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Cloning and characterization of a TNF-like protein of *Plecoglossus altivelis* (ayu fish)

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Abstract

Ayu TNF cDNA contains an open reading frame of 708 bp encoding 235 amino acids. Poly adeniration (A) signal and eight AU-rich sequences were present in 858 bp 3′ UTR. Southern blot analysis indicated that ayu TNF is single-copy gene. The genomic DNA sequence of ayu TNF, consisting of four exons and three introns, was shown to be conserved well throughout evolution from fish to mammals. The amino acid sequence of ayu TNF was shown to have 32–41% of amino acid identity to other known fish TNF, and about 30% of amino acid identity to mammalian TNFs. A phylogenetic analysis based on the amino acid sequence of TNF indicated that ayu has a distinctive evolutionary path. Also, two residues of cysteine important for the formation of the three-dimensional structure were conserved in ayu TNF. For the functional analysis, ayu TNF was inserted into expression vector pCold/TF, transferred into Chaperone Competent Cells BL21 (pKJE7); this produced soluble mature ayu recombinant TNF. Ayu recombinant TNF was shown to induce respiratory burst activity from ayu kidney. The above results indicate that ayu TNF plays an important role in phylaxis, as it does in mammals.

Keywords: TNF; Innate immunity; Phylaxis; Comparative immunology

1. Introduction

Currently, aquaculture accounts for 25% of the total world supply of fish and shellfish for human consumption, and the relative contribution is expected to increase with time. However, along with the growing global demand for aquaculture products have come certain negative impacts. The biggest issues are increasing problems due to high density breeding that enhance infectious disease processes and that cause deleterious changes in the environment. Up to now, antibiotics and chemical antimicrobial agents have been used to control infectious diseases in both animal husbandry and in aquaculture. However, it has been pointed out that overuse of antibiotics and chemical antimicrobials in food for animals are causing serious problems. They are creating risks to public health and causing environmental pollution. Thus, it is critically important to establish alternative new prophylactic strategies against infection.

Macrophages play a central role in innate immunity, and they have a wide range of functions. Activated macrophages produce a variety of cytokines, and this appears to be one of the major functions of macrophages in immune regulation. Therefore, activation of macrophages and induction of endogenous cytokines are also expected to be one of the techniques of phylaxis in fish. Of the cytokines produced by activated macrophages, one of the most remarkable is tumor necrosis factor (TNF), which can cause the necrosis of tumors (as is indicated by the name) (Carswell et al., 1975). It has also been shown that TNF has additional pluripotent biological activities. Depending on the target cells, TNF can induce cell proliferation, differentiation,

Abbreviations: TNF, tumor necrosis factor; LPS, lipopolysaccharide

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or apoptosis. In the immune system, it activates other immune cells or induces apoptosis in infected cells. Thus, TNF is considered to be an important mediator of macrophage function for phylaxis in mammals. In analyses with TNF knockout mice or with TNF neutralizing antibodies, TNF was reported to function as an essential effector molecule in infection by *Tuberculosis* or by *Listeria* (Rothe et al., 1993; Flynn et al., 1995; Bean et al., 1999; Roach et al., 2005).

Besides mammals, the TNF homologue gene has also been recently cloned in fish, a lower vertebrate, from homology of the TNF amino acid sequence (Hirono et al., 2000; Bobe and Goetz, 2001; Laing et al., 2001; Garcia-Castillo et al., 2002; Saeij et al., 2003; Praveen et al., 2006). Though the function of fish TNF has not been adequately clarified, it is reported to be induced in response to stimulation by LPS, as it is in mammals (Hirono et al., 2000; Laing et al., 2001). The construction of recombinants of the TNF homologue of rainbow trout (Zou et al., 2003a,b), gilthead seabream (Garcia-Castillo et al., 2004), and tilapia (Praveen et al., 2006) were performed, and they were functionally analyzed. Enhancement of phagocytosis and promotion of proliferation of thymocytes were reported in rainbow trout TNF (Zou et al., 2003a,b). Reinforcement of release of superoxide anion was reported in gilthead seabream (Garcia-Castillo et al., 2004), and cytotoxicity was reported in tilapia (Zou et al., 2003a,b). From these results, fish TNF is considered to be induced with macrophage activation during a bacterial infection and functions as a factor for phylaxis, as it does in mammals (Takahashi et al., 2000). We, therefore, isolated the ayu TNF gene, constructed the recombinant, and performed a functional analysis using the recombinant with an aim of determining the functions of TNF for phylaxis.

Ayu is a major fresh-water fish in Japan and belongs to the order Salmoniformes. It is characterized as an annual fish, which is unusual in the Salmoniformes. It is cultured widely in Taiwan and Japan. Infectious diseases, such as pseudomonas disease (pathogen *Pseudomonas plecoglossicida* (Park et al., 2000)) or cold water disease (*Flavobacterium psychrophilum* (Kondo et al., 2002)), cause illness during the culture of ayu. There is no useful drug against these diseases in ayu (Park and Nakai, 2003). For this reason there is a need for preventive methods that do not cause risks of environmental pollution or cause negative effects on humans.

In this study, we cloned an ayu TNF homologue by degenerative PCR, constructed a soluble recombinant of mature TNF to study its function for phylaxis, and determined that it had the capability to induce respiratory burst activity.

2. Materials and methods

2.1. Experimental animals

Ayu (*Plecoglossus altivelis*) (body weight ranging from 8 to 10 g) from an ayu farm which had no history of *Pseudomonas plecoglossicida* and *Flavobacterium psychrophilum* infections were used in this study. Fish were kept at 18 °C in 80-l glass tanks of water drawn from a well with a system of filtration.

Table 1
Primers used in experiments

Primer	Sequence (5′–3′)
DP-1	GCGAGIGCRAAIADNDDRAARAA
DP-2	GCGGTGTTTCAGCTGAATGAAGGGGAC
AYU-F1	TGGAAGAATTCGCGGCCGCTTAAGGGGGGGGGGC
AYU-F2	TGGAAGAATTCGCGG
AYU-F3	TGGAAGAATTCGCGGCCGCAGTTTTTTTTTTTTTT
AYU-F4	TGGAAGAATTCGCGG
AYU-R1	GATGAGGGAGACAGGTTGAAG
AYU-R2	ATGGGAGCTGTGTTCCTCAT
SP-1	GTGCAATGGAGTAAC GAGGT
SP-2	TTTACCAGATTCATCCTGCAG

2.2. Isolation of genomic DNA and RNA

Genomic DNA was isolated from fresh ayu liver with TE reagent containing 100 μg/ml Proteinase K (Wako Pure Chemical Industries, Osaka) and 1% SDS. The total RNA was extracted from ayu tissues using guanidine thiocyanate (Chirgwin et al., 1979).

2.3. Cloning of ayu TNF

The genomic DNA was used in Degenerative PCR amplification with DP-1 and DP-2 primers (see Table 1) designed against conserved motifs of rainbow trout and flounder TNF sequences. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into *E. coli* JM109 competent cells (Takara Shuzo, Tokyo) according to the standard protocol. Recombinants were identified through blue–white selection.

After obtaining a partial ayu TNF sequence, the 5′ and 3′ ends of the TNF gene were obtained by rapid amplification of cDNA ends (RACE) with several ayu specific primers (see Table 1). Total RNA was extracted from head kidney of ayu 3 h after LPS (Rist Biological Laboratories, USA) injection (1.5 mg/kg, i.p.). cDNA was synthesized from the total RNA using M-MLV Reverse Transcriptase (Invitrogen, Tokyo), and oligo $(dT)_{12–18}$ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as the primer.

2.4. Southern blot analysis of genomic DNA

Genomic DNA was extracted from ayu and digested at 37 °C overnight with one unit BamHI (Toyobo, Osaka) and HindIII (Roche Diagnostics, Tokyo) per μg DNA. Digested DNA (15 μg/lane) was electrophoresed on 0.8% agarose gels, and transferred to nylon membranes (Amersham, Hybond-N^+). Transferred DNA was hybridized with a 389 bp probe, generated by PCR using SP-1 and SP-2 primers (see Table 1), which span nucleotides from 457 to 846 of the TNF sequence and was connected with alkaline phosphatase using the Alkphos DIRECT (Amersham Pharmacia Biotech, Piscataway, NJ). PCR was done for 30 cycles at 60 °C of annealing temperature with Expand High Fidelity PCR System (Roche Diagnostics, Tokyo). The membranes were hybridized at 55 °C with the