Immune Expression Analysis and Recombinant Protein Production of a Fish Granulocyte Colony-Stimulating Factor (CSF3)

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ABSTRACT

Granulocyte colony-stimulating factor (CSF3) is a cytokine involved in the differentiation and maturation of neutrophils and its precursor cells in mammals. CSF3 orthologues has recently been identified in Japanese flounder (*po*CSF3-2), fugu (*tr*CSF3-1 and -2) and green-potted pufferfish (*tn*CSF3-1 and -2). *po*CSF3 was shown to be involved in Japanese flounder immunity, being basally well expressed in tissues and immediately up-regulated following immunostimulation. Here, we report that the CSF3 genes of fugu and green-potted pufferfish, which are in duplicates, also are constitutively expressed in several tissues but at varying levels. *po*CSF3 was further shown to be down-regulated by polyinosinic:polycytidylic acid (poly I:C), a known interferon inducer, in brain and kidney; up-regulated in PBLs (but only at 6 hr post-induction); and clearly induced in spleen. These results provide further evidence that teleost fish CSF3 is involved in the immune system. We likewise succeeded in producing a 26-kDa recombinant *po*CSF3 protein in a Japanese flounder cell line (Hirame Natural Embryo or HINAE) using a mammalian expression vector pCDNA4 HisMax C. This recombinant CSF3 fusion protein and the technique used are seen to be useful tools to further study teleost fish CSF3 function in relation to immunity.

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INTRODUCTION

The innate immune system of fish consists of nonspecific cellular responses that include monocytes/macrophages, granulocytes (neutrophils and eosinophils) and nonspecific cytotoxic cells (NCCs) (Iwama and Nakanishi, 1996). An important cytokine that is involved in the proliferation, survival, differentiation and maturation of neutrophils in mammals, granulocyte colony-stimulating factor (CSF3), has been recently and simultaneously identified in chicken and in 3 teleost fish species; Japanese flounder, *Paralichthys olivaceus* (*po*CSF3), fugu, *Takifugu rubripes* (*tr*CSF3-1 and -2), and pufferfish, *Tetraodon nigroviridis* (*tn*CSF3-1 and -2) (Santos *et al*., 2006). Chicken, *Gallus gallus* (*gg*CSF3) was shown to be the chicken Myelomonocytic Growth Factor (cMGF). On the other hand, the teleost fish CSF3 orthologues were confirmed and shown to be in duplicate or paralogous. In addition, teleost fish CSF3s have been shown to be more rapidly evolving than mammalian CSF3s, which appear to be undergoing purifying selection.

CSF3 in mammals has been reported to be produced by different kinds of cells including monocytes/macrophages and lymphocytes (Sallerfors, 1994), fibroblasts (Kaushansky *et al*., 1988), endothelial cells (Zsebo *et al*., 1988), astrocytes (Aloisi *et al*., 1992), bone marrow stromal cells (Fibbe *et al*., 1988a), T-lymphocytes (Ichinose *et al*., 1990) and polymorphonuclear granulocytes (Lindemann *et al*., 1989). The increase in production of CSF3 is very sharp and abrupt (having a half life of 4-10 h in circulation and 1-2 days in tissues) in response to endotoxins or secondary mediators such as tumor necrosis factor (TNF), interleukin-1 and interferon-γ (IFN-γ), suggesting that it is a vital regulator of granulocyte production during inflammation and immune responses (Demetri and Griffin, 1991). CSF3 could also be induced by PHA and PMA (Oster *et al*., 1989a), Interleukin 3 (Oster *et al*., 1989b), Interleukin 4 (Wieser *et al*., 1989), granulocyte-macrophage colony-stimulating factor (CSF2) (Sallerfors and Olofsson, 1991; Oster *et al*., 1989b) and macrophage colony-stimulating factor (CSF1) (Ishizaka *et al*., 1986). In teleost fish however, only the constitutive and mitogen responses of Japanese flounder *po*CSF3-2. *po*CSF3-2 has been shown to be highly expressed in major immune organs including kidney, peripheral blood leukocytes, gills and spleen and moderately in the brain. It is expressed strongly in kidney and peripheral blood leukocytes (PBLs) stimulated by lipopolysaccharides (LPS) and a combination of Concanavalin A (ConA) and Phorbol Myristate Acetate (PMA), indicating that like mammalian CSF3, it is intimately involved in the immediate immune response in Japanese flounder.

CSF3 is one of the successful cytokine therapeutics in humans and domestic mammals, which include interferons and hematopoietic growth factors (colony stimulating factors). Recombinant CSF3 protein is commercially available under the generic name filgrastim (brand name: Neupogen) or pegfilgrastim (brand name: Neulasta). These therapeutics are used for supporting cancer patients receiving chemotherapy or bone marrow transplants, patients under peripheral-blood-progenitor-cell collection and therapy, and patients with neutropenia (Vilcek and Feldmann, 2004). In lower vertebrates, particularly in teleost fish however, recombinant CSF3 has not been produced and studied for its function and potential therapeutic application.

In this study, we analyzed the constitutive expression of the CSF3 duplicate genes in fugu (*tr*CSF3-1 and *tr*CSF3-2) and pufferfish (*tn*CSF3-1 and *tn*CSF3-2). We also tested the *in*

vitro regulation of Japanese flounder *po*CSF3 in primary cultures of brain, kidney, peripheral blood leukocytes and spleen cells in response to polyinosinic:polycytidylic acid (poly I:C), a known interferon inducer. Lastly, we attempted to produce a recombinant *po*CSF3 protein in a Japanese flounder cell line (Hirame Natural Embryo or HINAE), in order to have enough material to confirm its activity, determine its structure or investigate its potential use as a stimulant to boost the fish immune system.

MATERIALS AND METHODS

1. Cell culture

HINAE cells were grown in Leibovitz's L-15 medium (Gibco-BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS) and 100 IU ml-1 penicillin G and 100 µg ml-1 streptomycin (Gibco-BRL, Grand Island, NY).

2. RT-PCR Analysis

2.1 Constitutive expression

Fugu and pufferfish samples were dissected for the following tissues: brain, eyes, gills, heart, intestine, kidney, liver, muscle, ovary, skin, spleen, and stomach. Total RNA was extracted using Trizol reagent (Invitrogen, USA). cDNA was synthesized in the following manner: 10 µg of total RNA, 1 µl of oligo (dT) (500 µg/ml), and 1 µl of dNTP mix were mixed together, heated to 65°C for 5 min then quick-chilled on ice; after which, 4 µl of 5x First-Strand buffer, 2 µl of DTT (0.1M), 0.25 µl of RNase inhibitor (TOYOBO, Japan) (40 U/µl) and MMLV RTase (Invitrogen, USA) (200 U/µl) was added, incubated at 37°C for 50 min, heated to 70°C for 15 min then cooled to 4°C.

cDNAs were PCR amplified using fugu and pufferfish CSF3 specific primers (Table 1). PCR conditions were: initial denaturation at 95°C for 5 min, 30 cycles (95°C - 30s, 55°C - 30s, 72°C - 1 min), and final elongation at 72°C for 5 min. β-actin was used as a control. PCR amplicons (5 µl) were visualized on a 1% agarose gel stained with ethidium bromide and photographed with a densitometer (Atto).

2.2 Immunostimulated expression

Japanese flounder brain, head kidney and spleen were dissected out, minced and filtered through a nylon mesh to a medium containing RPMI 1640 (Nissui, Japan), 1% streptomycin/penicillin (Gibco, USA) and 0.2% heparin (Sigma, USA). Primary PBL culture was prepared through density gradient centrifugation using Percoll solution (Pharmacia, USA). Centrifugation was done at 400x g at 4°C for 3 times with each PBS washing. The cells were cultured in RPMI 1640.

Head kidney and PBL primary cultures were treated with 0.5 mg/ml of poly I:C. The cells were then sampled at 1, 3 and 6 hrs post-induction. cDNAs were synthesized as above and PCR amplified using designed primers *po*CSF3-F2 and *po*CSF3-R (Table 1) and the following conditions: initial denaturation at 95°C for 5 min, 28 cycles (95°C - 30s, 55°C - 30s, 72°C - 1 min), and final elongation at 72°C for 5 min. β-actin was used as a positive control. Amplicons were visualized as above.

Table 1. Primers used for the RT-PCR analysis in this study.

Fish species	Primer name	Primer sequence (5' → 3')
Fugu (*Takifugu rubripes*)	trCSF3-1AF	TGAACATCCTGATTGTCCTCG
	trCSF3-1AR	ATCTGTCATCTGGTTCCTCGT
	trCSF3-2AF	ACAGACATGACCGACCTGACA
	trCSF3-2AR	TCCTGTAGGTGCTGATGGCT
Green-spotted pufferfiish (*Tetraodon nigroviridis*)	tnCSF3-1AF	CATGCACATCCTCATTGTCCT
	tnCSF3-1AR	TTATCTGCTTTGGTCCAGGCT
	tnCSF3-2AF	ACGGACAGACATGATCCACCT
	tnCSF3-2AR	TGCTGCTCCTGTAGCTGTTGA
Japanese flounder (*Paralichthys olivaceus*)	poCSF3-F2	ATGGACTCTGAGACAGTTGT
	poCSF3-R	CGGTAACTGCTTAGCGTGCA
	poCSF3-FProt	TTTGGATCCATGGACTCTGAGACAGTTGT
	poCSF3-RProt	TTTGATTCTTAGCGTGCACCTGCAGCTCGGC

3. Recombinant protein production

3.1 Plasmid construct

A DNA fragment containing the full *po*CSF3 ORF (containing 633 bp and a predicted mass of 21 kDa) and an added *Bam*H1 and an *Eco*R1 site was generated by PCR using designed primers *po*CSF3-FProt and *po*CSF3-RProt (Table 1). The mammalian expression vector pCDNA4/HisMax C and the PCR fragment were cut using *Bam*H1 and *Eco*R1 restriction enzymes, recovered using EASYTRAP ver. 2 (Takara, Japan) and ligated together using ligation high (TOYOBO, Japan) (Figure 1.).

3.2 Transfection and detection

HINAE cells seeded onto 35-mm tissue culture plates were transiently transfected with the pCDNA-HisMax-CSF3 construct using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's protocol. Two (2) each of 6 plates were incubated for 1, 2 and 3 days with pCDNA4-CSF3 and 2 plates were mock infected with blank pCDNA-HisMax C.

Transfected cells were harvested accordingly using a rubber scraper and then pelleted by centrifugation at 1,500 x g for 5 min. The 6 plates were separated into 2 sets, one for RT-PCR and the other for western blot. For RT-PCR analysis, the above method was used. For the recombinant protein, harvested cells were resuspended in 20 µl Phosphate Buffered Saline (PBS). This was then homogenized using a cell lysis buffer (50 mM Tris-HCl, pH 7.8; 150 mM NaCl; 1% Nonidet P-40). Samples were diluted with equal amounts of SDS sample buffer (125 nM Tris HCl, pH6.8; 4% SDS; 10% glycerol; 10% 2-mercaptoethanol; 0.004% bromphenol blue) and boiled for 10 min. The samples were resolved in 15% SDS PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with AntiXpress antibody (Invitrogen, USA) at a 1:10,000 dilution. Recombinant CSf3 protein was then detected by using anti-mouse IgG conjugated with alkaline phosphatase (Promega, USA) following the manufacturer's protocol.

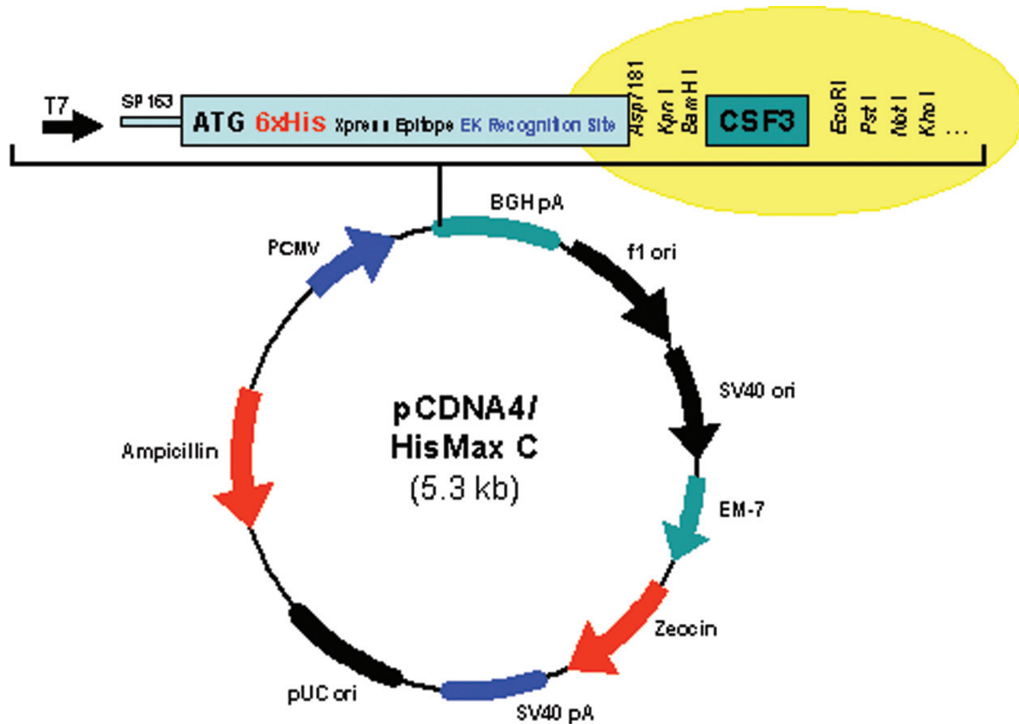

Figure 1. Mammalian expression vector pCDNA4-CSF3 construct map.

RESULTS

RT-PCR analysis revealed that both of the fugu CSF3 genes were expressed in tissues, albeit at varying degree; *tr*CSF3-1 in gills, kidney, ovary and skin while *tr*CSF3-2 in skin only. On the other hand, only one of the green spotted pufferfish CSF3 genes (*tn*CSF3-2) showed expression in gills, skin and ovary (Figure 2).

*po*CSF3-2 in response to poly I:C was also observed in primary cultures of brain, kidney, PBLs and spleen of Japanese flounder. *po*CSF3-2 expression was not detectable in the brain, only faintly observed in kidney at 1 and 3 hrs, highly expressed in PBLs at the 6th hr incubation and was inducible in spleen at 1 hr post-treatment (Figure 3).

Three clones of the pCDNA4-CSF3 construct (C6, C18, and C27) were confirmed to be in frame by sequencing (Figure 4). These same clones were transfected to HINAE cells and were able to produce the recombinant mRNA transcript of about 700 bp in size as shown by RT-PCR analysis (Figure 5A). pCDNA4-CSF3 construct C6 was selected and transfected again to HINAE cells for transient protein expression. Western blot analysis detected a 26-kDa protein (Figure 5B) and showed that its amount increased at the 3rd day of incubation. We tried to produce recombinant CSF3 with bacterial expression systems including pQE, pET32, pET28 and pNCMO2 for *Bacillus brevis* but with negative results (data not shown).

Figure 2. Expression of CSF3 genes in fugu (trCSF3-1 and -2) and green-spotted pufferfish (tnCSF3-1 and -2) in tissues.

Figure 3. Expression of Japanese flounder poCSF3 gene in brain, kidney, PBLs and spleen in vitro following poly I:C treatment at 1, 3 and 6 hrs-post stimulation.

DISCUSSION

At the transcriptome level, it appears that the fugu *tr*CSF3s and green spotted pufferfish *tn*CSF3-2 are likely to be functional while the *tn*CSF3-1 is not and might have become a pseudogene. *tn*CSF3-1 was previously reported to apparently lack the conserved Cys (C) residue, a missing probable receptor-binding-glutamic-acid residue (E_{19}), a rather long hydrophobic N-terminal region, an undetected conserved domain (Santos *et al.*, 2006). If this is the case, the pufferfish paralogs probably followed a nonfunctionalization event, the classical theory of gene duplicate evolution, which predicts that one of the duplicate genes becomes fixated as a null allele incapable of transcription, translation or biological functions (Ohno, 1970). On the other hand, the fugu CSF3 paralogs were

pCDNA4 vector region (5')

Xpress

EK Recog.

KpnI

BamHI

PstI

CSF 3 Open Reading Frame

EcoRI

pCDNA4 vector region (3')

Figure 4. Confirmation of pCDNA4-CSF3 insert construct by sequencing.

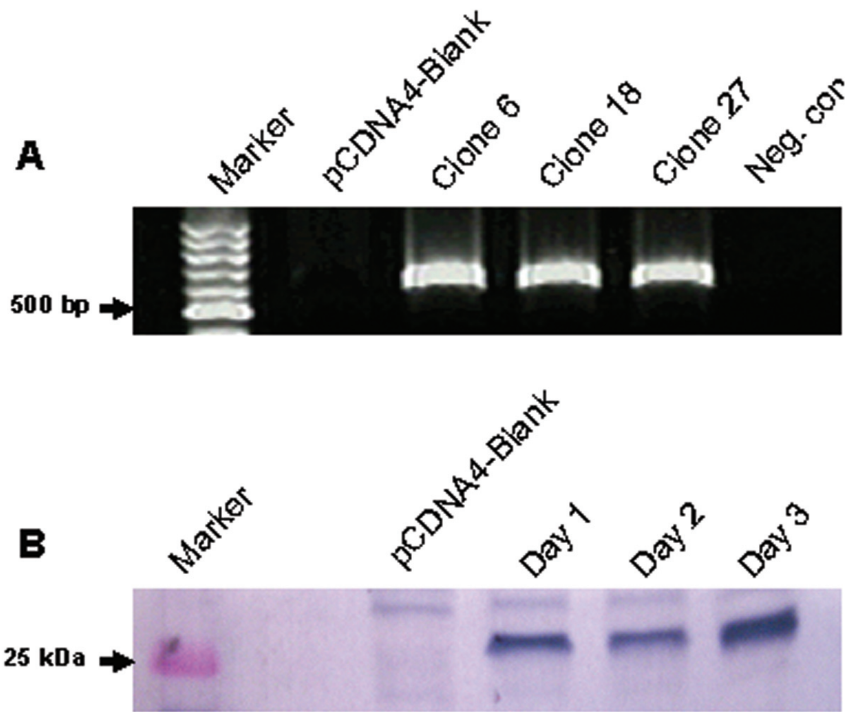

Figure 5. Recombinant Japanese flounder poCSF3-2 protein production using the mammalian expression vector pCDNA HisMax expressed in HINAE cells.
A) poCSF3-2 mRNA transient expression by 3 separate pCDNA4-CSF3 clones (C6, C18, C27) as shown through RT-PCR;

B) recombinant poCSF3-2 protein expression (Clone 6) at 1, 2 and 3 days-post transfection as detected by western blot using AntiExpress antibody.

expressed, although in different tissues. This suggests that these fugu genes undergone subfunctionalization, also called the duplication-degeneration-complementation (DDC) pathway (Force *et al.*, 1999). In the DDC pathway, the functions of both paralogs are complementary and have been preserved. The DDC pathway is not uncommon in fish. For example, evidence supporting the DDC theory has been observed in the *engrailed* genes of zebrafish. Another round of PCR (nested PCR) using some of the tissues for both fish yielded fragments of all four of the CSF3 orthologues (data not shown), indicating that all 4 mRNA transcripts are expressed and suggesting that pufferfish *tn*CSF3s is also undergoing positive selection. The functional evolution of the duplicate CSF3 genes in two species of the same fish family (fugu and green spotted pufferfish), which are thought to have diverged between 18 and 30 million years (Myr) ago (Hedges, 2002) needs to be clarified in future work.

*po*CSF3-2 production was observed in PBLs and spleen following poly I:C stimulation. In mammals, poly I:C has been reported to induce expression of CSF3 in endothelial and fibroblast cells (Fibbe *et al.*, 1988b and 1989), and in uterine epithelial cells (Schaefer *et al.*, 2005). In fish, poly I:C has been shown to stimulate immune-related genes such as

viperin in mandarin fish (Sun and Nie, 2004), and the inducible isoform of nitric oxide synthase (iNOS) in small spotted cat shark (Reddick *et al*., 2006). Interleukin 6 (IL6) of fugu, which belongs to the same protein family as CSF3, and which is called Pfam IL6/CSF/MGF because of their conserved protein domains, was also significantly upregulated in the spleen when fish were injected with poly I:C (Bird *et al*., 2005). It is, however, unclear why CSF3 was not detected in brain and kidney, and in PBLs (after 1 hr and 3 hrs of incubation). In fugu, IL6 may have been increased in the kidney by poly I:C injection but the increase was not statistically significant. Nevertheless, this study provides indirect evidence that fish CSF3 is regulated by interferon or interferon-induced genes.

We were successful in constructing a mammalian expression vector producing the recombinant CSF3 protein of Japanese flounder in a fish cell line *in vitro.* The implication of such is that the recombinant protein produced is fully functional. Why bacterial expression systems failed to produce recombinant CSF3 is unclear. The immune-related activity of Japanese flounder CSF3 against possible pathogens, together with an understanding of its regulatory regions, suggests that the recombinant CSF3 protein is a good candidate for enhancing immune responses against disease. For example in mice that have been vaccinated with HIV-1 *env* and *gag/pol*, co- delivery of recombinant mice CSF3 with macrophage colony-stimulating factor (MCSF) resulted in a moderate effect on serum antibody responses and T-helper cell proliferation, upregulated INF-γ production in antigen-stimulated splenocytes and increased the serum IgG_2/IgG_1 antibody isotype ratio (Kim *et al*., 1999).

CONCLUSIONS

Granulocyte colony-stimulating factor has recently been identified in teleost fish. Howerve, little is known about where it is expressed or how it is regulated. Our results show that duplicate CSF3 genes in fugu and pufferfish are differentially expressed, and that Japanese flounder *po*CSF3-2, aside from its up-regulation by LPS and conA-PMA stimulation as previously shown, is also involved in interferon-related mechanisms and pathways. Lastly, we successfully produced a recombinant Japanese flounder *po*CSF3-2 protein and this would allow for further studies of *po*CSF3-2 structure and function, and possible use in disease management.

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