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Transcription of histones H1 and H2B is regulated by several immune stimuli in gilthead seabream and European sea bass

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ABSTRACT

Histones (H1 to H4) are the primary proteins which mediate the folding of DNA into chromatin; however, and in addition to this function, histones have been also related to antimicrobial peptides (AMPs) activity in vertebrates, in fact, mammalian H1 is mobilized as part as the anti-viral immune response. In fish, histones with AMP activity have been isolated and characterized mainly from skin and gonads. One of most threatening pathogens for wild and cultured fish species nowadays is nodavirus (NNV), which target tissues are the brain and retina, but it is also able to colonize the gonad and display vertical transmission. Taking all this into account we have identified the *h1* and *h2b* coding sequences in European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) fish species and studied their pattern of expression under naïve conditions and NNV *in vivo* infection. The data obtained prompted us to study their role on the immune response of gonad and head-kidney leucocytes upon viral (NNV), bacteria (*Vibrio anguillarum* or *Photobacterium damselae*), pathogen-associated molecular patterns (PAMPs) or mitogens stimulation. The *h1* and *h2b* genes are expressed in a wide range of tissues and their expression is modify by infection or other immune stimuli, but further studies will be needed to determine the significance of these changes. These results suggest that *h1* expression is related to the immune response against NNV in the brain, while *h2b* transcription seems to be more important in the head-kidney. Moreover, the potential role of histones as anti-viral agents is suggested and further characterization is in progress.

1. Introduction

Histones are usually classified as core (H2A, H2B, H3 and H4) and linker histones (H1) due to their localization forming the basic units of the chromatin, the nucleosome. Thus, the nucleosome is formed by 146 base pairs of DNA wrapped around a protein octamer of two molecules of core histones. The linker H1 binds the DNA at the union sites whether it enters or exits the core nucleosome [1]. Histones, mainly core ones, are greatly conserved in eukaryotic organisms along evolution and therefore their functions might be also conserved. In addition to this function, they are also involved in other cellular functions and their implication in the epigenetic control of gene expression is nowadays in fashion. However, they have been also linked to immunity being their role as antimicrobial peptides (AMPs) the most described, which were first characterized in mammals long time ago [2]. Thus, histones and histone-derived fragments act as physiological barriers of cells exerting a variety of antimicrobial actions and functions, including bacterial cell membrane permeabilization, penetration into the membrane followed by binding to bacterial DNA and/or RNA, binding to bacterial lipopolysaccharide (LPS) in the membrane, neutralizing the toxicity of bacterial LPS, and entrapping pathogens as a component of neutrophil extracellular traps (NETs) [3].

In fish, the connections between histones and immunity have been established. First characterized was a catfish (*Ictalurus punctatus*) AMP isolated from the skin closely related to the H2B [4]. Since then, proteins highly homologous to histones or fragments derived by cleavage processes from histones (eg. Parasin I, hipposin) have been defined as histone-like proteins (HLPs) and

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identified in some fish species [5–9]. Most studies in fish have focused on the antimicrobial function of HLP-1 and HLP-2 proteins homologous to H2B and H1, respectively; and usually isolated from skin or gills [4,10–14]. However, other AMPs have been widely distributed among several tissues including immune-privileged tissues such as brain or gonads [15]. Recently, a H1-like protein has been isolated from acidified testis extracts (fH1LP) of olive flounder (*Paralichthys olivaceus*) and shown to be constitutively expressed in ovary and testis and to have antibacterial (Gram+ and Gram-) and antifungal activity [16]. In European sea bass (*Dicentrarchus labrax*), H2B and H1 coding genes were cloned and their expression levels have been reported to be altered under stress conditions [14], and also after *Vibrio anguillarum* infection [17].

Nodavirus (NNV) is a naked bipartite single stranded RNA virus which severely affects European sea bass larvae and juveniles provoking high mortality rates [18,19]. Nevertheless, other species such as the gilthead seabream (*Sparus aurata*) are infected without showing disease symptoms, acting as a natural reservoir for most of the virus strains [20]. NNV has demonstrated vertical transmission [21] and is able to colonize and replicate in very low levels into the European sea bass and gilthead seabream testis in order to not being detected by the immune response [22], altering the antimicrobial activities and pattern of expression of several AMPs [23].

In this study, we identify the complete sequences of H1 and H2B coding genes in European sea bass and gilthead seabream and study their pattern of expression in immune, reproductive and other important tissues in naïve specimens and under NNV infection. The results obtained, prompted us to analyse the modulation of both genes upon *in vitro* viral, bacterial infection, pathogen-associated molecular patterns (PAMPs) or mitogens stimulation of the immune response in gonad and/or head-kidney leucocytes (HKLs) in order to determine whether these two genes might have a role in the immune response of fish.

2. Material and methods

2.1. Animals

Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia) in 14 m^3 tanks with the water temperature ranging from 14.6 to 17.8 °C, flow-through circuit, suitable aeration, filtration systems and natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily. Juvenile specimens of both species with a mean body weight (bw) of 325 ± 37.5 g were used for the analysis of constitutive gene expression in naïve conditions (see below). Adult specimens of both species with a bw of 774 ± 93 g were used for *in vitro* treatments of the gonads (see below). Juvenile specimens of European sea bass (n = 50) or gilthead seabream (n = 50) with a mean bw of 200 ± 15 g, were transported to the University of Murcia (Spain) aquaria in order to perform *in vivo* infections (see below). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE). The protocol was approved by the Committee on the Ethics of Animal Experiments of the *Instituto Español de Oceanografía* (IEO) (Permit Number: 2010/02) and of the University of Murcia (Permit Number: A13150104).

2.2. Fish sampling

All specimens were anesthetized with 40 μl/l of clove oil before sampling, then weighed, completely bled and immediately decapitated. Blood was obtained from the caudal peduncle and the serum samples, obtained by centrifugation (10,000×g, 1 min, 4 °C), were immediately frozen in liquid nitrogen and stored at −80 °C until use.

In order to analyse the constitutive expression in naïve conditions, brain, gill, liver, skin, gonad, gut, head-kidney, spleen and thymus fragments from 6 independent fish were removed and immediately frozen in TRIzol® Reagent (Life Technologies) at −80 °C until used for RNA isolation. HKL suspensions were obtained as previously described [24]. In brief, fragments of head-kidney tissue were transferred to 7 ml of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride, 100 IU/ml penicillin (Life Technologies), 100 mg/ml streptomycin (Life Technologies) and 5% fetal bovine serum (FBS; Life Technologies)] under sterile conditions. Cell suspensions were obtained by forcing fragments of the organ through a 100 μm nylon mesh, washed twice by centrifugation [400×g, 10 min, room temperature (RT)], counted and adjusted to 10^7 cells/ml in sRPMI. In all cases, leucocyte viability was determined by the trypan blue exclusion test and resulted higher than 98%.

2.3. Viruses and bacteria

NNV (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line [19]. The SSN-1 cells were grown in Leibovitz's L-15 medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin (Gibco) at 25 °C using Falcon Primaria cell culture flasks (Becton Dickinson). Inoculated cells were incubated at 25 °C until the cytopathic effect (CPE) was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following a methodology previously described [25].

Pathogenic bacteria *Vibrio anguillarum* (*Va*) R-82 and *Photobacterium damselae* subsp. piscicida (*Pd*) were grown in sTSB [tryptic soy broth (Laboratorios Conda) supplemented with 1.5% NaCl] at 22 °C for 24 h. Absorbance at 600 nm was measured and used to know the concentration based on growth curves. Both bacterial cell cultures were washed in sterile 0.01 M phosphate-buffered saline (PBS, pH 7.4) by centrifugation (6,000 ×g, 15 min, 4 °C) and adjusted to 10^{10} bacteria/ml. For heat-killing, cultures were washed with PBS, incubated at 60 °C for 30 min, washed and adjusted to 10^{10} bacteria/ml with 0.01 M PBS.

2.4. In vivo infection

Once at the University of Murcia (Spain) facilities, juvenile specimens (n = 50) of both species were randomly divided into two tanks, kept in 450–500 L running seawater (28‰ salinity) aquaria at 25 °C and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the infection. The infection was performed by intramuscular injection of 100 μl containing 10^6 $TCID_{50}$/fish of NNV in SSN-1 culture medium, a mock-infected group was injected with 100 μl of SSN-1 culture medium since this route of infection has been proven to be the most effective [26]. Fish (n = 5 fish/group and time) were sampled 1, 7 or 15 days upon infection and gonad and brain were removed and immediately frozen in TRIzol® Reagent and stored at −80 °C for later RNA isolation as described below.

2.5. In vitro treatments

Fragments of European sea bass ovaries (n = 6) or testis (n = 6) or gilthead seabream gonads (n = 6) were removed, weighted and chopped into 1 mm^2 to culture them in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine, 100 IU./ml penicillin,