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Conference Paper

Bovine Neutrophil Antibiotic Peptides and Their Precursors: Structure and Role in Innate Immunity*

Domenico Romeo, Renato Gennaro, Margherita Zanetti, Alessandro Tossi, Barbara Skerlavaj, Paola Storici, Marco Scocchi, Laura Litteri and Donatella Verbanac#

Department of Biochemistry, Biopyhsics and Macromolecular Chemistry, University of Trieste and Department of Medical Sciences and Technology, University of Udine, Italy

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Four peptides were characterized in extracts of bovine neutrophil granules: an Arg-rich dodecapeptide, maintained in a cyclic structure by a disulfide bridge; a Trp-rich tridecapeptide named indolicidin; and two 43- and 59 amino acids long peptides, named Bac5 and Bac7, with frequent repeats of the triplets Arg-Pro-Pro and Pro-Arg-Pro, respectively.

The full length cDNA of the first three of these peptides was characterized recently. Sequence analysis showed that the prosequences of the predicted precursors of all the three peptides are highly identical and exhibited also a remarkable similarity to cathelin, a porcine inhibitor of cathepsin L. Purified proBac5 actually proved in *in vitro* assays to inhibit cathepsin L, but not other cysteine proteinases such as cathepsin B. Unlike proBac5, proBac7 is selectively chemotactic to monocytes.

Several fragments of Bac5 and Bac7 (from 6 to 35 residues) were synthesized by the Fmoc method. The results of antibacterial assays show that the N-terminal portion, the most cationic one in both Bac5 and Bac7, is essential for the antimicrobial activity and that the minimal length necessary to arrest the growth of susceptible bacteria is 18–20 residues.

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Permanent address: Department of Chemistry and Biochemistry, Medical Faculty, University of Rijeka, Braće Branchetta 22, 51000 Rijeka, Croatia.

INTRODUCTION

Cationic antimicrobial polypeptides are considered to play a significant role in host defense, both at the level of respiratory and intestinal epithelia and in phagocytic cells such as macrophages and neutrophils. Hirsch[1] was the first to describe a crude protein fraction of neutrophil granule extracts with bactericidal properties. A few years later, Zeya and Spitznagel[2,3] attempted a partial purification of bactericidal peptides from other cationic proteins of neutrophil granules, showing their selective activity on gram-positive and gram-negative organisms and *Candida albicans*.

Later on, a variety of these polypeptides were purified from neutrophils, macrophages, epithelia and body fluids and their antimicrobial properties were characterized (for reviews, see Refs. 4–10). This led to the coinage of definitions such as »endoantibiotics« or »antibiotics from within«,[4] as well as to the recognition of their importance as effectors in innate immunity. They have thus become a very challenging field of research at the crossroad of cell and molecular biology, immunology and, in general, modern biotechnology.

In particular, the role of the neutrophil antimicrobial peptides has been extended beyond that of simply combatting invading microorganisms. In fact, recent applications of recombinant DNA techniques, sequence comparisons and imaginative experiments have unravelled new functions for at least some of these »antibiotics« and their precursors. These roles include the regulation of LPS toxicity,[11,12] recruitment of monocycles to inflammatory sites[13–15] and inhibition of cathepsin L,[15] a major factor in inflammatory tissue degradation.

Antimicrobial Peptides of Bovine Neutrophils

Azurophil granules of neutrophils contain an arsenal of antimicrobial peptides.[4–6,8,10]

Bovine neutrophils also possess these classical azurophil antibiotic peptides.[16,17] However, in ruminant neutrophils, the azurophil granules are present in relatively small number.[18] Conversely, these cells contain a novel, abundant type of subcellular granule of a large size and high density, which appears to contain most of the antimicrobial factors.[19]

Thus far, in addition to azurocidin,[16] and defensins,[17] four novel cationic antimicrobial peptides of unique structure have been purified from extracts of bovine neutrophil granules: i) a dodecapeptide with four arginyl residues, maintained in a cyclic structure by a disulfide bond between the two cysteine residues;[20] ii) a tridecapeptide with five triptophanyl residues and a carboxyamidation at the *C*-terminal arginine, named indolicidin;[21] iii) a proline- and argininine-rich peptide, named Bac5, with 43 amino acid residues, a repeated motif Arg-Pro-Pro-large hydrophobic residue[22,23] and very

TABLE I

Antibiotic polypeptides of azurophil granules

BPI or CAP57 (Bactericidal/ Permeability Increasing Protein, 50–60 kDa)
Serprocidins (cathepsin G, elastase, proteinase 3, azurocidin or CAP37, 25–29 kDa)
Defensins (*ca.* 4 kDa)

likely a carboxyamidation at the *C*-terminal proline;[24] iv) a second, structurally different, proline- and arginine-rich peptide, named Bac7, with 59 amino acid residues and repeated Pro-Arg-Pro triplets (from position 10 to the *C*-terminus proline is present every second residue) and three tandemly repeated tetradecamers in the region between residues 15 and 56.[22,23]

Although with marked selectivity and different potency, all the four peptides are active on gram-negative and gram-positive organisms.[16,20–22] In general, Bac5 and Bac7 are much more active on enteric bacteria, such as *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae.* In addition, both peptides immobilize and kill *Leptospira interrogans* and *biflexa*, whereas they are inactive on another genus of spirochetes, *Borrelia burgdoferi.*[25] Finally, Bac7 neutralizes human herpes simplex virus (HSV1 and HSV2), but not a picornavirus (rhinovirus 1B).[26]

In gram-negative bacteria and leptospire, Bac5 and Bac7 very likely bind to LPS in the outer cell membrane.[16,25,27] Then, they rapidly move to the inner membrane, whose permeability is dramatically increased with a concomitant drop in the ATP content, loss of ability of macromolecular biosyn-

TABLE II

Novel antimicrobial peptides of bovine neutrophils

Dodecapeptide
R L C R I V V I R V C R
Indolicidin
I L P W K W P W W P W R R
Bac5
R F R P P I R R P P I R P P F Y P P F R P P I R P P I
F P P I R P P F R P L G P F P
Bac7
R R I R P R P P R L P R P R P R P L P F P R P G P R P I
P R P L P F P R P G P R P I P R P L P F P R P G P R P I P R P

TABLE III

Antimicrobial activity of Bac7 and Bac5 fragments

	MIC (μg/ml)				
	E. coli	*S.typhimurium*	*S. marcescens*	*B. megaterium*	*S. epidermidis*
Bac7(1–59)	5	5	5	10	10
1–35	1.2	0.6	5	2.5	10
1–23	1.2	0.6	5	5	10
1–18	2.5	1.2	40	20	40
1–15	40	80	≥320	320	≥320
1–13	≥320	>320	>320	>320	>320
5–23	60	320	>320	>320	>320
15–28	>320	>320	>320	>320	n.d.
29–56	>320	>320	>320	>320	n.d.
Bac5(1–43)	5	5	10	10	40
1–31	5	5	80	80	n.d.
1–25	2.5	2.5	80	160	320
1–21	2.5	10	320	160	>320
1–18	80	160	320	≥320	≥320
1–15	160	160	>320	>320	n.d.
3–18	>320	>320	>320	>320	n.d.
4–31	>320	>320	>320	>320	n.d.
19–43	>320	>320	>320	>320	n.d.

thesis and fall in the respiration linked proton motiv force.[27] To show susceptibility to the cyclic dodecapeptide, indolicidin, Bac5 or Bac7, bacteria do not need to be in a growing phase.

With the aim of finding the domain responsible for the antimicrobial activity of Bac5 and Bac7, several fragments, ranging from 6 to 36 residues, were synthesized using the automated Fmoc solid-phase method. The results of antimicrobial assays indicate that the *N*-terminal portion, the most cationic in both peptides, is essential for the antibacterial activity and that the minimal length of antimicrobial fragments is 18 to 20 residues. The tetradecamer, which is repeated three times in Bac7,[23] does not appear to represent a functional domain. Finally, all-D isomers are as active as their all-L counterparts, thus excluding a receptor-mediated mechanism for the antimicrobial effects.

For potential biotechnological applications (preservatives in food technology, antimicrobials in topical applications and skin wounds, *etc.*), we thus have a series of short synthetic peptides, from the dodecapeptide to indolicidin to the Bac5 or Bac7 fragments and their analogs, with a variable spectrum of antimicrobial activity.

Biosynthesis and Processing of Bac5 and Bac7 Precursors

Investigations utilizing the classical tools of cell biology have shown that Bac5 and Bac7 are synthesized in bone marrow cells, presumably at the myelocyte stage of cell maturation, as preproforms of 23.5 and 21 kDa, respectively.[28] PreproBac7 is then processed to a protein of 20 kDa, which is the granule storage, precursor form of Bac7, as suggested by Western blot analysis.[28] Conversely, preproBac5 is first converted to a polypeptide of 15.8 kDa. This is then processed (very likely in a post-ER compartment) to a 15 kDa polypeptide, which is the granule storage, precursor form of Bac5,[28] presumably by removal of the *C*-terminal tripeptide and concomitant amidation of the *C*-terminus.[24]

As shown by immunogold electron microscopy, the 20 kDa proBac7 and the 15 kDa proBac5 are specifically targeted at the matrix of the large granules.[28]

In vitro experiments conducted with purified proBac7 and proBac5 have indicated that these precursors can be stepwise converted to the corresponding mature antibiotic peptides by elastase.[30] This points to the need of cooperation between the large granules and the azurophils. Experiments carried out with phagocytosing neutrophils have indeed shown that such a cooperation occurs in the phagocytic vacuoles or extracellularly, under conditions favouring a concomitant release of proBac7/proBac5 and elastase.[31] Incidentally, detection of Bac7 and Bac5 antigens in phagocytic vacuoles by immunogold electron microscopy[31] provides experimental evidence that the precursor molecules are actually mobilized from the large granules and discharged into the vacuoles.

The proteolytic conversion of proBac7 and proBac5 to mature peptides is a requirement for manifestation of the antibiotic activity. In fact, purified proBac7 and proBac5 do not affect the viability of microorganisms susceptible to the corresponding mature peptides.[30] This might be due to the masking of the *N*-terminl moiety, which is required for full expression of the antibacterial activity (see above).

Molecular Cloning of Bovine Neutrophil Antimicrobial Peptides

Molecular cloning and sequencing of the full length cDNA of Bac5, indolicidin, and the dodecapeptide[25,29,32] have provided the following information: i) the predicted signal sequence comprises 29 amino acids and is practically identical in the three prepropeptides; ii) the three antimicrobial peptides are located at the carboxyl end of the corresponding precursors; iii) at the *C*-terminus of Bac5 there is a Gly-Arg-Arg sequence, which is generally considered a proteolysis/α-amidation signal;. the indolicidin precursor also terminates with a Gly, which is lost post-translationally for α-amidation of the terminal Arg; iv) the hydrophylic pro-region in the three precursors has a length of 101 residues, is highly identical and contains four invariant Cys.

Furthermore, it is characterized by a net negative charge, which may neutralize the highly cationic *N*-terminal domain of the antimicrobial peptides, thereby masking their activity.

The predicted pro-region shows a high identity with the proregion of two rabbit neutrophil proteins, CAP18 and p15, described as LPS-binding proteins.[33,34] In addition, the pro- region sequence is homologous with a pig neutrophil protein, which is a potent and specific inhibitor of the cysteine protease cathepsin L and was thus named cathelin.[35] Purified proBac5 was actually shown to inhibit *in vitro* cathepsin L, but not cathepsin B, with a K_i of 6×10^{-8} M.[15]

Suitable stimulants may cause release of proBac5 from the large granules into the extracellular fluid.[31] Since cathepsin L is a major factor in the degradation of inflamed tissue and may cause bone resorption as well as marked damage to the glomerular basement membrane, its potential inhibition by the precursors of large granule peptides is of great significance and points to a wider role of these proteins in the modulation of the inflammatory response.

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SAŽETAK

Antibiotički peptidi i njihovi prekursori izolirani iz neutrofila goveda: struktura i uloga u prirođenoj imunosti

Domenico Romeo, Renato Gennaro, Margherita Zanetti, Alessandro Tossi, Barbara Skerlavaj, Paola Storici, Marco Scocchi, Laura Litteri i Donatella Verbanac

Iz neutrofilnih goveđih granulocita do sada su određena četiri peptida: Arg-bogati dodekapeptid, s jednom disulfidnom vezom; Trp-bogati tridekapeptid, nazvan indolicidin; te dva polipeptida koja se sastoje od 43 odnosno 59 aminokiselina, nazvani Bac5 i Bac7, u kojima nailazimo na pravilno ponavljanje slijeda Arg-Pro-Pro odnosno Pro-Arg-Pro.

Nedavno je određena cjelokupna cDNA za prva od tri spomenuta peptida. Sekvencijska analiza pokazuje da je slijed pretpostavljenog prekursora za sva tri peptida visoko identičan i pokazuje značajnu sličnost s katelinom – inhibitorom katepsina L izoliranim iz svinja.

Pročišćeni proBac5 upotrebljen u *in vitro* eksperimentima, pokazuje inhibiciju katepsina L, ali ne i katepsina B druge poznate cisteinske proteaze, dok je proBac7 selektivno kemotaktičan za monocite.

Pojedini dijelovi peptida Bac5 i Bac7 (od 6 do 35 aminokiselinskih ostataka), sintetizirani su metodom Fmoc. Rezultati pokusa na bakterijama pokazuju da je upravo *N*-terminalni kraj najjači kationski dio kod Bac5 i Bac7, bitan za antibakterijsku aktivnost, a minimalna duljina lanca nužna za suzbijanje rasta bakterija iznosi 18 do 20 aminokiselinskih ostataka.