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Stable expression of bioactive recombinant pleurocidin in a fish cell line

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Abstract Pleurocidin (Ple), a linear cationic peptide of 25 amino acids, is a member of a larger family of antimicrobial peptides present in flatfish. Previous studies have shown that Ple displays a strong antimicrobial activity against a broad spectrum of bacteria and appears to play a role in innate host defence. In this work, the genomic sequence encoding the Ple prepropeptide has been isolated from *Limanda limanda* and cloned in a vector under the control of a non-viral promoter (the carp β-actin promoter). By using this construction, expression of bioactive Ple was demonstrated in transformed fish cell lines continuously growing for more than 2 years. Furthermore, the study of Ple processing, maturation and secretion (by using fusion with green fluorescence protein) and the high bactericidal activity of the secreted recombinant Ple (detectable in cell supernatants without any concentration) are all reported here, as no other recombinant Ple or fish antimicrobial peptide have been expressed before to that extent. Such an overexpression of recombinant Ple or any other related antimicrobial peptide might improve the chances to develop new antibiotic agents, as well as to provide essential information about the mechanism of action, range of activity and the role in the innate immune response of antibiotic peptides.

Introduction

Pleurocidins (Ple) are antimicrobial peptides (AMPs) isolated from flatfish in the late 1990s. AMPs are gene-encoded, natural peptide antibiotics of the innate defences of many organisms (Patrzykat et al. 2002, 2003). AMPs have a broad-spectrum activity against bacteria, fungi and/or enveloped viruses (Jia et al. 2000), and they act rapidly and do not easily select for resistant mutants (Hancock and Lehrer 1998; Hancock and Scott 2000; Simmaco et al. 1998). Because of these reasons, research efforts have focused on developing anti-infective treatments based on AMPs (Pearson 2002).

However, only a limited characterisation of AMPs has been accomplished beyond their in vitro assays because of difficulties in purifying AMPs from their natural sources (Yarus et al. 1996) or difficulties in producing AMPs from transformed eukaryotic cells. Thus, most of the reported studies analyse their expression only at the transcriptional, but not at the protein, level. On the other hand, chemical synthesis, when possible, compromises their antibiotic activity due to improper folding (Pore 2000; Yarus et al. 1996).

Because fish rely more heavily on their innate immune defences than mammals (Bly and Clem 1991; Douglas et al. 2003), they constitute a potentially rich source of vertebrate AMPs. However, few AMPs have been reported from fish. Among the mucus-derived fish AMPs, Ple is probably the best characterised. Ple constitute a large family of linear cationic peptides of 21–25 amino acids found in flatfish (Patrzykat et al. 2003). Ple was first isolated from the mucus of the winter flounder (WF) *Pseudopleronectes americanus* (Walbaum) (Cole et al. 1997, 2000). WF Ple was then found in cells of the intestine, eosinophils of the gills (Murray et al. 2003) and at different developmental stages of WF (Douglas et al. 2001). Ple have been also reported from other flatfish, like the American plaice (*Hippoglossoides platessoide*), Atlantic halibut (*Hipoglossus hipoglossus*) or yellowtail flounder (YT) (*Limanda ferruginea*) (Douglas et al. 2001, 2003; Patrzykat et al. 2003).

The genetic organisation of the characterised Ple genes consists of four exons and three introns (Cole et al. 1997; Douglas et al. 2003). They share highly conserved N-terminal signal peptide (pre) and C-terminal acid peptide (pro) sequences, in addition to the mature Ple sequences

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(Douglas et al. 2001; Patrzykat et al. 2003). This results in an initial pre-Ple-pro peptide, which is the precursor of mature and fully bioactive Ple. The prepeptide signal region is encoded by exons 1 and 2, the mature Ple peptide by exons 2, 3 and 4, and the propeptide by exon 4 (Cole et al. 1997). Polymerase chain reaction (PCR) products, obtained by using primers specific for the conserved sequences flanking WF Ple, have identified many other potential Ple-like peptides in the genomic DNA from different species of flatfish (Douglas et al. 2003; Patrzykat et al. 2003).

Mature Ple is predicted to form amphipathic α-helixes and to kill Gram-positive and Gram-negative bacteria (Cole et al. 2000; Syvitski et al. 2005) by forming pores in their membranes, causing cell lysis (Cole et al. 1997; Yoshida et al. 2001). Ple is effective against clinical isolates from human patients with *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* 8534 (resistant to conventional antibiotic treatments) and *Mycobacterium tuberculosis* (Burrowes et al. 2004; Cole et al. 2000). Furthermore, its microbicidal activity is salt-independent (Cole et al. 1997), which may prove beneficial for treatment of cystic fibrosis (Cole et al. 1997; Douglas et al. 2001). On the other hand, the administration of Ple protected salmons from infection by *Vibrio anguillarum* (Jia et al. 2000), suggesting a potential use of Ple as a therapeutic agent in aquaculture to replace conventional antibiotics.

Recombinant WF Ple has been recently produced in *Escherichia coli* as inclusion bodies. However, its specific antibacterial activity was low because it required extensive purification, as it was not secreted out of *E. coli* and it interfered with *E. coli* growth (Bryksa et al. 2006). A eukaryotic Ple in vitro expression system to produce bioactive Ple and to characterise its processing, maturation and secretion in eukaryotic cells has not been reported yet.

We describe here the characterisation, cloning and stable expression of Ple from the mud dab (MD) *Limanda limanda* (Linnaeus) in a carp cell line (*Epithelioma papulosum cyprini*, EPC), under the control of the carp β-actin promoter. Bactericidally active Ple could be produced and secreted to the cell culture media in transformed EPC cells, maintained continuously growing for more than 2 years. To our knowledge, this work is the fist report describing the stable eukaryotic expression of any recombinant Ple. Furthermore, with the use of a pre-Ple-pro-GFP fusion protein, the expression, processing, maturation and secretion of Ple could be easily followed and studied. This kind of study might be the first step to overexpress Ple as a possible therapeutic agent, to transfer Ple genes to other fish species of commercial importance to increase their resistance to infections, and/or to characterise and then use Ple promoters to express specific protein products of pharmaceutical interest in the fish mucus (Rocha et al. 2004b).

Materials and methods

Isolation of genomic DNA of Ple from the mud dab *L. limanda*

Genomic DNA was isolated from the skin of the MD *L. limanda* using the DNeasy Tissue Kit (Qiagen, CA, USA). Genomic DNA was amplified using the PL1/PL3′ primers (PL1, forward: 5′-GCCCACTTTGTATTCGCAAG-3′; PL3′ reverse: 5′-AAGCGTGCAGTCGATGA-3′), previously described on the conserved flanking regions of winter flounder Ple (Douglas et al. 2001). Whilst the primer PL1 was located outside the region coding for the prepeptide, the PL3′ was located inside the propeptide region (Douglas et al. 2001). PCR was performed using Taq DNA polymerase (Roche, Barcelona, Spain). The PCR amplification conditions were: 45 s at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 50.5 °C and 60 s at 72 °C. At the end of the last cycle, the samples were further incubated at 72 °C for 10 min. PCR products were resolved on a 1% agarose gel. Bands were excised from the gel, extracted using GeneClean (Bio 101, La Jolla, CA, USA) and then cloned into the PCR II-Topo vector (Invitrogen, CA, USA). The sequence of the clones was determined by Sistemas Genómicos (Valencia, Spain) using specific primers for the PCR II-Topo vector. Nucleotide sequence comparisons between WF Ple and MD Ple were performed using Basic Local Alignment Search Tool. Intron positions were identified by comparison of the MD Ple genomic sequence with the WF Ple genomic sequence. The amino terminal signal sequence (prepeptide) of Ple was predicted by using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The helical structure of the amino acid sequence of the mature Ple was modelled with the Agadir prediction algorithm (http://www.embl-heidelberg.de). The theoretical molecular weights (MW) were calculated by using protein sequence analysis software (http://pbil.univ-lyon1.fr).

Production of anti-mud dab Ple polyclonal antibodies in rabbit

Once the recombinant cloned Ple was sequenced, the mature sequence of the Ple peptide (GWKKWFKKATHVGKHVG KAALDAYL) was synthesised (Driverdrugs, Barcelona, Spain). The purity of synthetic Ple was >95%, as determined by high-performance liquid chromatography and mass spectrophotometry. To obtain polyclonal antibodies (PAbs), rabbits were first injected with 1 mg/ml of synthetic Ple diluted 1:1 in Freund's complete adjuvant. Four weeks later, a second injection with the same antigen in Freund's incomplete adjuvant was given. Blood was collected before injection (pre-immune serum) and 15 days after the second injection. The blood was then incubated at 4 °C for 2 h and centrifuged to obtain the serum.

Plasmids and plasmid constructs

The expression plasmids, $pQBI_{25}$ of 6.25 kbp (Quantum Biotechnologies, Quebec, Canada) and pGFP of 3.4 kbp (Clontech, CA, USA), both containing the green fluorescence protein (GFP) gene under the control of the Cytomegalovirus early promoter (CMV), and the pAE6 (6.2 kbp), containing the carp β-actin gene promoter (kindly provided by Dr. T.T. Chen, University of Connecticut, USA), were used. To select for permanently expressing cell clones, the plasmid pGEM-pac (Sanchez-Puig and Blasco 2000) was used as a source of puromycin N-acetyltransferase (*pac*) gene. Plasmid pMCV1.4 (ReadyVector, Madrid, Spain) (Rocha et al. 2004a) was used to subclone the *pac* gene before its transfer to the pAE6 plasmid.

To obtain the pAE6-GFP, the GFP cDNA sequence was first excised from the pGFP with the restriction enzymes *Kpn*I and *Xba*I and then subcloned into pAE6 digested with the same enzymes. To obtain the pAE6-Ple, digestion with the restriction enzymes *Kpn*I and *Xba*I was used to excise the DNA genomic sequence of Ple from the PCR II-Topo vector and then subcloned into pAE6 digested with the same enzymes. To obtain the Ple-GFP gene, the GFP cDNA sequence was excised from the pGFP vector with the restriction enzymes *Xba*I and *Not*I and the Ple genomic DNA sequence was excised from the PCR II-Topo with the restriction enzymes *Kpn*I and *Xba*I. At the same time, the pAE6 plasmid was linearised by *Kpn*I and *Not*I. The products were resolved on a 1% agarose gel; the DNA bands were extracted from the gel purified using GeneClean (Bio 101) and ligated.

To obtain the pAE6-*pac* construction, the puromycin resistance (*pac*) gene was first amplified from the plasmid pGEM-*pac* using oligonucleotides 5′-GACGGAGAATTCATGACCGAGTACAAG-3′ (*Eco*RI site underlined) and 5′-CTCAAGGGATCCTCAGGCACCGGGCTT-3′ (*Bam*HI site underlined). Plasmid pMCV1.4 linearised with *Eco*RI and *Bam*HI was ligated with the PCR amplified *pac* gene to obtain the pMCV1.4 *pac.* Then, the *pac* gene was excised from the pMCV1.4 *pac* vector with the restriction enzymes *Kpn*I and *Xho*I and subcloned into the fish vector pAE6 digested with the same enzymes.

Temporal expression assays in transfected EPC cells

Epithelioma papulosum cyprini cells, a fish cell line isolated from carp (Fijan et al. 1983), were grown in 24-well plates at 28 °C in a 5% CO_2 atmosphere with 500 µl of RPMI-1640 Dutch modified (Gibco, Invitrogen, UK) cell culture medium, containing 10% fetal calf serum (Sigma Chem., St. Louis, MO), 1 mM Piruvate (Gibco), 2 mM Glutamine (Gibco), 50 µg/ml gentamicin (Gibco) and 2 µg/ml fungizone. EPC cells were plated at 300,000 cells/ml, and the next day, 1 µg of plasmids complexed with 2 µl of FuGene 6 (Roche) were incubated for 15 min in 50 µl of RPMI-1640, 2 mM Cl_2Ca, and then added to the EPC cell monolayers in 400 µl of cell culture medium with 10% of fetal calf serum (Lopez et al. 2001; Rocha et al. 2004a). The cells were incubated during different times before the assays.

The efficiency of expression was calculated by the formula, number of cells expressing the transgene per well/total number of cells per well ×100. Both total number of cells and cells expressing the transgene were counted using a Leica model inverted fluorescence microscope (Leica, Cambridge, UK) provided with a digital camera.

Generation of transformed EPC cell lines stably expressing Ple

EPC cell monolayers in 6-well plates were co-transfected with 1.5 µg of pAE6-GFP, pAE6-Ple-GFP or pAE6-Ple plus 0.5 µg of pAE6-*pac*. After transfection, puromycin-resistant cells were selected by adding 20 µg/ml of puromycin (Sigma) to the cell culture media at 6 days. Resulting puromycin-resistant cells were seeded in 96-well plates at a density of 1–50 cells/well (limiting dilution) and grown in cell culture medium conditioned by the growth of non-transfected EPC cells to favour growth of isolated cells. Twenty-four hours later, the wells were screened for the presence of single cells. Two weeks later, single colonies were transferred to wells of 48-well plates and grown in conditioned medium. Cell lines expressing Ple were selected among the puromycin-resistant clones transfected with pAE6-Ple by immunofluorescence using anti-synthetic Ple PAb. Cell lines expressing GFP or Ple-GFP were selected among the puromycin-resistant clones transfected with pAE6-GFP or pAE6-Ple-GFP by GFP expression by using an inverted fluorescence microscope (Leica). Positive cell lines, one clone each for GFP, Ple-GFP and Ple, were selected for further work. They were grown in 96-well plates and gradually transferred into cell culture flasks. EPC cell lines have been maintained by continuous culture (about one subculture per week) in the absence of puromycin, as described above for non-transformed EPC cell monolayers for more than 2 years.

Detection of mRNA by reverse transcription-polymerase chain reaction in stably transformed EPC cell lines

Total RNA was isolated from EPC cell lines expressing GFP, Ple-GFP or Ple using the "Total RNA Isolation System" (Promega). The isolated RNA was treated with DNase (RQ1 RNAase-Free Dnase, Promega) and then reverse transcription (RT) was performed using Moloney murine leukaemia virus reverse transcriptase (Gibco) and a Poly-T primer (Applied Biosystems, Melbourne, Australian).

As forward primer, the PL5 (5′-ATGAAGTTCACTGCCACCTTC-3′), corresponding to the amino terminal part of the precursor polypeptide of WF Ple, and the reverse primer PL3 (5′-TTCATCGACTGCGCGCTT-3′) were used for amplification of Ple (Douglas et al. 2001) (Fig. 1). The GFP-forward primer 5′-ATGGTGAGCAAGGGCGAG

GAG-3′ and the GFP-reverse primer 5′-CCGCTTTACTTG TACAGCTCG-3′ were used for amplification of GFP. The forward primer PL5 and the GFP-reverse primer were used for amplification of the Ple-GFP. PCR was performed using Taq DNA polymerase (Roche). The PCR amplification conditions were: 1 min at 94 °C; 30 cycles of 30 s at 94 °C; 30 s at 60.5 °C and 30 s at 72 °C. At the end of the last cycle, the samples were further incubated at 72 °C for 5 min. The amplified products were resolved on a 1.5% agarose gel. Bands were excised, cloned and sequenced as described above.

Detection of recombinant Ple by Western blot in stably transformed EPC cell lines

Supernatants from confluent cultures of EPC cell lines expressing GFP, Ple-GFP or Ple were harvested, and the cells were frozen and defrozen, pelleted by centrifugation at 6,000×*g* for 45 min and resuspended in phosphate buffered saline (PBS) containing phenylmethanesulfonyl fluoride (100 μg/ml). Sodium dodecyl sulfate-polyacrylamide gels (4–20% Tris–Glycine gel, Novex pre-cast gels, Invitrogen) were loaded with 20 μl of samples in buffer containing mercaptoethanol. A synthetic Ple sample of 50 ng was also included in the gels. For Western blot, the proteins in the gel were transferred at 3 h at 125 V in 2.5 mM Tris, 9 mM glycine and 20% methanol to nitrocellulose membranes (BioRad, Richmond, VA, USA). The membranes were then blocked with 2% dry

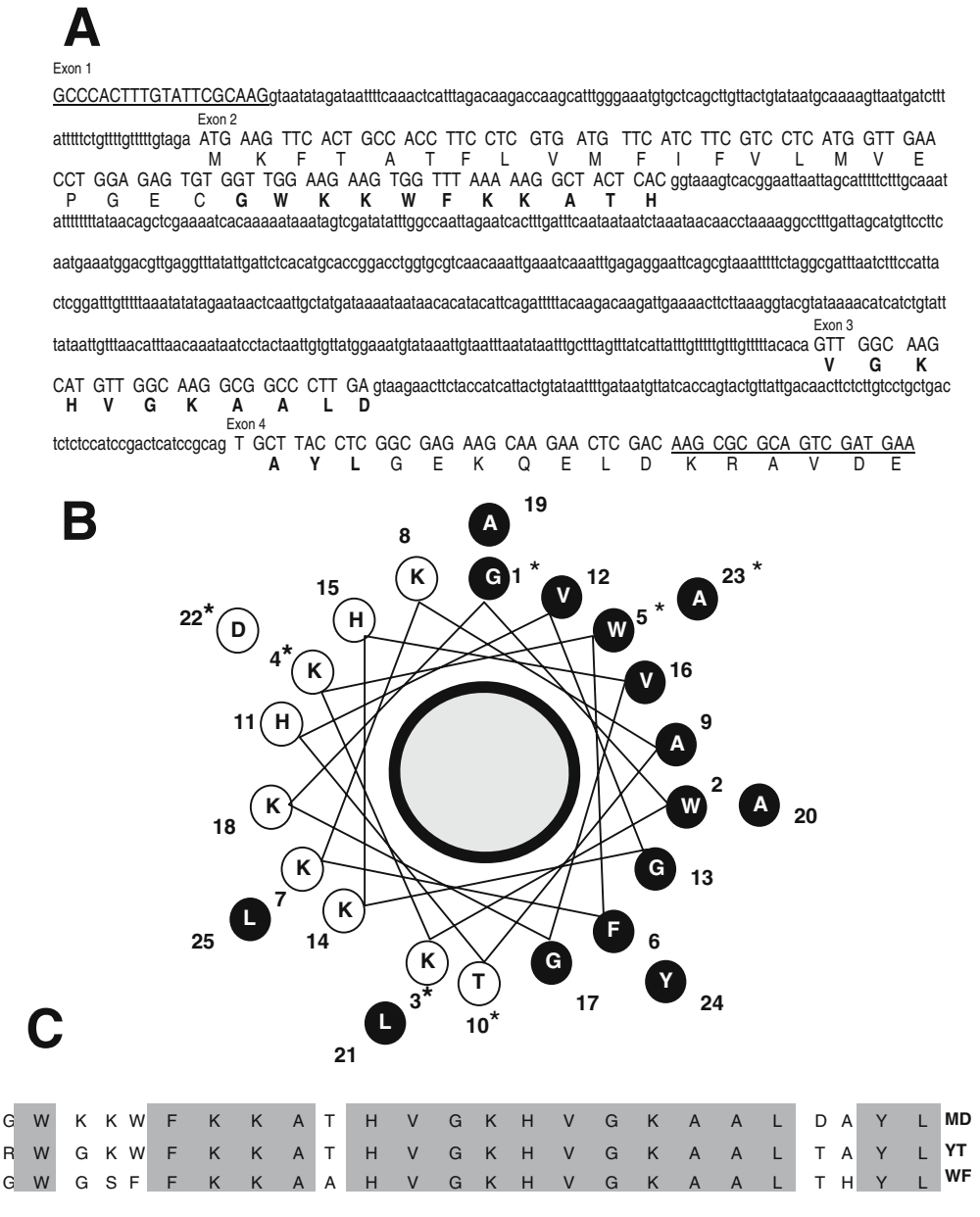

Fig. 1 Genomic sequence (**a**), helical wheel diagram (**b**) of MD Ple and amino acid sequence comparison among MD, WF and YT Ple. **a** The genomic sequence was obtained by PCR amplification using primers previously described for WF Ple (Douglas et al. 2001). *Lower case* are introns, *upper case* are coding sequences. Presented in *boldface* are amino acid sequences of mature Ple. *Underlined* are primers used for PCR amplification. **b** Helical wheel diagram of MD Ple. *Black circles* represent hydrophobic residues and glycines. *White circles* represent hydrophilic residues. The first amino acid (G) of the mature Ple peptide is placed at the *top* of the wheel. *Asterisks* mark positions that show variable amino acids in WF and YT Ple. **c** Alignment of amino acid sequences of mature Ple from MD, WF (Cole et al. 2000) and YT (Douglas et al. 2003). Amino acid conserved positions are *shadowed*

milk, 0.05% Tween-20 in PBS and incubated with a MAb anti-GFP (Santa Cruz Biotechnology, CA, USA) or with PAb anti-synthetic Ple before incubation with a peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit antibody (Nordic, Tilburg, The Netherlands), respectively. The peroxidase activity was detected by using the ECL chemiluminescence reagents (Amersham Biosciences, UK) and revealed by exposure to X-ray films (Amersham).

Time course of Ple-GFP release from stably transformed EPC cell lines

For the fluorometric analysis of GFP, 200 μl of cell culture supernatants from EPC cell lines, expressing GFP or Ple-GFP grown in 25 cm^2 culture flasks, were collected on days 0, 2, 4, 6, 8, 10 and 12 after cell plating. Fluorescence was measured at excitation 485 nm/emission at 520 nm in a fluorescence microplate reader (Fluorostar Galaxy BMG Labtechnologies, Gmbh, Germany). Relative fluorescence was expressed by the formula fluorescence in supernatants from the EPC cell line expressing GFP or Ple-GFP/ fluorescence in supernatants from non-transfected EPC cell cultures.

Cytotoxicity assay of synthetic Ple

The cytotoxic effects of synthetic Ple on EPC monolayers were determined by quantifying the EPC cell viability using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium]-based assay (Cell Titer 96; Promega, Mannheim, Germany). Cytotoxicity was examined following 2 (acute cytotoxicity) and 8 days (chronic cytotoxicity) of EPC cell monolayer exposures to 0.002 to 8 μg/ml of synthetic Ple.

Bactericidal assay of synthetic and recombinant Ple

E. coli (*E. coli* DH5-α strain) was employed in these assays. A single colony of *E. coli* was inoculated into Luria-Bertani (LB) media and cultured overnight at 37 °C. An aliquot of this culture was transferred to fresh LB media and incubated during 3 h at 37 °C to obtain mid-logarithmic-phase cells. Then, the bacteria were diluted again in fresh LB medium, and the number of colony-forming units (CFU) per milliliter was assayed in semisolid agar.

The minimal inhibitory concentration (MIC) of synthetic Ple was determined by incubating ~5×10^4 CFU of *E. coli* in 50 μl of LB media with serial dilutions of synthetic Ple in water (final volume of 50 μl) in wells of 96-well plates. Plates were incubated overnight at 37 °C, and the next day the *E. coli* growth was determined by measuring the absorbance at 620 nm. The lowest concentration of synthetic Ple that completely inhibited bacterial growth was defined as the MIC. Two independent experiments, each by duplicate, were performed to determinate the MIC of synthetic Ple.

To determine the anti-*E. coli* activity of the recombinant Ple expressed by the EPC cell lines, supernatant extracts had to be obtained free of serum and dialysed before performing the CFU assay because the serum present in the cell culture media could inhibit the activity of recombinant Ple (data not shown). Both non-transfected EPC cells and the EPC cell lines expressing Ple-GFP or Ple were grown into 25-cm^2 culture flasks as described before. Twenty-four hours after plating, the culture medium was removed, the cells washed with PBS and the serum-free medium was supplemented with L-glutamine added to the flasks. Flasks were then incubated at 28 °C, and 6 days later, cell-culture supernatants were clarified by centrifugation at 3,000×*g* for 15 min. The cell-culture supernatants were then dialysed against distilled water, using cellulose membranes of 1 kDa of cut-off, and were kept in aliquots that were frozen at −70 °C until they were used. The cell monolayers were washed with PBS and 2 ml of distilled water was added. Cells were frozen and defrozen, and supernatants from these cell lysates were clarified by centrifugation at 3,000×*g* for 10 min and kept in aliquots frozen at −70 °C until use.

Fifty microliters of dialysed culture supernatants or cell lysates from non-transfected EPC cells and EPC cell lines expressing Ple-GFP or Ple were mixed with 50 μl of *E. coli* suspensions containing 10^5, 10^4 or 10^3 CFU/ml and incubated for 1 h at 37 °C. One microgram of synthetic Ple, in the same loading volume, was used as positive control. Serial dilutions from each mixture were then plated on LB-agar plates, and the number of CFUs was determined after overnight incubation at 37 °C. *E. coli* CFUs, after incubation with dialysed supernatants or cell lysates, were calculated by the formula number of CFU after incubation of *E. coli* with supernatants or cell lysates/ number of CFU after incubation of *E. coli* in the absence of any additives ×100.

Injection of plasmids into trout skeletal muscle

Two micrograms each of PBS, pAE6, pAE6-GFP, pAE6-Ple-GFP and pAE6-Ple in 100 μl of PBS were injected into the skeletal muscle of four anaesthetised fingerling rainbow trout (about 10 cm length) per plasmid using a Microliter syringe (Hamilton, USA). Seven days later, 1×1×0.5 cm slides (about 100 mg of wet weight) of muscle tissue samples were taken from the injected area, homogenised in 300 μl of distilled water and clarified by centrifugation as described (Hwang et al. 2003). Protein was adjusted to 0.1 mg/ml by using the Bradford reagent (Biorad, Madrid, Spain) and frozen at −20 °C until use.

Determination of GFP and recombinant Ple expression in injected trout muscle by ELISA

One hundred microliters of muscle homogenates (about 10 μg of protein) were dried per well of 96-well polystyrene plates (Dynatech, Plochingen, West Germany) by incubation overnight at 37 °C. They were kept sealed, with blue silica gel, at a temperature of 4 °C until they were used. Before use, the coated plates were incubated for 1 h at room temperature with 3% dry milk in dilution buffer (0.24 mM merthiolate, 0.5 g of Tween 20, 50 mg of phenol red in PBS) and then washed. The plates were then incubated for 120 min at room temperature with 100 μl/well of the diluted antibodies (Abs). The MAb anti-GFP 1,000-fold diluted with dilution buffer was used to detect GFP expression. The PAb anti-synthetic Ple PAb described above was used 100-fold diluted to detect Ple. Then, the plates were washed once with distilled water and a peroxidase-labelled mouse anti-rabbit PAb (Nordic) 2,000-fold diluted in dilution buffer was added (100 μl/well). The plates were further incubated for 45 min and washed three times with distilled water. For colour development, 50 μl of substrate buffer (150 mM sodium citrate, 3 mM H_2O_2 and 1 mg/l *o*-phenylenediamine, pH 4.8) were pipetted per well, and the reaction was stopped after 30 min with 50 μl per well of 4 N H_2SO_4. The absorbance at 492 nm was used to estimate enzymatic activity and the absorbance at 620 nm to correct for individual-non-significant differences between wells.

Nucleotide sequence accession number The GeneBank accession number of the *L. limanda* Pleurocidin gene is DQ248966.

Results

Amplification and cloning of the MD Ple gene

A band of ~900 bp was amplified by PCR by using the primers PL1/PL3′ derived from the WF Ple. The extracted band was cloned into the PCR II-Topo vector and two independent clones were sequenced. The 966-bp sequences of the PCR products were coincident for the two independent clones (Fig 1a). By comparison with the published WF Ple gene, the MD Ple gene sequence consisted in four exons and three introns (Cole et al. 2000; Douglas et al. 2001) and encoded from exon 1 (from the transcriptional start site) to a truncated exon 4, which included the sequence of 13 aa of Ple or C-terminal peptide. Comparison between the nucleotide sequences, corresponding to the exons of mature WF and MD Ple, showed an 80% overall homology. Intron sequences and sizes varied slightly (data not shown).

The MW calculated for the Ple prepeptide (22 amino acids), mature Ple peptide (25 amino acids) and Ple propeptide (13 amino acids) were 2.5, 2.8 and 1.5 kDa, respectively. The theoretical MW of the Ple prepropeptide was, therefore, 6.8 kDa.

The first 22 amino acids of the open reading frame of MD Ple form a highly hydrophobic domain predicted to form a signal peptide similar to that of WF Ple that is not present in mature WF Ple (Douglas et al. 2001).

Figure 1b shows the predicted amino acid sequence of the mature MD Ple in an amphipathic α-helical conformation, indicating hydrophobic and hydrophilic residues on opposing sides of the helix. The hydrophilic surface was cationic as it was described for WF Ple (Cole et al. 2000; Douglas et al. 2001). Alignment among the amino acid sequences of most characterised Ple shows that the mature MD Ple sequence differs by six and three amino acids to the WF and YT mature Ple, respectively, in a total of seven different positions. This indicates a high degree of conservation among the amino acid sequences of mature Ple in flatfish (Fig. 1c). The seven positions showing amino acid variations corresponded to three similar locations defined by positions 1, 5 and 23 (hydrophobic side) and 4, 22, 3 and 10 (hydrophilic side) on the α-helix (positions labelled with an asterisk in Fig. 1b).

Generation of EPC cell lines stably expressing GFP, Ple-GFP and Ple

To select the most active promoter to be used in EPC cells to clone Ple, β-actin carp and CMV promoters were compared for GFP gene expression by using the pAE6-GFP (β-actin carp promoter) and $pQBI_{25}$-GFP (CMV promoter) plasmids. Figure 2a shows that the percentage of fluorescent cells was about threefold higher when GFP expression was driven by the β-actin carp promoter. Figure 2b,c compares the morphological aspects of the EPC cell monolayers transfected with the two plasmids. Therefore, the β-actin carp promoter was used to obtain transformed EPC cell lines expressing Ple.

To obtain transformed EPC cell lines, the pAE6-GFP, pAE6-Ple-GFP, pAE6-Ple and pAE6-*pac* plasmids (containing the GFP, Ple or *pac* genes under the control of the β-actin carp promoter) were first constructed as indicated in the "Materials and methods" section. Then EPC cell monolayers were co-transfected with pAE6-GFP, pAE6-Ple-GFP or pAE6-Ple and the pAE6-*pac*. Selection of the transformed EPC cells in the presence of puromycin allowed the isolation of at least one expressing clone for each pAE6-GFP, pAE6-Ple-GFP and pAE6-Ple plasmid. The whole process was similar for the three cases. For instance for pAE6-Ple, after puromycin selection and limiting dilution of the surviving cells, six different single colonies were isolated, grown and screened for Ple expression by immunofluorescence using anti-synthetic Ple PAb (not shown). Results demonstrated that four colonies were positive and that fluorescence was detected in 100% of the cells in the colonies, suggesting that they were true clones and that all their cells were expressing recombinant Ple. One of these colonies was selected and grown for further experiments. Each of the selected colonies expressing GFP, Ple-GFP and Ple clones has

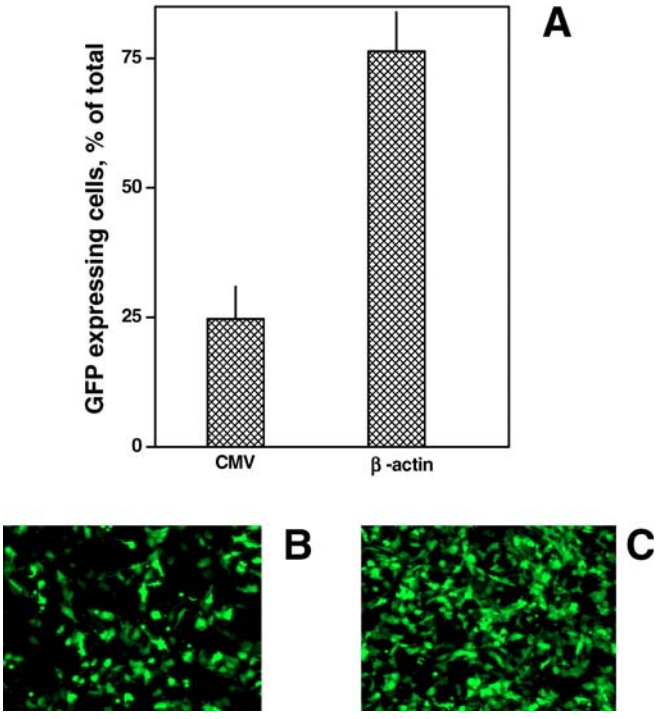

Fig. 2 Selection of the most active promoter in transiently transfected EPC cells. EPC cell monolayers were transfected with $pQBI_{25}$-GFP (CMV promoter) or pAE6-GFP (β-actin carp promoter. Forty-eight hours after transfection, fluorescent cells were counted (**a**) and photographed (**b**, **c**). The percentages of fluorescent cells were calculated by the formula: number of fluorescent cells per well/ total number of cells per well) ×100. Averages and standard deviations from two different experiments each by triplicate are given. **b** EPC cells transfected with $pQBI_{25}$-GFP photographed under the fluorescence microscopy. **c** EPC cells transfected with pAE6-GFP photographed under the fluorescence microscopy

been grown without interruption and has been propagated continuously by serial passages for more than 2 years.

To confirm the expression of recombinant proteins in the EPC cell lines selected and described above, specific mRNA expression was estimated by RT-PCR. The presence of ~700, ~900 and ~200 bp bands were obtained for GFP, Ple-GFP and Ple expressing EPC cell lines, respectively (Fig. 3a). The nucleotide sequence of the bands showed that the 200-bp band (Fig. 3a, lane 1) corresponded to the sequence of Ple without the intron sequences, indicating that Ple mRNAs were correctly processed in the corresponding EPC cell line. Similarly, the nucleotide sequences of the 700-bp (Fig. 3a, lane 2) and the 900-bp (Fig. 3a, lane 3) bands were coincident with the expected sequences of GFP and Ple fused to GFP, respectively.

Study of the processing, maturation and secretion of Ple-GFP

GFP was diffusely distributed in the cell cytoplasm and nucleus of the EPC cell line expressing GFP (Fig. 3b). Addition of the Ple genomic sequence to the N terminus of GFP caused a relocalisation of the fluorescence into the cells. Thus, 24 h after plating the EPC cell line expressing Ple-GFP, the bulk of the fluorescence appeared in the perinuclear region of the cell (Fig. 3c), where the trans-Golgi network is usually located. After 48 h, nearly all the cells exhibited a non-uniform granulate cytoplasmic distribution of the fluorescence (Fig. 3d). In contrast with the fluorescence in the EPC cell line expressing GFP, the fluorescence was excluded from the nuclear region in the EPC cell line expressing Ple-GFP. No effects of the Ple-GFP expression on EPC cell morphology or viability were detected.

Because the fluorescent granula within the cell cytoplasm could belong to secretory granules on their way to secretion, cell culture supernatants from the EPC cell line expressing GFP or Ple-GFP were collected every 2 days for 12 days and fluorescence was measured. Figure 4 shows that fluorescence of the cell culture supernatants from the Ple-GFP expressing EPC cell line showed a continuous increase with the time in culture as expected if there were a continuous secretion of Ple-GFP. In contrast, no significant variation in the fluorescence levels of the cell culture supernatants from the GFP-expressing EPC cell line was observed.

The intracellular accumulation (cell lysates) and extracellular secretion to the cell culture medium (culture supernatants) from the EPC cell lines expressing Ple-GFP or Ple were analysed by Western blot (Fig. 5) using anti-GFP (Fig. 5a) and anti-synthetic Ple (Fig. 5b) Abs.

With the anti-GFP, the expected ~29-kDa band corresponding to GFP was obtained in both cell lysates and cell culture supernatants obtained from the GFP-expressing EPC cell line (Fig. 5a lanes 1 and 3, respectively). The ~29-kDa band was more intense in cell lysates than in culture supernatants as expected, if GFP was not secreted. In both cell lysates and culture supernatants from the Ple-GFP expressing EPC cell line, two bands at ~34 and ~31 kDa were identified (Fig. 5a, lanes 2 and 4, respectively). The band at ~34 kDa might correspond to Ple-pro-GFP (2.8+1.5+29=33.3 kDa), whilst the band at ~31 kDa might correspond to pro-GFP (1.5+29=30.5 kDa). The bands were more intense in culture supernatants than in cell lysates as expected, if the Ple-pro-GFP and the pro-GFP were secreted.

With the anti-Ple, in both cell lysates and culture supernatants from the Ple-GFP expressing EPC cell line, the band at ~34 kDa was again stained (Fig. 5b, lanes 1 and 3), thus confirming that this band, detected by both anti-GFP and anti-Ple, most probably corresponded to Ple-pro-GFP. This result also suggests that the 31-kDa band in the anti-GFP Western blot mentioned above corresponded to pro-GFP, since it is not detected by the anti-Ple.

With the anti-Ple, synthetic Ple appeared as a ~6.5-kDa double band instead of appearing at its theoretical 2.8-kDa single band (Fig. 5b, lane 5). The same double band at ~6.5 kDa appeared in culture supernatants from the EPC cell line expressing Ple (Fig. 5b, lane 4). Only one ~6.5-kDa band appeared in cell lysates from the EPC cell line expressing Ple (Fig. 5b, lane 2) and a band >6.5 kDa

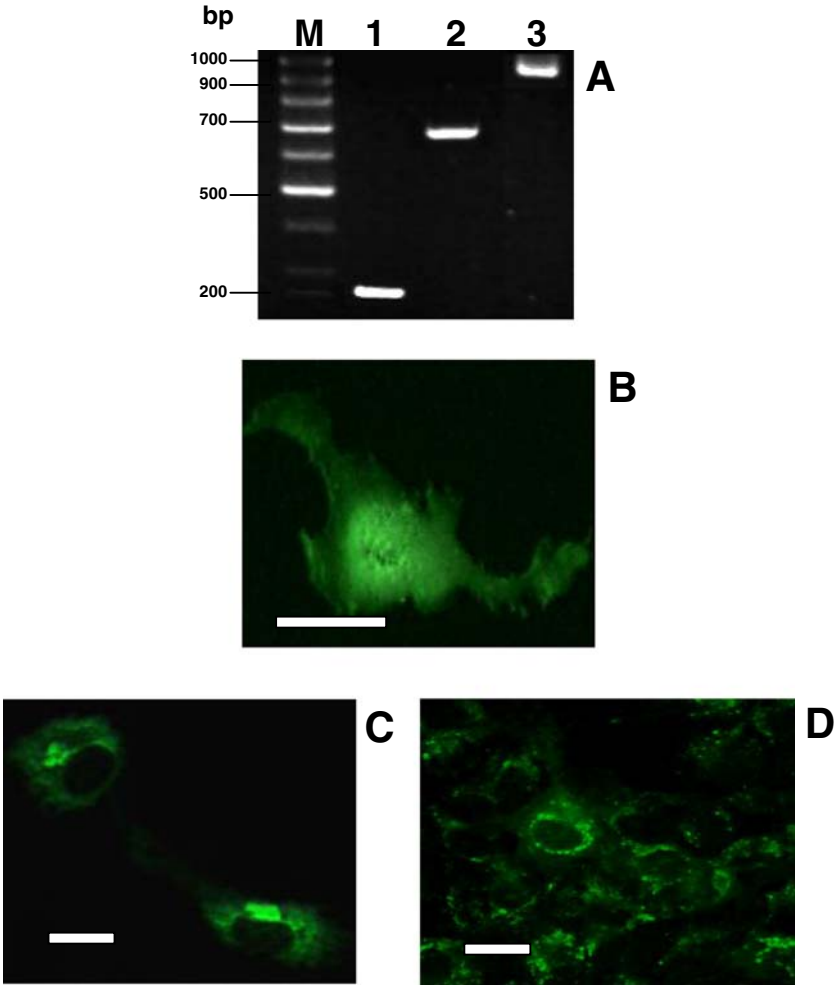

Fig. 3 Agarose gel of the RT-PCR from Ple, Ple-GFP and GFP expressing EPC cell lines (**a**) and fluorescence microscopy photographs of GFP (**b**) and Ple-GFP cell lines (**c**, **d**). **a** Total RNA was isolated from EPC cell lines expressing Ple, Ple-GFP or GFP. *Lane 1* Ple, *lane 2* Ple-GFP, *lane 3* GFP. **b** Cells from the EPC cell line expressing GFP photographed under the fluorescence microscopy. **b** Cells from the EPC cell line expressing Ple-GFP, photographed under the fluorescence microscopy **c** 24 and **d** 48 h after plating. *White bars* 10 μm

appeared in culture supernatants from the EPC cell line expressing Ple-GFP (Fig. 5b, lane 3). Bands corresponding to the expected 35.8 kDa of the pre-Ple-pro-GFP were not found by using anti-GFP or anti-Ple.

Bactericidal activity of recombinant Ple

First, the antimicrobial activity of synthetic Ple towards *E. coli* was tested and, as expected, synthetic Ple showed activity against *E. coli* with a MIC of 4.9±0.69 μM (data not shown). Then, to test the antimicrobial activity of recombinant Ple, *E. coli* suspensions were incubated with cell lysates or supernatants from EPC cell lines expressing Ple-GFP or Ple. The resulting CFU were counted with reference to *E. coli* growth in LB culture medium, which was regarded as 100%. Under those conditions, cell lysates and non-concentrated cell culture supernatants from EPC cell lines, expressing Ple-GFP or Ple, showed bactericidal activities that reduced to 50–60% the numbers of CFU, whilst cell lysates and cell culture supernatants from non-transfected EPC cells showed bactericidal activities that reduced the numbers of CFU by only ~10% (Fig. 6a,b).

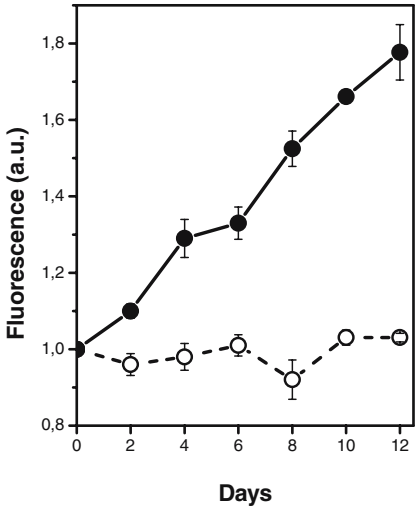

Fig. 4 Time-course of release of GFP fluorescence in cell culture supernatants from EPC cell lines expressing GFP and Ple-GFP. Culture supernatants from EPC cell lines expressing GFP and Ple-GFP were collected every 2 days during 12 days of incubation and the fluorescence measured. *Filled circles* represent culture supernatants from the Ple-GFP expressing cell line. *Open circles* represent culture supernatants from the GFP expressing cell line. *au* arbitrary units

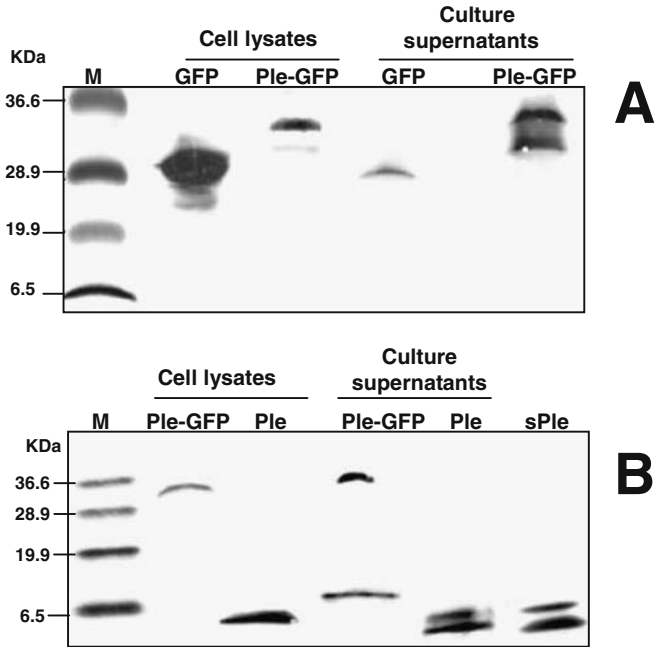

Fig. 5 Western blot of GFP, Ple-GFP and Ple expressing EPC cell line extracts The expression of GFP, Ple-GFP or Ple peptides was analysed in cell lysates and cell culture supernatants from EPC cell lines expressing GFP, Ple-GFP or Ple by Western Blot. **a** Western Blots obtained by using an anti-GFP MAb. **b** Western Blots obtained by using anti-synthetic MD Ple PAb. *M* MW standards, *GFP* EPC cell line expressing GFP, *Ple-GFP* EPC cell line expressing Ple-GFP, *Ple* EPC cell line expressing Ple, *sPle* 50 ng of synthetic Ple

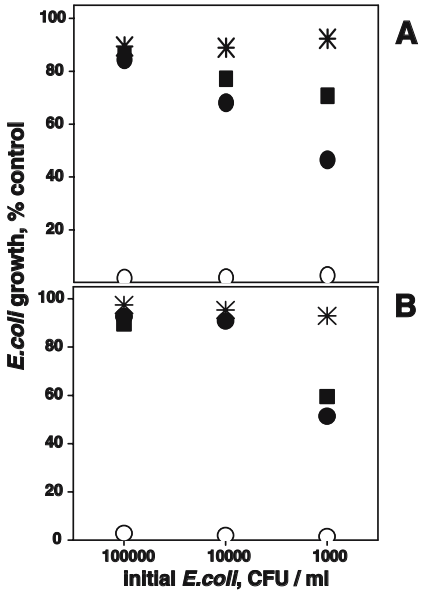

Fig. 6 Antimicrobial activity of cell lysates (**a**) or supernatants (**b**) from Ple-GFP and Ple expressing EPC cell lines. EPC cell lines expressing Ple-GFP, Ple or non-transfected were grown in 25 cm^2 culture flasks in serum-free medium during 6 days. Then, the cell culture supernatants were harvested and cell monolayers were lysed and both dialysed by using membranes with ~1 KDa of cut-off. Fifty µl of E.coli DH5-α suspensions mixed with 50 µl of lysates (**a**) or supernatants (**b**) were incubated during 1 h at 37 °C. One microgram of synthetic Ple in 50 µl was used as positive control. Serial dilutions were then plated on LB-agar plates, and the number of CFU were determined the following day. The percentage of *E. coli* colonies was calculated by the formula, (number of CFU after incubation with lysates or supernatants/number of CFU after incubation in the absence of any additives) ×100. The data presented in the figure are representative of two independent CFU assays. *Asterisks* non-transfected EPC cells, *filled circles* EPC cell line expressing Ple, *filled squares* EPC cell line expressing Ple-GFP, *open circles* synthetic Ple

Cytotoxicity and in vivo expression of Ple

The potential use of Ple in aquaculture will depend on both the absence of cytotoxicity and the in vivo activity in commercially important fish. For assaying Ple cytotoxicity, acute and chronic toxicities were tested by using synthetic Ple in EPC cell monolayers. For assaying in vivo activity, the rainbow trout was chosen as an example of commercially important fish.

Cytotoxicity was examined following 2 (acute cytotoxicity) and 8 (chronic cytotoxicity) days of exposure of EPC cell monolayers to 0.002 to 8 µg/ml of synthetic Ple. No cytotoxic effects were observed for any of the synthetic Ple concentrations and/or times used for the assay (data not shown).

In vivo activity was assayed by injecting skeletal muscle of rainbow trout with the constructs pAE6-GFP, pAE6-Ple-GFP and pAE6-Ple. All the injections resulted in levels of expression in muscle tissue that were detectable at three- to fourfold above background levels, 7 days after injection (Fig. 7).

Discussion

The four exons and three introns of the mud dab (MD) *L. limanda* L. Ple gene have been amplified, cloned, expressed in a fish cell line derived from carp (EPC) and characterised as a previous step to favour its possible applications.

The MD flatfish was chosen as a Mediterranean flatfish species of commercial value, widely used also as a sentinel species for in vivo chemical toxicity assays to compare it with the two other previously characterised Ple, those from *P. americanus* (winter flounder, WF) and *L. ferruginea* (yellowtail flounder, YF). For expression of the whole gene, the *Epithelioma papulosum cyprini* (EPC) cell line isolated from carp (Fijan et al. 1983) was chosen because it has been described as the best predictor of plasmid activity in transgenic fish (Moav et al. 1992), it can be readily transfected (Castric et al. 1992; Lopez et al. 2001; Moav et al. 1992; Rocha et al. 2004a) and it is suitable for the development of stable cell lines since its growth is less density-dependent as the growth of most mammalian cell lines (Collet et al. 2004; Collet and Secombes 2005).

On the other hand, to drive the expression of Ple in the EPC cell line, we selected the homologous β-actin carp promoter because it was more active than the heterologous Cytomegalovirus immediate early promoter (CMV), commonly used to drive expression of foreign genes in fish cells (Fig. 2). This result is in agreement with previous

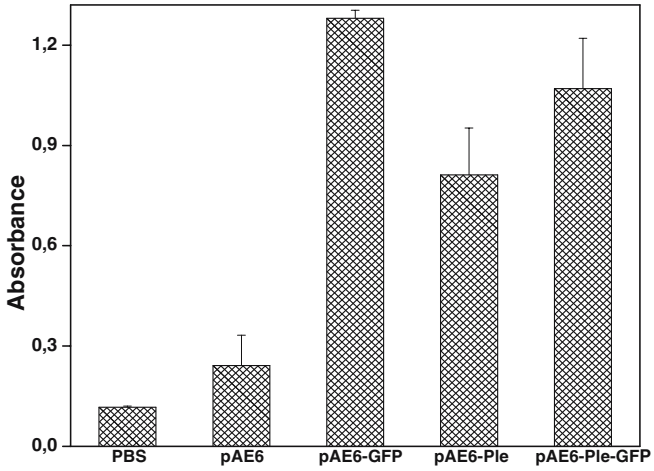

Fig. 7 Expression of recombinant peptides in skeletal muscle of rainbow trout injected with pAE6-GFP, pAE6-Ple-GFP or pAE6-Ple. Two micrograms each of phosphate buffered saline (PBS), pAE6, pAE6-GFP, pAE6-Ple-GFP and pAE6-Ple in 100 μl of PBS were separately injected into the skeletal muscle of four anaesthetised fingerling rainbow trout per plasmid. Seven days later, skeletal muscle tissue samples were taken from the injected area, homogenised in 300 μl of distilled water and clarified by centrifugation. One hundred microlitres of muscle homogenates (about 10 μg of protein) were dried per well. After washing, Abs were used to detect recombinant protein expression. A MAb anti-GFP was used to detect GFP expression in trout injected with pAE6-GFP, whilst the anti-synthetic Ple PAb was used to detect Ple in trout injected with the rest of the plasmids. Means and standard deviations from four trout per plasmid are represented

reports showing that regulatory sequences of fish origin might be more efficient than those of mammalian or viral origin in transgenic fish, depending on the gene to be expressed (Du et al. 1992; Dunham et al. 2002; Hwang et al. 2003). Furthermore, because viral promoters derived from human pathogenic virus should be restricted by safety considerations in their use in animals destined for human consumption (Alonso et al. 2003) or for production of pharmaceuticals (Rocha et al. 2003), the use of a homologous fish gene promoter is favoured for possible future applications. Most probably, the use of a homologous promoter in the system used for expression of MD Ple (the use of a carp cell line with a carp gene promoter) has contributed to the successful stable expression of the MD Ple in the EPC cell lines developed in this work.

The structure of the gene of MD Ple was similar to those previously described for WF Ple (Cole et al. 2000; Douglas et al. 2001). The mature MD Ple amino acid sequence differed by six and three amino acids with those of the WF and YT Ple, respectively, thus indicating a high degree of conservation among mature Ple peptides from any flatfish species. Furthermore, MD Ple might also fold into an amphipathic α-helix like the other two Ple. The three locations, which contain the positions showing amino acid variations among the Ple from MD, WF and YT, corresponded to similar relative positions on the amphipathic α-helix (Fig. 1). Those positions are from the area where positions 13 and 17, which are known to affect structure and bacterial cell selectivity of WF Ple, are located (Lim et al. 2004). Thus, it is possible that only the three locations showing amino acid variation are not essential for Ple structure and/or activity, although this has to be confirmed when more Ple structures from other flatfish species would be available.

Up to now, the expression of Ple and/or other antimicrobial peptides was only analysed at the transcriptional level or in tissue sections (Cole et al. 1997, 2000; Murray et al. 2003). Only the secretion of other AMPs, such as of insect cecropin in a fish cell line (Sarmasik and Chen 2003) and of human β-defensins in human cell lines (Carretero et al. 2004), were previously reported. In this report, the entire process of peptide synthesis, maturation and secretion of Ple in EPC cell lines stably expressing Ple under the control of a non-viral promoter has been studied. The complete sequence of the pre-Ple-pro was included into the plasmids used to transfect the EPC cells to assure that all the information required to process mature Ple would be available, and to investigate whether the pre- and propeptides could be processed for the Ple to be secreted out of the EPC cell lines expressing Ple. The EPC cell lines expressing Ple-GFP or Ple were transcribing RNAs with introns removed, as shown by the sequence analysis of the intermediate RT-PCR products. The addition of GFP to the carboxy terminal part of Ple did not alter its mRNA expression, processing and secretion.

The visualisation of intracellular GFP fluorescence in living EPC cell lines expressing GFP or Ple-GFP showed that, whilst GFP was homogeneously present throughout the whole cell, Ple-GFP had a different subcellular localisation. Ple-GFP was first seen in the perinuclear region of the cells most likely corresponding to the Golgi apparatus, and it was then dispersed in the cell cytoplasm with a granular appearance (Fig. 3). This granular appearance of fluorescence suggests the accumulation of the fusion protein inside vesicular structures along the secretory pathway. This result coincides with a previous report that localised the WF Ple in cytoplasmic granules from the mucus-producing cells of skin, goblet cells of small intestine (Cole et al. 2000) or in the eosinophilic granular cells of the gills (Murray et al. 2003) of WF. GFP fluorescence was recovered in the culture medium (Fig. 4), as expected, if the Ple-GFP was transported into secretory vesicles to fuse with the plasma membrane before being released into the culture medium. However, further studies are needed to reveal the precise nature of these intracellular organelles.

In a first attempt to further characterise the peptides secreted by the EPC cell line expressing Ple-GFP or Ple, those peptides were analysed in cell lysates and cell culture supernatants by Western blotting (Fig. 5). The results showed that the EPC cell line expressing Ple-GFP accumulated a major intermediate, the Ple-pro-GFP. This was suggested by the presence of a GFP-containing band with an apparent MW corresponding to the Ple-pro fused to GFP, produced by N-terminal proteolytic cleavage of the signal peptide (pre) from an initially synthesised pre-Ple-pro-GFP. The pre-Ple-pro-GFP peptide could not be detected most probably due to its short lifetime, as it

happened with human defensins precursors in human myeloid cells (Ganz et al. 1993). The presence of the corresponding cleavage peptides, pro-GFP and Ple peptides, further suggested further posttranslational processing involving the removal of the propeptide. However, Ple-pro-GFP seems to be only partially processed because the Ple-pro-GFP band was also detected in the cell culture supernatants. The secretion of a propeptide form has also been found in cells transduced with a virus carrying human preprodefensin cDNA (Ganz et al. 1993).

The appearance of a double band in synthetic Ple might occur because of the different electrophoretic behaviour of the two possible conformations of Ple, random coil and α-helical conformations (Syvitski et al. 2005). The higher apparent MW of synthetic Ple (apparent MW of ~6.5 kDa compared to a theoretic MW of 2.8 kDa) could be due to dimer formation, aggregation and/or an abnormal behaviour of low molecular peptides under the conditions of the cell culture medium and/or electrophoresis. Interestingly, in the EPC cell line expressing Ple, the intracellular recombinant Ple appeared as a single ~6.5-kDa band, whilst it appeared as a double band after it was secreted to the cell culture medium, thus confirming the possible artefactual appearance of Ple under some conditions. The band, greater than 6.5 kDa found in the culture supernatant from the EPC cell line expressing Ple-GFP, could correspond to pre-Ple, Ple-pro or pre-Ple-pro, since it was not present in the Western blot stained with anti-GFP. The precision of this technique is not high enough to decide among all those possibilities. Further studies are required to define details of the processing of the pre-Ple-pro peptides to yield extracellular Ple.

Most importantly, the Ple, secreted to the supernatants from the EPC cell line stably expressing Ple after being propagated without interruption for more than 2 years, still has antibacterial activity. The antibacterial activity could be detected in the cell culture supernatants even without further concentration in contrast to the required 100-fold concentration to detect activity of other AMPs obtained in transformed cell lines, such as it occurs with cecropin (Sarmasik and Chen 2003). On the other hand, MD Ple plasmids under the control of the carp β-actin promoter were expressed in rainbow trout muscle, therefore confirming that the Ple could be expressed in vivo by using the carp β-actin promoter in a fish species and in fish other than carp. The fact that the EPC cell lines accumulated bactericidally active recombinant Ple in the cell culture medium for a prolonged period of time and that injection of plasmids codifying Ple induced in vivo expression of Ple in trout muscle cells suggests that, on due time, Ple might also be produced in transgenic fish.

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