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Molecular cloning and characterization of Foxp3 in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Foxp3 is a T cell-specific transcription factor and plays a key role in the development of Treg cells and in the immune regulatory process during inflammation. Here we report cloning and characterization of the full-length cDNA of Atlantic salmon Foxp3, which possesses a Forkhead domain, a zinc finger domain and a leucine-zipper domain as its counterpart in mammals. Foxp3 is highly expressed in thymus. Furthermore, regulated expression was observed in head kidney cells in response to β-glucan and mitogens (LPS and ConA), and in the head kidney, spleen and liver after intraperitoneal injection of live *Aeromonas salmonicida*. In addition, transfection of CHSE-214 cells with salmon Foxp3 fused with a C-termial RFP tag, resulted in the expression of the transgene in and close to the nuclei upon stimulation. Taken together, these results suggest the presence of a Foxp3 gene in Atlantic salmon that may be an important transcription factor in immune regulation, and further research may reveal the existence of Treg-like T cells in this species.

1. Introduction

With the presence of the adaptive immune system during the evolution, the prevention of auto-immune responses and over-reaction to foreign molecules became required [1]. The idea of suppressive T cells, at present called regulatory T cells (Treg cells), was first proposed about 40 years ago, but the process of describing Treg cells turned out to be slow until the first Treg-cell marker, CD25, was identified, and was shown to be highly expressed in Treg cells in 1995 [2]. However, CD25 and other molecular markers, like CTLA-4 (cytotoxic T lymphocyte antigen 4) and GITR (glucocorticoid-induced tumor necrosis factor receptor family-related gene), were not solely restricted to Treg cells [2], which is a disadvantage in the study of the Treg cell lineage. The identification of Foxp3 exclusively expressed in Treg cells from Scurfy mice largely solved the problem [3,4].

In mammals, a reciprocally developmental model of Treg cells and Th17 cells has been deciphered [5]. In this model, TGF-β was shown to be required for the development of both types of cells. Moreover, in the presence of IL-6 Th17 cells developed whereas in the absence of IL-6, the development was skewed toward Treg cells. It has been suggested that IL-17s, RORγt and RORα (orphan nuclear receptor γt and α, respectively) were characteristic cytokines and key transcription factors of Th17 cells [6], whereas, in addition to the observation of Foxp3 as the master transcription factor of Treg cells, IL-10 was found to be highly expressed in Treg cells [7].

The TCR-based adaptive immune system appears after the agnatha [8], and in teleosts, molecules homologous to mammalian TGF-β, IL-17s, RORγ, IL-10 and Foxp3 have been characterized [9–14]. However, it remains unknown whether there exists a Treg-like T cell lineage in teleosts. In the present study, we cloned and characterized the cDNA of Foxp3 gene in Atlantic salmon. Also, its expression in primary immune cells, CHSE-214 cell line and selected tissues was carried out with or without stimulation.

2. Materials and methods

2.1. Cloning of salmon Foxp3

Three EST sequences (Acc No. DW573923, DW573924, CX358007) homologous to vertebrate Foxp3 were identified based on amino acid sequence through tBLASTn searches in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A pair of primers (Table 1) was designed based on three EST sequences for hybridization to a partial Foxp3 sequence from a salmon cDNA library. To obtain the full length of salmon Foxp3 cDNA, 3′- and 5′-RACE-ready cDNA were reversely transcribed from salmon spleen and kidney mRNA mix by using a SMARTer RACE cDNA amplification kit (Takara, Shiga, Japan, Cat No. 634923). One primer for 3′ terminal amplification and two primers for 5′ terminal (Table 1) were designed based on the partial sequence

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Table 1
List of Primers and their applications.

Primer name	Sequence (5′–3′)	Application
AsFoxp3-Fw1	TCATGAGCCAGGTCACCCAA	Partial sequence amplification
AsFoxp3-Rv1	TACGGAGGCCGGATGTTGTT	Partial sequence amplification
AsFoxp3-3-Fw2	AAGAGCCAGAGGAGCTGGCA	3′RACE
AsFoxp3-5-Rv2	TGGGGAGCGGCAGCGGGCCAATACT	5′RACE
AsFoxp3-5-Rv3	GGGGCAGGACTAGTGCCAGGCTGTGG	5′RACE
AsFoxp3-Fw3	GAAGACAGAAAGTGAGCAGGAGA	CDS verification
AsFoxp3-Rv4	CATTCACTGAAGGGGTGGCTATGT	CDS verification
AsFoxp3-Fw4	AGCTGGCACAGCAGGAGTAT	Real-time PCR
AsFoxp3-Rv5	CGGGACAAGATCTGGGAGTA	Real-time PCR
As18S-Fw	TGTGCCGCTAGAGGTGAAATT	Real-time PCR
As18S-Rv	CGAACCTCCGACTTTCGTTCT	Real-time PCR

obtained. The RACE PCR products were cloned with TOPO® TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA, Acc No.K4575-40). An additional pair of primers (Table 1) was synthesized to amplify the complete coding sequence (CDS).

2.2. Sequence analysis

The nucleotide sequences were assembled manually with BioEdit v7.0.5 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The protein sequence was deduced from the complete coding sequence using Translate tool (http://www.expasy.org/tools/dna.html) [15]. The cDNA sequence and corresponding protein sequence were further analyzed with BLAST methods in GenBank. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was applied to

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ggagaacaggaagacagaaagtgagcaggagatacagagtaagcccaggg  ATG CCA CAG ACT GAG AGT GAG    71
                                                      M   P   Q   T   E   S   E      7
AGG TTG AAG AGT GGG CGG CTG AAC AGT GGT AGA CAG CAG CAG CAG CGA GAT CGG CGG GGT   131
 R   L   K   S   G   R   L   N   S   G   R   Q   Q   Q   Q   R   D   R   R   G    27
GAA CAC GGA GAG GAG CCA GAC ACA CGT ACC AAG CCA AAT GAT TCT TTC TCA CCT CGG CTC   191
 E   H   G   E   E   P   D   T   R   T   K   P   N   D   S   F   S   P   R   L    47
TAT GCT CGT ACC TCT GTT ACT CAG ATG GGT TTC CCA TTG ATG ATT AGA CCT GGA CGT CCA   251
 Y   A   R   T   S   V   T   Q   M   G   F   P   L   M   I   R   P   G   R   P    67
CTT ATG GCC TCA TCT CAG CTC CAA AGC ATA CTG TTA CAG CAG TGT TCC AGT GAG GAG GAG   311
 L   M   A   S   S   Q   L   Q   S   I   L   L   Q   Q   C   S   S   E   E   E    87
GGG AAA CCG TTC CTG CAG CGG GTG TCT CGG TGC ACC CAG CTG AGC CAG CAC AGA CCC TCT   371
 G   K   P   F   L   Q   R   V   S   R   C   T   Q   L   S   Q   H   R   P   S   107
GTC CTC CGC CAG GGT GTC CTA GCT GCC CAC GTA CGA CCA CAG GCA GCG GCC GGT TCA GCT   431
 V   L   R   Q   G   V   L   A   A   H   V   R   P   Q   A   A   A   G   S   A   127
CAA CCC ATA TCA CTG TGC AAG GTG GAG GTG GAC ACA GGG AGC CGT GGA CAG TCC TCA CCC   491
 Q   P   I   S   L   C   K   V   E   V   D   T   G   S   R   G   Q   S   S   P   147
CCA CAC TCT GAG CAC TCT CCT GGG CCC ACT CGT CAC CCC TCG CCC CTG AGG AGA GCC AGT   551
 P   H   S   E   H   S   P   G   P   T   R   H   P   S   P   L   R   R   A   S   167
CCT AAG CAG AGC TCC ATC ATC ACC AGG CAT CAT GAG CCA GGT CAC CCA ACC CTA CGG AGC   611
 P   K   Q   S   S   I   I   T   R   H   H   E   P   G   H   P   T   L   R   S   187
TCC AGT GCT CTG TTT CTC AAC GGA CTC TGC TGC TGG CCA GGC TGC GAT GCG GTA TTT GAA   671
 S   S   A   L   F   L   N   G   L   C   C   W   P   G   C   D   A   V   F   E   207
GAA TTT CCC CGT TTT TTG AAA CAC CTC CAC TCT GAC CAT GGC CAT GGA GAC AGA AGC ATT   731
 E   F   P   R   F   L   K   H   L   H   S   D   H   G   H   G   D   R   S   I   227
GCA CAG TGG AGG GTA CAA CAA GAC ATG GTG CAA TAC ATG GAG ACC CAG CTG ACT GTG GAA   791
 A   Q   W   R   V   Q   Q   D   M   V   Q   Y   M   E   T   Q   L   T   V   E   247
AAA CAG AGA CTC TTT GCA ATG CAA CTC CAT CTG CAC CTG TCT GAA CAC AAG TCA ACT GGC   851
 K   Q   R   L   F   A   M   Q   L   H   L   H   L   S   E   H   K   S   T   G   267
ATG AAG GCA GGT TCA GAT TGG CCC TAC AGC CAC AGC CTG GCA CTA GTC CTG CCC CAG AAC   911
 M   K   A   G   S   D   W   P   Y   S   H   S   L   A   L   V   L   P   Q   N   287
CCT TCC CCG GCC CGG GCA GCA GAT GGA GTG CCA CGC TGC GCC ACT AAA GAG CCA GAG GAG   971
 P   S   P   A   R   A   A   D   G   V   P   R   C   A   T   K   E   P   E   E   307
CTG GCA CAG CAG GAG TAT TGG CCC GCT GCC GCT CCC CAC CAC CTA CTC CCA GAT CTT GTC  1031
 L   A   Q   Q   E   Y   W   P   A   A   A   P   H   H   L   L   P   D   L   V   327
CCG AGT GTT GAG TGT TAC AAA TAC AAC AAC ATC CGG CCT CCG TAC ACC TAC GCC TGC CTG  1091
 P   S   V   E   C   Y   K   Y   N   N   I   R   P   P   Y   T   Y   A   C   L   347
ATA AGA TGG TCT ATA ATG GAG ACT CCA GAC AAG CAG CGC TCT CTG AAT GAC ATC TAC AAC  1151
 I   R   W   S   I   M   E   T   P   D   K   Q   R   S   L   N   D   I   Y   N   367
TGG TTC ACC ACC ATG TTC TTC TAC TTC CGA CAC AAC ACG GCC ACA TGG AAG AAC GCT GTC  1211
 W   F   T   T   M   F   F   Y   F   R   H   N   T   A   T   W   K   N   A   V   387
CGT CAT AAT CTT AGC TTG CAC AAG TGT TTT GTG CGA GTG GAG GGA GGC AAG GGG GCT GTC  1271
 R   H   N   L   S   L   H   K   C   F   V   R   V   E   G   G   K   G   A   V   407
TGG ACA GTG GAT GAA ATG GAA TAT CAA AGG AGA AAG GGA CAA AAA TAT CAC AGG GAT CAT  1331
 W   T   V   D   E   M   E   Y   Q   R   R   K   G   Q   K   Y   H   R   D   H   427
CAT GTG AAA TGG TTG GCA CCC TTC TCC TTA TTC CGT CCA GAG GAA CCT TGA    acatagcca  1391
 H   V   K   W   L   A   P   F   S   L   F   R   P   E   E   P   *              443
ccccttcagtgaatgaaaactacaggaaaaggaagtcaccagggcctataactgaatcaggtatattataacagggctgg 1471
agcaaaagcctccatattagctctccaggcggagggttgaccagtcctggtctacagtatctacagcacccagcatcacc 1551
ttctttgttcagcagcagatggtgctataatacatagcatagccccggactcttttgcctctctagactctgatcatgaa 1631
gaagtgtgcatggctggaatggcaatatatttttgcagcctttgaaatacaaatatacacttaaaaagtatttgtcaataa 1711
aaacacagtgaaaaggtgaacaatcgttttgttttgtagtgttggctacatatgtgttacgcaagtaaaaatttgatatc 1791
tgtcaaaaattcataaaataattaattaaattaatatttgaaagtacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa    1865
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Fig. 1. Salmon Foxp3 cDNA sequence and the deduced protein. The CDS is presented in uppercase and the Polyadenylation signal (AATAAA) in 3′-UTR is underlined. The C2H2-type zinc finger and Winged helix/Forkhead domains, predicted with the online program PROSITE (http://ca.expasy.org/prosite/), are given as single strikethrough and boxed, respectively. As well, the observed leucine-zipper motif from alignment has double strikethrough.