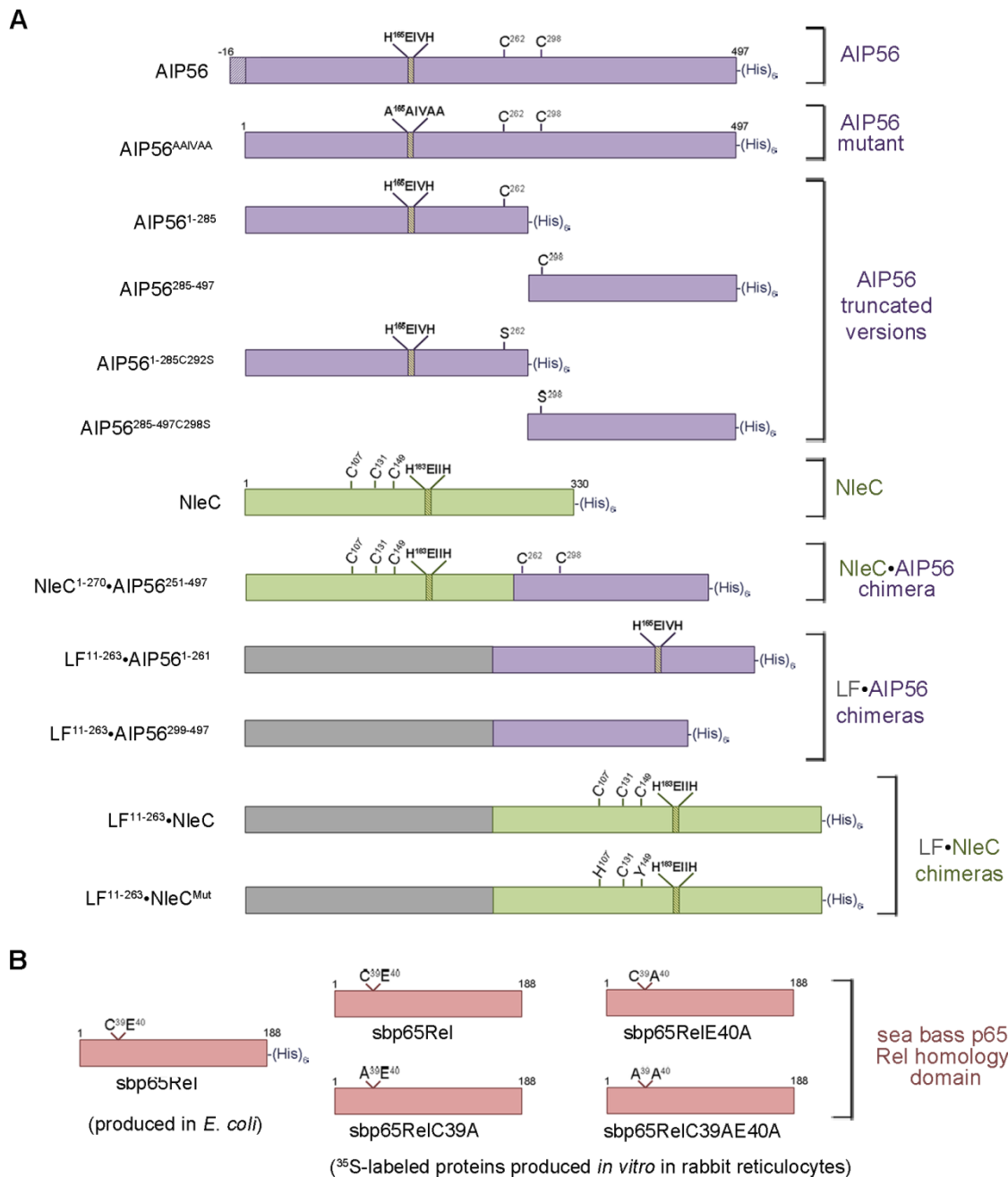


Figure 4.1. Schematic representation of recombinant proteins used in this study. A) Recombinant AIP56, NleC and their variants produced in *E. coli*. **B)** Recombinant sea bass p65 Rel homology domain and sea bass p65 Rel homology domain mutants produced in *E. coli* or *in vitro* in a rabbit reticulocyte system as ^{35}S -labelled proteins.

Recombinant proteins were expressed in *E. coli*, or *in vitro* in rabbit reticulocyte lysates as ^{35}S -labeled proteins. Those produced in *E. coli* contained a C-terminal 6xHis tag added for purification purposes. Conditions for protein expression in *E. coli* were individually established and are detailed in chapter III section 1.1. NleC, LF¹¹⁻²⁶³·NleC, LF¹¹⁻²⁶³·NleC^{Mut} and sbp65Rel were obtained in the soluble fraction of induced bacterial cells at 37 °C (not shown), while soluble AIP56, AIP56¹⁻²⁸⁵, AIP56²⁸⁶⁻⁴⁹⁷, AIP56^{286-497C298S}, LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷ were only obtained when protein expression was carried at 17°C (not shown). However, and despite our efforts to obtain soluble proteins, AIP56^{AAIVAA} and AIP56^{1-285C262S} were exclusively expressed as insoluble proteins and accumulated in bacterial cytoplasm as inclusion bodies (not shown). Hence, to obtain properly folded and functional proteins, refolding conditions were established using AIP56 inclusion bodies (obtained from *E. coli* BL21 (DE3) cells induced for 4 hours at 37 °C (do Vale et al, 2005)). Considering that the metalloprotease mutant was expected to not display catalytic activity, wild type AIP56 was used as control because the correct folding of the wild type protein could be assessed by testing the apoptogenic activity of the toxin in sea bass peritoneal leukocytes (not shown). The established protocol was applied to AIP56^{AAIVAA} and AIP56^{1-285C262S}, and the correct folding of the proteins was confirmed by comparing the biochemical and biophysical properties of the refolded proteins (determined by Native-PAGE, size exclusion chromatography and circular dichroism (CD) spectroscopy), to those of AIP56 or AIP56¹⁻²⁸⁵ as described in detail in the sections below.

The purification protocols were optimised for each protein. Considering that all recombinant proteins contained a C-terminal 6xHis-tag, nickel-affinity chromatography was the first purification step, followed by ion-exchange chromatography or size exclusion chromatography, depending on the molecular weight of protein contaminants. While NleC, its derived proteins and sbp65Rel are stable proteins and caused no major problems during purification, AIP56 and AIP56 derived proteins were unstable in solution, with high tendency to precipitate often leading to poor yields at the end of the purification process. These proteins were particularly prone to precipitate after affinity chromatography while in the presence of imidazole (the competitor ligand to protein elution). Thus, addition of imidazole to the bacterial fractions containing AIP56 was done just prior to loading the extract into the affinity chromatography column and its final concentration in eluted fractions was quickly reduced by dilution with imidazole-free buffer. Addition of glycerol to purification and/or storage buffers also successfully increased AIP56^{AAIVAA}, AIP56^{286-497C298S}, LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ and AIP56^{1-285C262S} stability. Final purification protocols of each individual protein are described in detail in chapter III section 1.3 and the purity of the produced recombinant

proteins was assessed by SDS-PAGE in Figure 4.2. Given the high purity and homogeneity requirements of protein samples to be used in crystallization trials, additional optimisations in the purification of AIP56 to be used for that purpose were undertaken (see section 5 of this chapter).

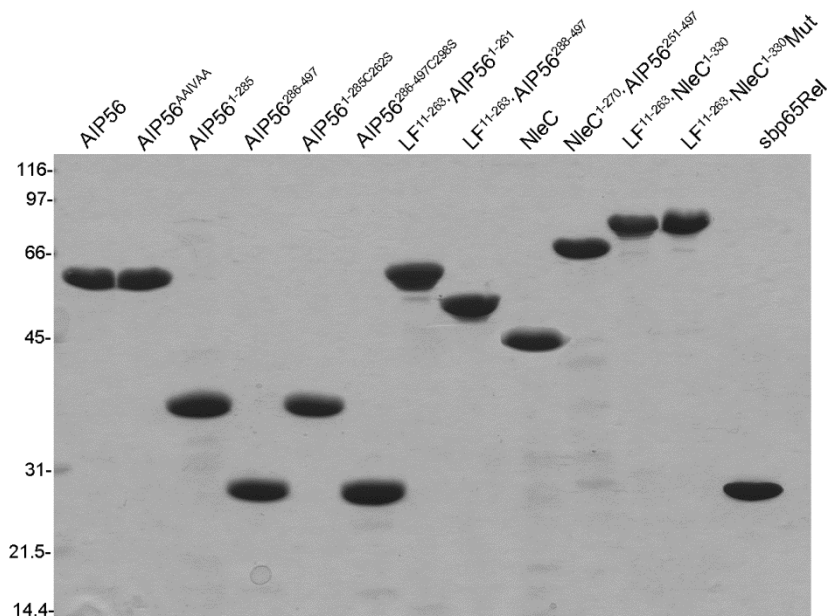


Figure 4.2. SDS-PAGE analysis of purified recombinant proteins produced in *E. coli*. Purity of the recombinant proteins was analysed by reducing SDS-PAGE, carried out on 12.5% separating gel and stained with Coomassie Blue. 10 µg of recombinant proteins were loaded onto the gel. Numbers on the left of the panels refer to the position and mass, in kDa, of the molecular weight markers

2. AIP56: a zinc metalloprotease targeting NF-κB p65

2.1. The metalloprotease signature of AIP56 is essential for its apoptogenic activity

Bioinformatic analysis of AIP56 primary structure revealed the presence of a conserved zinc-binding region signature, HEIVH, at positions 165-169 (do Vale et al, 2005; Jongeneel et al, 1989), suggesting that the toxin could be a zinc-metalloprotease. The HEIVH sequence corresponds to the consensus sequence HEXXH (where X is any amino acid) and contains all of the residues identified as part of the active site (Jongeneel et al, 1989). In zinc enzymes, the essential zinc ion is coordinated by the two histidines together with a more C-terminal residue (histidine, cysteine or glutamate) and a water molecule. In these enzymes, the hydrolysis is mediated by the nucleophilic attack of the activated water molecule on the carbonyl of the scissile bond (Vallee & Auld, 1990a; Vallee & Auld, 1990b).

During catalysis, the glutamate between the two histidine residues acts as nucleophile and activates the water molecule (Vallee & Auld, 1990a; Vallee & Auld, 1990b).

In order to study the role played by the putative zinc metalloprotease activity in the toxin's biological activity, a mutated version of the toxin (AIP56^{AAIVAA}) within the zinc-binding signature was produced (see section 1). Residues predicted to be involved in zinc ion coordination (His¹⁶⁵ and His¹⁶⁹) and in water molecule activation (Glu¹⁶⁶) together with a proximal histidine residue (His¹⁷⁰) were mutated to alanines. To ensure that no dramatic conformational changes occurred as a result of the introduced mutations, the oligomerization state, stokes radius and secondary structure content of the wild type and mutant toxin were compared by Native-PAGE, size exclusion chromatography and CD spectroscopy, respectively. No differences between wild type and mutant toxin were observed using the above mentioned techniques (Figure 4.3) indicating that, despite the introduced mutations, the overall folding of the toxin was undisturbed.

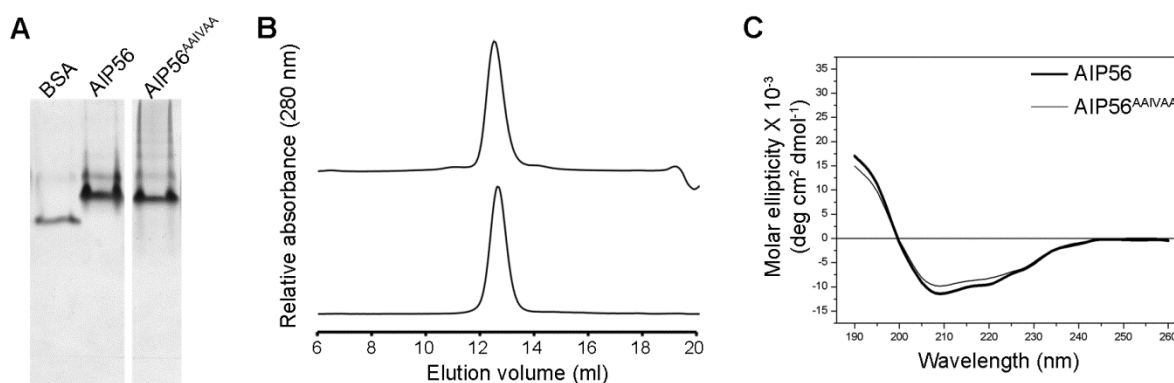


Figure 4.3. Disruption of the zinc-metalloprotease signature does not induce major structural changes in AIP56. (A) Native-PAGE analysis of AIP56 and AIP56^{AAIVAA}. BSA was run for control purposes. The depicted protein bands were from the same gel and were cut out and juxtaposed for presentation in the figure. (B) Size exclusion chromatography of AIP56 and AIP56^{AAIVAA} showing that disruption of the zinc-binding motif did not affect the monodispersity/stokes radius of the protein. (C) Far-UV CD spectra of wild-type AIP56 (thick line) and AIP56^{AAIVAA} (thin line) showing that the secondary structure content of the toxin was also unaffected by the introduced mutations.

In addition, to confirm that the residues composing the zinc-binding signature are in fact involved in zinc ion coordination the zinc content, of wild type and mutant AIP56 was determined by atomic absorption spectroscopy. Stoichiometric amounts of zinc are present in wild type AIP56 (0.93 ± 0.04 mol zinc/mol protein) while no zinc could be detected in AIP56^{AAIVAA}, which is in agreement with this region being the active site of the toxin. Hence, to evaluate if the disruption of the AIP56's metalloprotease signature would affect the toxin's apoptogenic activity, AIP56^{AAIVAA} was tested for its ability to induce apoptosis of sea bass phagocytes, using a previously established *ex vivo* model (Costa-Ramos et al, 2011; do Vale

et al, 2003) consisting of cells collected from inflamed fish peritoneal cavities. Apoptosis was assessed by analysis of typical apoptotic morphological characteristics (Hacker, 2000). The results showed that even when tested in doses of 50 $\mu\text{g/ml}$, AIP56^{AAIVAA} failed to induce apoptosis, in opposition to AIP56 that at 0.1 $\mu\text{g/ml}$ induced an increased number of cells with apoptotic morphology (Figure 4.4A). These results suggest that AIP56 is a zinc metalloprotease whose catalytic activity is essential for toxicity. It is worth noting that the AIP56 concentrations used in the present work are biologically relevant, since they are in the same range of the ones detected in the plasma of infected fish (Figure 4.4B).

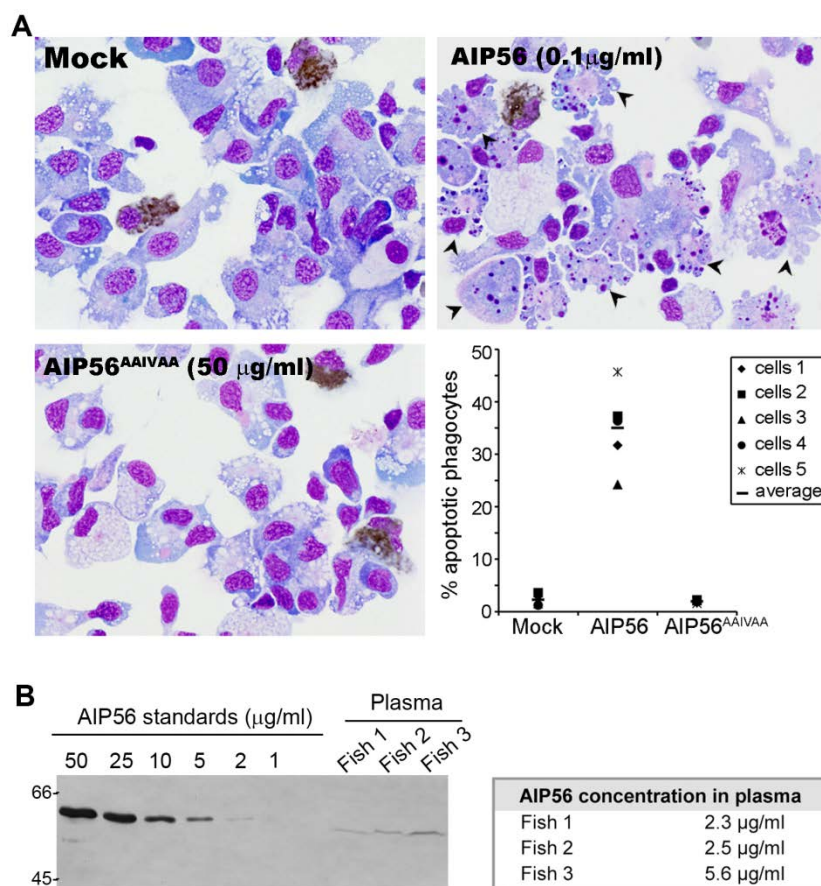


Figure 4.4. Disruption of the zinc-metalloprotease signature abolishes AIP56 apoptogenic activity. A) Sea bass peritoneal leukocytes collected from 5 animals were incubated with AIP56 or AIP56^{AAIVAA}, for 4 h at 22 °C. Mock-treated cells were used as controls. Images shown are representative cytospin preparations stained with Antonow's (Afonso et al, 1998; do Vale et al, 2002) 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 AIP56^{AAIVAA} and in mock-treated cells. The percentage of apoptotic phagocytes was determined by morphological analysis and is depicted in the dot plot. **B)** The AIP56 concentrations in the plasma of infected fish are in the same range as the ones used in the present work. The presence of AIP56 in plasmas (5 μl aliquots) from sea bass infected with a lethal dose of *Phdp* strain PP3 was determined by Western blotting and its concentration determined by densitometric analysis of the blot, and inferred from AIP56 standard curve (5 μl of AIP56 at different concentrations were loaded as standards). Numbers at the left refer to the position and mass, in kDa, of the molecular weight markers.

2.2. AIP56 cleaves NF- κ B p65 within the Rel homology domain

The AIP56's N-terminal homologue NleC, a type III secreted effector present in several enteric pathogenic bacteria, was recently shown to be a zinc dependent metalloprotease with activity towards NF- κ B p65 (Baruch et al, 2010; Muhlen et al, 2011; Pearson et al, 2011; Sham et al, 2011; Yen et al, 2010). The high degree of homology between AIP56 N-terminal region and NleC (Silva et al, 2010) prompted us to test if AIP56 had a similar activity.

The ability of AIP56 to cleave NF- κ B p65 was assessed *in vitro* using sea bass peritoneal cell lysates. Recombinant NleC (see section 1) was used as control. The obtained results show that AIP56 cleaved NF- κ B p65, similarly to NleC, with appearance of a lower molecular weight fragment (cl-p65), detected by Western blotting (Figure 4.5A). The p65 cleavage fragment, obtained after incubation with AIP56, was recognised by an antibody designed against a peptide located at the C-terminal region of sea bass p65, indicating that the AIP56-mediated p65 cleavage occurred at the N-terminal region, where the Rel-homology domain is located. By contrast, in cell lysates incubated with AIP56^{AAIVAA} or with AIP56 in the presence of the metalloprotease inhibitor 1,10-phenanthroline, p65 remained intact, confirming the involvement of the metalloprotease signature in the catalytic process. To investigate if the cleavage of p65 resulted directly from proteolysis by AIP56, a recombinant sea bass p65Rel domain (sbp65Rel) was produced in *E. coli* (see section 1). SDS-PAGE analysis of sbp65Rel incubated with AIP56 showed that the toxin cleaved recombinant sbp65Rel *in vitro* (Figure 4.5B) and N-terminal sequencing of the cleaved fragment identified the peptide EGRSA, revealing that AIP56 cleaved sbp65Rel at the Cys³⁹-Glu⁴⁰ peptide bond (the same residues previously identified as the cleavage site for NleC (Baruch et al, 2010)). This result was confirmed using ³⁵S-labeled sbp65Rel mutated within the identified cleavage site. Two single mutants (sbp65RelC39A, sbp65RelE40A) and a double mutant (sbp65C39AE40A) were produced *in vitro* using a rabbit reticulocyte system (see above section 1 Figure 4.1) and incubated with AIP56. Cleavage was assessed by autoradiography and the results show that, indeed, double mutation of Cys³⁹ and Glu⁴⁰ completely abolished p65 proteolysis by AIP56 and NleC (Figure 4.5C). Moreover, mutation in Glu⁴⁰ partially inhibited the cleavage mediated by AIP56, but not by NleC, while mutation of Cys³⁹ had no effect on the ability of either AIP56 or NleC to cleave p65 (Figure 4.5C). Taken together all the above presented results demonstrate that AIP56 cleaves NF- κ B p65 at the Cys³⁹-Glu⁴⁰ peptide bond in a process that is dependent on the metalloprotease activity of the toxin.

The fact that the identified cleaving residues are absolutely conserved among p65 of different species (Figure 4.5D), suggests that AIP56 could likely cleave mammalian p65. This suggestion was confirmed by incubating HeLa cell lysates with AIP56, and assessing p65 cleavage by Western blotting. The results presented in Figure 4.5E show that, similarly to NleC, AIP56 is able to cleave mammalian p65.

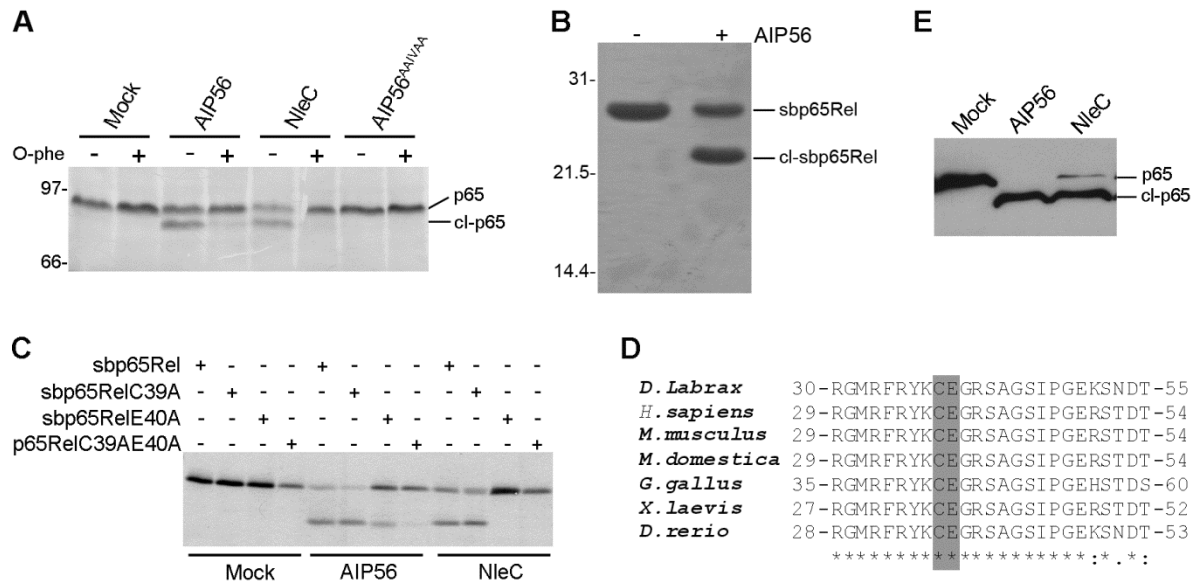


Figure 4.5. AIP56 is a zinc-metalloprotease that cleaves NF- κ B p65 at the conserved Cys³⁹-Glu⁴⁰ peptide bond. (A) Incubation of cell lysates with AIP56 and NleC resulted in p65 cleavage. Lysates were incubated for 2 h at 22 °C with 1 μ M of the indicated proteins in the presence or absence of the metalloprotease inhibitor 1,10-phenanthroline (O-phe), and p65 cleavage assessed by Western blotting. The positions of the full length and cleaved p65 (cl-p65) are indicated. Numbers on the left of the panel refer to the position and mass, in kDa, of the molecular weight markers. **(B)** AIP56 cleaves NF- κ B p65 at the Cys³⁹-Glu⁴⁰ peptide bond. Recombinant sea bass p65Rel was incubated in the presence or absence of 1 μ M 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. Numbers on the left of the panel refer to the position and mass, in kDa, of the molecular weight markers. **(C)** Mutation of both Cys³⁹ and Glu⁴⁰ of sbp65Rel inhibit proteolytic processing by AIP56 and NleC. ³⁵S-labeled sbp65Rel (Met¹-Arg¹⁸⁸) and sbp65Rel mutants (sbp65RelC39A, sbp65RelE40A or sbp65C39AE40A) were incubated for 2 h at 22 °C with 100 nM of the indicated proteins and cleavage assessed by autoradiography. **(D)** The AIP56 cleavage determining residues are highly conserved. Alignment of the p65 N-terminal region from different species (NCBI accession numbers: *Homo sapiens*, AAA36408; *Mus musculus*, NP_033071; *Gallus gallus*, NP_990460; *Xenopus laevis*, AAH70711; *Monodelphis domestica*, XP_001379658; *Danio rerio*, AAO26404). The AIP56 cleavage site is shadowed in grey. **(E)** AIP56 is able to cleave mammalian p65. HeLa cell lysates were incubated for 2 h at 22 °C with 1 μ M of the indicated proteins and p65 cleavage assessed by Western blotting using a commercially available antibody raised against a peptide mapping within the C-terminus of NF- κ B p65 of human origin. The positions of the full length and cleaved p65 (cl-p65) are indicated.

2.3. Cellular intoxication by AIP56 involves cleavage of the NF- κ B p65

To determine if cellular NF- κ B p65 is cleaved during AIP56 intoxication, sea bass peritoneal leukocytes were incubated with the toxin *ex vivo* and p65 proteolysis was assessed by Western blotting. Incubation of cells with AIP56 resulted in NF- κ B p65 depletion (Figure 4.6A), with the appearance of a cleavage fragment similar to that observed *in vitro* with cell lysates (Figure 4.5A). Additionally, and also in agreement with the *in vitro* results, no changes in p65 cellular levels could be observed upon incubation with the metalloprotease mutant AIP56^{AAIVAA} (Figure 4.6A). The fact that the AIP56 metalloprotease mutant lacking proteolytic activity was also unable to induce apoptosis suggests that the cleavage of NF- κ B p65 and the occurrence of apoptosis are related events.

It has been reported that activated caspase-3 can cleave p65 at its N-terminal region (Coiras et al, 2008; Kang et al, 2001). Considering that the AIP56's apoptogenic activity is caspase-dependent and involves activation of caspases-8, -9 and -3 (Costa-Ramos et al, 2011), the possible involvement of caspases in AIP56-mediated p65 cleavage was examined using a pan-caspase inhibitor (ZVAD-FMK) that has been previously shown to efficiently inhibit AIP56 induced caspase-3 activation and apoptosis (Costa-Ramos et al, 2011). In these experiments, ZVAD-FMK was effective in protecting cells from AIP56-induced apoptosis, but did not affect NF- κ B p65 cleavage (Figure 4.6B) indicating that AIP56-mediated p65 depletion is a caspase-independent event.

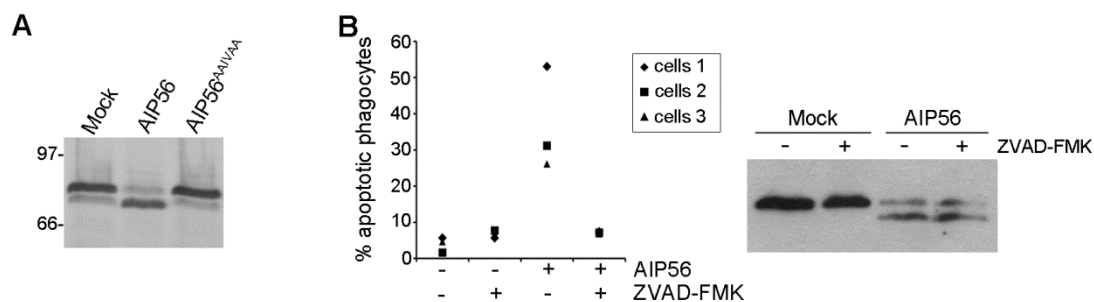


Figure 4.6. Cellular intoxication by AIP56 involves cleavage of the NF- κ B p65. (A) Incubation of leukocytes with AIP56, but not with AIP56^{AAIVAA} leads to p65 depletion. Leukocytes were incubated with 10 μ g/ml of the indicated proteins for 2 h at 22 $^{\circ}$ C and p65 cleavage was assessed by Western blotting. Numbers on the left of the panels refer to the mass, in kDa, of the molecular weight markers. (B) AIP56-mediated p65 cleavage is caspase-independent. Leukocytes were incubated with 10 μ g/ml (cells 1) or 2 μ g/ml (cells 2 and cells 3) of AIP56 in the presence or absence of 25 μ M of the pan-caspase inhibitor ZVAD-FMK for 4h at 22 $^{\circ}$ C. NF- κ B p65 cleavage was assessed after 2 h incubation by Western blotting and the occurrence of apoptosis accessed after 4 h incubation by morphological analysis of cytospin preparations. The blot shown refers to the analysis of p65 cleavage of "cells 3" samples.

3. AIP56 belongs to the AB toxin family

3.1. AIP56 has a two-domain structure

As reviewed in Chapter I, Blast analysis of AIP56 amino acid sequence revealed that the N-terminal region shares homology with a type III secreted effector present in several enteric pathogenic bacteria, and the C-terminal region is highly similar to an uncharacterized hypothetical protein of *Acrythosiphon pisum* bacteriophage APSE-2 (Degnan et al, 2009) and to the C-terminal part of a hypothetical protein of the monarch butterfly *Danaus plexippus* (Silva et al, 2010) (see Chapter I Figure 1.10). These observations suggested a two domain/subunit structure similar to the ones of diphtheria toxin and clostridial neurotoxins (Silva et al, 2010).

In order to gain insights about domain boundaries within AIP56 and to identify possible flexibility regions, limited proteolysis experiments were performed. SDS-PAGE analysis of the digests resultant from controlled incubation of AIP56 with trypsin, chymotrypsin and proteinase K, revealed that the toxin was highly resistant to trypsin digestion, whereas chymotrypsin and proteinase K cleaved AIP56 in two main fragments with approximately 32 and 24 kDa, consistent with the size of the two domains predicted by sequence analysis (Figure 4.7A). When analysed under non-reducing conditions, chymotrypsin-digestion fragments appeared as a single band co-migrating with non-digested AIP56, showing that, after cleavage, the fragments remained linked by a disulphide bridge (Figure 4.7B). N-terminal Edman sequencing of the two major fragments obtained with chymotrypsin revealed that cleavage occurred between Phe²⁸⁵ and Phe²⁸⁶, in the amino acid stretch between the two unique cysteine residues (Cys²⁶² and Cys²⁹⁸) of AIP56 (Figure 4.7C). These results are in accordance with the amino acid sequence alignment data and reveal the existence of two protease-resistant domains linked by an exposed region containing two cysteine residues involved in a disulphide bond.

To better understand the function of the two identified structural domains, constructs corresponding to AIP56's N- and C-terminal regions (AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷, respectively) were designed based on the chymotrypsin cleavage site and the proteins produced in *E. coli* (see section 1). SDS-PAGE analysis of the purified recombinant proteins under reducing and non-reducing conditions revealed that they display a major propensity to oxidize leading to the formation of DTT-sensitive dimers (Figure 4.8A), a phenomenon that could have a functional impact and complicate subsequent analyses. Therefore, mutated versions of AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷, where the single cysteine in each protein was replaced by a serine (AIP56^{1-285C262S} and AIP56^{286-497C298S}), were obtained by site directed mutagenesis and produced in *E. coli* (see section 1). Non-reducing SDS-PAGE confirmed

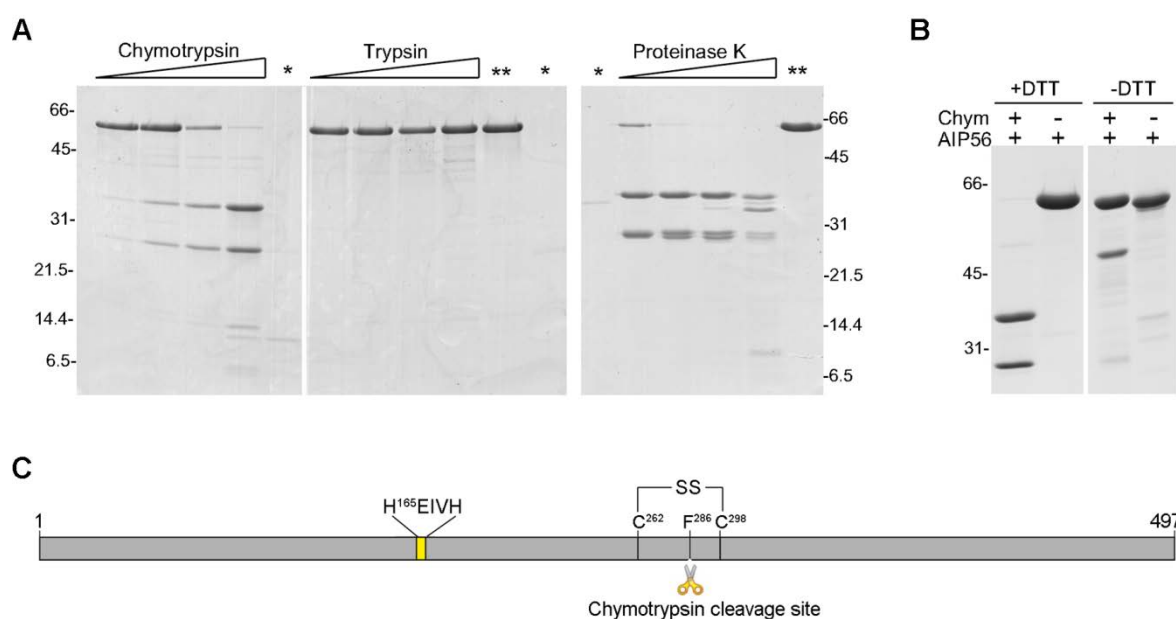


Figure 4.7. AIP56 is composed of two domains linked by a disulphide bridge. (A) Limited proteolysis of AIP56 with chymotrypsin and proteinase K originates two major fragments. AIP56 (0.6 mg/ml) was incubated with 0.25, 1.25, 6.25 or 25 µg/ml of chymotrypsin, trypsin and proteinase K for 30 min on ice and digests analysed by reducing SDS-PAGE. The proteases (marked as *) and undigested AIP56 (marked as **) were loaded as controls. The depicted protein bands were from the same gel and were cut out and juxtaposed for presentation in the figure. **(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. The depicted protein bands were from the same gel and were cut out and juxtaposed for presentation in the figure. Numbers to the left and right of the panels refer to the mass, in kDa, of the molecular weight markers. **(C)** Schematic representation of AIP56.

that cysteine mutation abolished dimer formation (not shown). CD spectroscopy was used to assess the impact of the mutations on the protein folding (Figure 4.8B and C), indicating that mutation of the cysteine residues to serine did not induce major structural rearrangements. CD analysis revealed that the predominant secondary structural element of the N-terminal portion is the α -helix whereas in the C-terminal part β -sheets are the predominant elements (Figure 4.8D). Furthermore, the weighted sum of the CD spectra of the recombinant N- and C-terminal portions reproduces the spectrum of the entire protein (Figure 4.8E), indicating that when produced independently, the two putative AIP56 functional domains maintain their native structures. Additionally, the position of the β -sheets and α -helices in AIP56 primary structure was predicted using bioinformatic tools (Figure 4.9). The predictions are in accordance with the CD results, and together with the above presented results, support the existence of two structurally distinct domains/subunits, which may play different roles in the intoxication process, linked by a disulphide bridge.

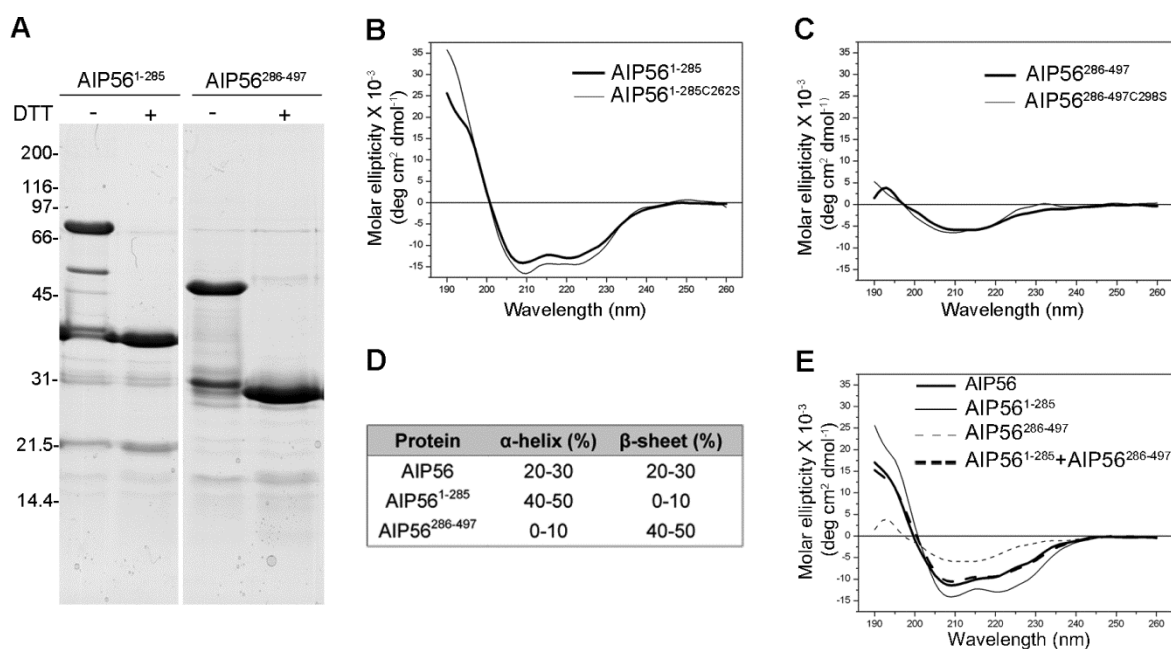
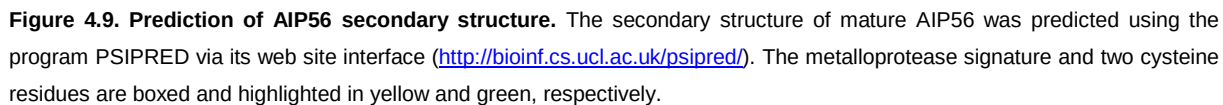


Figure 4.8. Structural analysis of the recombinant AIP56 truncated versions. (A) AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷ have a propensity to oxidize resulting in the formation of DTT-sensitive dimers. Reducing (DTT +) and non-reducing (DTT -) SDS-PAGE of purified AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷. Numbers on the left of the panels refer to the position and mass, in kDa, of the molecular weight markers. The depicted protein bands were from the same gel and were cut out and juxtaposed for presentation in the figure. (B) Secondary structure content of the N-terminal portion was unaffected by mutation of the single cysteine residue. Far-UV CD spectra of AIP56¹⁻²⁸⁵ (thick line) and AIP56^{1-285C262S} (thin line). (C) Secondary structure of the C-terminal portion was unaffected by mutation of the single cysteine residue. Far-UV CD spectra of AIP56²⁸⁶⁻⁴⁹⁷ (thick line) and AIP56^{286-497C298S} (thin line). (D) Secondary structure content of AIP56 and AIP56 truncates. (E) The weighted sum of the CD spectra of the recombinant N- and C-terminal portions reproduces the spectrum of the entire protein. 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).

Both wild type and cysteine mutant truncated versions of the toxin were used in functional assays (presented below) and similar results were obtained (not shown). Therefore, in this thesis only the results obtained using the cysteine mutants will be presented.



Considering the presence of the metalloprotease signature in the AIP56's N-terminal region and its similarity with the type III secreted effector NleC, we hypothesised that this region would play the catalytic role, while the C-terminal region would be involved in the binding/entry of the toxin in target cells.

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AIP56's C-terminus did not display proteolytic activity, whereas the N-terminal portion cleaved p65 as efficiently as the full-length toxin by a mechanism likewise inhibited by the zinc metalloprotease inhibitor 1,10-phenanthroline.

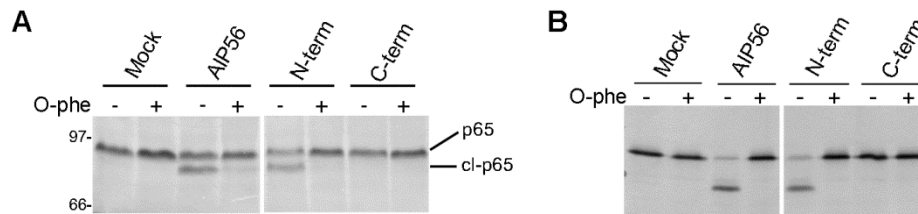


Figure 4.10. AIP56 N-terminal region displays proteolytic activity towards NF- κ B p65. (A) Incubation of cell lysates with AIP56 N-terminal portion resulted in p65 cleavage similar to the one induced by full length AIP56. Lysates were incubated for 2 h at 22 °C with 1 μ M of AIP56, AIP56^{1-285C262S} (N-term), or AIP56^{286-497C298S} (C-term) in the presence or absence of the metalloprotease inhibitor 1,10-phenanthroline (O-phe), and p65 cleavage assessed by Western blotting. The positions of the full length and cleaved p65 (cl-p65) are indicated. Numbers on the left of the panels refer to the position and mass, in kDa, of the molecular weight markers. The depicted protein bands were from the same membrane and were cut out and juxtaposed for presentation in the figure. (B) AIP56 N-terminal portion cleaves *in vitro* translated sea bass p65 Rel homology domain. ³⁵S-labeled sbp65Rel (Met¹-Arg¹⁸⁸) was incubated for 2 h at 22 °C with 100 nM of AIP56, AIP56^{1-285C262S} (N-term) or AIP56^{286-497C298S} (C-term) in the presence or absence of the metalloprotease inhibitor 1,10-phenanthroline (O-phe), and cleavage assessed by autoradiography.

Toxicity of the two putative AIP56 domains was assessed by testing their ability to induce apoptosis and to cleave NF- κ B p65 upon incubation with sea bass peritoneal leukocytes. Neither AIP56^{1-285C262S} nor AIP56^{286-497C298S} displayed apoptogenic activity, even when tested in doses 500 times higher than the ones used for the full length toxin (Figure 4.11A). Moreover, the same result was obtained when a mixture of both N- and C-terminal portions was tested (Figure 4.11A). Additionally, and in agreement with the observed lack of apoptogenic activity, no changes in p65 levels were observed in cells incubated with AIP56 truncated versions alone or mixed (Figure 4.11B). These results indicate that independently the two AIP56's domains are non-toxic and suggest that a concerted action of both regions as part of the same molecule is needed to elicit a biological effect.

Given the cytosolic location of NF- κ B p65, the lack of toxicity of the AIP56's catalytic region is most likely related to its inability to enter the cells and reach its target. Hence, a strategy to deliver the AIP56 N-terminal portion into the cell cytosol was delineated based on the *Bacillus anthracis* lethal factor (LF) delivery system. The N-terminal portion (first 263 residues) of LF was found to be sufficient for recognition and binding to the protective antigen (PA), the receptor-binding subunit for LF (Collier, 2009), and able to cause cellular uptake of fused polypeptides (Arora & Leppla, 1993). This strategy was previously used

successfully to deliver the catalytic domains of diphtheria toxin (DT), shiga toxin (Stx) and *Pseudomonas* exotoxin A (PE) into the cell cytosol (Arora & Leppla, 1993; Arora & Leppla, 1994; Milne et al, 1995).

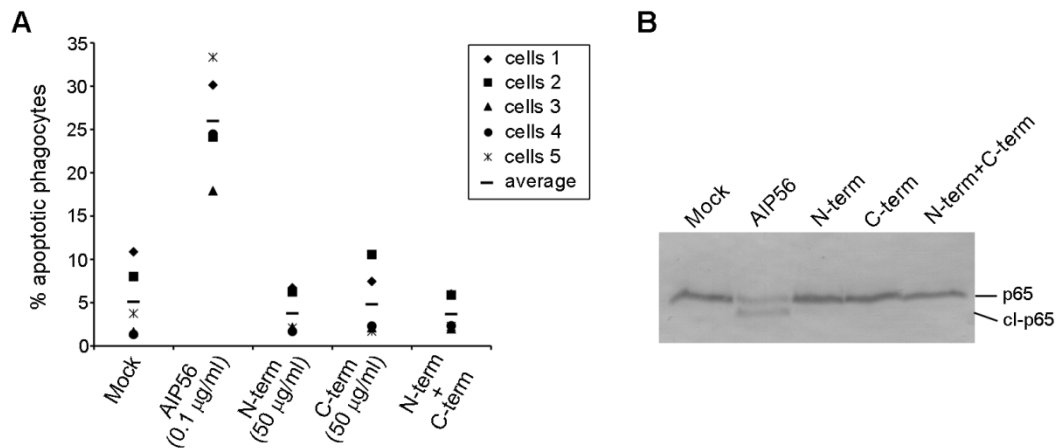


Figure 4.11. The two AIP56 structural domains need to be part of the same molecule to display toxicity. (A) Independently, recombinant AIP56 N- and C-terminal portions lack apoptogenic activity. Leukocytes collected from 5 animals were incubated with AIP56, AIP56^{1-285C262S} (N-term), AIP56^{286-497C298S} (C-term), or a mixture of AIP56^{1-285C262S} and AIP56^{286-497C298S} (N-term+C-term) (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)** Incubation of sea bass leukocytes with recombinant AIP56 N- and C-terminal domains did not result in NF-κB p65 depletion. Sea bass leukocytes were incubated as described in (A) and NF-κB p65 cleavage was assessed by Western Blotting. The positions of the full length and cleaved p65 (cl-p65) are indicated.

Therefore, chimeric proteins consisting of the LF N-terminal region fused to AIP56's N-terminus (LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹) or C-terminus (LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷) were produced (see section 1). Intoxication assays were performed by incubating sea bass peritoneal leukocytes with the LF·AIP56 chimeras in the presence of PA. In cells incubated with LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ the p65 levels were significantly reduced, confirming that LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ was effectively delivered into the cell cytosol, while no changes in p65 were observed in cells incubated with LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷ or with PA alone (Figure 4.12A). Accordingly, LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷ did not display apoptogenic activity, while incubation with LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ resulted in an increased number of cells with apoptotic morphology (Figure 4.12B), similar to what was observed in cells incubated with AIP56 (Figure 4.4). These results show that delivery of AIP56's N-terminal portion into the cytosol reproduces the toxic effect of the full length toxin and confirm that this region is the one responsible for the toxin's catalytic and apoptogenic activities.

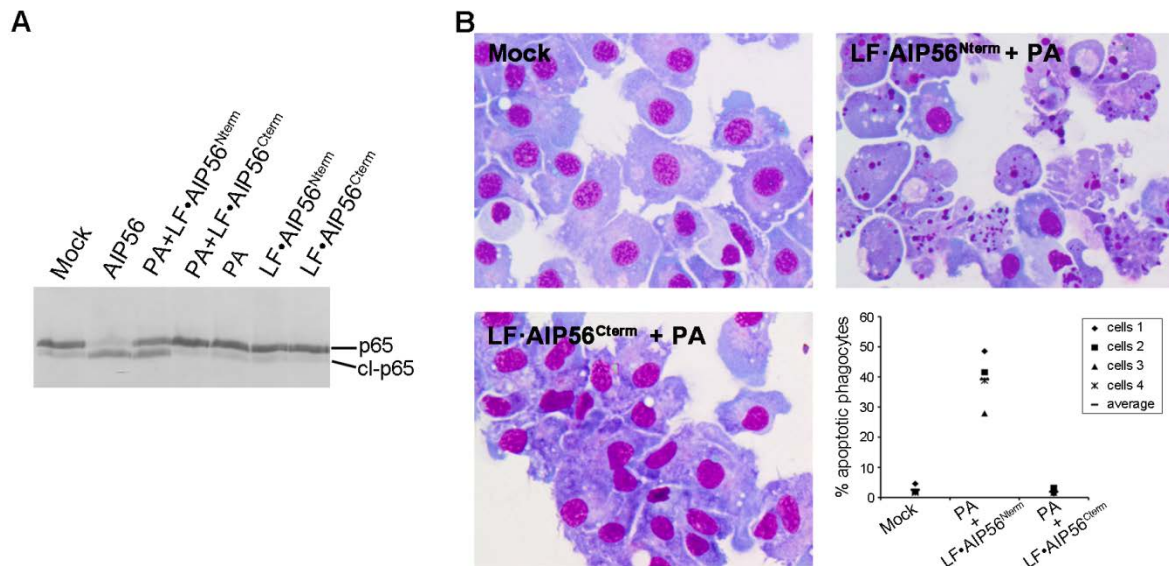


Figure 4.12. Delivery of the AIP56 N-terminal region into the cell cytosol, using the *B. anthracis* LF/PA system, reproduces the toxic effect of the full length toxin. (A) *B. anthracis* LF/PA system is able to deliver AIP56 N-terminal portion into the cell's cytosol. Leukocytes collected from 4 animals were incubated for 4 h at 22 °C with 20 nM LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ (LF·AIP56^{Nterm}) or LF¹¹⁻²⁶³·AIP56²⁹⁹⁻⁴⁹⁷ (LF·AIP56^{Cterm}) in the presence of 10 nM PA. Mock-treated cells and cells incubated with 35 nM (2 µg/ml) AIP56 were used as controls. Cleavage of NF-κB p65 was detected by Western blotting. The positions of the full length and cleaved p65 (cl-p65) are indicated. **(B)** Deliver of AIP56 N-terminal portion into the cell's cytosol is sufficient to induce apoptosis. Cells were treated as described in (A) and apoptosis assessed by morphological analysis. Images shown are representative cytopsin preparations stained with Hemacolor. The percentages of apoptotic phagocytes are depicted in the dot plot.

3.3. The C-terminal region mediates the interaction of the toxin with target cells

To investigate the role played by the two AIP56 domains in the interaction of the toxin with target cells, AIP56^{1-285C262S}, AIP56^{286-497C298S} and AIP56^{AAIVAA} were used as competitors for AIP56 activity. Both p65 cleavage and apoptosis induction were monitored in these experiments. Competitor:AIP56 ratios ranged from 40:1 to 400:1 (mol/mol). Mock treated cells and cells incubated with each of the competitors alone were used as negative controls. As expected, no apoptosis was observed in neither of the negative control conditions (Figure 4.13A). Moreover, AIP56^{286-497C298S} and AIP56^{AAIVAA} were found to compete with the full length toxin, inhibiting its apoptogenic activity in a dose dependent manner, whereas no effect was observed when AIP56^{1-285C262S} was used as a competitor (Figure 4.13A). Western blotting analysis of p65 levels in cells incubated with the highest dose of each competitor in the presence of AIP56 showed that AIP56^{AAIVAA} and AIP56²⁸⁶⁻⁴⁹⁷ inhibited AIP56-mediated p65 degradation (Figure 4.13B) indicating that, when used as competitors, these proteins efficiently blocked the arrival of the toxin to the cytosol. In accordance with the results

obtained in the apoptogenic activity assays (Figure 4.13A), no inhibition of the AIP56-dependent p65 cleavage was observed when AIP56^{1-285C262S} was used as a competitor (Figure 4.13B). These results implicate the C-terminal region in mediating the binding of the toxin to the cell surface and its entry into the cells.

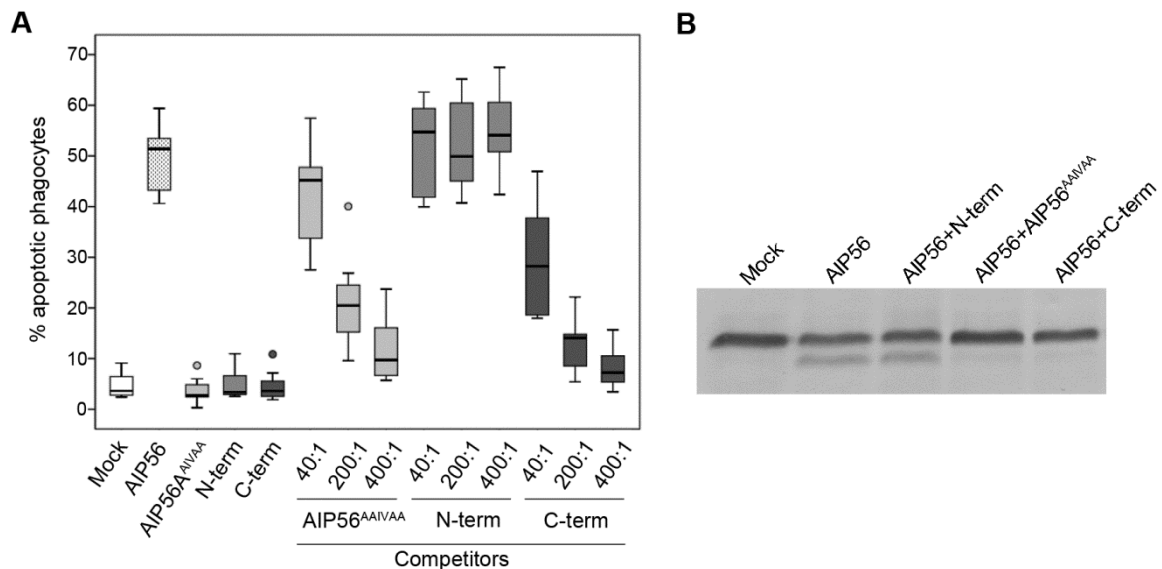


Figure 4.13. AIP56 C-terminal region plays a role in the toxin binding and entry into the target cells. (A) The metalloprotease mutant and the C-terminal portion, but not the N-terminal portion of AIP56, inhibits the toxin's apoptogenic activity in a dose dependent manner. Leukocytes collected from 7 fish were incubated with AIP56^{AAIVAA}, AIP56^{1-285C262S} (N-term) or AIP56^{286-497C298S} (C-term) at final concentrations of 0.35, 1.75 or 3.5 μ M for 15 min on ice, followed by further 15 min incubation on ice with 8.75 nM (0.5 μ g/ml) AIP56 in the presence of the competitors. The competitor:AIP56 ratios (mol:mol) are indicated. Cells incubated with AIP56 in the absence of competitors or with 3.5 μ M of each competitor alone were used as controls. Cells were washed, transferred to 22 $^{\circ}$ C and incubated for 4 h prior to determination of the percentage of apoptotic cells by morphological analysis of cytospin preparations stained with Hemacolor. The figure 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). **(B)** The metalloprotease mutant and the C-terminal portion, but not the N-terminal portion, inhibits AIP56-associated p65 cleavage. Cells were incubated as described in (A) and the inhibitory effect of the highest dose of each competitor on AIP56-mediated cleavage of NF- κ B p65 was assessed by Western blotting.

3.4. Translocation of AIP56 requires integrity of the Cys²⁶²-Cys²⁹⁸ linker

In toxins secreted as a single polypeptide chain, proteolytic cleavage at an exposed flexible region between two cysteine residues resulting in a toxin with an enzymatically active part linked to the rest of the molecule by a disulphide bond (commonly referred as nicking) is often associated with increased toxicity (Drazin et al, 1971; Knust et al, 2009; Krieglstein et al, 1991; Rupnik et al, 2005; Sandvig & Olsnes, 1981). The results obtained in experiments using the N- and C-terminal portions of AIP56 suggested that the two domains must be part of the same molecule to display toxicity. Therefore, in order to investigate whether the two

domains bound by a disulphide bridge were able to intoxicate cells, we reconstituted the full length toxin using the non-toxic AIP56¹⁻²⁸⁵ and AIP56²⁸⁶⁻⁴⁹⁷. To promote the formation the disulphide-bound heterodimer AIP56¹⁻²⁸⁵/AIP56²⁸⁶⁻⁴⁹⁷, the two proteins were mixed in equimolar amounts under denaturing and reducing conditions and subjected to refolding through extensive dialysis against sea bass PBS. Non-reducing SDS-PAGE confirmed that this reconstituted version of the toxin consists of disulphide-bound AIP56¹⁻²⁸⁵/AIP56²⁸⁶⁻⁴⁹⁷ along with small amounts of AIP56¹⁻²⁸⁵ and of AIP56²⁸⁶⁻⁴⁹⁷ homodimers and monomers (Figure 4.14A). Additionally, a chymotrypsin-nicked version of the toxin was also produced. AIP56's nicking and integrity of the disulphide bridge linking the two digestion fragments were confirmed by reducing and non-reducing SDS-PAGE (Figure 4.14A) and the structural impact of the cleavage was evaluated by CD spectroscopy and differential scanning fluorimetry (DSF). The Far-UV CD spectrum of the nicked toxin was found to be undistinguishable from that of AIP56 (Figure 4.14B), indicating that no changes in the secondary structure were induced by proteolysis. Moreover, when protein stability was evaluated by DSF, using the protein melting temperature as an indicator, we found that nicking of the toxin resulted only in a 1 °C decrease in the T_m (39±0.13 °C for AIP56 and 38±0.25 °C for nicked AIP56; mean±SD of 16 measurements in four independent experiments). Together, these results indicate that no major structural changes were induced by nicking.

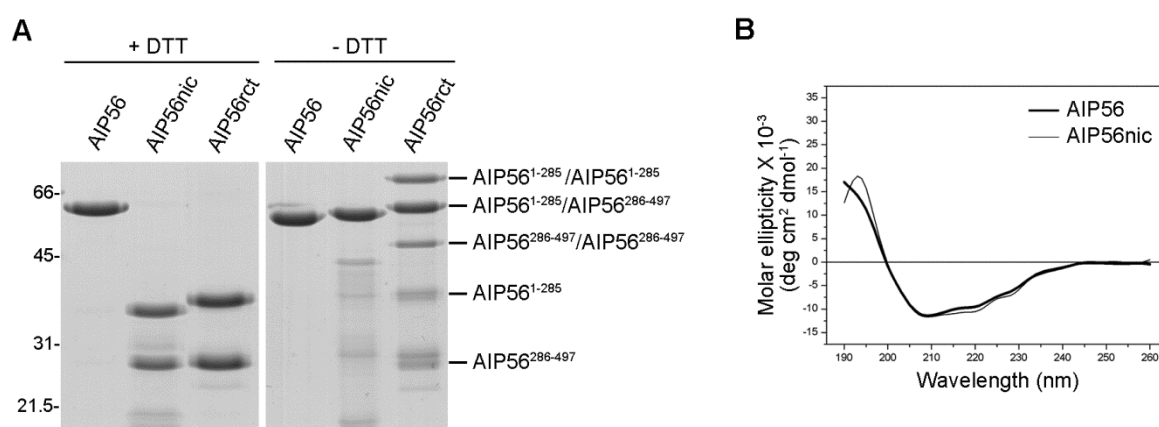


Figure 4.14. Structural analysis of nicked and reconstituted AIP56. (A) Reducing (+DTT) and non-reducing (-DTT) SDS-PAGE of AIP56, nicked AIP56 (AIP56nic) and reconstituted AIP56 (AIP56rcf). Numbers on the left of the panels refer to the position and mass, in kDa, of the molecular weight markers. The depicted protein bands were from the same gel and were cut out and juxtaposed for presentation in the figure. (B) Nicking of AIP56 does not affect its secondary structure content. Far-UV CD spectra of AIP56 (thick line) and nicked AIP56 (AIP56nic; thin line).

Toxicity of nicked and reconstituted AIP56 was tested by evaluating their ability to cleave NF- κ B p65 and induce apoptosis of sea bass leukocytes. Surprisingly, no apoptosis was observed with nicked or reconstituted AIP56, even when tested at very high doses (Figure 4.15A). Accordingly, no changes in p65 cellular levels were observed upon incubation with neither of the proteins (Figure 4.15B).

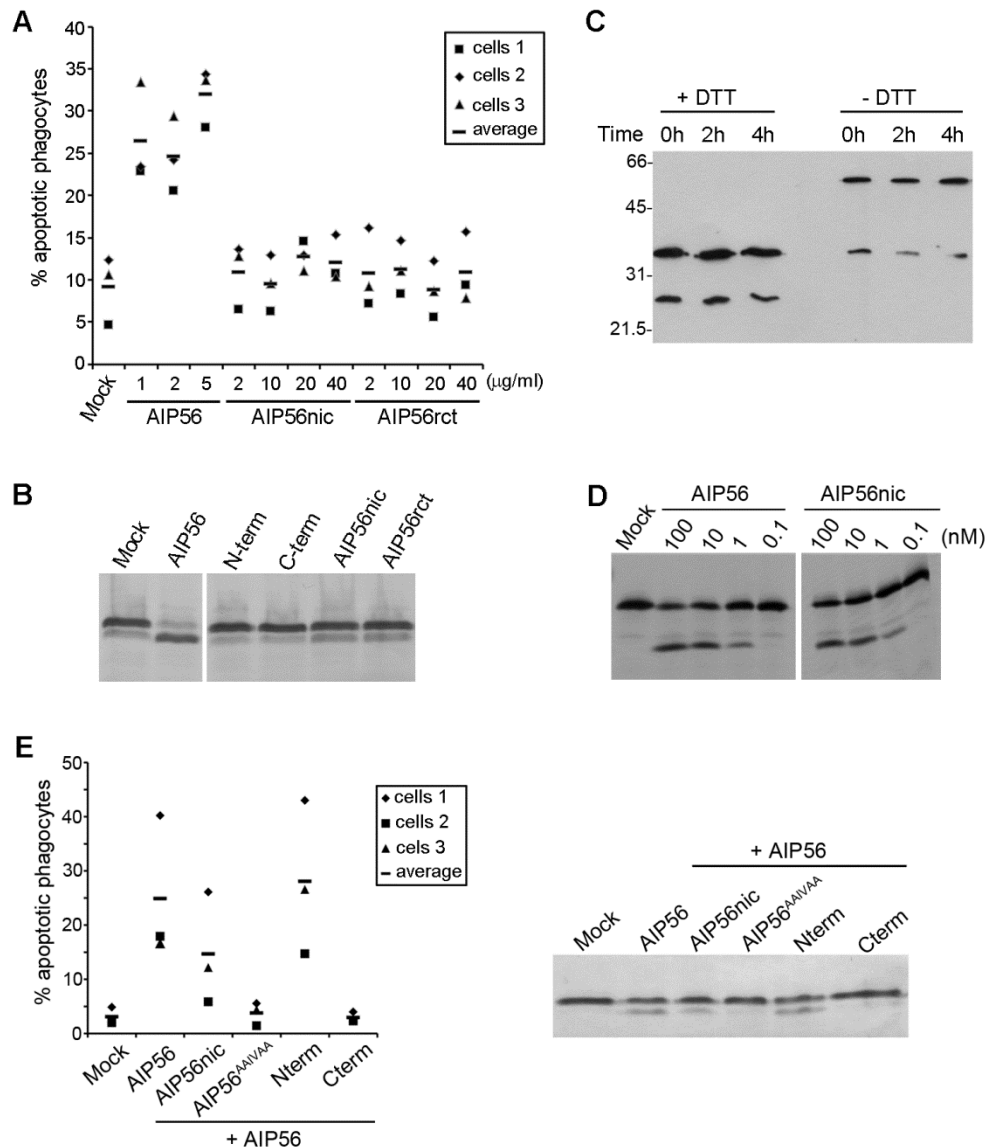


Figure 4.15. Integrity of the linker region, between the two cysteine residues, is essential for AIP56's toxicity (A) Nicked and reconstituted AIP56 lack apoptogenic activity. Sea bass leukocytes, collected from 3 fish, were incubated with nicked or reconstituted AIP56 (AIP56nic and AIP56rct, respectively) at the indicated doses 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)** No changes on p65 cellular levels were observed upon incubation with nicked or reconstituted AIP56. Sea bass leukocytes were incubated with 10 μg/ml of nicked or reconstituted AIP56 (AIP56nic and AIP56rct, respectively), for 2 h at 22 °C and the cleavage of p65 assessed by Western blotting. Mock treated cells and cells incubated with 10 μg/ml of AIP56, AIP56^{1-285C262S} (N-term) or AIP56^{286-497C298S} (C-term) were

used as controls. The depicted protein bands were from the same membrane and were cut out and juxtaposed for presentation in the figure. **(C)** The culture conditions do not reduce the disulphide bridge of nicked AIP56 (AIP56^{nic}). Nicked AIP56 was added to sea bass peritoneal cell suspension in supplemented L-15 medium and incubated up to 4 h at 22 °C. An aliquot of nicked toxin not incubated with cells (0) and aliquots of the cell culture supernatant collected 2 or 4 h after incubation with cells (all containing 50 ng of nicked toxin) were run in reducing and non-reducing SDS-PAGE and subjected to Western blotting using an anti-AIP56 rabbit serum (do Vale et al, 2005). Numbers on the left indicate the position and mass, in kDa, of the molecular weight markers **(D)** Nicked AIP56 (AIP56^{nic}) 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 or nicked AIP56 at the indicated concentrations and cleavage assessed by autoradiography. The depicted protein bands were from the same membrane and were cut out and juxtaposed for presentation in the figure **(E)** Nicked AIP56 competes with AIP56 and inhibits its toxicity. Leukocytes collected from 3 animals were incubated with 3.5 µM nicked AIP56 (AIP56^{nic}) for 15 min on ice followed by further 15 min incubation on ice with 8.75 nM (0.5 µg/ml) of AIP56. Mock-treated cells, cells incubated with 8.75 nM AIP56, or cells incubated with 3.5 µM of AIP56^{AAIVAA}, AIP56^{1-285C262S} (N-term) and AIP56^{286-497C298S} (C-term) 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 percentages of apoptotic cells were determined as described in (A).

To ensure that the incubation conditions were not leading to oxidation, and consequent disruption of the disulphide bridge linking the two protease-resistant domains, the oxidation state of nicked AIP56 during incubation with sea bass peritoneal cells was assessed. Supernatants of the sea bass peritoneal cell suspensions incubated with nicked AIP56 were analysed by reducing and non-reducing SDS-PAGE followed by Western blotting using an anti-AIP56 rabbit serum (do Vale et al, 2005). The results show that, even after 4 hour incubation, nicked AIP56 remain in its oxidised state (Figure 4.15C). Furthermore, the observed lack of toxicity was also not related with a loss of catalytic activity, since nicked AIP56 retained proteolytic activity *in vitro* in the same dose range as AIP56 (Figure 4.15D), nor was related with impairment of the cell binding ability, as indicated by the ability of the nicked toxin to inhibit the AIP56-mediated p65 cleavage and apoptosis in competition experiments (Figure 4.15E). Taken together, these results indicate that the lack of toxicity of nicked AIP56 is related with the inability of the AIP56's catalytic portion to reach the cytosol, suggesting that integrity of the linker region between the two cysteine residues is required for toxin internalization and arrival at the cytosol and pointing to a possible role of the linker region in the toxin's translocation process.

3.5. The disulphide bridge linking Cys²⁶²-Cys²⁹⁸ is not absolutely required for toxicity

In TeNT and BoNT type A, it has been shown that the disulphide bridge linking the A and B domains is essential for neurotoxicity (de Paiva et al, 1993; Schiavo et al, 1990). AIP56 contains only two cysteine residues that establish a disulphide bond linking the two functional regions. To evaluate the importance of the inter-cysteine bridge in the intoxication

by AIP56, we disrupted it by alkylating the cysteine residues. Disruption of the disulphide bridge was confirmed by SDS-PAGE analysis under reducing and non-reducing conditions (Figure 4.16A). Proteolytic activity of alkylated AIP56 was tested *in vitro* using ^{35} S-labeled sbp65Rel and the results show that alkylated AIP56 cleaved ^{35} S-labeled sbp65Rel in the same dose range as AIP56 (Figure 4.16B) indicating that alkylation did not compromise the catalytic activity of the toxin. Nevertheless, cysteine alkylation was found to partially compromise AIP56's toxicity. When tested *ex vivo* at the same doses, alkylated AIP56 exhibited a lower apoptogenic and p65 cleaving activity than AIP56 (Figure 4.16C and D). These results suggest that in AIP56, though not being an absolute requirement for toxicity, the disulphide bridge plays a role in the intoxication process.

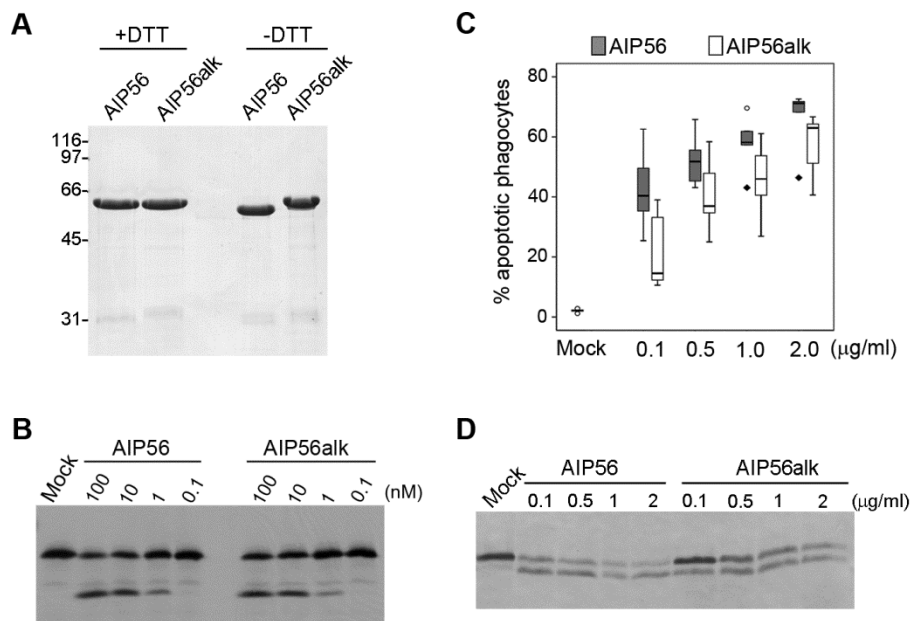


Figure 4.16. The disulphide bridge of AIP56 increases the efficiency of the intoxication process but is not an absolute requirement for toxicity. (A) Alkylation disrupted the disulphide bridge linking the two AIP56 cysteine residues. Reducing (+DTT) and non-reducing (-DTT) SDS-PAGE of AIP56 and alkylated AIP56 (AIP56alk). Numbers on the left of the panels indicate the position and mass, in kDa, of the molecular weight markers. (B) Alkylated AIP56 (AIP56alk) display proteolytic activity *in vitro* in the same dose range as AIP56. ^{35} S-labeled sbp65Rel was incubated for 2 h at 22 °C with wild type or alkylated AIP56 at the indicated concentrations and cleavage assessed by autoradiography. Part of this blot was included in Figure 4.15D. (C) and (D) Disruption of the disulphide bridge linking Cys²⁶² and Cys²⁹⁸ partially compromises AIP56 toxicity. Leukocytes collected from 5 animals were incubated with AIP56 or alkylated AIP56 (AIP56alk) for 4 h at 22 °C. The percentage of apoptotic cells was determined by morphological analysis of cytospin preparations stained with Hemacolor (C) and p65 cleavage assessed by Western blotting (D). The box plot (C) presents the 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 and diamonds signal extreme observations). When used at the same concentration, AIP56alk resulted in lower percentage of apoptotic cells than AIP56, except for the dose of 0.5 μ g/ml in which no statistical differences were observed.

4. AIP56 and NleC: structural and functional conservation but different unfolding properties

4.1. NleC and AIP56 catalytic region share structural similarity

The results presented so far revealed that, similarly to NleC, AIP56 is a zinc-dependent metalloprotease targeting the NF- κ B p65, with this cleaving activity attributed to its N-terminal region. However, and despite the pathological consequences of AIP56 and NleC catalytic activities are known (Baruch et al, 2010; do Vale et al, 2007a; do Vale et al, 2007b; do Vale et al, 2005; Muhlen et al, 2011; Pearson et al, 2011; Sham et al, 2011; Shames et al, 2011; Yen et al, 2010), structural data regarding these two proteins is still scarce and their crystal structures were not yet determined. The 47.0% similarity and the 30.5% identity of the primary structures of the AIP56 N-terminal region (Asn¹- Cys²⁶²) and NleC also suggests that, apart from function conservation, NleC and AIP56 catalytic region are structurally similar. Therefore, aiming at gathering partial structural information, the secondary structure of NleC and was analysed by CD. The results show that the NleC Far-UV spectrum is indistinguishable from the one of AIP56¹⁻²⁸⁵ (Figure 4.17A) and that both proteins fold predominantly as α -helices. In addition, *in silico* analysis using bioinformatics tools predicts conservation of the position of the α -helices and β -strands in the primary AIP56¹⁻²⁸⁵ and NleC sequences (Figure 4.17B). Thus, the high sequence conservation, together with the functional and structural data suggest that NleC and AIP56 catalytic region have a similar secondary structure content and may display a common fold and thus similar three-dimensional structures.

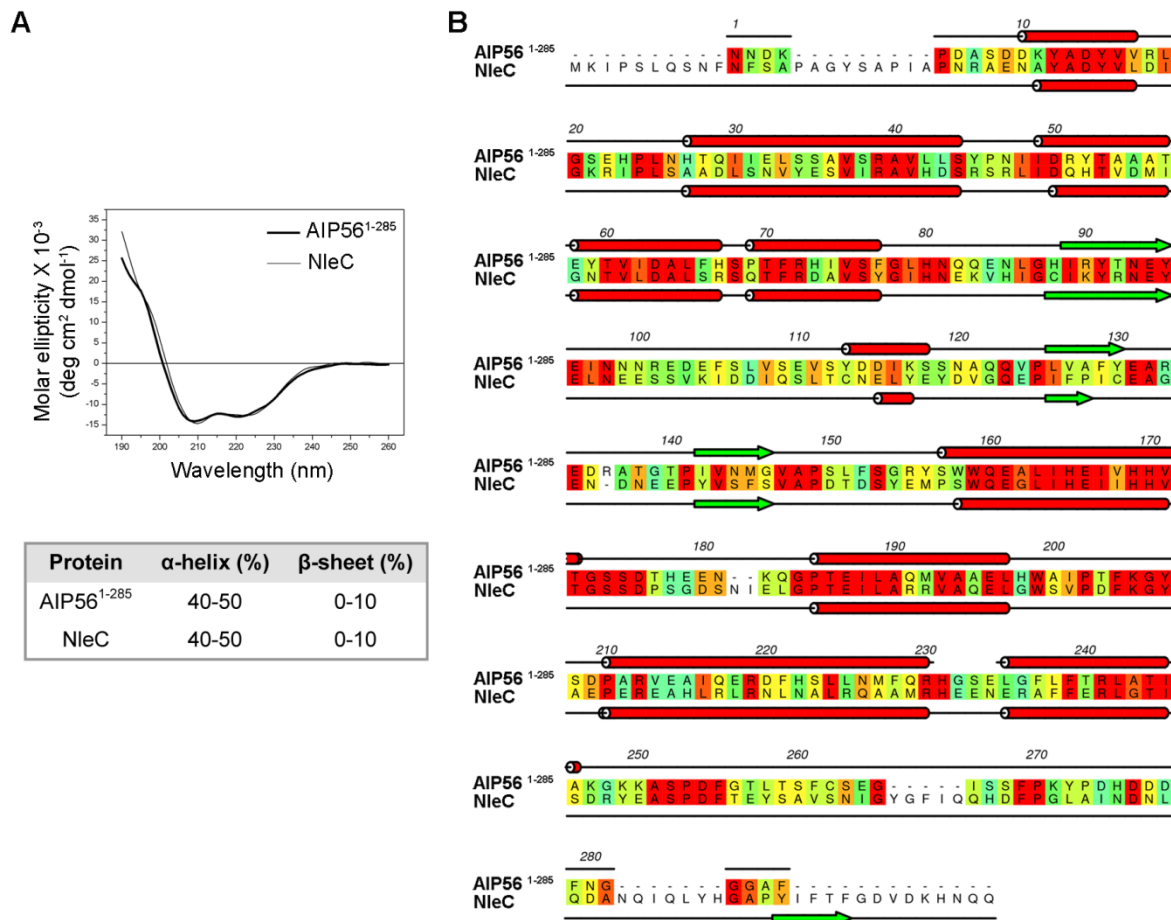


Figure 4.17. AIP56 N-terminal region and NleC share structural similarity. (A) Far-UV CD spectra of AIP56¹⁻²⁸⁵ and NleC and secondary structure content of AIP56¹⁻²⁸⁵ and NleC. **(B)** Sequence alignment of AIP56¹⁻²⁸⁵ and NleC showing the predicted secondary structures. α -helices are represented by red cylinders and β -sheets by green arrows. Sequence alignment was performed using ClustalW2 (Dunbrack, 2006) and secondary structures were predicted using PSIPRED (Jones, 1999; McGuffin et al, 2000).

4.2. NleC is not delivered into the cell cytosol by AIP56 C-terminal portion or LF/PA

Although AIP56 and NleC share the same intracellular target, these proteins differ in the mechanism used to enter target cells. While NleC is directly injected into the host cell cytoplasm through a type III secretion system (Dean & Kenny, 2009; Tobe et al, 2006), AIP56 has a chimeric structure, with an N-terminal catalytic region and a C-terminal region playing a role in binding and entry into target cells, conferring to the toxin an intrinsic ability to enter cells. To see whether the AIP56 C-terminus was able to deliver NleC into the cells, we produced a chimera in which the AIP56 N-terminal region (AIP56¹⁻²⁵⁰) was replaced by NleC¹⁻²⁷⁰ (see section 1). The rationale for this chimera was to join the NleC region that shares high similarity to AIP56, until the absolutely conserved motif ASPDF (see Figure 4.17) with the C-

terminal of AIP56 (AIP56²⁵¹⁻⁴⁹⁷) including the linker region, given the importance of those residues for the activity and delivery of the toxin (see above section 3.4). The delivery of NleC¹⁻²⁷⁰ into the cell cytoplasm of sea bass peritoneal leukocytes was tested using p65 cleavage as readout. No p65 cleavage could be observed upon incubation of NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ with cells, suggesting that the catalytic portion of the chimera (the NleC) was unable to reach the cytosolic compartment (Figure 4.18 A). The correct folding of the two fused protein domains was confirmed by testing NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ proteolytic activity *in vitro* against ³⁵S-labelled sbp65Rel (Figure 4.18B), and by using it as competitor for AIP56 apoptogenic activity (Figure 4.18C). The results show that the fused regions are not impaired in their functions, suggesting that the inability of the chimera to intoxicate cells is related to an inefficient delivery into the cytosol. It should be noted that the exact boundaries of the AIP56 binding/translocation domain were not yet established and, therefore, whether the lack of toxicity of the NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ chimera is related to the fusion of an incorrect AIP56 portion or to an inability of NleC¹⁻²⁷⁰ to translocate remains to be investigated.

Given the success of anthrax LF/PA system in delivering the catalytic domain of several toxins (Arora & Leppla, 1993; Arora & Leppla, 1994; Milne et al, 1995), including AIP56 (see above section 3.2), we decided to attempt the delivery of NleC into the cell cytoplasm using this system. A LF·NleC chimera, consisting on the N-terminal portion of LF (LF¹¹⁻²⁶³) fused with the full-length NleC, was produced (see section 1) and conservation of proteolytic activity towards p65 *in vitro* confirmed using ³⁵S-labelled sbp65Rel homology domain as substrate (Figure 4.18B). However, and in contrast with LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ chimera (Figure 4.12), incubation of cells with LF¹¹⁻²⁶³·NleC in the presence of PA did not result in p65 cleavage (Figure 4.18D) indicating that the LF/PA system was unable to translocate and deliver NleC into the cell cytoplasm.

A distinctive feature between AIP56 N-terminal region and NleC is the existence, in NleC, of three cysteine residues upstream the zinc metalloprotease signature, which are absent in AIP56 (Silva et al, 2010) (see chapter I Figure 1.10). Two of these cysteine residues (Cys¹⁰⁷ and Cys¹⁴⁹) are conserved among the NleC effectors from different enteropathogenic bacteria, suggesting that they may be involved in the formation of a disulphide bridge. Considering that translocation of protein cargos across the PA pores involves pH-driven unfolding of the passenger proteins, which can be impaired by the presence of disulphide bridges (Collier, 2009), the NleC putative disulphide bridge might constitute a structural restraint to protein unfolding required for translocation. Therefore, we produced a mutant chimera (LF¹¹⁻²⁶³·NleC^{Mut}) where the two conserved cysteine residues, Cys¹⁰⁷ and Cys¹⁴⁹, were mutated to their correspondent amino acid residue in AIP56 (a

histidine and a tyrosine, respectively) (see section 1). Nevertheless, although LF¹¹⁻²⁶³·NleC^{Mut} still retained the catalytic activity towards p65 *in vitro* (Figure 4.18B), it did not cleave p65 when added to cells in the presence of PA (Figure 4.18D), showing that the cysteine mutant LF·NleC chimera was also unable to translocate across the PA pores.

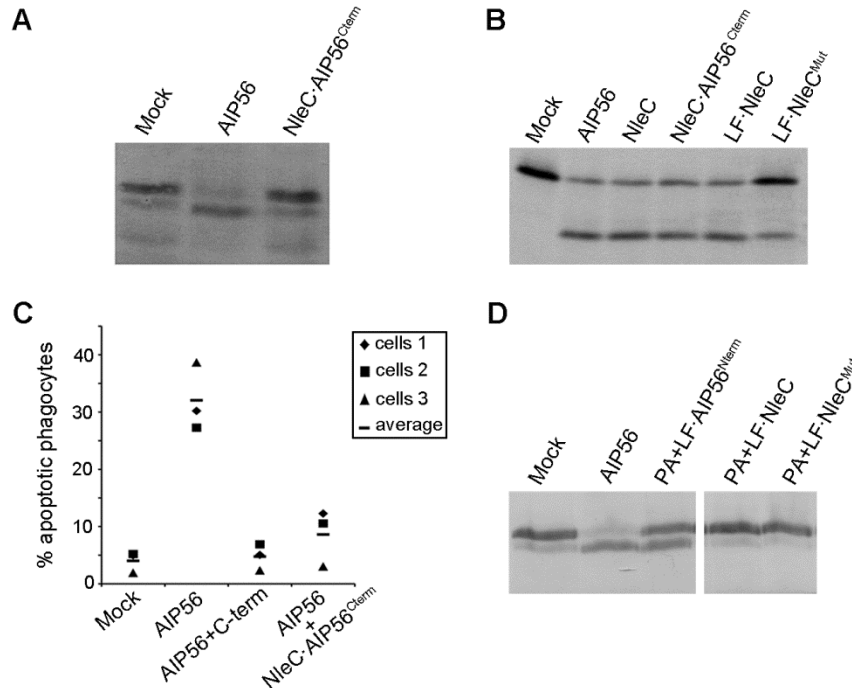


Figure 4.18. NleC-AIP56 and LF-NleC chimeras are catalytically active *in vitro* but do not cleave NF-κB p65 when incubated with cells. (A) NleC-AIP56 chimera does not cleave p65 when incubated with cells. Leukocytes were incubated with 10 µg/ml of NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ (NleC-AIP56^{Cterm}) for 2 h at 22 °C and p65 cleavage assessed by Western blotting. (B) NleC-AIP56 and LF-NleC chimeras are catalytically active *in vitro*. ³⁵S-labeled sbp65Rel (Met¹-Arg¹⁸⁸) was incubated for 2 h at 22 °C with 1 µM of AIP56, NleC, NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ (NleC-AIP56^{Cterm}), LF¹¹⁻²⁶³·NleC (LF-NleC) and LF¹¹⁻²⁶³·NleC^{Mut} (LF-NleC^{Mut}) and cleavage assessed by autoradiography. (C) NleC-AIP56 chimera competes with AIP56 and inhibits its apoptogenic activity. Sea bass leukocytes collected from 3 animals were incubated with 3.5 µM of NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ (NleC-AIP56^{Cterm}) for 15 min on ice followed by further 15 min incubation on ice with 8.75 nM of AIP56. Cells incubated with AIP56 in the absence of competitor, mock-treated cells and cells incubated with 3.5 µM AIP56²⁸⁶⁻⁴⁹⁷ (C-term) before incubation with AIP56 were used as controls. Cells were washed, transferred to 22 °C, incubated for 4 h and the percentage of apoptotic cells determined by morphological analysis of cytospin preparations stained with Hemacolor. (D) *B. anthracis* LF/PA system was not able to deliver NleC into the cells cytosol. Sea bass leukocytes were incubated for 4 h at 22 °C with 700 nM (48 µg/ml) of LF¹¹⁻²⁶³·NleC (LF-NleC) or LF¹¹⁻²⁶³·NleC^{Mut} (LF-NleC^{Mut}) in the presence of 10 nM of PA and cleavage of NF-κB p65 detected by Western blotting. Cells incubated with 2 µg/ml (35 nM) AIP56 or with 20 nM (1.2 µg/ml) LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ (LF-AIP56^{Nterm}) in the presence of 10 nM PA were used as positive controls. The depicted protein bands were from the same membrane and were cut out and juxtaposed for presentation in the figure. Part of this blot was included in Figure 4.12A.

4.3. AIP56¹⁻²⁶¹ and NleC have different stability towards unfolding

Considering that the ability to unfold in response to low pH is a requirement for proteins to translocate across the PA pores and in an attempt to explain the observed distinct

behaviour of AIP56¹⁻²⁶¹ and NleC in the LF/PA delivery, protein stability of LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·NleC and LF¹¹⁻²⁶³·NleC^{Mut} at different pH was assessed by differential scanning fluorimetry (DSF). Variation of the protein melting temperature (T_m) at different pH values was used as measure of the energy requirements for pH-driven unfolding. It was observed that at neutral pH, the thermo stability of LF¹¹⁻²⁶³·NleC is higher than the one of LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, as indicated by the 15.4 °C difference in their T_m (Figure 4.19). As expected, mutation of Cys¹⁰⁷ and Cys¹⁴⁹ of NleC, had a destabilizing effect with the T_m of LF¹¹⁻²⁶³·NleC^{Mut} being 8.0 °C below the one of LF¹¹⁻²⁶³·NleC. Still, LF¹¹⁻²⁶³·NleC^{Mut} is significantly more stable at pH 7.0 than LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, as denoted by the 7.8 °C difference in their T_m (Figure 4.19). Despite the differences in the thermo stability of the analysed proteins, in all of them, a marked effect of pH on the thermal stability was observed with the T_m decreasing as pH diminished. However, whereas LF¹¹⁻²⁶³·NleC was quite stable up to pH 5.0 (T_m of 36.4 °C at pH 5.0), LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ did not withstand pH values below 6.0 being already precipitated/unfolded at this pH (Figure 4.19). Despite LF¹¹⁻²⁶³·NleC^{Mut} was found to be more susceptible to pH-driven unfolding than LF¹¹⁻²⁶³·NleC (Figure 4.19), when compared with LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·NleC^{Mut} is significantly more stable at acidic pH values presenting at pH 6.0 and 5.5 T_m of 47.7 and 40.8 °C, respectively. Together, these results indicate that LF¹¹⁻²⁶³·NleC and LF¹¹⁻²⁶³·NleC^{Mut} have a significantly higher resistance to pH-induced unfolding than LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, a difference that may explain the inability of LF·NleC chimeras to translocate across the PA pore.

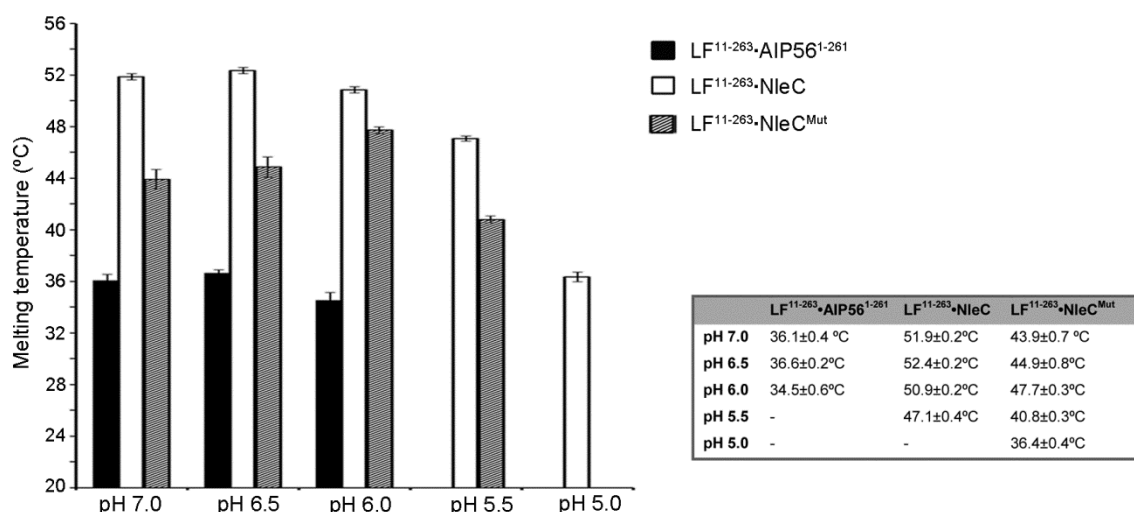


Figure 4.19. AIP56's N-terminal region and NleC have different unfolding properties. Melting temperatures of LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹, LF¹¹⁻²⁶³·NleC and LF¹¹⁻²⁶³·NleC^{Mut} at different pH values. The protein unfolding was monitored by DSF. The melting temperature (T_m) was calculated as the inflection point of the fluorescence curve. The results are the mean±SD of 7 measurements performed in two independent experiments. The melting temperatures for LF¹¹⁻²⁶³·AIP56¹⁻²⁶¹ at pH 5.5 and 5.0 and for LF¹¹⁻²⁶³·NleC^{Mut} at pH 5.0 could not be determined due to protein precipitation.

5. AIP56 crystallization

5.1. AIP56 purification and characterization

The complete characterization of a given protein involves the understanding of its biochemical, biophysical and structural features which are ultimately unravelled by knowing the protein's three-dimensional structure. The three-dimensional structures of AIP56 toxin or of its homologues are still unknown and their knowledge would provide an invaluable framework for detailed understanding of the molecular mechanisms of action of these molecules. X-ray crystallography was the method of choice to try to solve AIP56 three-dimensional structure, since it is a powerful technique that allows the visualization of macromolecular structures at atomic resolution. However, determining the structure of a protein by this technique entails growing high-quality crystals of the purified protein, which are highly dependent on protein sample purity, not only in terms of the lack of non-wanted proteins but also in terms of homogeneity and stability of the protein in solution (Dale et al, 2003; Durbin & Feher, 1996; Giergé R., 1992).

The protocol for purification of recombinant AIP56 described in section 1 (where the protein was purified by nickel affinity chromatography followed by anion-exchange chromatography, used as a polishing step) yield fairly pure but unstable protein, with high tendency to precipitate, that does not abide the high quality requirements for crystallization trials. Thus, improving AIP56 purification in order to obtain high amounts of highly pure and stable protein was imperative before proceeding to crystallization trials.

Taking into account that protein stability can also be significantly improved by changing buffer composition and knowing that AIP56 contains a zinc-binding signature (do Vale et al, 2005; Giergé R., 1992) addition of zinc chloride to cell lysis and affinity chromatography buffers was also attempted. However and contrary to what was expected, its presence in solution had a destabilizing effect and increased AIP56 precipitation. Pursuing the improvement of protein stability in solution, the effect of increasing glycerol and NaCl concentrations in the protein buffer were evaluated by Dynamic Light Scattering (DLS), using the polydispersity index (representative of the particle size distribution) as a measure of protein homogeneity and stability in solution (Table 4.1). High polydispersity index values were obtained for AIP56 in the majority of the tested buffer compositions. Yet, a decrease in the polydispersity index values, indicative of an increase in protein stability, was observed by increasing NaCl concentration (Table 4.1), while no significant improvement on the protein stability was achieved by addition of glycerol to the protein (Table 4.1). Thus, considering the observed stabilizing effect of NaCl, the concentration of this salt in cell lysis and affinity chromatography buffers was raised to 500 mM and the toxin was maintained at high NaCl

conditions whenever possible. Following this reasoning, substitution of the ion-exchange chromatography, by an alternative purification step would be desirable. However, the presence, in affinity chromatography eluted fractions, of contaminants with a molecular weight similar to that of AIP56 (not shown) dismissed the use of size exclusion chromatography as polishing step. Nonetheless, the time during which AIP56 was at lower NaCl concentrations was kept at minimum and by using a high resolution ion-exchange chromatography column, with higher affinity for AIP56, we were able to perform the binding of AIP56 to the chromatography column at a higher NaCl concentration and to improve protein purity. Additionally, reduced protein precipitation was achieved by performing the desalting of affinity chromatography elution fractions more rapidly using PD-10 columns instead for overnight dialysis (given the tendency of AIP56 to precipitate in the presence of imidazole, discussed in section 1), and by disrupting the bacterial cells by sonication instead of freezing/thawing cycles.

Table 4.1. Analysis of AIP56 stability in different solutions by Dynamic Light Scattering. Values are means $\pm$ SD of 3 independent measurements.

Condition	Polydispersity index
10 mM Tris-HCl pH 8.0, 100 mM NaCl	0.488 $\pm$ 0.022
10 mM Tris-HCl pH 8.0, 200 mM NaCl	0.364 $\pm$ 0.013
10 mM Tris-HCl pH 8.0, 500 mM NaCl	0.276 $\pm$ 0.014
10 mM Tris-HCl pH 8.0, 200 mM NaCl, 5% (v/v) glycerol	0.408 $\pm$ 0.003
10 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% (v/v) glycerol	0.426 $\pm$ 0.008
10 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol	0.382 $\pm$ 0.017
10 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% (v/v) glycerol	0.426 $\pm$ 0.012

(In terms of a protein analysis, a polydispersity index below 0.20 indicates that the sample is monodisperse (Borgstahl, 2007))

All the above mentioned adjustments to the purification procedure contributed to the improvement of the protein sample quality. Thirty milligrams of highly pure AIP56 (in 10 mM Tris-HCl pH 8.0, 200 mM NaCl), produced from three different batches of bacterial cell culture (average yield 2 mg/L pure AIP56) (Figure 4.20A), were obtained. Quality of AIP56 preparations was controlled in terms of purity (by SDS-PAGE) and in terms of homogeneity and monodispersity (by size exclusion chromatography, Native-PAGE and DLS) (Figure 4.20). Size exclusion chromatography and Native-PAGE analysis indicate a homogenous protein preparation with purified AIP56 eluting from size exclusion chromatography as a

single symmetrical peak (Figure 4.20B) and migrating as a single band in Native-PAGE (Figure 4.20C). However, DLS revealed the existence of high molecular weight species in the AIP56 preparation (Figure 4.20D). The fact that the scattering intensity is proportional to the particle molecular weight makes DLS a very sensitive technique to detect protein aggregation and/or the presence of large contaminants, even at very low concentrations undetectable in size exclusion chromatography, as it is the case. The measured AIP56 samples presented a polydispersity index above 0.3 and thus were considered as polydisperse. However, it should be taken into consideration that the polydispersity index is representative of the particle size distribution and therefore the high value obtained can be related not only to the presence of protein aggregates but also to a possible non-globular shape. Therefore, and even though AIP56 preparation did not fully meet all the high quality criteria, we decided to follow with crystallization trials.

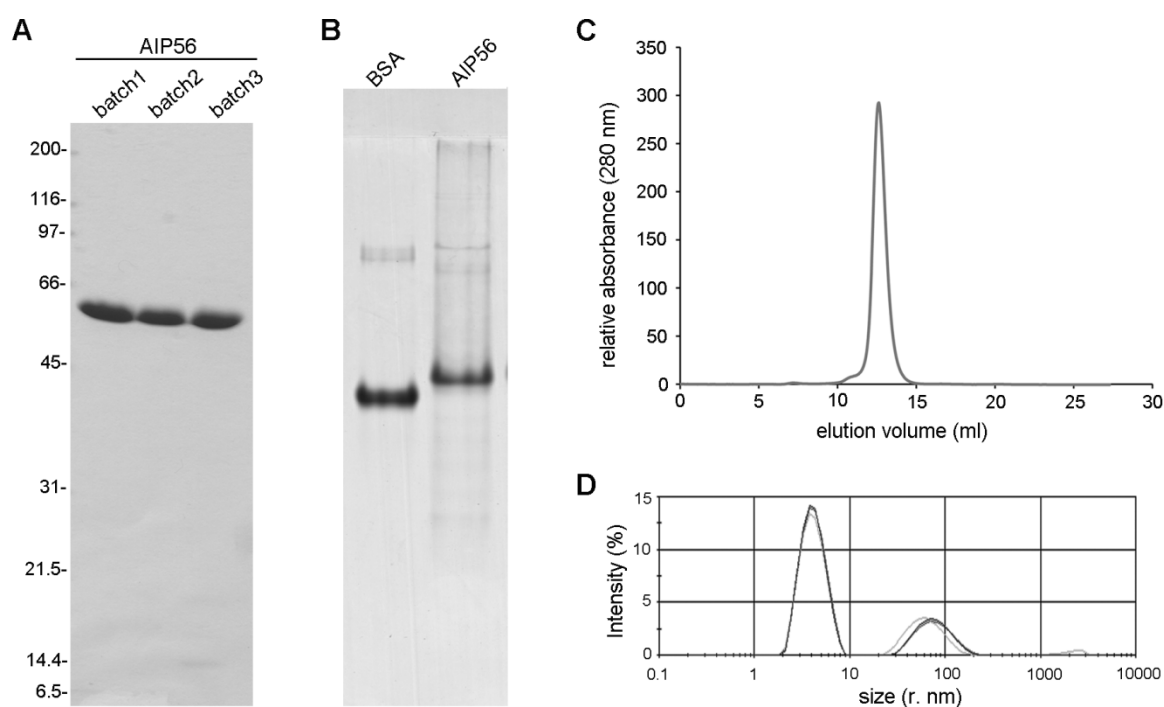


Figure 4.20. Purity and homogeneity of AIP56 preparations used in crystallization trials. **A)** Ten μ g AIP56 were analysed by SDS-PAGE (carried out in a 12.5% separating gel) and stained with Coomassie Blue. Numbers on the left of the panels refer to the position and mass, in kDa, of the molecular weight markers. **B)** Native-PAGE analysis of AIP56. BSA was run for control purposes. **C)** Analytical size exclusion chromatography of AIP56. Fifty μ g of AIP56 in 10 mM Tris pH 8.0, 200 mM NaCl was injected onto Superose 12 10/300 column (GE Healthcare) pre-equilibrated in the same buffer. Protein elution was monitored by measuring the absorbance at 280 nm. **D)** Dynamic light scattering analysis of AIP56. Scattered light intensity vs. particle radius plot. The first peak of intensity corresponds to a population of molecules with a hydrodynamic radius of 4.22 nm. Note the presence of a peak correspondent to high molecular weight species.

5.2. Crystallization trials

AIP56 crystallization screens were performed using the vapor diffusion method in sitting drops. Sparse-matrix commercial crystallization kits were used in the first screenings for crystallization conditions and formation of AIP56 microcrystals was observed in several conditions, (see annex 1 Table 4.2). An interesting observation was that in all crystallization conditions (with exception of condition I, see below), polyethylene glycol (PEG) was the precipitant agent. Conditions VIII, XV, XXVII, XXXII (see annex 1 Table 4.2) were selected for additional screening by varying pH of the crystallization solution, PEG molecular weight and concentration, salt concentration and addition of additives, as well as variation of drop volume and protein:crystallization solution ratio. Neutral or slightly alkaline pH were found to favour AIP56 crystallization. Furthermore, although variation of drop volume and ratio of crystallization solution:protein did not result in a significantly improvement of crystal growth, in most cases, variation of the polymeric range and concentration of the precipitant agent and the addition of glycerol significantly improved crystal size and singularity. The majority of the obtained crystals were composed of thin plates arranged in large clusters (Figure 4.21 A. B). Nonetheless, in some conditions, isolated crystals (mostly small thin crystal plates) (Figure 4.21 D, E and F) were obtained. These isolated crystals were flash-cooled and sent for testing at the European Radiation Facility (ESRF, Grenoble, France) (see annex 1 Table 4.3). Although some of the tested crystals looked promising they were found to be composed by very thin superimposed crystal layers and showed poor diffraction properties.

Flexibility in a protein and structural micro-heterogeneities adversely affect crystal growth, especially when structural heterogeneities concern regions involved in crystal packing. As presented above in section 3, AIP56 has a chimeric structure consisting in two disulphide-bound structural domains that are tethered by a flexible linker. While the two AIP56 domains are quite resistant to protease digestion in limited proteolysis experiments, the loop region is readily cleaved by chymotrypsin or proteinase K, originating the separation of two domains that are still held together through the disulphide bond (Figure 4.8). Hence, *in situ* proteolysis crystallization experiments, a technique proven to be successful in the crystallization of several multidomain proteins, which consists on crystallization of a protein in the presence of residual amounts of proteases (Bai et al, 2007; Dong et al, 2007; Mandel et al, 2006; Wernimont & Edwards, 2009), was attempted. Sparse-matrix crystallization kits were tested using a sample of AIP56 containing small amounts of chymotrypsin. However, no crystalline structures were obtained and formation of protein precipitates occurred in the vast majority of the conditions tested, even in those that yielded crystals with AIP56 alone. Additionally, in an attempt to control nucleation, and thus improve crystal size and singularity,

seeding techniques (Stura & Wilson, 1991) were employed. However, again, no significant improvements in crystal quality were achieved with crystal plate clusters obtained in most of the drops.

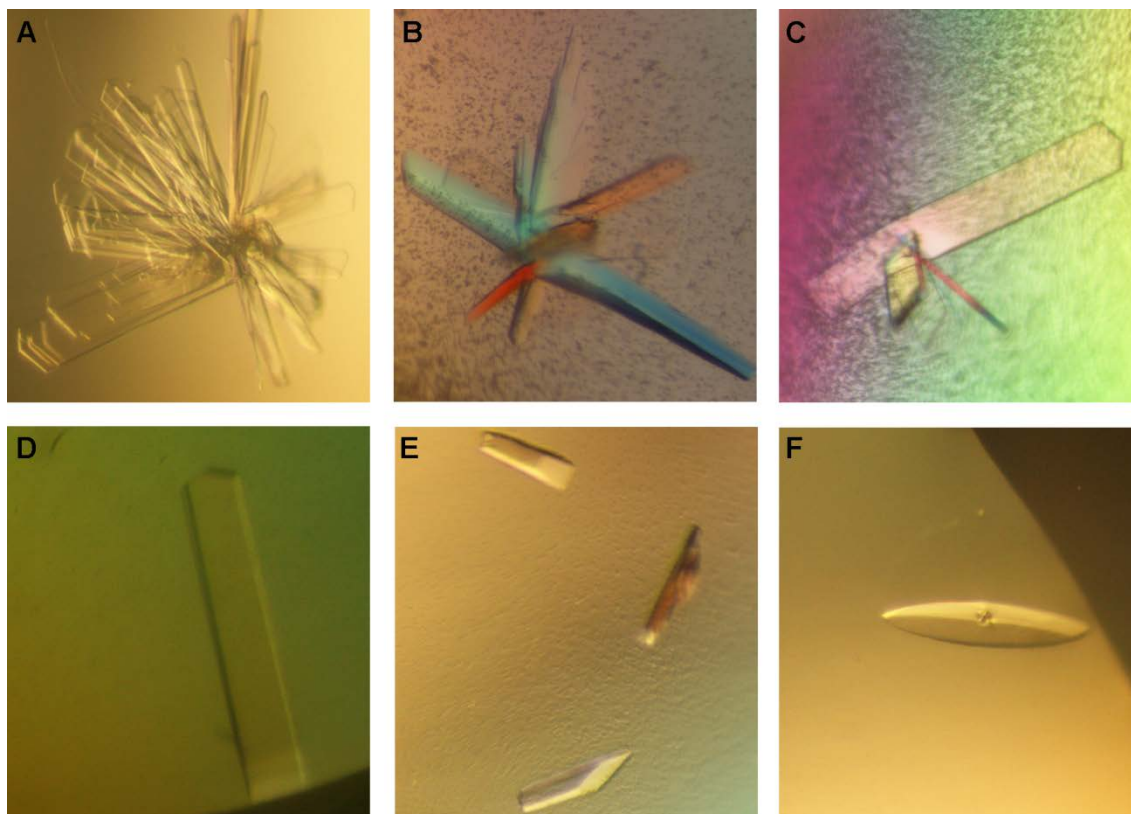


Figure 4.21. AIP56 crystals. (A) AIP56 crystal cluster grown in 0.2 M ammonium citrate tribasic pH 7.0, 15% (w/v) polyethylene glycol 3350. (B) AIP56 crystal cluster grown in 0.2 M potassium thiocyanate, 15% (w/v) polyethylene glycol 2000, 0.1 M HEPES pH 7.0, 1% glycerol. (C) AIP56 crystal plates grown in 0.2 M potassium thiocyanate, 15% (w/v) polyethylene glycol 2000, 0.1 M HEPES pH 7.0, 0.5% (v/v) glycerol. (D) AIP56 crystal plate grown in 0.2 M potassium thiocyanate, 15% (w/v) polyethylene glycol 2000, 0.1 M HEPES pH 7.0, 0.5% (v/v) glycerol (Crystal XVIII, table 4.3, annex 1). (E) AIP56 crystal plates grown in AIP56 crystal plate grown in 0.2 M potassium thiocyanate, 20% (w/v) polyethylene glycol 8000, 0.1 M HEPES pH 7.9. (Crystals XXXIII and XXXIV, table 4.3, annex 1). (F) AIP56 crystal plate grown in 0.2 M potassium thiocyanate, 20% (w/v) polyethylene glycol 6000, 0.1 M HEPES pH 7.9. (Crystal XXIV, table 4.3, annex 1).

As mentioned before, well-succeed protein crystallization, depends largely on the purity of protein samples, not only in terms of unwanted contaminating proteins but also in terms of structural and conformational homogeneity of the intended-protein (Dale et al, 2003; Giergé R., 1992). Structural micro-heterogeneities in the protein are known to adversely affect crystal growth, especially when structural heterogeneities concern domains involved in crystal packing (Durbin & Feher, 1996; Van der Laan et al, 1989). Thus, in the case of AIP56 crystallization, the observed AIP56 instability and heterogeneity are likely causes for

the multiplicity of the obtained AIP56 crystals and improvement of AIP56 stability seems to be imperative before undertaking new crystallization trials. Nevertheless, although we were unable to obtain AIP56 crystals suitable for performing X-ray diffraction studies, these studies allowed the identification of promising preliminary crystallization conditions and provided important knowledge regarding AIP56 stability and behaviour in solution useful for future AIP56 crystallization projects.

CHAPTER IV

RESULTS (ANNEX-1)

Table 4.2. List of conditions that produced crystals. (Conditions in bold were selected for optimisation)

Condition	Chemical composition	Result
I	0.1 M Bis-Tris pH 6.5, 3.0 M sodium chloride	small crystals
II	0.1 M Bis-Tris pH 6.5, 28% (w/v) polyethylene glycol 2000	microcrystals
III	0.2 M ammonium sulfate, 0.1 M HEPES pH 7.5, 25% (w/v) polyethylene glycol 3350	microcrystals
IV	0.2 M ammonium sulfate, 0.1 M Tris pH 8.5, 25% (w/v) polyethylene glycol 3350	small crystals
V	0.2 M lithium sulfate monohydrate, 0.1 M Tris-HCl pH 8.5, 25% (w/v) polyethylene glycol 3350	microcrystals
VI	0.2 M potassium sodium tartrate tetrahydrate, 20% (w/v) polyethylene glycol 3350	microcrystals
VII	0.2 M sodium malonate pH 7.0, 20% (w/v) polyethylene glycol 3350	microcrystals
VIII	0.2 M ammonium citrate tribasic pH 7.0, 20% (w/v) polyethylene glycol 3350	small crystals
IX	0.2 M sodium formate, 20% (w/v) polyethylene glycol 3350	microcrystals
X	0.15 M DL-malic acid pH 7.0, 20% (w/v) polyethylene glycol 3350	small crystals
XI	0.2 M sodium citrate tribasic dihydrate, 20% (w/v) polyethylene glycol 3350	microcrystals
XII	0.1 M potassium thiocyanate, 30% (w/v) polyethylene glycol 2000	microcrystals
XIII	0.15 M potassium bromide, 30% (w/v) polyethylene glycol 2000	microcrystals
XIV	0.2 M lithium sulfate monohydrate, 0.1 M Tris-HCl pH 8.5, 30% (w/v) polyethylene glycol 4000	microcrystals
XV	0.1 M HEPES pH 7.5, 10% (w/v) polyethylene glycol 6000, 5% (v/v) (+/-)-2-methyl-2,4-pentanediol	crystal plates
XVI	0.1 M HEPES pH 7.5, 8% (v/v) ethylene glycol, 10% (w/v) polyethylene glycol 8000	small crystals
XVII	0.1 M HEPES pH 7.5, 20% (w/v) polyethylene glycol 10000	microcrystals
XVIII	1.5 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5, 12% (v/v) glycerol	microcrystals
XIX	0.16 M magnesium chloride hexahydrate, 0.08 M Tris-HCl pH 8.5, 24% (w/v) polyethylene glycol 4000, 20% (v/v) glycerol	needle clusters
XX	0.17 M lithium sulfate monohydrate, 0.085 M Tris-HCl pH 8.5, 25.5% (w/v) polyethylene glycol 4000, 15% (v/v) glycerol	microcrystals
XXI	0.16 M magnesium acetate tetrahydrate, 0.08 M sodium cacodylate trihydrate pH 6.5, 16% (w/v) polyethylene glycol 8000, 20% (v/v) glycerol	microcrystals
XXII	0.17 M sodium acetate trihydrate, 0.085 M Tris-HCl pH 8.5, 25.5% (w/v) polyethylene glycol 4000, 15% (v/v) glycerol	microcrystals
XXIII	0.2 M potassium chloride, 20% (w/v) polyethylene glycol 3350	microcrystals
XXIV	0.2 M sodium iodide, 20% (w/v) polyethylene glycol 3350	microcrystals
XXV	0.2 M potassium iodide, 20% (w/v) polyethylene glycol 3350	microcrystals
XXVI	0.2 M sodium thiocyanate, 20% (w/v) polyethylene glycol 3350	microcrystals
XXVII	0.2 M potassium thiocyanate, 20% (w/v) polyethylene glycol 3350	small crystals

Table 4.2. List of conditions that produced crystals. (Cont)

Condition	Chemical composition	Result
XXVIII	0.2 M sodium formate, 20% (w/v) polyethylene glycol 3350	microcrystals
XXIX	0.2 M potassium formate, 20% (w/v) polyethylene glycol 3350	microcrystals
XXX	0.2 M sodium tartrate dibasic dihydrate, 20% (w/v) polyethylene glycol 3350	microcrystals
XXXI	0.2 M potassium sodium tartrate tetrahydrate, 20% (w/v) polyethylene glycol 3350	microcrystals
XXXII	0.2 M ammonium phosphate dibasic, 20% (w/v) polyethylene glycol 3350	small crystals
XXXIII	0.2 M lithium citrate tribasic tetrahydrate, 20% (w/v) polyethylene glycol 3350	small crystals
XXXIV	0.2 M sodium citrate tribasic dihydrate, 20% (w/v) polyethylene glycol 3350	small crystals

Table 4.3. List of AIP56 crystals measured

Crystal	Diffraction	Crystallization solution
I	4.3 Å	0.2 M ammonium phosphate dibasic, 15% (w/v) polyethylene glycol 8000, 0.1 M Tris-HCl pH 7.9
II	4.3 Å	
III	4.5 Å	
IV	4 Å	0.2 M ammonium phosphate dibasic, 15% (w/v) polyethylene glycol 8000, 0.1 M Tris-HCl pH 7.9, 0.2% (v/v) glycerol
V	3.5 Å	
VI	3.8 Å (anisotropic)	0.2 M ammonium phosphate dibasic, 15% (w/v) polyethylene glycol 8000, 0.1 M Tris-HCl pH 7.9, 1% (v/v) glycerol
VII	3 Å (anisotropic)	0.2 M ammonium phosphate dibasic, 15% (w/v) polyethylene glycol 6000, 0.1 M Tris-HCl pH 7.9
VIII	4 Å (anisotropic)	
IX	4 Å (anisotropic)	0.2 M ammonium phosphate dibasic, 15% (w/v) polyethylene glycol 6000, 0.1 M Tris-HCl pH 7.9, 0.5% (v/v) glycerol
X	7 Å	
XI	No diffraction	0.2 M ammonium phosphate dibasic, 15% (w/v) polyethylene glycol 6000, 0.1 M Tris-HCl pH 7.9, 1% (v/v) glycerol
XII	No diffraction	
XIII	3 Å	0.2 M potassium thiocyanate, 15% (w/v) polyethylene glycol 2000, 0.1 M HEPES pH 7.0, 0.2% (v/v) glycerol
XIV	3.1 Å	
XV	4 Å	
XVI	4 Å	
XVII	3.5 Å (anisotropic)	
XVIII	3.0 Å (anisotropic)	0.2 M potassium thiocyanate, 15% (w/v) polyethylene glycol 2000, 0.1 M HEPES pH 7.0, 0.5% glycerol
XIX	8 Å	
XX	3.5 Å (anisotropic)	
XXI	5 Å	
XXII	No diffraction	
XXIII	No diffraction	0.2 M potassium thiocyanate, 25% (w/v) polyethylene glycol 2000, 0.1 M HEPES pH 7.9
XXIV	5 Å	
XXV	4 Å	
XVI	8 Å	
XVII	No diffraction	
XVIII	No diffraction	0.2 M potassium thiocyanate, 20% (w/v) polyethylene glycol 6000, 0.1 M HEPES pH 7.9
XXIX	No diffraction	
XXX	No diffraction	
XXXI	No diffraction	
XXXII	3 Å (anisotropic)	
XXXIII	3 Å (anisotropic)	0.2 M potassium thiocyanate, 15% (w/v) polyethylene glycol 8000, 0.1 M HEPES pH 7.9

CHAPTER V

DISCUSSION

1. AIP56: a zinc metalloprotease targeting NF- κ B p65

Photobacterium damsela *piscicida* (*Phdp*) is a successful bacterial pathogen whose virulence strategy relies on the induction of apoptosis of the host professional phagocytes through the secretion of an exotoxin named AIP56 (Costa-Ramos et al, 2011; do Vale et al, 2007a; do Vale et al, 2005). By inducing apoptosis of both macrophages and neutrophils, AIP56 also causes a severe impairment of the host phagocytic defences, allowing the establishment of a septicemic infection (do Vale et al, 2007a; do Vale et al, 2003; do Vale et al, 2005). Another consequence of the lack of functional phagocytes is an insufficient clearance of apoptotic cells and apoptotic bodies that eventually lyse by secondary necrosis (do Vale et al, 2007a). This AIP56-induced phagocyte apoptosis proves to be a very effective mechanism of amplification of the etiopathogenicity, because it results in two concerted effects against the host: evasion of the pathogen from phagocytosis, a fundamental defence mechanism, and release of tissue harmful molecules by secondary necrosis of the apoptotic phagocytes (do Vale et al, 2007a). Although the pathology of the disease and the toxic effects of AIP56 are well characterized (Costa-Ramos et al, 2011; do Vale et al, 2002; do Vale et al, 2007a; do Vale et al, 2007b; do Vale et al, 2003; do Vale et al, 2005), at the beginning of this work the molecular mechanisms beyond AIP56 toxicity were still unknown.

Identification of host proteins targeted by bacterial virulence factors and characterization of their processing sites under relevant physiological conditions are essential for understanding the biological function of those factors in the context of an infection. Here, by showing that AIP56 is a zinc-dependent metalloprotease that cleaves the p65 subunit of the nuclear factor- κ B (NF- κ B) we extend the functional characterization of AIP56 and provide valuable information regarding the molecular mechanisms beyond the toxin's activity.

Several other bacterial toxins such as tetanus toxin (TeNT), botulinum toxin (BoNT) and anthrax lethal factor (LF) are zinc-dependent metalloproteases with a crucial role for the virulence of their producing bacteria (Moayeri & Leppla, 2004; Schiavo et al, 1994; Swaminathan, 2011; Tonello & Montecucco, 2009). The clostridial neurotoxins (TeNT and BoNT) cleave the SNARE proteins (soluble NSF-attachment protein receptors) (Schiavo et al, 1992a; Schiavo et al, 1992b; Schiavo et al, 1994), a group of proteins involved in the fusion of synaptic vesicles with the plasma membrane (Ramakrishnan et al, 2012), causing blockage of neurotransmitter release and leading to flaccid paralysis (Swaminathan, 2011). The anthrax LF cleaves the N-terminal tail of mitogen-activated protein kinase (MAPK) kinases, blocking MAPK signalling and thus causing an impairment of the host immune responses (Chopra et al, 2003; Duesbery et al, 1998; Klimpel et al, 1994; Tonello &

Montecucco, 2009; Vitale et al, 1998). One of the most prominent effects of the LF-mediated blocking of MAPK signalling pathways is the induction of apoptosis on macrophages (Park et al, 2002; Popov et al, 2002). By inducing macrophage apoptosis, LF prevents the release of chemokines and cytokines that would alert the immune system to the presence of infecting *Bacillus anthracis*, thereby facilitating the septicaemic spread of the infection (Park et al, 2002; Tournier et al, 2009). The described anthrax LF virulence mechanism resembles the one employed by AIP56 during *Phdp* infections (do Vale et al, 2007a; do Vale et al, 2003; do Vale et al, 2005; Silva et al, 2010).

Our data show that AIP56 cleaves the p65 subunit of NF- κ B within its Rel homology domain. Cleavage was observed upon incubation of sea bass cell lysates or recombinant p65 Rel homology domain with AIP56 and was dependent on the metalloprotease signature of the toxin. This proteolytic activity against p65 was found to play a relevant role in the intoxication of target cells with AIP56, since an AIP56 metalloprotease mutant lacking p65 cleaving activity did not induce apoptosis.

The NF- κ B transcription factors consist of homo- or heterodimeric assemblies of the Rel/NF- κ B proteins (NF- κ B 1 (p50), NF- κ B 2 (p52), RelA (p65), RelB and c-Rel) of which the p50/p65 heterodimer is the most abundant (Gilmore, 1999). These factors regulate the expression of hundreds of genes involved in the immunological response including several cytokines (such as IL-8, IL-6, IL-10, IL1- α , IL-1 β , TNF- α , IFN- γ) and their modulators, immune receptors, proteins involved in antigen presentation and cell adhesion molecules (Baeuerle & Henkel, 1994; Ghosh et al, 1998; Vallabhapurapu & Karin, 2009). In addition to immune related genes, the expression of several proteins involved in cell survival and proliferation are also regulated by NF- κ B (Burstein & Duckett, 2003; Gerondakis et al, 2012; Shishodia & Aggarwal, 2002). These proteins include regulators of apoptosis, both anti-apoptotic genes (such as cIAP-1 and cIAP-2, Bcl-2, Bcl- χ_L and Bfl-1), which are up-regulated upon NF- κ B activation, and pro-apoptotic genes (such as p53, TRAIL and Fas), which are down-regulated upon NF- κ B activation (Burstein & Duckett, 2003; Shishodia & Aggarwal, 2002), stress response genes, growth factors, growth factor ligands and their modulators (Baldwin, 2012; Gerondakis et al, 2012), among others. The expression of NF- κ B-regulated genes is signal-induced and highly regulated. Under normal physiological conditions, the NF- κ B complexes remain inactive in the cytosol through association with I κ B (inhibitors of NF- κ B) proteins that mask the nuclear localization signals on NF- κ B subunits. A variety of stimuli, including bacterial and viral products and cytokines acting via cellular receptors trigger signalling cascades that leads to degradation of the inhibitory I κ B proteins with rapid

activation and transport of the NF- κ B complexes to the nucleus (Oeckinghaus & Ghosh, 2009).

Conservation of the NF- κ B transcription factors from *Drosophila* to humans and the presence of a functional NF- κ B signalling cascade in the horseshoe crab *Carcinoscorpius rotundicauda* (known as a living fossil) suggest that the proteins involved and their mechanisms of action are evolutionarily conserved, underlining their pivotal role in biological responses (Gilmore & Wolenski, 2012; Wang et al, 2006). Although the knowledge about NF- κ B biological functions, transcriptional activity and regulation mechanisms in early vertebrates is still scarce, evidences point to preservation of function and activation mechanisms in fish. A number of NF- κ B/I- κ B family proteins, with a significant level of sequence homology and function conservation to their mammalian orthologues, have been identified in several fish species (Correa et al, 2004; Kong et al, 2011; Schlezinger et al, 2000; Tacchi et al, 2011; Wang et al, 2009). Noteworthy, in zebrafish, a fully conserved NF- κ B/I- κ B pathway activated by established NF- κ B inducers (such as LPS and UV-light) and cross compatible with mammalian orthologues was found (Correa et al, 2004). Moreover, indirect evidences also suggest that in carp, NF- κ B is involved in induction of expression of the inflammatory cytokines tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) (Saeij et al, 2003), and in rainbow trout, in the interferon signalling pathway (Collet et al, 2004). Furthermore, over-expression of olive flounder p65 in HINAE cells (olive flounder embryonic cell line) resulted in increased expression of TNF- α and I κ B- α mRNA, suggesting transcriptional control of immune-related genes and conservation of auto-regulative feedback pathways (Kong et al, 2011).

N-terminal sequencing of the p65 cleavage fragment, obtained upon incubation of recombinant sea bass p65 Rel homology domain with AIP56, revealed that the toxin cleaves NF- κ B p65 at the Cys³⁹-Glu⁴⁰ peptide bond. The identified AIP56 cleavage site within p65 is evolutionarily conserved and is similar to the cleavage site of the type III secreted effector NleC reported by Baruch and colleagues (Baruch et al, 2010). In addition to p65, NleC was also described to target c-Rel (Pearson et al, 2011) and the acetyltransferase p300 (Shames et al, 2011), and there are contradictory reports regarding its ability to cleave p50 (Muhlen et al, 2011; Pearson et al, 2011; Yen et al, 2010). The conservation of the identified AIP56 cleavage site (within p65) among the Rel/NF- κ B proteins together with the degree of homology between NleC and the N-terminal region of AIP56 suggest that other members of the NF- κ B family or the acetyltransferase p300 may also be targeted by AIP56. However, whether this hypothesis holds truth remains to be investigated.

Several key residues of p65 known to be involved in its interaction with DNA are located near the start of the Rel homology domain (Chen et al, 1998a; Chen et al, 1998b; Hoffmann et al, 2006) and, in the last decade, several reports revealed a key importance of Cys³⁸ of human p65 (Cys³⁹ in sea bass p65) for this interaction. This residue was shown to interact with the phosphate backbone of NF- κ B binding sites (Chen et al, 1998a) and its oxidation and nitrosylation were found to inhibit DNA binding (Kelleher et al, 2007). Furthermore, this particular cysteine is also the target of several NF- κ B inhibitors with reported anti-inflammatory and/or anticancer properties (Garcia-Pineros et al, 2001; Garcia-Pineros et al, 2004; Ha et al, 2009; Han et al, 2005; Harikumar et al, 2009; Laga et al, 2007; Liang et al, 2006; Sandur et al, 2006; Sethi et al, 2008; Straus et al, 2000; Watanabe et al, 2008) and more recently, Sen and colleagues showed that Cys³⁸ of human p65 plays a key role in regulating the anti-apoptotic actions of NF- κ B (Sen et al, 2012). These authors demonstrated that following TNF- α stimulation, Cys³⁸ of human p65 undergoes hydrogen sulphide-linked sulfhydration, enhancing the interaction with its co-activator, the ribosomal protein S3 (RPS3), and resulting in an increased binding to the promoters of several anti-apoptotic genes. They further showed that impairment of Cys³⁸ sulfhydration leads to the inability of p65 to bind to DNA and prevents the NF- κ B-mediated expression of anti-apoptotic genes, thereby sensitizing cells to TNF- α induced cell death (Sen et al, 2012). Therefore, cleavage of p65 by AIP56 at the Cys³⁹-Glu⁴⁰ peptide bond results in the removal of several crucial residues involved in 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- κ B dependent transcription (Baruch et al, 2010; Muhlen et al, 2011; Yen et al, 2010), it is likely that AIP56 also affects NF- κ B transcriptional activity. Therefore, and considering the anti-apoptotic functions of NF- κ B, and in particular, of its p65 subunit (Bachmeyer et al, 2001; Beg & Baltimore, 1996; Beg et al, 1995; Sen et al, 2012), the AIP56-mediated depletion of NF- κ B p65 is likely involved in the disseminated phagocyte apoptosis observed in *Phdp* infections that contributes to subvert the host immune response and determines the outcome of the infection. 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 (DeLeo, 2004; Lamkanfi & Dixit, 2010; Weinrauch & Zychlinsky, 1999).

Activation of NF- κ B is considered to be the central initiating event of host responses to invasion by microbial pathogens (Neish & Naumann, 2011; Rahman & McFadden, 2011) and therefore, it is not surprising that successful microbial pathogens have evolved complex

strategies to interfere with the NF- κ B signalling pathways. In the last years, a growing number of pathogenic bacteria were found to target the NF- κ B using different proteins that affect near all aspects of the NF- κ B signalling pathway (reviewed in detail in (Le Nègre, 2012; Neish & Naumann, 2011; Rahman & McFadden, 2011)). These pathogen-derived countermeasures have been selected to maintain a delicate balance between activation and inhibition of the NF- κ B pathway as a survival strategy (Rahman & McFadden, 2011). In the context of bacterial infections, inhibition of NF- κ B function usually leads to impairment of the inflammatory responses (Le Nègre, 2012; Rahman & McFadden, 2011). Findings made to date indicate that most bacterial effectors known to interfere with NF- κ B signalling target the IKK complex, some impair TLR- and MyD88-mediated activation of NF- κ B, while a few others interfere with NF- κ B-dependent transcription (Table 5.1). In addition to NleC, only the *Vibrio parahaemolyticus* VP1686, the *Chlamydia* protease-like activity factor (CPAF) and the *Chlamydia trachomatis* effector CT441 were reported to directly target NF- κ B p65. The VP1686 was found to physically interact with p65 and suppress NF- κ B activity (Bhattacharjee et al, 2006) whereas CPAF and CT441 were reported to cleave p65 at its C-terminal region (Christian et al, 2010; Lad et al, 2007a; Lad et al, 2007b). CT441 cleavage of p65 originates the C-terminal fragment p22 and an N-terminus-derived p40 fragment that, despite containing an intact Rel homology domain for I κ B α interaction and DNA binding, was found act as a dominant negative regulator of NF- κ B activation (Lad et al, 2007a; Lad et al, 2007b).

Although in many cell types, down-regulation of NF- κ B is not sufficient to trigger apoptosis, it is widely recognized that cells with inactivated NF- κ B are more prone to commit suicide in response to different stimuli including TNF- α and TLRs ligands (Beg & Baltimore, 1996; Haase et al, 2003; Hsu et al, 2004; Park et al, 2005; Van Antwerp et al, 1996; Wang et al, 1996). The induction of apoptosis by bacterial effectors through interference with NF- κ B activity has been described but, this is a fairly uncommon scenario. Examples are the *Yersinia* YopP/J (Monack et al, 1998; Ruckdeschel et al, 1998; Ruckdeschel et al, 2001) and its orthologue AopP from *Aeromonas salmonicida* (Jones et al, 2012), both inhibiting the degradation of the inhibitory I κ B proteins causing NF- κ B to remain inactive in the cytoplasm (Fehr et al, 2006; Mukherjee et al, 2006; Ruckdeschel et al, 1998; Zhang et al, 2005; Zhou et al, 2005), and the *V. parahaemolyticus* protein VP1686 (Bhattacharjee et al, 2006) that has been described to interact with and suppress DNA binding activity of NF- κ B (Bhattacharjee et al, 2006). In the case of *Yersinia* YopP/YopJ, the induction of apoptosis in macrophages was shown to require stimulation of TLR4 (Haase et al, 2003; Zhang & Bliska, 2003). Whether AIP56-mediated depletion of p65 is sufficient to induce apoptosis, in resemblance to what has been suggested for macrophage apoptosis induced by the *V. parahaemolyticus* type III

Table 5.1. Bacterial effectors that modulate NF- κ B activity.

Bacteria	Effector	Target	Type and mechanism of modulation	References
<i>Aeromonas salmonicida</i>	AopP	unknown	Inhibits NF- κ B activation by inhibiting IKK β phosphorylation.	(Fehr et al, 2006)
<i>Brucella melitensis</i>	TcpB/Btp1	MyD88 protein and TIRAP	Inhibits TLR-signalling and NF- κ B activation by binding to TIRAP and MyD88 inducing TIRAP polyubiquitination and degradation, and MyD88 interaction with TLR2/TLR4.	(Chaudhary et al, 2012 ; Radhakrishnan et al, 2009; Sengupta et al, 2010)
<i>Chlamydia trachomatis</i>	ChlADub1	I κ B α	Inhibits NF- κ B activation through binding to I κ B α preventing its ubiquitination and degradation.	(Le Negrate et al, 2008b)
	CT441	NF- κ B p65	Inhibits NF- κ B activity by cleaving NF- κ B p65 at C-terminal region.	(Lad et al, 2007a; Lad et al, 2007b)
<i>Chlamydia pneumoniae</i>	CP0236	ACT1	Inhibits NF- κ B activation by sequestering the NF- κ B activator ACT1.	(Wolf et al, 2009)
<i>Chlamydia trachomatis</i> and <i>Chlamydia pneumoniae</i>	CpAF	NF- κ B p65	Inhibits NF- κ B activity by cleaving NF- κ B p65.	(Christian et al, 2010)
Enterohemorrhagic <i>Escherichia coli</i> (EHEC)	NleH	RPS3	Inhibits NF- κ B activity by binding and sequestering RPS3 in the cytosol.	(Gao et al, 2009)
	NleB	unknown	Inhibits NF- κ B activation by inhibiting TNF- α -mediated I κ B degradation.	(Newton et al, 2010)
	NleC	NF- κ B p65	Inhibits NF- κ B activity by cleaving NF- κ B p65.	(Baruch et al, 2010; Muhlen et al, 2011; Pearson et al, 2011; Sham et al, 2011; Yen et al, 2010)
	NleD	JNK	Inhibits NF- κ B activation by cleaving JNK.	(Baruch et al, 2010)
Enteropathogenic <i>Escherichia coli</i> (EPEC)	NleE	TAB2/3	Inhibits NF- κ B activation by inhibiting IKK phosphorylation and I κ B degradation.	(Nadler et al, 2010; Newton et al, 2010; Vossenkamper et al, 2010; Zhang et al, 2011)
	NleH1 NleH2	unknown	Inhibits NF- κ B activation by inhibiting phospho-I κ B α ubiquitination.	(Royan et al, 2010)
	Tir	TRAF2	Inhibits NF- κ B activation by interacting with TRAF2 and inducing its proteasomal-independent degradation	(Ruchaud-Sparagano et al, 2011)
<i>Escherichia coli</i> CFT073	TcpC	MyD88 protein and TLR4	Inhibits TLR-mediated NF- κ B activation through direct binding to MyD88 .	(Ciri et al, 2008; Yadav et al, 2010)
<i>Escherichia coli</i> K1	OmpA	unknown	Inhibits NF- κ B activation through inhibition of I κ B- α phosphorylation.	(Selvaraj & Prasadaraao, 2005)
<i>Listeria monocytogenes</i>	InlC	IKK α	Inhibits TNF- α -mediated NF- κ B activation by interacting with IKK α and decreasing phosphorylation of I κ B.	(Gouin et al, 2010)

Table 5.1. Bacterial effectors that modulate NF- κ B activity. (cont)

Bacteria	Effector	Target	Type and mechanism of modulation	References
<i>Salmonella enterica</i> serovar <i>Typhimurium</i>	AvrA	I κ B α	Inhibits NF- κ B activation by deubiquitinating I κ B α .	(Ye et al, 2007)
	SseL	I κ B α	Inhibits NF- κ B activation by deubiquitinating I κ B α .	(Le Negrate et al, 2008a)
	SspH	PKN1	Inhibits NF- κ B-dependent gene expression by ubiquitinating PKN1.	(Haraga & Miller, 2006; Rohde et al, 2007)
<i>Salmonella enterica</i> serovar <i>Enteritidis</i>	TipA	unknown	Inhibits TIR-dependent NF- κ B activation	(Newman et al, 2006)
<i>Shigella boydii</i>	OspZ	unknown	Inhibits NF- κ B p65 nuclear translocation.	(Newton et al, 2010)
<i>Shigella flexneri</i>	IpaH9.8	IKK γ and ABIN-1	Inhibits NF- κ B activity by interacting and promoting degradation of IKK γ and ABIN-1.	(Ashida et al, 2010; Rohde et al, 2007)
	OspG	E2 enzymes	Inhibits NF- κ B activation by binding to ubiquitin-conjugating E2 enzymes and prevents proteasomal degradation of phosphorylated I κ B α .	(Kim et al, 2005)
<i>Vibrio parahaemolyticus</i>	VP1686/VopS	NF- κ B p65	Inhibits NF- κ B activity by physically interacting with NF- κ B p65.	(Bhattacharjee et al, 2006)
<i>Yersinia</i> spp.	YopJ/YopP	MAPK kinase, I κ B α , TRAF3, TRAF6, TAB1, and SUMO-1,	Inhibits MAPK signalling and NF- κ B activation by preventing activation of the IKK complex. YopJ acts as a cysteine protease deubiquitinase and acetyltransferase modifying several proteins involved in NF- κ B signalling.	(Haase et al, 2005; Mittal et al, 2006; Mukherjee et al, 2006; Orth, 2002; Orth et al, 1999; Sweet et al, 2007; Thieffes et al, 2006; Zhou et al, 2005)
	YspK	E2 enzymes	Homologous to OspG and presumed to inhibit NF- κ B activation by the same mechanism.	(Matsumoto & Young, 2006; Matsumoto & Young, 2009)
<i>Bordetella bronchiseptica</i> and <i>Bordetella pertussis</i>	BopN	unknown	i) Blocks nuclear translocation of NF- κ B p65. ii) Promotes nuclear translocation of NF- κ B	(Nagamatsu et al, 2009)
<i>Bordetella pertussis</i>	FHA	unknown	Dynamic, cell specific and time dependent NF- κ B modulation: (i) activation at early stages of infection and (ii) inhibition at late stages of infection (through inhibition of proteosomal activity).	(Abramson et al, 2008)
<i>Shigella flexneri</i>	OspF	MAPKs (ERK2 and p38)	i) Inhibits NF- κ B activity by blocking the phosphorylation of histone H3 and dephosphorylating MAPKs. ii) Activates NF- κ B and JNK pathways by phosphorylation of TAB1 disrupting a negative feedback loop regulation between p38 and TAK1.	(Arbibe et al, 2007; Reiterer et al, 2011)
<i>Legionella pneumophila</i>	LegK1	I κ B proteins	Activates NF- κ B by phosphorylating and activating I κ B proteins.	(Ge et al, 2009)
<i>Toxoplasma gondii</i>	TgIKK	I κ B α	Activates NF- κ B by phosphorylating and activating I κ B α .	(Molestina et al, 2003; Molestina & Sinai, 2005)

secreted effector VP1686 (Bhattacharjee et al, 2006), or if it requires a second stimulus remains to be investigated.

Almost all bacterial effectors known to modulate NF- κ B activity are injected directly into the host cell cytosol by type III or type IV secretion systems (see reviews by (Bhavsar et al, 2007; Neish & Naumann, 2011; Rahman & McFadden, 2011)) with the only reported exceptions being the proteases CT441 and CPAF of the obligatory intracellular *Chlamydia*, that contain predicted signal peptides for sec-dependent secretion (Chen et al, 2010a; Christian et al, 2010; Lad et al, 2007b), the *Bordetella pertussis* cell associated and secreted adhesin FHA (Abramson et al, 2008) and the *E. coli* membrane protein OmpA (Selvaraj & Prasadarao, 2005) (table 5.1). AIP56 is also an exotoxin likely secreted by a sec-dependent mechanism (do Vale et al, 2005) and acts at distance, without requiring contact of the bacteria with the host cell and, therefore, must have a mechanism to enter cells in order to cleave the cytosolic located NF- κ B p65.

2. AIP56 belongs to the AB toxin family

Bacterial protein toxins acting in the cell cytosol enter cells through multistep processes involving specific binding of the toxin to cell-membrane receptors followed by internalization via endocytic mechanism(s) and membrane translocation, with the release of the catalytic domain into the cytoplasmic compartment (Falnes & Sandvig, 2000; Montecucco et al, 1994). The structure of those toxins is adapted to their entry mechanisms and, to this end, these toxins are organized in a so-called AB-type structure consisting of a catalytic A subunit bound to a cell interacting B subunit(s) that mediate toxin binding to a cell receptor and delivery of the A subunit into the cytosol (Falnes & Sandvig, 2000; Montecucco et al, 1994). As discussed in chapter I, toxins belonging to the AB toxin family can be synthesized and assembled in different ways and the differences in the toxin architectures translate in differences in the molecular processes of toxin biogenesis and intoxication pathways (Montecucco & Papini, 1995). Diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE) and the clostridial tetanus and botulinum neurotoxins (TeNT and BoNT, respectively) typify those AB toxins secreted as a single polypeptide chain composed of three functional domains: a catalytic domain, a receptor binding domain and an intermediate membrane translocation domain (Collier, 2001; Turton et al, 2002; Wolf & Elsasser-Beile, 2009).

The work herein presented shows that similarly to DT, PE, TeNT and BoNT, AIP56 belongs to the AB toxin family, being composed of a single polypeptide chain with an N-terminal catalytic domain and a C-terminal domain involved in the toxin binding and entry into

target cells. The boundaries of the putative membrane translocation domain were not yet identified, but our data suggest that the linker region between the two cysteine residues is involved in toxin translocation into the cell cytosol.

AIP56 is synthesized and secreted by *Phdp* as a single polypeptide and analysis of AIP56 primary structure using bioinformatics tools provided the first evidences of a two-domain/subunit structural organization (Silva et al, 2010). Prediction of secondary structures together with limited proteolysis assays confirmed that AIP56 is organized into two structural protease-resistant domains linked by a disulphide bond. Truncated versions of the toxin produced based on the chymotrypsin cleavage site were used to disclose the role of the two identified structural domains. The recombinant AIP56 N-terminal portion (Asn¹-Phe²⁸⁵) exhibited proteolytic activity *in vitro* towards NF- κ B p65 and delivery of this region into the cell cytosol using the *Bacillus anthracis* PA/LF system (Arora & Leppla, 1993; Arora & Leppla, 1994; Milne et al, 1995) reproduced both the proteolytic and the apoptogenic activities of the full length toxin, while no toxic effects were observed upon delivery of AIP56 C-terminal portion. This not only attributes to the N-terminal region the catalytic role, but also reinforces the assumption discussed above that the cleavage of NF- κ B p65 is a determining event for the AIP56 induced-apoptotic cell death. The C-terminal portion of AIP56 exhibited no proteolytic activity *in vivo* or *in vitro* but, when used as a competitor, it efficiently prevented AIP56 toxic effects, demonstrating the involvement of this region in toxin binding/entry into target cells.

Nonetheless, despite sharing the same overall architecture, AIP56 diverges from DT, PE, TeNT and BoNT in some specific requirements of the intoxication process which may traduce relevant differences in the molecular mechanisms of intoxication. Upon secretion and at initial steps of cell intoxication, DT, TeNT and BoNT are cleaved by bacterial or host cell proteases (Ahnert-Hilger et al, 1990; DasGupta, 1971; Gill & Dinius, 1971; Tsuneoka et al, 1993; Weller et al, 1989). Cleavage occurs at an exposed region between two cysteine residues yielding a catalytic A chain disulphide-bounded to a binding/translocation B chain (Collier, 2001; Turton et al, 2002). In those toxins, proteolysis is required for toxicity and in toxin preparations where nicking by endogenous proteases was incomplete or inexistent, mild treatment with proteases resulted either in toxin activation or in increased toxin potency (Collier & Kandel, 1971; Drazin et al, 1971; Knust et al, 2009; Krieglstein et al, 1991; Rupnik et al, 2005; Sandvig & Olsnes, 1981). Furthermore, proteolytic activation was found not only to be needed for cell intoxication but also required for enzymatic activity *in vitro* (Collier & Kandel, 1971; Gill & Pappenheimer, 1971; Weller et al, 1988). Proteolytic processing of PE by host cell furin or cathepsin proteases, was also reported (El Hage et al, 2010; Inocencio et

al, 1994; Ogata et al, 1990), although recent findings indicate that it is not essential for toxicity (Morlon-Guyot et al, 2009). PE cleavage by these proteases is low pH-dependent and is presumed to take place at the endosomal compartment (El Hage et al, 2010; Inocencio et al, 1994). Cleavage occurs within the domain II (translocation domain) of PE (El Hage et al, 2010; Inocencio et al, 1994) and structural data reveals the furin cleavage site is not protease-accessible at neutral pH (Wedekind et al, 2001). Similarly to DT, TeNT and BoNT, PE is synthesized as a holotoxin and requires activation to display enzymatic activity. PE conversion into a catalytically active form *in vitro* requires toxin unfolding and disulphide-bond reduction (Leppla et al, 1978). By contrast, no evidences of proteolytic processed AIP56 in the bacterial culture supernatants or in the serum of infected fish were ever found (do Vale et al, 2005) and despite several attempts, including the use of ^{125}I -labelled AIP56 (not shown), we were unable to detect proteolytic processing of AIP56 upon its interaction with host cells. The lack of detection of processed AIP56 may result from the fact that a very small amount (not detectable in our experiments) of processed toxin is sufficient to intoxicate the cells, similarly to what has been described for other toxins (Falnes et al, 2000), or it may indicate that AIP56 is not proteolytically processed upon interaction with target cells. Furthermore, AIP56 does not requires proteolytic activation nor unfolding or reduction to display enzymatic activity *in vitro*, as shown by its ability to cleave p65 when incubated either with cell lysates or with recombinant sea bass p65 Rel homology domain (see chapter IV section 2.2). Another dissonant structural/functional aspect of AIP56 is that integrity of the region between the two cysteine residues linking the two functional subunits appears to be a requirement for toxicity. Chymotrypsin nicking of AIP56 (at the exposed flexible region linking Cys²⁷⁸ and Cys³¹⁴) rendered the toxin inactive, abolishing its ability to cleave p65 and to induce apoptosis upon incubation with sea bass cells. However, nicking did not affect the toxin's proteolytic activity *in vitro*, as demonstrated by the ability of nicked AIP56 to cleave ^{35}S -labelled sea bass p65Rel domain, nor did affect its capacity to interact with the host cell membrane, as deduced from the ability of nicked AIP56 to inhibit AIP56-mediated p65 cleavage and apoptosis in competition experiments. These findings suggest that the AIP56 linker region plays a role in the translocation of the toxin into the cell's cytosol and thus possibly be part of the yet unidentified translocation domain. Unpublished data from our laboratory show that similarly to DT, TeNT and BoNT, endosomal trafficking and pH-driven structural rearrangements play a crucial role in the AIP56 intoxication mechanisms. AIP56 is internalized by a process that requires endosome acidification since drugs that interfere with this process protected cells from intoxication. Accordingly, at low pH, AIP56 suffers a reversible conformational change with exposure of hydrophobic residues/regions that allows

interaction of the toxin with artificial lipid membranes and an external acidic pulse is sufficient to trigger translocation of cell-bound AIP56 across the host cell membrane. Within the linker region, the two phenylalanine residues constituting the chymotrypsin cleavage site (F²⁸⁵ and F²⁸⁶) are highly hydrophobic residues and are flanked N-terminally by an alanine (A²⁸⁴) and C-terminally by a leucine (L²⁸⁷), a proline (P²⁸⁸) and a serine (S²⁸⁹). This series of amino acid residues form an hydrophobic stretch which may be involved, or at least contribute, to membrane insertion and toxin translocation. Nonetheless, *in silico* secondary structure analysis suggests that this region does not share the α -helical folding typical of the translocation domains of DT, TeNT or BoNT (Montal, 2010; Murphy, 2011) and is predicted to be a random coil region (see figure 4.8). Additional data are required to identify the boundaries of the AIP56 translocation domain and to determine the precise role played by the linker region in the intoxication process.

Several studies have stated the importance of the conserved inter-chain disulphide bridge linking the A and B subunits in the toxicity of DT (Falnes & Olsnes, 1995; Papini et al, 1993), PE (Madshus & Collier, 1989), TeNT (Kistner et al, 1993; Schiavo et al, 1990) and BoNT (Fischer & Montal, 2007; Pirazzini et al, 2011; Simpson et al, 2004). Studies with BoNT have suggested that the disulphide bridge must remain intact during membrane translocation of the catalytic domain, being required during the low pH-driven structural change and for membrane insertion (Cai & Singh, 2001; Fischer & Montal, 2007; Pirazzini et al, 2011). In the case of AIP56, disruption of the disulphide bridge linking the two structural domains only partially compromised AIP56's toxicity, suggesting that the integrity of the disulphide bond is important, although not essential for intoxication. This disulphide bond may be involved in stabilizing the spatial relationship between the domains and/or be implicated in membrane insertion as reported for BoNT (Pirazzini et al, 2011).

Whether AIP56 suffers proteolytic processing during cell intoxication is still unknown. Considering the above mentioned structural requirements for AIP56 toxicity, either processing occurs at later steps of intoxication, possibly as the result of the action of a cytosolic protease, to promote the release of the catalytic domain after membrane translocation, or, similarly to what has been described for PE (Alami et al, 1998; Mere et al, 2005; Morlon-Guyot et al, 2009), AIP56 reaches the cytosol as an unprocessed protein. PE was shown to be able to translocate from the endosomal membrane to the cytosol in its unprocessed form through a process involving ATP hydrolysis (Alami et al, 1998; Mere et al, 2005; Morlon-Guyot et al, 2009). Alternatively, AIP56 may localize in an endomembrane with the catalytic domain facing the cytosolic compartment where it can interact with and cleave p65 similarly to what was described for anthrax edema factor (EF) (Guidi-Rontani et al,

2000). Studies from Guidi-Rontani and colleagues revealed that when EF or iodinated EF were incubated with RAW 264.7 macrophages in the presence of PA, catalytically active EF was found exclusively associated with the cell membrane fraction, in contrast with LF whose activity was mainly associated with the cytosolic fraction (Guidi-Rontani et al, 2000). These authors suggested that EF plays an active role in its own translocation, being associated with the endosomal membrane and exposing the catalytic domain to the cytosol. Also, membrane-bound EF is postulated to be responsible for the increase in cAMP in intoxicated cells (Guidi-Rontani et al, 2000). Additional studies are required to elucidate the structural determinants and the exact mechanism for AIP56 translocation. In this context, solving AIP56 crystal structure would provide valuable knowledge. However, and despite our efforts, we were unable to obtain crystals suitable for X-ray diffraction studies (see chapter IV section 5). Nevertheless, our crystallization studies allowed the identification of promising crystallization conditions and provided the foundations for future AIP56 crystallization projects.

The different homologies and the structural architecture of AIP56 suggest that the toxin has a chimeric structure. This mosaic structure is not an uncommon feature in the bacterial world and there are several examples of bacterial genes composed of diverse segments with different origins (Baldo et al, 2005; Davies et al, 2002; Haake et al, 2004; Krzywinska et al, 2004; Miold et al, 2001; Stavrinides et al, 2006). It is now recognized that acquisition of entire genes or portion of genes by horizontal gene transfer has a key role in generating diversity in pathogens by allowing them to acquire novel phenotypic characteristics (Haake et al, 2004; Krzywinska et al, 2004). Nevertheless, the actual transfer events that gave rise to AIP56 remain undisclosed and deserve further study.

Even though AIP56 and NleC share functional homology, these virulence factors use different mechanisms to intoxicate cells. While NleC is directly injected into the cell cytoplasm by its producing bacteria through a type III secretion machinery (Galan & Wolf-Watz, 2006), AIP56 has an intrinsic ability to reach the cytosol due to the presence of a “delivery module” in its C-terminal region. Notwithstanding the molecular effects of NleC injection into cells and its contribution to the dampening the host inflammatory response have been characterized, little is known about its structural features. Here we have extended NleC structural characterization and compared it with the one of the AIP56 catalytic region. Circular dichroism (CD) analysis revealed that the secondary structure content of NleC and AIP56 N-terminal portion is indistinguishable and *in silico* secondary structure prediction indicates conservation of the relative position of α -helices and β -sheets, suggesting that in addition to sharing functional homology, NleC and AIP56 catalytic region are structurally

similar. Nevertheless, these proteins were found not to be cross-compatible, since substitution of AIP56's N-terminus by NleC resulted in a non-toxic chimera. Still, it should be noted that the exact boundaries of the AIP56 binding/translocation domain were not yet established and, therefore, the lack of toxicity of the NleC¹⁻²⁷⁰·AIP56²⁵¹⁻⁴⁹⁷ chimera may be related either with an incorrect AIP56 portion or to an inability of NleC¹⁻²⁷⁰ to translocate. Moreover, the PA/LF delivery system, successfully used to deliver the catalytic domains of several AB toxins (Arora & Leppla, 1993; Blanke et al, 1996; Milne et al, 1995), including the AIP56's catalytic portion into the cell cytosol, failed to deliver NleC. We postulate that the distinct behaviour of AIP56 catalytic region and NleC is a consequence of their different unfolding dynamics in response to pH (AIP56 catalytic region was found to be more prone to unfold in response to acidification than NleC), because it is known that translocation of protein cargos across the PA pores requires pH-driven unfolding of the passenger proteins (Collier, 2009). These differences may have implications and should be taken into account when considering the use of these molecules as therapeutic agents.

3. AIP56 as a potential therapeutic agent

In recent years, the focus of medical research in the therapy field has shifted to new molecular therapeutics that target specific signalling pathways associated with a given disease with the intent of greater specificity coupled with reduced systemic toxicity. The major challenges in the development of such therapies concern not only the specificity and selectivity of the therapeutic agent, but also the efficiency of its delivery into the target cells. In this context, AB toxins, due to their intrinsic ability to translocate into the cytosol of eukaryotic cells and deliver protein cargos as well as to their high potency and specificity in modifying intracellular targets, have been emerging as attractive tools in pharmacology (Barth & Stiles, 2008; Odumosu et al, 2010). Strategies that are used to generate toxin-based therapeutics involve mixing A and B domains of various toxins to generate chimeras with newly desirable properties (Arora & Leppla, 1993; Arora & Leppla, 1994; Blanke et al, 1996; Krautz-Peterson et al, 2012; Liu et al, 2001; Madshus et al, 1991; Milne et al, 1995; Stenmark et al, 1991) or deleting toxin domains and substituting them with eukaryotic proteins such as specific antibodies (creating immunotoxins) (Pastan et al, 2007; Weldon & Pastan, 2011) or cytotoxic compounds (El Alaoui et al, 2007; Tarrago-Trani et al, 2006). Toxins such as cholera toxin (CT), shiga toxin (Stx) and heat labile toxin (LT) have been used as vectors to bring into cells proteins or epitopes that are then presented at the cell surface by MHC molecules (Haicheur et al, 2000; Lippolis et al, 2000; Loregian et al, 1999),

thus functioning as immunomodulators and antigen delivery systems in vaccination (Chen et al, 2010b; Facciabene et al, 2007; Zhou et al, 2009). In addition, DT- and PE-based immunotoxins are currently in clinical trials for the treatment of hematological malignancies and brain tumours (Chandramohan et al, 2012; Debinski, 2002; FitzGerald et al, 2011; Weldon & Pastan, 2011), and the B subunit of Stx associated with a topoisomerase I inhibitor (used in the treatment of colorectal carcinomas) or other cytotoxic compounds are also being presented as promising tools in anti-cancer therapy (Johannes & Romer, 2010).

Apart from their promising potential in pharmacology, over the last years AB toxins have also revealed themselves as extremely interesting tools in cell biology. Studies on the intracellular trafficking pathways of CT, Stx and PE have allowed the characterization of vesicular and protein transport pathways (Sandvig & van Deurs, 2000; Sandvig & van Deurs, 2002; Schiavo & van der Goot, 2001; Smith et al, 2006). Indeed, Stx was the first molecule observed to be transported from the cell membrane to the ER (Sandvig et al, 1992), revealing the existence of a yet undiscovered intracellular trafficking route (retrograde transport). Since then, studies on the intracellular trafficking of Stx and other retrogradely transported toxins, have been greatly contributing to the fine dissection of the molecular mechanisms underlying the fundamental steps of intracellular retrograde protein trafficking (Feng et al, 2004; Mallard et al, 1998; Mallard & Johannes, 2003; Mallard et al, 2002; Smith et al, 2006).

As discussed earlier, NF- κ B is one of the pivotal regulators of immune responses particularly involved in the regulation of the transcription of pro-inflammatory genes (Hayden et al, 2006; Vallabhapurapu & Karin, 2009). These include genes encoding several cytokines and chemokines, receptors involved in immune recognition and antigen presentation, receptors required for neutrophil adhesion and migration and also enzymes associated with the inflammatory response such as the inducible form of nitric oxide synthase (iNOS) and the inducible cyclooxygenase (COX-2) (Hayden et al, 2006; Vallabhapurapu & Karin, 2009). In addition, cytokines that are stimulated by NF- κ B, such as IL-1 β and TNF- α , can also establish a positive autoregulatory loop (through direct activation of the NF- κ B pathway) that amplify the inflammatory response and increase the duration of inflammation (Hayden et al, 2006; Vallabhapurapu & Karin, 2009). Therefore, it is not surprising that aberrant NF- κ B regulation is associated with several inflammatory diseases including atherosclerosis, asthma, arthritis, inflammatory bowel disease, muscular dystrophy and multiple sclerosis (Tak & Firestein, 2001; Yamamoto & Gaynor, 2001), with NF- κ B being an obvious target for the treatment/control of such diseases (DiDonato et al, 2012; Yamamoto & Gaynor, 2001). In addition to the association between NF- κ B and inflammatory pathologies, uncontrolled NF- κ B

activation has also been associated with cancer disease (DiDonato et al, 2012; Rayet & Gelinas, 1999; Staudt, 2010). Constitutive NF- κ B activation was found in many lymphoid or myeloid tumours (Lim et al, 2012) and in various solid tumours (Carbone & Melisi, 2012; Chen et al, 2011; Zubair & Frieri, 2012), with several well-characterized oncogenes, such as Ras, Rac and Bcr-Abl, known inducers of NF- κ B activity, being often implicated in the NF- κ B constitutive activation (DiDonato et al, 2012; Rayet & Gelinas, 1999; Staudt, 2010). The roles for NF- κ B in cancer appear to be complex but, given the importance of this transcription factor in controlling apoptosis, cell proliferation and angiogenesis, it is likely implicated in facilitating oncogenesis as well as metastasis (Baldwin, 2001; Shen & Tergaonkar, 2009; Staudt, 2010). Additionally, it has been reported that NF- κ B is activated in cancer cells by several chemotherapies and radiation, and that, in many cases, this response inhibits the ability of the cancer therapy to induce cell death (Baldwin, 2001; Wang et al, 1999a; Wang et al, 1999b).

A variety of drugs such as corticosteroids, acetylsalicylic acid and other non-steroidal anti-inflammatory drugs used in the treatment of several inflammatory conditions have an NF- κ B inhibitory effect (Olivier et al, 2006; Yamamoto & Gaynor, 2001). However, these drugs are nonspecific NF- κ B inhibitors, and the use of relatively high concentrations is needed to achieve effective inhibition (Olivier et al, 2006; Yamamoto & Gaynor, 2001). Some of the above mentioned anti-inflammatory drugs known to inhibit NF- κ B also function as cancer therapeutics and chemo-preventive agents (Olivier et al, 2006) and, in the last years, major efforts were put in the identification/development of NF- κ B inhibitors to be used as cancer therapeutics (Miller et al, 2010; Shen & Tergaonkar, 2009; Tsang et al, 2012). So far, only Bortezomib, a proteasome inhibitor capable of suppressing NF- κ B activation, has obtained FDA approval for treatment of multiple myeloma (Richardson et al, 2008). Although NF- κ B inhibitors alone hold great potential as cancer therapeutics, the combined use of NF- κ B inhibitors with cytotoxic chemotherapeutic drugs have been emerging as attractive new anti-cancer therapeutic strategies. Chemo-resistance is one of the major issues in cancer chemotherapy, and the development and use of new "combination therapies" offer the promise of enhancing treatment efficacy (Chen et al, 2011; Madonna et al, 2012; Olivier et al, 2006; Shen & Tergaonkar, 2009; Zubair & Frieri, 2012).

Given the indispensable role played by NF- κ B pathways in many important biological processes, the major concerns regarding NF- κ B-targeted therapies relate with the non-specificity of the utilized drugs and with the adverse effects of long-term global inhibition of NF- κ B pathways (Baud & Karin, 2009; Chen et al, 2011; Lim et al, 2012). Hence, development/discovery of new drugs/compounds that selectively target specific molecules of

the NF- κ B pathway and the development of new approaches that increase the specificity and efficiency of delivery of these molecules have great pharmacological interest.

In this context, by being an AB toxin targeting NF- κ B, AIP56 presents itself as a potential therapeutic agent to be used in the treatment of diseases associated with uncontrolled NF- κ B activation (such as cancer and inflammatory diseases). In addition, AIP56 might also be a valuable tool in cell biology studies, particularly in the study of the molecular details of the NF- κ B-mediated regulation of apoptosis and inflammation processes. When compared with NleC, AIP56 has an increased potential to be used as therapeutic tool, not only due to the intrinsic ability of AIP56 to reach the cytosol of target cells, but also because of the dissimilar unfolding characteristics of AIP56 and NleC proteins (discussed above), which might impact on their mechanism of cell delivery. The use of a given toxin/effector as a therapeutic agent requires a detailed knowledge of its molecular mechanism(s). In the case of AIP56, a complete understanding of the toxin structure/function relationships and interaction with cells is still missing. Hence, solving AIP56 three-dimensional structure, identifying the cell membrane receptor for the toxin and gaining in depth knowledge onto the toxin's intracellular pathways are crucial before considering the use of AIP56 as a therapeutic agent.

CHAPTER VI

CONCLUDING REMARKS

AIP56 was identified as an apoptogenic exotoxin and a key virulence factor of *Photobacterium damsela piscicida* in 2005 by do Vale and colleagues (do Vale et al, 2005). At that time, studies on the AIP56 toxin were mainly focused in understanding the consequences of the toxin's apoptogenic activity for the pathology of the disease. It was shown that AIP56 induces apoptosis of the host macrophages and neutrophils leading to a severe dampening of the host immune response, thus contributing the septicemic spread of the infectious agent (do Vale et al, 2005). Furthermore, the inefficient clearing of the apoptosing cells due to phagocyte depletion leads to post-apoptotic secondary necrosis, with cell lysis and consequent tissue damage contributing to the necrotic lesions found in the diseased animals (do Vale et al, 2007a; do Vale et al, 2007b; do Vale et al, 2003). Later, in 2011, it was shown that AIP56-induced phagocyte cell death is caspase-dependent, involving activation of caspase-8,-9 and -3 and the mitochondria, with loss of mitochondrial membrane potential, release of cytochrome c and over-production of ROS (Costa-Ramos et al, 2011).

Nevertheless, a detailed structural and functional characterization of AIP56 was still missing and, until the beginning of this work in 2008, very little was known about AIP56 structure and the mechanisms used by the toxin to reach its targets and kill the cells. With this study, by disclosing structural and functional features of AIP56, we gained a mechanistic insight into the toxin's mode of action. We report AIP56 as a zinc-metalloprotease that cleaves, and thus inactivates, the host cell NF- κ B p65. Taking into account the anti-apoptotic functions of NF- κ B, and in particular of its p65 subunit, the severe depletion of NF- κ B p65 found in AIP56 intoxicated cells likely explains the disseminated phagocyte apoptosis observed in *Phdp* infections (Costa-Ramos et al, 2011; do Vale et al, 2007a; do Vale et al, 2005).

The described AIP56 NF- κ B cleaving activity is similar to the one recently reported for the AIP56 N-terminal homologue NleC, a type III secreted effector produced by several enteropathogenic bacteria (Baruch et al, 2010; Muhlen et al, 2011; Pearson et al, 2011; Sham et al, 2011; Yen et al, 2010). Nevertheless, AIP56 differs from NleC by being an AB type toxin that is able to enter and reach the cytosol of host cells in an autonomous manner. We showed that while the N-terminal region of AIP56 is necessary and sufficient to cleave NF- κ B p65 and induce apoptosis once at the cell cytosol, the C-terminal region functions as a delivery module being involved in host cell binding and toxin internalisation. The boundaries of the A and B domains of AIP56 were not yet defined, and despite sharing the same overall architecture with known AB toxins secreted as a single polypeptide chain, our data revealed that AIP56 diverges from these toxins in some structural details of the

intoxication process. AIP56 does not require proteolytic, or any other kind of activation to display enzymatic activity *in vitro*. Furthermore, integrity of the region between the two cysteine residues linking the two putative AIP56 functional domains appears to be needed for toxicity. These data suggest that this region is involved in the translocation of the toxin into the cell's cytosol and may be part of a yet unidentified translocation domain and raises the hypothesis that proteolytic processing might not be required for cell intoxication. Nevertheless, the exact mechanism involved in AIP56 translocation across the lipid bilayer and the occurrence of proteolytic processing at the cytosol remain to be investigated.

NF- κ B is one of the pivotal regulators involved in a multitude of important cellular processes such as cell survival, differentiation and immune responses (Hayden et al, 2006; Vallabhapurapu & Karin, 2009). The extensive involvement of NF- κ B transcription factors in inflammation and cancer establishes them as targets for the treatment of such diseases (Kumar et al, 2004; Staudt, 2010; Tak & Firestein, 2001; Yamamoto & Gaynor, 2001). Thus, the results obtained during this work, open doors to the use of AIP56 as a therapeutic tool in several human pathologies associated to the uncontrolled activation of NF- κ B. Because the delivery of proteins into the cytosol of eukaryotic cells has a huge biotechnological interest, AB toxins such as AIP56, possessing intrinsic abilities to enter target cells, also have an increased potential to be used, not only as a tool in cell biology to study cellular processes such as endocytosis and intracellular transport, but also as a system to deliver different cargos inside target cells (Barth & Stiles, 2008; Pastan et al, 2007; Sandvig et al, 2010b). Therefore, the understanding of the intoxication mechanism of AIP56 transcends the context of a virulence factor operating in an economically important fish disease and represents a new prospect to the understanding and manipulation of a key transcription factor associated with several important biological processes often associated with severe human pathologies.

CHAPTER VII

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