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Characterization of spatial and temporal expression pattern of SCG10 during zebrafish development

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Abstract

SCG10 (Superior Cervical Ganglia 10, STMN2) is a member of the stathmin family of proteins. Stathmins regulate microtubule dynamics by inhibiting polymerization and promoting their depolymerization. SCG10 is believed to be a neuronal-specific stathmin that is enriched in the growth cones of developing neurons and plays a role in regulating neurite outgrowth. In all species examined so far, SCG10 is expressed in both the CNS and PNS.

We have cloned two zebrafish SCG10 homologues and have determined the temporal and spatial expression pattern of both of these genes by RT-PCR and *in situ* hybridization. RT-PCR shows that both transcripts are expressed maternally and zygotically through at least 5 days. *In situ* hybridization analysis reveals that both SCG10 orthologues have dynamic, spatial expression patterns that are nearly identical to each other. Initially, these orthologues are expressed in discrete areas of the forebrain, midbrain, and hindbrain, as well as in the anterior and posterior lateral line ganglia and transiently in the spinal cord Rohon-Beard neurons. From 48hpf onwards, the level of expression of both genes increases and becomes mainly restricted to the anterior CNS (the forebrain region, retina, optic tectum and hindbrain), and to the cranial ganglia. From 72 to 96 hpf, SCG10 genes are also expressed in the developing neurons in the gut and in the surrounding intestinal mesenchyme. Our results provide a starting point for future studies that will investigate the *in vivo* function of SCG10 orthologues in zebrafish neural development.

Keywords

SCG10; STMN2; zgc:92905; zgc:110132; stathmins; zebrafish; danio rerio; neuronal development; central nervous system; anterior lateral line ganglia; posterior lateral line ganglia; cranial ganglia; Rohon-Beard neurons; peripheral nervous system; enteric neurons; sympathetic chains

1. Results and discussion

The stathmin phosphoprotein family consists of four members: Stathmin (STMN1), Superior Cervical Ganglia, neural specific 10 (SCG10 or STMN2), SCG10-like-protein (SCLIP or STMN3) and stathmin-like-protein RB3 (STMN4) (Curmi et al., 1999). All stathmins have the ability to bind tubulin and depolymerize microtubules (MTs) (Charbaut et al., 2001). MT dynamics and function is critically important for both the developing and mature nervous

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system. In differentiated neurons microtubules function both as structural components of the cytoskeleton and play a critical role in intracellular transport (Dent and Gertler, 2003). In the developing nervous system regulated MT dynamics is required for neurite guidance, outgrowth and branching [for review see (Gordon-Weeks, 2004)]. Dynamic instability occurs at the ends of MTs as they are constantly extended or shortened by polymerization and depolymerization of the α - and β -tubulin heterodimeric subunits (Mitchison and Kirschner, 1984). All stathmins can sequester tubulin into a ternary 'T2SLD' complex, consisting of two tubulin and one stathmin protein and thus affect the rate of polymerization (Curmi et al., 1997; Jourdain et al., 1997). Stathmins can also destabilize the MTs by increasing the 'catastrophe frequency' (reaction causing the depolymerization of MTs) at MTs slow growing (minus) end (Howell et al., 1999; Manna et al., 2006; Morii et al., 2006; Nakao et al., 2004). In addition, SCG10 has been shown to increase the growth rate of the 'plus' end of the MTs *in vitro* (Manna et al., 2007).

SCG10 has three functional regions, N-terminal membrane anchoring domain, a central regulatory domain and a C-terminal coiled-coil domain (Grenningloh et al., 2004; Mori and Morii, 2002). The N-terminal domain anchors SCG10 to Golgi apparatus derived vesicles through palmitoylation. Subsequently, vesicles are transported to the neuronal growth cones (Di Paolo et al., 1997). This is believed to cause specific enrichment of SCG10 in the growth cones of neurites (Himi et al., 1994). The regulatory domain contains phosphorylation sites for MAP and PKA kinases that regulate the tubulin depolymerization activity of SCG10 (Antonsson et al., 1998; Tararuk et al., 2006). The C-terminal domain is thought to be a protein-protein interaction domain that permits SCG10 to bind tubulin and MT (Charbaut et al., 2001).

All members of the stathmin family are strongly expressed in neuronal tissue. In addition, STMN1 and SCLIP are more ubiquitously expressed with a higher abundance found in embryonic tissues and in tissues that continue to grow in adults. Low level of expression of SCG10 and RB3 proteins has also been reported in non-neural tissues, but this expression is minimal when compared to their abundant neuronal expression (Bieche et al., 2003; Koppel et al., 1990; Schubart et al., 1987; Sobel et al., 1989). SCG10 was originally identified as a marker for neural crest cells and early differentiating neurons (Anderson and Axel, 1985; Stein et al., 1988). To date, there has been no complete description of the precise temporal and spatial expression pattern of SCG10 during embryogenesis in any species, though partial descriptions exist for SCG10 in *Xenopus*, chicken, mouse, rat and human (Bieche et al., 2003; Groves et al., 1995; Himi et al., 1994; Okazaki et al., 1993; Ozon et al., 1997; Stein et al., 1988; Sugiura and Mori, 1995). From the published data, it appears that the basic pattern of SCG10 expression is conserved between these species. In all species SCG10 is highly expressed in the developing CNS neurons during the period of neurite extension. SCG10 is also strongly expressed in autonomic neurons during neurite extension. At later stages, SCG10 expression remains in the adult brain, but the level of expression is significantly reduced as compared to its embryonic expression (Bieche et al., 2003; Groves et al., 1995; Himi et al., 1994; Okazaki et al., 1993; Ozon et al., 1997; Stein et al., 1988; Sugiura and Mori, 1995). SCG10 mRNAs are also detected in several other tissues. As previously stated, the level of SCG10 expression in these non-neural tissues is low with the most significant expression being found in the small intestine, colon, adrenal glands, prostate and testis (Bieche et al., 2005). Furthermore, in chronic liver diseases, SCG10 transcripts are more abundant in this tissue (Bieche et al., 2005; Paradis et al., 2005). In this study we have characterized spatial and temporal expression of two SCG10 zebrafish orthologues (zgc:92905 and zgc:110132) during embryonic development.

1.1. Characterization of zebrafish SCG10 genes and proteins. Phylogenetic analysis

To identify zebrafish orthologues of SCG10 we undertook an Ensembl database blastp search (WU BLAST 2.0, default settings) with the human SCG10 protein sequence against the zebrafish peptides library. This search produced two significant hits. The first was zgc:92905, which we refer to as SCG10b (Tab. 1). The second was an annotated SCG10 orthologue (stmn2; zgc:110132; Tab. 1), which we refer to as SCG10a. We have ascribed these gene names based on the database annotation, our syntenic analysis and phylogenetic data. These indicate that SCG10a and SCG10b are duplicates that arose as a result of the proposed whole genome duplication event that occurred in teleosts lineage (Amores et al., 1998). SCG10a is located on Zebrafish Chromosome (Chr) 16 and has a 1 kb transcript that encodes a 175 amino acid (aa) protein. SCG10b is located on Chr 19 and has a 1.5kb transcript that encodes for a 180 aa protein. Zebrafish SCG10 proteins are 56% identical and 70% similar to each other. When compared to human orthologue SCG10a is 46% identical and 65% similar whereas SCG10b is 52% identical and 56% similar, respectively. The human and zebrafish proteins differ mainly in their C-termini (Fig. 1). The functional consequence of this difference is unknown. Zebrafish SCG10a and b transcripts and peptides show significant similarities also with SCG10 orthologues from other vertebrates. Table 1 shows the conservation of SCG10 nucleotide and amino acid sequences between zebrafish and human, rodents, avian and amphibian species. SCG10a has a higher similarity to other vertebrate homologues. Syntenic analysis shows that SCG10a is located on the segment of zebrafish Chr 16 that corresponds to the region of human Chr 8 where the human SCG10 orthologue is located (Barbazuk et al., 2000). Both the human and zebrafish SCG10 loci are linked to the adjacent Hey1 gene. Phylogenetic analysis revealed the divergence of SCG10b from SCG10a and other SCG10 orthologues (Fig. 2). Despite the more distant relationship of SCG10b to the other SCG10 orthologues, the analysis shows that SCG10b clusters within the SCG10 branch of the stathmin family, supporting our designation that this gene is a duplicate (Fig. 2).

1.2. Expression of SCG10a and SCG10b during zebrafish development

The temporal expression pattern of SCG10a and SCG10b were determined by RT-PCR. Both genes are expressed maternally and zygotically as transcripts can be detected at 0 hours post-fertilization (hpf) through 5 days post-fertilization – the latest stage examined in the present study (Fig. 3, 4). The SCG10a transcript is clearly more abundant through all the stages examined, potentially suggesting a greater requirement for SCG10a than SCG10b at the functional level.

To determine the spatial expression pattern of the zebrafish SCG10 orthologues during embryogenesis, whole mount *in situ* hybridizations were undertaken using riboprobes that complement the mRNAs for both genes. From 16 hpf, discrete patterns of expression were detectable. At this stage, expression of both genes is restricted to the posterior lateral line (PLL) ganglia and Rohon-Beard sensory neurons in the spinal cord. At 24 hpf, the expression patterns of both genes remains restricted to discrete locations within the anterior CNS (telencephalon, diencephalon, epiphysis), the anterior and posterior lateral line (ALL and PLL) ganglia (Fig. 4A-AD blue and red arrowheads), as well as all hindbrain rhombomeres and primary sensory neurons of the spinal cord (Fig. 4 G, H, K, L; arrows). At 48 hpf, expression becomes more abundant but is primarily restricted to the anterior CNS and cranial ganglia (Fig. 4M-P). SCG10a and SCG10b transcripts are clearly present in the forebrain region (olfactory bulb), retina, optic tectum, trigeminal, vagal ganglia, ALL and PLL ganglia, and the hindbrain (Fig. 4S). At 72, 96 and 120 hpf SCG10 transcripts remain expressed in the same CNS regions. Both genes are also expressed in the differentiated ALL and PLL ganglia, as well as in facial, glosso-pharyngeal and vagal cranial ganglia. At 72 and 96 hpf both SCG10 genes are also expressed in the enteric neurons (Fig. 4 R, T, W, Y; arrowheads and insets). Transverse sections through the midbrain and optic tectum (96 hpf) clearly show ubiquitous SCG10a expression throughout

the CNS. In the eye retina, SCG10a expression is restricted to the region posterior to the lens, close to the optic nerve head and the retinal ganglion cell layer (Fig. 5A). Transverse sections through the hindbrain at 96 hpf reveal a fairly ubiquitous expression of SCG10a however there is a conspicuous absence of expression in the hindbrain motor neurons (Fig. 5B). In addition, strong expression of SCG10a can be seen in the PLL and vagal ganglia, as well as in the sympathetic chains (Fig. 5B). A diffused wide spread expression of SCG10a can be seen in the mesenchyme surrounding the gastro-intestinal (GI) tract (Fig. 5B). By 120 hpf the overall level of SCG10a and SCG10b expression has decreased compared to earlier developmental ages. This decrease in SCG10 expression is consistent with the known requirement of SCG10 function in early stages of neurogenesis (Ozon et al., 1997).

1.3. Summary

Our study provides the first description of the spatial and temporal expression patterns of two zebrafish SCG10 orthologues. SCG10a and SCG10b have dynamic, spatially restricted patterns of expression. Their expression is initially confined to distinct regions of the CNS and cranial ganglia at early stages (16-24 hpf) of development, but later (48-120 hpf) their expression becomes more ubiquitous in the anterior CNS and in specific populations of peripheral neurons. Zebrafish SCG10 genes have highly conserved nucleotide and amino acid sequences when compared to other vertebrates. Furthermore, the overall pattern of expression of these two zebrafish orthologues is very similar to the pattern of SCG10 expression described in the CNS and PNS of other vertebrates. (Bieche et al., 2003; Groves et al., 1995; Himi et al., 1994; Okazaki et al., 1993; Ozon et al., 1997; Stein et al., 1988; Sugiura and Mori, 1995). Previously SCG10 has been used as a neuronal marker and as a marker for specific neural crest derivatives (Anderson and Axel, 1985; Stein et al., 1988). Our results suggest that SCG10 can also be used in zebrafish as a marker for developing enteric neurons, sympathetic and cranial ganglia. Our finding that SCG10 is not expressed in dorsal root ganglia (DRG) neurons is significant given SCG10's previously reported expression in avian and rat DRGs (Mason et al., 2002; Tsarovina et al., 2008). The significance of this difference is unclear but may reflect species-specific developmental differences. DRG development in zebrafish occurs much later in embryogenesis as compared to other species (An et al., 2002). Furthermore, SCG10 is expressed in the Rohon-Beard cells that act as the spinal cord primary sensory neurons in the zebrafish at early stages of embryogenesis (Reyes et al., 2004). Our observation that there is expression of SCG10a and b in the mesenchyme surrounding the GI tract, as well as in the regions of prospective liver and pronephros development is consistent with previous studies that have reported the presence of SCG10 transcript in other tissues than neurons (Bieche et al., 2005; Bieche et al., 2003; Paradis et al., 2005). This non-neuronal expression suggests that SCG10 expression has a function in regulating microtubule dynamics in other tissues. Future studies will investigate the *in vivo* function of the zebrafish SCG10 orthologues in both neurons and non-neuronal tissues.

2. Experimental procedures

2.1. Cloning of SCG10a and SCG10b

Both genes have been previously annotated in the zebrafish genome and complete cDNA sequences can be found in the public databases (<http://www.ncbi.nlm.nih.gov>; <http://www.ensembl.org/>; Genbank accession IDs: SCG10a - NM_001024222, SCG10b - NM_001005923). The candidates for zebrafish SCG10 orthologues were identified by blastp search with human SCG10 amino acid sequence and by subsequent blastn search with identified ESTs. The partial ORFs of both genes were cloned into the pCR TOPO II vector (Invitrogen) for the riboprobe synthesis. A fragment of 840 bp of SCG10a was amplified with 5'-CAGTCAAAGT- GGGTGTGGTT-3' forward and 5'-AACTTTGGGAGCAAGCTGTG-3' reverse primers. A fragment of 1067 bp of SCG10b was amplified with 5'-

TAAGCATCACCGCTCTCAA-3'forward and 5'-TTCACAGAACCCCAAACCTT-3' reverse primers. To determine the temporal expression of SCG10a and SCG10b, RT-PCR was carried out using One Step RT-PCR kit (Qiagen). In case of SCG10a, the same, aforementioned set of primers was used to amplify fragment encompassing exon 6 and 3' UTR of the gene. The forward 5'-AAATAAAAGG GCCTCAGGTCA-3' and reverse 5'-CAAACATCAGCCTTGAGTGC-3' primers were designed to amplify the region encompassing exons 2-6 in SCG10b gene. RT-PCR conditions were as followed: 30 min at 50°C, 15 min at 95°C, 30 cycles of 30 s at 94 °C, 30 s at 55°C and 30 s at 72°C followed by 10 min extension step at 72°C.

2.2. Phylogenetic analysis

All the BLAST searches were performed on the ENSEMBL website (<http://www.ensembl.org/>), which utilizes WU BLAST 2.0 (<http://blast.wustl.edu>). Protein sequence alignments were performed with the Clustal W algorithm (Thompson et al., 1994), using Jellyfish software (version 3.3.1; <http://www.jellyfishsoftware.com/>). The cladogram was constructed using Phylodendron software available online (<http://iubio.bio.indiana.edu/treeapp/>). Clustal W tree guide output files (<http://www.ebi.ac.uk/Tools/clustalw2/>) were used as the input data for Phylodendron, which utilizes DrawGram and DrawTree tree construction algorithms from Phylogeny Inference Package.

2.3. Animal maintenance

Zebrafish were kept and bred under standard conditions at 28.5°C (Westerfield, 1993). Embryos were staged and fixed at specific hours post fertilization (hpf) as described elsewhere (Kimmel et al., 1995). To better visualize *in situ* hybridization results, embryos were grown in 0.2 mM 1-phenyl-2-thiourea (Sigma) to inhibit pigment formation (Westerfield, 1993).

2.4. Whole mount *in situ* hybridization

Digoxigenin labeled riboprobes that complement SCG10a and SCG10b mRNAs were generated by linearization of pCR TOPO II vectors that contain partial ORFs of the genes. Plasmids were linearized with *EcoRV* (New England Biolabs) and subsequently transcribed the SP6 polymerase (Promega). Program used for probe synthesis reaction was as followed: 2 h incubation at 40°C, followed by the addition of 2 ul of RNase free DNase I for DNA template digestion. 1 µl of EDTA 0.5M, pH 8.0 was added to stop the reaction. Subsequently, probes were purified using mini Quick spin RNA columns (Roche). Whole mount *in situ* hybridization reactions were performed as described previously (Thisse et al., 1993)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Alignment of zebrafish SCG10a, SCG10b and human SCG10 amino acid sequences. A clustalw amino acid alignment generated by Jellyfish software using neighbor-joining method. Identical amino acids are crosshatched; dashes represent gaps in the alignment. Locations of the three functional domains are indicated.

Figure 2.

Cladogram of vertebrate stathmin family members. Cladogram was constructed by Phylo dendron software, using neighbor-joining method. Protein sequences were retrieved from Ensembl database (<http://www.ensembl.org>); h, human (*Homo sapiens*); m, mouse (*Mus musculus*); r, rat (*Rattus norvegicus*); g, chicken (*Gallus gallus*); x, frog (*Xenopus tropicalis*).

Figure 3. Temporal expression pattern of zebrafish SCG10a and SCG10b genes. RT-PCR was undertaken using RNA isolated from wild type embryos at 0, 6, 12, 20, 24, 36, 48, 72 and 96 hpf; A – SCG10a; B – SCG10b; C – β -tubulin; M – 100 bp DNA ladder (New England Biolabs)

Figure 4.

Temporal and spatial expression pattern of SCG10a and SCG10b genes. Wholemount *in situ* hybridized embryos hybridized with either an SCG10a (A, B, E, F, I, J, M, N, R, S, W, X, AA, AB) or an SCG10b (C, D, G, H, K, L, O, P, T, U, Y, Z, AC, AD) antisense riboprobes at the indicated developmental stages. The first and third columns show lateral views, while the second and fourth columns show dorsal views. Inserts in panels R, T, W, Y are close up views of the intestine. Anterior is to the left. Red arrowheads indicate posterior lateral line ganglia; blue arrows indicate the anterior lateral line ganglia; black arrowheads indicate developing enteric neurons in the gut; small black arrows (G, H, K, L) indicate Rohon-Beard neurons; pink arrows (R, T, W, Y) indicate enteric neurons expressing SCG10a and SCG10b. Abbreviations

in (T), al -anterior lateral line; t - trigeminal ganglia; f – facial ganglia; g – glossopharyngeal ganglia; v – vagal ganglia; pl - posterior lateral line ganglia; retina is indicated.

Figure 5.

Expression of SCG10a in the CNS. Transverse sections of 96hpf larvae hybridized with an SCG10a riboprobe. (A) section taken at the level of the eyes and the anterior optic tectum; pink arrowheads indicate retinal ganglion cell layer; black arrows indicate the region posterior to the lens, close to the optic nerve head; (B) section taken through the posterior hindbrain; black arrowheads indicate the posterior lateral line ganglia; blue arrowheads indicate the vagal ganglia; red arrowheads indicate the sympathetic ganglia chains; abbreviations: ot – optic tectum; ph – pharynx; mn – motor neurons region; oe – esophagus; m – mesenchyme.

Table 1
Accession IDs and sequence conservation of SCG10 in vertebrate species

A	GenBank acces.	SCG10a-DNA	SCG10a-protein	SCG10b-DNA	SCG10b-protein
	hSCG10	NM_007029	73.30/88.3%	46.20%	52.00/64.9%
	mSCG10	NM_025285	73.30/88.3%	37.60%	52.00/64.9%
	rSCG10	NM_053440	77.30/87.8%	37.90%	52.00/64.4%
	gSCG10	NM_205181	72.80/88.9%	42.20%	52.00/65.3%
	xSCG10	NM_001098442	70.60/86.1%	47.10%	56.50/73.9%
B	Zfin ID	NCBI Gene ID	GenBank access.	LG group	position (bp)
	SCG10a	zgc:110132	100003563	19	27,142,581 - 27,161,350
	SCG10b	zgc:92905	449651	16	11,902,413 - 11,919,210

Panel 'A' shows conservation of *Homo sapiens* (h), *Mus musculus* (m), *Rattus norvegicus* (r), *Gallus gallus* (g) and *Xenopus laevis* (x) SCG10 nucleotide and amino acid sequences compared to zebrafish SCG10a and SCG10b genes. Panel 'B' is a combination of zebrafish SCG10 genes accession IDs from Zfin and NCBI databases. Additionally, genomic positions of both zebrafish genes are given (assembly Zv7, April 2007).