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The Natural Antimicrobial Chromogranins/Secretogranins-Derived Peptides – Production, Lytic Activity and Processing by Bacterial Proteases

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1. Introduction

1.1 Multidrug antibiotic resistance and innate immunity

Multidrug-resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant enterococci (VRE) have important infection control implications in all healthcare settings. Multidrug antibiotic resistance is a worldwide crucial health problem and the production of new potent antibiotics, acting alone or in combination is urgent. In addition, a major factor in the emergence of antibiotic resistant organisms is the overuse of antibiotics in the hospital or the community. To overcome this abuse, numerous efforts are undertaken to reduce antibiotics prescription and/or promote synergistic effects by others molecules.

Indeed, stimulating organism defense is a promising way to struggle against pathogens. The innate immune system is, since 2 billion years, the primary defense in most living organisms and antimicrobial peptides (AMPs) are fundamental components of the innate immune defense of multicellular organisms, either animal or vegetal (Bulet et al., 2004; Aerts et al. 2008; Manners, 2007).

1.2 The antimicrobial peptides

The antimicrobial peptides (AMPs) have been well conserved throughout the evolution and they ensure the organism's defense against a large number of pathogens. They serve as endogenous antibiotics that are able to rapidly kill bacteria, fungi and viruses. Interestingly, they are not toxic for the host cells. Taking into consideration the diversity of the living beings, it is presumed that a large number of specific antibiotic peptides have been developed during evolution, allowing a protection of each organism in various conditions and the last years it has clearly appeared that many of these peptides, in addition to their direct antimicrobial activity, also have a wide range of functions in modulating both innate and adaptive immunity. Most of these are small molecules (less than 40 aminoacids) but some can be proteins. To date more than 1414 antibacterial, antifungal and 107 antiviral peptides have been

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identified, (antimicrobial peptides database <http://aps.unmc.edu/AP/main.php>), including peptides from several tissues and cell types from invertebrates, plants and mammals (Wang Z. & Wang G. 2004). Among them are found cytokines and chemokines, several neuropeptides and fragments derived from proteins exhibiting antimicrobial activity. They carry an average of 40-50 percent hydrophobic residues in such a structure that the folded peptide adopts an amphipathic profile. These properties are important for their microbial killing mechanism: the cationic character of AMPs induces an electrostatic attraction to the negatively-charged phospholipids of microbial membranes and their hydrophobicity aids the integration into the microbial cell membrane, leading to membrane disruption. Furthermore, the amphipathic structure also allows the peptides to be soluble both in aqueous environments and in lipid membranes (Yeaman & Yount 2003).

In mammals, the most well studied AMPs are human defensins and cathelicidins (Zanetti, 2004; Yang et al., 2002). Furthermore, some large proteins such as lysozyme, caseins, hemoglobin, lactalbumin, secretory phospholipase A2 and lactoferrin display antimicrobial activity against numerous microorganisms. Several of them, such as lysozyme and phospholipase A2 are ubiquitous and secreted by a large number of cells (*i.e.* epithelial cells, leukocytes and Paneth cells in the small intestine) (Keshav, 2006).

Because a large number of AMPs were identified in gut and skin, in the first part of this chapter we report a review of the well-studied AMPs expressed in these tissues and in the second part we present recent data relative to the new active CGs-derived peptides in relation with pathogens involved with intestine diseases, skin infections and sepsis.

1.3 Gut and antimicrobial peptides

Gastrointestinal mucosa is a large host-environmental interface, showing a remarkable organization (Figure 1) and operating several functions including the digestive absorptive processes and the nutrients peristalsis, but also a physical and immunological protection of the body against microbes and a reconnaissance between commensal and pathogenic microorganisms.

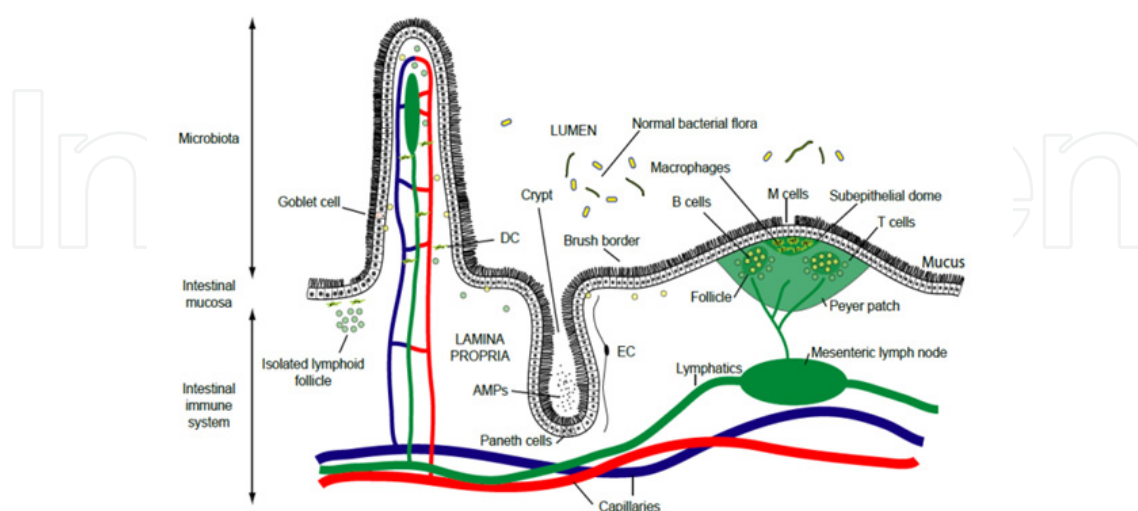


Fig. 1. Schematic representation of the gut epithelium. The different cellular actors involved in innate and/or adaptative immunity are represented. (DC: dendritic cells ; EC: enterochromaffin cells.)(According to Metz-Boutigue, M.H. et al., *Curr Pharm Des.*2010;16(9):1024-39).

Lamina propria is a conjunctive tissue composed of fibroblasts, immune cells and collagen. It also contains capillaries and lymphatic vessels. Epithelial cells, or enterocytes, are disposed on a single layer separating the *lumen* from the *lamina propria*. These cells are tightly bound by tight junctions forming an impermeable barrier to commensal flora and to pathogens. Brush-border *microvilli* are present on the apical surface of absorptive enterocytes, representing a large absorbing surface and allowing of microorganisms to the gut. In contrast, microfold cells are not present in *microvilli* (Figure 1); these cells express cathepsin E (a proteolytic enzyme) and Toll-like receptors able to secrete proinflammatory cytokines and chemokines. The main function of microfold cells is the transport of antigens from the lumen to the subepithelial lymphoid tissue and thus to the adaptive immune system.

Several anatomical structures are present along the gastrointestinal tractus. Peyer's patches are lymphoid structures containing B and T cells, macrophages and dendritic cells (Figure 1). Lieberkühn crypts are found in the small intestine and they constitute the basis of the intestinal *villi* (Figure 1). They contain multipotent stem cells and cells, involved in gastrointestinal immunity. Two other cellular types are also present in intestine: i) goblet cells synthesize and secrete large quantities of mucin, ii) enterochromaffin cells that originate from neural crest synthesize serotonin (5-HT) and numerous neuropeptides (Figure 1).

In addition to humoral and cellular immunity, non-immunological defense mechanisms represent an important line of intestinal defenses. Some of these protective factors have been amply documented: pancreatic and gastric juices, intestinal motility and intestinal flora (Sarker, 1992).

Mucosal epithelial cells and Paneth cells produce a variety of AMPs (defensins, cathelicidins, cryptdin related peptides, bactericidal/permeability increasing protein (BPI), chemokine CCL20 and bacteriolytic enzymes such as lysozyme and group IIA phospholipase A2 (Müller et al., 2005). In addition to their direct role in killing pathogenic microorganisms, AMPs are involved in attraction of leukocytes, alarming the adaptive immune system and neutralizing the proinflammatory bacterial molecules (Müller et al., 2005).

Lysozyme

Lysozyme is synthesized and secreted by Paneth cells, macrophages, neutrophils and epithelial cells (Mason & Taylor, 1975; Satoh et al., 1988). Its role and selectivity towards microbes are the same as in skin.

Lactoferrin

Lactoferrin (LF) exhibits a wide spectrum of antimicrobial and immunotropic properties (Artym et al., 2005). In contrast to caseins, LF is particularly resistant to proteolytic degradation in alimentary tract. LF is absorbed from the intestine by means of specific receptors located on brush border cells. Orally administered LF stimulates both local and systemic immune responses. It suppresses the growth of pathogenic bacteria, while promoting the multiplication of non-pathogenic *Lactobacillus sp.* and *Bifidobacterium sp.* (Artym et al., 2005).

Studies on mice showed LF to be protective against bacteremia and endotoxemia. LF inhibits the activity of proinflammatory cytokines, nitric oxide and reactive forms of oxygen. Furthermore, LF promotes the differentiation of T and B cells from their immature precursors and increases the activity of NK and LAK (lymphokine activated killer) cells (Artym et al., 2005).

Phospholipase A2

The hydrophobic layer of phosphatidylcholine (PC) overlies and protects the surface of the gastrointestinal (GI) tract, contributing to barrier integrity. In addition, phospholipase A2 is synthesized by Paneth cells and this enzyme hydrolyses bacterial membrane phospholipids to generate both free fatty acids and lysophospholipids. An important prerequisite for the action of phospholipase A2 is the successful binding to the phospholipids surface. *In vitro* studies utilizing recombinant enzymes and artificial phospholipids substrates have shown that phospholipases act on anionic phospholipids (phosphatidylglycerol, phosphatidylserine and phosphatidylethanolamine), but are inactive with phosphatidylcholine due to the lack of high affinity binding (Wu et al., 2010). During critical illness such as sepsis, gut barrier integrity may be compromised, which could be related to degradation of PC. Pretreatment with an orally active sPLA(2) inhibitor blocks the LPS-induced increase in GI permeability, and may suggest a new approach to reinforce the GI mucosal barrier and prevent complications from endotoxin in trauma in other septic conditions (Zayat et al., 2008).

Cathelicidins

Cathelicidin LL-37/hCAP18 is synthesized by neutrophils, where it was first identified (Romeo et al., 1988) and epithelial cells of the colon (Hase et al., 2002). *In vitro*, it has chemotactic properties for monocytes, macrophages and T cells (Koczulla et al., 2003). LL-37 is found in sites of inflammation where it modifies dendritic cells (DCs) differentiation, relying innate and adaptive immunity. *In vitro*, modified DCs had, among others characteristics induced by the peptide, enhanced secretion of Th-1 inducing cytokines and promoted Th1 responses (Davidson et al., 2004). LL-37 acts synergistically with IL-1-beta to increase the production of suppressive cytokines (IL-6 and IL-10) and chemokines MCP-1 and MCP-3 by macrophages (Yu et al., 2007). It acts *via* the transcription factor CREB and the activation of phosphorylation of the kinase Akt. In LPS-stimulated monocytes, LL-37 inhibits the release of TNF-alpha modulating inflammatory response induced by LPS, endotoxins and other agonists of TLRs (Mookherjee et al., 2006).

RNAses

Angiogenin-4 (Ang-4) is a member of the ribonucleases family. This protein is synthesized by Paneth cells and is similar to RNase 7 found in skin. Its secretion is stimulated by exposure to LPS. Ang-4 kills *E. faecalis* and *L. monocytogenes* at concentrations as low as 1 μ M, whereas its concentration in crypts can be 1000 times greater (Hooper et al., 2003). Similarly to defensins, it is sensitive to salt concentration and is potentially cytotoxic to eukaryotic cells (Saxena et al., 1992).

C-type lectins

C-type lectins HIP/PAP are synthesized in human by enterocytes and Paneth cells. The same protein exists in mouse and is named RegIII gamma. These lectins bind Gram-positive peptidoglycan and act by direct killing. Several members of this family are found in gastrointestinal tissues (Dieckgraefe et al., 2002).

Defensins

As in skin, defensins have a direct antimicrobial role as well as immunomodulatory function. Alpha-defensins are synthesized by Paneth cell in the gastrointestinal tractus (Porter et al., 2002). Alpha-defensin expression does not require microbe induction since

they are synthesized in germ-free conditions (Putsep et al., 2000) and/or prenatally (Mallow et al., 1996). In transfected mouse, it was shown that the alpha-defensin hBD-5 protects efficiently against *Salmonella typhimurium*, demonstrating the direct antimicrobial effect of this peptide. In mouse, alpha-defensins are named cryptdins and several families of peptides related to cryptdins are regrouped under the term CRS (Cryptdin Related Sequences). Interestingly, these CRS can form homo- or heterodimers, thus allowing a combinatorial diversity to struggle against pathogens (Hornef et al., 2004).

Beta-defensins are expressed in enterocytes of the small and large intestines. 28 beta-defensin encoding genes have been identified in human genome, but only 8 were found to be expressed. hBD-1 is constitutively expressed in absence of stimulus or bacterial infection (O'Neil et al., 1999), while some nutrients can stimulate its production in cell lines (Sherman et al., 2006). In mouse, an infection by the *Cryptosporidium* parasite resulted in a down-regulation of mBD-1 (Zaalouk et al., 2004), while *in vitro*, sporozoites are killed by this defensin. Some authors conclude on a unique and important regulation of hBD-1, during small intestine infections (Dann et Eckmann, 2007). hBD-2 is not constitutively expressed, but is induced by an infection or by proinflammatory stimuli (O'Neil et al., 1999). hBD-3 and -4 are inducible and particularly expressed in crypt regions (Fahlgren et al., 2004). Defensins can also act as chemotactic agents for immune cells in a similar way to that described for the skin.

Bactericidal Permeability Increasing protein

Bactericidal/permeability-increasing protein (BPI), a constituent of primary neutrophil granules, is a potent natural antibiotic and anti-BPI antibodies are detected during infectious enteritis. In addition, BPI is a target antigen for anti-neutrophil cytoplasmic autoantibodies in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Walmsley et al., 1997).

Neuropeptides

Enterochromaffin cells (EC) (Siddique et al., 2009) (Figure 1) are enteroendocrine cells present in the intestine, especially colon (Kuramoto et al., 2007) and containing large amounts of serotonin (5-HT). These cells can sense luminal content before its basolaterally release, and activate afferent neuron endings within *lamina propria*, allowing information exchange between gut and central nervous system (Hansen & Witte et al., 2008). Besides this important role, EC secrete also numerous other products, among which VIP (Zanner et al., 2004), Substance P (Heitz et al., 1976), CgA, CgB and secretogranin II/CgC (Cetin & Grube, 1991) and melatonin (Raikhlin et Kvetnoy, 1976).

Despite the crucial role of these cells, their sparse repartition and their low number did not allowed their extensive study. However, the BON cells were proposed as a model (Kim et al., 2001), that will enhance further research. When EC were stimulated by odors, they released serotonin, showing that these cells can also be stimulated by spices and fragrances (Braun et al., 2007). Moreover, a new method was proposed allowing isolating and purifying EC from biopsies (Modlin et al., 2006).

1.4 Skin and antimicrobial peptides

Mammal skin is an essential defense barrier against external aggressions, such as microbial pathogens, oxidant stress, chemical aggressions, mechanical insults, burns etc. For a long time,

skin was considered as a simple physical barrier, but it is in a process of continual regeneration and has its own immunological, histological and nervous responses to environment.

Skin is composed of three layers, from inside to outside (Figure 2): i) *hypodermis* or subcutaneous tissue, ii) *dermis*, or *corium*, with a 3 to 5 mm thickness, iii) *epidermis*, with a thickness varying from 0.06 to 0.8 mm. *Epidermis* can be subdivided itself into four layers : *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (Figure 2). The deeper layers are composed of keratinocytes, melanocytes, Langerhans cells, Merkel cells and malpighian cells (Figure 2). *Epidermis* is composed as a gradient of differentiated keratinocytes, synthesizing keratine in *stratum granulosum*, and losing nuclei and organelles.

Skin, and more specifically *stratum corneum*, acts as a barrier in several ways (Elias, 2007). Corneocytes and extracellular matrix represent a physical barrier (“brick wall” model) (Figure 2).

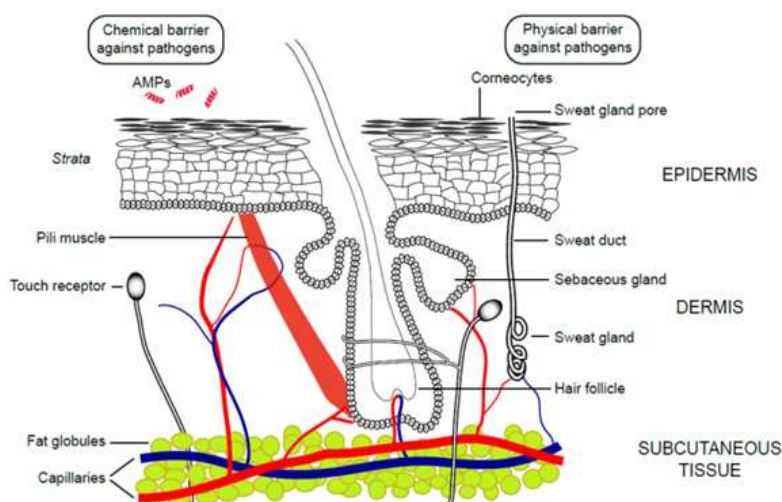


Fig. 2. Schematic representation of the different skin layers, adapted from different sources.

A: Epidermis, dermis and subcutaneous tissues are shown with the different structures composing them. (according to Metz-Boutigue, M.H. et al., *Curr Pharm Des.* 2010;16(9):1024-39).x

The slightly acidic surface (pH~ 5.0), as well as the low hydration level of the skin represents a hostile area for pathogens, such as *Staphylococcus aureus*. Lipids (ceramides, cholesterol, free fatty acids) and their metabolic products present in *stratum corneum* act also as antimicrobial defense. Last, constitutive (and/or inducible) expression of antimicrobial peptides and proteins helps to maintain skin integrity and to prevent pathogen colonization. On the contrary, the surface of healthy skin is ideal for the growth of the normal cutaneous microflora (*Micrococcacea*, i.e. *Staphylococcus epidermidis* and *Corynebacteriaceae*) that colonizes skin, competes with pathogens for nutrients and synthesizes antimicrobial compounds. These evolutionary conserved components of the innate immune system can act as direct antimicrobial agents and exert a role as immunomodulatory molecules in normal skin and during skin diseases, such as atopic dermatitis or psoriasis.

Lysozyme

Lysozyme is the first antimicrobial protein found in human skin. It was located in cytoplasm of epidermal cells in granular layers and in malpighian cells present in the *stratum spinosum* layer (Ogawa et al., 1971; Papini et al., 1982). Lysozyme is mainly active against Gram-

positive bacteria (*S. aureus*), but is also active against Gram-negative bacteria, acting probably as a control of bacterial growth. Still recently, the contribution of lysozyme to cutaneous defense was subjected to debate since it was not detected in *stratum corneum* as well as in washing fluid. However, it was recently detected in skin wash of adults, and lysozyme concentration was 5 times higher in newborn skin than in adult (Walker et al., 2008), confirming its status of antimicrobial molecule, as well as giving it an important role in preventing infections in newborn children.

Lactoferricin

Lactoferricin is an antimicrobial peptide originally produced by pepsin digestion of lactoferrin. It is active against Gram-positive, Gram-negative bacteria and also against *Candida albicans* (Bellamy et al., 1993). This molecule was also detected in skin wash of adult and newborn children (Walker et al., 2008). Synthesized by melanocytes, cutaneous lactoferrin is an iron-binding protein with antibacterial properties due to its ability to sequester iron in biological fluids or to destabilize bacterial membranes, limiting microorganism proliferation and adhesion. It has also immunomodulatory properties by up and down regulating immune cells involved in inflammatory processes (Legrand et al., 2005). The protective anti-inflammatory role of lactoferrin is due to its ability to bind free ferric ion acting as an anti-oxidant (Walker et al., 2008). It can bind to LPS and their receptors during an infection as well (Legrand et al., 2005). Expression of virulence factors of *S. aureus* is modulated by transferrin and lactoferrin (Kansal et al., 2005), demonstrating that these iron-binding proteins play an important role in the host-pathogen interaction in skin and in mucosal tissue probably by LPS or its receptors binding.

Dermcidin and its derived peptides

Dermcidin, is constitutively and specifically expressed in the eccrine sweat glands within the dermis of human skin, secreted into the sweat and transported *via* sweat to the epidermal surface (Schitteck et al., 2001). It is a 47 amino acids peptide produced from hydrolysis of a 9.3 kDa precursor by cathepsin D (Baechle et al., 2006). It possesses antibacterial properties at low concentration against *S. aureus*, *E. faecalis*, *E. coli* and *C. albicans*. The *in vivo* importance of DCD in prevention of infections has been demonstrated by its low expression in patients with atopic dermatitis. It was shown that dermcidin induces the production by SepA of *S. aureus*, a proteolytic virulence factor that cleaves and inactivates dermcidin (Lai et al., 2007). In the eccrine sweat, several proteolytically generated DCD fragments (DCD-1, DCD-1L) have been identified. DCD-1L is the most abundant antimicrobial peptide present in sweat, but other peptides derived from dermcidin by proteolysis are also found (Baechle et al., 2006; Rieg et al., 2006). The distribution of these peptides was found to be different according to the individuals. Most of them have 2 to 4 of the major DCD-derived peptides with the constant presence of at least one of the following peptides: DCD-1L (63-110), LEK-45 (66-110) and SSL-29 (63-91). The authors also showed that the distribution of these peptides is dependent on the body sites, which correlates with the presence of eccrine sweat glands and not with apocrine glands. Body parts in contact with pathogens (arms, face etc.) produce high levels of DCD-derived peptides. The molecular analysis of the antimicrobial activity of dermcidin-derived peptides showed that peptides like DCD-1L or SSL-23 do not disrupt bacterial membranes, but kill bacteria by still unknown mechanisms (Steffen et al., 2006).

Recently, by using a proteomic approach, a dermcidin precursor was found in human cervico-vaginal fluid (Shaw et al., 2007), together with haptoglobin, neutrophil defensin,

lysozyme and lactoferrin. Dermcidin precursor was also found in human gestational tissue (Lee Motoyama et al., 2007), where it is proposed to play a role in pregnancy by regulating trophoblastic functions.

Cathelicidin, LL-37, hCAP18

Cathelicidin is found in eccrine gland cells, but also released into circulation. The CAP18 precursor is produced in skin by keratinocytes and is processed within neutrophils, keratinocytes and mast cells by inflammation or injury. In circulation, the mature form is LL-37, after processing of CAP18 by neutrophil-derived elastase and proteinase-3, but other proteases can also produce KS-30 and RK-21, two peptides active against pathogenic bacteria. Cathelicidin expression is also regulated at the transcriptional level by bacterial LPS, cutaneous injury and pro-inflammatory mediators (IL-6, retinoic acid). The LL-37 is intensively studied and besides its wide antibacterial spectrum, it is considered as a mediator between innate and adaptive immunity (Kai-Larsen & Agerberth, 2008) and cell differentiation can also regulate its activity.

RNase A superfamily

Eight known functional RNase A ribonucleases genes are encoding small polypeptides of 15 kDa (Dyer & Rosenberg, 2006). Besides their well-documented ribonuclease activity, some of these proteins display unexpected antimicrobial activities unrelated to their primary function. Eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic peptide (ECP/RNase 3) are proteins secreted by eosinophilic leukocytes and were primarily tested for their toxic role against parasites. *In vitro*, ECP has also an activity against Gram-positive and Gram-negative bacteria (Lehrer et al., 1989).

RNase A7 was identified as a major agent of the innate immune response of the skin acting on Gram-positive and Gram-negative bacteria and also on *C. albicans* (Harder & Schröder, 2002). RNase 7 transcripts were induced in keratinocyte culture by addition of TNF-alpha, interferon-gamma, interleukin-1 beta and in the presence of bacteria (Harder & Schröder, 2002). More recently, RNase 5 was added to the list of antimicrobial molecules present in skin (*stratum corneum*), and the same authors showed that skin proteases are involved in inhibition of RNases 5 and 7 (Abtin et al., 2009).

Psoriasin (S100A7)

Psoriasin belongs to the S100 family of calcium-binding proteins. This family is composed of 21 genes and 11 proteins that have been found to be expressed in human epidermis or in cultured keratinocytes. Langerhans cells and melanocytes (Boni et al., 1997; Broome et al., 2003) express S100B and Meissner's corpuscles (sensorial receptors localized in the upper part of dermis) express S100P (Del Valle et al., 1994). These proteins possess two EF hands (helix-loop-helix calcium binding domains) and they act probably as calcium sensors. Several functions have been proposed for S100 proteins in keratinocytes, the main role being an implication in skin inflammatory processes (Jinquan et al., 1996). Another role could be keratinocytes membrane remodeling, that occurs during differentiation: psoriasin and another member of the S100 family, calgranulin-A (S100A9), have been shown to have their expression correlated with the degree of keratinocyte differentiation, suggesting that they are involved in this process (Martinsson et al., 2005). A third role could be an involvement in the formation of calcium channels, in conjunction with annexins. Other postulated roles

concern S100 proteins as substrate for transglutaminase, resulting in an incorporation of S100 in the cornified envelope; a last role could be a response to exogenous agents that modulate S100 proteins distribution and consequently their function (Eckert et al., 2004). Psoriasin has been found to be overexpressed in psoriasis. It is produced in *stratum corneum* by keratinocytes (Martinsson et al., 2005) and its basal expression is influenced by extracellular calcium level. Its expression in normal adult tissue is low, but high expression levels were detected in fetal skin, as for transferrin, suggesting a protective role in innate immunity. Psoriasin was found to be the main *E. coli*-cidal agent in the skin. It is a chemotactic agent for neutrophils and CD4⁺ T cells (Jinquan et al., 1996). Moreover, psoriasin mediates the production of several inflammatory cytokines and chemokines from neutrophils *via* MAPK p38 and ERK activation. It also induces reactive oxygen species production and the exocytosis of alpha-defensins from neutrophils (Zheng et al., 2008).

Defensins

To date 4 defensins (hBD-1 to -4) in neutrophils and 2 defensins (hBD-5 and hBD-6) produced by Paneth cells were identified. The first inducible human defensin, hBD-2, was identified in psoriatic lesions as the most abundant AMP. It was found to be expressed in terminally differentiated keratinocytes, in a structure located in *stratum corneum*, lamellar bodies that contain lipid-rich secretory granules. It is probably released with lipid-like content of these lamellar bodies (Oren et al., 2003). hBD-2 is also up-regulated locally by infections (Radek & Gallo, 2007) or wounds (Butmarc et al., 2004). It has preferential bactericidal properties against Gram-negative bacteria (Harder et al., 1997) and like LL-37, its effect is sensitive to the concentration of NaCl. hBD-2 derived from neutrophils, promotes prostaglandins production and histamine release from mast cells, playing a role in allergic response (Bals et al., 1998). hBD-2 has also chemotactic properties for immature dendritic cells and memory T cells; it was described to bind to CCR-6, the receptor for macrophage inflammatory protein 3 alpha. In monocytes, hBD-2 expression is stimulated by several cytokines (Ganz, 2003; Kanda & Watanabe, 2008) and Il-1 seems to be the major inducer of hBD-2 production. Bacteria can also stimulate the expression of hBD-2 by epithelial cells, in a cytokine-independent pathway. *P. aeruginosa* is a powerful inducer of hBD-2 by primary keratinocytes (Schroeder & Harder, 2006).

hBD-1 was considered as a constitutively expressed antimicrobial peptide and in particular not induced by proinflammatory cytokines. However, its production can be induced by peptidoglycan or LPS exposure (Sorensen et al., 2005). It is expressed in malpighian layer and in *stratum corneum* (Ali et al., 2001) and this expression is induced by increasing concentration of calcium (Harder et al., 2004), condition that provokes keratinocyte differentiation *in vitro* (Lichti et al., 2008).

hBD-3 has its expression induced by EGF that provokes keratinocytes proliferation in skin wounds (Sorensen et al., 2006).

It has chemotactic properties for monocytes (Garcia et al., 2001). While its expression is not induced by infection, hBD-3 displays a broad spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, as well as against fungi (Harder et al., 2001). Regarding the adaptive immune system, hBD-2, 3 and 4 stimulate expression of proinflammatory cytokines, IL-10 and MCP-1 (Niyonsaba et al., 2007). They also stimulate the phosphorylation of STAT-1 and STAT-3 that induce keratinocytes migration and proliferation.

Neuropeptides in skin immunity

It was reported that neuropeptides display antimicrobial activities, linking together nervous and immune system (Radek & Gallo, 2007; Sternberg, 2006). Both systems can influence each other: brain and peripheral nervous system directly influence the activity of innate and adaptive immune system. Immune system can relay signals to the nervous system *via* the production of growth factors and cytokines. For example, stress can induce alterations in the immune response (Webster et al. 2002), or can be elicited by infection or injury with release of neuropeptides (Brogden et al., 2005).

Exchange between both systems can occur at systemic, as well as at regional or local levels (Sternberg, 2006). The first, global level gathers sympathetic nervous system, the hypothalamic-pituitary-adrenal axis and circulating AMPs. The second, local level, is composed of nervous endings, neuropeptide-releasing cells and receptors-exhibiting cells.

At the skin level, important structures, such as Merkel cells (Lucarz & Brand, 2007) localized at the basement membrane, separating epidermis from dermis, are neuropeptide-producing cells, cutaneous nervous cells and target cells. Merkel cells have characteristics of both epidermal and neuroendocrine cells. They are connected to nervous system with terminal sensory synapses and dense-core granules contain CGRP (Calcitonin Gene Related Peptide), VIP (VasoIntestinal Peptide), and CgA (Chromogranin A)-derived peptides (Hartschuh et al., 1989a; Hartschuh et al., 1989b).

Alpha-melanocyte-stimulating hormone (alpha-MSH), a 13 amino-acid peptide, is synthesized by keratinocytes, melanocytes, monocytes and astrocytes (Wikberg et al., 2000). This peptide derives from the pro-opiomelanocortin (POMC) after a processing by a proteolytic cascade (Pritchard & White, 2007), producing also five other peptides. Alpha-MSH acts as an AMP by inhibiting *S. aureus* and *C. albicans* growth at picomolar concentration (Cutuli et al., 2000). Interestingly, the tripeptide KPV (alpha-MSH 11-13) exhibited similar antimicrobial properties (Hiltz & Lipton, 1990; Mandrika et al., 2001; Mugridge et al., 1991), without effect on melanocytes (Sawyer et al. 1990). Alpha-MSH acts in two ways; it has a direct antimicrobial effect at very low concentration and reduces inflammatory responses associated with UV induced epithelial injury (Radek & Gallo, 2007).

2. Structural and biological properties of the antimicrobial peptides derived from chromogranins/secretogranins

2.1 Introduction

Chromogranins/secretogranins (CGs/SGs) constitute the granin family of genetically distinct acidic proteins present in secretory vesicles of nervous, endocrine and immune cells (Helle, 2004). The natural processing of bovine CGs is well described in granules of sympathoadrenal medullary chromaffin cells, where the resulting peptides are co-secreted with the catecholamines (Metz-Boutigue et al., 1993). The numerous cleavage sites are consistent with the specificity of prohormone convertases (PC1/3 and PC2) and carboxypeptidase E (CPE), that reside within chromaffin granules (Metz-Boutigue et al., 1993; Seidah & Chretien 1999). Secretogranin II (SgII), the third member of the chromogranin family is also processed to generate several natural fragments (Metz-Boutigue et al., 1993; Anouar et al., 1998; Marksteiner et al., 1993; Yajima et al., 2004). The discovery

that pancreastatin, a chromogranin A (CGA)-derived peptide inhibits insulin secretion from pancreatic beta-cells, initiated the concept of prohormone (Eiden, 1987; tatemoto et al., 1986). The release of these CGs-derived peptides from chromaffin cells results from the nicotinic cholinergic stimulation and regulates several neuroendocrine functions (Helle & Serck-Hanssen, 1975).

Numerous cleavage products of the granins have been characterized, among which some display biological activities (Tatemoto et al., 1986; Aardal et al., 1993; Curry et al., 1992; Fasciotto et al., 1993; Lugardon et al., 2001; Mahata et al., 1997; Strub et al., 1996a,b). Neuroendocrine activities are reported from *in vivo* studies, with modulations of homeostatic processes, such as calcium regulation and glucose metabolism (Helle et al., 2007), cardiovascular functions (Brekke et al., 2002; Corti et al., 2004), gastrointestinal motility (Amato et al., 2005; Ghia et al., 2004a), nociception (Ghia et al., 2004b) tissue repair (Gasparri et al., 1997; Ratti et al., 2000), inflammatory responses (Ceconi et al., 2002; Corti et al., 2000) and as host defense agents during infections (Radek et al., 2008). During the past decade, our laboratory has characterized new antimicrobial CGs-derived peptides (Strub et al., 1996a,b; Metz-Boutigue et al., 1998; Lugardon et al., 2000, 2001; Briolat et al., 2005; Helle et al., 2007) (Figure 3).

Peptide	Location	Sequence	Net charge
CGA			
VS-I	1-76	LPVNSPMNKGDTVMKC*IVEVISDTLSKPSMPVSKEC*FETLRGDERILSILRHQNLKELQDLALQGAKERTHQQ	+3
NCA	4-40	NSPMNKGDTVMKC*IVEVISDTLSKPSMPVSKEC*FE	-1
CHR	47-66	RILSILRHQNLKELQDLAL	+1.5
Chrom	173-194	<u>Y</u> PGPQAKEDSEG P SQGPASREK	-1
CAT	344-364	RSMRLSFRARGYGFRGPGLQL	+5
CCA	418-427	LEKVAHQLEE	-2
ProChrom	79-431	HSS <u>Y</u> EDELSEVLEK.....	-37
CGB			
Chromb	564-626	SAEFPDFYDSEEQMS <u>P</u> QHTAENEEKAGQGVLTETEEKELENLAAMDLELQKIAEKFSGTRRG	-12
SEC	614-626	QKIAEKFSGTRRG	+3
CGC			
Rrf	131-138	RKLKHMRF	+4.5
Kvk	430-443	KVLSRLPYGPRGSK	+4

Fig. 3. The antimicrobial bovine CGs-derived peptides according to the sequence of CGA (P05059), CGB (P23389) and CGC ((P20616) For each antimicrobial peptide the sequence, the location and net charge are indicated. *, cysteine residues of the disulfide bridge; phosphorylated residue are underlined and the glycosylated residue is in bold.

They act at micromolar range against bacteria, fungi, yeasts and are non-toxic for mammalian cells. They are recovered in biological fluids involved in defense mechanisms (serum, saliva) and in secretions of stimulated human neutrophils (Briolat et al., 2005; Lugardon et al., 2000).

These new AMPs are integrated in the concept that highlights the key role of the adrenal medulla in the immunity (Sternberg, 2006) as previously reported for adrenaline and neuropeptide Y that regulate immunity systemically once released from the adrenal medulla. Furthermore, the adrenal medulla contains and releases large amounts of IL-6 and TNF-alpha in response to pro-inflammatory stimuli such as LPS, IL-1 alpha and IL-1 beta (Metz-Boutigue et al., 1998). The discovery of the presence of TLRs on the adrenal cortex cells raises the interesting possibility that the adrenal gland might have a direct role in the response to pathogens, activation of innate immune response and clearing of infectious agents (Sternberg, 2006).

2.2 Antimicrobial peptides derived from chromogranin A

Several new antimicrobial peptides isolated from the granules of chromaffin cells of the bovine adrenal medulla correspond to CGA-derived peptides (Figure 3). The corresponding sequences are highly conserved in human. Interestingly, the main cleavage site in position 78-79 of bCGA and the subsequent remove of the two basic residues K77 and K78 by the carboxipeptidase H (Metz-Boutigue et al., 1993) produces two antimicrobial fragments: vasostatin-I (VS-I; bCGA1-76) (Lugardon et al., 2000) and prochromacin (Prochrom; bCGA79-431) (Strub et al., 1996b). For these N- and C-terminal domains with antimicrobial activities several shorter active fragments were identified: for VS-I, bCGA1-40 (N CgA; NCA) (Shooshtarizadeh et al., 2010), bCGA47-66 (chromofungin; CHR) and for ProChrom, bCGA173-194 (Chromacin; Chrom) (Strub et al., 1996b) and bCGA344-364 (Catestatin; CAT) (Shooshtarizadeh et al., 2010). The unique disulfide bridge of bCGA is present in VS-I and NCA sequences. Two post-translational modifications are important for the expression of the antibacterial activity of Chrom: the phosphorylation of Y173 and the O-glycosylation of S186 [130] (Strub et al., 1996a). Furthermore, it is important to point out that a dimerization motif GXXXG similar to that reported for Glycophorin A (Brosig & Langosch, 1998) is present in the Chrom sequence (G184-G188).

Vasostatin-I

Vasostatin-I (VS-I) displays antimicrobial activity against (i) Gram-positive bacteria (*Micrococcus luteus* and *Bacillus megaterium*) with a minimal inhibitory concentration (MIC) in the range 0.1-1 μ M; (ii) against filamentous fungi (*Neurospora crassa*, *Aspergillus fumigatus*, *Alternaria brassicola*, *Nectria haematococca*, *Fusarium culmorum*, *Fusarium oxysporum*) with a MIC of 0.5-3 μ M and (iii) against yeast cells (*Saccharomyces cerevisiae*, *Candida albicans*) with a MIC of 2 μ M (Lugardon et al., 2000). However VS-I is unable to inhibit the growth of *Escherichia coli* SBS363 and *Escherichia coli* D22. VS-I (Figure 3) possesses structural features specific for antimicrobial peptides, such as a global positive charge (+3), an equilibrated number of polar and hydrophobic residues (20:23) and the presence of a helical region CGA40-65 characterized to be a calmodulin-binding sequence (Lugardon et al., 2001; Yoo, 1992). The loss of the antibacterial activity of CGA7-57 suggests that the N- and C-terminal sequences are essential, nevertheless CGA7-57 is less efficient than VS-I against fungi. Besides, the disulfide bridge is essential for the antibacterial, but not the antifungal property. Altogether, these data suggest that antibacterial and antifungal activities of VS-I have different structural requirements (Lugardon et al., 2001). Interestingly, two helix-helix dimerization motifs important for the interaction with membranes such as LXXXXXXL,

present in DAT and dopamine transporter sequences (Torres et al., 2003) are present in the bovine and human VS-I sequences (L42-L49; L57-L64).

Surface interaction of rhodamine-labelled bCGA1-40 was demonstrated using confocal microscopy after incubation of the labeled peptide with *Aspergillus fumigatus*, *Alternaria brassicicola* and *Neurospora crassa* (Blois et al., 2006). In addition, the interaction of bCGA1-40 with monolayers of phospholipids and sterols, as models for the interaction with mammalian and fungal membranes was investigated by the surface tension technique (Blois et al., 2006; Maget-Dana et al., 1999). These studies demonstrated that the N-terminal bCGA1-40 fragment interacts with model membrane phospholipids in a manner consistent with an amphiphilic penetration into membranes in a concentration range relevant for biological activity in mammalian tissue (Blois et al., 2006).

Chromofungin

When VS-I was treated with the endoprotease Glu-C from *Staphylococcus aureus*, one of the generated peptide, chromofungin (CHR), is the shortest active VS-I-derived peptide with antimicrobial activities (Figure 3). It is well conserved during evolution and displays antifungal activity at 2-15 μM against filamentous fungi (*Neurospora crassa*, *Aspergillus fumigatus*, *Alternaria brassicicola*, *Nectria haematococca*, *Fusarium culmorum*, *Fusarium oxysporum*) and yeast cells (*Candida albicans*, *Candida tropicalis*, *Candida neoformans*) (Lugardon et al., 2001). Since this peptide was generated after digestion of the material present in chromaffin secretory vesicles by the endoprotease Glu-C from *S. aureus*, it may be hypothesized that it is produced during infections by this class of pathogens.

The 3-D structure of CHR has been determined in water-trifluoroethanol (50:50) by using $^1\text{H-NMR}$ spectroscopy. This analysis revealed the amphipathic helical structure of the sequence 53-56, whereas the segment 48-52 confers hydrophobic character (Lugardon et al., 2001). The importance of the amphipathic sequence for antifungal activity was demonstrated by the loss of such activity against *N. crassa* when two proline residues were substituted for L61 and L64, disrupting the helical structure, the amphipathic character and the dimerization motif helix-helix L57-L64 (Lugardon et al., 2001).

Catestatin

Two CGA-derived fragments bCGA333-364 and bCGA343-362 were characterized after the extensive processing of bCGA by prohormone convertases (PC 1/3 or 2) in chromaffin granules (Taylor et al., 2000). More recently, it was shown that cathepsin L colocalizes with CGA in chromaffin granules. *In vitro* it is able to generate after digestion of recombinant hCGA, a catestatin (CAT)-derived fragment hCGA360-373 (Biswas et al., 2009). In addition to the inhibitory effect of CAT on catecholamine release from chromaffin cells (Mahata et al., 1997), we have shown for this peptide and its shorter active sequence bCGA344-358 (cateslytin, CTL), (Figure 3) a potent antimicrobial activity with a MIC in the low-micromolar range against Gram-positive bacteria (*Micrococcus luteus*, *Bacillus megaterium* at concentration of 0.8 μM), Gram-negative bacteria (*Escherichia coli* D22 at concentration of 8 μM), filamentous fungi (*Neurospora crassa*, *Aspergillus fumigatus*, *Nectria haematococca* at concentration of 0.2-10 μM) and yeasts (*Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida neoformans* at concentration of 1.2-8 μM). The sequence of CAT (Figure 3) has been highly conserved during evolution (Briolat et al., 2005). The two human variants P370L and

G364S display antibacterial activity against *M. luteus* with a MIC of 2 and 1 μM , respectively, and against *E. coli* with a MIC of 20 and 10 μM , respectively (Briolat et al., 2005). However, the most active peptide corresponds to the bovine sequence. Bovine CTL, a cationic sequence with a global net charge of +5 (R344, R347, R351, R353, R358) and five hydrophobic residues (M346, L348, F360, Y355, F357) (Figure 3), is able to completely kill bacteria at concentration lower than 10 μM even in the presence of NaCl (0-150 mM) (Briolat et al., 2005). The C-terminal sequence bCGA352-358 is inactive, whereas the N-terminal sequences bCGA344-351 and bCGA 348-358 are antibacterial at 20 μM .

C-terminal CGA-derived fragment

CCA, the C-terminal CGA-derived fragment bCGA418-427 (Figure 3), with a remarkable net charge of -2, displays antifungal activity and belongs to the less abundant anionic AMPs family. It is well conserved during evolution and is homologous to the C-terminal sequence of CGB and the antibacterial peptide SEC (secretolytin) [(Strub et al., 1996b). This peptide was generated *in vitro* after digestion, by the protease Glu-C from *S. aureus*, of the material present in chromaffin secretory vesicles. As previously postulated for CHR, CCA could be generated during infections induced by this pathogen.

2.3 Antimicrobial peptides derived from bovine chromogranin B

To date, the natural C-terminal fragment of bovine CGB (CCB; bCGB 564-626), isolated from chromaffin granules of the adrenal medulla, was found to display antibacterial activity against both *M. luteus* and *E. coli*. The complete inhibition of bacterial growth was observed at a concentration around 1.8 μM (Strub et al., 1996b). This large fragment contains the natural short antibacterial peptide secretolytin (SEC, bCGB614-626) with a net positive charge (+3) (Figure 3). We observed the natural formation of a pyrrolidone glutamic acid at the N-terminal end of SEC and both forms displayed antibacterial activity against *M. luteus*, reaching 100% of growth inhibition at 2 μM (Strub et al., 1996ab). A structure-activity analysis suggests that an alpha-helical amphipathic structure common to SEC and cecropins may account for the antibacterial activity (Strub et al., 1996b).

2.4 Antimicrobial peptides derived from bovine secretogranin II

Because bSGII is weakly expressed in the intragranular matrix of chromaffin secretory vesicles (2% of total proteins), the detection of endogenous AMPs by classical methods was unsuccessful. After *in-silico* analysis, two synthetic peptides with cationic amphipathic sequences were prepared: Rrf and Kvk, which correspond to the sequences bSGII131-138 and bSGII430-443 with respective net charges of +4.5 and 4 (Figure 3). Rrf, completely inhibits the bacterial growth of *M. luteus* and *B. megaterium* with a MIC of 5 and 15 μM , respectively, and Kvk displays antifungal properties at 19 μM against *N. crassa* (Shooshtarizadeh et al., 2010).

3. Interaction of antimicrobial chromogranins-derived peptides with bacterial proteases

The AMPs avoidance mechanisms deployed by bacteria include the proteolytic degradation of the active forms by the bacterial proteases. In order to examine the effects

of bacterial proteases on the isolated AMPs derived from CGs, we have tested the effects of *Staphylococcus aureus* V8 protease Glu-C and several supernatants from *S. aureus*, *Salmonella enteretica*, *Klebsiella oxytoca*, *Shigella sonnei* and *Vibrio cholera*. By using biochemical methods we have analyzed the degradation of the peptide in presence of bacteria.

Interaction of antimicrobial CGs-derived peptides with proteases from diarrheogenic bacteria
Bacteria were isolated from patients of the Strasbourg Civil Hospital by the Bacteriology Institute, University of Strasbourg, (EA-4438). The four strains have a clinical interest because apart from inducing diarrhea, they may cause other infections.

Thus, *Klebsiella* was involved in the occurrence of post-antibiotic diarrheas (Gorkiewicz 2009). Many studies show that *Klebsiella oxytoca* is also involved in nosocomial infections for newborns or adults (Biran et al., 2010) *Klebsiella* infections may also be commensal (Tsakris et al., 2011). *Klebsiella oxytoca* has also been associated with hemorrhagic colitis (Hoffmann et al., 2010) and intercurrent colitis in Crohn's disease (Plessier et al. 2002). *Salmonella* destroys infected cells and the infection continues through blood (sepsis) or through lymphatic vessel (typhoid fever). *Salmonella* cause also gastrointestinal infections. *Shigella sonnei* and *Vibrio cholera* non O1 cause inflammation of the intestinal mucosa by producing the Shiga toxin.

Klebsiella oxytoca, *Salmonella enterica*, *Shigella sonnei*, and *Vibrio cholera* develop phenomena of antibiotic resistance. Thus, *Salmonella* was reported to be resistant for the action of Ciprofloxacin (Medalla et al., 2011) and Ceftriaxone (Su et al., 2011).

Concerning CgA, we have tested bovine, rat and human CAT corresponding to the sequences bCgA344-364, rCgA6344-364 and hCgA352-372, bovine CTL located at bCgA344-358, two short fragments hCgA360-372 and the conserved tetrapeptide LSFR (bCgA348-351). In addition, we have tested a scrambled peptide relative to the sequence of bovine CAT and the procatenastatin fragment bCgA332-364.

We have found antimicrobial activities only for the bovine CAT and CTL, showing that CTL is the shorter active fragment and that it corresponds to the active domain of CAT. Procatenastatin was inactive in similar experimental conditions. Bovine CAT and CTL were active against *Klebsiella oxytoca*, *Salmonella enterica* and *Vibrio cholera* at 100 μ M and 50 μ M respectively and against *Shigella Sonnei* at 50 μ M and 25 μ M. In addition, CHR and the C-terminal fragment (CgA387-431) were inactive for concentration up to 100 μ M. In contrast, CTL is active at 30 μ M against the four pathogens.

Three CgB-derived peptides (CgB58-62, CgB279-291, and CgB547-560) and secretoneurin corresponding to SgII189-254 were examined against the four strains in order to analyse their degradation by bacterial proteases. By using HPLC we have compared the profiles of the peptide alone and the peptide with the inoculated medium.

These experiments show that except CTL all the peptides are completely degraded by the bacteria. To illustrate these data, we present on Figure 4, the profiles relative to CAT and CTL in presence of buffer with *Salmonella enterica*. The complete peptide and the processed form are analysed by sequencing and mass spectrometry (MALDI-TOF).

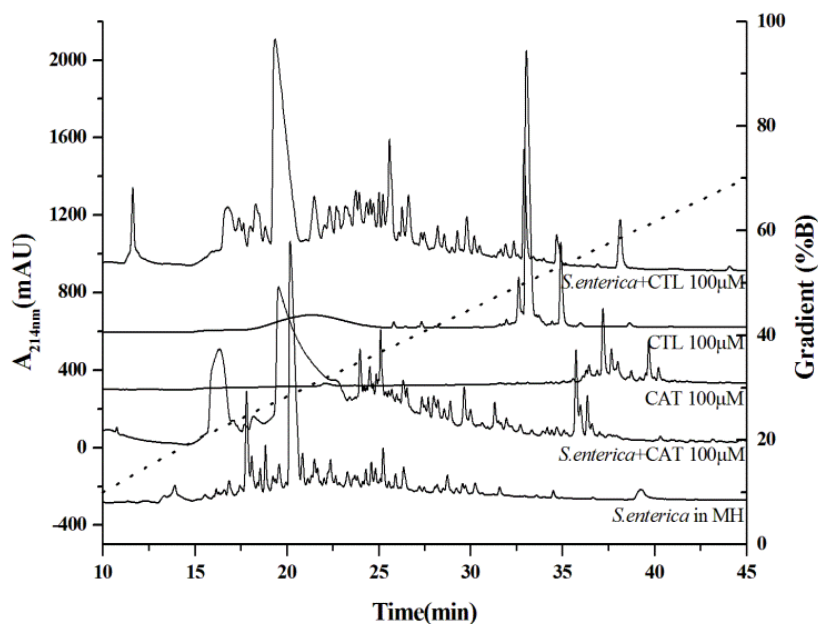


Fig. 4. Analysis by HPLC of the catestatin (CAT) and cateslytin (CTL) degradation by *Salmonella enterica*. The HPLC system is composed by a Dionex chromatogram, Germerong, Germany), using a Nucleosil 300-5 C18 column (4×250 mm, particle size 5 μm , porosity 300 \AA ; Macherey Nagel, Düren, Germany). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile in milliQ water (solvent B). Elutions were performed at a flow rate of 700 $\mu\text{L min}^{-1}$ using the gradient indicated on the chromatogram.

3.1 Interaction of antimicrobial Cgs-derived peptides with proteases from *Staphylococcus aureus*

After incubation with *S. aureus* V8 protease Glu-C of the proteic intragranular material of chromaffin cells present in the adrenal medulla, 21 new peptides were isolated by HPLC and analysed by sequencing and mass spectrometry. These peptides were tested against Gram positive bacteria (*Micrococcus luteus* and *S. aureus*), Gram negative bacteria (*Escherichia coli*), fungi (*Neurospora crassa*) and yeast (*Candida albicans*). They are not antibacterial but 5 peptides corresponding to CgA47-60, CgA418-426 and CgB 279-291, CgB 450-464 and CgB470-486 display antifungal activity at the micromolar range against *N. crassa*. Thus, *S. aureus* subverts innate immunity to degrade the antibacterial Cgs/Sgs-derived peptides and produce new antifungal peptides (manuscript in preparation).

Four antimicrobial CgA derived peptides (CHR CgA47-66, bovine CAT CgA344-364, human CgA352-372 and CTL CgA344-358) were incubated in presence of staphylococcal supernatants from S1 (a Methicillin resistant strain) and S2 (a non-resistant strain). CTL, the active domain of CAT, is able to completely kill *S. aureus* at 30 μM , but the two others peptides are inactive. By using a proteomic analysis (HPLC, sequencing and mass spectrometry) we demonstrated that CHR and CTL were not degraded by supernatants, whereas bovine and human CAT are processed to produce several fragments (Figure 5).

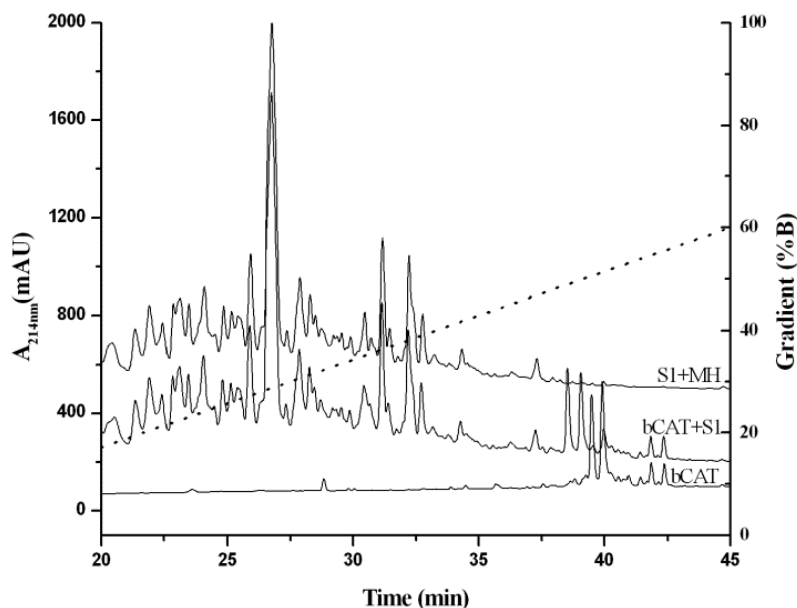


Fig. 5. Analysis by HPLC of the catestatin (CAT) degradation by a methicillin resistant *Staphylococcus aureus* (MRSA). The HPLC system is composed by a Dionex chromatograph, (Germerong, Germany), using a Nucleosil 300-5 C18 column (4×250 mm, particle size 5 μm , porosity 300 \AA ; Macherey Nagel, Düren, Germany). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile in milliQ water (solvent B). Elutions were performed at a flow rate of 700 $\mu\text{L min}^{-1}$ using the gradient indicated on the chromatogram.

4. Synergy of the combination of antimicrobial peptides with antibiotics

The emergence of multi-drug resistant bacteria (MDR), with therapeutic failure against *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* have paved the way to develop new therapeutic agents by the help of the synergism. In addition the highly toxic effects of antibiotics have shifted the research focus to discover new peptides with broad spectrum of activity and less toxicity. Synergy is the combined activity of two antimicrobial agents that can never be attained by any one of them singly (Serra et al., 1977). Numerous AMPs demonstrate broad spectrum of activity against pathogens, interacting directly with membranes or acting with a specific mode. They represent interesting candidates to synergistically act with antibiotics.

4.1 For *Staphylococcus aureus* MRSA

Most of the patients can prone to serious bacterial infections caused mainly by the multi-resistant microorganisms, *Staphylococcus aureus* coagulase negative spp are one of them. These coagulase negative strains (MRSA) have got approximately 90% of methicillin resistance due to β -lactam resistance (Silva et al., 2011). Story just not stopped here, but still it continues, some of the staphylococcal strains got resistance to the other drugs such as Vancomycin, which was previously widely used against the MRSA infections, and to treat

infections of central nervous system, bone infections and sometimes for the pulmonary infections which require a more concentrations to get treated (Dehority, 2010). *S. aureus* have also developed resistance to the Vancomycin due to the use at low level concentrations and recently, *S. aureus* was isolated that had got the *VanA* gene from the *Enterococcus spp* (Sievert et al., 2008) which leads to drug resistance.

In our group, we have examined the synergically effects of three CGA-derived peptides (CAT, CTL and CHR) with Minocyclin, Amoxicillin and Linezolid. To demonstrate that antimicrobial peptides are able to reduce the doses of antibiotics used and to potentiate the activity of antibiotics, antimicrobial tests were carried out by combining the antibiotic peptides at doses below the MIC. The comparison was made with the antibiotic or peptide separately at the same doses.

Minocyclin has a MIC of 2 $\mu\text{g}/\text{ml}$ alone against the *S. aureus* ATCC 49775, but when it was combined with CTL at a concentration corresponding to 75% of the MIC, the, concentration of Minocycline was lowered to 0.5 $\mu\text{g}/\text{ml}$. Similar data were obtained by the use of the two others peptides (Figure 6). Thus we demonstrate that amidated bCTL acts synergistically with Minocycline against *S. aureus*. In addition CTL acts synergistically with Voriconazole against *Candida albicans* and *Candida tropicalis*.

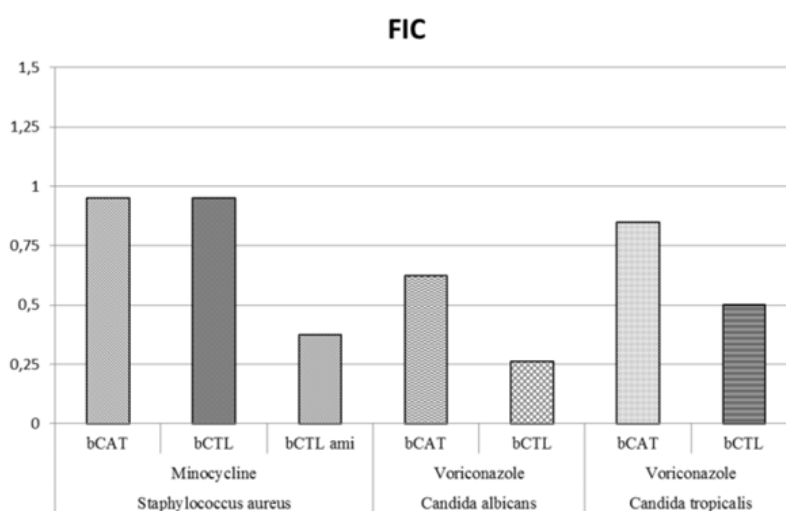


Fig. 6. Fractional inhibitory concentration (FIC) of the chromogranin derived peptides combined with the antimicrobials (Minocycline against the *Staphylococcus aureus* and Voriconazole against *Candida albicans* and *Candida tropicalis*). FIC in range of ≤ 0.5 gives a synergistic effect, $\leq 0.5 - < 2$ is an additive effect but if more than 2 have an antagonistic effect.

4.2 From *Shigella*

Some of the strains of *Shigella* got resistance to antibiotics. A 9-year study of shigellosis in Malaysia, show that 58.4% of the studied strains were resistant to tetracyclin and 53.8% to trimetropin-sulfamethoxazol (Banga Singh et al., 2011). In China, another study establish for *Shigella* the resistance to aztreonam (30,8%), ampicillin (92,3%), piperacilline (61,5%), ceftazidime (30,8%), cefotaxime (30,8%), gentamicine (53,8%) (Zhang et al., 2011). Furthermore, *Vibrio cholera* was also described to develop several resistances against antibiotics (Lamrani et al., 2010).

In conclusion these studies show that CGA-derived AMPs potentiate the effects of antibiotic drugs. One could imagine a mechanism in which the peptide would favor the destabilization of the membrane allowing the antibiotic to rapidly penetrate inside the bacterial cells and thus to reach its site of action.

5. Chromogranin A, a new marker of severity

In clinical practice, CGA has been used as a marker of pheochromocytomas (O'Connor et al., 1984), carcinoid tumors (O'Connor & Deftos, 1986; Syversen et al., 1993), neuroblastomas (Hsiao et al., 1990), neuroendocrine tumors (Berruti et al., 2005), and neurodegenerative diseases (Rangon et al., 2003). Recent data have shown CGA to be a useful prognostic indicator in patients with chronic heart failure (Omland et al., 2003, suggesting that CGA may have some association with cardiovascular diseases. Furthermore, a pilot study has shown CGA to be a predictor of mortality in patients with acute myocardial infarction (Estensen et al., 2006). Characterization of the severity of organ failures and prediction of patient outcome are of major importance for physicians who care for critically ill patients. Multiple organ failure (MOF) remains the main problem in intensive care because of its impact on morbidity, mortality, and resources (Baue et al., 1998). MOF can develop as a consequence of multiple causes, such as infection, systemic inflammatory response syndrome (SIRS), myocardial infarction, septic shock, leading to the activation of various endogenous cascades, cellular dysfunction and death (Baue et al., 1998).

In a recent study we have evaluated whether unselected critically ill patients at ICU (Hautepierre Hospital, Strasbourg, France) admission demonstrate increased plasma CGA concentrations and whether CGA can be of any interest in the care of patients at high risk of death. Patients older than 18 years were recruited consecutively over 3 months during 2007. Exclusion criteria included: duration of stay >24 h and conditions known to increase CGA concentrations independently of acute stress [i.e., a history of documented neuroendocrine tumors (O'Connor & Deftos, 1986) or chronic treatment with proton pump inhibitors before admission (Giusti et al., 2004). Patients who required surgical interventions were also excluded. Of the 120 participants included in the study, 70 patients had a primary diagnosis severe infection, and 50 had a SIRS. Serum CGA concentrations were measured with a commercial sandwich RIA kit (a gift of Cisbio Bioassays, Marcoule, France. In the central 95% of the healthy population, serum CGA concentrations range from 19 µg/L to 98 µg/L. In neuroendocrine system tumors, the CGA serum concentration varies from the typical range up to 1200 µg/L, depending on the biological and structural characteristics of the tumor, as well as on the extent of tumor spread (Degorce et al., 1999). As a control Procalcitonin (PCT) concentrations were measured on the Kryptor system (Brahms Diagnostic) with the time-resolved amplified cryptate emission methodology in accordance with the manufacturer's recommendations. The Simplified Acute Physiological Score II (SAPS II) and the Logistic Organ Dysfunction System (LODS) score were calculated at admission according to published standards (Levy et al., 2003; Le Gall et al., 1993). Our data show that CGA concentration was positively but weakly correlated with age, PCT concentration, creatinine concentration, SAPS II, and LODS score ($P < 0.001$ for all variables) and was correlated with CRP concentration (Zhang et al., 2008). Thirty-three deaths occurred during the median follow-up time of 23 days. The death rates for CGA and PCT are shown by quartiles in Figure 7. Statistical analysis revealed a significant difference in death rates between CGA quartile 4 and CGA quartiles 1, 2, and 3 ($P < 0.001$, log-rank test). The death rate for CGA quartile 3 was also significantly different from that of CGA quartile 1 ($P = 0.033$).

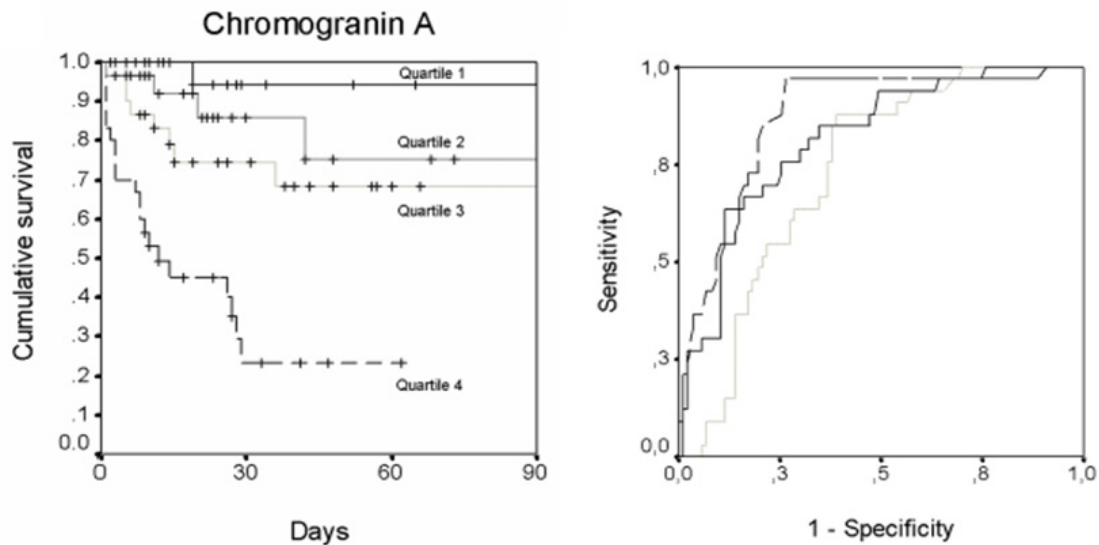


Fig. 7. Kaplan–Meier analysis: cumulative incidence of death by CGA and PCT quartiles.

(A), Median (interquartile range) for CGA concentration data: quartile 1, 35 $\mu\text{g/L}$ (30–53 $\mu\text{g/L}$); quartile 2, 84 $\mu\text{g/L}$ (77–94 $\mu\text{g/L}$); quartile 3, 174 $\mu\text{g/L}$ (151–197 $\mu\text{g/L}$); quartile 4, 563 $\mu\text{g/L}$ (355–974 $\mu\text{g/L}$). Each quartile includes 30 patients.

(B), ROC curve to test the ability of CGA (black line), SAPS II (black dashed line), and PCT (gray dashed line) to predict outcome.

ROC curves for CGA, PCT, and SAPS II are shown in Figure 7. To assess the best positive likelihood ratio, we chose the cutoff value that was associated with the best specificity. For CGA, we chose a cutoff value of 255 $\mu\text{g/L}$, which produced a sensitivity of 0.63 and a specificity of 0.89 (positive likelihood ratio, 5.73; negative likelihood ratio, 0.42; AUC, 0.82). A cutoff value of 65 for SAPS II produced a sensitivity of 0.61 and a specificity of 0.85 (positive likelihood ratio, 4.07; negative likelihood ratio, 0.46; AUC, 0.87). For a PCT cutoff value of 4.82 $\mu\text{g/L}$, sensitivity and specificity were 0.60 and 0.71, respectively (positive likelihood ratio, 2.07; negative likelihood ratio, 0.56; AUC, 0.73). To conclude, in this clinical study of critically ill nonsurgical patients, we demonstrate that plasma CGA is a strong and independent prognostic in consecutive critically ill nonsurgical patients. The over expression of complete CGA suggests that for these patients the processing machinery to produce antimicrobial peptides is not correct.

6. Insertion of synthetic antimicrobial chromogranins-derived peptides in biomaterials

The surface of medical devices is a common site of bacterial and fungal adhesion, first step to the constitution of a resistant biofilm leading frequently to chronic infections. In order to prevent such complications, several physical and chemical modifications of the device surface have been proposed. In a previous study, we experimented a new type of topical antifungal coating using the layer-by-layer technique. The nanometric multilayer film obtained by this technique is functionalized by the insertion of a CgA-derived antifungal peptide (CGA 47-66, Chromofungin). We show that the embedded peptide keeps its

antifungal activity by interacting with the fungal membrane and penetrating into the cell. *In vitro* studies demonstrated that such an antifungal coating is able to inhibit the growth of yeast *Candida albicans* by 65% and completely stop the proliferation of filamentous fungus *N.crassa*. The cytotoxicity of such a coating was also assessed by growing human gingival fibroblasts at its surface. Finally, the antifungal coating of poly (methylmethacrylate), a widely used material for biomedical devices, is successfully tested in an *in vivo* oral candidiasis rat model (Etienne et al., 2005).

7. Conclusions

CGs family emerges as prohormones able to modulate homeostatic processes in response to excessive stimulations such as microbial infections. The studies concerning the expression of CGs and their antimicrobial peptides in patients with inflammatory diseases and the correlation with the proteolytic processes occurring in these pathologies vs. controls are crucial to understand the involvement of these prohormones and their derived peptides in innate and adaptive immunities. Calcium is a universal secondary messenger involved in many cellular signal transduction pathways, regulating crucial functions such as secretion, cell motility, proliferation and cell death. The calcium-dependent immunomodulatory properties of CHR and CAT are important for the understanding of their involvement in inflammatory mechanisms. In sum, these linear peptides may represent prototypic lead molecules useful for the development of new therapeutic agents and also biomaterials.

8. Acknowledgement

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