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Identification of two Stat3 variants lacking a transactivation domain in grass carp: New insights into alternative splicing in the modification of teleost Stat3 signaling

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ABSTRACT

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family in response to cytokines and growth factors. In mammals, alternative splicing of *STAT3* generates STAT3α and STAT3β, which have distinct and overlapping functions. In the previous study, we have identified two spliceforms of Stat3α (Stat3α1 and Stat3α2) possessing all functional domains of Stat3 in grass carp (*Ctenopharyngodon idella*). In the present study, two Stat3β variants (Stat3β1 and Stat3β2) without C-terminal transactivation domain were isolated from this species, and their transcripts were ubiquitously expressed in all examined tissues with the highest levels in liver. Further studies showed that Stat3β1/2 had the ability to translocate into the nucleus upon activation, indicating their roles in transcriptional regulation. In support of this notion, grass carp Stat3β1 and Stat3β2 displayed the abilities to inhibit Interleukin-10 (Il-10) signaling and competitively impaired the transcriptional activities of Stat3α1/2. In particular, similar to their mammalian counterparts, grass carp Stat3β1 and Stat3β2 could enhance Stat3α1/2 phosphorylation upon cytokine stimulation. Interestingly, *stat3β1* and *stat3β2* transcripts were also found in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*), and each variant in these teleosts is generated through similar alternative splicing events, including exon skipping and intron retention. This highlights a conserved splicing event of *stat3* gene during vertebrate evolution and indicates a potential physiological significance of generating unique Stat3 variants in fish. These results, along with the findings regarding Stat3α1/2, demonstrate the existence of Stat3 isoforms with functional diversity and redundancy in teleosts. It leads to the hypothesis that teleost-specific spliceforms of *Stat3* gene may contribute to the complexity of Stat3 signaling in fishes, thereby benefiting them to adapt to evolution and environmental changes.

1. Introduction

Signal transducer and activator of transcription 3 (STAT3) is a member of the DNA-binding transcription factors family. It consists of an N-terminus domain, a coiled-coil domain, a DNA-binding domain, an SH2 (Src homology 2) domain and a C-terminal transactivation domain (TAD) [1]. The N-terminus domain plays roles in nuclear translocation and protein interactions; The coiled-coil domain is involved in nuclear export and the regulation of tyrosine phosphorylation; The DNA-binding domain can recognize and bind to the STAT-responsive element ($TTCN_{3-4}GAA$) in the promoters of target genes; The SH2 domain can mediate the interaction with phospho-tyrosines; The C-terminal TAD mediates transactivation [2]. In mammals, at least four isoforms of STAT3 generated by alternative splicing or proteolytic cleavage have been revealed: full-length STAT3α (92 kDa), and the truncated forms, STAT3β (83 kDa), STAT3γ (72 kDa) and STAT3δ (64 kDa) [3,4]. Among these isoforms, only STAT3β is generated by alternative splicing using an alternative acceptor site embedded in exon 23 that leads to a truncated isoform lacking the TAD replaced by seven unique amino acids. Because STAT3β protein lacks the TAD including S727, whose phosphorylation can stimulate transcriptional activity [5], it was initially thought to be a functional dominant negative regulator of STAT3 signaling [6]. As an example, STAT3β can revert STAT3α-induced promoter activities of Bcl-XL, $p21^{WAF1/CIP1}$ and cyclin D1 [7,8]. However, the facts that STAT3β can rescue the embryonal lethality of a STAT3 null mutation and induce the expression of STAT3 target genes indicate that STAT3β is not only a dominant negative factor. Moreover, STAT3β-deficiency in mice leads to impaired recovery from endotoxic shock and stimulation of a subset of endotoxin-inducible genes, implying that STAT3β acts as an off-switch in systemic inflammation [9]. A distinct

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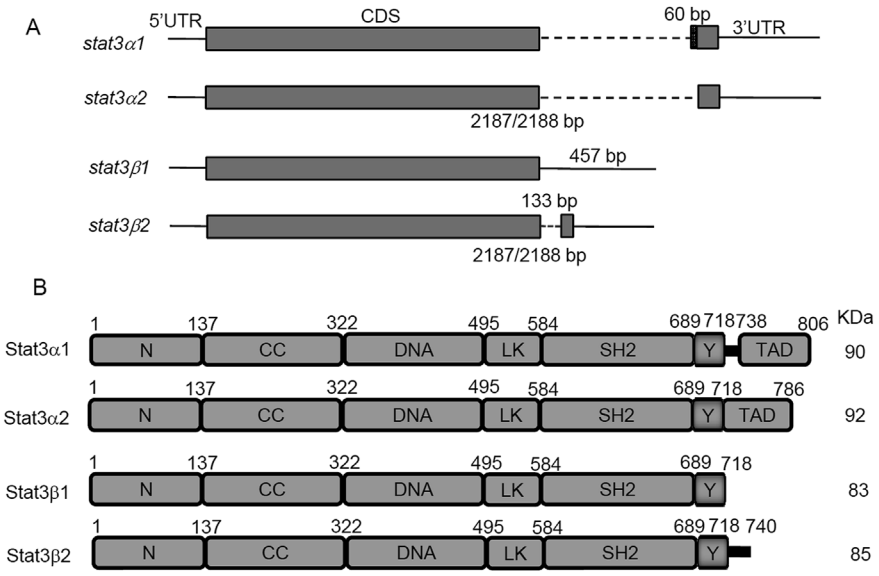

Fig. 1. (A) Schematic representation of the cDNA sequences of grass carp *stat3α1/2* and *stat3β1/2*. The position and length (bp) of alternatively spliced sequences are shown. (B) Schematic diagram of protein domains of grass carp Stat3α1/2 and Stat3β1/2. N, N-terminal domain; CC, coiled-coil domain; DNA, DNA-binding domain; LK, linker region; SH2, Src homology 2 domain; Y, Tyrosine phosphorylation site; TA, transcription activation domain.

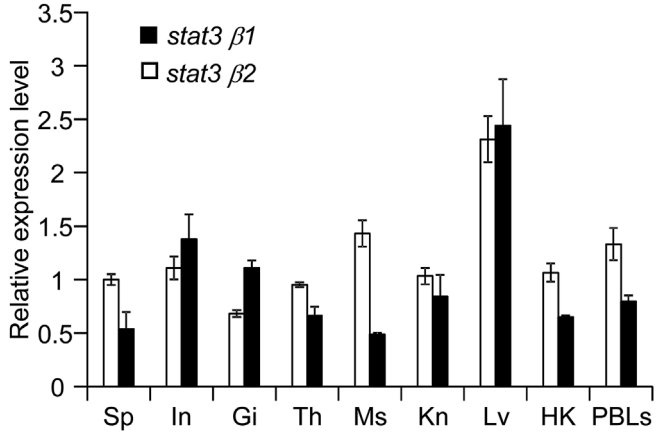

Fig. 2. Constitutive expression of grass carp *stat3β1/2* mRNA in the selected tissues and PBLs. Total RNAs were extracted from the selected tissues and PBLs of six grass carp, and the mRNA levels of *stat3β1/2* and *β-actin* were detected by RT-qPCR. The mRNA levels of *stat3* variants were normalized against *β-actin* and expressed as the ratios to the *stat3β2* mRNA level in spleen. Sp, spleen; In, intestine; Gi, gill; Th, thymus; Ms, muscle; Kn, kidney; Lv, liver; HK, head kidney; PBLs, peripheral blood lymphocytes.

set of genes specifically regulated by STAT3β under basal conditions and after cytokine stimulation are revealed via comparing transcriptome profiling of STAT3-deficient cells expressing either STAT3α or STAT3β. Taken together, STAT3β is not simply a truncated form of STAT3α with dominant-negative properties and its unique biological functions enrich the complexity of STAT3 signaling. Indeed, it has been demonstrated that STAT3α and STAT3β have distinct and overlapping functions in mammals [10]. These findings not only show the complexity of STAT3 signal transduction pathways, but also highlight the unique regulatory patterns of STAT3 signaling based on alternatively spliced variants. It raises the question of whether *STAT3* gene also undergoes alternatively splicing in non-mammalian vertebrates, e.g. teleosts, which are the largest and most diverse species of vertebrates and contain a single *stat3* gene.

In several fish species, only one *stat3* transcript has been reported [11–16]. Recently, we demonstrated the existence of *stat3α1* and *stat3α2* in grass carp (*Ctenopharyngodon idellus*) and found that the deduced proteins of them possess conserved functional domains compared to their mammalian homologs [17]. In the present study, two additional Stat3 variants lacking TAD (gcStat3β1 and gcStat3β2) were revealed in grass carp. Their ability to translocate into the nucleus as transcription factors was shown. Moreover, overexpression of gcStat3β1 and gcStat3β2 suppressed the expression of *suppressor of cytokine signaling 3* (*socs3*) induced by Interleukin-10 (Il-10) or overexpression of Stat3α1/2 in grass carp cells. Intriguingly, gcStat3β1/2 overexpression augmented the phosphorylation of gcStat3α1/2 triggered by Il-10, showing their interaction in this species. Notably, these two variants of *stat3β* were also found in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*). Bioinformatics analysis showed these variants were generated through the same splicing patterns. These findings along with conserved splicing patterns of *stat3α1/2* in fish species [17] reinforce the existence of teleost-specific alternative splicing of *stat3* gene. Given that *stat3* emerges as the first *stat* gene during teleost evolution [18,19], our data provide an evolutionary perspective of how the spliced variants drive flexibility of Stat3 signaling. Taken together, our results provide evolutionary information for generating *stat3* variants, and are beneficial to understanding of the complexity of Stat3 signaling in fish.

2. Materials and methods

2.1. Animals

Healthy grass carp weighing ~0.75 kg were purchased from Chengdu Tongwei Aquatic Science and Technology Company (Chengdu, China). Goldfish and zebrafish were obtained from local aquaria. The fishes were maintained in glass aquaria for about one week. Then the fish were sacrificed for the subsequent experiments. All animal experiments were in conformity with the Regulation for Animal Experimentation of Sichuan province, China and approved by the ethics committee of the University of Electronic Science and Technology of China.

2.2. Molecular cloning and sequence analysis of stat3β1/2

Trunk kidneys were collected from goldfish, zebrafish and grass carp, and about 70 mg of each tissue were homogenized in 1 ml TriPure Isolation Reagent, separately (Roche, Indianapolis, USA). Total RNAs were extracted from these tissues and about 2 μg total RNA was subjected to reverse transcription using M-MLV Reverse Transcriptase (Promega, Madison, WI) and Olig $(dT)_{18}$ as the primer. Partial sequences of grass carp and goldfish *stat3β1/2* were obtained by PCR using the degenerated primers (Supplementary Table 1). The full-length cDNAs of *stat3β1/2* in grass carp and zebrafish were pulled out using 3′-