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Oyster hemocytes express a proline-rich peptide displaying synergistic antimicrobial activity with a defensin

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Abstract:

A cDNA sequence that encodes a 61-amino acid polypeptide precursor with homologies to proline-rich antimicrobial peptides (AMPs) was identified in the oyster *Crassostrea gigas*. After release of a hydrophobic signal peptide, the resulting 37-amino acid peptide, *Cg*-Prp, is composed of an acidic region and a cationic proline-rich region. To evaluate the biological properties of *Cg*-Prp, multiple proline-rich peptides corresponding to putative processing of the full-length *Cg*-Prp were synthesized. A limited antimicrobial activity was observed for two of them, which also showed strong synergistic antimicrobial activity with *Cg*-Def, a defensin from *C. gigas*. To our knowledge, this is the first evidence of synergy between a defensin and another AMP in an invertebrate. By *in situ* hybridization, the expression of *Cg-prp* was found to be restricted to hemocytes and induced following bacterial challenge. *Cg-prp* transcripts were also detected in hemocytes infiltrating mantle, where *Cg*-Def is expressed. Additionally, by immunocytochemistry, we showed that *Cg*-Prp or one of its variants is present in some hemocytes together with defensins. In conclusion, we described here the first proline-rich AMP from mollusk. From our study, it is likely to provide a first line of defense against bacterial invasion by acting through synergy with defensins.

Keywords: Pacific oyster; Innate immunity; Invertebrate; Synergy; Mollusk; Antimicrobial peptide

Introduction

Marine invertebrates including bivalve mollusks have evolved in the continuous presence of microorganisms. Thus, the oysters, such as *Crassostrea gigas,* harbor a diverse microflora both on their surface and inside the body cavities and hemolymph. As filter feeders, they are exposed to a constant challenge by pathogenic and opportunistic bacteria naturally present in its environment. Therefore, the capacity to overcome infections is essential for oyster survival.

Although lacking an adaptive immune system, invertebrates fight microbes with effective mechanisms that include the generation of gene-encoded antimicrobial peptides (AMPs). AMPs are important components of the innate immune system, which have been conserved during evolution (Brogden, 2005; Yang *et al.*, 2002). They form a key line of host defence against pathogens in plants and animals (Bachère *et al.*, 2004; Boman, 2003; Bulet *et al.*, 2004). In animals, AMPs are particularly abundant in tissues that are likely to come in contact with microorganisms, such as at mucosal surfaces (Zasloff, 2006a, b) and within immune cells (Bals, 2000; Zhao *et al.*, 2007). AMPs can be classified into three major groups: (i) linear peptides that can form amphipatic α–helices, (ii) peptides containing cysteine residues engaged in internal disulphide bonds, and (iii) peptides with one or two amino acids over-represented (Pro, Arg, Gly or His) (Brogden, 2005). AMPs from those different groups are often encountered within one organism, as shown in *Drosophila* (Lemaitre and Hoffmann, 2007). Despite their great diversity in terms of size, primary structure and amino acid composition, most AMPs are characterized by a high content in cationic and hydrophobic amino acids (Bulet *et al.*, 2004; Jenssen *et al.*, 2006). The resulting amphipathic structure is considered to be required for the interaction of the peptide with the membrane of the sensitive microorganisms, leading commonly to the disruption of the membrane integrity. Membrane permeabilization is proposed to be one major mechanism by which AMPs kill their target cells, which include bacteria, fungi, parasites and enveloped viruses (Brogden, 2005).

Proline-rich AMPs have a dissimilar mode of action and act without membrane permeabilization. These peptides are characterized by repeated proline-containing motifs and are active predominantly against Gram-negative bacteria. Although relying on unresolved mechanisms, translocation of proline-rich AMPs across bacterial membranes occurs without significant membrane lysis. Then, the peptides reach an intracellular target causing cell death (Brogden, 2005). For example, PR39, a mammalian proline-rich AMP kills Gram negative bacteria by inhibition of DNA and protein synthesis (Gennaro *et al.*, 2002). Similarly, the

insect pyrrhocidin, drosocin and apidaecin enter the target cells and specifically bind to DnaK, a heat shock protein involved in chaperone-assisted protein folding. Pyrrhocidin inhibits the ATPase activity of DnaK, which results in the accumulation of misfolded proteins and subsequent cell death (Kragol *et al.*, 2001). Several proline-rich AMPs have been isolated from invertebrates (Jiravanichpaisal *et al.*, 2007; Markossian *et al.*, 2004; Rabel *et al.*, 2004; Stensvag *et al.*, 2008), but none in mollusks. To date, mollusks AMPs have been only reported in the sea hare *Dolabella auricularia* (Iijima *et al.*, 2003), the mussels *Mytilus edulis* (Charlet *et al.*, 1996) and *M. galloprovincialis* (Hubert *et al.*, 1996), the scallop *Argopecten irradians* (Zhao *et al.*, 2007), and the oysters *C. virginica* (Seo *et al.*, 2005) and *C. gigas* (Gonzalez *et al.*, 2007; Gueguen *et al.*, 2006). All of the AMPs described, which include several defensins, belong to the group of cysteine-containing peptides.

In this study, we describe the first proline-rich AMP from mollusks. A sequence with homologies to proline-rich AMPs was identified by screening the *C. gigas* hemocyte EST library (http://www.ifremer.fr/GigasBase (Gueguen *et al.*, 2003)) for Pro-Arg-Pro motives. Based on the sequence of the proline-rich peptide identified in *C. gigas* (*Cg*-Prp) and putative processing sites, we synthesized several proline-rich peptides. Limited antimicrobial activity was detected when the peptides were used alone, but a strong synergistic antimicrobial activity was evidenced with the defensin previously characterized from *C. gigas* mantle (Gueguen *et al.*, 2006). Furthermore, *in situ* hybridization revealed that the expression of *Cg*-Prp is induced in circulating and in infiltrated hemocytes of the oyster following a bacterial challenge. Altogether, these data strongly argue in favor of the implication of this peptide in the oyster defense reactions.

Materials and methods

Animals, tissue collection and immune challenge

Adult C*rassostrea gigas* were purchased from a local oyster farm in Palavas (Gulf of Lion, France) and kept in sea water at 15°C. Oysters were challenged by adding heat-killed bacteria *Micrococcus lysodeikticus, Vibrio splendidus* and *V. anguillarum* (5×10^8 bacteria/litre) in sea-water tanks. Hemolymph was collected at different times (0, 15, and 72h) in anti-aggregant Modified Alsever Solution (MAS) (Bachère *et al.*, 1988). Hemocytes were collected by centrifugation (700 g, 10 min, 4°C) and fixed in 4% paraformaldehyde for immunofluorescence. Oyster tissues were harvested by dissection for *in situ* hybridization.

Peptides

Chemical synthesis of *Cg*-Prp_{20-36}, *Cg*-Prp_{22-36}, *Cg*-Prp_{26-36} (cationic putative mature peptides), and *Cg*-Prp_{1-19} (anionic putative proregion) was performed on an Abimed AMS 422 synthesizer by Fmoc chemistry (Gausepohl *et al.*, 1992). Peptides were deprotected and released from the Rink amide resin (Novabiochem) by trifluoroacetic acid treatment in the presence of appropriate scavengers. Peptides were lyophilized and then purified on a preparative C_{18} reverse-phase HPLC column (SymmetryPrep, Waters) by elution with a mixture of water/acetonitrile containing 0.1% (v/v) trifluoroacetic acid (gradient from 0 to 60%). Peptide mass was assessed by MALDI-TOF mass spectrometry. The full-length peptide *Cg*-Prp (from Ile1 to Gly37) was purchased from EPYTOP (Nîmes, France). *Cg*-Prp_{20-36}, *Cg*-Prp_{22-36}, *Cg*-Prp_{26-36} carried an amidated C-terminal Glu36 (putative removal of the C-terminal glycine residue). The amino acid sequences of the different synthetic peptides used in this study is shown in Table 1.

Recombinant expression and purification of *Cg*-Def, the defensin from *C. gigas* mantle, was performed as described in (Gueguen *et al.*, 2006).

Polyclonal antibodies and Immunofluorescence

Antibodies against *Cg*-Def were raised in New-Zeland rabbits by Eurogentec (Belgium). Antibodies against *Cg*-Prp_{20-36} were raised in mice. Briefly, two milligrams of synthetic *Cg*-Prp_{20-36} were coupled to ovalbumin (Sigma) in a 1:1 mass ratio after activation with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Pierce). Balb/c mice (4 weeks old) received three subcutaneous injections (50 µg each) of ovalbumin-coupled *Cg*-Prp_{20-36} at day 0 with complete Freund's adjuvant, and at day 15 and 30 with incomplete Freund's adjuvant.

Mouse tumor cells (5 x 10^6 cells in 500 µl RPMI 1640, Gibco) were injected in the mouse peritoneal cavity at day 30. Ten days after cell injection, ascitic fluid was withdrawn and clarified by centrifugation (800 x *g*, 15 min, 4°C). For both immune sera, immunoglobulins G (IgG) were purified on a Hitrap protein G sepharose column (Pharmacia). Reactivity and specificity of purified IgG were tested by ELISA against recombinant *Cg*-Def and synthetic *Cg*-Prp_{20-36}.

Immunofluorescence analyses were carried out using polyclonal antibodies specific for *Cg*-PRP_{22-36} and *Cg*-Def, according to the method of Gonzalez and colleagues (Gonzalez *et al.*, 2005). Briefly, hemocytes fixed with paraformaldehyde were cytocentrifuged (900 rpm, 10 min) on L-polyLysine slides. Hemocytes were then permeabilized with 0.1% Triton X-100 and incubated overnight (4°C) with both anti-*Cg*-Def (2.3 µg/ml) and anti-*Cg*-Prp_{20-36} (2.1 µg/ml) antibodies. After three washes in phosphate buffer saline (PBS), a second incubation was performed for 1 h at room temperature with anti-mouse FITC-conjugated and anti-rabbit Texas red-conjugated goat IgG (Pierce) diluted at 1:500 in PBS. Slides were washed three times in PBS and observed by confocal microscopy Leica TCS 4D.

Antimicrobial Assays

Determination of Minimal Inhibitory Concentrations (MICs) - Antimicrobial activity was assayed against several bacteria including the Gram-positive *Micrococcus lysodeikticus* (CIP5345), *Brevibacterium stationi* (CIP 104 228 T) and *Microbacterium maritypicum* (CIP 105 733 T), the Gram-negative *E. coli* SBS363 (a Trp+ galU129 derivative of *E. coli* K12 strain D22, generous gift of P.L. Boquet), *Enterobacter cloacae, Erwinia carotovora and Klebsiella pneumonia* (generous gift from IBMC Strasbourg collection). The activity of the peptides was also investigated against the following filamentous fungi *Fusarium oxysporum* (generous gift from Alain Veil, INRA, Saint Christol-les-Alès, France), and *Botrytis cinerea* (generous gift from Institut Universitaire de Montpellier, France). Minimum inhibitory concentrations (MICs) were determined in duplicate by the liquid growth inhibition assay based on the procedure described by Hetru and Bulet (1997). Poor broth (PB: 1% bactotryptone, 0.5% NaCl w/v, pH 7.5) nutrient medium was used for standard bacteria, and saline peptone water (1.5% peptone, 1.5% NaCl, pH 7.2) was used for marine bacteria. Antifungal assay was performed in potato dextrose broth (Difco, Sparks, USA) at half strength supplemented with tetracycline (10 µg/ml final concentration). Growth was monitored spectrophotometrically at 620 nm on a Multiscan microplate reader colorimeter (Dynatech).

Determination of Fractional Inhibitory Concentrations (FICs) - Synergy between *C. gigas* antimicrobial peptides was tested using the checkerboard microtiter assay (Rabel *et al.*, 2004) (Patrzykat *et al.*, 2001). To detect a possible reduction of the MIC values of each peptide when used in combination, 2-fold serial dilutions of one peptide were tested against 2-fold serial dilutions of the other peptide. Results were expressed as the fractional inhibitory concentration (FIC) index according to the following formula: $FIC = (A)/MIC_A + (B)/MIC_B$ where MIC_A and MIC_B are the MICs of peptides A and B tested alone and (A) and (B) are the MICs of the two peptides tested in combination. An FIC index of 0.5 indicates strong synergy (representing the equivalent of a fourfold decrease in the MIC of each compound tested), while an FIC index of 1.0 indicates that the antimicrobial activity of the two compounds are additive (i.e. a twofold decrease in the MIC of each compound tested).

In situ hybridization

The cDNA clone (Genbank™ BQ426670) was used as a template for the riboprobe preparation. [^{35}S] UTP-labelled antisense and sense riboprobes were generated from linearized cDNA plasmids by *in vitro* transcription using RNA transcription kit, T_3 RNA polymerase (Roche, Meylan, France) and [^{35}S] UTP (Amersham, Saclay, France). After hybridization, by contact with the autoradiographic emulsion, the emissions from the [^{35}S]-riboprobe produce silver grains, the number of which is proportional to the hybridization signal. Preparation of *C. gigas* tissues (serial sections) and hemocytes from a pool of five oysters, as well as *in situ* hybridization analyses, were performed as described (Munoz *et al.*, 2002). Hybridization signals were observed after a 36-h- (hemocytes) or 72-h-exposure (tissues). Controls consisted in replacing the antisense riboprobe with the sense riboprobe.

Biocomputing

Homology searches were performed with the BLAST software (http://www.ncbi.gov./Blast). Signal peptide prediction was performed with the SignalIP software (http://www.cbs.dtu.dk/services/SignalP/). Specific search for the Pro-Arg-Pro motive were performed using the Motifs Pattern program from the GCG Wisconsin Package (http://www.accelrys.com/products/gcg/). Deduced amino acid sequences were aligned by ClustalW (www.ebi.ac.uk/clustalw).

Results

Identification of an oyster cDNA encoding a proline-rich AMP

A 455bp-cDNA sequence (Genbank™ **BQ426670**) was identified in the oyster hemocyte EST library previously published (Gueguen *et al.*, 2003). The deduced 61-amino acid sequence starts with a predicted 24-amino acid hydrophobic signal peptide (Fig. 1). The 37-amino acid polypeptide resulting from signal peptide release, named *Cg*-Prp, displays a calculated molecular mass of 4.24 kDa and a calculated pI of 10.53. It is composed of an anionic N-terminal region, *Cg*-Prp$_{1-19}$ (ILENLLARSTNEDREGSIF), and a cationic C-terminal region, *Cg*-Prp$_{20-37}$ (DTGPIRRPKPRPRPRPEG), separated by a chymotrypsin cleavage site at position 19 (Phe19) (Fig. 1). The effectiveness of *Cg*-Prp cleavage was demonstrated *in vitro* on synthetic *Cg*-Prp using commercial chymotrypsin (data not shown).

The C-terminal region of *Cg*-Prp beyond the chymotrypsin site, *Cg*-Prp$_{20-37,}$ contains a high number of Pro and Arg residues (6 and 5, respectively over 18 residues). Amino acid sequence alignment shows that it is homologous to invertebrate proline-rich AMPs with two Pro-Arg-Pro tripeptides conserved in the alignment (Fig. 2).

Cg-Prp has synergistic antimicrobial activity with the oyster defensin *Cg*-Def

In order to investigate the antimicrobial properties of the identified peptide, the full-length *Cg*-Prp, and its putative C-terminal cationic fragments *Cg*-Prp$_{20-36}$, *Cg*-Prp$_{22-36}$, *Cg*-Prp$_{26-36}$, as well as the N-terminal anionic region *Cg*-Prp$_{1-19}$ (Table 1) were chemically synthesized. The cationic fragments (putative mature forms) were C-terminally amidated assuming Gly elimination, a posttranslational modification frequently observed in AMPs (Destoumieux *et al.*, 1997; Jiravanichpaisal *et al.*, 2007; Mitta *et al.*, 1999). These truncated forms were selected as follow: (i) *Cg*-Prp$_{20-36}$ because of the presence of a chymotrypsin-sensitive site (Phe19), (ii) *Cg*-Prp$_{22-36}$ in order to increase peptide cationicity, and (iii) *Cg*-Prp$_{26-36}$, because of the presence of the dibasic dipeptide Arg-Arg that may represent a trypsin-sensitive site. The peptides did not show significant antimicrobial activity against the tested microorganisms, which included Gram-positive and Gram-negative bacteria, as well as filamentous fungi. Only *Cg*-Prp$_{20-36}$ and *Cg*-Prp$_{22-36}$ presented a weak activity (100 to 200 µM) against filamentous fungi (Table 2). *Cg*-Prp$_{22-36}$ was also antibacterial at 100 µM against *M. lysodeikticus* (Table 2).

We then investigated possible synergistic interactions between *Cg*-Prp and *Cg*-Def, a defensin characterized from *C. gigas* (Gueguen *et al.*, 2006). Using the checkerboard titration assay, a strong synergy was observed between the full-length synthetic *Cg*-Prp and the

recombinant *Cg*-Def against *E coli* (FIC = 0.29) and *M. lysodeikticus* (FIC = 0.54) (Table 3). Thus, in the presence of 10 µM *Cg*-Def, a concentration three times below the MIC value against *E. coli* (35 µM), only 3.1 µM *Cg*-Prp were sufficient to have 100% growth inhibition. In contrast, in the absence of *Cg*-Def, 200 µM *Cg*-Prp were totally inactive. Similar strong synergy was obtained with the cationic *Cg*-Prp_{22-36} (FIC = 0.32) and *Cg*-Prp_{26-36} (FIC = 0.33) against *M. lysodeikticus* but not with the longer *Cg*-Prp_{20-36} nor with the anionic *Cg*-Prp_{1-19} (Table 3).

Cg-prp gene expression is restricted to oyster hemocytes and induced upon bacterial challenge.

The length of the *Cg-prp* mRNA was estimated at 470 bp by Northern blot analysis of oyster hemocyte total RNA. One single reactive band was detected on the blot (data not shown). *Cg-prp* expression was analyzed by *in situ* hybridization in tissues from unchallenged (control) and challenged (15-h and 72-h) oysters. In challenged oysters, strong hybridization signals were seen in circulating and in infiltrated hemocytes only (Fig. 3). No hybridization was detected in epithelial cells of any tissue. Therefore, numerous *Cg-prp*-positive hemocytes were evidenced infiltrating mantle, intestine (Fig. 3) but also the digestive gland, and gills (data not shown). In unchallenged oysters, autoradiographic signal was not quantified since none or very low hybridization signal was observed. No hybridization signal was observed with the *Cg-prp* sense probe, revealing that the detection of *Cg-prp* transcripts with the antisense riboprobe was specific (data not shown). These results indicate that expression of *Cg-prp* is restricted to the hemocytes and induced following a bacterial challenge.

_Cg_-Prp is present in hemocytes with oyster defensins

Immunocytochemistry on cytocentrifuged hemocytes using anti-*Cg*-Prp_{22-36} polyclonal antibodies showed the presence of *Cg*-Prp or one of its putative processed forms within hemocytes (Fig. 4). Although not all hemocytes were positive, a strong granular-like staining was observed intracellularly in positive hemocytes. At this point, we could not determine whether the peptides are stored in cytoplasmic granules. Interestingly, some hemocytes positive for anti-*Cg*-Prp_{22-36} were also positive for anti-*Cg*-Def staining (Fig. 4C), indicating the presence of defensins and *Cg*-Prp variants in one single cell. In other cases, hemocytes were only positive for one antibody, either anti-*Cg*-Prp_{22-36} or anti-*Cg*-Def (Fig. 4F).

Discussion

Results showed that hemocytes from the oyster *C. gigas* express a proline-rich peptide displaying synergistic antimicrobial activity with a defensin. To our knowledge, *Cg*-Prp is the first proline-rich AMP identified in a mollusk. The *Cg*-Prp cDNA encodes a 61-amino acid polypeptide precursor composed of (i) a 24-amino acid hydrophobic signal peptide, (ii) an 19-amino acid acidic region (pI = 4.4) ending with a Phe residue (a putative chymotrypsin clivage site), and (iii) an 18-amino acid cationic proline-rich region (pI = 11.83) ending with a Gly residue (Fig. 1). The cationic region has significant similarities with AMPs from the proline-rich family (Otvos, 2002). It also contains two repeats of the Pro-Arg-Pro tripeptide, a motif characteristic of the insect proline-rich AMPs (Otvos, 2002).

The overall structure of the *Cg*-Prp precursor suggests that *Cg*-Prp is synthesized as a prepropeptide that would be processed by a signal-peptidase and other proteinases resulting in the release of the anionic proregion and of the C-terminal Gly (amidation of the preceding Glu). Processing by elimination of an anionic proregion has been described for many AMPs from both vertebrates and invertebrates. For example, the mouse intestine α-defensins (cryptdins) are synthesized as a prepropeptide cleaved by signal peptidase and the matrix metalloproteinase-7 (MMP-7 matrilysin) to generate the mature α-defensin (Ayabe *et al.*, 2002). The second cleavage enables the release of the anionic proregion from the cationic AMP. Similarly, in invertebrates, the *Drosophila melanogaster* proline-rich drosocin (Bulet *et al.*, 1993) and the *Mytillus galloprovinciallis* defensins are processed from their precursor by elimination of a N-terminal signal peptide and a C-terminal anionic region (Mitta *et al.*, 1999). Interestingly, the anionic and cationic regions of *Cg*-Prp are separated by a chymotrypsin cleavage site at Phe19 (Fig. 1), which was found to be functional *in vitro* using commercial chymotrypsin (data not shown). However, based on the antimicrobial data discussed below, we expect additional processing after release of the anionic region. Such a multi-step processing has been described for the proline-rich AMP astacidin 2, from the crayfish *Pacifastacus leniusculus*. Astacidin 2 is indeed synthesized as a prepropeptide, which after signal-peptide elimination, is processed at both ends for elimination of a N-terminal tetrapeptide (chymotrypsin cleavage site) and a C-terminal Gly-Lys dipeptide resulting in peptide amidation (Jiravanichpaisal *et al.*, 2007). As with many C-terminally amidated AMPs including astacidin 2 (Jiravanichpaisal *et al.*, 2007), mussel defensins (Mitta *et al.*, 1999), and penaeidins (Destoumieux *et al.*, 1997), we expect a C-terminally amidated Glu for *Cg*-Prp upon elimination of the C-terminal Gly. Such a modification would increase the peptide cationic properties.

The antimicrobial activities of synthetic *Cg*-Prp variants (full-length peptide and putative mature forms shown in Table 1) were evaluated *in vitro* against a panel of bacteria and fungi. Surprisingly, no antimicrobial activity was detected for the different peptides except for *Cg*-Prp$_{22\text{-}36}$ and *Cg*-Prp$_{26\text{-}36}$ (between 100 and 200µM), which in turn could correspond to the actual mature forms of *Cg*-Prp. One cannot rule out the possibility that the poor antimicrobial activity observed results from the use of synthetic peptides. Indeed, previous works have shown that the antimicrobial activity of proline-rich AMPs depends on (i) the conformation of the peptide bond at the N-terminal side of Pro residues (Luders *et al.*, 2005), (ii) the chirality (Gennaro *et al.*, 2002) and (iii) the glycosylation state. For instance, the glycosylated forms of drosocin and formaecin are more active than their unglycosylated analogs (Otvos, 2002). Isomerization of the proline peptide bond(s) by a peptidylproline *cis-trans*-isomerase was also reported to be necessary for the activation of a synthetic proline-rich AMP (Luders *et al.*, 2005). The occurrence of such posttranslational modifications in *Cg*-Prp could not be evidenced by the molecular biology approach used in this study.

Interestingly, three synthetic variants of *Cg*-Prp displayed a strong antimicrobial activity in synergy with *Cg*-Def, a defensin characterized from *C. gigas* oyster, against the Gram-positive *M. lysodeikticus* and the Gram-negative *E. coli* SBS363. These include the full-length *Cg*-Prp, but also the short cationic *Cg*-Prp$_{22\text{-}36}$ and *Cg*-Prp$_{26\text{-}36,}$ which displayed antimicrobial activity. Such a synergy with *Cg*-Def is of particular interest. Indeed, while both vertebrate and invertebrate defensins have been shown to play a key role in the defense against infections (Salzman *et al.*, 2003; Tzou *et al.*, 2002), to our knowledge, only defensins from vertebrates have been reported to have synergistic activity with other antimicrobials (Bals *et al.*, 1998; Levy *et al.*, 1994). Thus, from our data, *Cg*-Prp would be an oyster defense peptide that acts through synergy with AMPs such as *Cg*-Def rather than through direct killing of microbes. This is reminiscent of MPAC, the proline-rich prodomain of *Drosophila* attacin, which has poor antimicrobial activity alone but acts synergistically with *Drosophila* cecropin (Rabel *et al.*, 2004).

Our demonstration of synergistic antimicrobial activities between the two AMPs *in vitro* may reflect an important determinant of their overall effectiveness since our results indicate that the two peptides can be present in the same tissues or cells. Thus, *in situ* hybridization revealed that *Cg*-Prp expression is induced in circulating hemocytes and in hemocytes infiltrating tissues including mantle (Fig. 3) following a bacterial challenge. Interestingly, previous studies have demonstrated that *Cg*-Def is continuously expressed in the mantle (Gueguen *et al.*, 2006). Besides, *Cg*-Prp or one of its variants were shown here to

be present in some hemocytes together with defensins. In a previous work, we showed that hemocyte defensins are continuously expressed and that their sequence is 79-84% identical to that of mantle *Cg*-Def (Gonzalez *et al.*, 2007). Altogether, these data support the hypothesis of a synergistic activity between both classes of AMPs *in vivo*. In other species, several examples of co-localization of antimicrobial compounds have been reported in various tissues and cell types, as well as specific evidence of synergistic activity (Lauth *et al.*, 2005; Patrzykat *et al.*, 2001; Rosenfeld *et al.*, 2006; Yan and Hancock, 2001).

In conclusion, our results showed that oyster proline-rich peptides and defensins are (i) present in the same tissues (either hemocytes or mantle through infiltrating hemocytes), and (ii) display strong synergistic antimicrobial activity. This may reflect an interesting feature of the oyster innate immune system. This mechanism, in which the inducible *Cg*-Prp acts synergistically with the constitutively expressed *Cg*-Def, would strongly increase the oyster antimicrobial defense upon infection.

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References

Ayabe, T., Satchell, D.P., Pesendorfer, P., Tanabe, H., Wilson, C.L., Hagen, S.J., Ouellette, A.J., 2002. Activation of Paneth cell alpha-defensins in mouse small intestine. J Biol Chem. 277, 5219-5228.

Bachère, E., Chagot, D., Grizel, H., 1988. Separation of *Crassostrea gigas* hemocytes by density gradient centrifugation and counterflow centrifugal elutriation. Dev. Comp. Immunol. 12, 549-559.

Bachère, E., Gueguen, Y., Gonzalez, M., de Lorgeril, J., Garnier, J., Romestand, B., 2004. Insights into the antimicrobial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. Immunol. Rev. 198, 149-168.

Bals, R., 2000. Epithelial antimicrobial peptides in host defense against infection. Respir Res. 1, 141-150.

Bals, R., Wang, X., Wu, Z., Freeman, T., Bafna, V., Zasloff, M., Wilson, J.M., 1998. Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. J Clin Invest 1998 Sep 1;102(5):874-80. 102, 874-880.

Boman, H.G., 2003. Antibacterial peptides: basic facts and emerging concepts. J Intern Med. 254, 197-215.

Brogden, K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 3, 238-250.

Bulet, P., Dimarcq, J.-L., Hetru, C., Lagueux, M., Charles, M., Hegy, G., Van Dorsselaer, A., Hoffmann, J.A., 1993. A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. J. Biol. Chem. 268, 14893-14897.

Bulet, P., Stöcklin, R., Menin, L., 2004. Anti-microbial peptides: from invertebrates to vertebrates. Immunol Rev. 198, 169-184.

Charlet, M., Chernysh, S., Philippe, H., Hétru, C., Hoffmann, J.A., Bulet, P., 1996. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. J. Biol. Chem. 271, 21808-21813.

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., Bachère, E., 1997. Penaeidins: A new family of antimicrobial peptides in the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. 272, 28398-28406.

Gausepohl, H., Boulin, C., Kraft, M., Frank, R.W., 1992. Automated multiple peptide synthesis. Pept Res. 5, 315-320.

Gennaro, R., Zanetti, M., Benincasa, M., Podda, E., Miani, M., 2002. Pro-rich antimicrobial peptides from animals: structure, biological functions and mechanism of action. Curr Pharm Des. 8, 763-778.

Gonzalez, M., Gueguen, Y., Desserre, G., de Lorgeril, J., Romestand, B., Bachere, E., 2007. Molecular characterization of two isoforms of defensin from hemocytes of the oyster Crassostrea gigas. Dev Comp Immunol. 31, 332-339.

Gonzalez, M., Romestand, B., Fievet, J., Huvet, A., Lebart, M.C., Gueguen, Y., Bachere, E., 2005. Evidence in oyster of a plasma extracellular superoxide dismutase which binds LPS. Biochem Biophys Res Commun. 338, 1089-1097.

Gueguen, Y., Cadoret , J.P., Flament, D., Barreau-Roumiguiere, C., Girardot , A.L., Garnier, J., Hoareau, A., Bachère, E., Escoubas, J.M., 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. Gene. 16, 139-145.

Gueguen, Y., Herpin, A., Aumelas, A., Garnier, J., Fievet, J., Escoubas, J.M., Bulet, P., Gonzalez, M., Lelong, C., Favrel, P., Bachere, E., 2006. Characterization of a defensin from the oyster Crassostrea gigas. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. J Biol Chem. 281, 313-323.

Hetru, C., Bulet, P. (1997) Strategies for the isolation and characterization of antimicrobial peptides of invertebrates. In *Methods in Molecular Biology* (Edited by Shafer W. M.), Vol. 78, p. 35-49. Humana Press Inc., Totowa, NJ.

Hubert, F., Noël, T., Roch, P., 1996. A member of the arthropod defensin family from the edible Mediterranean mussels (*Mytilus galloprovincialis*). Eur. J. Biochem. 240, 302-306.

Iijima, R., Kisugi, J., Yamazaki, M., 2003. A novel antimicrobial peptide from the sea hare Dolabella auricularia. Dev Comp Immunol. 27, 305-311.

Jenssen, H., Hamill, P., Hancock, R.E., 2006. Peptide antimicrobial agents. Clin Microbiol Rev. 19, 491-511.

Jiravanichpaisal, P., Lee, S.Y., Kim, Y.A., Andren, T., Soderhall, I., 2007. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish Pacifastacus leniusculus: characterization and expression pattern. Dev Comp Immunol. 31, 441-455.

Kragol, G., Lovas, S., Varadi, G., Condie, B.A., Hoffmann, R., Otvos, L., Jr., 2001. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry. 40, 3016-3026.

Lauth, X., Babon, J.J., Stannard, J.A., Singh, S., Nizet, V., Carlberg, J.M., Ostland, V.E., Pennington, M.W., Norton, R.S., Westerman, M.E., 2005. Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and in vivo hepatic response to bacterial infections. J Biol Chem. 280, 9272-9282.

Lemaitre, B., Hoffmann, J., 2007. The host defense of Drosophila melanogaster. Annu Rev Immunol. 25, 697-743.

Levy, O., Ooi, C.E., Weiss, J., Lehrer, R.I., Elsbach, P., 1994. Individual and synergistic effects of rabbit granulocyte proteins on Escherichia coli. J Clin Invest. 94, 672-682.

Luders, T., Birkemo, G.A., Nissen-Meyer, J., Andersen, O., Nes, I.F., 2005. Proline conformation-dependent antimicrobial activity of a proline-rich histone h1 N-terminal Peptide fragment isolated from the skin mucus of Atlantic salmon. Antimicrob Agents Chemother. 49, 2399-2406.

Markossian, K.A., Zamyatnin, A.A., Kurganov, B.I., 2004. Antibacterial proline-rich oligopeptides and their target proteins. Biochemistry (Mosc). 69, 1082-1091.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. J. Cell Sc. 112, 4233-4242.

Munoz, M., Vandenbulcke, F., Saulnier, D., Bachère, E., 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimps. Eur. J. Biochem. 269, 2678-2689.

Otvos, L., Jr., 2002. The short proline-rich antibacterial peptide family. Cell Mol Life Sci. 59, 1138-1150.

Patrzykat, A., Zhang, L., Mendoza, V., Iwama, G.K., Hancock, R.E., 2001. Synergy of histone-derived peptides of coho salmon with lysozyme and flounder pleurocidin. Antimicrob Agents Chemother. 45, 1337-1342.

Rabel, D., Charlet, M., Ehret-Sabatier, L., Cavicchioli, L., Cudic, M., Otvos, L., Jr., Bulet, P., 2004. Primary structure and in vitro antibacterial properties of the Drosophila melanogaster attacin C Pro-domain. J Biol Chem. 279, 14853-14859.

Rosenfeld, Y., Barra, D., Simmaco, M., Shai, Y., Mangoni, M.L., 2006. A synergism between temporins toward Gram-negative bacteria overcomes resistance imposed by the lipopolysaccharide protective layer. J Biol Chem. 281, 28565-28574.

Salzman, N.H., Ghosh, D., Huttner, K.M., Paterson, Y., Bevins, C.L., 2003. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature. 422, 522-526.

Seo, J.K., Crawford, J.M., Stone, K.L., Noga, E.J., 2005. Purification of a novel arthropod defensin from the American oyster, Crassostrea virginica. Biochem Biophys Res Commun. 338, 1998-2004.

Stensvag, K., Haug, T., Sperstad, S.V., Rekdal, O., Indrevoll, B., Styrvold, O.B., 2008. Arasin 1, a proline-arginine-rich antimicrobial peptide isolated from the spider crab, Hyas araneus. Dev Comp Immunol. 32, 275-285.

Tzou, P., Reichhart, J.M., Lemaitre, B., 2002. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc Natl Acad Sci U S A. 99, 2152-2157.

Yan, H., Hancock, R.E., 2001. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrob Agents Chemother. 45, 1558-1560.

Yang, D., Biragyn, A., Kwak, L.W., Oppenheim, J.J., 2002. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol. 23, 291-296.

Zasloff, M., 2006a. Defending the epithelium. Nat Med. 12, 607-608.

Zasloff, M., 2006b. Inducing endogenous antimicrobial peptides to battle infections. Proc Natl Acad Sci U S A. 103, 8913-8914.

Zhao, J., Song, L., Li, C., Ni, D., Wu, L., Zhu, L., Wang, H., Xu, W., 2007. Molecular cloning, expression of a big defensin gene from bay scallop Argopecten irradians and the antimicrobial activity of its recombinant protein. Mol Immunol. 44, 360-368.

Figure legends

Fig. 1: *Cg*-Prp cDNA and its deduced amino acid sequences. The putative signal peptide is underlined. The atg and tga start and stop codons are in bold. The chymotrypsin cleavage site that separates the anion and cationic regions of *Cg*-Prp is indicated by an arrow.

Fig. 2: **Amino acid sequence alignment of** *Cg*-Prp_{20-37} **with invertebrate proline-rich AMPs.** Amino acids from *Cg*-Prp_{20-37} conserved in other proline-rich AMPs are shaded in grey. Pro-Arg-Pro tripeptides are in bold. Pro-Arg-Pro tripeptides in boxes are conserved between Cg-PRP_{20-37} and other proline-rich AMPs. Z stands for pyroglutamic acid and underlined Thr residues carry an *O*-glycosylation. Oyster *Crassostrea gigas* (*Cg*-Prp_{20-37} [**BQ426670**]); fruit fly *Drosophila melanogaster* (MPAC for mature pro-domain-attacin C [**Q95NH6**], Metchnikowin-A1 [**Q24395**] and Drosocin [**NP_523744**]; sap-sucking bug *Pyrrhocoris apterus* (Pyrrhocoricin [**P37362**]); green shield bug *Palomena prasina* Metalnikowin 1 [P80408]; honey bee *Apis mellifera* (Apidaecin-1a [**P35581**]); tobacco budworm *Heliothis virescens* (Heliocin [**P83427**]); spider crab *Hyas araneus* (Hyasin [**ABI74600**]); domestic silkworm *Bombyx mori* (Lebocin 1 [**NP_001037468]**); green crab *Carcinus maenas* (Crab 6.5kDa [**P82964**]); red bulldog ant *Myrmecia gulosa* (Formaecin-2 **[P81437**]); crayfish *Pacifastacus leniusculus* (Astacidin 2 [**ABH05920**]); spider crab *Hyas araneus* (arasin-1 [**ABI74601**]). The nomenclature derived from the SwissProt database and the accession numbers are in brackets.

Fig. 3: Expression of *Cg*-Prp is restricted to hemocytes both circulating and infiltrating tissues. Paraffin-embedded sections of *C. gigas* oyster were analyzed by ^{35}[S] *in situ* hybridization using a *Cg-prp* antisense riboprobe. In challenged oysters, hybridization signals were observed in hemocytes infiltrating intestine (B), mantle (C) and in the blood vessels (D). In unchallenged oysters, no signal was observed in hemocytes infiltrating intestine (A). Control sections with the sense riboprobe were devoid of labelling. (Scale bars, 50µm).

Fig. 4: Detection of *Cg*-Prp (or one of its variants) and *Cg*-Def in circulating hemocytes of *C. gigas* by immunofluorescence. FITC staining (green; A, D) was used for *Cg*-Prp detection, and Texas Red staining (red; B, E) for *Cg*-Def detection. Immunostained cells were photographed under epifluorescence confocal microscopy. Merged confocal microscope images show that *Cg*-Prp and *Cg*-Def are present in different hemocytes (F), but also inside the same hemocytes (C). (Scale bars 10µm).

Table 1: Amino-acid sequences of the different synthetic peptides used in the study.

Peptide	Sequence	pI
Cg-Prp	ILENLLARSTNEDREGSIFDTGPIRRPKPRPRPRPEG	10.53
Cg-Prp$_{20-36}$	DTGPIRRPKPRPRPRPE*	11.83
Cg-Prp$_{22-36}$	GPIRRPKPRPRPRPE*	12.18
Cg-Prp$_{26-36}$	RPKPRPRPRPE*	12.00
Cg-Prp$_{1-19}$	ILENLLARSTNEDREGSIF	4.41

The calculated isoelectric points (pI) of the different peptides are indicated on the right.
* stands for C-terminal amidation

Table 2: Antimicrobial activity of synthetic *Cg*-Prp peptides.

	Antimicrobial activity (MIC, µM)*					
	***Cg*-Prp**	***Cg*-Prp$_{20-36}$**	***Cg*-Prp$_{22-36}$**	***Cg*-Prp$_{26-36}$**	***Cg*-Prp$_{1-19}$**	***Cg*-Def****
Bacteria						
Gram-positive						
Micrococcus lysodeikticus	>200	>200	100	>200	>200	0.01
Brevibacterium stationis	>100	>200	>200	NT	NT	0.2
Microbacterium maritypicum	>100	>200	>200	NT	NT	1
Gram-negative						
Escherichia coli 363	>200	>200	>200	>200	>200	35
Enterobacter cloacae	>100	>200	>200	NT	NT	NT
Erwinia carotovora	>100	>200	>200	NT	NT	NT
Klebsiella pneumonia	>100	>200	>200	NT	NT	NT
Filamentous fungi						
Fusarium oxysporum	>200	200	125	>100	>200	9
Botrytis cinerea	>200	100	100	>100	>200	>20

***** MIC values refer to the minimal inhibitory concentration of antimicrobial peptides required to achieve 100% growth inhibition.
** Results from (Gueguen *et al.*, 2006).
NT, not tested

Table 3: FIC index* for *Cg*-Prp peptides and *Cg*-Def against selected bacteria.

	FIC index[a]				
Species	***Cg*-Prp +*Cg*-Def**	***Cg*-Prp$_{20-36}$ +*Cg*-Def**	***Cg*-Prp$_{22-36}$ +*Cg*-Def**	***Cg*-Prp$_{26-36}$ +*Cg*-Def**	***Cg*-Prp$_{1-19}$ +*Cg*-Def**
M. lysodeikticus	0.54	1.5	0.32	0.33	2
E. coli 363	0.29	NT	0.78	NT	NT

***** *FIC index = [A]/MICA + [B]/MICB, where MICA and MICB are the MICs of peptides A and B alone and [A] and [B] are the MICs of peptides A and B in combination. The MICs for the peptides alone are as given in Table II. When MIC values are higher than 200μM, the highest concentration we tested, we chose this value as the MIC in the calculation of the FIC index.*
FIC index were interpreted as follows: ≤ 0.5, strong synergy; 0.5-1 synergy; ≥ 1; additive effect; ≥ 2, antagonism.

NT, not tested

Figure 1

Fig. 1

Fig. 2


Cg-Prp20-37 DTGPIRRPKPRPRPRPEG
Metalnikowin-1 VDKPDYRPRPRPPNM
Apidaecin-1a GNNRPVYIPQPRPPHPRL
Drosocin GKPRPYS-PRPTSHPRPIRV
Crab 6.5kDa VPYPRP-PRPPIGPRPLPFPGGGRPFQS
Arasin-1 SRWPSPGRPRPFP-GRPKPIFRPRPCNCYAPPCPCDRW
Heliocin ZRFIHPTYRPPPQPRRPVIMR
Hyasin WQRPLTRPRPFSRPRPY-RPNYG
Astacidin SLGYRPRPNYRPRPIYRPGK
Pyrrhocoricin VDKGSYLPRPTP-PRPIYNRN
MPAC ZRPYTQPLIYYPPPPTP-PRIYRA
Lebocin DLRFLYPRGKLPVPTP-PPFNPKPIYIDMGNRY
Metchnikowin-A1 HRHQGPIFDTRPSPFNPNQPRPGPIY
Formaecin-2 GRPNPNNKPTPHPRL

Figure 3
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Fig. 3

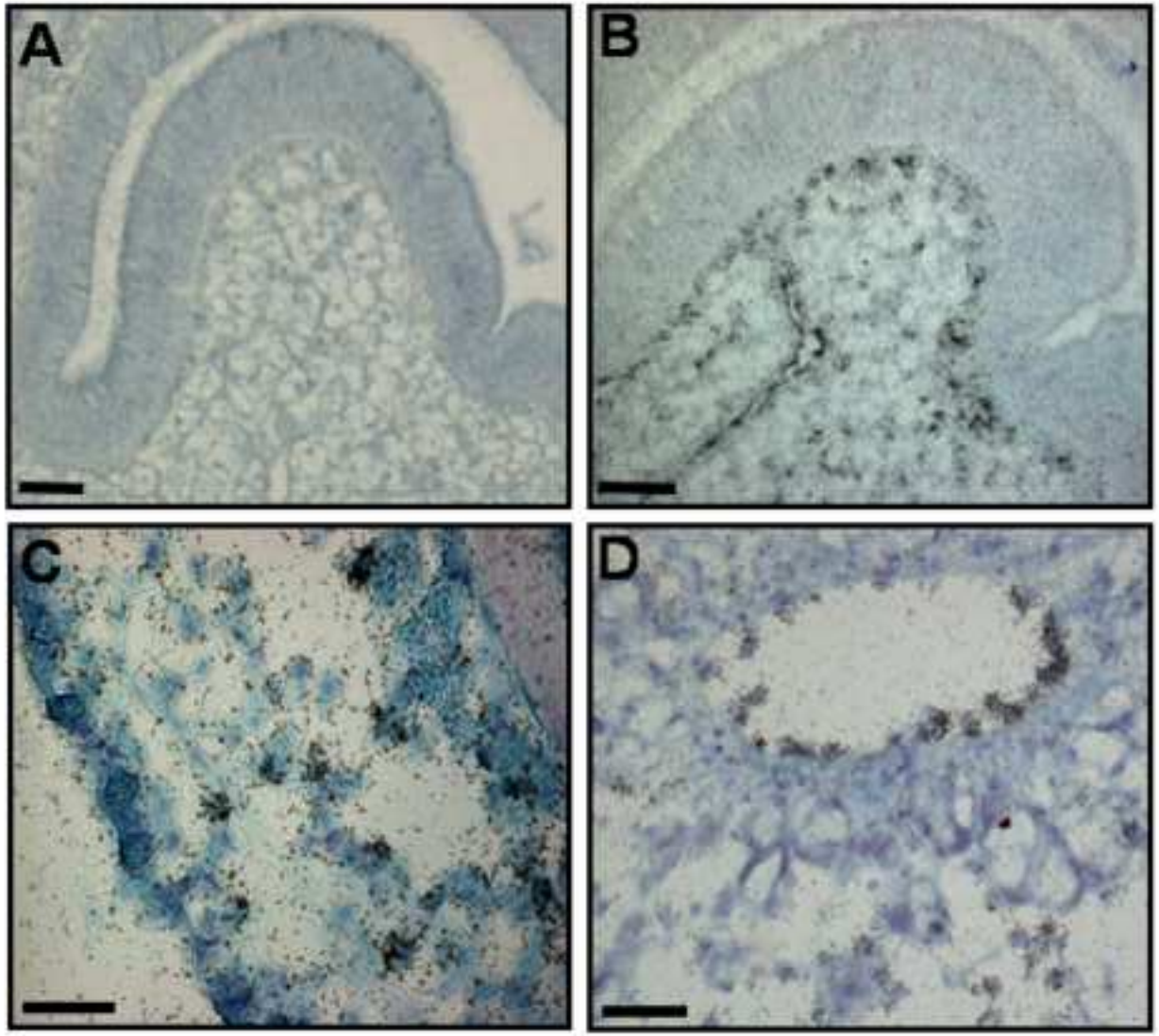

Figure 4
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Fig. 4