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Tollip, a negative regulator of TLR-signalling, is encoded by twin genes in salmonid fish

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Abstract The factor Tollip is known to dampen TLR2- and TLR4-mediated signalling in mammals. No negative regulator of the piscine TLR-signalling cascade has been described so far, albeit a sizable collection of factors contributing to this ancient pathogen-sensing system are known from fish to date. We identified two closely related Tollip-encoding genes in Atlantic salmon (*Salmo salar*) and the respective ortholog mRNA molecules in rainbow trout (*Oncorhynchus mykiss*). The salmonid Tollip genes are segmented into 6 exons, similar to the human orthologous gene. The protein-encoding sequences are homologous to >97% among the twin factors and also between the species. Both encoded proteins contain a C2 domain and an ubiquitin system component, which are also characteristic features of the mammalian Tollip factor. We analysed the expression of these genes in trout. Both Tollip-encoding genes are ubiquitously and also equally expressed, as indicated by similar mRNA concentrations of both factors in any one tissue. Tollip expression was found to be up-regulated by viral infection. Our data suggest that the Tollip genes were duplicated before salmon and trout were evolutionary separated. Moreover, pathways dampening the activity of the TLR-cascade may have been conserved from lower vertebrates to mammals since Tollip, as a respective key factor has been highly conserved from fish to human.

Abbreviations: AA, amino acid; EST, expressed sequence tag; IRAK, interleukin receptor associated kinase; MyD88, myeloid differentiation factor 88; nt, nucleotide; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, PCR following reverse transcription of RNA; TLR, toll-like receptor; Tollip, toll-interacting protein; UTR, untranslated region of the mRNA; VHSV, viral haemorrhagic septicaemia virus.

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Introduction

Toll-like receptors (TLRs) eventually alert vertebrates about the presence of pathogens [1]. Thirteen different TLRs are known in mammals [2]. They recognize molecular components (PAMPs) that are usually not present in host cells. Some TLRs also recognize danger signals from the host, like components of heat shock proteins, heparan sulphate or fibrinogen, to name only some [3—5]. Ligand binding sets in motion a train of events [6] to ultimately activate relevant transcription factors. The best known example of these are the transcription factor complex NF-κB, a proinflammatory key activator [7], regulating a wealth of genes contributing to immune defence [8,9]. Moreover, evidence is accumulating that TLRs may also signal *via* the MAP kinase pathway to eventually activate the transcription factors cAMP response element binding protein (CREB) or the activator protein 1 (AP1) factor complex [10].

Ligand specific signalling of the transmembrane TLR2 and TLR4 factors are perhaps the best studied examples of TLR-dependent activation of gene expression in mammals. Regarding the down-regulation of the TLR-signalling cascade, it was shown that a cellular factor named Tollip interacts with factors contributing to the TLR-signalling pathway [11]. It inhibits NF-κB activation in TLR2- and TLR4-mediated signalling [12,13]. Tollip suppresses the phosphorylation activity of IRAK-1 thereby blocking its kinase activity. Consequently, Tollip limits the production of proinflammatory mediators during inflammation and infection. Tollip-mediated dampening of the intensity of TLR-dependent signalling is a reversible process. A key molecule in this process is the interleukin-1 receptor-associated kinase (IRAK-1) that phosphorylates Tollip [14]. Both factors dissociate and subsequently ubiquitin is tethered to the C-terminus of Tollip, supposedly leading to its degradation in proteasomes [15]. The ubiquitin system component or CUE is required to conjugate Tollip with mono-ubiquitin as a regulatory signal for proteasome activation [16,17].

Over-expression of a Tollip mutant lacking a type II C2 motif (spanning residues 54—186 on human cDNA sequence) fails to evoke an inhibitory effect on LPS-induced NF-κB activation [18]. Tollip binds preferentially to 3′-phosphorylated phosphatidylinositides *via* its C2 domain implying that the phosphoinositide-3 (PI3) kinase regulates its function.

The key features of the TLR-signalling cascade are ancient. Many factors contributing to the activation of the innate immune system following pathogen contact have already been described in teleost fish [19,20]. Four different TLR types have been published for salmonid fish so far [21—26] including our own description of the twin TLR22 receptors in rainbow trout. However, no negative regulators of this cascade have been characterized from teleost fish.

We show in this study that the salmonid species Atlantic salmon and rainbow trout both encode two very closely related Tollip factors and that viral infection eventually leads to increased abundance of the mRNA molecules encoding these factors.

Materials and methods

Fish

Healthy adult rainbow trout (*Oncorhynchus mykiss*) weighing 80—100 g were purchased from a local rainbow trout breeding farm (Uckermark-Fisch GmbH, Germany). The fish were fed with commercial dry pellets and maintained at 19 °C on a simulated natural photoperiod.

Cloning of rainbow trout Tollip cDNA

We cloned the Tollip cDNA using total RNA extracted from livers of rainbow trout as a template. Livers from freshly slaughtered fish were snap frozen in liquid nitrogen. Tissue was powdered under nitrogen and TRIzol® reagent (Invitrogen, Karlsruhe, Germany) was used to extract total RNA. RNA was quantified with the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA). To derive Tollip-specific oligonucleotide primer sequences we used the human Tollip cDNA sequence to identify *via* BLAST analyses of the EST database GRASP (*G*enome *R*esearch on *A*ll *S*almonids *P*roject) the rainbow trout EST 1RT62M09_A_G05, [27] as sharing highest identities with mammalian Tollip sequences. Total RNA (500 ng) from rainbow trout liver was reverse transcribed using Superscript II (Invitrogen, USA) to generate a cDNA template for the subsequent PCR amplification of the rainbow trout Tollip cDNA, essentially as described in Ref. [28]. Rainbow trout specific 3′-RACE primers were 5′-GGACAGGTATACATTGGGGAG-3′ and the 3′-RACE nested gene-specific primers 5′-TTGCCTCAA GACTTCCTGCGT-3′. PCR was performed by proof reading FastStart Taq polymerase (Roche, Mannheim, Germany); PCR conditions were: 94 °C, 1 min; 1 min at the specific annealing temperature; 70 °C, 2 min for 25 cycles.

Amplicons were subcloned into the pGEM®-T Easy Vector (Promega, La Jolla, CA) and sequenced with the universal T7 and SP6 sequencing primers and the LICOR 4000L sequencer. Each cDNA was sequenced at least three times.

BAC library screening and sequence analysis

Tollip-specific oligonucleotides were derived from the rainbow trout Tollip I and Tollip II sequences and used as probes to screen the BAC library CHORI-214, a resource for the genome of the Atlantic salmon. This library was established by the Children's Hospital of the Oakland Research Institute (CHORI, Oakland, USA). It includes a total of 305,557 clones, which are spotted in duplicates on high-density filters, each carrying 18,432 clones. We used the sequence 5′-GTACAGCCTCAGCCTCAGTG-3′ and 5′-GTGGTA CAGCCTCAGCGGCA-3′ to identify Tollip I and Tollip II, respectively. Twenty-five ng of the respective oligonucleotide was labelled with [α-^{32}P]-CTP, using the Readiprime II random prime labelling system, as prescribed (65 °C for 10 min) by the manufacturer (GE Healthcare, Freiburg, Germany). BAC filters were prehybridised (65 °C for 10 min) in a solution containing 150 mM NaCl, 15 mM sodium citrate, 0.1% SDS. Probes were denatured (10 min, 100 °C) before adding to hybridization buffer ('Church buffer', 1 mM