Stanniocalcin Has Deep Evolutionary Roots in Eukaryotes

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Abstract

Vertebrates have a large glycoprotein hormone, stanniocalcin, which originally was shown to inhibit calcium uptake from the environment in teleost fish gills. Later, humans, other mammals, and teleost fish were shown to have two forms of stanniocalcin (STC1 and STC2) that were widely distributed in many tissues. STC1 is associated with calcium and phosphate homeostasis and STC2 with phosphate, but their receptors and signaling pathways have not been elucidated. We undertook a phylogenetic investigation of stanniocalcin beyond the vertebrates using a combination of BLAST and HMMER homology searches in protein, genomic, and expressed sequence tag databases. We identified novel STC homologs in a diverse array of multicellular and unicellular organisms. Within the eukaryotes, almost all major taxonomic groups except plants and algae have STC homologs, although some groups like echinoderms and arthropods lack STC genes. The critical structural feature for recognition of stanniocalcins was the conserved pattern of ten cysteines, even though the amino acid sequence identity was low. Signal peptides in STC sequences suggest they are secreted from the cell of synthesis. The role of glycosylation signals and additional cysteines is not yet clear, although the 11th cysteine, if present, has been shown to form homodimers in some vertebrates. We predict that large secreted stanniocalcin homologs appeared in evolution as early as single-celled eukaryotes. Stanniocalcin’s tertiary structure with five disulfide bonds and its primary structure with modest amino acid conservation currently lack an established receptor-signaling system, although we suggest possible alternatives.

Key words: hormone evolution, calcium regulation, ancient hormone, voltage-dependent calcium channel.

Stanniocalcins (STCs) are large (approximately 250 amino acids) glycoprotein hormones in vertebrates whose classical function was established to be the inhibition of calcium uptake from the environment in teleost fish gills (Wagner et al. 1986). Initially, orthologs of STC1 were identified and isolated in a variety of actinopterygian (ray-finned) fish species, including salmonids (Wagner et al. 1986, 1988; Lafeber et al. 1988), eels (Butkus et al. 1987), and basal ray-finned fishes (Marra et al. 1995; Amemiya et al. 2002; Amemiya and Youson 2004). Mammalian orthologs were also identified in humans (Chang et al. 1995; Wagner et al. 1995; Olsen et al. 1996) and mice (Chang et al. 1996), with an expanded repertoire of functions including phosphate regulation (Haddad et al. 1996; Olsen et al. 1996), bone formation, growth, and neuroprotection (Yoshiko and Aubin 2004). A second hormone, STC2, was more recently identified in both mammals (Chang and Reddel 1998; DiMattia et al. 1998; Ishibashi et al. 1998) and teleost fishes (Luo et al. 2005; Shin and Sohn 2009). Although not as well characterized as STC1, functions of STC2 include roles in growth (Gagliardi et al. 2005; Chang et al. 2008), ovarian development (Luo et al. 2005), and cancer (Chang et al. 2003). The roles of the stanniocalcin hormones in vertebrates have expanded with the discovery of novel forms, but a common theme that has constantly reappeared is the regulation of calcium and phosphate homeostasis by STC1 and phosphate by STC2 (Ishibashi et al. 1998; Yahata et al. 2003).

The calcium ion, $\text{Ca}^{2+}$, has been a critical second messenger from the earliest stages of life. Regulation of calcium has been important in the evolution of prokaryotes, as high concentrations can disrupt intracellular homeostasis by aggregating proteins and nucleic acids, disrupting membranes, and precipitating phosphates (Case et al. 2007). As such, the cytosolic concentration of $\text{Ca}^{2+}$ is strictly maintained within all cells at a level at least one-thousand times lower than the concentration of the ion in saltwater (Williams 2006). Calcium regulatory machinery is found in bacteria, which possess ubiquitous polymeric complexes (poly-$3$-hydroxybutyrate/polypolyphosphate) forming specific voltage-gated calcium channels for influx into the cell (Reusch et al. 1995). Prokaryotes also bear homologs of major components of the calcium regulatory system in eukaryotes.
including a simplified one-domain voltage-dependent calcium channel (VDCC) compared with the four-domain channel in eukaryotes (Durell and Guy 2001). In addition to these influx channels, and Ca\(^{2+}\) exchangers and pumps, members of the calcium-binding EF-hand protein family (which includes calmodulin) have also been identified in some bacteria (Michiels et al. 2002).

Considering the role of STC1 in regulating extracellular calcium influx in the vertebrates, the question of its phylogenetic roots bears strong merit. Previously, we had discovered homologs of STC1/STC2 in two species of invertebrate chordates, the tunicate Ciona intestinalis and the amphioxus Branchiostoma floridae, establishing the history of stanniocalcin as predating the evolution of vertebrates (Roch and Sherwood 2010). A few studies also provided immunological evidence, using vertebrate antibodies, to suggest the presence of stanniocalcin homologs in a snail (Wendelaar Bonga et al. 1989) and a leech (Tanega et al. 2004). After phylogenetic analysis of currently available genomic and expressed sequence tag (EST) databases, we discovered new STC homologs from a diverse array of invertebrates and unicellular eukaryotes, including members of almost all major taxonomic groups with the notable exception of plants and algae. These putative STCs have low primary sequence conservation but retain the critical pattern of ten cysteine positions previously established in all stanniocalcins. The identification of stanniocalcin in unicellular organisms raises the question of function for this large (minimally 150 amino acids) secreted protein, structurally conserved from the early evolution of eukaryota. Given the lack of an identified STC receptor, we suggest that the interaction of stanniocalcin with the VDCC is one possibility.

**Materials and Methods**

**Searching for Stanniocalcin Homologs**

Stanniocalcin homologs were identified by a combination of BLAST (Altschul et al. 1997) and HMMER v2.3.2 (http://hmmer.janelia.org/) homology searches. Protein, genomic, and EST databases at National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and the Joint Genome Institute (JGI; http://genome.jgi-psf.org) were initially probed for stanniocalcin homologs using previously established vertebrate and later putative protostome homologs listed in table 1 with blastp (established and predicted proteins) and tblastn (genomic and EST libraries). Default search values were relaxed (PAM30 or BLOSUM45 matrix, word size 2, E-value 100) to retrieve highly divergent sequences. In addition, hidden Markov models (HMMs) were constructed from various combinations of STC homologs from the vertebrates, protostomes, fungi, and protists and were employed to search proprietary protein and translated EST databases using hhmsearch with default values. Theoretical consensus protein sequences were also generated from these HMMs using hmmemit (default values) and these were used to search the public databases with Blast. Retrieved amino acid sequences were aligned with MUSCLE v3.7 (Edgar 2004) and visually inspected for characteristic features including protein length (150–300 amino acids typically), conserved cysteine residues, and other areas of primary sequence similarity. Predicted protein sequences and translated ESTs were then trimmed for the best open reading frame start and stop sites.

**Phylogenetic Analysis**

Sequences that were confirmed as STC homologs by primary structure and used for further phylogenetic analysis in this study are listed in table 1. Representative amino acid sequences from a diverse array of eukaryotes were aligned by MUSCLE v3.7 and output in BioEdit v7.0.5 (Hall 1999) to produce the protein alignment and amino acid identity matrix in figures 1 and 2. Phylogenetic analysis of all STC homologs listed in table 1 was performed by the maximum likelihood method of PhyML 3.0 (Guindon et al. 2010). Protein sequences were trimmed to include the region encompassing the ten highly conserved cysteine residues, excluding the N-terminus (including signal peptide) and C-terminus, and partially degapped to produce an alignment of 117 amino acids in length. This alignment is provided in the Supplementary material (Supplementary material). The tree was then constructed with PhyML with the parameters LG + I + G after selecting the best model for the alignment using PROTEST 2.4 (Abascal et al. 2005). The following variables were employed: the LG substitution model, four rate categories, an estimated gamma distribution parameter, an estimated proportion of invariant sites, subtree pruning and regrafting tree topology searches, an optimized starting tree topology using BioNJ, and aLRT branch support (SH-like). Remaining options were default, including using the model’s equilibrium of amino acid frequencies. The resulting tree was visualized in MEGA v4.1 (Tamura et al. 2007) to produce figure 3. It is presented as an unrooted radial tree due to the lack of a useful outgroup for stanniocalcin sequences.

**Peptide Motif Analysis**

Stanniocalcin homologs presented in table 1 were analyzed for putative signal peptides. The signal peptide analysis was conducted on full-length amino acid sequences using SignalP 3.0 (Emanuelsson et al. 2007), employing both neural networks and HMMs for eukaryote sequences. Only sequences with predicted signal peptides from both methods were listed as having signal peptides in table 1. Additionally, for the sequences shown in figure 1, Signal-3L (Shen and Chou 2007) and Philius (Reynolds et al. 2008) were used to predict signal peptides, and a consensus prediction was chosen based on the three methods and highlighted in the figure. The sequences in figure 1 were also analyzed using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) for N-glycosylation motifs.
| Group     | Phylum     | Common name | Species name | Sequence | Source    | Type       | Accession # | cDNA | Signal peptide |
|-----------|------------|-------------|--------------|----------|-----------|------------|-------------|------|----------------|
| Fish      | Vertebrata | Anguilla australis | Anguilla australis | STC2 | NCBI, gene model | AAB93483 | X | X |
| Elephant  | Vertebrata | Ganothorax petenii | Ganothorax petenii | STC1 | NCBI, gene model | BAD99609 | X | X |
| Toad      | Vertebrata | Bufo bufo | Bufo bufo | STC1 | NCBI, gene model | BAB36987 | X | X |
| Frog      | Vertebrata | Rana pipiens | Rana pipiens | STC2 | NCBI, gene model | AC016621 | X | X |
| Gar       | Vertebrata | Lepisosteus osseus | Lepisosteus osseus | STC1 | NCBI, gene model | BAB68464 | X | X |
| Human     | Vertebrata | Homo sapiens | Homo sapiens | STC2 | NCBI, gene model | NP_003146 | X | X |
| Mouse     | Vertebrata | Mus musculus | Mus musculus | STC1 | NCBI, gene model | NP_003621 | X | X |
| Opossum   | Vertebrata | Monodelphis domestica | Monodelphis domestica | STC1 | NCBI, gene model | NP_0003705 | X | X |
| Porcupine | Vertebrata | Hylocoerus jayakari | Hylocoerus jayakari | STC1 | NCBI, gene model | NP_00107786 | X | X |
| Osteichthyes | Chondrichthyes | Canis lupus | Canis lupus | STC1 | NCBI, gene model | XP_00107786 | X | X |
| Cephalochordata | Chordata | Amphioxus proteus | Amphioxus proteus | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Xenoturbellida | Platyhelminthes | Turbellaria | Turbellaria | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Nemertodrilus | Nemertea | Nemertodrilus | Nemertodrilus | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Nematode | Nematoda | Caenorhabditis briggsae | Caenorhabditis briggsae | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Nematode | Nematoda | Caenorhabditis elegans | Caenorhabditis elegans | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Nemertina | Nemertea | Caenorhabditis japonica | Caenorhabditis japonica | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Cnidaria | Cubozoa | Hydractinia echinata | Hydractinia echinata | STC1 | NCBI, gene model | NP_0004697 | X | X |
| Porifera | Spongia | Leucosolenia sp. | Leucosolenia sp. | STC1 | NCBI, gene model | NP_0004697 | X | X |
Results and Discussion

Stanniocalcins with Ten Conserved Cysteines are Found across the Eukaryotic Spectrum

Putative homologs of stanniocalcin from invertebrate protostomes were identified with BLAST and HMMER homology searches using various STC protein sequences as queries. A number of genomic resources were investigated, including the NCBI protein and EST databases and several JGI draft genomes. An Interpro model for the stanniocalcin family (IPR004978) was identified that included sequences from the nematode *Caenorhabditis elegans*, the abalone *Haliotis diversicolor*, the fungi *Coccidioides immitis* and *Laccaria bicolor*, and the ciliate *Tetrahymena thermophila*. In our study, sequences representing potential stanniocalcins were identified in a diverse range of animals including sponges, cnidarians, and protostomes (table 1). A unique paralog of the hormone was also identified in the amphioxus *B. floridana* (STCc) bearing structural hallmarks more similar to protostome homologs. Two additional STC-like gene models containing sequences similar to amphioxus STCc were also identified, however, their close proximity to STCc on the same scaffold and lack of corresponding ESTs led us to conclude they were most likely tandem duplications that had been pseudogenized, and they were not included in this study. Earlier, we characterized two amphioxus STCs that were more closely related to the vertebrate hormones (Roch and Sherwood 2010). An STC sequence similar to amphioxus STCc was reconstructed using two partial ESTs from the acorn worm (hemichordate) *Saccoglossus kowalevskii*, another deuterostome invertebrate. As well, several homologs were identified in unicellular eukaryotes, including a large group of fungi, the slime mold *Polysphondylium pallidum* and the amoeboid flagellate *Naegleria gruberi*. Combined, these organisms represent almost every major group of eukaryotes, except for plants and algae. Prospective stanniocalcin sequences were identified from the ESTs of a few plants including rice and tobacco (NCBI accession numbers CA754792 and AM786515, respectively), however, a lack of genomic support suggests these sequences may have been derived from contamination. A few predicted STC homologs were identified from bacteria as well (NCBI accession numbers NP_968019 and YP_782912) but given their novelty amongst the plethora of sequenced bacteria and discordant phylogenetic grouping (not shown), they may be artifacts of horizontal gene transfer and were not considered further in this analysis.

Every STC protein displays the conserved ten-cysteine pattern necessary for proper disulfide bridging, with the sole exception of most nematode sequences (fig. 1). The disulfide linkages have been empirically mapped for both salmon (Hulova and Kawauchi 1999) and human (Trindade et al. 2009) STC1. Stanniocalcin homologs were identified in five nematode species, and four of them (*Caenorhabditis sp.*) lack the first and third conserved cysteine residues, which form the first disulfide bond in other stanniocalcins. This is a feature unique to these animals, the only representatives of the ecdysozoan protostomes found to possess the hormone. Also, all of the sequences in figure 1 possess the signal peptide necessary for secretion.

As seen in figure 1, the tenth cysteine residue in the newly identified sequences is present in amphioxus STCc, the
FIG. 1.—Alignment of stanniocalcin homologs from a variety of eukaryotes. Putative full-length protein sequences from representative species were chosen and aligned by MUSCLE v3.7, with additional manual alignment at the N-terminus and C-terminus outside of the conserved cysteine core region (indicated by a line). Salient features were highlighted as follows: black—conserved cysteine residues, green—predicted signal peptides, blue—predicted N-glycosylation sites, and yellow—unique hydrophobic region. Cysteine residues in addition to the ten conserved cysteines are highlighted in orange, and the 11th cysteine residues in vertebrates, which are dimerization sites, are highlighted in red. The accession numbers of the displayed sequences are as follows: Human STC1 (NCBI NP_003146), Human STC2 (NCBI NP_003705), Zebra fish STC1 (NCBI NP_956833), Zebra fish STC2 (NP_001014827), Amphioxus STCa (NCBI XP_002603782), Amphioxus STCc (NCBI FE592411), Annelid STC (JGI 227571), Mollusc STC (JGI 165566), Nematode STC (NCBI NM_001083148), Cnidarian STC (NCBI XP_002157469), Sponge STC (NCBI GO093006), Fungus STC (NCBI XP_001891263), and Ciliate STC (NCBI XP_001015058).

| Human STC1 | Human STC2 | Zebrafish STC1 | Zebrafish STC2 | Amphioxus STCa | Amphioxus STCc | Annelid STC | Mollusc STC | Nematode STC | Cnidarian STC | Ciliate STC |
|------------|------------|----------------|---------------|----------------|---------------|-------------|-------------|--------------|-------------|-------------|
| 10         | 10         | 10             | 10            | 10             | 10            | 10          | 10          | 10           | 10          | 10          |
| 20         | 20         | 20             | 20            | 20             | 20            | 20          | 20          | 20           | 20          | 20          |
| 30         | 30         | 30             | 30            | 30             | 30            | 30          | 30          | 30           | 30          | 30          |
| 40         | 40         | 40             | 40            | 40             | 40            | 40          | 40          | 40           | 40          | 40          |
| 50         | 50         | 50             | 50            | 50             | 50            | 50          | 50          | 50           | 50          | 50          |
| 60         | 60         | 60             | 60            | 60             | 60            | 60          | 60          | 60           | 60          | 60          |
| 70         | 70         | 70             | 70            | 70             | 70            | 70          | 70          | 70           | 70          | 70          |
| 80         | 80         | 80             | 80            | 80             | 80            | 80          | 80          | 80           | 80          | 80          |

Fig. 1.—Alignment of stanniocalcin homologs from a variety of eukaryotes. Putative full-length protein sequences from representative species were chosen and aligned by MUSCLE v3.7, with additional manual alignment at the N-terminus and C-terminus outside of the conserved cysteine core region (indicated by a line). Salient features were highlighted as follows: black—conserved cysteine residues, green—predicted signal peptides, blue—predicted N-glycosylation sites, and yellow—unique hydrophobic region. Cysteine residues in addition to the ten conserved cysteines are highlighted in orange, and the 11th cysteine residues in vertebrates, which are dimerization sites, are highlighted in red. The accession numbers of the displayed sequences are as follows: Human STC1 (NCBI NP_003146), Human STC2 (NCBI NP_003705), Zebrafish STC1 (NCBI NP_956833), Zebrafish STC2 (NP_001014827), Amphioxus STCa (NCBI XP_002603782), Amphioxus STCc (NCBI FE592411), Annelid STC (JGI 227571), Mollusc STC (JGI 165566), Nematode STC (NCBI NM_001083148), Cnidarian STC (NCBI XP_002157469), Sponge STC (NCBI GO093006), Fungus STC (NCBI XP_001891263), and Ciliate STC (NCBI XP_001015058).
nematode STC, and sponge STC, as well as in the previously reported human STC1, STC2 (Chang and Reddel 1998), and amphioxus STCa (Roch and Sherwood 2010). Although the C-terminal region is poorly conserved compared with the core of the protein located between the conserved ten cysteines, the 11th cysteine is responsible for hormone dimerization in human STC1 (Trindade et al. 2009), a condition present in almost all vertebrate stanniocalcins characterized thus far (Wagner and Dimattia 2006). Dimerization may play a role in the function of some of these newly identified stanniocalcins, although this has not been tested. Additional cysteines within the conserved core of the protein also occur in several species including the sponge and slime mold (fig. 1). The nematode sequence has an additional three cysteine residues, another unique feature conserved within the entire group of nematode STC homologs.

Another major hallmark of previously characterized vertebrate stanniocalcins is the conserved N-glycosylation motif, shown in figure 1, which results in glycosylation of the hormone and has been detailed in actinopterygian fishes and mammals. Although the glycosylation site is not conserved, several of the newly identified stanniocalcins, although this has not been tested. Additional cysteines within the conserved core of the protein also occur in several species including the sponge and slime mold (fig. 1). The nematode sequence has an additional three cysteine residues, another unique feature conserved within the entire group of nematode STC homologs.

An extended selection of STC protein homologs was aligned, trimmed to the region including only the conserved ten cysteines, and degapped to produce a maximum likelihood tree, presented in figure 3. As shown, the vertebrate STC1 and STC2 paralogs group together, with the invertebrate chordate tunicate and amphioxus STCa and STCb sequences branching basally. The ciliate sequences branch further away from the chordate sequences, in an unusual position not congruent with the rest of the unicellular STCs, indicating the difficulty of phylogenetic reconstruction of these highly divergent sequences. The protostome STCs, acorn worm (hemichordate) STC and amphioxus STCc all cluster together, as do the sponge, cnidarian and remaining unicellular (fungi, slime mold and amoeboflagellate)

To contrast the conserved structural features with primary sequence similarity, an amino acid identity matrix was produced from the alignment and is displayed in figure 2. When the core regions (between and including the conserved first and tenth cysteines) are compared, the homology of any pair of sequences besides the vertebrate orthologs is low, between 13% and 37% amino acid identity. This comes as no surprise as the identity between vertebrate paralogs is comparatively low, with human STC1 and STC2 only 39% identical. Considering the low identity between stanniocalcin sequences, their conserved cysteines are critical for identifying homologs and suggest that function is based on tertiary rather than primary structure.

**Phylogenetics Reveals an Evolutionary Transition of Stanniocalcin in Amphioxus**

An extended selection of STC protein homologs was aligned, trimmed to the region including only the conserved ten cysteines, and degapped to produce a maximum likelihood tree, presented in figure 3. As shown, the vertebrate STC1 and STC2 paralogs group together, with the invertebrate chordate tunicate and amphioxus STCa and STCb sequences branching basally. The ciliate sequences branch further away from the chordate sequences, in an unusual position not congruent with the rest of the unicellular STCs, indicating the difficulty of phylogenetic reconstruction of these highly divergent sequences. The protostome STCs, acorn worm (hemichordate) STC and amphioxus STCc all cluster together, as do the sponge, cnidarian and remaining unicellular (fungi, slime mold and amoeboflagellate).
sequences, forming two major clusters. This supports the hypothesis that the evolution of stanniocalcin started early in eukaryotic history, with homologs present in single-celled organisms from diverse taxonomic groups. This is shown in figure 4, which also displays other components of the calcium-signaling toolkit found in the major groups of eukaryotes.

Amphioxus presents a possible transition in the evolution of stanniocalcin, as it possesses a homolog (STCc) more similar to the protostome STCs as well as homologs (STCa/b) that group basally to vertebrate STC1/STC2. As seen in the phylogenetic tree presented in figure 3, the node-containing branch with amphioxus STCc has a low aLRT support value (58). This raises the question of whether STCc should be considered truly homologous with the protostome stanniocalcins or whether its placement in the tree was due to long-branch attraction from sequence homoplasies. This latter possibility seems unlikely, however, considering the node representing the ancestral partition of the nematode STCs from other protostome invertebrate deuterostome STCs (including amphioxus STCc) has a high support value (91). Also, amphioxus STCc has the additional hydrophobic amino acid region that all identified protostome sequences contain, in contrast to amphioxus STCa/b, tunicate STC, and the vertebrate STCs (fig. 1). Finally, the putative STC found in the acorn worm, a member of the hemichordates that are a group of deuterostomes separate from the chordates, also shares this structural addition (not shown) and groups phylogenetically with the protostomes in figure 3. Amphioxus STCa/b and tunicate STC may represent the ancestral gene that was duplicated within the vertebrates to STC1 and STC2. Supporting evidence for the timing of the vertebrate STC1/STC2 duplication could be provided by the identification of a stanniocalcin homolog(s) from the jawless fishes. To date, STC orthologs have not been identified in the sea lamprey Petromyzon marinus.

**Are There Common Activation Signals for Stanniocalcin in All Eukaryotes?**

The presence of stanniocalcin in unicellular and multicellular organisms implies that there may be common activation signals that upregulate STC. The activation signal has been addressed in teleost fish and humans where an increase in
extracellular calcium was responsible for the upregulation of STC1 mRNA in a primary culture from trout corpuscles of Stannius (Wagner and Jaworski 1994), in zebra fish embryos (Tseng et al. 2009) and in various human immortalized cell lines (Chang et al. 1995). Additionally, flounder adapted to seawater rather than freshwater expressed three times as much STC1 mRNA (Hang and Balment 2005). The mechanism by which extracellular calcium increases STC transcription is not well characterized, although one study reported an association between activation of the extracellular calcium-sensing receptor (calcium-sensing receptor [CaSR]) and STC secretion (Radman et al. 2002). The C family of G protein-coupled receptors (GPCRs) contains CaSR along with GPRC6A, a newly identified receptor that also senses Ca\(^{2+}\), other cations and amino acids (Pi et al. 2005) and receptors for taste, pheromones, glutamate, and gamma-aminobutyric acid (GABA). However, neither the CaSR nor any of its distantly related receptors in the C family of GPCRs is likely to be responsible for the activation of any unicellular stanniocalcins (fungi, slime molds, ciliates, and amoeboflagellates), as none of these organisms have receptors of this type according to our analysis, with the exception of GABA-like receptors in slime mold (not shown). The lack of CaSRs in unicellular organisms makes it less likely that CaSRs activate STC in multicellular animals but does not rule it out. Indirect evidence shows that CaSR mimetics can activate STC secretion in salmon (Radman et al. 2002). If a common mechanism is used in all eukaryotes, the activation of STC transcription in response to elevated calcium levels is not yet clear.

**Are There Common Functions of Stanniocalcin in All Eukaryotes?**

A common function of STC in eukaryotes would depend in part on whether STC is secreted to the outside of the cell or acts inside. There is a strong probability for secretion of most STC sequences reported in the present study, as shown by the prediction of a signal peptide in the homologs (table 1 and fig. 1). The presence of a signal peptide is compatible with endocrine, paracrine, autocrine, and intracrine actions by STC, whereas the lack of a signal peptide suggests an intracrine action. Intracrine function may occur if the signal peptide is not encoded or alternative splicing removes it. Also, the secreted hormone can reenter a cell and initiate an intracrine function. STC qualifies as a possible intracrine in the sense that it has been shown in mammals to bind to mitochondria and increase the respiratory rate (Wagner and Dimattia 2006). Examples of paracrine and autocrine functions mediated by stanniocalcin are known in mammals and include the differentiation of ovarian granulosa cells (Luo et al. 2004) and bone formation (Yoshiko et al. 2002; Wu et al. 2006). Given the wide expression of STC1 and STC2 throughout many tissues in mammals (Chang et al. 1996; Chang and Reddel 1998; DiMattia et al. 1998; Ishibashi et al. 1998) and actinopterygian fish (McCudden et al. 2001; Amemiya and Youson 2004), it is possible that stanniocalcins act in a paracrine, autocrine or intracrine manner in all organisms in which they are present.

The mechanism by which STC inhibits calcium from entering cells remains unanswered, especially for understanding a common function in eukaryotes. In teleost fish, this canonical function begins when stanniocalcin is released from secretory cells in the corpuscles of Stannius and travels to the gills where it reduces uptake of calcium by an unknown mechanism (Wagner and Dimattia 2006). Exogenous STC1 was found to decrease gill-based calcium uptake when injected into trout (Wagner et al. 1986) and goldfish (Olsen et al. 1996), and a knockdown of stc1 in zebra fish embryos resulted in increased Ca\(^{2+}\) uptake and whole-body content (Tseng et al. 2009). However, a stanniocalcin receptor has never been identified. Based on the conservation of cysteine patterns and assuming a conserved folding structure, it is likely that the STC homologs in all identified eukaryote species share conserved binding sites, but the question as to what this structure is and how it affects calcium influx remains.

Calcium is a critical second-messenger throughout eukary-ya, as it is involved in fundamental processes such as cell division, motility, transcription, exocytosis, and apoptosis (Albrand et al. 1995). Even unicellular eukaryotes contain a basic calcium-signaling system, composed of plasma membrane influx channels, endoplasmic reticulum influx and efflux channels, cation exchangers, and Ca\(^{2+}\) ATPases (Berridge et al. 2003; Cai 2008; Bencina et al. 2009). Of particular relevance to stanniocalcin are the voltage-dependent calcium channels (VDCCs) identified in many species across the range of eukaryotes (fig. 4). A study of cultured rat cardiomyocytes determined that exogenous STC1 blocked the influx of calcium across L-type VDCCs (Sheikh-Hamad et al. 2003). A potential common target of vertebrate and unicellular stanniocalcins may be this voltage-gated channel. If the direct target of stanniocalcin is the VDCC, there are two suggestions for a potential mechanism of interaction. The first is to directly block the pore of voltage-gated ion channels and inhibit the influx of calcium. This method is used by a diverse group of peptide toxins, including the conotoxins secreted by cone snails and other toxins by snakes and spiders (Albrand et al. 1995; Mouhat et al. 2004; Kubista et al. 2007). The second potential mechanism is for STC to bind inside the cell to the tail of the VDCC. This method is used by the intracrine members of the fibroblast growth family (FGF 11–14); these specific FGFs also lack a receptor but bind directly to the cytoplasmic tail of the sodium voltage-gated channel to alter its functional characteristics (Goldfarb et al. 2007; Goetz et al. 2009; Itoh and Ornitz 2010). Both the mechanisms of blocking the VDCC pore outside the cell...
and binding to its tail inside the cell require experimental proof for STC but provide a testable suggestion for the mechanism of action.

The Stanniocalcin Family Is Missing in Some Species

Given the diversity of eukaryotes from which stanniocalcin homologs were identified, it is interesting that some major lineages appear to lack a gene encoding the hormone. Homologs were not identified in plants or related green algae. This distinct group of eukaryotes appears to have evolved novel approaches to calcium signaling, as plants lack VDCCs altogether and representative algal species lack other components of the conserved eukaryotic calcium-signaling toolkit (Wheeler and Brownlee 2008; Verret et al. 2010). Choanoflagellates, arthropods, echinoderms, yeast (model fungi), and Dictostylium sp. (model slime molds) also lack STC. These groups have well-characterized genomes, so stanniocalcins would have been detected by homology searches unless their structures are very divergent. For Saccharomyces yeast and Dictostylium, however, this seems unlikely as related species of Aspergillus fungi and dictyostelid slime molds possessed homologs.

It appears, then, that stanniocalcin is not essential to every lineage and has been lost at different periods of time by distinct groups of organisms. A different regulator may have replaced stanniocalcin within the lineages missing the hormone (fig. 4). A double knockout of STC1 and STC2 in mice resulted in a phenotype in which the only significant change was an increase in weight (Chang et al. 2008).

In summary, we have identified novel stanniocalcin homologs from a variety of organisms spanning unicellular eukaryotes through invertebrate animals. Although these STC putative homologs share low primary sequence identity, they all retain the conserved ten cysteines necessary for proper disulfide bridging in vertebrates. A receptor for stanniocalcin has not been structurally identified, which suggests a different mechanism from the usual hormone-receptor system. A potential target, the VDCC, does have homologs throughout most eukaryote lineages, with the exception of plants. Future work will determine the molecular relationship between VDCCs and STC, specifically as to whether the hormone can directly block or affect the channel. STC now appears to be one of the largest and most diverse peptide hormones in both multicellular and unicellular organisms, suggesting it evolved very early in the eukaryotic lineage.

Supplementary Material

Supplementary material is available at Genome Biology and Evolution online (http://www.oxfordjournals.org/our_ journals/gbe/).

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