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MARCH5 gene is duplicated in rainbow trout, but only fish-specific gene copy is up-regulated after VHSV infection

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ABSTRACT

Ubiquitination regulates the activity, stability, and localization of a wide variety of proteins. Several mammalian MARCH ubiquitin E3 ligase proteins have been suggested to control cell surface immunoreceptors. The mitochondrial protein MARCH5 is a positive regulator of Toll-like receptor 7-mediated NF-κB activation in mammals. In the present study, duplicated MARCH5-like cDNA sequences were isolated from rainbow trout (*Oncorhynchus mykiss*) comprising open reading frames of 882 bp (MARCH5A) and 885 bp (MARCH5B), respectively. Trout MARCH5A and MARCH5B-encoding sequences share only 65% sequence identity. Phylogenetic analyses including an additionally isolated MARCH5-like sequence from whitefish (*Coregonus maraena*) suggest that teleosts possess an additional MARCH5 gene copy resulting from a fish-specific whole genome duplication. Coding sequences of MARCH5A and MARCH5B genes from trout are distributed over six exons. Hypothetical MARCH5 proteins from trout comprise four transmembrane helices and a single motif similar to a RING variant domain (RINGv) including eight highly conserved cysteine and histidine residues. A 'reverse-northern blot' analysis revealed furthermore a MARCH5B Δexon5 transcript variant. Both MARCH5 genes from trout show a strain-, tissue- and cell-specific expression profile indicating different functional roles. Fish-specific MARCH5A gene for instance might be involved in defense mechanisms, since *in vivo*-challenge with the viral pathogen VHSV caused a significant 1.7-fold elevated copy number of the respective gene in gills four days after infection, whereas MARCH5B transcript level did not increase.

1. Introduction

Ubiquitination is the posttranslational attachment of the polypeptide ubiquitin to target proteins, reviewed in [1]. This multi-enzyme process is carried out by a cascade of concerted reactions involving ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-protein ligase E3, reviewed in [2]. Unlike to E1 and E2, E3 ubiquitin ligases display substrate specificity. Eleven members of the MARCH (membrane-associated RING-CH) ubiquitin E3 ligase family have been identified in human so far [3]. The majority shares an N-terminal C_4HC_3-type RING (really interesting new gene) finger [4]. Furthermore, two transmembrane spans are characteristic for MARCH proteins, although MARCH5 bears 4 and MARCH6 even 13 transmembrane domains, whereas MARCH7 and MARCH10 lack the respective domain.

The precise physiological function of the MARCH family remains as yet unknown, but there is evidence for an association with immune defense [5]. Therefore, MARCH family members have also been termed as MIR (modulators of immune recognition). It has been hypothesized that MARCH-dependent ubiquitination allows internalization, recycling or lysosomal degradation of cell surface immunoreceptors, reviewed in [6]. Human MARCH4 and MARCH9 were suggested to influence surface expression of MHC-I (major histocompatibility complex, class I) molecules [4]. Similarly, human MARCH1 and MARCH8 proteins have been identified as potent regulators of MHC-II surface expression [7]. Furthermore, human MARCH8 is reported to influence the expression of the co-stimulatory molecule CD86 (B7-2) [8]. Both MHC and CD86 molecules are essential for antigen presentation and/or the subsequent

Abbreviations: aa, amino acid; EST, expressed sequence tag; mAb, monoclonal antibody; MARCH, membrane-associated RING-CH; MIR, modulator of immune recognition; MHC, major histocompatibility complex; ORF, open reading frame; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; RING, really interesting new gene; RINGv, RING-variant; TLR, Toll-like receptor; VHSV, viral hemorrhagic septicaemia virus.

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activation of effector immune cells. MARCH9 has been suggested to control expression of the intercellular adhesion molecule ICAM1, which is important for the onset and manifestation of inflammatory responses [9]. However, TLR (Toll-like receptor)-signaling and other dendritic cell maturation signals are known to counteract ubiquitination of immunoreceptors [5]. It might be noteworthy that the MARCH-homolog MIR proteins have been initially identified in double-stranded DNA virus KSHV (Kaposi's sarcoma-associated herpes virus) [10] as down-modulators of MHC-I.

Human MARCH5 is reported to participate in the regulation of mitochondrial networks [11]. It has been shown that MARCH5 acts as a critical regulator of mitochondrial division and interconnection in mammals [12]. Most likely the MARCH5-dependent ubiquitination and subsequent degradation of the dynamin 1-like protein and further members of the mitochondrial scission complex plays a central role. Furthermore, it has been hypothesized that MARCH5 also interacts with the membrane protein mitofusin 2 promoting mitochondrial fusion. The balanced regulation of mitochondrial fission and fusion rates contributes to the cellular fitness concerning for example essential mechanisms like Ca^{2+} buffering [13]. Recently, it has been shown that human MARCH5 protein positively regulates TLR7 signaling by ubiquitination of TANK (TRAF family member-associated NF-κB activator) [14]. TANK is known as an inhibitor of TLR-dependent NF-κB activation by suppressing the autoubiquitination of the downstream factor TRAF6 (TNF receptor-associated factor 6).

In this manuscript, MARCH5-like genes from the salmonid fishes rainbow trout and maraena whitefish are characterized including bioinformatic analyses and expression profiles in healthy trout and after infection with the single-stranded RNA rhabdovirus VHSV (viral hemorrhagic septicaemia virus), causing severe hemorrhages in different organs and tissues. VHSV is a serious threat for salmonid aquaculture industry with high mortality rates predominantly among rainbow trout and Atlantic salmon, reviewed in [15].

2. Materials and methods

2.1. Sampling and nucleic acid extraction

Two-year old farmed rainbow trout (*Oncorhynchus mykiss*) of the imported strain TCO steelhead II-WA or of the local selection steelhead strain Born (BORN), and one-year old maraena whitefish (*Coregonus maraena*) were purchased from Binnenfischerei Mecklenburg GmbH Schwerin (Frauenmark, Germany).

In order to isolate MARCH5 cDNA and genomic DNA sequences as well as to investigate its tissue-specific expression, tissues (adipose tissue, gills, head kidney, heart, intestine, liver, muscle, and spleen) from six healthy imported trout and six healthy BORN trout as well as liver tissue from three healthy maraena whitefish were collected and immediately frozen in liquid nitrogen. For RNA isolation, flash-frozen tissue samples were homogenized individually in 1 ml TRIzol Reagent (Invitrogen, Karlsruhe, Germany). RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with in-column DNase treatment for 30 min. DNA was isolated from flash-frozen liver tissue using QIAamp DNA Micro Kit (Qiagen). DNA and RNA quantity was assessed at the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The integrity of RNA was determined by agarose gel electrophoresis.

2.2. Isolation of MARCH5 sequences from rainbow trout and maraena whitefish

Subsequent to BLAST searches, we designed oligonucleotides (Sigma–Aldrich, Taufkirchen, Germany) specific for MARCH5A utilizing two ESTs with the GenBank accession numbers CB490059 and CX722962 as well as for MARCH5B utilizing three overlapping ESTs with the accession codes CA343783, CR376203, and BX085190 (Table 1).

RNA was extracted from livers of steelhead rainbow trout and maraena whitefish. 5 μg of total RNA were reverse-transcribed using Superscript II™ (Invitrogen) to generate a cDNA template for PCR amplification of MARCH5A and 5B fragments. In order to obtain full-length trout MARCH5A cDNA sequence, 5′- and 3′-RACE experiments were conducted using the Gene Racer Super Script™II RT Module (Invitrogen) according to a touchdown-PCR protocol. This included a 5-min pre-incubation at 95 °C, a denaturing step at 94 °C for 30 s, an annealing step at temperatures decreasing from 68 to 59 °C during the first 10 cycles (temperature decrement of 1 °C per cycle) for 30 s, and an elongation step at 72 °C for 2 min, followed by 30 cycles with 30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C, and final elongation at 72 °C for 7 min. A total of 41 cycles was performed.

Coding MARCH5 mRNA sequence of maraena whitefish was generated using primer pairs CM_MARCH_f1, -r1; and CM_MARCH_f2, -r2 (Table 1) according to a 30-cycle PCR protocol.

Introns of both MARCH5 genes were generated in touchdown-PCRs using genomic trout DNA as template and the oligonucleotides listed in Table 1. In order to amplify intron 1, a genomic walking library from rainbow trout DNA was established using the BD GenomeWalker™ Universal Kit (BD Biosciences, Erembodegem, Belgium).

Each nucleotide position of both cDNA and genomic DNA fragments was sequenced at least four times.

2.3. Southern blot analysis on RACE PCR fragments

3′-RACE PCR products from spleen and gill tissue amplified with OM_MARCH5B_f2 gene-specific oligonucleotide (Table 1) were run together with a PCR-generated MARCH5B fragment as a positive control on 2% agarose gel and washed twice in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and twice in neutralization solution (0.5 M Tris–HCl [pH 7.5], 1.5 M NaCl). DNA was transferred on a positively charged nylon membrane (Roche, Mannheim, Germany) by overnight capillary blotting in 20× SSC and eventually UV-cross-linked. In parallel, a 169-bp digoxigenin-labelled probe for hybridization reaction, which corresponds to exon 4 of trout MARCH5 was synthesized using the oligonucleotides OM_MARCH5-SB_f and -r (Table 1) and the PCR DIG Probe Synthesis Kit (Roche). Prehybridization and overnight hybridization were carried out at 43 °C in DIG Easy Hyb solution (Roche). Membranes were washed twice in low stringency buffer (2× SSC, 0.1% SDS) at room temperature for 5 min each and then twice in high stringency buffer (0.1× SSC, 0.1% SDS) at 43 °C for 20 min each. The blots were visualized according to the DIG Nucleic Acid Detection Kit (Roche).

2.4. Flow-sorting of trout lymphocytes

Leukocytes from head kidney, spleen, gills, and intestine were prepared and incubated with anti-CD8α monoclonal antibodies (mAb) in mixed medium (MM), i.e. Iscove's DMEM/Ham's F-12 (Sigma–Aldrich, Steinheim, Germany) at a ratio of 1:1, supplemented with 10% fetal bovine serum (FBS) and 0.1% sodium azide for 30 min. The cells were washed twice with MM and incubated with FITC-conjugated goat anti-rat IgG (H + L) (Jackson ImmunoResearch, Newmarket, England) for 20 min. Flow-sorting was performed with a MoFlo™ high speed cell sorter (Dako, Eching, Germany). The lymphocytes were sorted into two populations, anti-CD8α mAb-positive and anti-CD8α mAb-negative cells. After