Mammalian Defensins – New Insights from Ancient Peptides

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Introduction

The defensins are an ancient family of peptides found in plants and animals where they form an important part of the innate immune system (1–5). In mammals, their role as a first line of defense against infections is highlighted by their presence in leukocytes, mucosal surfaces and epithelia, settings that are constantly challenged by microorganisms. Although diverse in sequence and size, mammalian defensins share some overall features that include a β-sheet structure stabilised by three disulfide bonds and a large number of positive charges. Generally defensins have broad antimicrobial activity against bacteria as well as viruses and fungi, and thus can directly target invading microbes. However, they also have other roles, including linking the innate and adaptive immune system through chemoattraction of lymphocytes to sites of infection.

Structural Features of α-, β-and θ-defensins

Structurally, the mammalian defensins can be subdivided into three classes, α-, β-and θ-defensins (**Fig. 1**), all of which have been extensively characterised by crystallography and NMR spectroscopy approaches (e.g. (6–8)). The α-defensins consist of 29 to 35 amino acids with their three disulfides connected in a Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{IV}, Cys^{III}-Cys^{V} arrangement that stabilises a central triple-stranded β-sheet. Although their sequences are highly diverse, hallmarks include the invariant disulfide array, an Arg-Glu salt-bridge, a conserved Gly residue important for the β-sheet structure, and high Arg relative to Lys content. The β-defensins comprise a similar central triple-stranded b-sheet but are larger at 35 to 45 residues, and possess a Cys^{I}-Cys^{V}, Cys^{II}-Cys^{IV}, Cys^{III}-Cys^{VI} disulfide array. The extended N-terminal region in β-defensins can adopt an additional structural feature in the form of a short α-helix (7). The θ-defensins are structurally distinct, comprising only 18 residues with the striking feature of having their N- and C-termini linked together in a head-to-tail fashion, creating a complete circle of peptide bonds (9). NMR spectroscopy studies have shown that the circle is elongated to form an anti-parallel β-sheet that is cross-braced by three disulfides linked in a Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{V}, Cys^{III}-Cys^{IV} fashion (8). θ-defensins are referred to in the article by Daly *et al.* in this Showcase on Research as part of the emerging class of gene-encoded cyclic peptides.

Sites of Expression, Biological Activity and Functions

Defensins have been found in all mammals investigated and are abundant in host-defense settings (1). The first α-defensins were purified from granules of polymorphonuclear leukocytes. After these immune cells identify and ingest invading microbes, the granules are fused to their phagocytic vacuoles, releasing the defensins to directly act on the microbe (1). In addition to leukocytes, α-defensins are found in secretory granules in Paneth cells of the small intestine (10). These cells are located at the bottom of narrow intestinal pits referred to as crypts and defensins are released into the crypts both constitutively and in response to

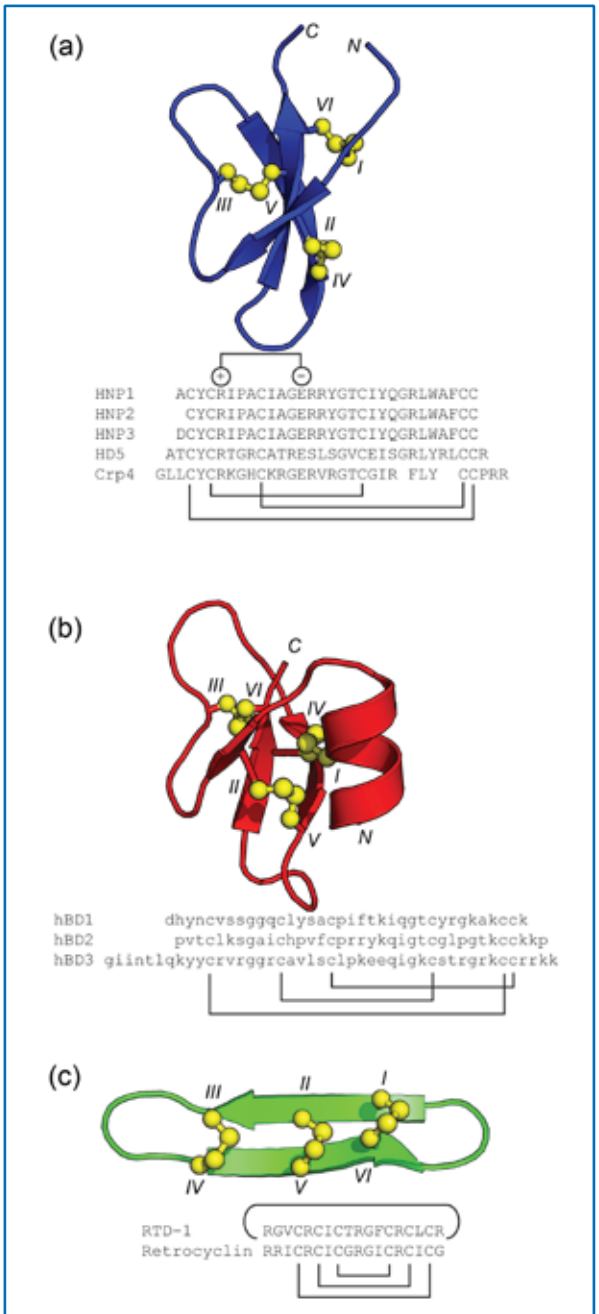


Fig. 1. Structural features of mammalian defensins. Representative structures and selected sequences of (**a**) α-defensins, (**b**) β-defensins and (**c**) θ-defensins. Cysteines are labeled with roman numbers in order of appearance in the sequence. Connecting lines above and below the sequences highlight the conserved disulfide bonds, a conserved salt-bridge in α-defensin and the head-to-tail backbone cyclisation of θ-defensins.

cholinergic or bacterial stimuli to control the microbial flora of the gut (10). The first β-defensin was isolated from bovine tracheal epithelium and they have since been identified in various epithelial cells and leukocytes, where their expression is either constitutive or inducible (2). The θ-defensins were first discovered in Rhesus monkeys where they are only found in neutrophils and monocytes (2). Interestingly there are considerable differences in the occurrence of defensins in various cell lineages in different species, e.g. both rats and mice have numerous epithelial and Paneth cell defensins, but although rats also have leukocyte defensins, mice do not (1,2).

Most defensins show antimicrobial activity against bacteria and fungi *in vitro*, in particular when tested under 'ideal' conditions with low levels of salt, plasma proteins or interfering substances, where their activity is generally in the low micromolar range (1,2). Although their *in vitro* activity is significantly reduced under more 'physiological conditions', this is well compensated for by the high local concentrations achieved *in vivo* in settings such as phagocytic vacuoles and intestinal crypts. Direct evidence for the importance of defensins for intestinal immunity comes from transgenic mice studies. Knockout mice lacking MMP-7, the enzyme responsible for processing and activation of Paneth cell mouse defensins, are significantly more susceptible to oral bacterial infections (11). In contrast, knockin mouse expressing an additional human Paneth cell defensin, HD5, are fully immune to such challenges (12).

In addition to the direct bactericidal effect, defensins are able to bridge the innate and adaptive immune systems and promote longer lasting responses by recruiting various lymphocytes. Human neutrophil defensins (HNPs) have been shown to be chemotactic for monocytes, T cells and dendritic cells, while the human β-defensins 2-4 (hBD2-4) are able to recruit memory T cells, dendritic cells and monocytes via directly binding to the chemokine receptors 2 and 6 (CCR2 and CCR6) (2,4). β-defensins can also bind to toll-like receptors on antigen-presenting cells (4). In addition, defensins have numerous other physiological roles, including in proinflammatory suppression under certain conditions, wound healing, sperm maturation, and cancer (4). The link between dysfunction of the intestinal innate immunity and inflammatory bowel disease is one area that has received recent attention (13).

Evolution and Biosynthesis

Families of small disulfide-rich antimicrobial peptides, some of which are referred to as defensins, are found throughout the vertebrates, invertebrates, plants, fungi and myxobacteria (3). Although the evolutionary relationship between 'modern' mammalian defensins and these distant cousins is unclear, sequences and structural features suggest a genetic link to the invertebrate 'big defensin' family (3), the first member of which was identified in horseshoe crabs. The 'big defensins' comprise a C-terminal domain with an identical size and disulfide structure to β-defensins, thus it seem likely that the β-defensins that are today widespread in fish, reptiles, birds and mammals have all evolved from this class of peptides (3). The α-defensin genes, which are only found in mammals and marsupials, have evolved from the β-defensins through further gene duplication and diversification, but generally remain outnumbered by β-defensins in most species. For example, humans have over 30 β-defensin genes but only five α-defensin genes (3). The θ-defensins are encoded by mutated α-defensin genes that produce truncated α-defensin precursors due to the insertion of a stop-codon after the third cysteine. From two such precursors, nine residue fragments are excised and subsequently ligated head-to-tail to create the 18-residue circular backbone (9). θ-defensins are only found in non-human primates, as human and their closest relatives chimpanzee, gorilla and bonobo have inherited defective pseudogenes comprising a second stop-codon preventing production (5). Chemical synthesis of what would have been the products of human θ-defensin genes, had they actually been produced, revealed that these peptides termed 'retrocyclins' are potent antiviral agents, including against HIV-1 (5). In the light of the AIDS epidemic, it seems a rather cruel twist of fate that humans actually do have a powerful 'built-in' HIV-1 protection system, but that the genes encoding it are dysfunctional.

α-Defensins are produced as prepropeptides comprising a signalling sequence, an anionic prodomain, and a cationic mature domain. After removal of the signal sequence, the peptides remain inactive, presumably due to a charge neutralisation of the two domains that may help reduce intracellular toxicity and aid folding. Final processing requires proteolytic removal of the prodomain, which in mouse Paneth cells is done by MMP-7 prior to peptide packing into granules (11). In contrast the human HD5 is packed into granules in its inactive form and only processed by trypsin during or after secretion (14). β-Defensins lack or have only a short prodomain, thus differ in their processing. Very little is known about the complex proteolytic cleavage events and subsequent dual ligation required for the biosynthesis of θ-defensins from their two parent propeptides.

Structure–activity Relationships and Mechanism of Action

The notion that the biological function of a protein is intimately linked to its three-dimensional structure has long been the driving force behind structural biology efforts. However, several studies on defensins have shown that this notion may not always be true. The overall fold of the defensins is clearly primarily determined by the positioning and connectivities of its cysteine residues. Thus when it was demonstrated that chemically forcing hBD3 to adopt non-native disulfide linkages, or replacing all its cysteine residues with alanines, had little effect on its antimicrobial activity (15), it came as a surprise. Similarly, removal of the disulfides in the mouse Paneth cell α-defensin cryptdin4 (Crp4), which was confirmed by solution NMR spectroscopy to result in a completely unstructured peptide, did not decrease its antibacterial action (16). In fact, for both peptides, if anything a slight increase in antimicrobial activity was seen against several bacterial strains (15,16). Strikingly, this behaviour of defensins is not just a quirky feature observed *in vitro*, rather it can be physiologically relevant. hBD1 has been considered an enigma in that this defensin, which is ubiquitously expressed in all human epithelia, has very

poor antimicrobial activity *in vitro*. However, at sites of low oxygen partial pressure such as the colonic lumen, the metabolism of anaerobic microbes creates a reducing redox potential. Under these conditions hBD1, which co-localises with the redox protein thioredoxin, can be reduced, unmasking a dramatic increase in antimicrobial activity to the same levels as the most potent defensins (17). Thus reduced – and unstructured – hBD1 plays an important role in protecting the healthy colonic epithelium, and likely also in other anaerobic niches such as oral cavities, wounds and sites of infection (17).

If the overall structure is dispensable, then what features are important for the bactericidal activity of defensins? Their cationic nature is critical, with charge reversals significantly reducing their antimicrobial activity (18). Antimicrobial peptides generally kill microbes by interacting with and permeabilising the bacterial cell membrane, and the defensins are no exception (3). It is well known that this interaction relies heavily on the cationic nature of these peptides, which targets bacterial membranes that are rich in anionic lipids. The events that follow this initial interaction differ between different peptides, and indeed between different defensins, in that it may involve formation of ordered pore structures or passage through the membrane to bind to an intracellular target. The fact that the overall structure is not of importance for Crp4, hBD1 and hBD3 suggests that at least for these peptides, the primary mechanism of action is accumulation at the cell membrane surface and membrane disruption in a non-specific detergent-like mechanism. In contrast, the human neutrophil α-defensins HNP1-3 appear to form more ordered pore structures of defined size, suggested to arise from defensin multimeric structures (3).

So if the disulfides, and the tightly folded β-sheet structure that they confer, which are the hallmarks of the defensin family, are not required for the antimicrobial activity of defensins – then why are they conserved? There are several answers to this question. Defensins do have physiological functions other than directly killing microbes through non-specific interactions with membranes. Functions such as chemotaxis require binding to receptors and such protein-protein interactions can be expected to be highly dependent on a defined structure. Indeed, hBD1 is chemotactic for immature dendritic cells and T-lymphocytes via direct binding and activation of the chemokine receptor CCR6, but removal of its disulfide bonds completely abolished this function (15). In addition, several defensins, including retrocyclin-2 and human α-defensins HNP1 and HNP3, have been shown to self-associate, and at least for some peptides this dimerisation is functionally relevant (19). The ability to self-associate into ordered multimers relies heavily on a specific structure of the individual monomers. Finally, the disulfide array provides the defensin structure protection against proteolysis, with a number of studies revealing that disulfide-less defensins are rapidly degraded (16,20). Resistance against proteolysis is a key factor for *in vivo* function, in particular as α-defensins require proteolytic processing for activation and Paneth cell defensins exert their biological function in an environment designed to break down proteins to allow absorption of amino acids. The α-defensins have developed additional protection from degradation in the form of a stabilising conserved salt-bridge. Although this feature can be removed with minimal effect on the overall structure, this results in the flexibility of the fold increasing to the point where any mutated analogue becomes readily digested by proteases (21–24).

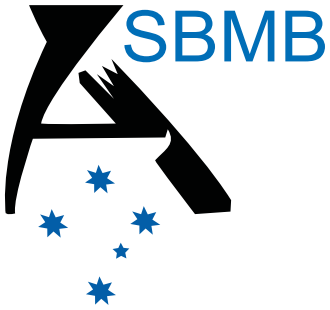
Defensins as Novel Antibiotics?

Much of the intense studies of defensins and other antimicrobial peptides in recent years have at least in part been spurred on by the rapid decrease in effectiveness of antibiotics currently used clinically. Although antimicrobial peptides have been hailed as a potential solution, so far the results of clinical trials have been rather disappointing (25). Inherent problems are poor stability due to their peptidic nature and toxicity at the rather high doses required for systemic administration to reach sufficient local concentrations for efficacy. However, with a number of recent developments in peptide design, manufacturing, and formulation, the outlook is again bright. Strategies for reducing toxicity on eukaryotic cells often involve reducing hydrophobicity. Increasing activity against microbes can include creating oligomers or targeting sequences directing the peptide to specific strains. Increasing stability can be achieved through introduction of cyclisation through disulfides or incorporation of non-natural amino acids. Finally, the delivery of peptides can be improved through efficient formulation or controlled release strategies (25). Thus it appears likely that within the next ten years the defensins or analogues thereof will be ready to provide host defensins not only as endogenous peptides but also as exogenously delivered antibiotics.

References

1. Ganz, T. (2003) *Nat. Rev. Immunol.* **3**, 710-720
2. Selsted, M.E., and Ouellette, A.J. (2005) *Nat. Immunol.* **6**, 551-557
3. Lehrer, R. I., and Lu, W. (2012) *Immunol. Rev.* **245**, 84-112
4. Semple, F., and Dorin, J.R. (2012) *J. Innate. Immun.* in press
5. Penberthy, W. T., Chari, S., Cole, A. L., and Cole, A. M. (2011) *Cell Mol Life Sci.* **68**, 2231-2242
6. Hill, C. P., Yee, J., Selsted, M. E., and Eisenberg, D. (1991) *Science* **251**, 1481-1485
7. Sawai, M. V., Jia, H. P., Liu, L., Aseyev, V., Wiencek, J. M., McCray, P. B., Jr., Ganz, T., Kearney, W. R., and Tack, B. F. (2001) *Biochemistry* **40**, 3810-3816
8. Daly, N. L., Chen, Y. K., Rosengren, K. J., Marx, U. C., Phillips, M. L., Waring, A. J., Wang, W., Lehrer, R. I., and Craik, D. J. (2007) *Biochemistry* **46**, 9920-9928
9. Tang, Y.Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C.J., Ouellette, A.J., and Selsted, M.E. (1999) *Science* **286**, 498-502
10. Ayabe, T., Satchell, D.P., Wilson, C.L., Parks, W.C., Selsted, M.E., and Ouellette, A.J. (2000) *Nat. Immunol.* **1**, 113-118
11. Wilson, C.L., Ouellette, A.J., Satchell, D.P., *et al.* (1999) *Science* **286**, 113-117
12. Salzman, N.H., Ghosh, D., Huttner, K.M., Paterson, Y., and Bevins, C.L. (2003) *Nature* **422**, 522-526

References continued on page 9



References continued from page 12

13. Gersemann, M., Wehkamp, J., and Stange, E.F. (2012) *J. Intern. Med.* **271**, 421-428
14. Ghosh, D., Porter, E., Shen, B., Lee, S.K., Wilk, D., Drazba, J., Yadav, S.P., Crabb, J.W., Ganz, T., and Bevins, C.L. (2002) *Nat. Immunol.* **3**, 583-590
15. Wu, Z., Hoover, D.M., Yang, D., Boulegue, C., Santamaria, F., Oppenheim, J.J., Lubkowski, J., and Lu, W. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 8880-8885
16. Maemoto, A., Qu, X., Rosengren, K.J., Tanabe, H., Henschen-Edman, A., Craik, D.J., and Ouellette, A.J. (2004) *J. Biol. Chem.* **279**, 44188-44196
17. Schroeder, B.O., Wu, Z., Nuding, S., Groscurth, S., Marcinowski, M., Beisner, J., Buchner, J., Schaller, M., Stange, E.F., and Wehkamp, J. (2011) *Nature* **469**, 419-423
18. Tanabe, H., Qu, X., Weeks, C. S., Cummings, J. E., Kolusheva, S., Walsh, K. B., Jelinek, R., Vanderlick, T. K., Selsted, M. E., and Ouellette, A. J. (2004) *J. Biol. Chem.* **279**, 11976-11983
19. Pazgier, M., Wei, G., Ericksen, B., Jung, G., Wu, Z., de Leeuw, E., Yuan, W., Szmacinski, H., Lu, W.Y., Lubkowski, J., Lehrer, R.I., and Lu, W. (2012) *J. Biol. Chem.* **287**, 8944-8953
20. Kamdar, K., Maemoto, A., Qu, X., Young, S.K., and Ouellette, A.J. (2008) *J. Biol. Chem.* **283**, 32361-32368
21. Wu, Z., Li, X., de Leeuw, E., Ericksen, B., and Lu, W. (2005) *J. Biol. Chem.* **280**, 43039-43047
22. Rosengren, K.J., Daly, N.L., Fornander, L.M., Jonsson, L.M., Shirafuji, Y., Qu, X., Vogel, H.J., Ouellette, A.J., and Craik, D.J. (2006) *J. Biol. Chem.* **281**, 28068-28078
23. Rajabi, M., de Leeuw, E., Pazgier, M., Li, J., Lubkowski, J., and Lu, W. (2008) *J. Biol. Chem.* **283**, 21509-21518
24. Andersson, H.S., Figueredo, S.M., Haugaard-Kedstrom, L.M., Bengtsson, E., Daly, N.L., Qu, X., Craik, D.J., Ouellette, A.J., and Rosengren, K.J. (2012) *Amino Acids* in press
25. Eckert, R. (2011) *Future Microbiol.* **6**, 635-651