Stanniocalcin Has Deep Evolutionary Roots in Eukaryotes

Graeme J. Roch, and Nancy M. Sherwood*

Department of Biology, University of Victoria, Victoria, British Columbia, Canada V8W 3N5

*Corresponding author: E-mail: nsherwoo@uvic.ca.

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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, 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 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 ubiquitious polymeric complexes (poly-3-hydroxybutyrate/polyphosphate) 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,

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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 cysteines 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 hmmsearch 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 PROTTEST 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.

Table 1
Stanniocalcin Homologs Identified and Used for Analyses in this Study

Group	Phylum	Common name	Species name	Sequence	Source	Type	Accession #	cDNA	Signal peptide
Vertebrates	Chordata	Arawana	*Osteoglossum bicirrhosum*	STC1	NCBI	gene model	BAB43868	X	X
		Bowfin	*Amia calva*	STC1	NCBI	gene model	BAC66163	X	X
		Butterflyfish	*Pantodon buchholzi*	STC1	NCBI	gene model	BAD99601	X	X
		Chicken	*Gallus gallus*	STC1	NCBI	gene model	XP_425760	-	X
				STC2	NCBI	gene model	XP_414534	-	X
		Eel	*Anguilla australis*	STC2	NCBI	gene model	AAB91483	X	X
		Elephantfish	*Gnathonemus petersii*	STC1	NCBI	gene model	BAD99600	X	X
		Flounder	*Paralichthys olivaceus*	STC1	NCBI	gene model	AB164157	X	X
				STC2	NCBI	gene model	ACJ06521	X	X
		Frog	*Xenopus laevis*	STC1	NCBI	gene model	NP_001086522	X	X
				STC2	NCBI	gene model	NP_001016004	X	X
		Gar	*Lepisosteus osseus*	STC1	NCBI	gene model	BAC66164	X	X
		Human	*Homo sapiens*	STC1	NCBI	gene model	NP_003146	X	X
				STC2	NCBI	gene model	NP_003705	X	X
		Mouse	*Mus musculus*	STC1	NCBI	gene model	NP_033311	X	X
				STC2	NCBI	gene model	NP_035621	X	X
		Opossum	*Monodelphis domestica*	STC1	NCBI	gene model	XP_001373050	-	X
				STC2	NCBI	gene model	XP_001370619	-	X
		Platypus	*Ornithorhynchus anatinus*	STC1	NCBI	gene model	XP_001506746	-	X
		Pufferfish	*Fugu rubripes*	STC1	NCBI	gene model	NP_001072056	X	X
				STC2	NCBI	gene model	NP_001072057	X	X
		Trout	*Oncorhynchus mykiss*	STC1	NCBI	gene model	NP_001117762	X	X
		Zebrafish	*Danio rerio*	STC1	NCBI	gene model	NP_956833	X	X
				STC2	NCBI	gene model	NP_001014827	X	X
Urochodates	Chordata	Tunicate	*Ciona intestinalis*	STC	NCBI	gene model	XP_002130163	X	X
Cephalochordates	Chordata	Amphioxus	*Branchiostoma floridae*	STCa	NCBI	gene model	XP_002603782	X	X
				STCb	NCBI	gene model	XP_002607913	X	X
				STCc	NCBI	EST	FE592411	X	X
Hemichordates	Hemichordata	Acorn worm	*Saccoglossus kowalevskii*	STC	NCBI	EST	FF655958+FF660405	X	-
Protostomes	Annelida	Earthworm	*Lumbricus rubellus*	STC	NCBI	EST	DR696791	X	X
		Leech	*Helobdella robusta*	STC	JGI	gene model	167713	-	X
		Marine worm	*Capitella teleta*	STCa	JGI	gene model	227571	X	X
				STCb	JGI	gene model	220807	X	X
		Tubeworm	*Pomatoceros lamarckii*	STC	NCBI	EST	GR310789	X	I
	Mollusca	Abalone A	*Haliotis asinina*	STC	NCBI	EST	GD272434	X	X
		Abalone B	*Haliotis diversicolor*	STC	NCBI	gene model	ABY87355	X	X
		Freshwater snail	*Biomphalaria glabrata*	STC	NCBI	EST	FC855621	X	X
		Land snail	*Nesiohelix samarangae*	STC	NCBI	EST	DC604854	X	X
		Limpet	*Lottia gigantea*	STCa	JGI	gene model	165566	X	X
				STCb	JGI	gene model	170051	-	X
		Pond snail	*Lymnaea stagnalis*	STC	NCBI	EST	CN810154	X	X
		Sea hare	*Aplysia californica*	STC	NCBI	EST	GD231158	X	X
	Nematoda	Nematode A	*Caenorhabditis brenneri*	STC	NCBI	EST	FF102834	X	I
		Nematode B	*Caenorhabditis briggsae*	STC	NCBI	gene model	XP_002639944	-	I
		Nematode C	*Caenorhabditis elegans*	STC	NCBI	gene model	NM_001083148	X	X
		Nematode D	*Caenorhabditis japonica*	STC	NCBI	EST	FF120232	X	I
		Nematode E	*Meloidogyne hapla*	STC	NCBI	EST	BM902155	X	X
Cnidarians	Cnidaria	Hydra	*Hydra magnipapillata*	STCa	NCBI	gene model	XP_002157469	X	X
				STCb	NCBI	gene model	XP_002164639	X	X
		Sea anenome	*Metridium senile*	STC	NCBI	EST	FC847465	X	I
Sponges	Porifera	Sponge A	*Leucetta chagosensis*	STC	NCBI	EST	GO093006	X	X
		Sponge B	*Oscarella carmela*	STC	NCBI	EST	EC374232	X	I

Table 1
Continued

Group	Phylum	Common name	Species name	Sequence	Source	Type	Accession #	cDNA	Signal peptide
Fungi	Ascomycota	Mold A	*Aspergillus clavatus*	STC	NCBI	gene model	XP_001270777	-	X
		Mold B	*Aspergillus fischerianus*	STC	NCBI	gene model	XP_001263713	-	X
		Fungal pathogen A	*Coccidioides immitis*	STC	NCBI	gene model	XP_001244930	X	X
		Fungal pathogen B	*Coccidioides posadasii*	STC	NCBI	gene model	EER29681	X	X
		Fungal pathogen C	*Microsporum canis*	STCa	NCBI	gene model	EEQ31957	-	X
				STCb	NCBI	gene model	EEQ33677	-	X
		Fungal pathogen D	*Penicillium marneffei*	STC	NCBI	gene model	XP_002146208	-	X
		Fungal pathogen E	*Talaromyces stipitatus*	STC	NCBI	gene model	XP_002480983	-	X
		Fungal pathogen F	*Trichophyton rubrum*	STC	NCBI	EST	EL789447	X	X
	Basidiomycota	Deceiver mushroom	*Laccaria bicolor*	STCa	NCBI	gene model	XP_001891263	X	X
				STCb	NCBI	gene model	XP_001881732	-	X
Protists	Mycetozoa	Slime mold	*Polysphondylium pallidum*	STCa	NCBI	gene model	EFA77206	-	-
				STCb	NCBI	gene model	EFA77207	-	-
	Ciliophora	Ciliate	*Tetrahymena thermophila*	STCa	NCBI	gene model	XP_001017802	-	X
				STCb	NCBI	gene model	XP_001015058	X	X
	Percolozoa	Amoeboflagellate	*Naegleria gruberi*	STC	NCBI	gene model	XP_002680178	-	X

Note.—Previously identified sequences from the vertebrates, tunicates, and cephalochordates are listed with stanniocalcin orthologs identified in this study. Accession numbers are listed and species are organized and color-coded according to phylogenetic group. Only proteins with known gene models and/or ESTs are included in the figure. The presence of corresponding ESTs is indicated by an "X" in the cDNA column and the confirmation of a predicted signal peptide from SignalP 3.0 is indicated by an X in the signal peptide column. An "I" indicates an incomplete gene model or EST from which a signal peptide cannot be predicted. Accession numbers for gene models refer to protein sequences.

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. floridae* (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 amoeboflagellate *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

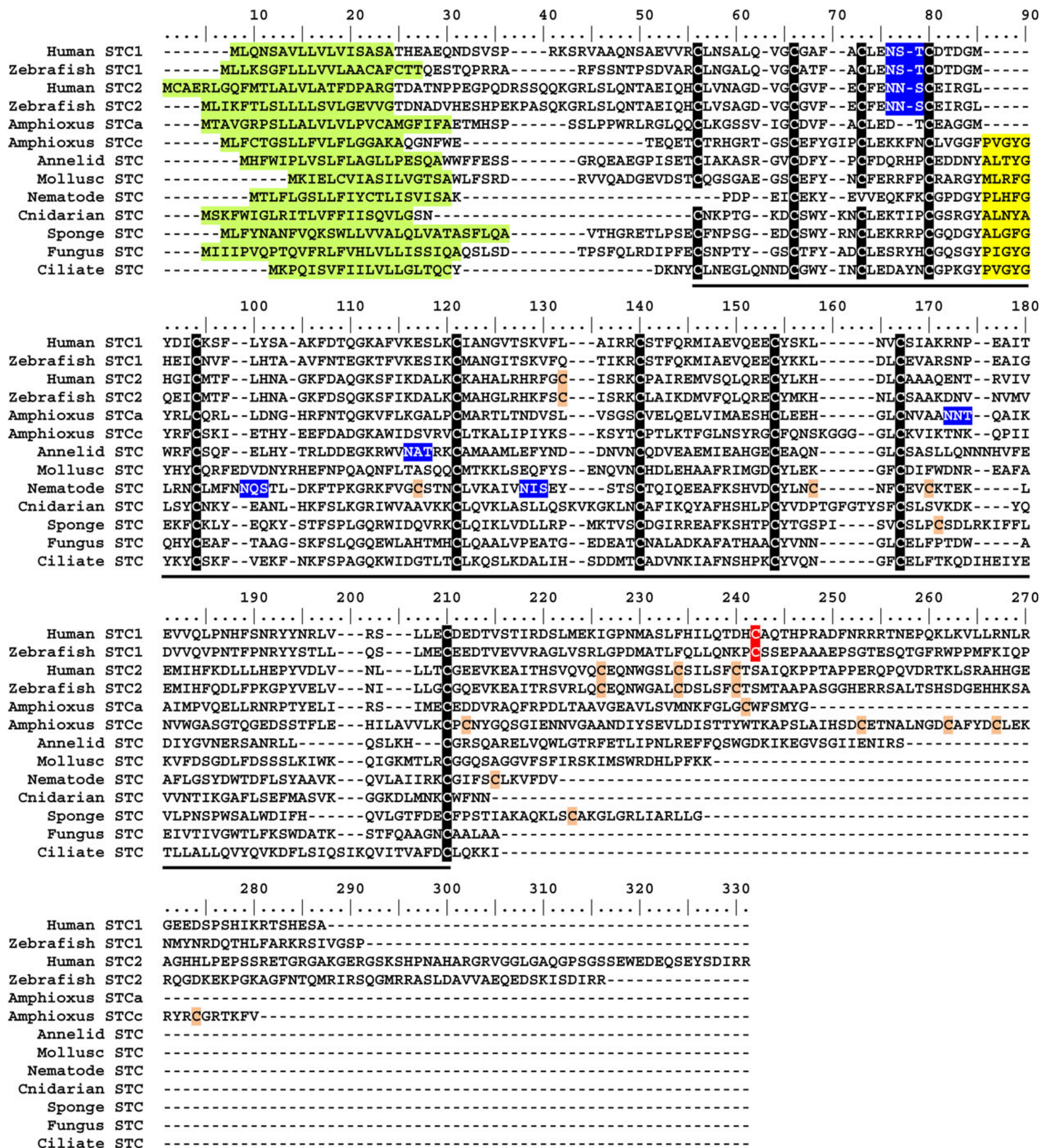


Fig. 1.—Alignment of stanniocalcin homologs from a variety of eukaryotes. Putative full-length protein sequences from representative species were chosen and aligned by MUSCLE v3.7, with additional manual alignment at the N-terminus and C-terminus outside of the conserved cysteine core region (indicated 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	Zebrafish STC1	Human STC2	Zebrafish STC2	Amphioxus STCa	Amphioxus STCc	Annelid STC	Mollusc STC	Nematode STC	Cnidarian STC	Sponge STC	Fungus STC
Zebrafish STC1	71											
Human STC2	39	35										
Zebrafish STC2	44	37	78									
Amphioxus STCa	35	37	33	33								
Amphioxus STCc	18	17	20	19	21							
Annelid STC	18	20	17	17	20	23						
Mollusc STC	19	18	19	23	23	24	23					
Nematode STC	17	18	18	18	16	24	17	25				
Cnidarian STC	16	15	17	18	13	23	18	24	25			
Sponge STC	18	16	16	16	13	24	24	23	27	36		
Fungus STC	16	15	18	18	17	25	21	26	27	30	28	
Ciliate STC	19	17	15	16	15	25	20	23	23	28	32	33

Fig. 2.—Amino acid identity matrix of stanniocalcin homologs. Putative full-length protein sequences from representative species were aligned by MUSCLE v3.7 and an amino acid identity matrix was then produced in BioEdit 7.0.5. Values correspond to the percentage of identical sites between two sequences using only the core region between conserved cysteines 1 and 10. The accession numbers of the displayed sequences are listed in the legend of figure 1.

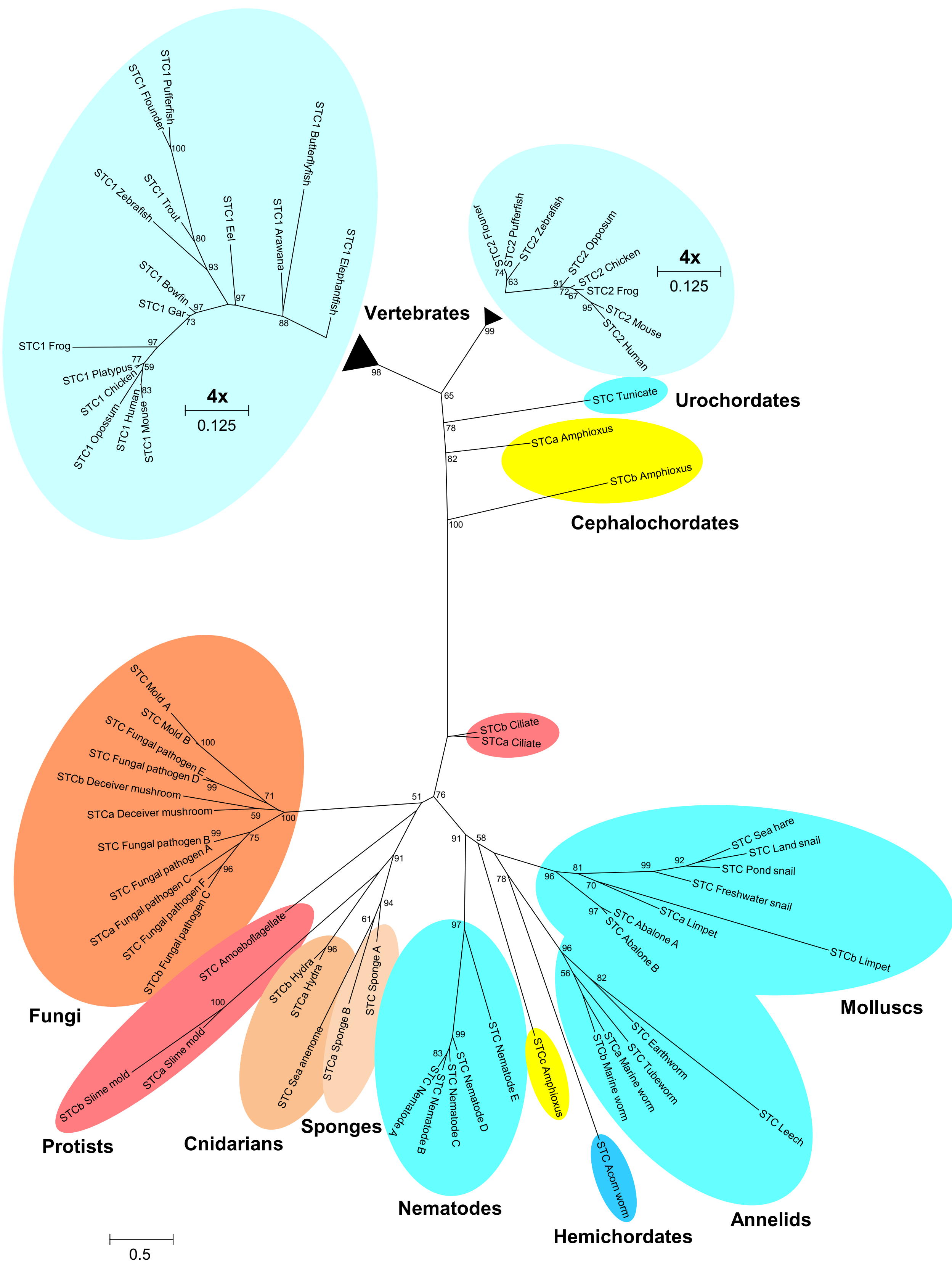
nematode STC, and sponge STC, as well as in the previously reported human STC1, STC2 (Chang and Reddel 1998), and amphioxus STCa (Roch and Sherwood 2010). Although the C-terminal region is poorly conserved compared with the core of the protein located between the conserved ten cysteines, the 11th cysteine 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 sequences contain potential *N*-glycosylation sites, including those of annelid and nematode. If glycosylation occurs for some of these proteins, the signal has significantly more variance than for previously described vertebrate stanniocalcins. A striking and unique shared feature amongst the newly identified STC homologs, including amphioxus STCc, is a hydrophobic region of six amino acids between the fourth and fifth conserved cysteines, of unknown importance (fig. 1).

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)


Vertebrates
Urochordates
Cephalochordates
Fungi
Protists
Cnidarians
Sponges
Nematodes
Hemichordates
Annelids
Molluscs
STC1 Pufferfish
STC1 Flounder
STC1 Butterflyfish
STC1 Zebrafish
STC1 Trout
STC1 Eel
STC1 Arawana
STC1 Elephantfish
STC1 Bowfin
STC1 Gar
STC1 Frog
STC1 Platypus
STC1 Chicken
STC1 Opossum
STC1 Human
STC1 Mouse
4x
0.125
STC2 Flounder
STC2 Pufferfish
STC2 Zebrafish
STC2 Opossum
STC2 Chicken
STC2 Frog
STC2 Mouse
STC2 Human
STC Tunicate
STCa Amphioxus
STCb Amphioxus
STCb Ciliate
STCa Ciliate
STC Mold A
STC Mold B
STC Fungal pathogen E
STC Fungal pathogen D
STCb Deceiver mushroom
STCa Deceiver mushroom
STC Fungal pathogen B
STC Fungal pathogen A
STC Fungal pathogen C
STCa Fungal pathogen C
STC Fungal pathogen F
STCb Fungal pathogen C
STC Amoeboflagellate
STCb Slime mold
STCa Slime mold
STCb Hydra
STCa Hydra
STC Sea anemone
STCa Sponge B
STC Sponge A
STC Nematode A
STC Nematode B
STC Nematode C
STC Nematode D
STC Nematode E
STCc Amphioxus
STC Acorn worm
STC Sea hare
STC Land snail
STC Pond snail
STC Freshwater snail
STCa Limpet
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0.5

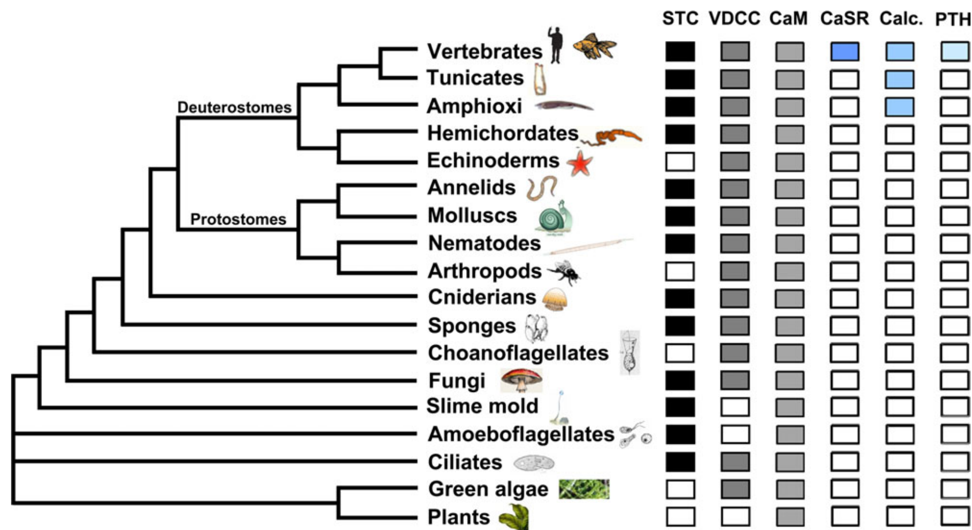

Fig. 4.—Diagram of the evolution of several proteins related to calcium control in eukaryotes. Stanniocalcin (STC) has a remarkable conservation across eukaryotes whereas calcitonin (Calc) and parathyroid hormone (PTH) are restricted, to date, to the chordates or vertebrates, respectively. Only proteins with known gene models and/or ESTs are included in the figure. Organisms with VDCCs are included only if they have four-domain calcium channels, which includes a variety of unicellular organisms. Organisms such as amoeboflagellates and ciliates do not have four-domain VDCCs but do have one- or two-domain calcium channels. Two important calcium sensors, calmodulin (CaM) and the calcium-sensing receptor (CaSR) are included in the figure. CaM is ubiquitous in eukaryotes but CaSR has been verified only in vertebrates, although distantly related receptors that are also in family C of the GPCRs are present throughout metazoans.

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 deuterstome 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

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Fig. 3.—Phylogenetic tree of stanniocalcin homologs from multicellular and unicellular eukaryotes. Stanniocalcin homologs listed in table 1 were aligned by MUSCLE v3.7, trimmed to the region only including the ten conserved cysteine residues, and degapped (for the alignment file, see Supplementary material, Supplementary material online). Maximum likelihood trees were then constructed using PhyML 3.0 with modifications outlined in Methods. The unrooted tree is shown with the vertebrate STC1 and STC2 clades compressed and expanded 4× adjacent to the main tree in order to provide viewable branch lengths. The scale bars represent the substitutions per site, and the numbers at each node are approximate likelihood ratio test for branches (aLRT, SH-like) support values. Support values under 50 were removed.

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 mitochrondria 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 eukarya, 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 *Dictyostelium* 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 Saccharomycetes yeast and *Dictyostelium*, 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). Clearly, compensatory mechanisms exist suggesting the importance of calcium and phosphate regulation.

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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Literature Cited

Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21:2104–2105.

Albrand JP, Blackledge MJ, Pascaud F, Hollecker M, Marion D. 1995. NMR and restrained molecular dynamics study of the three-dimensional solution structure of toxin FS2, a specific blocker of the L-type calcium channel, isolated from black mamba venom. Biochemistry 34:5923–5937.

Altschul SF, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Amemiya Y, Marra LE, Reyhani N, Youson JH. 2002. Stanniocalcin from an ancient teleost: a monomeric form of the hormone and a possible extracorpuscular distribution. Mol Cell Endocrinol. 188:141–150.

Amemiya Y, Youson JH. 2004. Primary structure of stanniocalcin in two basal Actinopterygii. Gen Comp Endocrinol. 135:250–257.

Bencina M, Bagar T, Lah L, Krasevec N. 2009. A comparative genomic analysis of calcium and proton signaling/homeostasis in Aspergillus species. Fungal Genet Biol. 46(Suppl 1):S93–S104.

Berridge MJ, Bootman MD, Roderick HL. 2003. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 4:517–529.

Butkus A, et al. 1987. Purification and cloning of a corpuscles of Stannius protein from Anguilla australis. Mol Cell Endocrinol. 54:123–133.

Cai X. 2008. Unicellular Ca^{2+} signaling 'toolkit' at the origin of metazoa. Mol Biol Evol. 25:1357–1361.

Case RM, et al. 2007. Evolution of calcium homeostasis: from birth of the first cell to an omnipresent signalling system. Cell Calcium. 42:345–350.

Chang AC, Dunham MA, Jeffrey KJ, Reddel RR. 1996. Molecular cloning and characterization of mouse stanniocalcin cDNA. Mol Cell Endocrinol. 124:185–187.

Chang AC, et al. 2008. The murine stanniocalcin 2 gene is a negative regulator of postnatal growth. Endocrinology 149:2403–2410.

Chang AC, et al. 1995. A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol Cell Endocrinol. 112:241–247.

Chang AC, Jellinek DA, Reddel RR. 2003. Mammalian stanniocalcins and cancer. Endocr Relat Cancer. 10:359–373.

Chang AC, Reddel RR. 1998. Identification of a second stanniocalcin cDNA in mouse and human: stanniocalcin 2. Mol Cell Endocrinol. 141:95–99.

DiMattia GE, Varghese R, Wagner GF. 1998. Molecular cloning and characterization of stanniocalcin-related protein. Mol Cell Endocrinol. 146:137–140.

Durell SR, Guy HR. 2001. A putative prokaryote voltage-gated Ca(2+) channel with only one 6TM motif per subunit. Biochem Biophys Res Commun. 281:741–746.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP, and related tools. Nat Protoc. 2:953–971.

Gagliardi AD, Kuo EY, Raulic S, Wagner GF, DiMattia GE. 2005. Human stanniocalcin-2 exhibits potent growth-suppressive properties in transgenic mice independently of growth hormone and IGFs. Am J Physiol Endocrinol Metab. 288:E92–E105.

Goetz R, Dover K, Laezza F, et al. 2009. Crystal structure of a fibroblast growth factor homologous factor (FHF) defines a conserved surface

on FHFs for binding and modulation of voltage-gated sodium channels. J Biol Chem. 284:17883–17896.

Goldfarb M, Schoorlemmer J, Williams A, et al. 2007. Fibroblast growth factor homologous factors control neuronal excitability through modulation of voltage-gated sodium channels. Neuron 55:449–463.

Guindon S, et al. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 59:307–321.

Haddad M, Roder S, Olsen HS, Wagner GF. 1996. Immunocytochemical localization of stanniocalcin cells in the rat kidney. Endocrinology. 137:2113–2117.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 41:95–98.

Hang X, Balment RJ. 2005. Stanniocalcin in the euryhaline flounder (Platichthys flesus): primary structure, tissue distribution, and response to altered salinity. Gen Comp Endocrinol. 144:188–195.

Hulova I, Kawauchi H. 1999. Assignment of disulfide linkages in chum salmon stanniocalcin. Biochem Biophys Res Commun. 257:295–299.

Ishibashi K, et al. 1998. Molecular cloning of a second human stanniocalcin homologue (STC2). Biochem Biophys Res Commun. 250:252–258.

Itoh N, Ornitz DM. 2011. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J Biochem. 149:121–130.

Kubista H, et al. 2007. CSTX-1, a toxin from the venom of the hunting spider Cupiennius salei, is a selective blocker of L-type calcium channels in mammalian neurons. Neuropharmacology. 52:1650–1662.

Lafeber FP, et al. 1988. Identification of hypocalcin (teleocalcin) isolated from trout Stannius corpuscles. Gen Comp Endocrinol. 69:19–30.

Luo CW, Kawamura K, Klein C, Hsueh AJ. 2004. Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors. Mol Endocrinol. 18: 2085–2096.

Luo CW, Pisarska MD, Hsueh AJ. 2005. Identification of a stanniocalcin paralog, stanniocalcin-2, in fish and the paracrine actions of stanniocalcin-2 in the mammalian ovary. Endocrinology. 146:469–476.

Marra LE, Groff KE, Youson JH. 1995. The corpuscles of Stannius in arawana (Osteoglossum bicirrhosum), an ancient teleost. Tissue Cell. 27:425–437.

McCudden CR, Kogon MR, DiMattia GE, Wagner GF. 2001. Novel expression of the stanniocalcin gene in fish. J Endocrinol. 171:33–44.

Michiels J, Xi C, Verhaert J, Vanderleyden J. 2002. The functions of Ca(2+) in bacteria: a role for EF-hand proteins? Trends Microbiol. 10:87–93.

Mouhat S, Jouirou B, Mosbah A, De Waard M, Sabatier JM. 2004. Diversity of folds in animal toxins acting on ion channels. Biochem J. 378:717–726.

Olsen HS, Cepeda MA, Zhang QQ, Rosen CA, Vozzolo BL. 1996. Human stanniocalcin: a possible hormonal regulator of mineral metabolism. Proc Natl Acad Sci U S A. 93:1792–1796.

Pi M, Faber P, Ekema G, et al. 2005. Identification of a novel extracellular cation-sensing G-protein-coupled receptor. J Biol Chem. 280: 40201–40209.

Radman DP, McCudden C, James K, Nemeth EM, Wagner GF. 2002. Evidence for calcium-sensing receptor mediated stanniocalcin secretion in fish. Mol Cell Endocrinol. 186:111–119.

Reusch RN, Huang R, Bramble LL. 1995. Poly-3-hydroxybutyrate/polyphosphate complexes form voltage-activated Ca^{2+} channels in the plasma membranes of Escherichia coli. Biophys J. 69:754–766.

Reynolds SM, Käll L, Riffle ME, Bilmes JA, Noble WS. 2008. Transmembrane topology and signal peptide prediction using dynamic Bayesian networks. PLoS Comput Biol. 4:e1000213.

Roch GJ, Sherwood NM. 2010. Genomics reveal ancient forms of stanniocalcin in amphioxus and tunicate. IntegrCompBiol.50:86–97.

Sheikh-Hamad D, Bick R, Wu GY, et al. 2003. Stanniocalcin-1 is a naturally occurring L-channel inhibitor in cardiomyocytes: relevance to human heart failure. Am J Physiol Heart Circ Physiol. 285:H442–H448.

Shen HB, Chou KC. 2007. Signal-3L: a 3-layer approach for predicting signal peptides. Biochem Biophys Res Commun. 363:297–303.

Shin J, Sohn YC. 2009. cDNA cloning of Japanese flounder stanniocalcin 2 and its mRNA expression in a variety of tissues. Comp Biochem Physiol A Mol Integr Physiol. 153:24–29.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24:1596–1599.

Tanega C, Radman DP, Flowers B, Sterba T, Wagner GF. 2004. Evidence for stanniocalcin and a related receptor in annelids. Peptides. 25:1671–1679.

Trindade DM, Silva JC, Navarro MS, Torriani IC, Kobarg J. 2009. Low-resolution structural studies of human Stanniocalcin-1. BMC Struct Biol. 9:57.

Tseng DY, et al. 2009. Effects of stanniocalcin 1 on calcium uptake in zebrafish (Danio rerio) embryo. Am J Physiol Regul Integr Comp Physiol. 296:R549–R557.

Verret F, Wheeler G, Taylor A, Farnham G, Brownlee C. 2010. Calcium channels in photosynthetic eukaryotes: implications for evolution of calcium-based signalling. New Phytol. 187:23–43.

Wagner GF, Dimattia GE. 2006. The stanniocalcin family of proteins. J Exp Zool A Comp Exp Biol. 305:769–780.

Wagner GF, et al. 1988. Comparative biochemistry and physiology of teleocalcin from sockeye and coho salmon. Gen Comp Endocrinol. 72:237–246.

Wagner GF, Guiraudon CC, Milliken C, Copp DH. 1995. Immunological and biological evidence for a stanniocalcin-like hormone in human kidney. Proc Natl Acad Sci U S A. 92:1871–1875.

Wagner GF, Hampong M, Park CM, Copp DH. 1986. Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen Comp Endocrinol. 63:481–491.

Wagner GF, Jaworski E. 1994. Calcium regulates stanniocalcin mRNA levels in primary cultured rainbow trout corpuscles of stannius. Mol Cell Endocrinol. 99:315–322.

Wendelaar Bonga SE, Lafeber FP, Flik G, Kaneko T, Pang PK. 1989. Immunocytochemical demonstration of a novel system of neuroendocrine peptidergic neurons in the pond snail Lymnaea stagnalis, with antisera to the teleostean hormone hypocalcin and mammalian parathyroid hormone. Gen Comp Endocrinol. 75:29–38.

Wheeler GL, Brownlee C. 2008. Ca^{2+} signalling in plants and green algae—changing channels. Trends Plant Sci. 13:506–514.

Williams RJ. 2006. The evolution of calcium biochemistry. Biochem Biophys Acta. 1763:1139–1146.

Wu S, Yoshiko Y, De Luca F. 2006. Stanniocalcin 1 acts as a paracrine regulator of growth plate chondrogenesis. J Biol Chem. 281:5120–5127.

Yahata K, et al. 2003. Regulation of stanniocalcin 1 and 2 expression in the kidney by *klotho* gene. Biochem Biophys Res Commun. 310:128–134.

Yoshiko Y, Aubin JE. 2004. Stanniocalcin 1 as a pleiotropic factor in mammals. Peptides. 25:1663–1669.

Yoshiko Y, Aubin JE, Maeda N. 2002. Stanniocalcin 1 (STC1) protein and mRNA are developmentally regulated during embryonic mouse osteogenesis: the potential of stc1 as an autocrine/paracrine factor for osteoblast development and bone formation. J Histochem Cytochem. 50:483–492.