Developmental and Comparative Immunology 56 (2016) 57–66

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Recognition of purified beta 1,3/1,6 glucan and molecular signalling in the intestine of Atlantic salmon

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ARTICLE INFO

Article history:
Received 17 May 2015
Received in revised form
11 November 2015
Accepted 13 November 2015
Available online 22 November 2015

Keywords:
Atlantic salmon
Beta-1,3/1,6 glucan
MacroGard®
C-type lectin receptor genes
Tagln
Actb
Psma2

ABSTRACT

Atlantic salmon was orally intubated with a highly purified β-glucan product (MacroGard®) to study the recognition of the molecule by the receptor genes, the regulation of the downstream signalling genes and global proteins, and the micromorphological changes in the intestine.

The β-glucan receptor genes of Atlantic salmon, *sclra*, *sclrb*, *sclrc* and *cr3*, seem to recognize the molecule, and initiate the downstream ITAM-motif signalling, as evident from the significantly high mRNA levels of *ksyk*, *mapkin2*, *il1b* and *mip2a* levels. Among the altered proteins, the Apoa4 (involved in carbohydrate and lipid metabolism); Tagln, Actb (uptake of β-glucan); Psma2 (associated with substrate recognition); and Ckt (energy metabolism-related) were the overexpressed ones. The underexpressed proteins included the Uk114, Rpl9, Ctsb and Lgal that are connected to proliferation, LPS-stimulation, Il1b and lactose recognition, respectively. Furthermore, the mRNA levels of *igt* and the number of immune cells in the distal intestine were found to increase upon β-glucan uptake by the fish. This study provides some clues on the mechanisms by which the β-glucan evokes response in Atlantic salmon, particularly at the intestinal level.

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1. Introduction

Immunomodulatory feed additives are relied on to enhance the performance and health of farmed animals, including fish. The purified β-glucan derived from yeast is considered as an additive that supports the immune system and improves the health of the host (Mantovani et al., 2008; Volman et al., 2008). These molecules are not digested and absorbed in the gut of animals, but are recognized by the surface receptors of leukocytes; mainly by Dectin-1 and the Toll-like receptors (TLRs), and to a certain extent by others including the complement receptor 3 (CR3) (Chan et al., 2009; Kim et al., 2011). The receptors are known to act singly or in combination with ligands. Dectin-1, a C-type lectin belonging to group V has a calcium (Ca)-independent carbohydrate recognition domain (CDR), an extracellular stalk region, a transmembrane region, a short cytoplasmic tail and an immunoreceptor tyrosine-based activation (ITAM)-like motif (Carter, 2013; Goodridge et al., 2009; Huysamen and Brown, 2009). Once the pattern recognition receptor of a host identifies a fungal pattern, Src kinases phosphorylates tyrosine in the ITAM-like motif to cause the transduction of the downstream signalling (Brown, 2006). Additionally, two phosphotyrosines bind to the spleen tyrosine kinase (SYK) and induce cellular responses (Brown, 2006).

Group V C-type lectins, which are the main fungal pattern recognition receptors (C-type lectin receptor, CLR) in mammals have not been identified in bony fish. Instead, in teleosts, group II members have been characterized, e.g. salmon C type lectin receptors a, b, c – Sclra, Sclrb and Sclrc in Atlantic salmon (Soanes et al., 2004). While CLRs and TLRs can recognize fungal patterns directly, CR3 identifies pathogen recognition receptor (PRR)-coated fungal particles (Brown, 2006). Collaborative action of Dectin-1 and TLRs induces inflammatory responses (Brown, 2006), and β-glucans are capable of initiating the production of inflammatory mediators such as TNFα and MIP-2 (Abel and Czop, 1992). Furthermore, the Dectin-1–dependent pathway initiated by β-glucans activates the transcription of the proinflammatory cytokine IL-1β (Kankkunen et al., 2010). The TLR pathway starts with the

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http://dx.doi.org/10.1016/j.dci.2015.11.007
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recognition of the yeast pattern by TLR 2 or TLR 6, after which the association of the key signalling cytosolic domain of TLR, Toll/IL-1R domain (TIR) with the adaptor protein, Myd88 is initiated, leading to the activation of mitogen-activated protein kinases, MAPKs (O'Neill and Bowie, 2007). Furthermore, as mentioned before, Src family kinase-induced phosphorylation of tyrosine causes, among others, MAP kinase signalling (Goodridge et al., 2009; Huysamen and Brown, 2009). Additionally, teleost IgT that is associated with gut mucosal surfaces has immunoprotective roles (Zhang et al., 2011), and in mammals immunomodulins induce TGF-β, APRIL and BAFF to simulate lymphocytes to produce IgA (Preidis and Versalovic, 2009).

Although it is accepted that dietary β-glucan exerts immunomodulatory effects in fish, their mechanism of action has not been uncovered. When included in feeds containing multiple ingredients, it would be difficult to single out the mode of action of β-glucan. Therefore, an oral intubation study with a purified beta 1,3/1,6 glucan product was performed on Atlantic salmon to precisely examine the ensuing intestinal stimulation. The recognition of the molecule by the receptor genes (*sclra*, *sclrb*, *sclrc*, *cr3*) and the downstream signalling based on gene transcriptional changes (of *srckin*, *ksyk*, *myd88*, *mapkin2*, *il1b*, *mip2a*, *igt*) were studied. The changes in the proteome and the micromorphology of the intestine were also considered to obtain a better understanding of the physiological processes at the molecular level.

2. Materials and methods

2.1. Fish and rearing conditions

Hatchery produced Atlantic salmon (*Salmo salar*, AquaGen strain), procured as smolts (from Cermaq, Bodø, Norway) and maintained on commercial feeds in the indoor rearing facilities of the Research Station, University of Nordland (UiN), Bodø, Norway were used for the study. Zero-year class of healthy fish (av. wt. 275 g) were transferred to 500 L experimental tanks and allowed to acclimatize for 2 weeks. Two replicate tanks, each with 20 fish, were set up for the two treatments. The water temperature of the flow-through seawater system was 7 °C and the oxygen saturation was above 90%. The experiments were conducted with the approval of the National Animal Research Authority (Forsøksdyrutvalget, FDU; ID-5595) in Norway. The fish were handled by authorized personnel and the procedures were in accordance with the guidelines of FDU.

2.2. Preparation of the β-glucan suspension

The commercial product MacroGard® containing highly purified beta 1,3/1,6 glucans from *Saccharomyces cerevisiae* (Biorigin, Lençóis Paulista, Brazil) was employed in the study. An appropriate amount of the product was suspended in 5 ml of sterile phosphate-buffered saline (PBS), and sonicated (Vibra-Cell VC 750, Sonics and Materials Inc., Newtown, USA) for 3 min at a pulse rate of 20 s. The resulting suspension was employed for intubating the fish.

2.3. Oral intubation of fish

The oral intubation study was conducted on 2 groups of fish, which were starved for 2 days ahead of the procedure. The beta 1,3/1,6 glucan-intubated fish (at the rate of 15 mg/kg fish) constituted the treatment group (NL), while the PBS-intubated group served as the control group (CO). To perform the intubation, individual fish were netted out from each tank and sedated using MS-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, Redmond, USA; 80 mg/l), approximately 4 min prior to initiating the intubation process. After ensuring that the fish were sedated, each fish was intubated with 500 μl of either the beta 1,3/1,6 glucan suspension or the saline using a Buster Cat Catheter 1.3 × 130 mm (Jorgen Kruuse A/S Denmark) connected to 1 ml syringe. Following the intubation, the fish were allowed to recover from sedation. Then, they were transferred to the original holding tanks for the rest of the experimental period (7 days).

2.4. Intestinal tissue collection

At 1 and 7 days post intubation (dpi), 10 fish each from the study groups CO and NL were sampled to isolate the entire distal intestine. Immediately after the dissection, the distal intestinal region was divided into anterior, mid and posterior parts. The anterior and mid segments were snap-frozen in liquid nitrogen and stored at −80 °C prior to RNA/protein extractions, respectively. The posterior portion was used for the histological studies (see Section 2.7).

2.5. Assaying the expression of the target genes

The genes of the β-glucan receptors (salmon C type lectin receptors A, B, C – *sclra, sclrb, sclrc,* complement receptor 3, *cr3*); the genes involved in the downstream signalling pathway (Src kinase, *srckin*; spleen tyrosine kinase, *ksyk*); and other relevant immune genes (myeloid differentiation primary response gene 88, *myd88*; mitogen-activated protein kinase, *mapkin2*; interleukin 1b, *il1b*; macrophage inflammatory protein-2-alpha, *mip2a*; immunoglobulin T, *igt*) were studied.

All the qPCR reactions were performed in duplicate and the attributes of the gene specific primers used are presented in Table 1. The primers were designed flanking the intron-exon border to confirm the primer specificity. The total RNA was extracted from the distal intestine following the TRI-reagent method (Sigma, St. Louis, MO, USA), as described earlier (Lokesh et al., 2012). The RNA quality was assessed on 1% (W/V) agarose gels and subsequently quantified using Qubit® 2.0 Fluorometer and Quant-iT RNA assay kit (Life Technologies, Carlsbad, CA, USA). Total RNA (1000 ng) was reverse transcribed to complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The resulting cDNA was then diluted 50 times to perform quantitative real time PCR (qPCR) on StepOnePlus™ Real-Time PCR system (Applied Biosystems, Carlsbard, CA, USA). The reaction mixture for qPCR (10 μl) contained 4 μl of diluted cDNA, 5 μl of the Fast SYBR® Green PCR Master mix (Applied Biosystems) and 1 μl of gene specific primer mix (5 pM each of forward and reverse). Conditions set for the qPCR reaction were: initial holding at 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 3 s and isothermal annealing and extension at 60 °C for 30 s. A melt curve analysis was performed to confirm the amplification specificity of the PCR products from each primer pair. Further, the amplicons generated by each of the gene specific primers were sequenced to confirm the specificity of the primers. Two negative controls, namely, water (control for cDNA template) and minus reverse transcriptase (i.e., pooled RNA treated with DNase) were also included. Additionally, 3-fold dilutions (1:1–1:243) of cDNA template (pooled) was used to prepare standard curves included in every qPCR reaction plate to evaluate the amplification efficiency (E) of each gene specific primer using the formula: $E = (10^{-1/slope} - 1) \cdot 100$.

Four reference genes – elongation factor 1 AB (*ef1ab*), hypoxanthine phosphoribosyltransferase 1 (*hprti*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and ubiquitin (*ubi*) – were run on all the samples. Quantification cycle values (Cq) obtained for every sample within a particular gene were converted to relative quantities. Finally, the geNORM (Vandesompele et al., 2002) was

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