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Identification and Molecular Characterization of the Stanniocalcin Family Gene from the Inshore Hagfish, *Eptatretus burgeri*

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Stanniocalcin (STC) is a glycoprotein hormone that was first isolated from teleost corpuscles of Stannius. STC is involved in the decrease in plasma Ca^{2+} level in teleosts. Comparative studies between invertebrate chordate STC and two paralogous vertebrate STCs suggest that the chordate ancestral gene of STC diverged into two paralogous genes in the evolution of vertebrates. However, this evolutionary scenario remains unclear. To clarify the diversification process of vertebrate STC, we made an attempt to isolate orthologs of STC in extant jawless fishes (cyclostomes) located most distantly in the vertebrate tree of life. In the present study, we searched the STC–like sequence by performing degenerate PCR in the inshore hagfish *Eptatretus burgeri*. We identified a STC homolog, designated as Eb–STC. Our molecular phylogenetic analysis confirmed that Eb–STC is a member of the STC family. The comparison of amino acid sequences revealed that Eb–STC is similar to gnathostome STC1 and 2, in that Eb–STC shares the 10 conserved cysteine residues that form intramolecular disulfide bonds. Eb–STC and gnathostome STCs possess the conserved *N*–glycosylated site. However, typical features discriminated between STC1 and 2, such as the distinct position of the cysteine residue associated with the intermolecular dimer, were not observed in Eb–STC. Tissue distribution analysis revealed that Eb–STC is expressed in various organs, but not in the brain. This expression pattern resembles that of teleost STC1 and 2, suggesting that Eb–STC acts as a paracrine factor. To the best of our knowledge, this is the first report on the identification and molecular characterization of STC in cyclostomes.

Key words: stanniocalcin, hagfish, cyclostomes, calcium homeostasis

INTRODUCTION

Stanniocalcin (STC) is a glycoprotein hormone first isolated from teleosts (Wagner and Dimattia, 2006; Wagner *et al*., 1992; Wagner *et al*., 1988; Wagner *et al*., 1986). In teleosts, an increase in the plasma calcium level elicits the release of STC from the corpuscles of Stannius (CS) (Wagner *et al*., 1998; Wagner and Jaworski, 1994; Wagner *et al*., 1991), which induces a decrease in the plasma Ca^{2+} level via the gill and intestine. In gills, STC stimulates the excretion of Ca^{2+}(Lafeber *et al*., 1988a; Lafeber *et al*., 1988b; Wagner *et al*., 1986). The chum salmon STC inhibits the Ca^{2+} uptake in perfused Atlantic cod intestine (Sundell *et al*., 1992). STC is also associated with phosphate metabolism. Treatment with coho salmon STC results in stimulation of inorganic phosphate (Pi) reabsorption in the primary cultured flounder renal proximal tube cells (Lu *et al*., 1994). To date, STC has been observed in a wide range of species including vertebrates, invertebrate deuterostomes, protostomes, and unicellular eukaryotes (Roch and Sherwood, 2010; 2011; Schein *et al*., 2012). The paralogous genes of STC1 and STC2 have been identified in various organisms, from teleosts to mammals (Luo *et al*., 2005; Shin and Sohn, 2009). Studies of vertebrate STC have uncovered its molecular characteristics. The vertebrates STC1 and STC2 have in common the *N*–glycosylated site and contain 10 cysteine residues forming five intramolecular disulfide bonds (Hulova and Kawauchi, 1999). Additionally, they form a homodimer via an intermolecular disulfide bond that is located in a different position in each STC paralog (Roch and Sherwood, 2011). In vertebrate STC1, the conserved 11th cysteine is involved in the formation of a homodimer (Hulova and Kawauchi, 1999). On the other hand, vertebrate STC2 shares the 3 conserved cysteine residues, one of which is expected to form an intermolecular disulfide bond (Roch and Sherwood, 2011). Furthermore, a histidine–rich sequence is conserved in vertebrate STC2 (Roch and Sherwood, 2011). Comparisons of the sequences of STC in invertebrate chordates including ascidian and amphioxus demonstrate that the STCs possess prototypical features of vertebrate STC1 and 2, implying that vertebrate STC1 and 2 are derived from an ancestral STC of primitive chordates (Roch and Sherwood, 2010). To obtain insight into the more precise evolutionary process, we focused on the cyclostomes including hagfish and lamprey. Cyclostomes are jawless vertebrates and

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phylogenetically located in the basal position in vertebrates (Kuraku, 2013; Shimeld and Donoghue, 2012). However, no STC gene has been identified in cyclostomes.

In the present study, to elucidate the evolutional diversity of vertebrate STC1 and 2, we explored the STC gene from the inshore hagfish, *Eptatretus burgeri*. We cloned and determined the sequence of the STC–like gene, designated as *E. burgeri* STC (Eb–STC). Furthermore, its molecular characteristics and tissue distribution pattern were clarified.

To the best of our knowledge, this is the first identification of STC in cyclostomes.

MATERIALS AND METHODS

Animals

Inshore hagfish (*E. burgeri*) were obtained from near the Noto–Jima Island by a fisherman. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Kanazawa University.

Molecular cloning of STC

The digestive tract was removed from adult hagfish. First–strand cDNA was synthesized using the SuperScript® Ⅲ reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and oligo–$(dT)_{20}$ primer following extraction of total RNA. The 371–bp fragment of putative STC cDNA was obtained by degenerate reverse transcription polymerase chain reaction (RT–PCR) using the forward primer 5'–SANGYNGGITGYGSIDYITT–3' and the reverse primer 5'–TCITSIYCRCAIBYIADNA–3'. Additionally, we determined the open reading frame (ORF) of STC by 5' and 3'rapid amplification of cDNA ends (RACE) using the GeneRacer® kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA library was synthesized from 2 μg total RNA using the oligo–(dT) adapter primer and was amplified using the adapter primer and the gene–specific complementary first primers (5'–RACE first primer: 5'–TTGAGGTGTTGGAAGGTGAGGGAGAATG–3'; 3'–RACE first primer: 5'–GTGAAACGGGAGGTCTCCATGCCATCTG–3'). The first PCR product was amplified using the 5' or 3' nested adapter primer and each second gene–specific primer (5'–RACE second primer: 5'–CGCCACCGTCTCATCATCGCACTTAATC–3'; 3'–RACE second primer: 5'–GGAGATGGTGGTACAACTGCAGCGTCAT–3'). The second PCR products were subcloned and sequenced by the ABI PRISM™ 3130 Genetic Analyzer with a Big–Dye Sequencing Kit version 3.1 (Thermo Fisher Scientific) using the universal primers SP6 and T7.

Molecular phylogenetic analysis of STC

Molecular phylogenetic analysis was performed as described previously (Sekiguchi *et al*., 2016; Sekiguchi *et al*., 2009). In brief, the full–length amino acid sequences of chordate STC were aligned using the CLUSTAL X program (Higgins and Sharp, 1988), and the alignment was manually checked using BioEdit version 7.0.5.3. After the removal of gaps, phylogenetic trees were inferred using alignments by the neighbor–joining method with MEGA version 7.0 (Saitou and Nei 1987; Tamura *et al*., 2013). The accession numbers of the protein sequences used in this analysis are as follows: human STC1 (NP_003146), chicken STC1 (XP_425760), turkey STC1 (XP_010721360), platypus STC (ABI64157), American alligator STC1 (XP_006274704), pig STC1 (NP_001096682), cattle STC1 (NP_788842), mouse STC1 (AAP47156), coelacanth STC1 (XP_005991938), African clawed frog STC1 (AAH76749), green anole STC1 (XP_008118056), spotted gar STC–like (XP_006625371), bowfin STC (BAC66163), zebrafish STC1b (AAI22229), medaka STC1b (ENSORLP00000013724), stickleback STC1b (ENSGACP00000014284), zebrafish STC1a (AAH66540), medaka STC1a (ENSORLP00000000399), Japanese flounder STC1 (ACJ06521), elephant nose fish STC (BAD99600), ghost shark STC–like–a (SINCAMP00000001885), skate STC (FF600059), ghost shark STC–like–b (SINCAMP00000001117), Japanese flounder STC2 (ACJ06521), medaka STC2a (ENSORLP00000009199), stickleback STC2a (ENSGACP00000024367), zebrafish STC2a (NP_001014827), spotted gar STC2 (XP_006631778), coelacanth STC2 (XP_005992572), tropical clawed frog STC2 (AAI21339), cattle STC2 (XP_005899841), pig STC2 (NP_001103643), human STC2 (NP_003705), mouse STC2 (NP_035621), green anole STC2 (XP_008102897), chicken STC2 (XP_414534), turkey STC2 (XP_003210363), American alligator STC2 (XP_006270913), and ascidian (*Ciona intestinalis*) STC–like (XP_002130163).

Reverse–transcription (RT)–PCR of STC and GAPDH mRNAs in various tissues in hagfish

The brain, gill, heart, liver, gallbladder, digestive tract, and kidney of the hagfish were removed. Total RNA (1 μg) was extracted and reverse–transcribed using the SuperScript® Ⅲ reverse transcriptase (Thermo Fisher Scientific) and oligo–$(dT)_{20}$ primer. Next, we performed PCR of STC using the primer set GGTGTTCAACAGAACGGTGC (forward primer) and ATGACGCTGCAGTTGTACCA (reverse primer). PCR of GAPDH was performed as an internal control of each tissue using the primer set GTCTGGTGACCAGGGC (forward primer) and CAATCCCAAAGTTATCATGG (reverse primer). We checked the fragment size of PCR products by agarose gel electrophoresis. Moreover, sequences of the PCR fragments were directly determined by the ABI PRISM™ 3130 Genetic Analyzer, using specific PCR primers of STC and GAPDH. This experiment was independently performed in three individuals.

RESULTS

Molecular characterization of the STC ortholog in hagfish

To identify the orthologous gene of STC in hagfish, we cloned and determined the full–length ORF sequence of STC from a first–strand cDNA library of the digestive tract. First, the 371–bp sequence was determined by

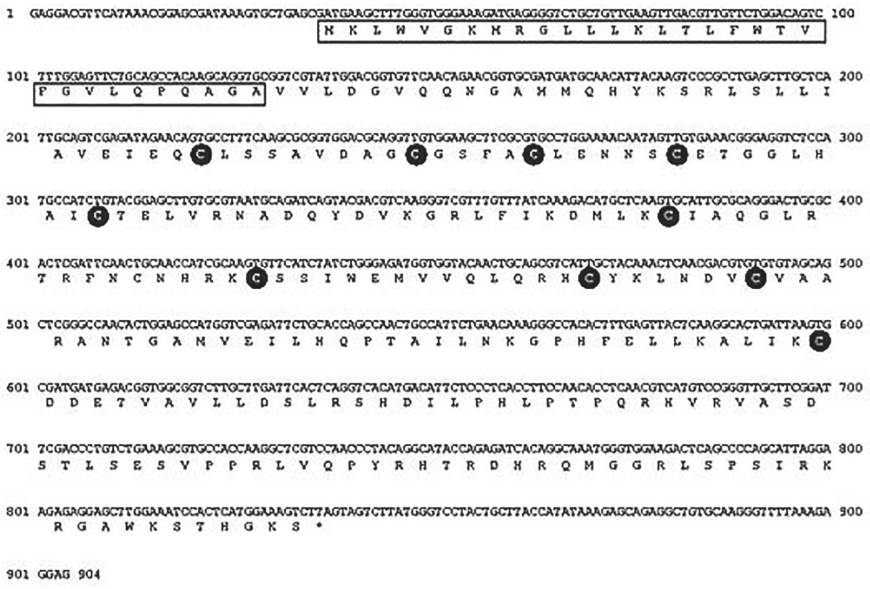

Fig. 1. Nucleotide and deduced amino acid sequences of hagfish STC.
The predicted signal peptide is shown by the open box. Black circles show cysteine residues observed in vertebrate STC. The stop codon is depicted by an asterisk. These data are available under GenBank accession no. LC191307.

PCR analysis using degenerated primers designed from conserved sequence stretches in gnathostome STC. Moreover, an ORF sequence of 801 bp was determined using the 5'– and 3'–RACE methods. The deduced amino acid sequence of 266 residues possessed the putative 31–amino acid signal peptide, which was predicted by the Signal IP4 server (Petersen *et al.*, 2011) (Fig. 1). Ten cysteine residues are detected in this protein (Fig. 1).

In an attempt to elucidate whether this protein is orthologous to gnathostome STC, we performed molecular phylogenetic analysis. As shown in Fig. 2, the molecular phylogenetic tree of chordate STC demonstrated that the vertebrate STC family is divided into two groups, namely STC1 and STC2, with bootstrap values of 99 and 63, respectively. The hagfish STC–like protein showed a higher proximity to STC2 and was placed outside the gnathostome sequences in accordance with the phylogenetic position of the hagfish (Fig. 2). Therefore, we designated this sequence as *E. burgeri* STC (Eb–STC).

In order to evaluate the molecular characteristics of Eb–STC, we compared the amino acid sequence of Eb–STC with that of various chordate STCs. Multiple amino acid sequence alignment analyses of Eb–STC, gnathostome STCs, and ascidian STC demonstrated that 10 cysteine residues are conserved in STCs of all chordates (Fig. 3). Furthermore, the *N*–glycosylation site is conserved in vertebrate STC. However, the 11th cysteine residue that is responsible for dimer formation of gnathostome STC1 was not detected in Eb–STC (Fig. 3). Moreover, the three cysteine residues that are conserved in gnathostome STC2s were not observed in hagfish STC (Fig. 3). No gnathostome STC2–specific histidine–rich sequence was detected in hagfish STC (Fig. 3).

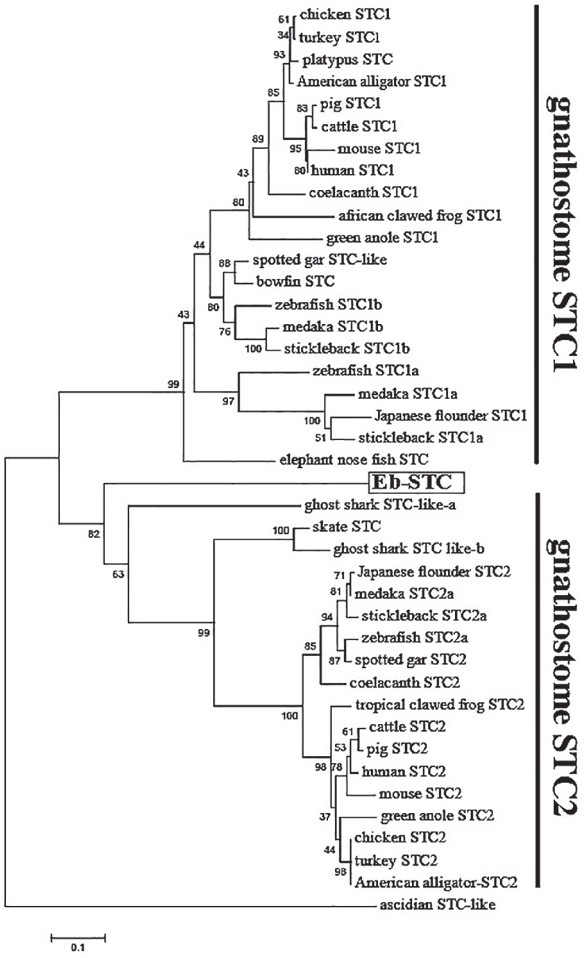

Fig. 2. Molecular phylogenetic tree of STC in chordates.
Molecular phylogenetic tree prediction was performed using the neighbor–joining method. The bootstrap value is shown beside each branch. The unrooted tree is depicted as a root tree. The scale bar represents an evolutionary distance of 0.1 amino acid substitutions per protein.

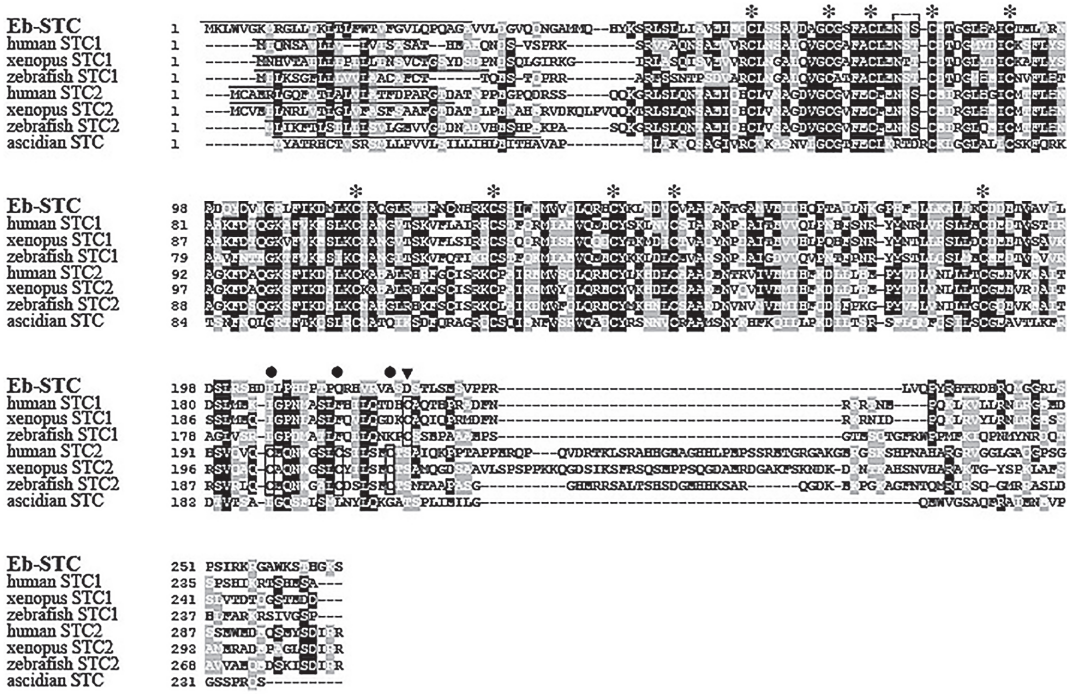

Fig. 3. Amino acid comparison of Eb–STC with chordate STC1 and 2.
Identical and similar amino acids among half of the total members are depicted by black and gray boxes, respectively. The 10 conserved cysteine residues among STCs are indicated by asterisks. The cysteine residue conserved in the gnathostome STC1s is indicated by an open box and arrowhead. The three cysteine residues conserved in the gnathostome STC2s are indicated by open boxes and closed black circles. The *N*–glycosylation site is indicated by a dotted box. Signal peptides are shown by over bars. Signal peptides are shown in overbar.

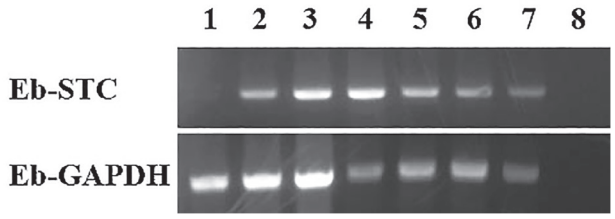

Fig. 4. RT–PCR analysis of Eb–STC mRNA.
The distribution of Eb–STC was examined using RT–PCR in the brain (lane 1), gill (lane 2), heart (lane 3), liver (lane 4), gallbladder (lane 5), digestive tract (lane 6), and kidney (lane 7). No product was detected in the absence of first–strand cDNA (DW, lane 8). Eb–GAPDH represents an internal control.

Expression analysis of Eb–STC mRNA in various tissues

To evaluate the tissue distribution pattern of Eb–STC, we performed RT–PCR analysis in various tissues of *E. burgeri*. The Eb–STC transcript was detected in the gill, heart, liver, gallbladder, digestive tract, and kidney (Fig. 4).

DISCUSSION

Plasma Ca^{2+} levels are stably maintained by various hormones, given that Ca^{2+} plays a central role in the physiological function of vertebrates (Norris and Carr, 2013). STC is a hormone involved in calcium homeostasis in teleosts and has been identified in various species (Roch and Sherwood, 2011). In the present study, we identified the STC gene from the hagfish *E. burgeri* and confirmed its orthology to the gnathostome STC family member and its molecular characteristics.

Hagfish STC named as Eb–STC possesses the signal peptide that is necessary for secreting peptides and 10 cysteine residues typically observed in STC (Fig. 1). Molecular phylogenetic analysis revealed that gnathostome STCs were divided into two clades as previously reported (Wagner and Dimattia, 2006). Eb–STC was placed in proximity to the clade formed by gnathostome STC2s, suggesting that Eb–STC is orthologous to gnathostome STC2. However, owing to limited information on cyclostomes and elasmobranchs, the possibility that Eb–STC is the common ancestor of both gnathostome STC1 and STC2 cannot be ruled out. To uncover the precise phylogenetic position of Eb–STC, explorations of the Eb–STC paralogous gene in *E. burgeri* and the STC gene in lamprey are currently underway.

An amino acid sequence comparison among chordate STCs indicates that Eb–STC shares a common sequence with vertebrate STC. The *N*–glycosylation site and 10 cysteine residues are conserved among vertebrates including hagfish (Fig. 3) (Roch and Sherwood, 2011). On the other hand, the gnathostome STC1–specific 11th cysteine and three cysteine residues conserved in gnathostome STC2 were not detected in Eb–STC (Fig. 3). In addition, no 11th cysteine residue was

observed after the 10 conserved cysteine residues. These findings suggest that Eb–STC functions as a monomer, like arowana and ascidian (Amemiya *et al*., 2006; Roch and Sherwood, 2010). In amphioxus, two STC types were identified (Roch and Sherwood, 2010). One STC is presumed to act as a monomer, and the other STC is a putative homodimer type, indicating that the homodimerized type of STC is conserved in chordates. Taken together, these findings imply that hagfish also possesses the other STC gene forming the homodimer.

Tissue distribution analysis demonstrated ubiquitous expression of Eb–STC in all organs except the brain (Fig. 4). In bony fish, STC1 and 2 are commonly expressed in various tissues including the CS (Hang and Balment, 2005; Shin *et al*., 2006; Shin and Sohn, 2009). The expression pattern of euryhaline flounder STC is coincident with that of Eb–STC (Hang and Balment, 2005). Altogether, these findings imply that the tissue distribution pattern of Eb–STC is similar to that in gnathostomes. Moreover, it is suggested that Eb–STC acts as a paracrine factor. One possible function of Eb–STC is Ca^{2+} ion homeostasis. The hagfish is an osmoconformer. The osmotic pressure and univalent ion (such as Na^+, Cl^-, and K^+) levels of their internal environment are the same as those of seawater. However, the Ca^{2+} and Mg^{2+} ion levels in hagfish serum are stably maintained at lower levels than in seawater (Sardella *et al*., 2009). The extract from the eel CS stimulates the release of Ca^{2+} from the gill of *Eptatretus cirrhatus*. These issues suggest that Eb–STC induces Ca^{2+} release in the gill (Forster and Fenwick, 1994). Additionally, Pi metabolism is observed in the Pacific hagfish, *Eptatretus stoutii*, and the Pi transporter Slc34a is expressed in the gill, skin, intestine, and kidney (Schultz *et al*., 2014). Taking into account the involvement of STC on Pi homeostasis in teleosts (Lu *et al*., 1994), it is suggested that Eb–STC is also associated with Pi homeostasis. To uncover STC functions, further functional research is needed.

In the present study, we first identified the STC gene in cyclostomes. This study provides insight into the evolutionary biology of the STC gene.

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