Fish & Shellfish Immunology 35 (2013) 532–538

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Differential regulation of cathelicidin in salmon and cod

Daniela C. Broekman, Guðmundur H. Guðmundsson [1], Valerie H. Maier*,[1]

Institute of Biology, University of Iceland, Sturlagata 7, 101 Reykjavik, Iceland

ARTICLE INFO

Article history:
Received 29 October 2012
Received in revised form
29 April 2013
Accepted 6 May 2013
Available online 28 May 2013

Keywords:
Innate immunity
Cell culture
Gene expression
Cell signalling
Cathelicidin

ABSTRACT

Antimicrobial peptides (AMPs) are an important component of innate immunity in vertebrates. The cathelicidin family of AMPs is well characterized in mammals and has also been reported in several fish species. In this study we investigated the regulation of cathelicidin expression in a gadoid and a salmonid cell-line in order to dissect the signalling pathways involved. For this, fish cells were treated with microbial lysates, purified microbial components and commercial signalling inhibitors and expression of cathelicidin was assessed with quantitative real-time PCR (qPCR). We found that cathelicidin expression was induced in both cell lines in response to microbial stimuli, but the response patterns differed in these evolutionary distant fish species. Our data suggest that in salmonids, pattern recognition receptors such as TLR5 may be involved in the stimulation of cathelicidin expression and that the signalling cascade can include PI3-kinase and cellular trafficking compartments. A detailed knowledge of the regulating factors involved in AMP-related defence responses, including cathelicidin, could help in developing strategies to enhance the immune defence of fish.

1. Introduction

Antimicrobial peptides (AMPs) are present in living organisms including bacteria, plants and animals [1]. In animals they demonstrate abundant expression in epithelial and immune cells [2,3]. AMPs are able to kill Gram-negative and Gram-positive bacteria, fungi, viruses and parasites [4] and therefore have an important role in immune defence. In mammals, AMPs are multifunctional molecules that not only directly kill pathogens, but also have other functions related to tissue repair and immune modulation like promotion of wound healing, angiogenesis, recruitment of T-cells, neutralization of proinflammatory cytokines or anti endotoxin activity [5,6]. In fish, multifunctionality in the form of bactericidal and immunomodulatory activity of AMPs has also been suggested [7,8]. The importance of AMPs in fish immunity has been demonstrated in studies with administration of synthetic AMPs to fish or transgenic fish expressing additional AMPs, which led to improved survival during infection challenges [9,10].

AMPs can be constitutively expressed, or alternatively their expression can be induced through a stimulus. This induction can be direct with a stimulated receptor leading to the upregulation of the AMP gene, or indirect, where a stimulus leads to synthesis of proteins which then elicit the transcription of AMPs [6,11]. The direct induction in some AMPs can be triggered by pathogen-associated molecular patterns (PAMPs) through toll-like receptors (TLR) or other pattern recognition receptors (PRR). In *Drosophila*, AMPs are upregulated differentially through bacterial and fungal stimuli [12]. In fish, AMPs have also been shown to be upregulated due to bacterial infection [13–19]. In mammals, only a few studies have shown the increased expression of AMPs in response to bacteria. Examples are the intranasal administration of flagellin upregulating CRAMP (cathelin-related antimicrobial peptide) expression in mouse lung [20] and the human β-defensins 1-3 being upregulated upon stimulation with live staphylococci [21]. It has been shown that the expression of the human cathelicidin antimicrobial peptide LL-37 is increased through vitamin D [22], butyrate [23], bile salts [24], or lithocholic acid [25], all endogenous products of metabolism. Vitamin D and lithocholic acid represent effectors of direct induction, while butyrate and it's derivate phenylbutyrate are indirect inducers causing upregulation of the human cathelicidin LL-37 as a secondary response [26].

The emergence of antibiotic resistant pathogens has led to the increased focus on antimicrobial peptides to fight infections [1]. Initially it was assumed that pathogens cannot develop resistance to AMPs, however exposure of a bacterium to sublethal concentrations of a single AMP has been shown to lead to resistance [27]. Since AMPs are a vital part of the immune system, the emergence of

Abbreviations: AMP, antimicrobial peptide; TLR, toll-like receptor; PI3K, phosphoinositid-3-kinase; PAMPs, pathogen associated molecular patterns; h, hours; p.s., post stimulation; ECP, extracellular product.

* Corresponding author. Tel.: +354 525 5230; fax: +354 525 4069.
E-mail address: valerie@hi.is (V.H. Maier).

[1] Contributed equally.

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.05.005

pathogen resistance to AMPs, leading to the loss of their effectiveness, could be devastating. Therefore, provoking the emergence of AMP resistant pathogens through the use of synthetic AMPs is not recommendable. Taking advantage of the benefits of AMPs while avoiding the emergence of resistance could be achieved by inducing endogenous AMP expression with selected stimuli, thereby upregulating a protective innate immune response as done in mammalian systems before [28–30].

Cathelicidins, together with defensins, are the best described AMPs in mammals [31]. The importance of cathelicidin in mammalian immunity has been demonstrated in studies showing that the absence of cathelicidin in knock-out mice had a weakening effect on the innate immune defence against infection [32,33], while transgenic mice expressing an additional cathelicidin showed increased defences [34].

In fish, cathelicidins have been identified in many fish species including Atlantic hagfish (*Myxine glutinosa*) [35], different salmonids [15,17,36], *ayu* (*Plecoglossus altivelis*) [16] and Atlantic cod (*Gadus morhua*) [37]. Several studies have suggested a role of cathelicidins in fish immunity due to their increased expression upon stimulation with bacteria [13–17] and the antibacterial activity of the peptide [15,16,38].

In the present study, we identified different microbial compounds that induced cathelicidin expression in a salmon and a cod cell line and studied the signalling pathways involved. Knowledge on these pathways could be applied in future in aquaculture settings to identify compounds that increase the endogenous defence of the fish and thereby help the fight against infection.

2. Materials and methods

2.1. Cell culture

The Chinook salmon (*Oncorhynchus tshawytscha)* embryo cell line (CHSE-214) and the Atlantic cod (*G. morhua*) larvae cell line (ACL) [39] were cultured either in MEM (Earles media containing GlutaMAX™-1 and 25 mM HEPES) or L-15 medium (Leibovitz' medium) supplemented with 10% foetal bovine serum, 25 μ/ml penicillin and 25 mg/ml streptomycin, 1% non-essential amino acids and 1% sodium bicarbonate (all Gibco/Invitrogen). Cells were maintained at 16 °C in closed 75 cm^2 flasks and split into 25 cm^2 flasks for experiments. Cells were passaged at 2–3 week intervals.

2.2. Bacterial and fungal culture and preparation

Pseudomonas aeruginosa (strain PA01) was cultured in LB medium at 37 °C. *Aeromonas salmonicida* subsp. *achromogenes* was cultured in brain-heart infusion at 25 °C. *Vibrio anguillarum* was cultured in marine broth at 25 °C. *Escherichia coli* (D21) was cultured in LB medium at 37 °C. A *Lactobacillus* species originally isolated from Atlantic salmon was cultured in tryptic soy broth at 20 °C. *Candida albicans* was cultured in yeast maltose broth at 37 °C.

Microorganisms were grown until early stationary phase and the density was measured at 600 nm. The concentration of microorganisms was determined according to $OD_{600} = 1$ equals 5.5×10^8 bacteria/ml. The cells were pelleted by centrifugation at $2000 \times g$. The pelleted bacteria were inactivated by resuspension and incubation in 70% ethanol overnight followed by 3 washes in PBS and subsequent exposure to UV light overnight. The microbial cells were then disrupted in order to expose intracellular PAMPs by sonication at 40% amplitude with a Vibra Cell sonicator (Sonics) in intervals of 20 s 10–20 times. Subsequently inactivation of microorganisms was confirmed by testing growth on agar plates. Preparations were stored frozen until needed.

2.3. Stimulation and inhibition

Fish cells were stimulated with a range of 100–200 dead bacteria/cell in serum- and antibiotic-free medium for 24 h unless otherwise stated. For the comparison of different microorganisms the volumes of inactivated bacteria used for stimulations were adjusted according to their density to assure equal application of microbial fragments and corresponded to approximately 250 bacteria/cell.

The following stimulants were used at the indicated concentrations: sodium-4-phenylbutyrate (Tocris): 4 mM, 2,6-diaminopimelic acid (Sigma): 100 μg/ml, β-D-glucan (Sigma): 100 μg/ml, poly(I:C) (Tocris): 10 and 100 μg/ml, LPS (Sigma, derived from *P. aeruginosa*): 10 μg/ml, flagellin (isolated from *P. aeruginosa*): 5.7 μg/ml, DNA (isolated from *P. aeruginosa*) at 115 ng/ml. Flagellin and bacterial DNA were isolated as described previously [40,41] and the purity of these components was confirmed by SDS-PAGE and NanoDrop ND-1000 UV/Vis-spectroscopy.

For the experiments with inhibitors the compounds were applied at the following concentrations: brefeldin A (Sigma) (Golgi apparatus inhibitor): 10 μg/ml, nocodazole (Sigma) (microtubule inhibitor): 10 μg/ml, chloroquine (Sigma) (inhibitor of lysosomal function and TLR9): 10 μM and wortmannin (Sigma) (PI3K inhibitor): 1 and 5 μM. For the assays, fish cells were preincubated with inhibitors for 2 h at 16 °C prior to addition of the microbial stimulus and subsequent incubation for 24 h at 16 °C.

2.4. RNA isolation and real-time qPCR

Total RNA from fish cells was isolated with TRI Reagent (Sigma Aldrich) according to the manufacturer's instructions except that the extraction step was repeated once in order to assure maximal RNA purity. The RNA pellet from one 25 cm^2 flask of cells was dissolved in 40 μl RNase-free water. RNA quality and quantity was examined on a NanoDrop ND-1000 UV/Vis spectrometer. Five hundred ng of RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences) following the manufacturer's instructions. The absence of genomic DNA was confirmed by preparing samples without addition of reverse transcriptase (-RT). The cDNA was subsequently diluted 1:10 in water and qPCR was performed. The Chinook salmon cathelicidin primers were designed with PerlPrimer [42] (forward primer: 5′-ATGGGAAACGAATGATGTGC-3′, positioned over the exon 2/3 boundary and reverse primer: 5′-CGGTCAGTGTTGAGGGTATT-3′). The reference genes in CHSE-214 cells were RPS20 and EF-1α with primer sequences published previously [43]. Primer sequences for Atlantic cod cathelicidin, the reference genes RPS9 and ubiquitin have been published previously [13,44]. Efficiencies for all primer sets were calculated and shown to lie between 90 and 110%. All qPCRs were performed using Power SYBRGreen with ROX (Applied Biosystems) according to the manufacturer's instructions with the exception that 10 μl final reaction volume was used. The qPCR runs were performed on an ABI 7500 real-time PCR System (Applied Biosystems) and the thermal cycling parameters were 50 °C for 2 min, followed by a 95 °C hot start for 10 min, subsequently the amplification was performed with 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Every sample was assayed in duplicate and each experiment was performed two times or more as stated in the figure legends. In order to exclude contamination a no-template control (H2O) and a -RT sample were run for each primer pair in every experiment. A dissociation step (60 °C–95 °C) was performed at the end of the amplification phase to identify a single, specific melting temperature for each primer set. The Ct values of the reference genes were stable over time and during infections of the cells, indicating the suitability of these genes as reference genes.