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Characterization of two putative cytokine receptors, gp130 and ciliary neurotrophic factor receptor, from terrestrial salamanders

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Cytokines of the gp130 family are fundamental regulators of immune responses and signal through multimeric receptors to initiate intracellular second-messenger cascades. Here, we provide the first characterization of two full-length gp130 cytokine receptors from the cDNA of the red-legged salamander (*Plethodon shermani*). The first, gp130 (2745 bp), is a common signaling receptor for several multi-functional cytokines in vertebrates. We also isolated the full-length (1104 bp) sequence of the ciliary neurotrophic factor receptor (CNTFR), which forms a heteromeric signaling complex with gp130. The open reading frames of both receptors were predicted to contain many of the conserved features found in mammalian gp130s, such as cytokine binding homology regions and residues known to form disulfide bonds. Finally, we used RT-PCR to show that gp130 and CNTFR were expressed in most *P. shermani* tissues, including brain, intestine and muscle. The expression profiles, along with the structural predictions, show that gp130, CNTFR, and their cytokine ligands are parts of the immune system of *P. shermani* and other caudate amphibians.

Key words: interleukin-6 signal transducer, molecular evolution, *Plethodon shermani*

The glycoprotein 130 (gp130) cytokine family is found in all vertebrates and regulates important aspects of immune and immuno-endocrine physiology (Bravo and Heath, 2000; Boulanger and Garcia, 2004). Gp130 cytokines function in cellular proliferation, growth, and differentiation in a range of tissues and developmental stages (e.g., ciliary neurotrophic factor - neural tissue, cardiotrophin - heart muscle; Bravo and Heath, 2000). This group of cytokines exhibits functional redundancy and pleiotropy that is likely caused by the specificity and binding capabilities of their multi-subunit receptors (Ishihara and Hirano, 2002). The receptors can cross-signal and co-regulate signaling cascades (e.g., Jak/STAT), allowing specialized and overlapping responses in different tissues. Consequently, mammalian gp130 cytokines and their receptors are models for studies of cytokine-multimeric receptor interactions (Chow et al., 2001) and multifunctionality (Ishihara and Hirano, 2002; Huising et al., 2006).

Every member of the gp130 cytokine family signals by binding with at least one molecule of gp130, a transmembrane receptor subunit also known as the interleukin-6 signal transducer. This receptor is activated by multiple cytokines and expressed in most human cell types (Bravo and Heath, 2000). Some gp130 cytokines bind to one gp130 subunit and to additional structurally related signaling receptors in the gp130 family to form heteromeric signaling complexes (Bravo and Heath, 2000). These receptors include other signaling receptors such as leukemia inhibitory factor receptor (LIFR), oncostatin M receptor (OSMR), and colony stimulating factor 3 receptor (CSF3R) as well as soluble non-signaling receptors (e.g., ciliary neurotrophic factor receptor [CNTFR], interleukin-6 receptor alpha [IL6RA], IL-11 receptor alpha [IL11RA]) (Boulanger and Garcia, 2004).

Mammals have been the primary research system for the study of cytokine/gp130 interactions but little is known about gp130 cytokines or their receptors in non-mammalian vertebrates. In this study, we focused on amphibians because they are especially useful for comparative analyses and can help define early tetrapod

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structure and function, yet many of the fundamental components of the amphibian immune system have not yet been described. In addition, the immune system is of growing interest to amphibian biologists because of the unprecedented demographic declines that have occurred due to emerging pathogens in many amphibian populations (e.g., Berger et al., 1998; Lips et al., 2006).

In this study, we isolated two receptors from our focal species, the red-legged salamander (*Plethodon shermani*). We isolated (1) gp130, as it is the signaling receptor for at least nine biologically important cytokines and (2) CNTFR, a non-signaling receptor in the gp130 receptor family that is used for multiple types of neuroendocrine-immune interactions (Gerez et al., 2007). Adult animals were collected from natural populations (035°10′48″N 083°33′38″W) and sacrificed by decapitation before tissue collection, in accordance with the standards of the Institutional Animal Care and Use Committee at Oregon State University (ACUP 3549 to Lynne D. Houck). Tissue was removed from RNAlater® (Invitrogen, Carlsbad, CA, USA) and immediately submerged in Trizol® (Invitrogen). The RNA was extracted according to the manufacturer's protocol. RNA was reverse-transcribed using the ImProm-II™ reverse transcription system (Promega, Madison, WI, USA) into cDNA. The cDNA was used as a template for the subsequent reactions, which were all preformed with GoTaq DNA polymerase (Promega). First-strand 5'RACE-ready cDNA was synthesized with the FirstChoice® RLM-RACE kit (Ambion, Austin, TX, USA) and 3' RACE-ready cDNA was made using the ImProm-II™ reverse transcription system (Promega). Degenerate primers were designed to align with two regions of gp130 that are conserved in most vertebrates (degenerate gp130 forward and reverse; Table 1). These primers were used to obtain the first fragment of gp130 from *P. shermani*. Subsequent overlapping *P. shermani*-specific primers (not shown) were then designed from the previously amplified *P. shermani* sequences. These sets of species-specific primers were used to obtain regions closer to the 5' and 3' ends of the open reading frame (ORF) using a 'primer walking' technique. The complete ORF was then obtained from 5' and 3' RACE using standard reaction conditions. All amplicons from all species were cloned using the pGEM®-Teasy vector system (Promega). After propagation of the bacteria on Luria Broth agar medium containing ampicillin, single colonies were chosen using blue/white screening. These colonies were cultured overnight in ampicillin-containing LB medium. Plasmid DNA was isolated from each culture with the Qiaprep spin miniprep kit (Qiagen, Valencia, CA, USA) and sent to the Nevada Genomics Center for sequencing using the SP6 and T7 universal primers, and/or unique sequencing primers.

The length of the *P. shermani* gp130 ORF (GenBank accession number FJ824846) was 915 amino acids and comparable to that of other vertebrates (chicken and human: 918 amino acids). In humans, alternative splicing can produce a soluble form of gp130 that functions as a disease-associated autoantigen in individuals with rheumatoid arthritis (Tanaka et al., 2000). We did not, however, amplify any alternative splice variants that may represent a soluble form of gp130 from *P. shermani*. SignalP3.0 (Bendtsen et al., 2004) predicted a signal peptide of 19 amino acids for the *P. shermani* gp130 (Fig. 1A), a size similar to the 22 amino-acid signal peptide in human gp130 (Hibi et al., 1990). The predicted molecular weight of the mature *P. shermani* unglycosylated gp130 was approximately 98 kDa (www.basic.northwestern.edu/biotools/proteincalc.html). The *P. shermani* gp130 5' and 3' untranslated regions (UTRs) were 142 bp and 427 bp, respectively. We used the BLASTP (with default parameters) to make pairwise alignments and report the amino acid identities between the two sequences. The *P. shermani* gp130 shared 53% amino acid identity with

Table 1. Primers used in this study

Primer name	Sequence (5'→3')
degenerate gp130 forward	TCA AGR TCY GAR TCH ACH CAG CC
degenerate gp130 reverse	ACW CTY TGY GGS AWR TAA CTT TT
salamander gp130 forward	GGV GGV TCI TCR TGY ATG TC
salamander gp130 reverse	YTG YGG IAD STA ACT TTT
P. shermani gp130 tissue forward	GGA TAT TGG AGT GAT TGG AG
P. shermani gp130 tissue reverse	CCT AAA ACT TTC CCG TTG GC
degenerate CNTFR forward	TTC TAC TGC AGC TGG CAY CT
degenerate CNTFR reverse	GCC ACC TGG ATG ATG TAY TCC T
P. shermani CNTFR tissue forward	TTC TAC TGC AGC TGG CAC CT
P. shermani CNTFR tissue reverse	GGT GAC CAC CAC AGT CAC CC
P. shermani actin forward	CTG GCA CCT AGC ACA ATG AA
P. shermani actin reverse	TGT TTA GAA GCA TTT ACG GTG

chicken, 49% with human, and 31% with zebrafish.

The crystal structure of human gp130 shows that the mature protein contains six extracellular domains that are necessary for receptor functionality: one with an immunoglobulin-like conformation, two comprising the cytokine-binding homology region (CHR), and three fibronectin type III repeats (Bravo et al., 1998; Bravo and Heath, 2000). This domain architecture is predicted by amino acid sequence homology to be conserved in the gp130s of most vertebrates (Bazan, 1990; Liongue and Ward, 2007). The salamander gp130 was predicted to contain these six functional domains by the NCBI domain prediction tool (http://www.ncbi.nlm.nih.gov/cdd). The sequence also contained a conserved amino-acid motif WSXWS found in all gp130 cytokine receptors, as well as the four cysteines necessary for functionality of the CHR (Fig. 1A). Within the CHR, residues located at critical structural interfaces of human gp130 with IL-6 and/or

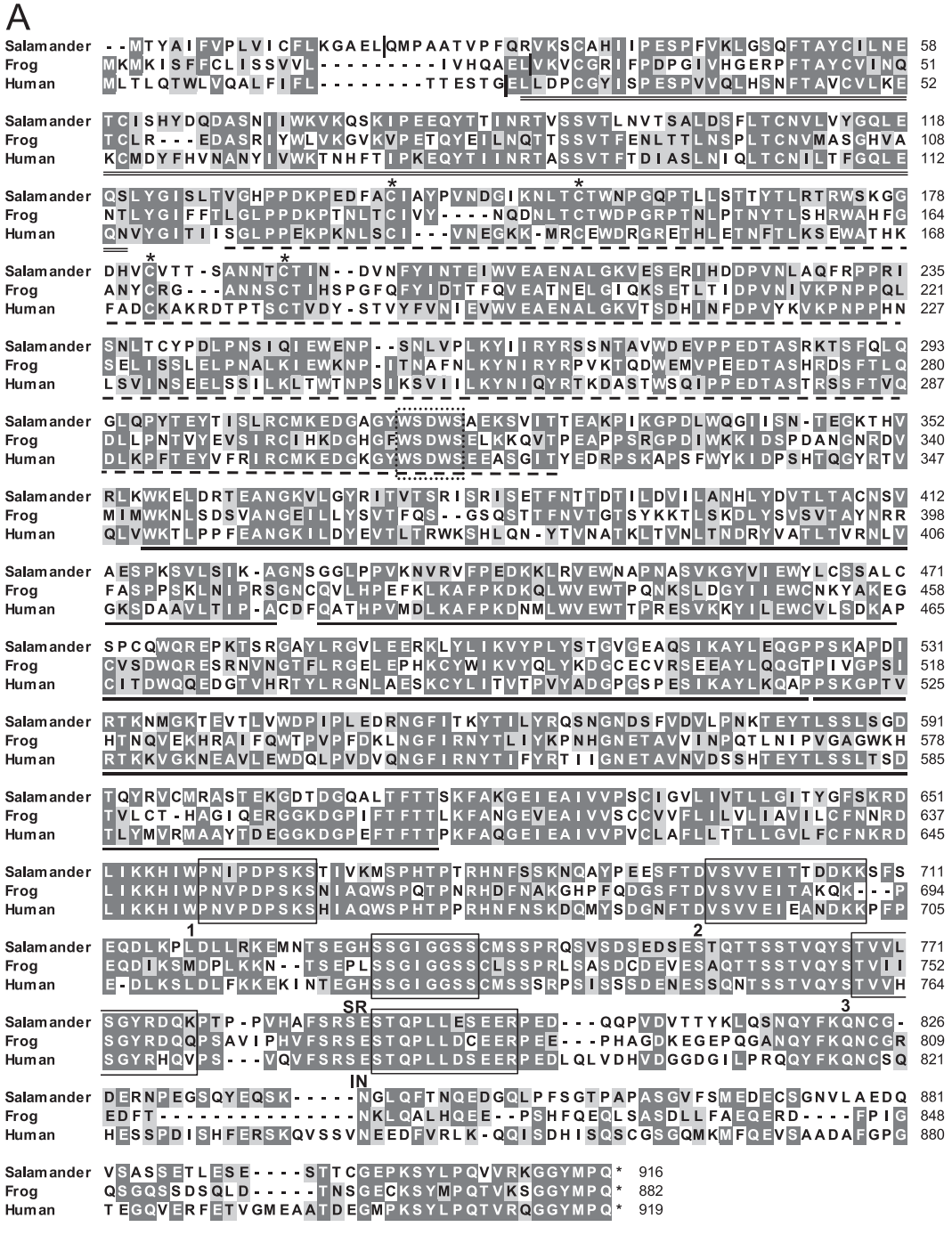

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Fig. 1. Continued

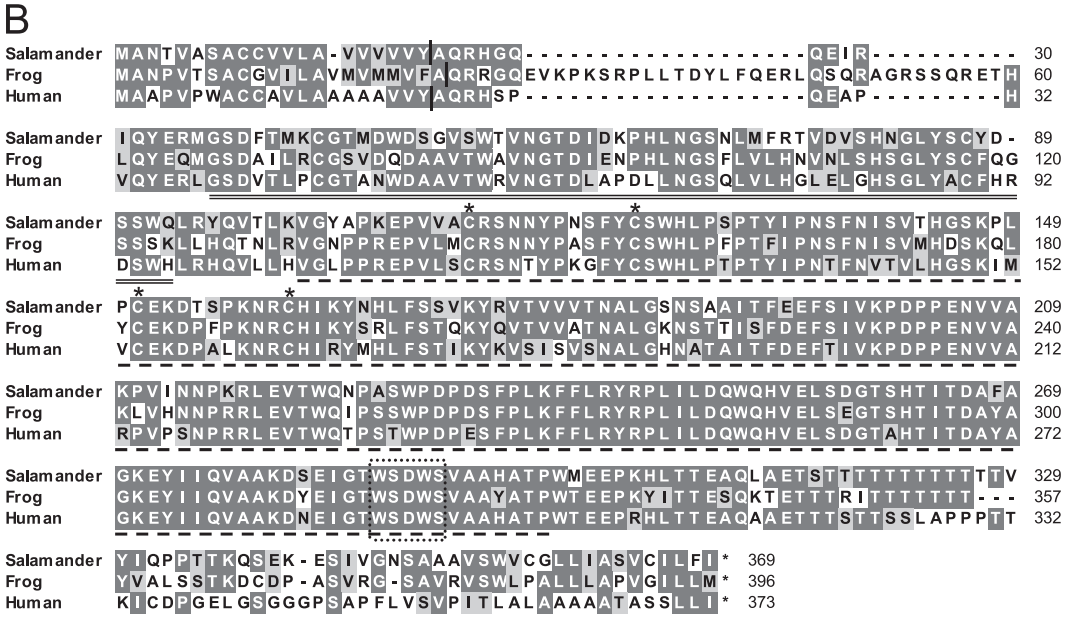

Fig. 1. Full-length deduced amino acid sequence alignment of (A) gp130 and (B) CNTFR (see Fig. 3 for GenBank accession nos.) from salamander (*P. shermani*), frog and human. The symbols indicate features of the amino acid sequences: vertical lines represent the signal peptide boundaries; double lines underline the immunoglobulin-like domain; dashed lines underline the cytokine-binding homology region; solid lines indicate the three fibronectin type III repeats; dotted box contains the WSXWS motif; solid boxes indicate intracellular motifs/domains (1 = Box1, 2 = Box2, 3 = Box3, SR = serine rich, IN = internalization); and asterisks mark the conserved cysteines. Dark gray shading indicates identical amino acids and light gray shading indicates conservative substitutions.

IL6RA (Boulanger et al., 2003) are conserved in *P. shermani* (Q^{116}, L^{117}, W^{174}). The presence of these conserved residues in both human and amphibian gp130 suggests that the majority of sites on IL-6 used in receptor binding may also be conserved between mammals and amphibians.

The intracellular region of gp130 is responsible for transducing the cellular signal and three cytoplasmic motifs, in particular, are important for initiating signal transduction. The first, Box1, was identical in both human and *P. shermani* except for a single conservative substitution of valine to isoleucine (Fig. 1A). Similarly high levels of amino acid conservation were seen in Box2 and Box3 (which contained the YXXQ sequence, a motif that interacts with the second messenger, STAT3). The serine rich and internalization domains were also highly conserved (Fig. 1A). We observed that the intracellular region of gp130 was more conserved among vertebrate species than was the extracellular region. For example, the extracellular regions of *P. shermani* and *Xenopus laevis* shared 41% amino acid identity while the intracellular regions shared 53% identity. The consistent pattern of sequence conservation in the three intracellular box motifs of gp130 may be because these regions associate with highly conserved signaling molecules, such as Jaks (Yamaoka et al., 2004) and STATs (Stahl et al., 1995).

To begin to determine gp130 diversity within salamanders, we amplified a ~500 bp region of gp130 from two other species from the family Plethodontidae (*P. vehiculum*, *Aneides flavipunctatus*) and one from a second salamander family, Salamandridae (*Taricha granulosa*) using primers that annealed to a region within the last exon in human gp130 (salamander gp130 forward and reverse; Table 1). We collected ~5 mm of posterior end of the tail from adult animals (for genomic DNA extraction) from various sites within the USA: *P. vehiculum* (044°29′49″N 123°34′05″W), *A. flavipunctatus* (042°05′18″N 123°01′39″W), and *T. granulosa* (044°28′49″N 123°39′35″W). Genomic DNA was extracted from the tail tips by an overnight digestion with proteinase K, followed by standard phenol/chloroform extraction. DNA was precipitated with 5 M NaCl and ethanol, washed with 70% ethanol, dried and re-dissolved in sterile ddH_2O.

The 500–510 bp encoded protein fragments that shared many of the conserved residues necessary for function in other species (deposited in GenBank under the accession nos. GQ176294–GQ176296). The fragment sequenced included the Box3 domain (necessary for activation of the signal transduction cascade). The Box3 domain of the other three salamanders was identical to that of *P. shermani* except for a substitution of serine to threonine in *T. granulosa*. Amino acid identity in the region varied from 94% between closely related *P. shermani* and *P.*

vehiculum, to 62% between *P. shermani* and the most distantly related species, *T. granulosa*.

In order to gain a broader understanding of the evolution of the gp130 family of cytokine receptors, we used the same RT-PCR strategy used for gp130 to amplify an 1104 bp CNTFR-like sequence from *P. shermani*. First, a fragment of *P. shermani* CNTFR was amplified using the primers degenerate CNTFR forward and degenerate CNTFR reverse (Table 1). After obtaining the initial fragment of CNTFR, *P. shermani*-specific primers were designed and 5' and 3' RACE was used to obtain the entire ORF. The predicted protein was composed of 368 amino acids (GenBank accession no. GQ904193). The *P. shermani* CNTFR 5' and 3' UTRs were 326 bp and 28 bp, respectively. The CNTFR contains two of the same modules as gp130, the immunoglobulin and the CHR, but lacks an intracellular domain and is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (Davis et al., 1991). The *P. shermani* amino acid sequence contained the two modules, as well as conserved motifs within these domains (e.g., WSXWS; Fig. 1B). Potential GPI-modification sites were identified in the *P. shermani* (N^{347}) CNTFR using the big-PI Predictor 3.0 (Eisenhaber et al., 1999). The *P. shermani* CNTFR amino acid sequence shared 64% identity with *X. laevis*, 70% with chicken, and 62% with human, and 37% identity with zebrafish. CNTFR was more conserved at the amino acid level across the vertebrate lineage than was gp130. For example, the CNTFRs of *P. shermani* and *X. laevis* were 64% identical, while the gp130s were only 45% identical.

To investigate the expression levels of both receptors in different *P. shermani* tissues, we collected approximately 50 mg of each tissue from one female and one male and immediately placed in 500 µl of RNAlater®. We sampled olfactory mucosa (containing both olfactory and accessory olfactory epithelia), skin (from the neck), brain, muscle, liver, kidney, eye, spleen, and intestine from 5 adults (3 males and 2 females). RNA was extracted with Trizol and used to generate first-strand cDNA with the ImProm-II™ reverse transcription system. For each tissue type, 175 ng of RNA was added to each reverse-transcription reaction. One µl of the resulting cDNA was used as a template to amplify fragments of (a) gp130, with the primers *P. shermani* gp130 tissue forward and reverse (Table 1), (b) CNTFR, with the primers *P. shermani* CNTFR tissue forward and reverse or (c) β-actin (GenBank accession no. FJ824845), using the primers *P. shermani* actin forward and reverse, to control for the efficiency of reverse transcription. All primer pairs were tested for specificity of product amplification via cloning and sequencing before tissue RT-PCR was preformed.

The RT-PCR analysis showed that gp130 and CNTFR were both expressed in most tissues in both male and female adult *P. shermani* (Fig. 2). Overall, the expression patterns were very similar between the sexes and gp130 and CNTFR RNA were detected in almost every tissue examined (Fig. 2). The salamander gp130 expression pattern was consistent with the ubiquitous expression occurring in different mammalian cell lines (Hibi et al., 1990). In addition, salamanders showed CNTFR expression in tissues also documented to express the receptor in rats. Both salamanders and rats had high levels of CNTFR in the brain and muscle and low levels in the skin (Davis et al., 1991).

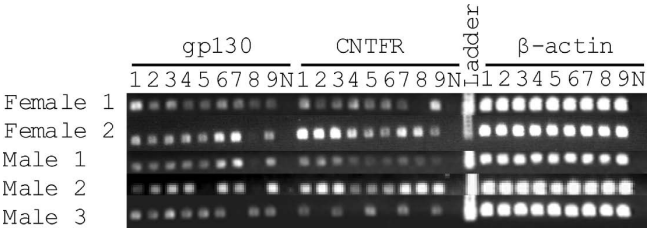

Fig. 2. Expression of gp130, CNTFR, and β-actin in female and male *P. shermani* tissues and organs. Tissues and organs tested: brain (1), eye (2), intestine (3), kidney (4), muscle (5), liver (6), olfactory (7), skin (8), and spleen (9). PCR amplification was carried out for 28 cycles of 30 sec at 95°C, 45 sec at 50°C and 30 sec at 73°C. No template controls for each primer pair were simultaneously run (N). Three µl of each reaction was run on a 1.75% agarose gel and visualized under UV light.

To determine the phylogenetic relationships of the salamander gp130 and CNTFR, we compiled an alignment that contained salamander receptors and other members of the gp130 receptor family accessioned from GenBank. We translated the full-length cytokine receptor sequences to amino acid sequences and then aligned them using the T-Coffee alignment program (Notredame et al., 2000) on the EMBL- EBI Server (http://www.ebi.ac.uk/Tools/msa/tcoffee/) with default parameters. A tree of vertebrate cytokine receptors was generated from the amino acid alignment using the minimum evolution method (with Poisson correction for amino acids) implemented in MEGA 4.0 (Tamura et al., 2007). All positions containing gaps and missing data were eliminated from the dataset and bootstrapping was conducted with 5000 replicates. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The analysis grouped the *P. shermani* gp130 with other gp130s, rather than LIFR, OSMR, or CSF3R, and the *P. shermani* CNTFR with the chicken and human CNTFRs, suggesting the salamander receptors were orthologous to those identified in other vertebrates (Fig. 3). The subtree containing amphibian, avian and mammalian gp130s was generally consistent with the accepted vertebrate phylogeny (Fig. 3), as was the clade of CNTFRs.

Studies of clawed frogs (*Xenopus* and *Silurana*) and salamanders (*Ambystoma*) have demonstrated that amphibians possess the many of the elements found in

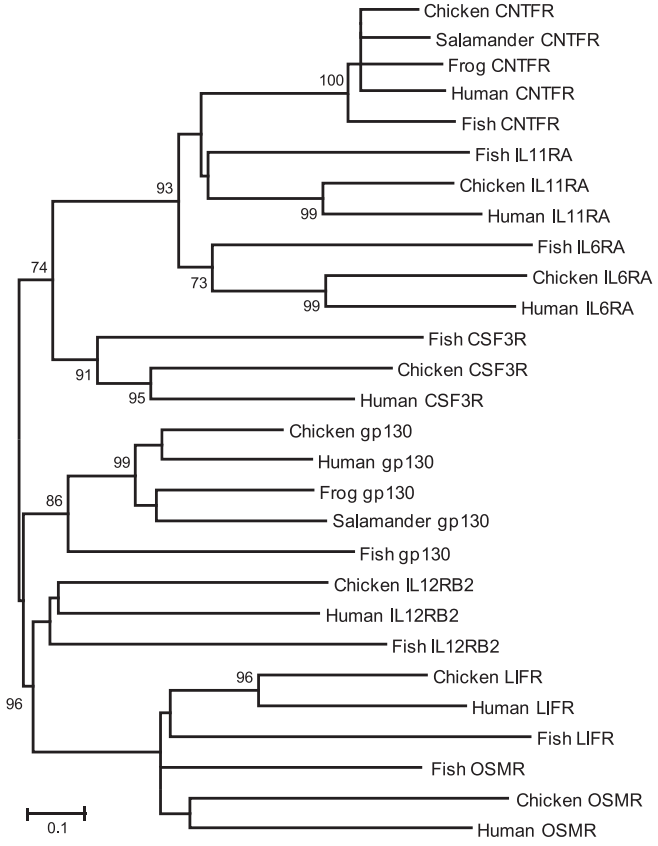

Fig. 3. Phylogenetic tree of vertebrate cytokine receptors. Bootstrap values above 70% are shown as percentages next to the branches. Scale bar represents 0.1 amino acid substitutions per site. GenBank accession numbers for Chicken (CNTFR: NP_990359; CSF3R: NP_001026069; IL11RA: BAD72064; IL12RB2: CAF21997; IL6RA: NP_001038140; gp130: NM_204871; LIFR: NP_989906; OSMR: XP_425020), Human (CNTFR: NP_671693; CSF3R: Q99062; IL11RA: NP_001136256; IL12RB2: NP_001550; IL6RA: NP_000556; gp130: BC117402; LIFR: NP_001121143; OSMR: NP_003990), Fish (CNTFR: NP_001091712; CSF3R: NP_001106848; IL11RA: NP_001106971; IL12RB2: NP_001106977; IL6RA: NP_001107790; gp130: BAH47260; LIFR: NP_001107204; OSMR: XP_001920274) and Frog (CNTFR: NM_001087091; gp130: AF041845).

mammalian immune systems, including cytokines (Carey et al., 1999; Robert and Ohta, 2009). However, sequence data on gp130 cytokines and their receptors in amphibians is extremely limited. Our characterization of two gp130 receptors from a salamander has provided insight into the evolution and structural conservation of these receptors. Finding highly conserved regions such as the CHR (with the four cysteines and WSXWS motif) in both gp130 and CNTFR implies that these sites are likely crucial for correct conformation and binding in all vertebrates (Bazan, 1990). The gp130 family of cytokine receptors diversified in the common ancestor of vertebrates, with subsequent processes, such as tandem duplication, shaping the receptor repertoire of specific lineages (Huising et al., 2006; Liongue and Ward, 2007). Our comparative study corroborates this process by showing that gp130 and CNTFR from representative amphibians have the same domain architecture as their orthologs in other vertebrates.

The redundancy in receptor binding of the gp130 cytokines leads us to infer that salamanders likely possess most of the gp130 cytokine receptors and their cognate ligands so far identified in mammals. In addition to IL-6 and CNTFR, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, cardiotrophin-1, IL-11, IL-27 and cardiotrophin-like cytokine/novel neurotrophin-1/B cell stimulating factor-3 all signal via gp130 in mammals (Bravo and Heath, 2000). Additionally, since the CNTFR is present in *P. shermani*, the LIFR is probably present as well because LIFR is part of the CNTF signaling complex. LIFR, in turn, is used by leukemia inhibitory factor, oncostatin M, cardiotrophin-1, and cardiotrophin-like cytokine/novel neurotrophin-1/B cell stimulating factor-3 (Bravo and Heath, 2000). Finally, the grouping of fish receptors with corresponding human and chicken orthologs (Fig. 3), strongly suggests that salamander genomes contain orthologs of all these cytokine receptors. The presence of these important cellular signaling pathways will hopefully lead to more studies on the functional roles of these molecules in amphibian immunity.

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