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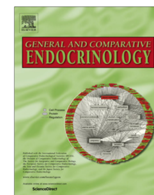

Research paper

Identification of three somatostatin genes in lampreys

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ABSTRACT

Somatostatins (SSs) are a structurally diverse family of neuropeptides that play important roles in the regulation of growth, development and metabolism in vertebrates. It has been recently proposed that the common ancestor of gnathostomes possessed three *SS* genes, namely *SS1*, *SS2* and *SS5*. *SS1* and *SS2* are still present in most extant gnathostome species investigated so far while *SS5* primarily occurs in chondrichthyes, actinopterygians and actinistia but not in tetrapods. Very little is known about the repertoire of SSs in cyclostomes, which are extant jawless vertebrates. In the present study, we report the cloning of the cDNAs encoding three distinct lamprey SS variants that we call SSa, SSb and SSc. SSa and SSb correspond to the two SS variants previously characterized in lamprey, while SSc appears to be a totally novel one. SSa exhibits the same sequence as gnathostome SS1. SSb differs from SSa by only one substitution ($Thr^{12} \rightarrow Ser$). SSc exhibits a totally unique structure (ANCRMFYWKTMAAC) that shares only 50% identity with SSa and SSb. SSa, SSb and SSc precursors do not exhibit any appreciable sequence similarity outside the C-terminal region containing the SS sequence. Phylogenetic analyses failed to clearly assign orthology relationships between lamprey and gnathostome SS genes. Synteny analysis suggests that the *SSc* gene arose before the split of the three gnathostome genes *SS1*, *SS2* and *SS5*.

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1. Introduction

Somatostatin (SS) is a tetradecapeptide that was originally isolated from the ovine hypothalamus due to its ability to inhibit growth hormone secretion from rat anterior pituitary cells (Brazeau et al., 1973). Since then, many SS variants have been identified in vertebrates where they play important roles in the regulation of growth, development and metabolism (Gahete et al., 2010; Sheridan and Hagemeister, 2010). In gnathostomes (jawed vertebrates), it has been shown that all these variants are the products of at least six paralogous genes named *SS1*, *SS2* (in mammals generally called *SS* and *cortistatin*, respectively), *SS3*, *SS4*, *SS5* and *SS6* (de Lecea et al., 1996; Liu et al., 2010; Tostivint et al., 1996, 2008). *SS1* and *SS2* have been found in most gnathostome species investigated so far. In contrast, *SS3* and *SS6* are known only in actinopterygians, *SS4* in teleosts and *SS5* in chondrichthyes, actinopterygians and actinistia (Conlon et al., 1997; Liu et al., 2010; Tostivint et al., 2008, 2013, 2014; Yun et al., 2015).

The current model of evolution of the *SS* gene family is based on the view that vertebrates underwent two rounds of whole-genome duplications (1R and 2R) early during their history, approximately 500 million years (My) ago (Dehal and Boore, 2005; Putnam et al., 2008; Van de Peer et al., 2010). According to this model (referred to as the 2R hypothesis), 1R and 2R led to the emergency of the *SS1*, *SS2* and *SS5* genes (Liu et al., 2010; Tostivint et al., 2013, 2014). The *SS4* gene was subsequently duplicated from the *SS1* gene in the teleost fish tetraploidization (3R), about 350 My ago, while the *SS3* and *SS6* genes arose by tandem duplication of the *SS1* and *SS2* genes, respectively, more or less around the same period (Liu et al., 2010; Tostivint et al., 2004, 2008, 2013, 2014). The implication of these findings is that the gnathostome ancestor possessed three *SS* genes, namely *SS1*, *SS2* and *SS5*, generated in the 2R (Liu et al., 2010; Tostivint et al., 2013, 2014).

Study of the SS system in lampreys could be of particular interest to test this view because cyclostomes represent the most basally branching lineage of vertebrates (Heimberg et al., 2010;

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Smith et al., 2013). Comparisons between cyclostomes (living jawless vertebrates) and gnathostomes (jawed vertebrates) have yielded important insights into the origin and evolution of vertebrate features (McCauley et al., 2015). However, whether lampreys diverged from the gnathostome lineage prior to 1R, between 1R and 2R, or after 2R has long been debated (Kuraku et al., 2009; Mehta et al., 2013; Smith et al., 2013; Smith and Keinath, 2015), and this question is still far from being resolved.

Previous investigations in lampreys have led to the purification and characterization of two SS variants from three species, *Petromyzon marinus*, *Lampetra fluviatilis* and *Geotria australis* (Andrews et al., 1988; Conlon et al., 1995a,b; Sower et al., 1994; Wang et al., 1999). The first one was identical in structure to SS1, while the second exhibited one substitution Thr → Ser at position 12. Several molecular forms of the latter variant (ranging from 14 to 37 residues depending on the species and the tissue) were isolated but they all derive from the same precursor. The occurrence of a SS1-like peptide has also been reported in the hagfish, another cyclostome (Conlon et al., 1988).

The aim of the present study was to determine which SS paralogs are present in lampreys as well as to reveal their orthology relationships with other vertebrate SSs which have never been investigated so far. For this purpose, the genomes of two lamprey species, namely sea lamprey *P. marinus* (Smith et al., 2013) and Japanese lamprey, *Lethenteron japonicum* (Mehta et al., 2013) and the transcriptome of one lamprey species, European river lamprey, *L. fluviatilis* (Guérin et al., 2009), were examined. Here, we report the identification of the cDNAs encoding three lamprey SS variants we propose to name SSa, SSb and SSc. We show that SSa and SSb correspond to the two SS variants already known in lampreys while SSc appears to be a totally novel one. However, we failed to clearly assign orthology relationships between lamprey and gnathostome SS genes.

2. Materials and methods

2.1. Characterization of lamprey somatostatin-related sequences

2.1.1. From L. japonicum

Genome assembly of the Japanese lamprey (at http://jlampreygenome.imcb.a-star.edu.sg/) was searched by TBLASTN using the SS1, SS2 (from human and zebrafish) and SS3 (from stickleback and fugu) sequences as queries. Regions that showed similarity were searched against the NCBI NR database by BLASTX to identify *SS* genes in the lamprey genome. Our Blast searches identified fragments of three *SS* genes, *SSa*, *SSb* and *SSc* respectively on scaffold_71405, scaffold_646 and scaffold_89. The coding sequences of these genes were incomplete due to gaps in the genome assembly. We completed the coding sequences by doing 5′RACE and RT-PCR using cDNA prepared from the total RNA of the Japanese lamprey brain tissue. The primer sequences used are given in Table S1. The protocols for the extraction of total RNA from the Japanese lamprey brain and RACE and RT-PCR have been previously described (Ravi et al., 2016). Protein domains in the Japanese lamprey *SS* sequences were predicted using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/) and InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search).

The Japanese lamprey sequences reported in this study have been deposited in GenBank under accession numbers KU522234 (*SSa*), KU522235 (*SSb*) and KU522236 (*SSc*).

2.1.2. From P. marinus

The sea lamprey genome database (http://www.ensembl.org/Petromyzon_marinus/Info/Index) was searched by TBLASTN using the human and zebrafish SS1 sequence as query. A fragment encoding a putative SS gene that shares highest sequence similarity for the Japanese lamprey SSc was identified. The complete sequence of the *P. marinus SSc* cDNA was cloned by rapid amplification of cDNA ends (RACE)-PCR. For this purpose, total RNAs were extracted from adult sea lamprey brains using RNAble (Eurobio, Courtaboeuf, France). Poly(A^+) RNAs were purified from total RNAs with Dynabeads mRNA Purification Kit (Invitrogen, Saint Aubin, France). 5′ and 3′ RACE-ready cDNAs were constructed from 1 μg of poly(A^+) RNAs using the SMARTer RACE cDNA Amplification kit (Clontech, Saint-Germain-en-Laye, France). The 5′ and 3′ ends of the sea lamprey *SSc* cDNA were amplified by nested PCR using the Advantage 2 PCR kit (Clontech) under the following conditions: (1) 94 °C for 3 min; (2) 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and (3) 72 °C for 7 min. For the 5′ end amplifications, the primers used were PmSSc Rev × Universal Primer A Mix (UPM), then PmSSc Rev Nest1 × Nested Universal Primer A Mix (NUP) and PmSSc Rev Nest2 × NUP, while for the 3′ end amplifications, they were PmSSc For × UPM then PmSSc For Nest1 × NUP, and PmSSc For Nest2 × NUP (see Table S1 for sequence of primers). The PCR products were separated by agarose gel electrophoresis and purified using the Minelute Gel Extraction Kit (Qiagen, Courtaboeuf, France). The DNA fragments were then subcloned into the pGEM-T vector (Promega, Charbonnières-les-Bains, France) and sequenced (Value Read Sequencing at MWG Biotech, Ebersberg, Germany).

The coding sequence of the *P. marinus SSc* cDNA has been deposited in the GenBank database under the accession number KU529940.

2.1.3. From L. fluviatilis

An adult brain/eye cDNA library was constructed in the pSPORT1 vector using the Superscript plasmid system with Gateway technology (Invitrogen) (Guérin et al., 2009). The library was plated, arrayed robotically and submitted to large-scale EST sequencing on an ABI3730xl by the Genoscope (Evry, France). The resulting EST database was searched by TBLASTN using the human and zebrafish SS1 sequence as query. A fragment encoding a putative SS gene that shares highest sequence similarity for the Japanese lamprey SSb was identified.

The coding sequence of the *L. fluviatilis SSb* cDNA has been deposited in the GenBank database under the accession number KX345282.

2.2. Sequence comparisons

The percent deduced amino acid identity between the lamprey prepro-SSs (PSSs) identified in the present study and PSSs from two representative gnathostome species (human and spotted gar) were determined by pairwise analysis using a Clustal Omega alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.3. Phylogenetic analysis

The amino acid sequences of the lamprey prepro-SSs (PSSs) were aligned with selected PSS sequences of other vertebrates using the MAFFT algorithm (http://www.ebi.ac.uk/Tools/msa/mafft/) and then manually optimized.

Phylogenetic trees were constructed using maximum likelihood and Bayesian methods. The maximum likelihood analysis was carried out in PhyML (Guindon and Gascuel, 2003) via the Seaview version 4 software (Gouy et al., 2010). The best amino acid substitution model for the alignment was determined to be JTT + G + I using ProtTest (Abascal et al., 2005) on the University of Vigo server (http://darwin.uvigo.es/software/prottest2_server.htm.l). The robustness of the tree was assessed by the bootstrap procedure with 1000 replications. Sequence data was also analysed using

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