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A piscidin-like antimicrobial peptide from the icefish *Chionodraco hamatus* (Perciformes: Channichthyidae): Molecular characterization, localization and bactericidal activity

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ABSTRACT

Antimicrobial peptides (AMPs) are considered one of the most ancient components of the innate immune system. They are able to exert their protection activity against a variety of microorganisms, and are widely distributed in both vertebrates and invertebrates. In this paper we focused on an AMP identified in the Antarctic teleost *Chionodraco hamatus*, an icefish species. The cDNA sequence of the AMP, named chionodracine, is comprised of 515 bp and translates for a putative protein precursor of 80 amino acids, with a signal peptide of 22 amino acids. The structural features evidenced in the primary sequence of chionodracine lead to the inclusion of the peptide in the antimicrobial family of piscidins. The analysis by real-time PCR of the basal gene transcripts of chionodracine in different icefish tissues showed that the highest expression was found in gills, followed by head kidney. The chionodracine expression levels in head kidney leukocytes were up-regulated *in vitro* both by LPS and poly I:C, and *in vivo* by LPS. A putative chionodracine mature peptide was synthesized and employed to obtain a polyclonal antiserum, which was used in immunohistochemistry of gills sections and revealed a significant positivity associated with mast cells. The bactericidal activity of the peptide was investigated and found significant against Antarctic psychrophilic bacteria strains (*Psychrobacter* sp. TAD1 and TA144), the Gram-positive *Bacillus cereus*, and at a lesser extent against the Gram-negative *Escherichia coli*. Interestingly, the haemolytic activity of chionodracine was tested *in vitro* on human erythrocytes and no significant lysis occurred until peptide concentration of 50 μM.

1. Introduction

The antimicrobial peptides (AMPs) are considered as fundamental components of the innate immune system of eukaryotes, where they contribute actively to the control of microbial invasion [1–3]. The specificity of AMPs against pathogens is extremely wide towards species and strains of bacteria, fungi, parasites and viruses [4]. Due to their considerable genetic variability, AMPs cannot be easily classified on the base of size, structure or physico-chemical parameters, thus several categories have been described as peptides having common structures such as α-helices, β-sheets, extended structures and loop structures [5,6]. The expression of AMPs is usually high in primary barriers tissues of the organism, such as the skin in mammals, to prevent colonization by pathogens [4,7,8]. The potential use of AMPs as antimicrobial drugs can be significant [9,10], but it is actually limited by the knowledge of their mechanisms of action that has not been fully understood [11].

During recent years the research on fish AMPs showed an exponential increase, and a large number of AMPs sequences have been cloned and characterized (for a review see Ref. [12]). Among these it should be mentioned pleurocidin from winter flounder (*Pleuronectes americanus*) [13], piscidin or moronecidin from hybrid striped bass (white bass, *Morone chrysops*, female, x striped bass, *M. saxatilis*, male) [14] and dicentracine from sea bass (*Dicentrarchus labrax*) [15]. Fish AMPs have been shown to be biologically active against both bacterial and viral fish pathogens [16,17] and, therefore, could be of importance in fish farming for their impact on the immune system responses [18]. The tissue localization of fish AMPs has been performed in sea bream, where piscidin were demonstrated to be primarily present in mast cells and professional

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phagocytic granulocytes [19]. Other AMPs from fish, like hepcidins, have been shown to also function as iron regulators [20], thus suggesting others possible functional roles of these molecules.

The icefish *Chionodraco hamatus* is a teleost species belonging to the Channichthyidae family, one of the six extant families of Antarctic Perciformes [21]. These fishes display interesting physiological adaptations, like antifreeze glycoproteins [22] and peculiar immunoglobulins [23,24]. In addition, icefishes show a complete absence of haemoglobin and of functional blood erythrocytes [25]. Such peculiarities make *C. hamatus* an interesting model of study for the immune system adaptation to low environmental temperatures.

In this paper we describe in *C. hamatus* the identification of a cDNA coding for a piscidin-like AMP, named chionodracine, and investigated levels of gene expression in different tissues, as well as the *in vitro* and *in vivo* gene regulation after stimulation with LPS or poly I:C. Moreover, a polyclonal antibody has been produced against the putative mature sequence of the peptide and used to investigate the presence of chionodracine in the gills, a tissue in direct contact with the outside. Indeed, the gills are the major route of entry for pathogen invasion after epithelial damage [19,26–28] and thus are considered important immune organs capable of mounting robust immune responses [29]. Finally, we evaluated the bactericidal activity of chionodracine against both fish and human bacteria, for a possible use of the peptide as an antimicrobial drug.

2. Materials and methods

2.1. Cloning of an AMP from C. hamatus

C. hamatus specimens were collected with nets in January–February 2011 during the XXVI Italian Antarctic Expedition organized by the National Program of Antarctic Research (P.N.R.A.) at the Italian Antarctic Base, Terra Nova Bay, Ross sea (Mario Zucchelli Station). After collection, fish were placed in tanks, transported to aquaria and kept in running seawater.

Head kidney leukocytes were obtained from *C. hamatus* after disrupting the organ by teasing on a 100 μm cell strainer and total RNA was isolated from leukocytes using Trisure (Bioline). The RNA was resuspended in DEPC-treated water and cDNA prepared with the BioScript RNase H minus (Bioline) enzyme using the protocol described in Ref. [30]. Two degenerate primers (PISFW 5′-CT(T/C) TCTTGTGCT GTCGATGGT-3′ and PISRV 5′-GCA(A/T/G)GA(G/T/C) CAG(C/G)AAG(A/C)(T/G)C (G/A/T)GC-3′) corresponding to conserved regions of known AMP genes were used in PCR on the cDNA. Controls for the presence of DNA contamination were performed using the cDNA as template and with β-actin primers that span an intron. Reactions were conducted using a Mastercycler (Eppendorf). The cycling protocol was one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 48 °C for 45 s, 72 °C for 45 s, followed by one cycle of 72 °C for 10 min. PCR products (15 μl) were visualized on 1% (w/v) agarose gels containing ethidium bromide (10 ng/ml) using hyperladder IV (Bioline) as size marker. The PCR product was purified using a QIAquick Gel Extraction Kit (QIAgen), inserted into the pGEM-T Easy vector (Promega) and transformed into competent JM109 *Escherichia coli* cells. Plasmid DNA from at least five independent clones was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced using MWG DNA Sequencing Services. Sequences generated were analysed for similarity with other known sequences using the BLAST [31] program.

Further primers were designed based on the initial icefish AMP sequence for 5′- and 3′-rapid amplification of cDNA ends (RACE-PCR) (3′AMFW: 5′-CTTTCTTGTGCTGTCGATGGT-3′ and 5′AMRV: 5′-GCAAGACCAGCAAGAGCGGC-3′). cDNA was synthesized from head kidney RNA with a First-strand cDNA Synthesis kit (GE Healthcare) following the manufacturer's instructions. For 3′ RACE-PCR, cDNA was transcribed using an oligo-dT adaptor primer (5′-CTCGA-GATCGATGCGGCCGCT$_{15}$-3′). PCR was performed with the 3′AMFW primer and the oligo-dT adaptor primer. For 5′ RACE-PCR, cDNA was transcribed from total RNA using the oligo-dT primer, treated with *E. coli* RNase H (Promega), purified using a PCR Purification Kit (QIAgen), and tailed with poly(C) at the 5′ end with terminal deoxynucleotidyl transferase (TdT, Promega). PCR was performed with 5′AMRW primer and an Oligo-dG primer (5′-GGGGGGIGG-GIIGGGIIG-3′). Sequencing of amplified products and similarity searches were performed as described above. The icefish AMP sequence was analysed for the presence of a signal peptide using SignalP software [32]. Alignment of the icefish AMP amino acid sequence to other known molecules from other species was carried out using the BioEdit sequence alignment package. A phylogenetic tree was constructed by the "neighbor joining" method using MEGA 4.1 Software [33] on full-length amino acid sequences using the normal default parameters and as test of reliability the bootstrap test with 10,000 replications and 60,000 random seeds. The identified AMP was named chionodracine.

2.2. Basal expression of chionodracine

To study the chionodracine basal expression, four icefishes were sampled and leucocytes from different tissues (spleen, head kidney (HK), gills, liver, gut) obtained as described above. Total RNA was isolated from each tissue separately with Trisure (Bioline), resuspended in DEPC-treated water and used for real-time quantitative PCR without pooling the tissue samples coming from the different fishes. For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used with the protocol described in Ref. [30]. The expression level of chionodracine transcripts was determined with an Mx3000P™ real-time PCR system (Stratagene) equipped with version 4.1 software and using the Brilliant SYBR Green Q-PCR Master Mix (Agilent Technologies) following the manufacturer's instructions, with ROX as internal passive reference dye. Specific PCR primers were designed for the amplification of about 200 bp products from chionodracine (AMPFW: 5′-CTTGTGCTGTCGATGGTGGT-3′; AMPRV: 5′-CTGCACCTTCATCGCT TCCC-3′) and 18 S ribosomal RNA (18SFW: 5′-CCAACGAGCTGCTGACC-3′; 18SRV: 5′-CCGTTACCCGTG-GTCC-3′), used as a house-keeping gene. Ten nanogram of cDNA template was used in each PCR reaction. The PCR conditions were: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Triplicate reactions were performed for each template cDNA and the template was replaced with water in all blank control reactions. The analysis was carried out using the endpoints method option that causes the collection of the fluorescence data at the end of each extension stage of amplification. A relative quantitation has been performed, comparing the levels of the target transcript (chionodracine) to a reference transcript (calibrator, the tissue with the lowest chionodracine expression, in this case the liver). A normalizer target (18 S ribosomal RNA) is included to correct for differences in total cDNA input between samples. The results are expressed as the mean ± SD of the results obtained from the four considered fishes.

2.3. In vitro and in vivo expression of chionodracine after stimulations

The *in vitro* chionodracine expression was studied using leukocytes isolated from four icefishes (100 g of weight) head kidney (HK) cells cultured in L-15 medium (Gibco) containing 10% FCS, adjusted to 1×10^5 cells/ml and incubated for 4 h and 24 h with: (1) 5 μg ml^{-1} of lipopolysaccharide (LPS from *E. coli* 0127:B8, Sigma); (2) 50 μg ml^{-1} poly I:C (Sigma). The cell control samples were

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stimulated with L-15 alone. Total RNA was isolated with Tripure (Roche), resuspended in DEPC-treated water and used for real-time quantitative PCR without pooling the samples coming from the different fishes. The primers and the real-time PCR conditions were the same as described in the above section, except that the calibrator for this experiment was the time 0 control.

The *in vivo* chionodracine expression was studied after intraperitoneally injection of 500 μl of L-15 medium (Gibco) brought to 600 mOsm kg^{-1}, that is the osmolarity of Antarctic Teleosts as determined in previous experiments and due to their particular adaptation to low temperatures, and containing 100 μg of LPS in four icefishes. The four control fishes were injected with L-15 only. After 24 h, leukocytes were isolated from head kidney and total RNA extracted as described above. The primers and the real-time PCR conditions were the same as described in the above section, except that the calibrator for this experiment was the time 0 control.

The results of all the experiments were expressed as the mean ± SD of the results obtained from four fishes and the differences from the control were considered significant if $p < 0.05$ using the two-way ANOVA analysis followed by the Bonferroni's post-test for the *in vitro* stimulations and the one-way ANOVA followed by the Bonferroni's post test for the *in vivo* stimulation.

2.4. Production of a polyclonal antibody, and enzyme-linked immunosorbent assay (ELISA)

Three Balb/C mice (eight weeks old from Charles River Laboratories, Lecco, Italy) were acclimatized for one week in the animal facilities of the University of Tuscia (Viterbo, Italy). These mice were immunized by intraperitoneal injection of the selected chionodracine peptide (22 amino acids long) conjugated with the carrier protein KLH (Primm srl, Milano, Italy). The immunization procedure was as follows: (1) $t = 1$ day injection of 50 μg of KLH-peptide diluted with 50 μl of PBS (Sigma) plus 50 μl of complete Freund's adjuvant; (2) $t = 13$ days and $t = 25$ days injection of 50 μg of KLH-peptide diluted with 50 μl of PBS (Sigma) plus 50 μl of complete Freund's adjuvant; (3) $t = 36$ days and $t = 47$ days injection of 50 μg of KLH-peptide diluted with 50 μl of PBS (Sigma); $t = 60$ days bled of the mice. The sera isolated by centrifugation contain the polyclonal antibodies against the chionodracine peptide. The sera from the three different mice were successively analysed by enzyme-linked immunosorbent assay (ELISA) to investigate their activity against the peptide. The procedure was similar as previously described [34]. Briefly, the 96-well microtitres plates (Cellstar, Greiner Bio-One) were filled with 100 μl/well of 50 mM carbonate–bicarbonate buffer (pH 9.4) containing 0.2 μg of the chionodracine peptide and coated overnight at 4 °C. After washing and blocking procedures, 100 μl of serial dilutions of mice serum (from 1:10 to 1:100) were added to the wells and incubated for 16 h at 25 °C. After washing, antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1: 200 dilution) using a colouration with H_2O_2 and 4-chloro-1-napthol. The results were recorded by reading the optical density (OD) value at 492 nm by an automated ELISA reader (Labsystems Multiskan MS). Each point was performed in triplicate.

2.5. Immunohistochemistry

Gills from three different fish were fixed for 7 h at 4 °C in Bouin's fixative. After embedding in paraplast, blocks were serially sectioned at a thickness of 7 μm. Some sections were stained with May-Grümwald Giemsa while others were used for the immunohistochemistry.

Immunohistochemistry (IHC) was performed by ABC-peroxidase with nickel enhancement as previously described [35]. In summary, serial sections 7 μm-thick were incubated for 18 h at room temperature with the mouse polyclonal antiserum named Pab Ch1 (diluted 1:100 in PBS 0.1 M, pH 7.3 containing 0.1% sodium azide) that recognizes the selected mature chionodracine peptide. Pre-immune serum substituted the primary antibody in negative controls. Thereafter, sections were incubated for 60 min with biotinylated horse anti-mouse IgG serum (Vector Labs., Burlingame, USA) diluted 1:1000 with PBS containing 0.1% sodium azide and 1% BSA, followed by incubation for 60 min with avidin-biotinylated peroxidase complex (ABC, Vectastain Elite, Vector). After rinses and staining (diaminobenzidine and nickel enhancement), sections were dehydrated, mounted and examined under bright-field illumination.

In each specimen, multiple sets of consecutive sections were differentially immunostained with the antibody above mentioned. Cell measurements were obtained using a computer-assisted image analysis system which includes a Zeiss microscope equipped with a colour video camera (Axio Cam MRC, Arese, Milano Italy) and a software package (KS 300 and AxioVision). Measurements of cell diameter (major axis) were performed in 5 specimens and pooled. Mean and SD were calculated.

2.6. Antibacterial activity assays

The antimicrobial activity of the chionodracine peptide was examined against four Gram-negative bacterial strains (*Psychrobacter* sp. TAD1, *Psychrobacter* sp. TA144, *Pseudomonas aeruginosa*, *E. coli* BL21(DE3)) and one Gram-positive bacterial strain (*Bacillus cereus*). The strains were kindly provided by the Department of Organic Chemistry and Biochemistry, University of Naples and the Institute of Protein Biochemistry, CNR, Naples. Luria Bertani (LB) medium was used for the growth of the four Gram-negative bacteria while peptone 1.5% was used for the growth of *B. cereus*. All bacteria were grown aerobically and cultured at different temperatures: 15 °C (*Psychrobacter* sp. TAD1, *Psychrobacter* sp. TA144) for 48 h, 25 °C (*E. coli* and *B. cereus*) for 48 h and 37 °C (*E. coli*, *B. cereus* and *P. aeruginosa*) for 16 h.

The peptide was dissolved in sterile deionized water to reach a final concentration of 1 mg/ml. The minimum inhibitory concentration (MIC) was determined by using serial dilutions of the peptide, with LB medium or Peptone 1.5%, using a volume of 500 μl per tube. The final concentration of the peptide ranged from 1.25 to 20 μM. Two internal controls were used: an *E. coli* BL21 (DE3) culture without the peptide and an *E. coli* culture with the peptide pOVA (as internal control) that has no antimicrobial properties. The tubes were inoculated with an appropriate bacterial cell suspension to reach a final concentration of 10^5 cfu/ml for each strain. After incubation at different temperatures (as reported above), the MIC was defined as the lowest concentration of the peptide that totally inhibited the growth.

To measure the minimum bactericidal concentration (MBC), an aliquot (200 μl) of the cell suspension was taken from the tubes above the MIC and the cell suspension was plated on an LB agar plate after incubation at the different temperatures (see above) for 48 h. MBC was defined as the lowest concentration of the peptide at which more than 99.9% of the cells were killed compared with a non-treated control.

2.7. Haemolytic activity assay

The haemolytic assay was performed as indicated by Beloko-neva et al. [36]. In brief, a 2.5% (v/v) suspension of human red blood cells from healthy donors in PBS (Gibco) was incubated with serial

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dilutions of the selected peptide. Red blood cells were counted by a haemocytometer and adjusted to approximately to about 8.0×10^6 cells/ml. Erythrocytes were successively incubated at 37 °C for 2 h with the chionodracine peptide in distilled water (positive control), PBS (negative control) and with the different concentrations of peptide (from 50 μM to 0.5 μM with seven dilutions). Each point has been made in triplicate. The supernatant was separated from the pellet with a centrifugation at 1500 × *g* for 5 min; the absorbance was measured at 570 nm. The relative OD compared to that of the positive control defined the percentage of haemolysis.

3. Results

3.1. Chionodracine cloning and sequence analysis

The first PCR was made with primers PISFW and PISRV and gave only one product of the expected size (146 bp) that, after sequencing, showed homology with other known AMP sequences in Teleosts (data not shown). 3′-RACE-PCR performed with primer 3′AMFW (based on the initial 146 bp sequence) and oligo-dT adaptor primer gave a product of 407 bp. 5′-RACE-PCR performed with 5′AMRW (based on the initial 146 bp sequence) and the Oligo-dG primer gave a product of 289 bp. The full-length cDNA (EMBL accession number FR718953) of the chionodracine is comprised of 515 bp from the three overlapping products with a coding sequence of 240 bp and was confirmed by PCR using primers that amplify the complete coding sequence (data not shown). The 3′-UTR contained a polyadenylation signal (AATAAA) 18 bp upstream of the poly(A) tail. The presence of a putative signal peptide of 22 amino acids (cleavage site between Ala^{22} and Phe^{23}) and no N-glycosylation and O-glycosylation sites was evidenced using predictive methods.

A multiple alignment of the chionodracine amino acid sequence with other known AMP sequences from Teleosts was assembled (Fig. 1) to investigate the conserved amino acid residues. The chionodracine, due to its primary structure, should be included in the antimicrobial family of piscidins, which comprises moronecidins and pleurocidins [37]. Pleurocidin was found in the skin mucus of winter flounder (*P. americanus*) [13] and was predicted to assume an amphipathic alpha-helical conformation [38]. These AMPs are produced as pre-pro-peptides and, after secretion, the mature peptides should be obtained due to the processing of the pro-peptides by local proteases, as it happens in mammals [39]. From the alignment in Panel A of Fig. 1, it has been evidenced that only few amino acids are conserved between all the pleurocidin and moronecidin sequences and these amino acids are mainly located in the signal peptide region. In the Panel B of Fig. 1, the chionodracine has been aligned only with the components of the Moronidae family and, in this case, the number of conserved amino acids is much higher and they are present all along the sequence.

Phylogenetic analysis (Fig. 2) performed using amino acid sequences showed that chionodracine grouped with the AMP sequences of the other Teleosts belonging to the Perciformes Order. Another branch comprises the Teleosts of the Pleuronectiformes Order, whereas the two representatives of the Scorpaeniformes and Gadiformes Orders are in a separate cluster.

3.2. Basal and in vitro and in vivo chionodracine expression

Chionodracine mRNA basal levels have been analysed in different organs and tissues of *C. hamatus* specimens (Fig. 3). The products of real-time PCR were loaded on agarose gels to exclude the presence of non-specific amplicons and single bands of the expected sizes were obtained. The highest chionodracine expression was found in gills, followed closely by head kidney. The lowest mRNA levels were evidenced in liver, followed by gut. Chionodracine expression was studied on icefish leukocytes from head kidney after 4 h and 24 h of stimulation with LPS and poly I:C. The stimulation with LPS (Panel A, Fig. 4) shows a slight decrease of chionodracine expression after 4 h and a slight increase after 24 h, both being not statistically significant. The stimulation with poly I:C (Panel A, Fig. 4) shows a slight increase of chionodracine transcripts levels after 4 h and a much higher increase at 24 h, both being statistically significant. Differently, the *in vivo* treatment with LPS

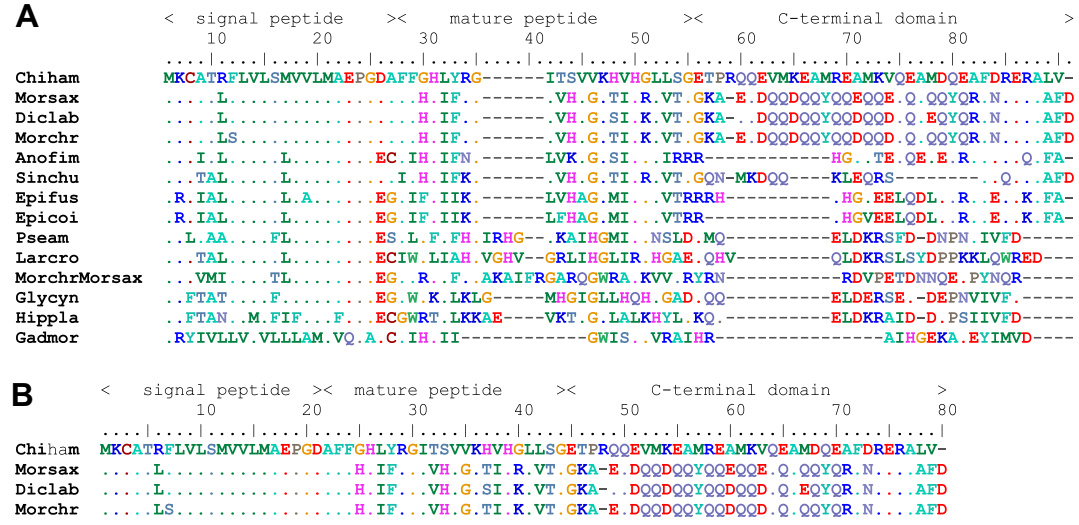

Fig. 1. Alignment of the predicted icefish AMP amino acid sequence with other known AMP molecules from Teleost fishes. The conserved amino acid residues in the different sequence are indicated by dots. The different identified domains are evidenced above the alignment. Accession numbers: dicentracine (sea bass, *Dicentrarchus labrax*) AAP58960; moronecidin (white bass, *Morone chrysops*) AAL57318; moronecidin (striped sea bass, *Morone saxatilis*) AF385583; moronecidin (Mandarin fish, *Siniperca chuatsi*) AAV65044; chionodracine (icefish, *Chionodraco hamatus*) FR718953; piscidin-like (brown-marbled grouper, *Epinephelus fuscoguttatus*) ADE06665; epinecidin (orange-spotted grouper, *Epinephelus coioides*) AY705494; dicentracin (sablefish, *Anoplopoma fimbria*) ACQ58110; piscidin-4 (*Morone chrysops* × *Morone saxatilis*) ADP37959; piscidin (Atlantic cod, *Gadus morhua*) FJ917596; pleurocidin-like (witch flounder, *Glyptocephalus cynoglossus*) AY273177; pleurocidin-like (Atlantic halibut, *Hippoglossus hippoglossus*) AAP55801; pleurocidin-like (winter flounder, *Pseudopleuronectes americanus*) AY282498; piscidin-like (large yellow croaker, *Larimichthys crocea*) EU741827. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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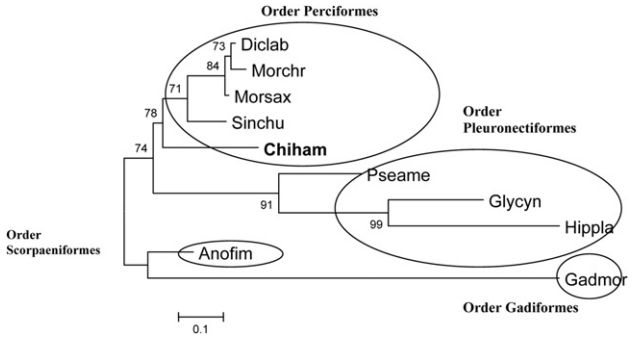

Fig. 2. Phylogenetic tree showing the relationship between chionodracine sequence with other known Teleosts AMP molecules. The tree was constructed by the "neighbour-joining" method using the bootstrap test with 10,000 replications. 0.1 Indicates the genetic distance.

(Panel B, Fig. 4) shows a significant and relevant increase of chionodracine expression after 24 h of stimulation.

3.3. Immunohistochemical detection of chionodracine in gills

The ELISA assay performed on sera of the three mice immunized with chionodracine revealed that mice responded to immunization with a medium serum titre of 1:100 (data not shown), and the one showing highest positivity in ELISA (Pab Ch1) was used for immunohistochemical detection in icefish gills.

The general morphology of the *C. hamatus* gill was characterized by thin and slender filament (or primary lamellae) with elongated secondary lamellae. An epithelial sheet covered filaments and secondary lamellae, in particular a pluristratified primary epithelium covered the filament while a monostratified secondary epithelium covered the secondary lamellae. In the primary and secondary epithelia among the different cell types could be observed immune cells such as lymphocytes and mast cells (Fig. 5). These latter were predominantly found in the primary lamellae and in the interlamellar regions (Fig. 5). They were also distributed in the secondary epithelium. Chionodracine-positive cells were identified by comparing serial sections of gills that were stained with May-Grümwald Giemsa or Pab Ch1 antibody. This comparison evidenced that mast cells were consistently chionodracine-positive and were localized within the primary lamellae, in the interlamellar regions and in the secondary lamellar epithelia (Fig. 5). These mast cells were very numerous large cells (cell diameter 8.43 ± 1.13) in which the immunostaining was distributed throughout the cytoplasm (Fig. 5). No reaction was observed in the control sections (Fig. 5).

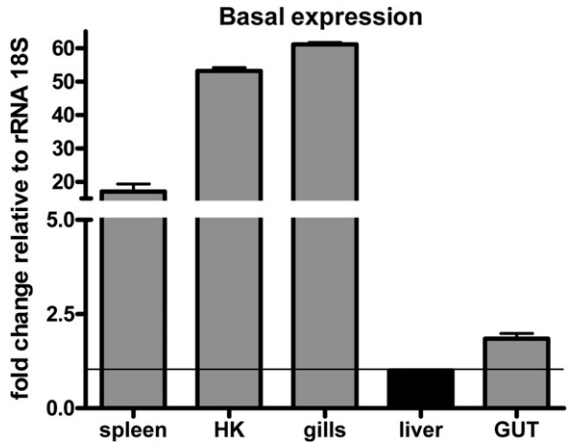

Fig. 3. Chionodracine basal expression in different tissues. Chionodracine mRNA levels were expressed as a ratio relative to rRNA 18 S levels in the same samples after real-time PCR analysis using the tissue with the lowest expression (liver) as calibrator. Data were expressed as the mean ± SD.

3.4. Antibacterial activity assays

The concentrations of the selected chionodracine peptide required to inhibit and to kill the bacterial strains (MIC) are summarized in the Table 1. Among all bacterial strains tested, *E. coli* and *B. cereus*, grown at 25 °C, were the most susceptible to the peptide followed, very closely, by psychrophilic bacteria. At 37 °C, the MIC value for *E. coli* and *B. cereus* were 4-fold and 2-fold higher, respectively, compared to their growth at 25 °C. MBC values were also determined and showed in Table 1. These values were the same of the corresponding MIC values except for *E. coli*. This indicates that the chionodracine peptide is able to exert bacteriostatic rather than bactericidal activity against these two strains.

3.5. Haemolytic activity assay

The haemolytic effect of the selected chionodracine peptide has been tested on human erythrocytes to investigate its capacity to induce membrane lysis. Seven concentrations have been used (starting from a concentration of 50 μM with successive dilutions) that correspond for the first point to 120 μg of peptide (Fig. 6). The percentage of haemolysis is very low for all tested concentrations and it reaches the maximum (0.8%) with the highest concentration value (50 μM).

4. Discussion

The resistance of pathogens to antibiotics is an increasing threat to public health, leading to a demand of new classes of antibiotics that may overcame this problem [11]. In turn, to avoid or delay as much as possible microbial resistance, it could be a strategy to search for antibiotic substances in environments where the ecology of microbial interactions are markedly different. In this view, and considering that different AMPs have already been discovered in fish species, the aim of our work was to investigate the presence of antibacterial peptides in Antarctic species, namely in the icefish *C. hamatus*.

We have thus identified in this species, by homology cloning, an AMP that we named chionodracine, belonging to the antimicrobial family of piscidins. The peptide displays a relative high sequence identity to its homologous in striped bass, sea bass and white bass.

Investigating basal expression analysis of chionodracine mRNA we evidenced highest levels in the gills. This is in agreement with the consideration that in fish the gills are a main portal for entry of pathogens [7] and, therefore, a strong local immune response is needed in this site to try to avoid the development of a disease. Comparing these results with other Teleosts, it could be evidenced that dicentracine in sea bass [15], epinecidin in orange-spotted grouper [16] and a piscidin from mandarin fish [37] appeared to be highly expressed in head kidney, whereas piscidin-4 peptides are present in high concentrations in gills of striped bass [40].

The expression of chionodracine was also studied *in vitro* and *in vivo* in leukocytes, and obtained results gave us the first clues on the possible biological activity of chionodracine. Indeed, we observed a significant up-regulation after 24 h of stimulation with LPS, a component of the external membrane of the *E. coli*, indicating an involvement of chionodracine in the innate immune responses against bacteria. In addition, also a stimulation with poly I:C, that mimics a double-stranded RNA molecule, was observed after 24 h,

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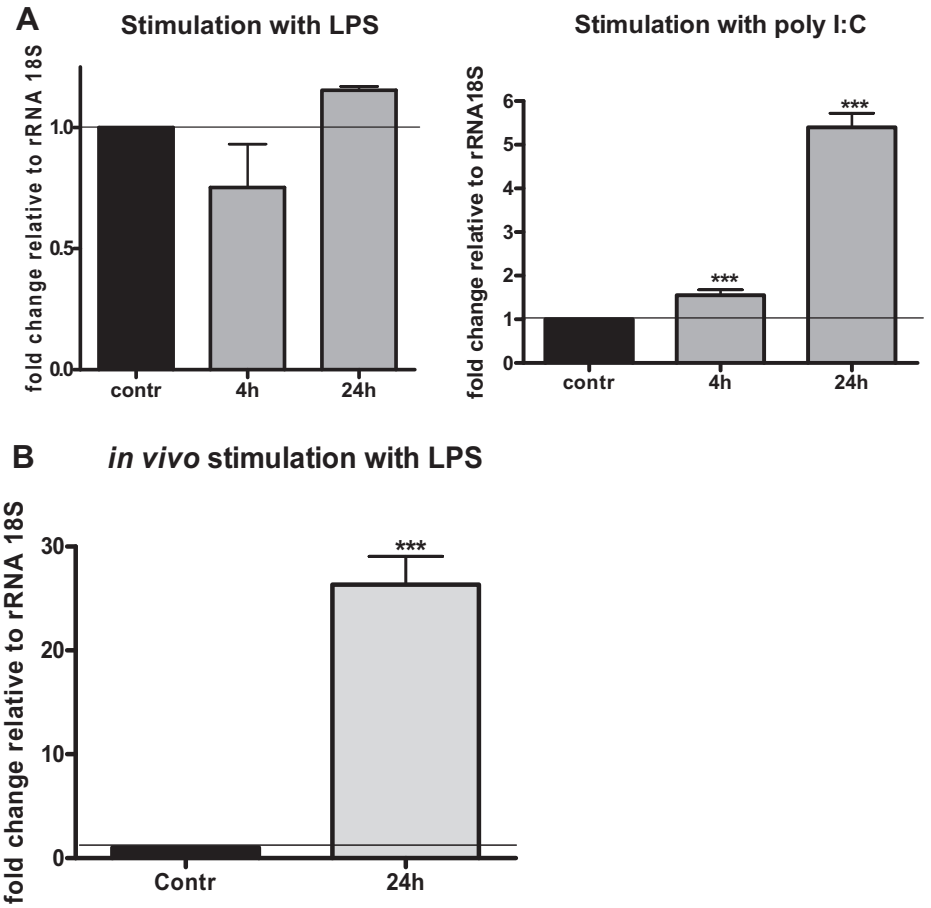

Fig. 4. Chionodracine expression analysis after *in vitro* stimulation with LPS and poly I:C and *in vivo* stimulation with LPS. Panel A: Chionodracine mRNA levels expressed as a ratio relative to rRNA 18 S levels in the same samples after real-time PCR analysis of HK leukocytes stimulated with L15 (control), with 5 μg/ml LPS for 4 and 24 h, and with 50 μg/ml of poly I:C for 4 and 24 h, and normalized against the non-stimulated 0 h control. Data were expressed as the mean ± SD and three asterisks indicates when $p < 0.001$ with respect to the time 0 control. Panel B: Chionodracine mRNA levels expressed as a ratio relative to rRNA 18 S levels after real-time PCR analysis of HK leukocytes stimulated *in vivo* with L15 (control) and with 100 μg of LPS for 24 h and normalized against the non-stimulated 0 h control. Controls and data expression are the same as indicated for Panel A.

thus suggesting an involvement in innate responses against viruses. Similar experiments have been performed *in vivo* in the orange-spotted grouper for epinecidin [16] and in the mandarin fish for a piscidin [37] and in both species a significant increase of expression was found after 6 h of stimulation.

After these encouraging results, we decided to investigate more in detail the putative amino acid sequence of chionodracine to evidence a possible mature antimicrobial peptide. In the alignment of Fig. 1 a prediction of putative mature AMP from the different species is evidenced, although a mature sequence is known only for winter flounder, where pleurocidin was purified from skin [13]. However, some structural features are well defined, as usually antimicrobial peptides are highly amphipathic molecules with hydrophobic and hydrophilic moieties present in different localizations on the surface of the peptide [41]. Therefore, we decided to analyse the primary sequence of our putative mature peptide using some web resources. The server for the prediction of antimicrobial peptides in a protein sequence (AntiBP server http://www.imtech.res.in/cgibin/antibp/antibp1.pl) should give an overall accuracy of about 92% in finding and designing peptide based antibiotics [42] and when used on our sequence the obtained score was quite high (0.817), suggesting a putative high antibacterial activity. Subsequently, the mature sequence has been investigated for its capacity to form an amphipathic α-helical conformation using the server http://rzlab.ucr.edu/scripts/wheel/wheel.cgi created by Don Armstrong and Raphael Zidovetzki. The obtained helical wheel projection is shown in Fig. 7. The chionodracine mature peptide shows two positive charged amino acids, which is a common aspect of most antimicrobial peptides, and it shows in the upper right side of the wheel hydrophobic and hydrophilic amino acids, whereas in the lower left side potentially charged amino acids: therefore the primary sequence possesses all the characteristics needed to form an amphipathic α-helical structure.

The results from structural analyses encouraged the synthesis of a mature peptide to be used for the production of a polyclonal antibody and for other studies. Thus, mice were immunized and a serum was obtained and employed to test by IHC the positivity to chionodracine in gills sections. As expected by the high mRNA expression data, a significant positivity was detected by IHC, and the chionodracine-positive cells resulted to be very similar to mast cells (MC) localized within the primary lamellae, in the inter-lamellar regions and in the secondary lamellar epithelia. This observation is in agreement with previous results, where AMPs were identified in teleost gill epithelial goblet cells [14,43] as well as in immune cells, including neutrophils [44], rodlet cells [45] and mast cells [46,47].

To better understand these results, it should be remembered that in teleosts, MC have been recognized to be important components of not specific immune defence, having populations heterogeneity with varied morphologies, granular content and

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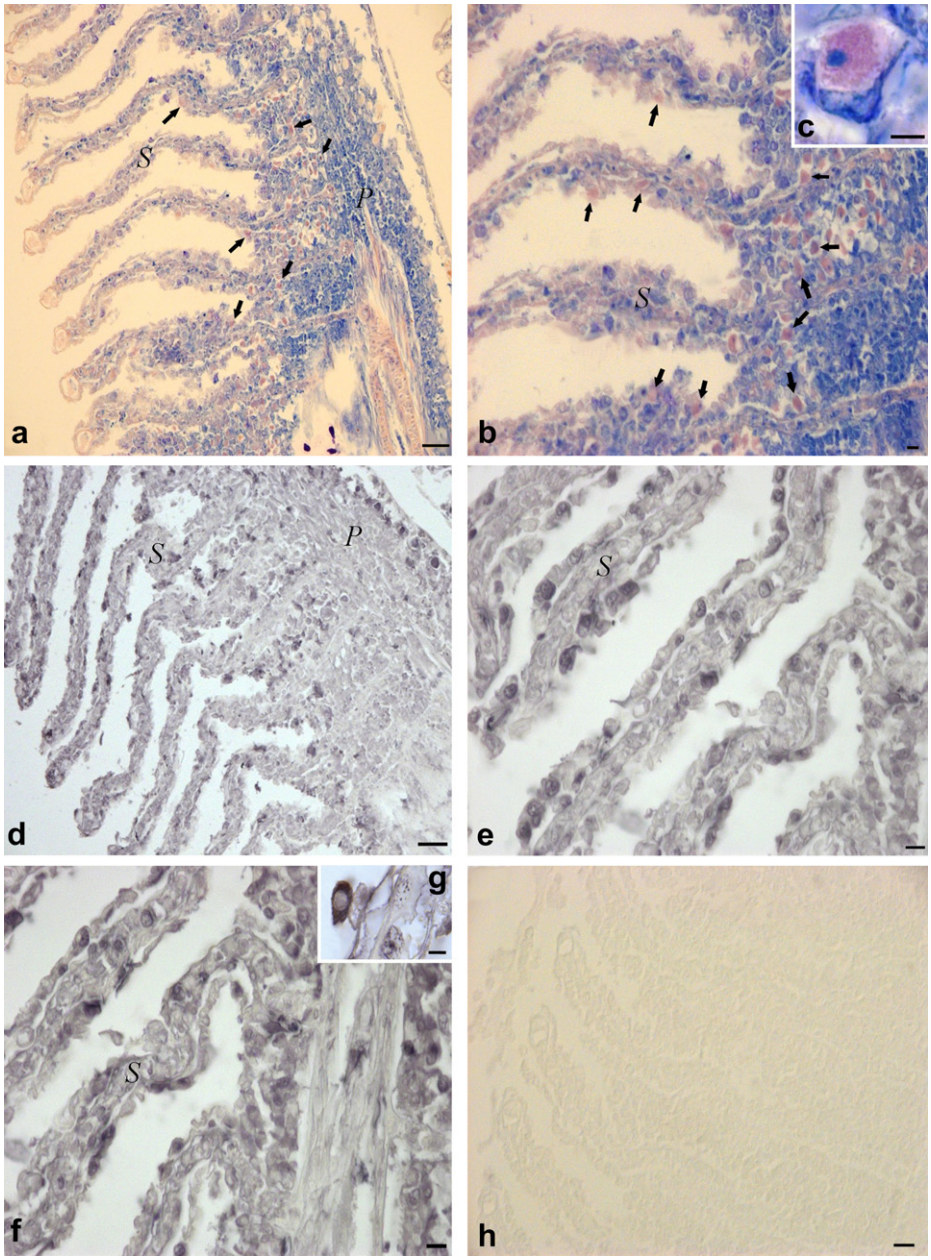

Fig. 5. Histological sections and immunohistochemical localization of chionodracine in the gills of *Chionodraco hamatus*. (a) May-Grümwald Giemsa (MGG) of gill showing within the primary and secondary lamellae several mast cells (arrows). Scale bar = 50 μm. (b) Higher magnification of figure (a) showing the mast cells within the interlamellar regions and in the secondary epithelium (arrows). Scale bar = 10 μm. (c) A mast cell is shown at higher resolution in the insert. Scale bar = 5 μm. (d) Chionodracine-positive cells in the primary and secondary lamellae (arrows). Scale bar = 50 μm. (e) Immunopositive cells prevalent localized in the secondary epithelium. Scale bar = 10 μm. (f) Immunoreactive cells in the interlamellar regions. Scale bar = 10 μm. A chionodracine-positive cell is shown at higher resolution in (g). Scale bar = 2 μm. (h) Negative control showing absence of chionodracine immunoreactivity in gill tissue treated with pre-immune serum. Scale bar = 20 μm. *Key*: *S*: secondary lamella; *P*: primary lamella.

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the chionodracine mature peptide against mesophilic and psychrophilic bacteria at the three different tested temperatures.

Temperature	Species	Gram	MIC (μM)	MBC (μM)
15 °C	*Psychrobacter* sp. TAD1	–	10	10
15 °C	*Psychrobacter* sp. TA144	–	15	15
25 °C	*Escherichia coli*	–	5	30
25 °C	*Bacillus cereus*	+	5	5
37 °C	*Escherichia coli*	–	20	30
37 °C	*Bacillus cereus*	+	10	10
37 °C	*Pseudomonas aeruginosa*	–	ND	ND

response to stimuli, and diverse mediator molecules [48]. The MC have been identified in all vertebrate classes as single-lobed cells containing variable amounts of membrane-bound secretory granules, which store a large series of mediators that in teleost fish are tryptase and histamine. In particular histamine was detected in MC of Perciformes [19], while was absent in Pleuronectiformes, Salmoniformes, Anguilliformes, Cypriniformes and Lepidosireniformes, corroborating the high heterogeneity of MC populations. In addition, increasing evidence supported that in fish the MC are able to secrete AMPs among which the piscidins are the prototypical AMP present in piscine MC [8,14,19,40,43,45,49,50].

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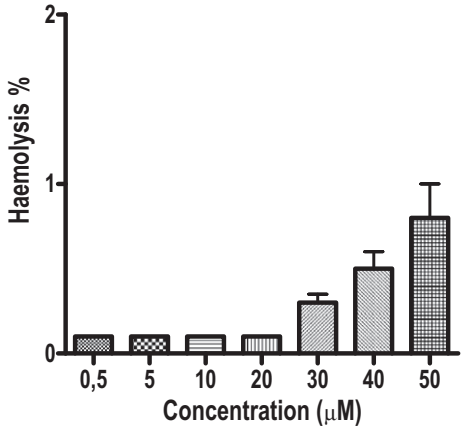

Fig. 6. Haemolytic activity of chionodracine selected peptide against human erythrocytes. Seven different concentrations have been tested, starting from 50 μM with successive dilutions. The values represent the mean ± SD ($n = 3$). A positive control was determined using distilled water and was considered as 100% of haemolysis.

The chionodracine peptide was also employed to investigate a possible biological activity as antibiotic substance, and thus we performed specific antimicrobial assays against two strains of psychrophilic bacteria (*Psychrobacter* sp. TAD1 and *Psychrobacter* sp. TA144), the Gram-negative bacteria *E. coli* and the Gram-positive bacteria *B. cereus* (see Table 1). Taking into consideration the low-temperature Antarctic environment, to study the chionodracine activity we decided to perform further assays growing the *E. coli* and *B. cereus* at lower temperatures than usual (25 °C instead of 37 °C). The result showed that the peptide was more active against both bacteria under these conditions. This could be a first indication that either the peptide is adapted to low temperatures, or that the membranes of the two bacteria change their conformations at low temperatures and could be therefore more easily affected by the peptide. These speculations need more experiments to better elucidate observed activities.

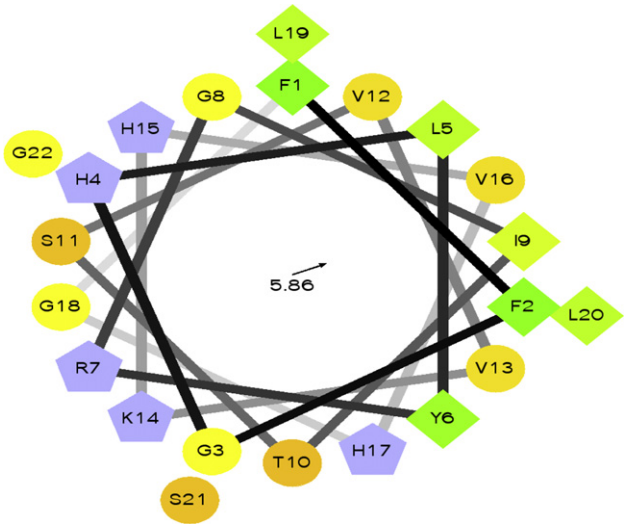

Fig. 7. Helical wheel projection of the chionodracine mature peptide. The hydrophilic residues are presented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is colour coded: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. The black arrow stands for the helical hydrophobic moment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For comparison with fish antibacterial peptides, piscidins have potent, broad-spectrum antibacterial and antifungal activity and have strong antiparasitic activity [8,45,49]. With regard to their mechanism of action, piscidins are thought to inhibit the synthesis of the cell wall, nucleic acids, and proteins or even inhibit enzymatic activity of pathogens [51]. Chionodracine, belonging to the family of piscidins, could be then directly involved in the destruction of pathogens as reported for the pleurocidin by Murray et al. [52]. The AMPs in fact have been recognized in regions of active inflammatory response especially due to parasitic and bacterial infections [47,53,54] and in this sense the gills can be considered one of the tissues first exposed to environmental challenges and pathogens, whose ability to mount an immune defence is crucial [8,52].

In the near future, the capacity of the peptide to kill microbes will be further studied on other fish bacteria (like *Vibrio anguillarum*, *Photobacterium damselae* subsp. *piscicida*, etc.) and viruses (like Betanodavirus, etc.) to test its possible use in aquaculture. Furthermore, its activity will be investigated on human bacteria (other than the already tested *P. aeruginosa*, which was not affected by the peptide), virus and fungi specific pathogens to verify the utility as a new antimicrobial drug. This idea was supported by the haemolytic activity assays, showing that the mature peptide did not induced membrane lysis on human erythrocytes at the tested peptide concentrations, thus providing a first pre-requisite for its possible use as antibiotic molecule.

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