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Review

Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda)

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Abstract. The production of antimicrobial peptides represents a first-line host defense mechanism of innate immunity that is widespread in nature. Only recently such effectors were isolated in crustacean species, whereas numerous antimicrobial peptides have been characterized from other arthropods, both insects and chelicerates. This review presents findings on a family of antimicrobial peptides, named penaeidins, isolated from the shrimp *Penaeus vannamei*. Their structure and antimicrobial properties as well as their immune function will be discussed through analyses of penaeidin gene expression and peptide distribution upon microbial challenge.

Key words. Crustacean; shrimp; innate immunity; antimicrobial peptides; structure; activity spectrum; blood cells; expression.

Introduction

The past few years brought significant advances in crustacean immunity, with a focus on antimicrobial peptides. These molecules, which are found in both the plant and animal (vertebrate and invertebrate) kingdoms, act as endogenous antibiotics and as such are considered to be a key element of innate immunity.

A wide variety of antimicrobial peptides have been described to date. They are classified into three distinct groups based on amino acid sequences, secondary structures and functional similarities [1]. The first and largest group is composed of peptides stabilized by intramolecular disulfide bonds, and the two latter groups are linear peptides and polypeptides characterized by (i) α-helical structures or (ii) a high content of proline residues and/or a high percentage of glycine residues. Despite variations in size and structure, most of these molecules are amphiphilic, displaying both cationic and hydrophobic surfaces. Different mechanisms of action have been described. In most cases, antimicrobial peptides were shown to disrupt microbial membranes by a pore-forming action or by a detergent effect [2]. However, other peptides, such as the insect attacins, were found to block bacterial membrane biosynthesis, resulting in cell death [3].

Antimicrobial peptides are produced in phagocytic cells of vertebrates [4], invertebrates [5–7], and tunicates [8, 9], and are also found in various tissues, for instance in the mammalian intestinal epithelium [10] or in the insect fat body (a functional equivalent of the mammalian liver), which was found in this group to be the main site for antimicrobial peptide synthesis [11].

Depending on the species or tissues, their expression is known to follow different modes of regulation. In

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arthropods, peptide synthesis is rapidly induced in the insect fat body upon in vivo microbial challenge, but also aseptic injury [6]. Two pathways have been identified, namely *Toll* and *Imd*, which control the transcription of *Drosophila melanogaster* antifungal and antibacterial peptides, respectively [12]. In horseshoe crab hemocytes (invertebrate blood cells), antimicrobial peptides appear to be constitutively expressed, and further stored in cytoplasmic granules from which they are released, after contact with microbial endotoxins, by regulated exocytosis (degranulation) [13]. Both the insect and chelicerata systems are believed to end in an increased quantity of antimicrobial peptides in the plasma (cell-free hemolymph), resulting in a greater ability to fight microbial infection.

Until recently, the contribution of antimicrobial peptides to crustacean defense was only suspected. Bactericidal activities were observed in *Homarus americanus* plasma [14] and hepatopancreas [15], but were shown to be absent from the plasma of the crab *Carcinus maenas*, even after microbial challenge. From these observations, White et al. [16] have suggested that in crustaceans, elimination of invading organisms from the bloodstream is more likely to be mediated by hemocytes (through phagocytosis and further killing by cellular molecules) than by the presence of plasma-soluble antimicrobial factors.

Potent antibacterial activities in *C. maenas* hemocyte lysates that were not due to agglutinins or associated with the prophenoloxidase (proPO) activating system reinforced the idea of the existence of cellular antibacterial factors [17]. Similar observations were also made in the hemocytes of several other crustacean species [18], and over the last 4 years several peptidic factors displaying antimicrobial activities were isolated in crabs and shrimp. To date, antibacterial peptides and polypeptides were extracted from the hemocytes of the crabs *C. maenas* [19] and *Callinectes sapidus* [20], and two of them, a 6.5-kDa peptide and the 3.7-kDa callinectin, respectively, were partially characterized. In addition, a family of antimicrobial peptides displaying both antifungal and antibacterial activities was isolated from the hemolymph of the shrimp *Penaeus vannamei*, and were named penaeidins [21]. The full characterization of penaeidins at the biochemical and nucleic acid level led to production of both recombinant peptides and specific molecular probes to study penaeidin antimicrobial activities and expression in shrimp subjected to microbial infection. Taken together, these new data provide clues to understanding the role of antimicrobial peptides in crustacean immune response.

Penaeidin structure

Mature peptides

Three penaeidins were purified from the hemolymph of the shrimp *P. vannamei*. The peptides were isolated in their active form (5.48–6.62 kDa) in acid extracts from both plasma and hemocyte organelle-rich fractions of shrimps obtained from intensive Ecuadorian culture farms. The peptides were fully characterized at the amino acid (Pen-1, -2 and -3a) and nucleic acid (pen-2, -3a, -3b and -3c) levels, by a biochemical approach and complementary DNA (cDNA) cloning from a hemocyte library, respectively. Like most of the antimicrobial peptides hitherto described, penaeidins are highly cationic molecules with calculated isoelectric points ranging from 9.34 to 9.84. The bioactive molecules show high levels of similarities in their amino acid sequences. They are composed of an NH_2-terminal proline-rich region as observed for the other crustacean antimicrobial peptides [19, 20], followed by a COOH-terminal domain stabilized by three intramolecular disulfide crosslinks (fig. 1a). This unique structure led to the identification of a new family of antimicrobial peptides. Besides this chimeric-like overall structure, penaeidins display posttranslational modifications. In particular, Pen-2 and -3a are COOH-terminally amidated in their mature form, but such a modification is still unknown in Pen-1, for which the cDNA sequence has not been established. Other marine invertebrate antimicrobial peptides, including the horseshoe crab tachyplesins [22] and polyphemusins [23], are also COOH-terminally amidated. In addition, Pen-3a is blocked at the NH_2-terminus by a pyroglutamic acid which has also been reported in the insect hymenoptaecin [24] or some bovine β-defensins [25].

Peptide processing

The amino acid sequences deduced from the cDNA revealed that the penaeidins isolated from hemocytes are synthesized as precursor molecules consisting of a signal peptide (19–21 amino acids) immediately preceding the bioactive molecule [21]. Pen-2 and -3a signal peptides are fully identical with the exception of two additional amino acids (Glu-Ala) at the COOH-terminal position in the Pen-2 signal sequence (fig. 1b). Since a potential cleavage site by signal peptidase is predicted at the NH_2-terminal position of the glutamic acid residue [26], we can speculate that Pen-2 maturation involves an additional processing step compared with Pen-3a, possibly by a dipeptidyl amino peptidase (DPAP) (fig. 2). Such DPAPs are able to cleave at the COOH-terminal position of X-Pro and X-Ala dipeptides (as found in Pen-2), and their involvement in processing precursor molecules was previously shown for other antimicrobial peptides such as cecropins or mellitins [27]. As for Pen-2, cecropin and sarcotoxin IIA mature peptides are separated from their signal sequence by one or two X-Pro and X-Ala dipeptides [27, 28]. Similar maturation events, including elimination of signal sequence and cleavage by DPAPs, are believed to process the horseshoe crab tachyplesins [29], a family of antimicrobial peptides synthesized and stored within hemocytes after maturation, indicating that such a process may be at work in penaeidin processing in the shrimp blood cells.

Recombinant penaeidins

In order to produce large amounts of peptides for further structural studies or biological assays, penaeidins were synthesized in a heterologous expression system. Along with baculoviruses [30], yeasts are good candidates for heterologous expression of antimicrobial peptides containing disulfide-linked cysteine residues [31–33]. Penaeidin-2 and -3a cDNAs were fused with the β-glucanase signal peptide and α-mating factor pheromone propiece coding sequences, and this construct was used to tranfect *Saccharomyces cerevisiae* TGY [34]. In this system, mature penaeidins were secreted into the culture medium at concentrations of about 1 mg/l. Since the antimicrobial activities of the recombinant penaeidins were almost indistinguishable from those of the native molecules, they were most likely correctly folded in yeast.

Further characterizations showed that recombinant penaeidins differ from the native molecules by the presence of an additional glycine residue at the COOH-terminus, instead of the naturally occurring α-amide. Moreover, two distinct Pen-3a isoforms were also obtained either with an unprocessed glutamine residue or with the natural pyroglutamate at position 1. Finally, about 50% of the recombinant molecules were shown to be O-substituted by a dimannosyl moiety on Thr-6 and -8 (predicted by Netoglyc 2.0 software as potential

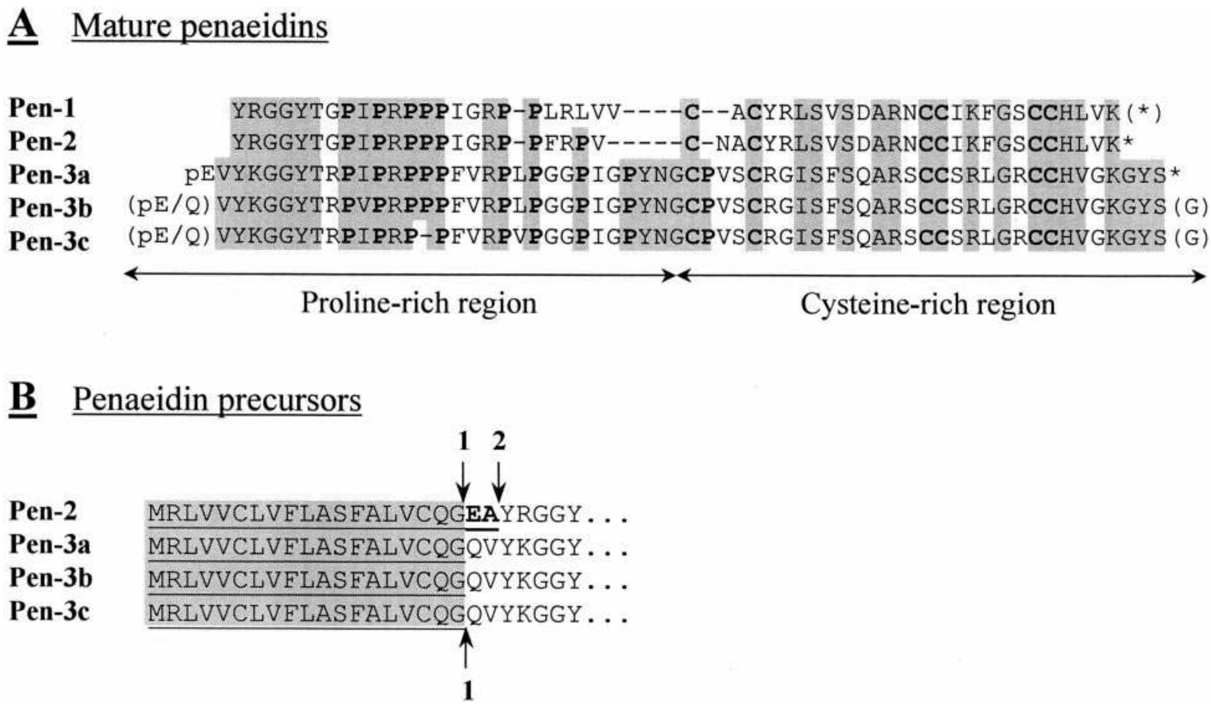

Figure 1. Sequence comparison of penaeidins. (*A*) Mature penaeidin primary sequences obtained by biochemical characterization (Pen-1, Pen-2, Pen-3a) and/or cDNA cloning data (pen-2, pen-3a, pen-3b, pen-3c) were aligned. Gaps were introduced to optimize the alignment. Cysteine and proline residues are in boldface, and identical amino acids are in boxes. Stars indicate COOH-terminal amidation, and pE stands for pyroglutamic acid. Assumed posttranslational modifications are in brackets. (*B*) The NH_2-terminal sequences of penaeidin precursors obtained by amino acid deduction from cDNA sequences were compared. Underlined amino acids show sequences absent in the mature molecules. Arrows indicate the position of the predicted cleavage sites by signal peptidase. 1 and 2 stand respectively for preferential and secondary cleavage sites according to SignalP IV.1 software. Identical residues are in boxes, and the dipeptide Glu-Ala (EA) found in Pen-2 precursor sequence exclusively is in boldface.

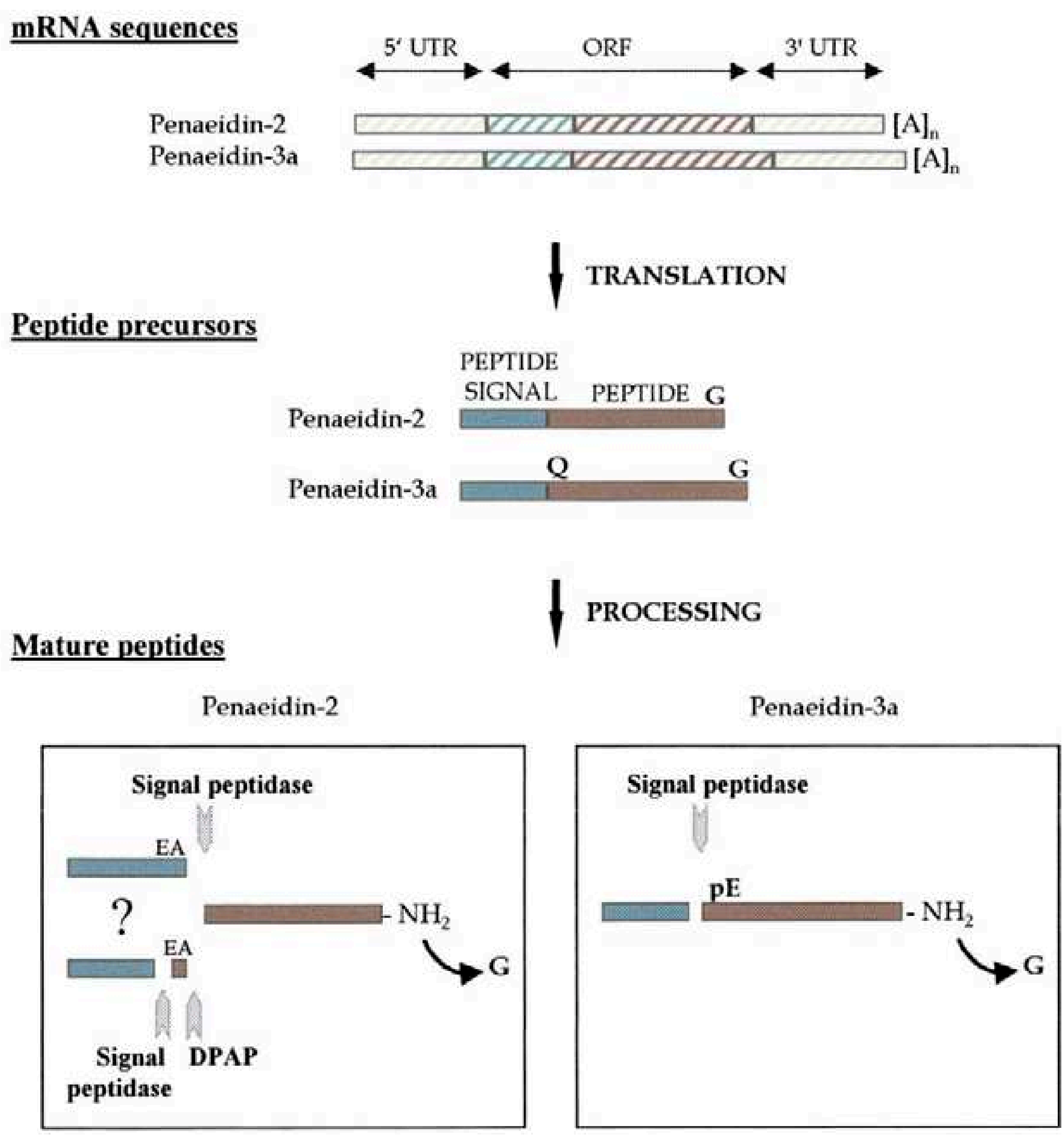

Figure 2. Penaeidin processing in *Penaeus vannamei* hemocytes. Pen-2 and -3a synthesis from mRNA to mature peptides is represented. After transcription and translation, the two molecules undergo posttranslational modifications including elimination of signal peptide, and COOH-terminal amidation by elimination of a glycine residue. In Pen-3a, an additional step involving formation of a pyroglutamic acid by cyclization of a glutamine residue occurs. Two different processing pathways leading to the removal of the 21 first amino acids of Pen-2 precursor are represented with (i) elimination of a 21-residue signal peptide, or (ii) a two-step process including elimination of a 19-residue signal peptide and subsequent removal of a Glu-Ala dipeptide by a dipeptidyl aminopeptidase (DPAP).

glycosylation sites) for Pen-2 and -3a, respectively. The availability of the different recombinant molecules allowed investigation of the role of post-translational modifications in antimicrobial peptide activity (see below). Finally, because the nonsubstituted recombinant molecules displayed activity similar to native ones, they were further used to enlarge activity spectrum of penaeidin.

Antimicrobial properties of penaeidins

Preliminary antimicrobial assays performed with native penaeidins purified to homogeneity showed that penaeidins had both antibacterial and antifungal activities [21]. To determine the activity spectrum in more detail, large quantities of peptides were required and, hence, recombinant penaeidins were employed.

Antifungal activity

Pen-2 and -3a have broad-spectrum fungicidal activity against filamentous fungi with minimum inhibitory concentrations (MIC) below 10 μM, but are inactive against yeast such as *S. cerevisiae* or *Candida albicans* [34]. Interestingly, penaeidins are active against the shrimp pathogen *Fusarium oxysporum*, which is responsible for infections in different *Penaeus* species [35, 36], whereas such infections remain unreported in *P. vannamei*. Many phytopathogenic fungal strains such as *Nectria haematococca*, *Alternaria brassicola*, *Neurospora crassa* and *Botritys cinerea* were shown to be sensitive to the peptides, indicating that penaeidins could have applications in agronomy as therapeutic agents. Penaeidin fungicidal activity against the different strains was shown to be associated with inhibition of spore germination. At lower concentrations, the peptides cause growth inhibition of fungal hyphae, resulting in morphological abnormalities.

Antibacterial activity

Penaeidin antibacterial activity was found to be rather specific and is essentially directed against Gram-positive bacteria via a strain-specific inhibition mechanism. We observed that depending on the strains penaeidins displayed a rapid killing activity or bacteriostatic properties [34]. This suggests that penaeidins could act through multiple modes of actions. Incubation of *Bacillus megaterium* strain with Pen-2 or Pen-3 at concentrations 10-fold higher than their MIC values (MIC = 2.5–5 μM) resulted in their immediate killing. On the other hand, a noticeable potent growth inhibition (MIC = 0.3–0.6 μM for Pen-3a) associated with slow killing of the target bacteria (10% of bacteria are still alive after a 24-h incubation) was observed on *Aerococcus viridans*, which is reported to be highly pathogenic in crabs and lobsters, in which it induces rapid septicemia and subsequent death [37]. Finally, penaeidins were found to display little activity against Gram-negative strains, including the *Vibrio* species commonly responsible for many crustacean bacterial infections [38, 39].

Despite better efficiency of Pen-3a on most of the strains tested, similar activity spectra and inhibition mechanisms are observed for both Pen-2 and -3a (at concentrations above the MIC, sensitive strains are killed or inhibited with identical time courses). No synergistic effect was observed between the two molecules against sensitive microorganisms. Nevertheless, because other antimicrobial peptides are likely to be present in shrimp, as evidenced by reversed-phase high-performance liquid chromatography (HPLC) of hemolymph acid extracts and subsequent antimicrobial assays [21], such a synergism could exist between penaeidins and other putative antimicrobial peptides present in shrimp hemolymph, in a manner similar to that proposed in other organisms [13, 24, 40, 41].

Effect of posttranslational modifications on bioactivity of penaeidins

The role of posttranslational modifications observed in native penaeidins (COOH-terminal amidation and NH_2-terminal pyroglutamic acid in Pen-3a) was studied with the set of penaeidin variants obtained by recombinant expression (see above). These modifications were found to have little effect on penaeidin antimicrobial properties [34], but possibly they increase the stability of penaeidins, which are highly resistant to proteolysis [21].

NH_2-terminal blocking has no effect on penaeidin antimicrobial properties, as reported for the plant antifungal peptide *Rs*-AFP2 isolated from *Raphanus sativus* [33]. Moreover, when COOH-terminal amidation is replaced by an extra glycine residue, penaeidin antifungal properties are unaltered, and antibacterial activity is decreased only twofold. Similar to reports on insect cecropins [42, 43], the partial loss of antibacterial activity that occurs in nonamidated penaeidins may be due to the loss of a positive charge at the COOH-terminus, and consequently to less efficient interaction of the peptide with bacterial membranes.

Several antimicrobial peptides, such as the proline-rich *Drosophila melanogaster* drosocin [44] or the glycine-rich *Phormia terranovae* diptericin [45], occur naturally as O-glycosylated molecules, and substitution is necessary for full biological activity of these peptides [1]. In contrast, no member of the penaeidin family has been extracted from shrimp in a substituted form, and O-glycosylation of recombinant penaeidins induced a two to fourfold decrease in penaeidin activity against half of the microorganisms tested [34]. In drosocin, it was proposed that the carbohydrate moiety might be involved in conformational changes beneficial to the stereospecific recognition of a target molecule on bacterial membranes [44]. However, a recent report by McManus et al. on the three-dimensional (3D) structure of drosocin revealed no substantial difference in the structure of drosocin with or without the sugar part [46]. The elucidation of the 3D structure of penaeidin would clarify the possible implication of the glycan part in destabilizing the secondary structure of the recombinant O-glycosylated molecule.

Role of penaeidin NH_2- and COOH-terminal regions

Penaeidins combine two domains in their overall structure, one proline-rich and the other cysteine-rich, usually observed in distinct groups of antimicrobial peptides. Similarly, the big horseshoe crab defensin [47] is composed of (i) a NH_2-terminal domain without evident homologies with other antimicrobial peptides and displaying anti-Gram-positive activity, and (ii) a COOH-terminal domain with three disulfide bonds displaying anti-Gram-negative properties. Thus, the overall biological activity of penaeidin may be associated with distinct properties of their two characteristic regions.

NH_2-terminal region

The penaeidin NH_2-terminal proline-rich region shares sequence similarities with other proline-rich antimicrobial peptides. In particular, penaeidins display 70–76% identity over 17 residues, with the 6.5-kDa peptide isolated from the crab *C. maenas* [19]. This region also shares 60% identity over 10 residues with the bovine bactenecin-7 (Bac7), and contains a Pro-Ile-Pro-Arg-Pro motif, repeated three times in the Bac7 sequence [48] (fig. 3a).

A synthetic peptide corresponding to the NH_2-terminal proline-rich domain of penaeidin (residues 1–20) was produced and found to be inactive against both bacterial and fungal strains [unpublished data]. Similar observations were also made with the Bac7 repeated peptide homologous to the penaeidin NH_2-terminal region [49]. Since it was demonstrated that Bac7 is able to tightly bind artificial membranes through electrostatic interaction, we can speculate that the penaeidin proline-rich region is similarly involved more in target cell interaction than in direct antimicrobial activity. This is also consistent with the spectrum of penaeidin antimicrobial activity, which is directed mainly against fungi and Gram-positive bacteria [34], in contrast to most of the known proline-rich peptides, which have essentially anti-Gram-negative properties [1].

COOH-terminal region

Since the penaeidin NH_2-terminal region failed to exhibit antimicrobial activity, it is tempting to speculate that the COOH-terminal region mediates penaeidin antimicrobial properties. In addition, most of the antimicrobial peptides with intramolecular disulfide bridges, such as defensins, have antimicrobial properties similar to those observed for penaeidins. Defensins are found in a large range of animal species, both in vertebrates and invertebrates [50], and show a highly conserved cysteine motif. On the basis of the position and bonding of the six conserved cysteine residues, defensins from vertebrates are divided into two categories, α- and β-defensins [51], whereas insect defensins fit into a distinct group [52]. The penaeidin cysteine-spacing—which consists of two cysteine doublets conserved between the different molecules, and two additional cysteine residues separated by one, two and three amino acids in Pen-1, -2 and -3, respectively (fig. 1a)—does not correspond to any of the motifs hitherto described, and the cysteine connectivity remains to be determined by 3D-structure analysis.

The penaeidin COOH-terminal region was found to display partial conservation of a repeated motif common to several chitin-binding proteins of the hevein family, such as wheat germ agglutinin [53] (fig. 3b). Interestingly, this feature is also found in two other antimicrobial peptides: (i) the plant antimicrobial protein-2 (*Ac*-AMP2) isolated from *Amaranthus caudatus* [54, 55], which also contains six cysteine residues and has significant sequence similarities with penaeidins (fig. 3b); and (ii) tachycitin (10 cysteines), a peptide isolated from horseshoe crab hemocytes [41]. Their partial conservation of a chitin-binding motif is associated with an ability to attach chitin (a component of the fungal cell wall) tightly, and as penaeidins, these peptides display both antibacterial and antifungal activities. Chitin binding has also been demonstrated for intact penaeidins but was absent for their synthetic NH_2-terminal domain (20 amino acids) [56]. Thus we believe that binding of penaeidins to chitin, and consequently their antifungal activity, is mediated by their COOH-terminal domain.

Penaeidin chimeric-like structure

Penaeidins consist of two structurally distinct regions whose functions remain unstudied. Two families of plant proteins were shown to have a similar chimeric-like structure: (i) the tobacco extracellular matrix proteins CELPs—for cysteine-rich extensin-like protein [57]—that have a proline-rich extensin-like domain and a cysteine-rich domain with a highly charged COOH-terminus; and (ii) the potato lectins, which have an extensin domain rich in glycosylated hydroxyproline residues, fused with a lectin domain containing partial conservation of a repeated motif common to the chitin-binding proteins of the hevein family [53]. These chimeric proteins might be multifunctional [57] with roles in plant growth, development and defense. Interestingly, penaeidins display homologies with CELPs (fig. 4) and thus, as proposed for these molecules, penaeidin overall structure could also be associated with several functions. In particular, as crustaceans also contain chitin as a major cuticle component, penaeidin chitin-binding ability could also be involved in wound healing or chitin assembly during the moulting process. Experimental comparison of the two characteristic penaeidin regions in terms of activity should help to clarify their involvement in the properties of the entire molecule but also to aid in understanding the role of penaeidins in the shrimp defense system.

A Penaeidin NH_2-terminal region

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C. maenas 6,5 kDa peptide        XXVPYPRPFPRPP-IGPRP-LPFPGGGPFQS...

Penaeidin-1                      YRGGYTGPIPRPPPIG-RPPLR-LVV--------
Penaeidin-2                      YRGGYTGPIPRPPPIG-RPPFR-PV---------
Penaeidin-3a                  pEVYKGGYTRPIPRPPPFV-RP-L--PGG-PIGPYNG
Penaeidin-3b                  pEVYKGGYTRPVPRPPPFV-RP-L--PGG-PIGPYNG
Penaeidin-3c                  pEVYKGGYTRPIPRPP-FV-RP-V--PGG-PIGPYNG
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Bactenecin-7   RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPRPGPR-PIPRPLPFPRPGPRPIPRP

Penaeidin-1                         YRGGYTGPIPRPPPIGRPPLR-LVV----
Penaeidin-2                        YRGGYTGPIPRPPPIGRPPFR-PV-----
Penaeidin-3a                    pEVYKGGYTRPIPRPPPFVRPLPGGPIGPYNG
Penaeidin-3b                    pEVYKGGYTRPVPRPPPFVRPLPGGPIGPYNG
Penaeidin-3c                    pEVYKGGYTRPIPRP-PFVRPVPGGPIGPYNG
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B Penaeidin COOH-terminal region

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                                             MOTIF
Chitin-binding domain           xxCGxxx[CxxxxxCCSxxGxCGxxxxxC]xxxCxxxxC

Penaeidin-3a       GGPIGPYNGCPV--SC--RGISFSQARSCCSRLGRCCHVGKGYS*
Ac-AMP2                     V-GECV-RGRCPS--GMCCSQFGYC-GKGPKYCG
Tachycitin               RCGRYSPCLDDGPNVN-LYSCCS-FYNC-HKCLARLE
Hevein                AEQCGR-----QAGGKLCPNNLCCSQWGWC-GSTDEYCS
WGA (D3)       GGPCRADIKCG---SQ--SGGKLCPNNLCCSQWGFC-GLGSEFCG
WGA (D4)                KPCG-----KDAGGRVCTNNYCCSKWGSC-GIGPGYCG
PL-C                   YNRCG-----KEFGGKECHDELCCSQYGWC-GNSDGHCG
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Figure 3. Sequence comparison of penaeidin NH_2- and COOH-terminal regions with other peptides/proteins, (*A*) The penaeidin NH_2-terminal proline-rich region was aligned with the NH_2-terminal sequence of the *Carcinus maenas* 6.5-kDa peptide [19], and with bactenecin-7 from bovine neutrophils [48]. Gaps were introduced to optimize the alignment. Identical amino acids and conservative replacements are shaded, and the PIPRP motif, repeated three times in bactenecin-7, is in boldface. Nonidentified amino acids are represented by X. (*B*) The penaeidin COOH-terminal sequence was compared with the chitin binding domain (boldface, amino acids) of lectins and antifungal peptides isolated from plants. The amino acids belonging to the consensus pattern are shaded as well as amino acid conservative replacements. Stars indicate a carboxy-terminal amidation. The two antimicrobial proteins *Ac*-AMP2 and tachycitin, which also display partial conservation of the chitin binding motif, are included in the alignment. The following sequences are aligned: *Ac*-AMP2, *Amaranthus caudatus*, antimicrobial peptide [54]; tachycitin, antimicrobial peptide from *Tachypleus tridentatus* [41]; hevein, antifungal peptide from *Hevea brasiliensis* and WGA, wheat germ agglutinin [73]; PL-C, *Phytolacca americana* lectin C [74].

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Penaeidin-3      pEVYKGGYTRPIPRPPPFV--RPLPGGPIGPYNGCP
CELP                      RPYPCPPPRP--RPCPPPPPPP---CP
PTL1                      RPMPKPPSTIPKRPPPKGPLKPP
EXTN_SORVU(1)       YGGGYPTPTPKPPAKGP-KP
EXTN_SORVU(2)           YTPPVSHTPSSPP--PP
PR33_DAUCA              YTPPVHKPPSEY--KPP
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Figure 4. Comparison of penaeidin NH_2-terminal domain with plant extensins. CELP, cysteine-rich extensin-like protein from *Nicotiana tabacum* [57]; PTL1, extensin-like protein from *Antirrhinum majus* [75]; EXTN_SORVU(1) residues 30–48 and EXTN_SORVU(2) residues 263–279, extensin from *Sorghum vulgare* [76]; PR33_DAUCA, extensin from *Daucus carota* [77]. Conservative replacements of identical amino acids are shaded. A VYK motif involved in intermolecular cross-links in extensins [78] is boxed.

Penaeidin expression and immune function

In arthropods, antimicrobial peptide synthesis and tissue localization were studied in horseshoe crabs, and regulation of their gene expression in response to injuries was studied in insects. Our recent studies devoted to penaeidins provide information about the immune reaction mediated by antimicrobial peptides in crustaceans.

Expression in unchallenged animals

Comparison of penaeidin expression levels in various cells and tissues has demonstrated that hemocytes are the main site for penaeidin synthesis. The intense hybridization signals observed by Northern blot experiments [56] were consistent with the high percentage of positive clones previously obtained during screening of the shrimp cDNA library (168 positive clones over 500,000 pfu) [21]. Besides their active transcription of penaeidin genes, hemocytes were also shown to store peptides. In fact, penaeidins represent the bulk of proteinaceous material observed by reversed-phase HPLC [21] in acid extracts from organelle-rich hemocyte fractions, and some hemocyte populations are highly reactive to penaeidin immunostaining [56].

On the basis of morphological observations, shrimp hemocytes are classified into two distinct groups, (i) the hyaline cells (devoid of cytoplasmic electron-dense granules), and (ii) the hemocytes, with large granules (LGH) or small granules (SGH) [58]. Immunogold experiments clearly demonstrated that penaeidins are contained in the cytoplasmic granules of the LGH (fig. 5) and to a lesser extent in SGH [56], but that hyaline cells are devoid of stored penaeidins. Thus it is believed that after synthesis penaeidins undergo different posttranslational modifications in the endoplasmic reticulum (elimination of the signal peptide) and in the Golgi (COOH-terminal amidation and NH_2-terminal cyclization of a glutamine residue in Pen-3a), before being addressed and stored in hemocyte cytoplasmic granules. This scheme is similar to that proposed in horseshoe crab hemocytes [5] or mammalian neutrophils [59, 60]. In other respects, the absence of penaeidin immunoreactivity in hyalinocytes does not indicate that they do not express the peptides. This cell type could directly secrete the newly synthesized penaeidins into the plasma as do antimicrobial peptides in the insect fat body cells [61].

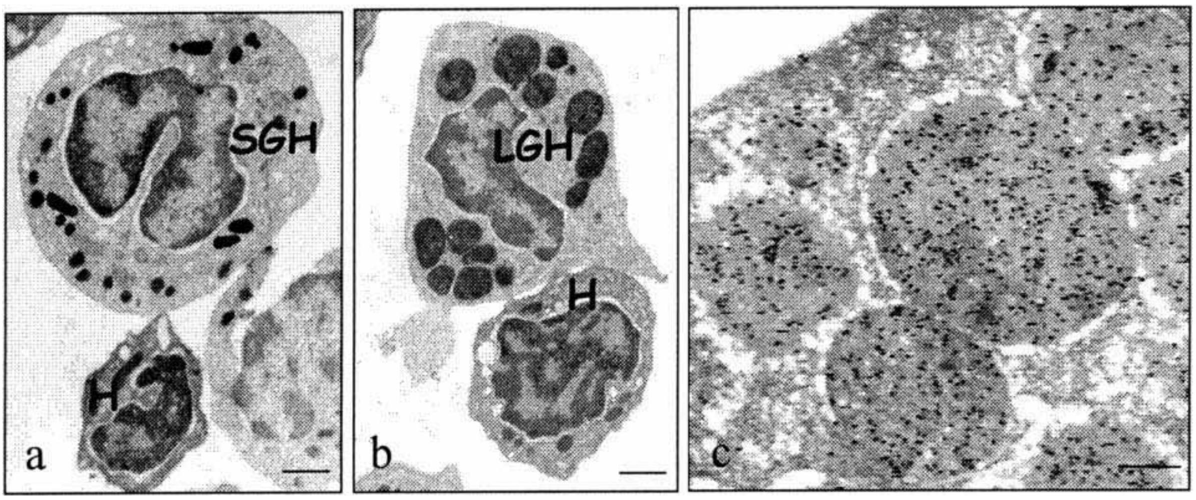

Figure 5. Ultrastructural localization of penaeidins in *Penaeus vannamei* hemocytes. Transmission electron micrographs of (*a*) granular hemocyte with large granules (LGH) and hyaline hemocyte (H) and (*b*) granular hemocyte with small granules (SGH) (bar, 1 µm). (*c*) Immunolocalization of penaeidins by immunogold labelling in cytoplasmic granules of haemocyte with large granules (bar, 500 nm).

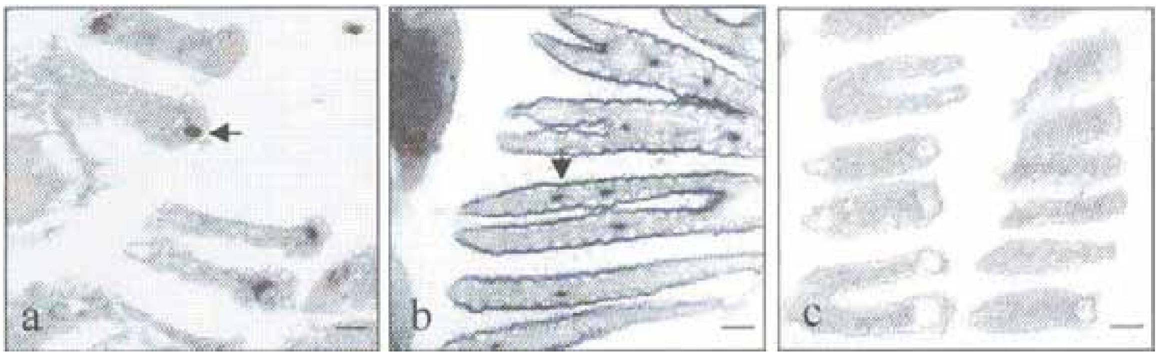

Figure 6. Penaeidin immunoreactivity in gill filaments. (*a*) Immunoreactivity is observed in hemocytes located in gill efferent blood vessels of unchallenged shrimp. (*b*) Penaeidin immunoreactivity, as observed after microbial challenge (24 h), is found at the level of gill cuticle when (*c*) no labelling is noticed in control (nonimmune serum) (bar, 10 μm).

In situ hybridization experiments on hemocyte populations should help to resolve this issue.

Expression following microbial challenge

In vivo microbial challenge experiments (injection of heat-killed bacteria and fungi in shrimp) demonstrated that penaeidin transcription is not upregulated in shrimp hemocytes [56] as observed for antimicrobial peptides in the insect fat body [12], but induces an increase in the penaeidin concentration in plasma. Thus, although other tissues could contribute to penaeidin release into the bloodstream, we can speculate that along with other components, penaeidins are released from hemocyte cytoplasmic granules in response to microbial stimulation. Similar release of antimicrobial substances has previously been shown by in vitro stimulation of horseshoe crab hemocytes (in contact with bacterial endotoxins) [62]. Thus, as proposed for *Limulus*, regulated exocytosis of antimicrobial peptides from shrimp granular hemocytes could help to circumvent microbial infections by increasing plasma antimicrobial activity in the contact areas between microbes and hemocytes.

Secretion of antimicrobial peptides was also described for human neutrophils that release granule-stored cathelicidin hCAP-18 into the bloodstream upon challenge [63]. Within the same cells, the α-defensins HNP1–4 are stored in a nonsecretory granule type, and act intracellularly after fusion of the granules to the microbe-containing phagosome [64]; penaeidins could act in a similar manner during phagocytic and post-phagocytic events. Granule-containing hemocytes are phagocytic cells [65], and they are also involved in nodule formation, where small-granule hemocytes (found in the most central part of the nodule) undergo phagocytosis and lose their cytoplasmic granules [66]. Thus penaeidin release into the bloodstream could result from localized degranulation events within multicellular structures where high penaeidin concentrations could be reached in contact with the microbes.

Besides the high transcription levels observed in hemocytes, significant concentrations in penaeidin messenger RNA (mRNA) were observed by Northern blot analysis in tissues rich in hemocytes such as the heart or gills. It was consequently supposed that in these tissues the observed signals were due to the hemocyte ability to infiltrate tissues. Penaeidin immunocytochemical patterns observed in response to microbial challenge are consistent with this hypothesis. In gills, in particular, whereas penaeidins are immunolocalized in granulocytes observed in the filament blood vessels of unchallenged shrimp, a noteworthy immunoreactivity appears on the gill cuticle surfaces following a microbial stimulation (fig. 6), and no more penaeidin-reactive hemocytes are observed in the efferent blood vessels [56]. Hence, after release into the plasma, penaeidins could attach to the shrimp cuticle through their chitin-binding properties and ensure optimal protection of the whole animal.

Recently it has been demonstrated in insects that the surface epithelia of gut tissue [67], trachea [68] or reproductive tract [69] synthesize antimicrobial peptides. In addition, the mammalian intestine was also found to produce defensins [70, 71]. It now appears worthwhile to investigate the expression of penaeidins in epithelial barriers where they could prevent infections from exterior microorganisms. Such a study will be of special importance for a better understanding of crustacean immune defense reactions in conjunction with their aquatic environment, which is rich in potentially pathogenic microorganisms.

Conclusion

The recent discovery of antimicrobial peptides in crustaceans provides new clues for fundamental understanding of crustacean immunity. The studies on the shrimp penaeidins have largely contributed to this knowledge as they are the first antimicrobial peptides fully characterized in a crustacean and for which expression studies were performed.

The well-established involvement of hemocytes in shrimp defense reinforces the hypothesis of a major role of penaeidins as effectors of shrimp immune defense. On the one hand, by their presence and their ability to migrate to tissues, hemocytes are likely to insure penaeidin transport to sites exposed to environmental pathogenic microorganisms. However, even though we still do not have evidence of such a phenomenon, penaeidin expression in physiological barriers such as epithelia will be an important question to address. These two defense reactions, which would mediate local protection to limit invasion by foreign microbes, are most likely relayed by systemic protection as suggested by penaeidin release into the plasma, thus providing a second line of defense to circumvent microbial infection.

Besides their antimicrobial properties, penaeidins may be involved in additional functions as suggested by their chimeric-like structure. In particular, penaeidin chitin-binding ability may constitute one of the most interesting aspects of these peptides. Not only is this property likely to be essential for penaeidin antifungal activity, but penaeidins could also be involved in chitin assembly or wound healing and as such be essential in shrimp protection during the moulting cycle.

Knowledge of penaeidin functions will be particularly important for further establishment of disease control in penaeid shrimp production [72]. Moreover, from their unusual features both at the structural level and in terms of biological activity, penaeidins could represent, along with other antimicrobial peptides, a new generation of therapeutic agents and find potential applications in aquaculture or agronomy.

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