Study of new antimicrobial peptides in chromaffin granules from bovine adrenal medulla: new aspects of innate immunity.

Marie-Hélène Metz-Boutigue, Anne-Estelle Kieffer, Yannick Goumon, Karine Lugardon and Dominique Aunis

Inserm U575, IFR37, Strasbourg, France.

Secretory granules of chromaffin cells contain with catecholamines several antimicrobial peptides, which are secreted in the extracellular medium following exocytosis. In this report we decided to focus on three active peptides Chromofungin (chromogranin A 47-66), Enkelytin (proenkephalin-A 209-237 and Ubifungin (ubiquitin 65-76). Using confocal laser microscopy we have shown that Chromofungin and Ubifungin are able to cross the cell membranes and penetrate into fungi and yeasts, whereas the N-terminal fragment ubiquitin 1-34 was stopped at the fungal cell wall level. At the intracellular level Chromofungin and Ubifungin are able to inhibit calmodulin-dependent calcineurin, a crucial enzyme for fungal growth. Finally, all together these peptides constitute a mixture of potent antimicrobial peptides, which might represent molecules useful for the development of new therapeutic agents.

Correspondence*:* Dr. Marie-Hélène Metz-Boutigue, Inserm U575, 'Physiopathologie du Système Nerveux', IFR37, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France.
Phone: 33-3-88456609; **Fax:** 33-3-88600806; **Email:** metz@neurochem.u-strasbg.fr

Cell Biology of the Chromaffin Cell
R. Borges & L. Gandía Eds.
Instituto Teófilo Hernando, Spain, 2004

Antimicrobial peptides are present in the hemolymph of insects and are also stored in the secretory granules of immune cells found within mammals and birds[1-3]. The importance of these molecules is clearly established in the innate immunity of invertebrates by the microorganism-induced antibacterial activity, that can be detected in the hemolymph[4]. In vertebrates, these peptides complete adaptive immunity by acting as a first line of defense against pathogens and by controlling natural flora[5]. Similarities have been highlighted between pathogen recognition, signalling pathways and effector mechanisms of innate immunity in Drosophila and mammals[6].

Numerous natural antimicrobial peptides from mammals have been previously characterized in several tissues[5]. In addition to antibacterial peptides, some antifungal molecules were characterized. A first group acts by lysis that occurs after destabilization of the membrane, formation of aqueous pores and intracellular mechanism, whereas the second group interferes with cell-wall synthesis or the biosynthesis of glucan or chitin [6,7].

Secretory granules from adrenal medullary chromaffin cells contain with catecholamines a complex mixture of peptides derived from chromogranins (CGs), proenkephalin-A (PEA) and others precursors. During the past decade, our laboratory has characterized the processing of chromogranin A (CGA)[8], chromogranin B (CGB)[9] and proenkephalin-A (PEA)[10] in chromaffin granules from bovine adrenal medulla and we have identified other unexpected peptides derived from ubiquitin (Ub)[11], that are secreted with catecholamines during stimulation of the chromaffin cells and possess antimicrobial activity[9,11-17]. Interestingly, they are recovered in biological fluids implicated in defense mechanisms, for example, abcess fluids and secretions of stimulated polymorphonuclear neutrophils (PMNs)[13, 14]. These peptides have been hypothesized to play a role in stress situations, acting as an immediate protective shield against pathogens[12].

In this review we focus on three natural antimicrobial peptides derived from CGA, PEA and Ub and their synthetic active domains. They display common structural features that are believed to be important for the molecular mechanisms implicated in their antimicrobial activities.

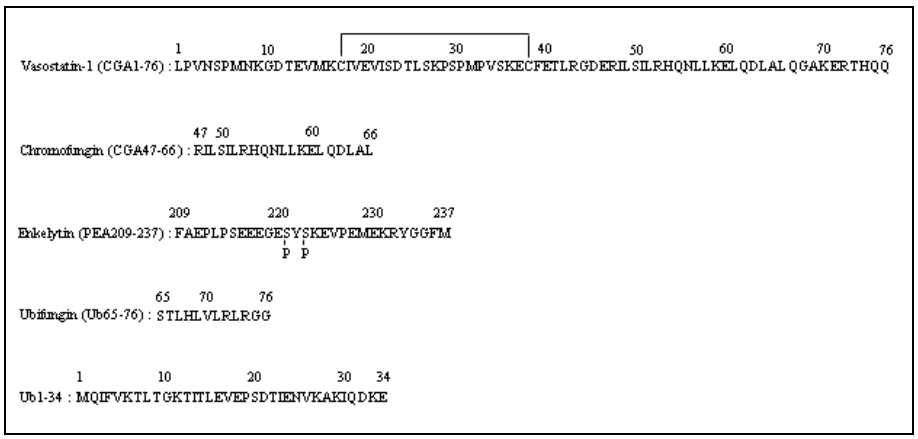

Figure 1: Antimicrobial peptides isolated from chromaffin secretory granules of bovine adrenal medulla. The disulfide bridge and the phosphorylated residues are indicated.

Vasostatin-I and Chromofungin: potent inhibitors of fungi and yeast. Following separation of the soluble material present in chromaffin granules by HPLC, antibacterial activity against *M. luteus* can be detected in several fractions. One of these fractions has been identified as Vasostatin-I (CGA1-76), the highly conserved N-terminal domain in which the disulfide bridge and the sequence 50-62 (SILRHQNLLKELQ) are strictly unchanged[14, 15] (Figure 1). Natural bovine Vasostatin-I is selectively active against *M. luteus* and *B. megaterium* at micromolar range[14], but activity is not detectable against pathogenic Gram-positive and Gram negative bacteria. In contrast, Vasostatin-I was found to be strongly active against a large variety of filamentous fungi, including pathogenic strains and yeasts[14]. Interestingly, this peptide is inactive against erythrocytes and others mammalian cell-types[14]. These experiments were extended by analysis of the recombinant-derived fragments of human Vasostatin-I, that are able to display antifungal activity. An active peptide corresponding to the fragment CGA47-60 was identified by sequencing and MALDI-TOF mass spectrometry. The shortest active peptide with maximun global hydrophobicity and amphipathic features corresponded to CGA47-66 (Figure 1) and was named Chromofungin[15]. The three

dimensional structure of Chromofungin has been determined in water-trifluoroethanol (50:50) using ^{1}H NMR spectroscopy. This analysis revealed the amphipathic helical character of the C-terminal part of the sequence 53-66.

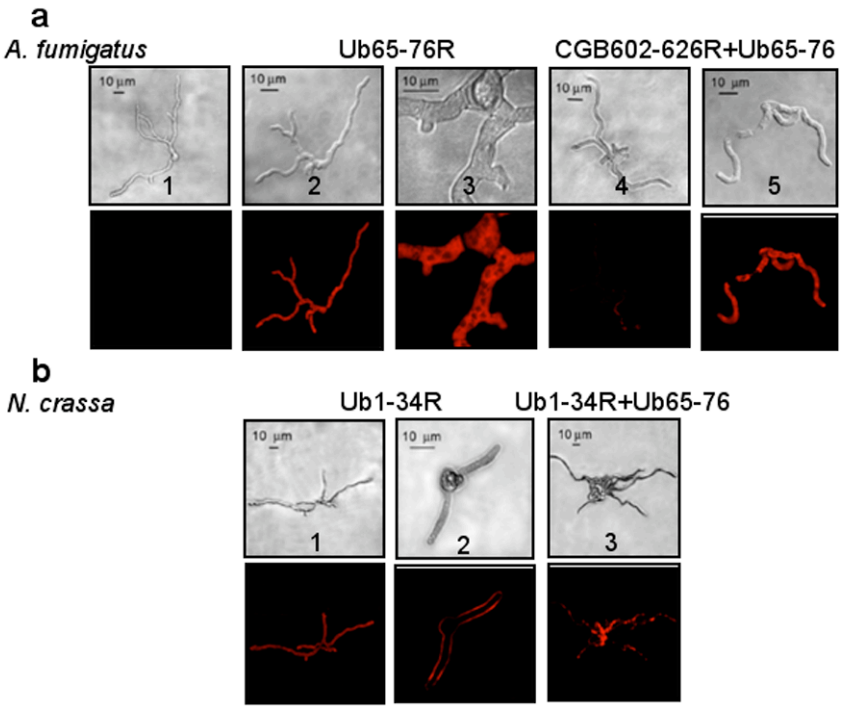

Figure 2: Phase contrast and fluorescence confocal laser micrographs of *Aspergillus fumigatus*, *Neurospora crassa* and *Candida albicans* with rhodamine-labeled synthetic peptides Chromofungin, Ubifungin and Ub1-34. a) 1, *A. fumigatus* in the absence of peptide; 2, *A. fumigatus* after 60 min incubation with Chromofungin (10 µM); 3, *A. fumigatus* after 60 min incubation with rhodminated inactive peptide CGB602-626 (10 µM). **b)** 1, *A. fumigatus* after 2 min incubation with Ubifungin (2 µM); 2, at higher magnification, the core of fungi and unlabeled vacuoles and septum are clearly visible; 3, *N. crassa* was incubated with 5 µM Ub1-34 for 15 min: note the labelling along the cell membrane; 4, *A. fumigatus* was first incubated with 20 µM unlabeled Ubifungin for 45 min before a final incubation with 5 µM rhodaminated Ub1-34 for 15 min. Note the fluorescence within cells revealing Ub1-34 penetration due to membrane destabilization evoked by Ubifungin.

The importance of the amphipathic sequence for antifungal activity was demonstrated by the loss of such activity when two proline residues were substituted for Leu61 and Leu64, disrupting the helical structure and amphipathic character of the peptide[15]. Thus, Chromofungin is a cationic amphipathic molecule with a helical structure and is able to interact with inner and outer membranes to reach intracellular targets, as shown by confocal laser microscopy (Figure 2).

Enkelytin potent inhibitor of Gram-positive bacteria. The processing of PEA has been extensively studied in chromaffin cells obtained from bovine adrenal medulla[10] and proceeds through an orderly series of steps, starting with the removal of the C-terminal domain (PEA209-239). The bisphosphorylated form of PEA209-237 (Figure 1) displays a potent antibacterial activity against Gram positive bacteria including the pathogenic *S. aureus* in the 0.2-4.5 micromolar range. In contrast, to the bisphosphorylated form, the non-modified peptide displays a low antibacterial activity, indicating that the two phosphorylated residues S221 and S223 play an important conformational role[10, 13]. Furthermore, the synthetic modified peptide PEA209-237 with three E residues in place of S215, S221 and S23 conserves antibacterial activity, suggesting the importance of negative charges in the expression of such activity. In contrast to cationic antimicrobial peptides, Enkelytin possess a negative charge (-7) and because of this might be compared with polyaspartic acid peptides identified in secretions from the lung[18]. To characterize the biological function of Enkelytin several fluids and PMN secretions from injured animals with infection have been analyzed[13]. Following secretion at the inflammatory area, Enkelytin was quantified by sequencing and its concentration in bovine periarthritis abscess fluid was estimated from 0.5 to 1 micromolar. As a continuation of these studies, its presence in secretions of PMNs has been demonstrated[13].

PEA has been reported to be significantly expressed in the immune system and might provide a basis for neuroimmune interactions[19]. The local inflammatory response initiates the synthesis and secretion of opioid peptides by immune cells. Therefore, Enkelytin degradation by neuropeptide degrading endopeptidase

(NEP) and angiotensin converting enzyme (ACE) present in granulocytes generate Met-enkephalin and its derived peptides[10]. Met-enkephalin enhances the immune reaction and this pentapepide can bind opiod receptors present in peripheral tissues with inflammation to mediate an analgesic effect[20]. Taken together the major bisphosphorylated form of PEA209-237 and Met-enkephalin would provide a highly beneficial survival strategy for the proinflammatory process.

Ubiquitin, its N- and C-terminal fragments. Ubiquitin is a peptide of 76 residues (Figure 1) found in all eukatyotic cells that display well-conserved sequences from protozoa to vertebrates[21]. Recently, we have reported the subcellular localization of free Ub in bovine adrenal chromaffin cells[11]. Ub is present in secretory granules and secreted with catecholamines after chromaffin cells stimulation. In addition, we have shown that free Ub displays *in vitro* antimicrobial activities and inhibits the growth of *M. luteus* and *B. megaterium* at a MIC of 60 micromolar. At a concentration of 100 micromolar, Ub completely inhibits the growth of *N. Crassa*[11]. Then, we have shown that the C-terminal fragment Ub65-76 is crucial for the expression of the antifungal activity. This peptide named Ubifungin is active against Gram positive, pathogenic *E. coli* and various fimalentous fungi and yeasts[11]. Interestingly, it is inactive against erythrocytes[11]. Because microorganism growth has not resumed after 48 hours, this peptide is believed to exert a lytic mechanism.

The N-terminal fragment Ub1-34 adopts a beta-hairpin (residues 1-17) followed by an alpha helix (residues 18-34)[11] the latter being important for membrane interactions. Antimicrobial assays of Ub1-34 have indicated that this peptide displays weak activity against *N. crassa* at a concentration of 100 micromolar. Interestingly, when Ub1-34 was added to Ub65-76 the two peptides acted synergistically to inhibit the growth of several filamentous fungi[11].

Confocal laser microscopy has allowed analysis of the interaction of Chromofungin, Ubifungin and Ub1-34 with fungal membranes of pathogenic strains of *A. fumigatus* and *C. albicans* (Figure 2). Labeled Ubifungin was visible at the level of the cell wall and in the inner part of the fungi after two minutes of incubation. In addition, Ub1-34 was visible at the level of cell wall but not within

cells. However, when the fungi was treated with unlabeled Ubifungin before incubation with rhodaminated Ub1-34, an intense fluorescence was observed, indicating that Ubifungin destabilizes the cell wall, allowing the peptide Ub1-34 to actively penetrate the fungi.

Chromofungin and Ubifungin: inhibition of the phosphatase activity of calcineurin. In addition, to destabilizing the cell wall, Chromofungin and Ubifungin might also exert activity on intracellular targets. Indeed we have shown that these two peptides are able to inhibit the phosphatase activity of calcineurin a calmodulin dependent enzyme[11,15] crucial for the fungal growth.

CONCLUSIONS

Chromofungin, Enkelytin, Ubifungin and Ub1-34 correspond to highly conserved peptides and their antimicrobial activities probably occurred early in evolution. They are widely distributed in nervous, endocrine, neuroendocrine and immune cells. Their liberation from cells indicates that they probably play a role in inflammatory processes. Therefore, we suggest that in stress situation these peptides might provide a highly beneficial strategy against pathogenic invasion. They might be used together or in combination with classical antifungal molecules to increase their efficiency.

ACKNOWLEDGEMENTS

This work was funded by Inserm and supported by grants from the Ligue contre le Cancer (to MHMB and DA), the Fondation pour la Recherche Médicale (to AEK and MHMB) and from the Meiji Company (Odawara, Japan to DA). G. Nullans and C. Gasnier (Inserm U575) provided excellent technical assistance.

REFERENCES

1. Gennaro, R., et al., *Neutrophil and eosinophil granules as storage of 'defense' proteins*. In Blood Cell Biochemistry. JR Harris Eds 335-368. Plenum Press. New York, NY, 1991.
2. Weiss, J. *Leukocyte-derived antimicrobial proteins.* Curr Opin Hematol, 1994. **1:**78-84.
3. Lehrer, R.I., A.K. Lichtenstein and T. Ganz, *Defensins: antimicrobial and cytotoxic peptides of mammalian cells.* Annu Rev Immunol, 1993. **11:**105-112.
4. Hoffmann, J.A., J.M. Reichhart and C. Hetru, *Innate immunity in higher insects.* Curr Opin Immunol, 1996. **8:**8-13.

5. Zasloff, M. *Antimicrobial peptides of multicellular organisms.* Nature, 2002. **415:**389-395.
6. Debono, M. and R.S. Gordee, *Antibiotics that inhibit fungal cell wall development.* Annu Rev Microbiol, 1994. **48:**471-497.
7. De Lucca, A.J. and T.J. Walsh, *Antifungal peptides: novel therapeutic compounds against emerging pathogens.* Antimicrob Agents Chemother, 1999. **43:**1-11.
8. Metz-Boutigue, M.H., et al., *Intracellular and extracellular processing of chromogranin A. Determination of cleavage sites.* Eur J Biochem, 1993. **217:**247-257.
9. Strub, J.M., et al., *Processing of chromogranin B in bovine adrenal medulla. Identification of secretolytin, the endogenous C-terminal fragment of CGB614-626 with antibacterial activity.* Eur J Biochem, 1995. **229:**356-368.
10. Goumon, Y., et al., *Processing of proenkephalin-A in bovine chromaffin cells. Identification of natural derived fragments by N-terminal sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry.* J Biol Chem, 2000. **275:**38355-38362.
11. Kieffer, A.E., et al., *The N- and C-terminal fragments of ubiquitin are important for the antimicrobial activities.* FASEB J, 2003. **17:**776-778.
12. Metz-Boutigue, M.H., et al., *Antibacterial peptides are present in chromaffin cell secretory granules.* Cell Mol Neurobiol, 1998. **18:**249-266.
13. Goumon, Y., et al., *Characterization of antibacterial COOH-terminal proenkephalin-A-derived peptides (PEAP) in infectious fluids. Importance of enkelytin, the antibacterial PEAP209-237 secreted by stimulated chromaffin cells.* J Biol Chem, 1998. **273:**29847-29856.
14. Lugardon, K., et al., *Antibacterial and antifungal activities of vasostatin-I, the N-terminal fragment of chromogranin A.* J Biol Chem, 2000. **275:**10745-10753.
15. Lugardon, K., et al., *Structural and biological characterization of chromofungin, the antifungal chromogranin A-(47-66)-derived peptide.* J Biol Chem, 2001. **276:**35875-35882.
16. Goumon, Y., et al., *The C-terminal bisphosphorylated proenkephalin-A (209-237) peptide from adrenal medullary chromaffin granules possesses antibacterial activity*. Eur J Biochem, 1996. **235:**516-525.
17. Kieffer, B., et al., *Solution conformation of the synthetic bovine proenkephalin-A 209-237 by ^{1}HNMR spectroscopy.* J Biol Chem, 1998. **273:**33517-33523.
18. Brogden, K.A., M. Ackermann and K.M. Huttner, *Small, anionic and charge-neutralizing propeptide fragments of zymogens are antimicrobial.* Antimicrob Agents Chemother, 1997. **41:**1615-1617.
19. Plotnikoff, N.P., et al., *Methionine enkephalin: a new cytokine-human studies.* Clin Immunol Immunopathol, 1997. **82:**93-101.
20. Stein, C., et al., *Local analgesic effect of endogenous opioid peptides*. Lancet, 1993. **342:**321-324.
21. Schlesinger, D.H., G. Goldstein and H.D. Niall, *The complete amino acid sequence of ubiquitin, an adelynate cyclase stimulating polypeptide probably universal in living cells*. Biochemistry, 1975. **14:**2214-2218.