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Identification of suppressor of cytokine signalling (SOCS) 6, 7, 9 and CISH in rainbow trout *Oncorhynchus mykiss* and analysis of their expression in relation to other known trout SOCS☆

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ABSTRACT

Four new members of the SOCS family of molecules in rainbow trout (*Oncorhynchus mykiss*), CISH and SOCS6, 7 and 9, are described for the first time in this species. The genes had a wide tissue distribution in trout, and were detected in gills, skin, muscle, liver, spleen, head kidney, intestine and brain, with brain having the highest expression levels. Stimulation of a rainbow trout leucocyte cell line, RTS-11, (mononuclear/macrophage-like cells) with LPS or Poly I:C had no effect on the expression of these genes, although in both cases the previously identified SOCS1-3 genes were up-regulated. Similarly, stimulation of RTS-11 or RTG-2 (fibroblasts) cells with the trout recombinant cytokines IFN-γ or IL-1β had no effect on CISH or SOCS6, 7 and 9 expression. However, PMA stimulation did impact on SOCS6 and SOCS9 expression, and LPS stimulation of primary cultures or bacterial infection (*Yersinia ruckeri*) increased significantly CISH expression (as well as SOCS1 and SOCS2 or SOCS3 respectively). It is apparent that the type II SOCS genes (CISH, SOCS1-3) are particularly relevant to immune regulation in fish, although the intriguing expansion of the SOCS4/5 subgroup in fish requires further investigation as to their role and functional divergence.

1. Introduction

Many important cytokines have been identified in teleost fish in the last decade. These have included pro-inflammatory cytokines such as interleukin-1β (IL-1β) [1], IL-6 [2,3], IL-8 [4,5], IL-11 [6] and tumour necrosis factor-α (TNF-α) [7,8], with studies of the recombinant proteins in general confirming a similar function in fish to their counterparts in mammals [9–11], although exceptions exist [12]. Important initiators and effectors of adaptive immune responses have also been cloned, such as IL-2 [13], IL-12 [14], IL-15 [15], IL-17 [16,17], IL-18 [18], IL-21 [13], IL-22 [19,20], interferon-γ (IFN-γ) [21] and molecules with relatedness to Th2-type cytokines [22,23], with protein studies beginning to validate their functional relevance [15,24,25]. However, rather less is known about the control of cytokine signalling in fish. Two important cytokines known to be negative regulators of adaptive immune responses have been cloned, namely transforming growth factor-β1 (TGF-β1) and IL-10 [26–28], with rTGF-β shown to down-regulate the nitric oxide response of TNF-α activated macrophages [29]. In addition a novel IL-1 family member has been discovered in fish that can antagonise the activity of IL-1β [30], although it remains to be determined if it is a receptor antagonist that has evolved independently of the IL-1ra known in mammals. Another group of molecules has also been discovered that can regulate cytokine action in mammals, called the suppressors of cytokine signalling (SOCS) molecules [31–33], and these have also now been discovered in fish.

SOCS molecules comprise eight proteins in mammals, SOCS1 to 7 and cytokine inducible SRC homology 2 (SH2) – containing protein (CISH)[33]. All of these proteins possess an SH2 domain, a C-terminal SOCS box and a variable N-terminal extended SH2 subdomain (ESS). They are induced by cytokines and inhibit signal transduction from type I and II cytokine receptors in a classical negative-feedback loop. Whilst it is known that they all bind phosphorylated tyrosine residues via the SH2 domain, different SOCS function in different ways to inhibit cytokine signalling. For example, SOCS1 and SOCS3 can inhibit JAK activity via a kinase inhibitory region (KIR) domain that acts as

☆ The nucleotide sequence data will appear in the EMBL/DDBJ/GenBank nucleotide sequence database under the following accession numbers: AM903340 (CISH), AM903342 (SOCS6), AM903344 (SOCS6 gene), AM903343 (SOCS7) and AM903341 (SOCS9).

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a pseudo-substrate. In contrast, SOCS2 and CISH appear to act as competitors of STAT proteins. In addition, the SOCS box acts to target substrate proteins for ubiquitination and proteasomal degradation, via E3 ligases [32]. In this way SOCS molecules can regulate the half life of a wide range of proteins. In fish, SOCS1-3 molecules were initially described in a number of fish species [34–36], and most recently a thorough analysis of fish genomes has revealed a larger number of SOCS family members are present in teleost fish than are seen in mammals [37]. In this study we have discovered genes for four new members of the SOCS family in rainbow trout (*Oncorhynchus mykiss*), by analysis of ESTs, that were identified as CISH, SOCS6, 7 and 9. We have analysed their expression in different tissues and, in comparison to the already identified trout SOCS1, 2 and 3 genes, after infection for the first time in fish. In addition we have studied their expression in trout cell lines after stimulation with LPS, Poly I:C, PMA and two recombinant cytokines.

2. Materials and methods

2.1. Molecular identification of trout CISH, SOCS5, 6 and 7

An initial search of rainbow trout (*O. mykiss*) ESTs with homology to mammalian CISH and SOCS molecules was conducted using the TBLASTN program (http://www.ncbi.nlm.nih.gov/BLAST) [38]. The ESTs BX863093 (CISH), CU066723 and BX312152 (SOCS6), BX878085 and CA376519 (SOCS7), and CA387203 (SOCS9) were identified and used for primer design (Table 1) to amplify the full-length cDNAs.

3′- and/or 5′-RACE was conducted to obtain the complete coding regions as described previously [36,39]. The nucleotide sequences generated were assembled and analysed with the AlignIR programme (LI-COR, Inc.). The protein identification was carried out on http://www.expasy.org/tools/ [40]. The domain structure was predicted using the SMART6 program (http://smart.embl-heidelberg.de/smart/) [41]. Global sequence comparison was performed using the MatGAT program [42]. Phylogenetic trees were created by the neighbour-joining method using the MEGA program (V3.1)[43] based on a CLUSTAL multiple alignment and were bootstrapped 5000 times. The analyzed protein sequences were selected from eleven vertebrate species, including human, mouse possum, chicken, frog, tetraodon, fugu, zebrafish, stickleback, medaka and rainbow trout. The accession numbers are detailed in Table 2. A more detailed analysis of the trout SOCS9 molecule with other known fish SOCS5 and SOCS9 molecules was also undertaken by multiple alignment.

2.2. Real-time PCR quantification of gene expression

The expression of trout SOCS family members as well as a common reference gene, elongation factor-1α (EF-1α), was quantified by real-time PCR using CYBR green (Invitrogen) and a LightCycler 480 real-time PCR system (Roch) as described previously [15,36]. The primers used for real-time PCR are given in Table 1 and were pre-tested to ensure that each primer pair could not amplify genomic DNA using the real-time PCR protocols. For comparison of the relative expression level of different members of the SOCS family, a standard was constructed with a mixture of equal mole amounts of PCR products of each SOCS gene amplified from cloned plasmids. A serial dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as a reference for quantification. The expression level of each gene was calculated as arbitrary units that had been normalized to the expression of EF-1α. A fold change was also calculated as the expression level of each treatment divided by that of the corresponding control.

Table 1
Primers used for cloning and expression.

Gene	Primer	Sequence (5′ to 3′)	Application
CISH	CISH F1	CAGACCGTCTATTAACATATGGGATATTC	3′-RACE
	CISH F2	CGGACACAACTTCTCAACTGAACAG	3′-RACE
	CISH F	CATTCTACCTTGATACCTCAGGCTGGT	Real-time PCR
	CISH R	CTGTACTGGATCGCTATACTGGTGG	Real-time PCR
SOCS1	SOCS1 F2	GATTAATACCGCTGGGATTCTGTG	Real-time PCR
	SOCS1 R1	CTCTCCCATCGCTACACAGTTCC	Real-time PCR
SOCS2	SOCS2 F2	GACGCGTGGGAGAAGTGTATTTGAGTC	Real-time PCR
	SOCS2 R1	CGAGTCTCGTCGGAATCTACAACC	Real-time PCR
SOCS3	SOCS3 F1	GAACAACACAAGATATCAAGCTCAAGG	Real-time PCR
	SOCS3 R1	GAAGGTCTTGTAACGGTGAGGCAG	Real-time PCR
SOCS6	SOCS6 F	GTTGAAAAACATACTGTAGAAAGGGATGAC	Real-time PCR
	SOCS6 F7	CCCACAATGCAAGCGTCATGT	Genomic PCR
	SOCS6 R1	CCTTTGCTTTGAGGTTGGGAGTATAAC	5′-RACE & genomic PCR
	SOCS6 R2	GTTGAACAGATCACTAGATAGGGAGACA	5′-RACE
	SOCS6 R3	CTGTGGTACAATATCCTTTCCCTCCTC	Real-time PCR
SOCS7	SOCS7 F3	CCTCTGGTGTCACCCAAAGTTTG	3′-RACE
	SOCS7 F4	GAACTGGAAAAATGTGGCTGGT	Real-time PCR & 3′-RACE
	SOCS7 R1	GACATGTACATCTCCTCCTCAG	5′-RACE
	SOCS7 R2	GGAGCTCCTGGATGTGGTCT	5′-RACE
	SOCS7 R5	GACACCAGAGGCTGAAGGTTCC	Real-time PCR
SOCS9	SOCS9 R2	GCCTGCCTCCATAGTGTTAAGTTAG	5′-RACE
	SOCS9 R3	GTAGCCTAGTTGTGGTTCACTTCC	5′-RACE
	SOCS9 R5	CGGCATCTCTTCTCTCCTCAGACC	Real-time PCR
	SOCS9 F3	CAGCAGCTGCACCACCTACGA	3′-RACE
	SOCS9 F4	ACTAACTAGCTATCTTTTGGAGAGCAGCA	Real-time PCR
	SOCS9 F5	AGCCTCAAGTGACTGACAGTGATGC	3′-RACE
EF-1α	EF-1aF	CAAGGATATCCGTCGTGGCA	Real-time PCR
	EF-1aR	ACAGCGAAACGACCAAGAGG	Real-time PCR

Table 2
Accession numbers of selected sequences of the SOCS family used in this study. The rainbow trout SOCS members identified in this study are highlighted.

Gene	Species	Accession#
CISH/SOCS8		
CISH	Human	Q9NSE2
CISH	Mouse	Q62225
CISH	Opossum	XP_001378784
CISH	Chicken	NP_989957
CISH	Frog	AAI57221
CISH	Tetraodon	EF195753
CISH	Fugu	EF195746
CISH	Zebrafish	EF195760
CISH	Stickleback	EF371369
CISH	Medaka	EF544576
SOCS8	Tetraodon	EF195758
SOCS8	Zebrafish	EF195766
SOCS8	Fugu	EF195751
SOCS8	Stickleback	EF382413
SOCS8	Medaka	EF544585
CISH	**Rainbow trout**	AM903340
SOCS1		
SOCS1	Human	AAY87931
SOCS1	Mouse	NP_034026
SOCS1	Opossum	XP_001376339
SOCS1	Chicken	XP_414929
SOCS1	Frog	AAH88083
SOCS1	Tetraodon	DQ643956
SOCS1	Zebrafish	DQ350479
SOCS1	Fugu	DQ643957
SOCS1	Stickleback	DQ923044
SOCS1	Medaka	EF544577
SOCS1	Rainbow trout	AM748721
SOCS2		
SOCS2	Human	AAH10399
SOCS2	Mouse	AAI06154
SOCS2	Opossum	XP_001363646
SOCS2	Chicken	ZP_989871
SOCS2	Frog	EF544587
SOCS2	Tetraodon	EF195754
SOCS2	Zebrafish	EF195761
SOCS2	Fugu	EF195747
SOCS2	Stickleback	EF371370
SOCS2	Rainbow trout	AM748722
SOCS3		
SOCS3	Human	O14543
SOCS3	Mouse	O35718
SOCS3	Opossum	XP_001371144
SOCS3	Chicken	Q90X67
SOCS3	Frog	Q6DJC0
SOCS3a	Tetraodon	DQ333314
SOCS3a	Zebrafish	DQ333315
SOCS3	Fugu	DQ335254
SOCS3a	Stickleback	EF544595
SOCS3a	Medaka	EF544579
SOCS3b	Tetraodon	EF544574
SOCS3b	Zebrafish	DQ345761
SOCS3b	Stickleback	EF382411
SOCS3b	Medaka	EF544580
SOCS3	Rainbow trout	AM748723
SOCS4		
SOCS4	Human	Q8WXH5
SOCS4	Mouse	NP_543119
SOCS4	Chicken	EF544591
SOCS4	Frog	AAI23972
SOCS4	Tetraodon	EF371365
SOCS4	Zebrafish	EF371367
SOCS4	Fugu	EF371366
SOCS4	Stickleback	EF371368
SOCS4	Medaka	EF544581
SOCS5/SOCS9		
SOCS5	Human	NP_054730
SOCS5	Mouse	AAB96648
SOCS5	Opossum	XP_001375574
SOCS5	Chicken	EF544592
SOCS5	Frog	NP_001016844
SOCS5	Tetraodon	EF195755
SOCS5a	Zebrafish	EF195763
SOCS5b	Zebrafish	EF195762
SOCS5	Fugu	EF195748
SOCS5	Stickleback	EF382412
SOCS5	Medaka	EF544582
SOCS9	Tetraodon	EF195759
SOCS9	Zebrafish	EF195767
SOCS9	Fugu	EF195752
SOCS9	Stickleback	EF371372
SOCS9	Medaka	EF544586
SOCS9	**Rainbow trout**	AM903341
SOCS6		
SOCS6	Human	Q8WUM3
SOCS6	Mouse	NP_061291
SOCS6	Opossum	XP_001365964
SOCS6	Chicken	EF544593
SOCS6	Frog	EF544589
SOCS6	Tetraodon	EF195756
SOCS6	Zebrafish	EF195764
SOCS6	Fugu	EF195749
SOCS6	Stickleback	EF371371
SOCS6	Medaka	EF544583
SOCS6	**Rainbow trout**	AM903342
SOCS7		
SOCS7	Human	O14512
SOCS7	Mouse	Q8VhQ2
SOCS7	Opossum	XP_001366804
SOCS7	Frog	EF544590
SOCS7	Tetraodon	EF195757
SOCS7	Zebrafish	EF195765
SOCS7	Fugu	EF195750
SOCS7	Stickleback	EF544575
SOCS7	Medaka	EF544584
SOCS7	**Rainbow trout**	AM903343

2.3. Expression of CISH, SOCS6, 7 and 9 in vivo

The selection of tissues, RNA preparation, cDNA synthesis and real-time PCR analysis of gene expression was described previously [36] and the same set of 6 cDNA samples from 6 individual fish were used in this study. The real-time quantification of gene expression was as described above.

2.4. Modulation of expression of trout SOCS family members in vivo by bacterial infection

Rainbow trout (Mean ± SEM = 54.2 ± 1.4 g) were challenged with a pathogenic strain (MT3072) of *Yersinia ruckeri*, the causative agent of enteric red mouth disease (ERM). The bacteria were prepared from a tryptic soy agar plate (Fluke) inoculated with MT3072 for 2 days at 22 °C, by washing the bacteria three times with PBS (GIBCO) and re-suspending in PBS at a concentration of 2×10^6 cfu ml^{-1}. Two tanks of fish (30 fish/tank) were acclimatised to the aquarium facilities for two weeks before the experiment. Fish from one tank were injected intraperitoneally (i.p.) with bacteria (0.5 ml/fish) prepared as above and fish in the second tank were injected i.p. with phosphate buffered saline (PBS) (0.5 ml/fish) as controls. The water temperature throughout the experiment was 15 °C, the fish were fed with commercial trout pellets twice a day and the waste water was sterilized by ozonation. Six fish from each treatment were sampled at 24 h and 48 h post-injection. Spleen tissue was collected and used for total RNA preparation using TRIzol (Invitrogen).

2.5. Cell lines

A mononuclear/macrophage-like cell line RTS-11 [44], and a fibroid cell line RTG-2 [45] were cultured in L-15 medium supplemented with either 30% foetal calf serum (FCS) for RTS-11 cells, or 10% FCS for RTG-2 cells and 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin.

2.6. LPS and poly I:C stimulation of RTS-11 cells and splenocytes

To minimize the potential effects of serum protein on LPS, RTS-11 cells were collected by centrifugation (200 g, 5 min), resuspended in maintenance medium (L-15 plus 0.5% FCS), and then seeded into 6-well plates at 2×10^6 cells/well in 3 ml of maintenance medium. The cells were incubated at 20 °C overnight before any treatments. The cell culture supernatant was replaced with maintenance medium containing *E. coli* LPS (strain 055:B5, Sigma) at 25 μg ml^{-1}, Poly I:C (Sigma) at 50 μg ml^{-1} or medium alone as control, and incubated for a further 4, 11 and 24 h. The treatment was terminated by dissolving the cells in Trizol (Invitrogen). Similarly, freshly prepared splenocyte primary cultures were stimulated with LPS or Poly I:C as above, and sampled along with control, unstimulated cells 4, 8 and 24 h later.

2.7. Stimulation of RTG-2 and RTS-11 cells by PMA and trout rIL-1β and rIFN-γ

For PMA treatment, cells were seeded in L-15 medium containing 10% FCS and incubated at 20 °C overnight. PMA (Sigma) was prepared at 1 mg ml^{-1} ethanol and diluted with cell culture medium before addition to the cell cultures to achieve final concentrations of 0, 0.5, 5, 50 and 500 ng ml^{-1} PMA. The treatment was terminated at 8 h by dissolving the cells in Trizol. As the carrier ethanol concentration was diluted at least 2000-fold and no apparent effect on SOCS gene expression was seen in pilot studies even at the highest concentration, cells without any treatment were used as controls. The trout rIL-1β and rIFN-γ treatment of RTG-2 and RTS-11 cells was as described previously [36].

2.8. Statistical analysis

Real-time quantitative PCR measurements were analyzed using the nonparametric Mann–Whitney test within the SPSS package 17.0 (SPSS Inc. Chicago, Illinois), with $P < 0.05$ between treatment groups and control groups considered significant.

3. Results

3.1. Molecular identification and analysis of trout CISH, SOCS6, 7 and 9

3.1.1. CISH

Search of the EMBL database revealed that the translation of EST BX863093 had homology to the N-terminus of mammalian CISH sequences. Primers CISH F1/F2 (Table 1) designed to the 5′-UTR of this EST were used for 3′-RACE PCR using SMART cDNA prepared from splenic tissue [39]. A 1.3 kb 3′-RACE product was cloned and sequenced and contained part of the 5′-UTR, the complete coding region and the 3′-UTR of the trout CISH gene (Accession No. AM903340). The trout CISH gene encoded for a protein of 225 amino acids, with a theoretical molecular weight of 24.8 kDa and p*I* of 9.44. The trout CISH protein had highest homology to CISHs (including SOCS8) from other fish species (47–65% identity), with lower homology to higher vertebrate CISH (37–41% identity) (Table 3).

Table 3
Summary of characteristics and homology analysis of rainbow trout SOCS members identified in this study relative to other known SOCS molecules. Values in bold are those with the highest homology.

Trout		CISH	SOCS9	SOCS6	SOCS7
Accession number		AM903340	AM903341	AM903342	AM903343
Amino acids		225	544	515	652
Molecular weight(kDa)		24.8	60.6	58.1	71.5
p*I*		9.44	6.67	6.07	6.66
Species	Molecules	Homology (Similarity/Identity%)			
Human	CISH	**51.9/38.6**	24.6/14.7	25.4/16.1	19.3/12.7
Mouse	CISH	**51.0/37.0**	23.3/14.7	25.0/15.2	19.6/12.9
Chicken	CISH	**57.0/40.0**	20.8/11.7	26.6/16.4	19.0/11.9
Frog	CISH	**54.1/41.2**	21.7/13.9	26.4/17.2	21.8/13.9
Tetraodon	CISH	**80.4/65.3**	19.9/13.1	22.1/13.0	16.6/11.2
Zebrafish	CISH	**63.6/52.2**	19.9/12.3	18.6/11.9	16.4/10.9
Trout	CISH	**100/100**	18.6/13.1	21.9/12.8	17.5/12.1
Tetraodon	SOCS8	**58.7/46.5**	19.9/10.8	21.0/11.5	17.3/9.9
Zebrafish	SOCS8	**62.2/47.6**	21.0/12.8	19.4/12.6	15.5/10.3
Human	SOCS5	20.1/11.8	**61.6/43.5**	39.2/20.9	31.4/18.4
Mouse	SOCS5	20.9/11.8	**60.5/44.8**	40.1/20.2	31.9/18.3
Chicken	SOCS5	20.3/13.4	**61.8/44.4**	38.6/19.9	33.1/19.6
Frog	SOCS5	20.9/14.2	**60.7/42.9**	38.5/20.1	32.2/18.3
Tetraodon	SOCS5a	19.1/14.1	**58.5/41.8**	36.1/21.1	32.8/19.2
Zebrafish	SOCS5a	21.2/14.9	**59.8/43.1**	34.6/20.3	31.7/18.9
Zebrafish	SOCS5b	20.1/13.3	**58.8/41.8**	37.7/21.4	30.8/18.2
Tetraodon	SOCS9	20.4/13.7	**80.7/71.7**	36.5/22.3	30.1/18.4
Zebrafish	SOCS9	20.1/14.8	**74.8/62.8**	39.6/20.7	31.1/19.2
Trout	SOCS9	19.9/12.3	**100/100**	36.3/20.6	35.3/19.9
Human	SOCS6	20.4/12.4	38.6/19.1	**75.3/63.9**	38.3/25.8
Mouse	SOCS6	20.8/12.1	37.5/19.8	**73.9/60.9**	36.0/26.1
Chicken	SOCS6	20.2/12.8	38.8/20.6	**77.3/65.5**	35.7/24.0
Frog	SOCS6	20.3/12.6	38.2/19.5	**75.6/63.4**	36.0/24.8
Tetraodon	SOCS6	21.8/13.2	38.2/20.0	**75.1/63.5**	36.5/25.1
Zebrafish	SOCS6	20.3/12.8	39.5/20.7	**79.7/67.0**	37.0/24.9
Trout	SOCS6	21.9/12.8	36.3/20.6	**100/100**	37.7/26.2
Human	SOCS7	17.4/11.5	34.9/21.5	38.9/25.9	**53.2/40.9**
Mouse	SOCS7	17.6/11.5	35.1/21.2	38.3/25.9	**52.8/40.1**
Frog	SOCS7	21.8/13.9	33.8/20.1	40.6/26.3	**48.2/39.8**
Tetraodon	SOCS7	18.7/12.2	34.9/19.5	41.2/27.3	**51.2/40.1**
Zebrafish	SOCS7	20.0/13.0	34.6/17.7	42.5/28.0	**57.5/46.1**
Trout	SOCS7	17.5/12.1	35.3/19.9	37.7/26.2	**100/100**

3.1.2. SOCS6

A 2.4 kb 5′-RACE product was obtained using primers SOCS6 R1/R2 (Table 1) designed against the ESTs CU066723 and BX312152. When sequenced it was found to contain 635 bp of 5′-UTR, the complete coding region and a partial 3′-UTR of the SOCS6 cDNA (Accession No. AM903342). The trout SOCS6 cDNA encoded a protein of 515 amino acids with a theoretical molecular weight of 58.1 kDa and p*I* of 6.07. The trout SOCS6 protein shared similar homology to SOCS6s from other vertebrate species (61–67% identity) (Table 3). A 2.6 kb PCR product amplified from genomic DNA using primers SOCS6 F1/R1 was also sequenced, and revealed that the trout SOCS6 gene had a single intron of 130 bp in the 5′-UTR (Accession No. AM903344). The determination of the intron position allowed the design of the cDNA specific primer SOCS6 F, that was subsequently used for expression studies by real-time PCR.

3.1.3. SOCS7

A 1.4 kb 3′-RACE product was obtained using primers SOCS7 F4/F3 (Table 1), designed to the ESTs BX878085 and CA376519. When sequenced the product was found to contain the C-terminus of the trout SOCS7 molecule, together with the complete 3′-UTR including a poly A signal 16 bp upstream of the poly A tail. A 1.9 kb 5′-RACE product was amplified using primers SOCS7 R1/R2 (Table 1) extending the cDNA sequence to 2951 bp. The trout SOCS7 cDNA (Accession No. AM903343) encoded a protein of 652 amino acids with a theoretical molecular weight of 71.5 kDa and p*I* of 6.66. The trout SOCS7 protein had 40–46% identity to SOCS7s from other vertebrate species (Table 3).

3.1.4. SOCS9

A1.9 kb 5′-RACE product was obtained using primers SOCS9 R2/R3 (Table 1) designed against the EST CA387203. When sequenced the product was found to contain the 5′-UTR, the complete coding region and a partial 3′-UTR of the SOCS9 molecule. A further 3′-RACE product of 0.7 kb was obtained using primers SOCS9 F4/F3 (Table 1) and extended the SOCS9 cDNA sequence to 2.38 kb. The trout SOCS9 cDNA (Accession No. AM903341) encoded a protein of 544 amino acids, with a theoretical molecular weight of 60.6 kDa and p*I* of 6.67. The trout SOCS9 protein had 42–45% identity to SOCS5s from other vertebrate species but higher homology (63–72% identity) to SOCS9 molecules that appear to be fish specific [37] (Table 3).

3.2. Sequence analysis and classification of fish SOCS genes

Phylogenetic tree analysis (Fig. 1) of the protein sequences supported the homology analysis, in ascribing the new SOCS genes as trout CISH, SOCS6, SOCS7 and SOCS9. The CISH clade, including the CISH related SOCS8 genes, grouped together with the SOCS1, 2 and 3 molecules, in what has previously been termed the Type II SOCS subfamily [37]. Trout SOCS6 and SOCS7 grouped with the other known SOCS6 and SOCS7 as expected, and together form a subgroup within the Type I subfamily of SOCS molecules. The SOCS4, 5 and 9 subgroup is more complicated, and whilst the trout sequence was clearly related to SOCS9 molecules, their relationship to SOCS4 and SOCS5 has still to be clarified.

A multiple alignment of the trout SOCS protein sequences was also generated, in combination with a representative of all the SOCS family molecules now known in fish, using the stickleback sequences for this purpose (Fig. 2). This revealed clear areas of conservation within the SH2 domain and SOCS box within all SOCS molecules, and the more variable N-terminus that contains the N-terminal extended SH2 subdomain (ESS) and kinase inhibitory region (KIR) seen in SOCS1 and SOCS3. The alignment presented did not extend to the full N-terminus of the type I SOCS molecules in particular due to the difficulty of aligning sequences of very different lengths in this region (N/B the SOCS1 molecules were only +42 and +57 for the stickleback and trout molecules respectively, in a region that was highly serine rich). Another notable feature seen in the alignment was an extended C-terminus in many of the Type I SOCS family members, including the trout SOCS7 and SOC9 molecules. A more detailed analysis of the SOCS5 and SOCS9 family members was also performed, using all known fish molecules and representative tetrapod SOCS5 molecules, to shed light on the relatedness between these two SOCS groups. This analysis (Fig. 3) showed that the C-terminal domain containing the SH2 and SOCS box domains (indicated above the alignment) are highly conserved among all the SOCS5 and SOCS9 molecules, whilst SOCS5-specific and SOCS9-specific features can be seen at the N-terminus.

3.3. Tissue distribution of the expression of the trout SOCS genes

The expression of the trout CISH and SOCS6, 7 and 9 genes was examined in eight tissues, the gills, skin, muscle, liver, spleen, head kidney, intestine and brain from six healthy trout by real-time PCR as described previously [15]. The expression of all the four SOCS genes was detectable in all the tissues examined, with the brain having the highest levels. In contrast, the liver expressed the lowest level of SOCS6, 7 and 9, whilst the intestine had the lowest expression level of CISH. The expression of CISH was higher than the other three genes in all the tissues examined (Fig. 4). Immune tissues (gills, head kidney, spleen) had considerable levels of expression of all four genes examined.

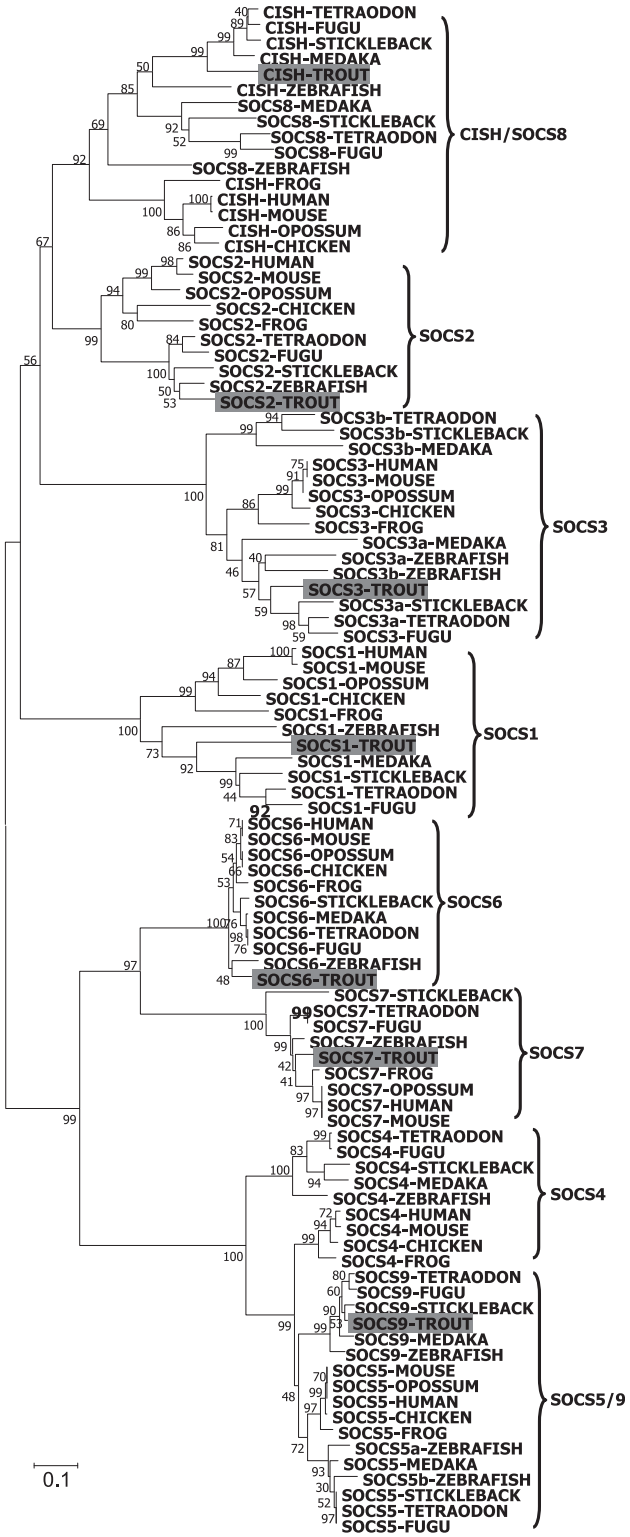

Fig. 1. An unrooted phylogenetic tree of 98 selected members of the SOCS family constructed using the neighbour-joining method within the MEGA program. The sequences analyzed and their accession numbers are given in Table 2, and are chosen from 3 mammals, one bird, one amphibian and 6 fish. The neighbour-joining tree was produced from a multiple alignment of full-length amino acid sequences using CLUSTAL. The complete deletion of gaps/missing data and JTT matrix (Jones–Taylor–Thornton) amino acid model options were used. Node values represent percent bootstrap confidence derived from 5000 replicates. The trout SOCS molecules are highlighted.

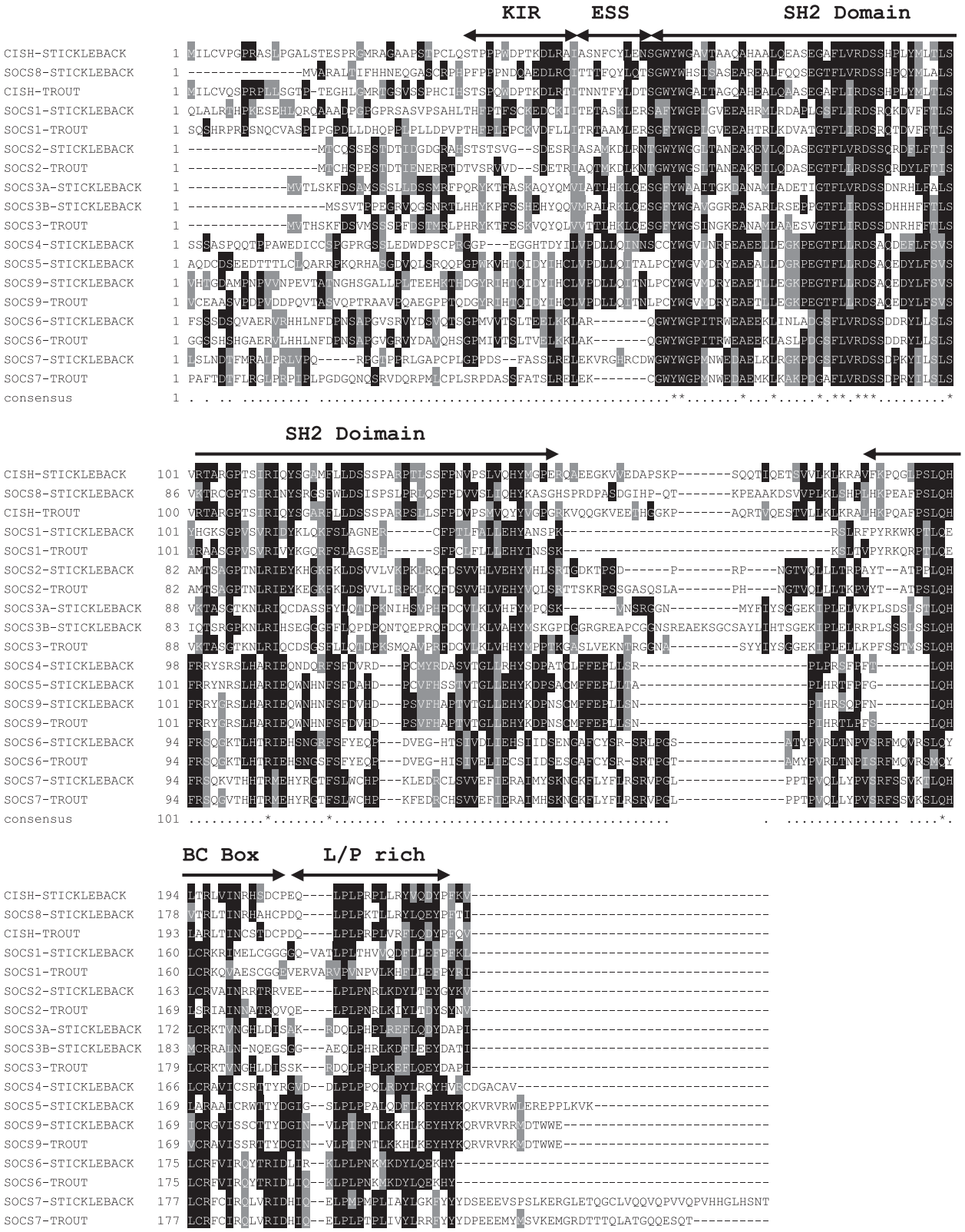

Fig. 2. Multiple alignment of the C-terminal domain of the trout SOCS molecules with the equivalent region of all known fish SOCS molecules from a representative fish species (stickleback). The multiple alignment was produced using ClustalW. The C-terminal domain contains the SH2 and SOCS box domains (indicated above the alignment), as well as the KIR and ESS domains in some molecules (eg SOCS1 and SOCS3), and were predicted using the SMART6 program [41]. Dashes (−) indicate gaps in the alignment, asterisks (*) indicate complete conservation and dots (.) indicate similarity.

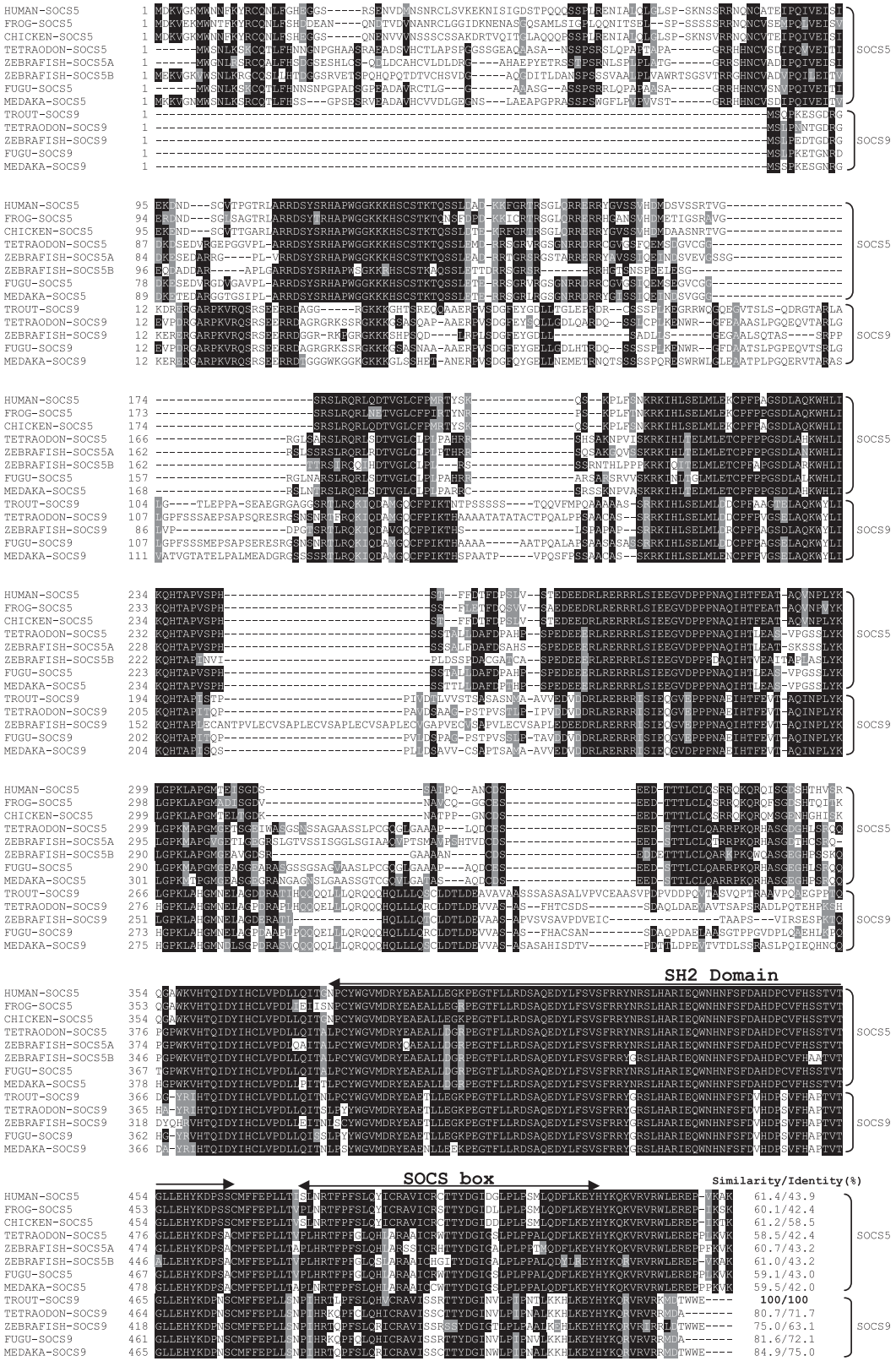

Fig. 3. Multiple alignment of SOCS5 and fish specific SOCS9 molecules. The multiple alignment was produced using ClustalW and boxshaded. Dashes (−) indicate gaps in the alignment. The SOCS5-specific and SOCS9-specific features are observed at the N-terminus of the molecules but the C-terminal region containing the SH2 and SOCS box domains (indicated above the alignment) are highly conserved among all the SOCS5 and SOCS9 molecules. The amino acid sequence similarities/identities of trout SOCS9 to the SOCS5 and SOCS9 molecules from other species are indicated at the end of the alignment.

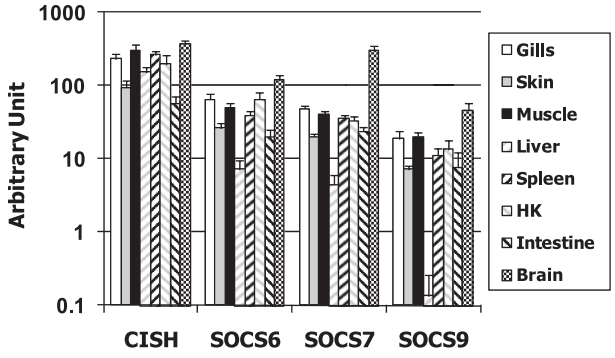

Fig. 4. *In vivo* transcript expression of trout CISH, SOCS6, 7 and 9. The relative expression level of each SOCS gene in different tissues was expressed as arbitrary units that were normalized to the expression level of EF-1α. The results are presented as the mean + SEM of six fish.

3.4. Differential regulation of trout SOCS gene expression by bacterial infection

To investigate if SOCS gene expression could be modulated by bacterial infection, trout were injected intraperitoneally with PBS (control) or *Y. ruckeri*, the causative agent of enteric red mouth disease, and SOCS gene expression studied in the spleen 24 and 48 h later. Although the expression of all the SOCS genes was significantly higher at 48 h compared to 24 h, even in the PBS injected control group, only the expression of CISH and SOCS1 and 3 was specifically up-regulated by bacterial infection (Fig. 5). The expression level of CISH, SOCS1 and 3 in samples from the infected fish were some 5, 34 and 44 fold that of the PBS injected control group at 48 h, respectively. Lastly, the expression levels of all the SOCS genes in the spleen were significantly higher than in normal unhandled fish (data not shown), and suggests a link between the operational procedures (injection, handling) and SOCS gene expression, perhaps contributing to subsequent immune inhibitory effects often associated with stress.

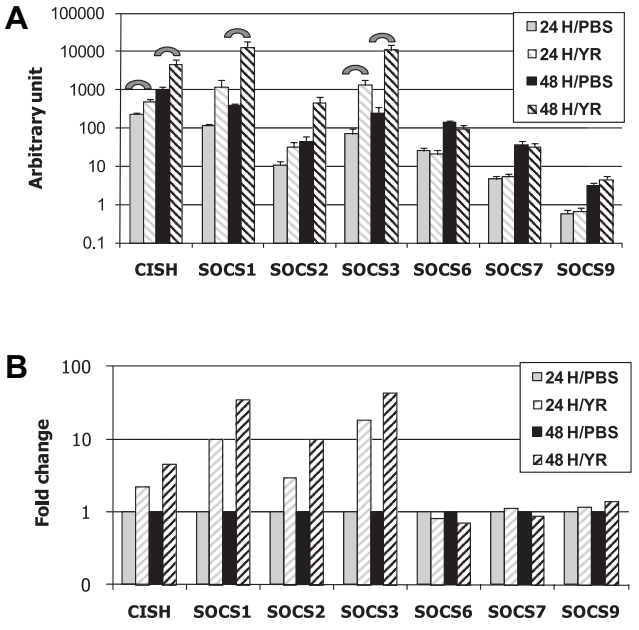

Fig. 5. Modulation of SOCS gene expression in the spleen after *Yersinia ruckeri* infection. Rainbow trout were injected intraperitoneally with either 0.5×10^6 cfu *Y. ruckeri* (YR) or phosphate buffered saline (PBS), and six trout from each group were sample at 24 h, and 48 h post-injection. The relative expression level of each SOCS gene in the spleen was expressed as arbitrary units normalized to the expression of EF-1α (A). Fold changes were calculated by dividing the average expression level of each *Y. ruckeri* challenged group by the PBS-challenged group at the same time point (B). The results are presented as the mean + SEM of six fish. Symbols above the bars indicate significant differences ($p < 0.05$) relative to the respective controls at the same time points.

3.5. Differential regulation of trout SOCS genes in the monocyte/macrophage cell line RTS-11 and primary splenocyte cultures by LPS and Poly I:C

The expression levels of different SOCS family members in RTS-11 cells varied, with SOCS3 having the highest expression level and CISH the lowest (Fig. 6). There were no significant changes in expression level of any of the SOCS family members in the control cells over the time course of the experiment (i.e. 4–24 h of culture). Poly I:C was a strong stimulator of SOCS1 expression in RTS-11 cells, giving a 24–357 fold increase. It also significantly up-regulated the expression of SOCS2 and 3 at the later time points, albeit to a lesser extent, but significantly down-regulated the expression of SOCS7 and 9 (by 27% in the latter case) after 11 h of treatment. LPS also up-regulated the expression of SOCS1, 2 and 3 in RTS-11 cells, with expression levels some 14, 3 and 6 fold higher than control samples at peak values. Curiously, SOCS1 expression peaked at 4 h, whereas SOCS3 expression peaked at 11 h and SOCS2 expression at 24 h after LPS treatment. LPS had no effect on the expression of CISH, SOCS6, 7 or 9 in RTS-11 cells.

In primary splenocyte cultures LPS significantly increased the expression of CISH, SOCS1 and SOCS2 24 h post-stimulation, with SOCS1 also increased at 4 h and 8 h post-stimulation (Fig. 7). A significant decrease in expression of the other SOCS genes was also seen at the early time points relative to unstimulated cells. In contrast, Poly I:C stimulation had no effect on splenocyte SOCS expression.

3.6. Differential expression and modulation of trout SOCS genes in RTG-2 and RTS-11 cell lines by PMA

As with RTS-11 cells, RTG-2 cells expressed constitutively all seven SOCS genes, with SOCS3 expression being the highest and CISH/SOCS2 the lowest (Fig. 8). PMA was a good stimulator of SOCS gene expression in RTG-2 cells after 8 h of incubation (Fig. 8A and C), significantly increasing the expression of SOCS1 (8–18 fold), SOCS2 (2–4 fold) and SOCS3 (11–24 fold) at all doses (0.5–500 ng ml^{-1}) tested. The modulation of SOCS gene expression by PMA in RTS-11 cells was more widespread and also apparently more dose-dependent. As with RTG-2 cells, the expression of SOCS1, 2 and 3 was significantly up-regulated at the lowest (0.5 ng ml^{-1}) and highest (500 ng ml^{-1}) doses used, but was significantly down-regulated by incubation with 5 ng ml^{-1}. The lowest dose (0.5 ng ml^{-1}) also significantly up-regulated SOCS6 and 9 expression, as did the higher doses of 50 and 500 ng ml^{-1} for SOCS9 expression. The fold increase in gene expression were comparable between the lowest and highest doses used, although generally higher using 0.5 ng ml^{-1}. For example, SOCS1, 3 and 9 expression increased 4, 9 and 9 fold using 0.5 ng ml^{-1} PMA and 2, 5 and 6 fold using 500 ng ml^{-1} PMA. Whilst the expression of SOCS1, 2 and 3 was significantly decreased after treatment with 5 ng ml^{-1} PMA, the expression of SOCS6, 7, 9 and CISH was unaffected.

Both IFN-γ and IL-1β were shown previously to up-regulate the expression of SOCS1, 2 and 3 in RTG-2 cells, but only IFN-γ could up-regulate these three genes in RTS-11 cells [36]. In this study neither IFN-γ or IL-1β had any significant effects on the expression of CISH, SOCS6, 7 and 9, in RTG-2 or RTS-11 cells (data not shown).

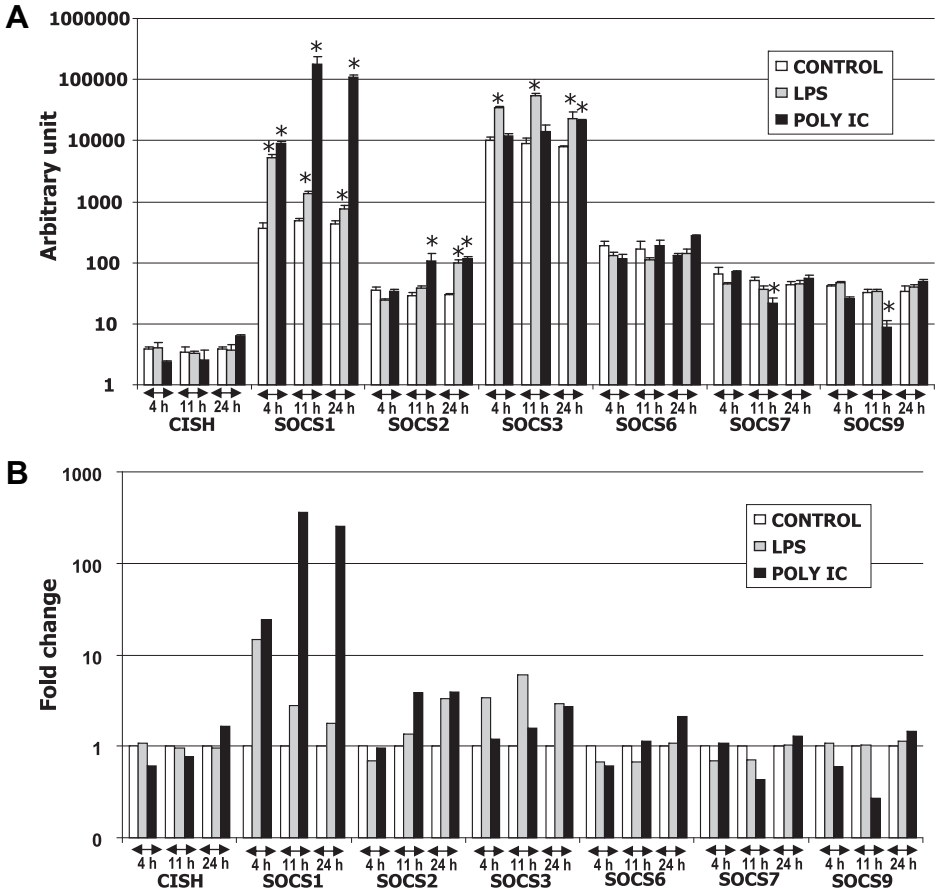

Fig. 6. Modulation of SOCS gene expression by LPS and Poly I:C in RTS-11 cells. RTS-11 cells were incubated with LPS (25 μg ml^{-1}) or Poly I:C (50 μg ml^{-1}), or remained unstimulated (Controls), and were sampled 4, 11 and 24 h later. The relative expression of the trout SOCS genes was detected by real-time PCR, and expression level expressed as arbitrary units normalized to the expression of EF-1α (A). The mean of three independent replicates is shown for each treatment, and bars indicate the standard error of the means. Fold changes were calculated by dividing the average expression level of each treatment by that of the respective control (B). Asterisks above the bars indicate significant differences ($p < 0.05$) relative to the respective controls at the same time points.

4. Discussion

In this paper we describe for the first time in rainbow trout another four members of the SOCS family of molecules, and study where they are expressed and whether expression can be modulated by commonly used stimulants and by infection. The SOCS family members identified were CISH and SOCS6, 7 and 9. The classification of these new trout molecules seemed clear but the exact relationships of some of the recently described fish specific SOCS molecules remains to be fully determined. For example, Jin et al. [37] describe for the first time SOCS3b, 8 and 9, and whilst the SOCS3b and SOCS8 genes are clearly related to SOCS3 and CISH, and probably arose via additional gene duplication events from a common ancestor, the origin of SOCS9 is less clear and complicated by the clustering of the fish SOCS4 molecules to the base of the tetrapod SOCS4 and SOCS5, and fish SOCS5 and SOCS9 clades in phylogenetic tree analysis. Nevertheless, the evolutionary history of these SOCS molecules as proposed by Jin et al. is quite plausible as they state, even if the SOCS3b, SOCS4 and SOCS5 genes are found to be absent from trout. Lastly, in zebrafish an additional SOCS gene duplication is apparent [37], where two SOCS5 genes are present and to date this appears to be a species specific duplication.

The new trout molecules have various features that are typical of the different SOCS family subgroups. For example, trout SOCS7 and 9 have a C-terminal extension beyond the SOCS box, as is common in type I molecules (with fish SOCS9 being in the SOCS4/5 subgroup), that is lacking in CISH. This appears to be influenced by the interaction of SOCS molecules with Elongin B/C, required for linkage to E3 ligase and subsequent ubiquitination of target proteins for degradation. Crystal structure determination of type I and II SOCS molecules within this complex has shown that in type II molecules the C-terminus is buried, allowing exposure of the N-terminal KIR domain (in SOCS1/3) and results in an absolute limit on the C-terminal length [32]. In contrast it is the N-terminus that is buried in type I molecules. Trout SOCS9 also has an HTQIDYIHC motif N-terminal of the ESS domain, in common with other SOCS5/9 molecules, and this region in mammals has been postulated to contain a possible KIR domain [46], similar to the situation with SOCS1 and 3. Interestingly, the 3 N-terminal amino acids of this domain differ in SOCS9 compared to the SOCS5 molecules in mammals/fish, being WKV in the latter and usually YRI in the former (as seen in trout and stickleback SOCS9 in Figs. 2 and 3), and it could be speculated that this may have functional consequences for these two SOCS. Mammalian SOCS5 is known to be expressed in Th1 cells and can inhibit IL-4 signalling and Th2 differentiation [47]. In addition it inhibits EGF receptor signalling [48]. Thus there is potential for these functions to be performed separately in fish, or that Th polarisation is more complex and has additional control pathways.

As with SOCS1-3, CISH and SOCS6, 7 and 9 had a wide tissue distribution in trout, with liver typically having the lowest expression level (with the exception of CISH). In Tetraodon particularly high levels of CISH and SOCS9 were found in the head kidney [37], with

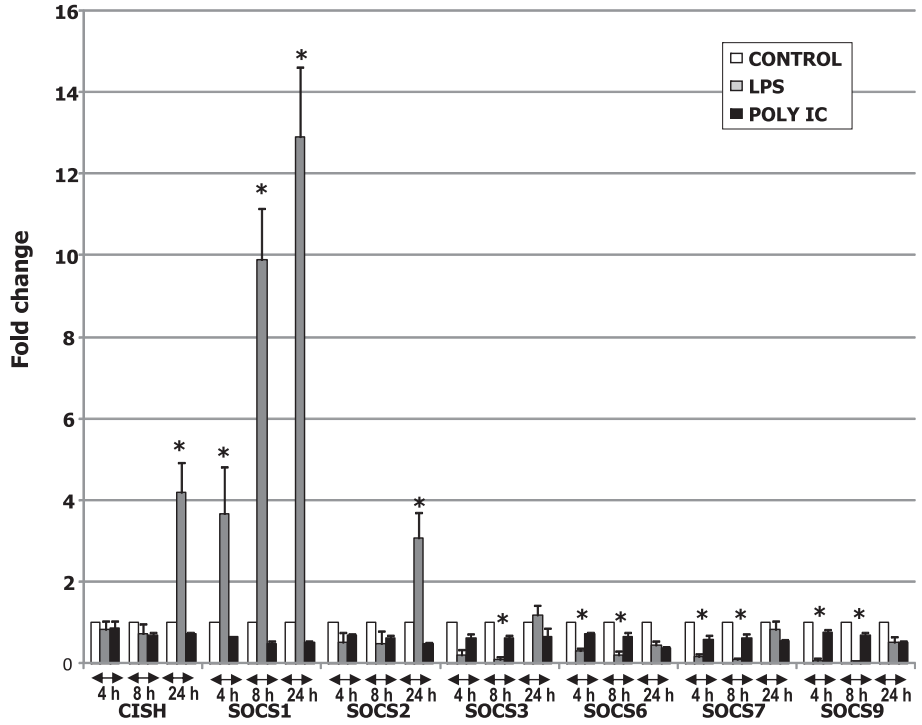

Fig. 7. Modulation of SOCS gene expression by LPS and Poly I:C in splenocytes. Freshly prepared trout splenocytes were incubated with LPS (25 μg ml^{-1}) or Poly I:C (50 μg ml^{-1}), or remained unstimulated (Controls), and were sampled 4, 8 and 24 h later. The relative expression of the trout SOCS genes was detected by real-time PCR as above, and expression level was normalized to that of EF-1α and expressed as fold change, where the expression in the control was defined as 1 at each time point for each fish. The mean of three independent replicates (fish) is shown for each treatment, and bars indicate the standard error of the means. Asterisks above the bars indicate significant differences ($p < 0.05$) relative to the respective controls at the same time points.

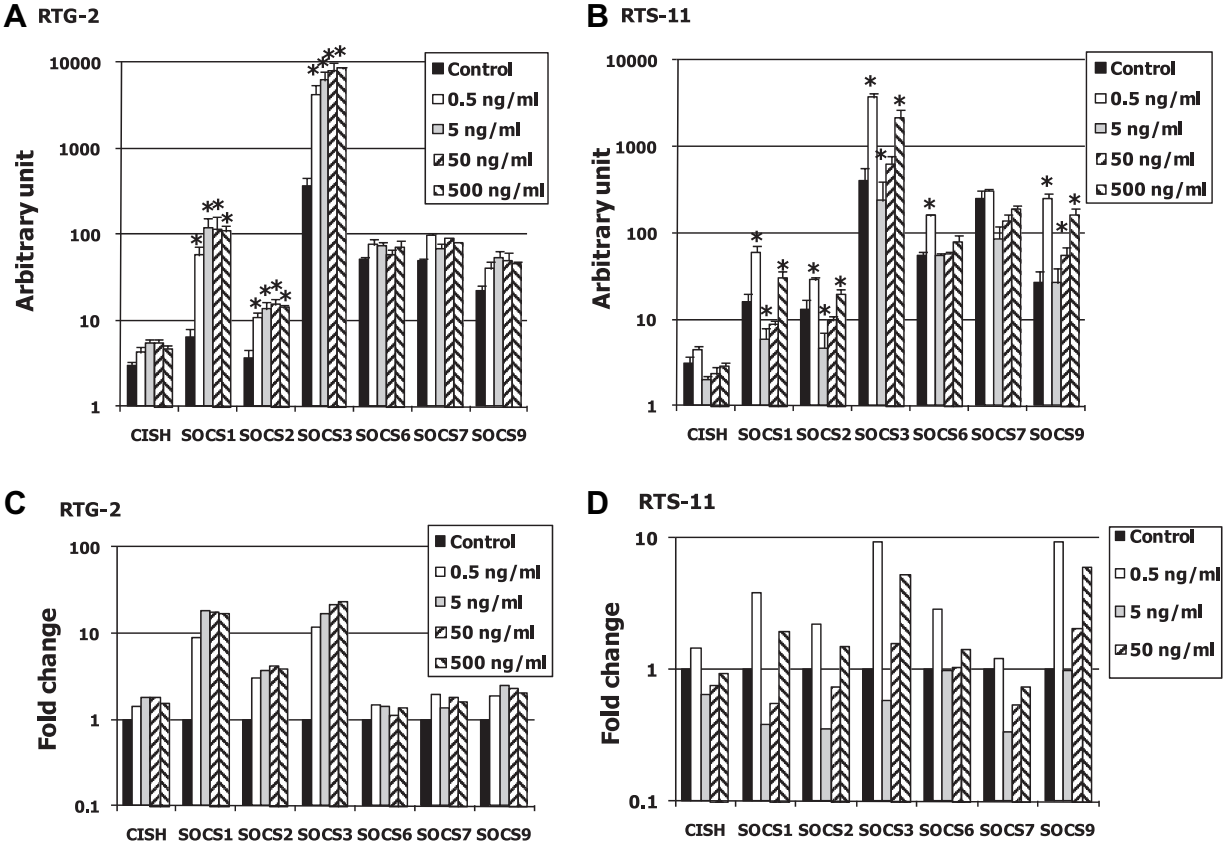

Fig. 8. Modulation of SOCS gene expression by PMA in RTG-2 (A, C) and RTS-11 (B, D) cell lines. Different concentrations of PMA, 0 (control) and 0.5–500 ng ml^{-1}, were added to RTG-2 cells and RTS-11 cells for 8 h prior to RNA extraction. The relative expression (A and B) of the trout SOCS genes, was detected by real-time PCR. Expression level of each SOCS gene was expressed as arbitrary units normalized to the expression of EF-1α. Fold changes (C and D) were calculated by dividing the average expression level of each treatment by that of the respective control. The mean of three replicates is shown for each treatment, and bars indicate the standard error of the means. Asterisks above the bars indicate significant differences ($p < 0.05$) relative to the respective controls.

SOCS6 highest in the gonad and SOCS7 being lowly expressed in all tissues. This was not the case in trout where head kidney expression levels were similar to most other tissues, and SOCS7 was expressed at moderate levels, being approximately equal to SOCS6 and higher than SOCS9. Injection of Tetraodon with LPS increased the expression levels of CISH and SOCS9 (amongst others) in head kidney but had no effect on SOCS6 (with SOCS7 not expressed in this tissue)[37]. In our study, where *in vitro* stimulation of RTS-11 cells or splenocytes was used, LPS only significantly affected trout CISH and SOCS1-3 expression, with no stimulatory effect on any of the newly discovered trout SOCS. Similarly Poly I:C significantly increased trout SOCS1-3 expression in RTS-11 cells, but had no positive effects on the expression of any SOCS molecules in splenocytes. However, with PMA stimulation, whilst a similar result was found for stimulation of RTG-2 cells, a more complex picture emerged with RTS-11 cells, with high and low doses having a stimulatory effect on the expression of SOCS1-3 but also on SOCS6 and SOCS9, indicating that cell type specific differences exist in relation to SOCS expression. Lastly, unlike the previously reported stimulatory effect on SOCS1-3 expression of incubation of RTG-2 and RTS-11 cells with rIFN-γ or RTG-2 cells with rIL-1β [36], no effect of these cytokines on the expression of the newly discovered trout SOCS was apparent in either cell line.

To date no studies have looked at the impact of infection on SOCS gene expression in fish. Here a Gram negative bacterium was used for this purpose, and following injection into fish SOCS expression was analysed in the spleen 24 h and 48 h later. This showed that it was the type II SOCS molecules that were modulated by infection, with CISH and SOCS3 expression significantly increased at both time points, and SOCS1 expression increased by 48 h post-injection. Whilst not significant, in fact SOCS2 expression also showed a similar trend. This result was not particularly surprising since in mammals it is known that SOCS1, SOCS3 and CISH can be induced by TLR stimulation [31], where they serve to limit inflammatory responses, although pathogens can also induce SOCS expression as a means of immune evasion [33]. This was also in agreement with the *in vitro* stimulation experiments undertaken here, where with mixed cell suspensions (i.e. splenocytes) it was clear that CISH as well as SOCS1 and 2 are up-regulated by LPS. Finally, it was also apparent that the expression of all the SOCS molecules increased with time post-injection, and that this may have been a consequence of the handling procedures, and hence linked to a potential sensitivity of SOCS genes to mediators involved in other physiological pathways.

In conclusion, we have discovered another four members of the trout SOCS gene family, and studied their expression in relation to the already known SOCS1-3 in this species. It is apparent that the type II SOCS genes are particularly relevant to immune regulation in fish, although the intriguing expansion of the SOC4/5 subgroup in fish requires further investigation as to their role and functional divergence.

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