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Dynamic Expression Patterns of Zebrafish *1G5* (*1G5z*), a Calmodulin Kinase-Like Gene in the Developing Nervous System

Minho Won,[1†] Hyunju Ro,[1†‡] Hae-Chul Park,[2] Kyoon E. Kim,[3] Tae-Lin Huh,[4] Cheol-Hee Kim,[1] and Myungchull Rhee[1*]

Evolutionarily well-conserved Ca^{2+}/calmodulin-dependent protein kinase (CaMK) proteins are known for their role as Ca^{2+} signaling mediators. *1G5* encodes a CaMK like protein, which belongs to a calmodulin (CaM) kinase gene family. Here, we report the isolation of zebrafish homologue of mammalian 1G5, which we named 1G5z. 1G5z is composed of three major domains: (1) an N-terminal serine/threonine kinase domain, (2) a central calmodulin-binding domain, and (3) a C-terminal alanine-rich domain, the 1G5z-specific domain. 1G5z shares 83~84% homology with other vertebrate 1G5 proteins. Spatiotemporal expression studies found that *1G5z* is expressed by means of zygotic transcription and appears in various neuronal tissues from the 20-somite stage. *1G5z* transcripts are more regionalized in the brain and spinal cord at 24 hr postfertilization (hpf). At 35 hpf, *1G5z* transcripts are exclusively present in the anterior trunk spinal cord as well as in the hindbrain, tegmentum, hypothalamus, and telencephalon. This expression pattern lasts until 48 hpf but ceases in the trunk. At 72 hpf, *1G5z* is abundantly transcribed particularly in the specific region of the tectum and eye. We further observed that the number of *1G5z*-positive cells is dramatically increased in the mindbomb mutant embryos but abolished in the trigeminal ganglion and caudal trunk sensory neuron of the *neurogenin1* morphant at 24 hpf. In addition, bromodeoxyuridine staining further confirmed that the *1G5z*-positive cells were postmitotic sensory and interneurons. *Developmental Dynamics 235:835–842, 2006.* © 2006 Wiley-Liss, Inc.

Key words: zebrafish; CaMK; 1G5z; neurons; *mindbomb*; *neurogenin1* morphant

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INTRODUCTION

Calcium (Ca^{2+}) is essential for the regulation of a wide variety of biological processes such as contraction, secretion, fertilization, cell proliferation, apoptosis, learning, and memory (Berridge et al., 1998). Calmodulin (CaM) is primarily known as a Ca^{2+} binding protein in all eukaryotic cells and plays a critical role as a Ca^{2+} mediator (Cheung, 1980; Klee et al., 1980). CaM is highly abundant in the vertebrate central nervous system (CNS) and spatially enriched in postsynaptic membranes, postsynaptic densities, and synaptic vesicles (Sola et al., 2001). Cellular Ca^{2+} signaling induced by CaM requires an interaction between CaM and specific proteins. Among the several CaM binding proteins, the Ca^{2+}/CaM-dependent protein kinase II (CaMK-II) is important in numerous neural functions. CaMK-II is highly conserved from *Drosophila* to

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human. This well-conserved serine/threonine protein kinase CaMK-II is encoded by at least four genes (α, β, γ, and δ) in vertebrates (Tombes et al., 2003). In contrast to that in vertebrates, *Drosophila* CaMK-II is encoded by a single gene (Griffith and Greenspan, 1993). In the brain, the α and β isoforms of CaMK-II constitute between 0.25% and 2% of the total protein and is especially abundant in the hippocampus (Klee, 1991; Braun and Schulman, 1995; Sola et al., 2001). In contrast to the α and β isoforms, γ and δ are widely expressed in various tissues (Tobimatsu and Fujisawa, 1989). Other CaMKs abundant in neurons include members of the CaMK cascade: CaMKI, CaMKIV, and CaMKK (CaMK kinase; Means, 2000). A previous report showed the exclusive expression of CaMK-II in the CNS in *Drosophila* embryos (Rachidi et al., 1999). Another report also demonstrated that a CaMK-II–like gene, *1G5*, was predominantly expressed in the rat brain, including olfactory bulb, cerebral cortex, caudate nucleus, hippocampus, hypothalamus, and midbrain, and mild expression was observed in pons, medulla, and spinal cord but was not detected in the non-neural tissues (Godbout et al., 1994). Recently, Thisse et al. reported the zebrafish *CaMK-IID* expression pattern to the ZFIN (unpublished data; http://zfin.org/cgi-bin/webdriver?MIval=aa-xpatview.apg&OID=ZDB-XPAT-041008-109). Of interest, the expression of *CaMK-IID* began at the late segmentation stage and became confined in brain tissues throughout development. However, detailed studies of the expression pattern of CaMK or CaMK-like genes during vertebrate embryonic development have not been conducted.

Here, we report the isolation and expression of a *CaMK*-like gene, the zebrafish homologue of mammalian *1G5* (*1G5z*). *1G5z* expression starts long after primary neurogenesis begins and is restricted to the particular neuronal cells of the developing CNS. At 3 days postfertilization, *1G5z* expression is further restricted in the brain hierarchy, particularly in the eye. The number of *1G5z*-positive sensory neurons and interneurons are greatly enhanced in the *mindbomb* mutant embryos at 24 hpf. However, *1G5z* transcripts in the trigeminal ganglion and trunk sensory neurons are completely abolished in *neurogenin1* morphants. By using the bromodeoxyuridine (BrdU) incorporation assay, we further identified that *1G5z*-expressing cells were terminally differentiated sensory and interneurons. The application of *1G5z* as a marker gene for neurons and brain is discussed.

RESULTS AND DISCUSSION

Molecular Cloning and Characterization of 1G5z

We isolated a partial cDNA through the in situ screening for genes with tissue-specific expression patterns during zebrafish embryonic development. The isolated cDNA fragment encodes a novel member of vertebrate *1G5* homologue. In an attempt to obtain a complete open reading frame (ORF), we initially searched the zebrafish homologue of *1G5* gene by using Ensemble Zebrafish Genome Server Genescan (http://www.ensembl.org/Danio_rerio) and NCBI Blast the Zebrafish Genome EST Blast Search (http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html) programs. As a result, we found several DNA fragments from the database enough to encompass the full ORF of zebrafish *1G5* homologue. After connecting the DNA fragments, we could deduce putative ORF. The expected ORF was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) and verified by sequencing analysis. The zebrafish homologue of 1G5 shared high amino acids sequence homology with the proteins from several species, including rat (Godbout et al., 1994), mouse (Strausberg et al., 2002), and human (Fig. 1A), and was designated as *1G5z* (*1G5* of zebrafish; accession no. AY450361). *1G5z* encoding 436 amino acids contains major three domains: (1) a serine/threonine kinase domain (catalytic domain) between residues 24 and 286, (2) a calmodulin-binding domain between residues 309 and 325, and (3) a region between residues 334 and 398 that contains an alanine-rich domain (Fig. 1B). A phylogenetic tree generated from a CLUSTALW multiple amino acid sequence alignment (Thompson et al., 1994) of 1G5z with other mammalian homologues shows that 1G5z shares highest sequence homology with rat 1G5 (Fig. 1C). A previous report suggested that 1G5 could be classified as a subfamily of CaMK-II (Godbout et al., 1994). However, based upon the accumulated sequence data, we identified that 1G5 shares most sequence homology with CaMK-I (Fig. 1C). Rat 1G5 shows 67% sequence homology with rat CaMK-I but shares only 57% of sequence similarity with rat CaMK-II. But the serine/threonine kinase domain and the calmodulin-binding domain of all CaMK members are relatively well matched to each other.

Whereas 1G5z protein shares 83–84% amino acid sequence homology with other vertebrate homologues, the C-terminal alanine-rich domain of 1G5z does not show significant homology, which makes it the 1G5z-specific domain. As we mentioned above, the amino terminal region of 1G5z encompassing the serine/threonine kinase motif and calmodulin-binding domain shows high sequence similarity with CaMK-II, like as mouse 1G5 does (Fig. 1B,C; Godbout et al., 1994). This sequence information suggests that 1G5z might have calmodulin-binding and serine/threonin kinase activities. However, 1G5z lacks the consensus autophosphorylation site (RXXS/T) that is commonly found in other vertebrates and located between the catalytic and calmodulin-binding domains in CaMK-II (Miller et al., 1988; Godbout et al., 1994). Previous studies showed that the autophosphorylation of CaMK-II is important for maintenance of its kinase activity, without prolonged calcium signal stimulation (Hanson and Schulman, 1992). This difference between CaMK-II and 1G5/1G5z suggests that 1G5/1G5z is possibly involved in short-term calcium-dependent signaling. In addition, the high sequence homology between zebrafish and its mammalian homologues suggests that they are evolutionarily well conserved and play important roles in vertebrate development.

Spatial and Temporal Expression Patterns of *1G5z*

In *Drosophila* early embryonic stage, *CaMK-II* gene was expressed throughout the CNS at the first instar larvae (Rachidi et al., 1999). As development

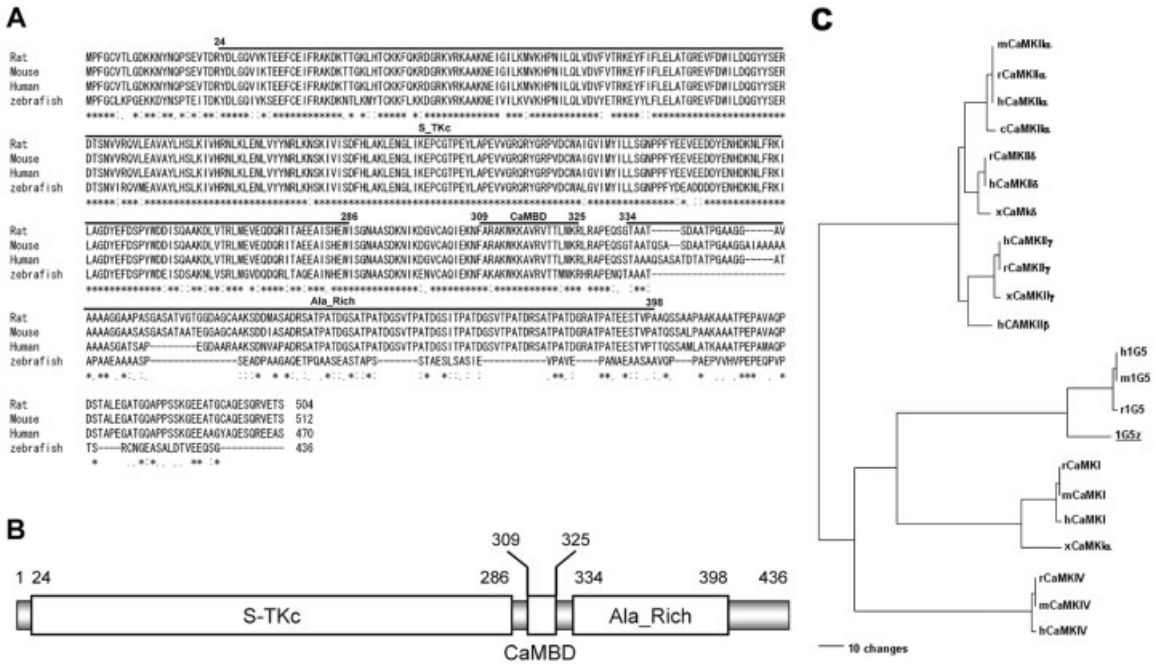


Fig. 1. Amino acid sequence analysis of 1G5z. **A:** Alignment of deduced amino acid sequence of 1G5z with other vertebrate 1G5 proteins. The asterisk represents the amino acid residues identical to each other; the: symbol represents high similarity, and the symbol shows weak similarity. The continuous lines indicate the putative serine/threonine kinase domain (S_TKc; amino acids 24-286), calmodulin-binding domain (CaMBD; amino acids 309-325), and alanine-rich region (Ala-Rich; amino acids 334-398) of 1G5z. The overall similarity is 84%, 83%, and 83% between 1G5z and rat 1G5, mouse 1G5, and human 1G5, respectively. Alignment was performed with ClustalW algorithm. **B:** Diagram depicting the three major domains of 1G5z. The amino acid residues are numbered. **C:** Phylogenetic tree of 1G5 proteins with the Ca^{2+}/calmodulin–dependent protein kinase gene family (CaMKI, II, and IV). The tree was constructed using sequences from the catalytic domain and regulatory domain. GenBank accession numbers for the sequences included in the analysis are as follows: 1G5z (AY450361), h1G5 (Human 1G5; NM_024046), r1G5 (Rat 1G5; NM_024000), m1G5 (Mouse 1G5; NM_145621), hCaMKI (Human CaMKI; NM_003656), rCaMKI (Rat CaMKI; NM_134468), mCaMKI (Mouse CaMKI; NM_133926), xCaMKI (*Xenopus* CaMKI; BC084930), hCaMKII (Human CaMKII; NM_015981), hCaMKII (Human CaMKII; NM_001220), hCaMKII (Human CaMKII; NM_172171), hCaMKII (Human CaMKII; NM_172127), mCaMKII (Mouse CaMKII; NM_177407), cCaMKII (chicken CaMKII; NM_204295), rCaMKII (Rat CaMKII; NM_012920), rCaMKII (Rat CaMKII; NM_133605), xCaMKII (*Xenopus* CaMKII; AF233630), rCaMKII (Rat CaMKII; NM_012519), xCaMKII (*Xenopus* CaMKII; AF233629), hCaMKIV (Human CaMKIV; NM_001744), rCaMKIV (Rat CaMKIV; NM_012727), mCaMKIV (Mouse CaMKIV; BC070420). CaMKII family was used as an outgroup.

progresses, *CaMK-II* signals were restricted in several ganglia tissues and eye imaginal disc (Rachidi et al., 1999). In mice, Godbout et al. (1994) demonstrated that *1G5* was exclusively detected in brain and eye and not in the non-neural tissues using Northern blot analysis. However, the detailed expression pattern of CaMK and/or CaMK-like genes throughout vertebrate development has not been investigated.

To determine the spatiotemporal expression profile of *1G5z*, we performed both RT-PCR and whole-mount in situ hybridization (WISH). RT-PCR analysis showed that the *1G5z* transcripts are initially detectable at 24 hpf, and the transcriptional level is constantly maintained until 48 hpf (Fig. 2A) but not detectable before the 18-somite stage (Fig. 2B). In the embryos at 19.5 hpf (20 somites), cells expressing *1G5z* are located in the developing neurons along the trigeminal ganglion, hindbrain, and spinal cord (Fig. 2C). Thus, these data suggested that *1G5z* is not transcribed until the onset of primary neurogenesis.

At 24 hpf, *1G5z* is highly expressed in the neuronal cells at the trunk neural tube (Fig. 2D). Transverse section through the spinal cord resolved a more detailed expression pattern of *1G5z* in the interneuron and sensory neuron (Fig. 2G–J). It is of interest to note that *1G5z* is differentially expressed through the anterior to posterior trunk spinal cord. *1G5z* is expressed in the interneuron and sensory neuron from anterior to medial trunk (Fig. 2G–J). Beyond the yolk sac extension, the expression domain of *1G5z* is restricted in the sensory neurons. At this stage, *1G5z* is newly transcribed in the brain region (Fig. 2E). Horizontal sections of the 24-hr-old zebrafish brain revealed the *1G5z* expression in the diencephalon and trigeminal ganglion (Fig. 2F). We further determined the precise location of *1G5z* expression domain with two-color WISH using *isl1* (*islet1*), *isl2* (*islet2*), *th* (*tyrosine hydroxylase*), *lim1*, and *krox20* marker genes. *is1* family (*isl1*, *isl2*), and LIM/homeodomain-type transcription factors are the markers for the cranial and trunk motor neurons as well as caudal Rohon–Beard (RB) neurons (Inoue et al., 1994; Appel et al., 1995; Tokumoto et al., 1995; Segawa et al., 2001). *th* and *krox20* are the markers for dopaminergic neurons (Holzschuh et al., 2001) and the rhombomere (r) 3 and 5 (Oxtoby and Jowett, 1993), respectively. In 24 hpf, *lim1* mRNA is expressed in the brain and spinal cord (Toyama and Dawid, 1997). *1G5z* is partly coexpressed with *isl-1* in the telencephalon, anterior–ventral diencephalons and trigeminal ganglion

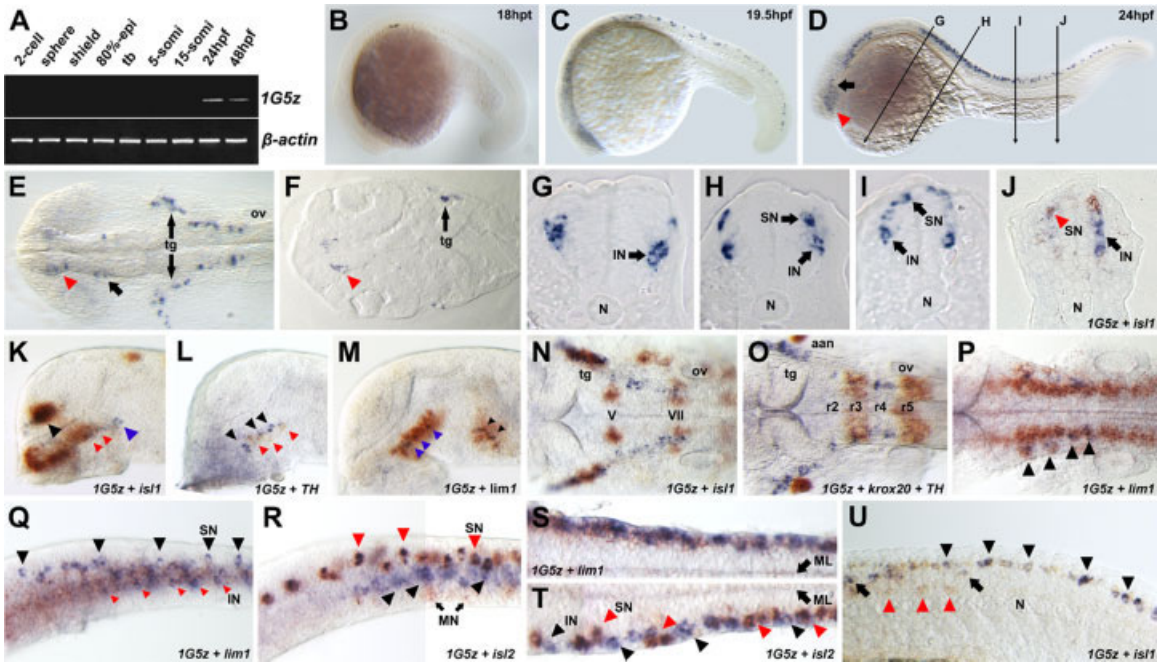
(Fig. 2J,K). Of interest, *1G5z*-expressing cells in the diencephalon form three different neuronal cell clusters (Fig. 2K–M). To clarify the identity of these cell clusters, we carried out two-color WISH using *1G5z* and *th* as in situ probes. At 24 hpf, *th* is specifically expressed in the diencephalic catecholaminergic cluster (Holzschuh et al., 2001). Bicolor WISH experiments found that *1G5z* and *th* are expressed in the similar regions of diencephalon but not overlapping with each other (Fig. 2L). It suggests that *1G5z* may not be expressed in the diencephalic dopaminergic neurons. *1G5z* expression domains in the diencephalon and tegmentum were further analyzed by bicolor WISH with the *lim1* marker gene. As shown in Figure 2M, *lim1* expression domain completely encompasses *1G5z*-expressing cells in the diencephalon and tegmentum. In the hindbrain, *1G5z* appeared in the lateral margin of r2, 3, and 4 (Fig. 2N–P). *1G5z* expression pattern in the trunk region is also confirmed by two-color WISH using *isl1*, *isl2*, and *lim1* markers. In the trunk spinal cord, *lim1* demonstrates restricted expression pattern in the interneurons, whereas *isl1* and *isl2* are expressed in the sensory (RB) and motor neurons. As shown in Figure 2Q,R, *1G5z* transcripts in the anterior trunk region are specifically detected in the RB neurons and internuerons but not in the ventral motor neurons where *isl2* is exclusively expressed. In the posterior trunk and tail region, *1G5z* is also colocalized with *isl1* in the RB neurons, but the expression in the interneuron gradually fades as it reaches the end of tail (Fig. 2U). Transverse section of spinal cord after staining with *1G5z* and *isl1* further clarified RB and interneuron-specific expression of *1G5z* (Fig. 2J).

By 35 hpf, *1G5z* transcripts appear in the anterior neural tube as well as in the hindbrain, tegmentum, hypothalamus, and telencephalon (Fig. 3A,B). It is worth noting that *1G5z* transcripts no longer exist in the trunk (data not shown) at 48 hpf, whereas the overall expression pattern in the brain hierarchy has not been altered after 35 hpf (Fig. 3C,D). At 72 hpf, new transcripts of *1G5z* appear in the tectum, are more distinct in the eyes, but gradually vanished in the rhombencephalon (Fig. 3E,F). Transverse sections resolved a more detailed expression pattern of *1G5z* in the tectum, lateral margin of tegmentum, hypothalamus, proximal region of inner nuclear layer, and some ganglion cells of retina (Fig. 3G–I). In particular, *1G5z* appears to be restricted in the amacrine cells of the inner nuclear layer (Fig. 3H). Of interest, the distinct expression pattern of *1G5z* in the trigeminal ganglia and eye is reminiscent of the expression profile of *Drosophila CaMK-II* at the late embryonic stage (Rachidi et al., 1999). In *Drosophila*, the CaMK-II expression is restricted to the thoracic and cephalic ganglia of the CNS and the eye imaginal disc at the white pupae stage (Rachidi et al., 1999). *Drosophila* CaMK-II is encoded by a single gene, and its expression is initiated from very early in CNS development (Griffith and Greenspan, 1993; Rachidi et al., 1999). On the other hand, *1G5z* is not expressed at the onset of primary neurogenesis. These observations suggest that *CaMK* or *1G5*-like genes other than *1G5z* could be expressed in the early primary neurons of zebrafish embryos.

Because *1G5z* expression is restricted to the specific neuronal cells in the developing embryos, we analyzed its expression pattern in the *mindbomb* mutant and *neurogenin 1* morphant. The *mindbomb* mutant is characterized by a severe neurogenic phenotype, with overproduction of differentiating neurons that results from deficits in Notch signaling (Jiang et al., 1996; Itoh et al., 2003). The num-

Fig. 2. Spatiotemporal distribution of *1G5z* during early embryogenesis. **A:** *1G5z* expression levels in the embryos at various developmental stages were analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR). A β-*actin*-specific primer set was used to generate internal control for the RT-PCR. **B:** At the 18 hours postfertilization (hpf) stage, lateral view. **C:** The 20-somite stage, lateral view. Note that the first *1G5z* transcripts appeared at this stage. **D:** At 24 hpf, lateral view. Diagram showing the plane of cryostat section (10 μm) in G, H, I, and J. **E:** Highly magnified dorsal view of brain region of D. Before taking pictures, the embryos were flat-mounted. The red arrowhead and arrow indicate the anterior–ventral diencephalon and the tegmentum, respectively. **F:** Horizontal section of E. The red arrowhead indicates the restricted expression pattern of *1G5z* in the diencephalon. **G–I:** *1G5z* mRNAs are detected in interneuron and sensory neuron. **J:** Two-color whole-mount in situ hybridization (WISH) with *isl1* (orange color) shows interneuron- and sensory neuron-specific expression of *1G5z* (blue color) in the medial spinal cord. The red arrowhead indicates overlapping expression of *1G5z* and *isl1* in the sensory neuron. The black arrow indicates interneuron expression of *1G5z*. K–U: Bicolor WISH with *isl1*(K,N,U), *th* (L), *lim1* (M,Q,S) *isl2* (R,T) *th* plus *krox20* (O) in orange (INT-BCIP substrate) and *1G5z* in blue (NBT-BCIP substrate) was performed. K–M: Lateral view of the forebrain. **K:** *1G5z* and *isl1* are colocalized in the telencephalon (black arrowhead) but partly overlap in the diencephalon (red arrow). Note that the blue arrowhead indicates a nonoverlapping region. **L:** *1G5z*-expressing neural cell clusters (black arrowhead) are not colocalized with *th*-positive cells (red arrowhead). **M:** *lim1*-expressing domains in the diencephalon (blue arrow) and mesencephalon (black arrow) encompass *1G5z*-positive neural cell clusters. N–P: Dorsal view of the hindbrain. **N:** *1G5z* and *isl1* are colocalized in the trigeminal ganglion (tg). **O:** *1G5z* and *krox20* are colocalized in the rhombomere3 (r3). **P:** *lim1* expression domain completely encompasses *1G5z* expressing hindbrain cells. Q–T: Anterior trunk spinal cord. Q,R,U: Lateral view of trunk spinal cord. **Q:** *1G5z* and *lim1* are colocalized in the interneurons (red arrowhead) but not in the sensory neurons (black arrowhead). **S:** Dorsal view of Q. **R:** *1G5z* and *isl2* are colocalized in the sensory neurons (red arrowhead) but not in the interneurons (*1G5z*-specific expression; black arrowhead) and motoneurons (*isl2*-specific expression; black arrow). **T:** Dorsal view of R. **U:** In the medial–caudal trunk region, *1G5z* and *isl1* are colocalized in the sensory neurons (black arrowheads) but not in the interneurons (black arrows). Red arrowheads indicate motor neurons in which *isl1* is exclusively detected. The anterior part of all embryos is oriented to the left. aan, arch-associated neuron; epi, epiboly; IN, interneuron; ML, midline; MN, motor neuron (motoneuron); N, notochord; ov, otic vesicle; r, rhombomere; SN, sensory neuron; tb, tail bud; tg, trigeminal ganglion; V, fifth cranial nerve; VII, seventh cranial nerve.

Fig. 3. Distribution of *1G5z* transcripts during late embryogenesis. **A:** At 35 hours postfertilization (hpf). Lateral view. The red arrowhead indicates the hypothalamus; and black arrowhead indicates the telencephalon. **B:** Dorsal view of the brain domain of A. The arrow indicates new messages in the retina. **C:** At 48 hpf. Lateral view. **D:** At 48 hpf. Dorsal view. **E:** At 72 hpf. Lateral view. **F:** At 72 hpf. Dorsal view. G–I: Transverse section of the brain along the white lines in E. **G,I:** *1G5z*-expressing cells are detected in the optic tectum (black arrow), lateral part of tegmentum (G, I, red arrowhead), and hypothalamus (G, I, black arrowhead). **H:** High-magnification view of the eye of G. *1G5z* transcripts are detected in the proximal part of the inner nuclear layer (black arrow) and some ganglion cells (red arrowhead). Anterior part of all embryos is oriented to the left. ep, epiphysis; gc, ganglion cell layer; H, hypothalamus; In, inner nuclear layer; Ip, inner plexiform layer; MeS, mesencephalon; ov, otic vesicle; Tg, tegmentum; tg, trigeminal ganglion.



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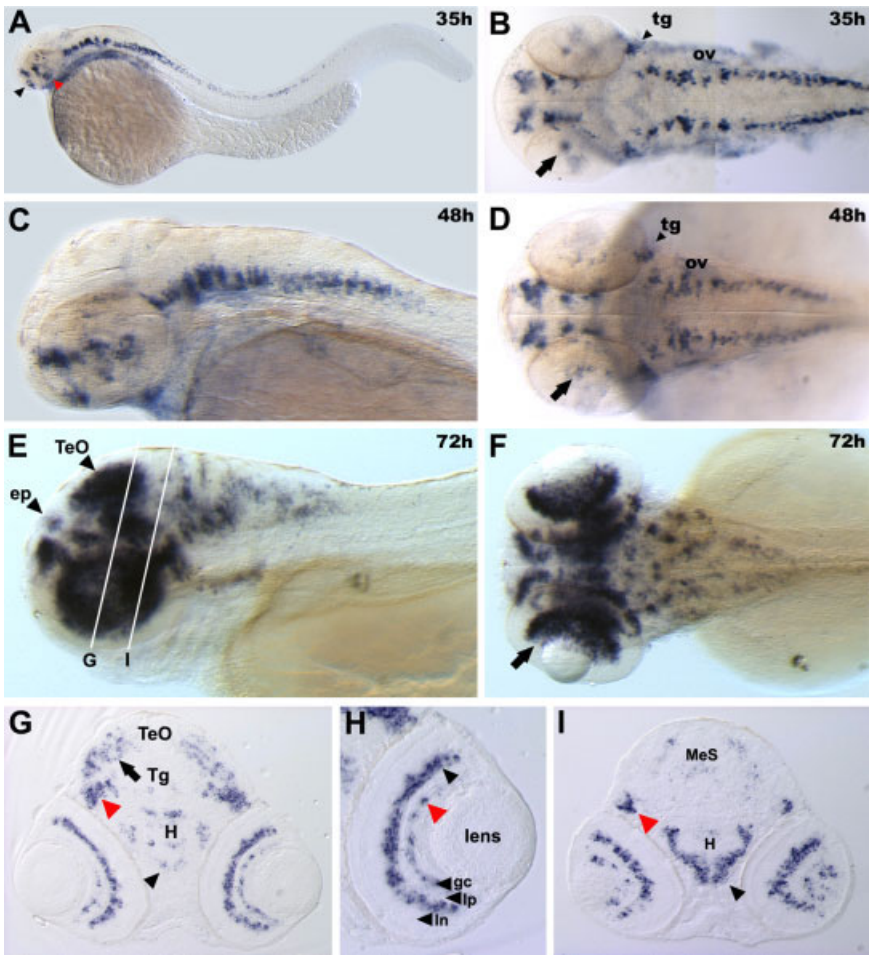

Fig. 3.

ber of *1G5z*-expressing cells is remarkably increased in the *mindbomb* mutant at 24 hpf when compared with wild-type embryos (Fig. 4A,B). Ectopic expression of *1G5z* in the neuronal tissues in *mindbomb* mutant embryos is obvious at the 20-somite stage, when *1G5z* transcription begins (data not shown). However, lack of *1G5z* expression in the ventral motor neurons in *mindbomb* further supports the distinct expression pattern of *1G5z* in the developing sensory and interneurons of the spinal cord. This result suggests that *1G5z* expression is directly or indirectly regulated by *mindbomb* during the zebrafish embryonic neurogenesis. The correlation between the function of *1G5z* with Delta-Notch signaling remains to be investigated further.

Previous reports showed that *neurogenin1* (*ngn1*) depletion caused complete loss of sensory ganglia and trunk sensory neurons without affecting interneurons and motor neurons in zebrafish embryos (Andermann et al., 2002; Cornell and Eisen, 2002). To further specify the *1G5z* expression domains in developing neuronal tissues, we injected *ngn1* morpholino (MO) and then analyzed the *1G5z* expression pattern. As shown in Figure 4C,D, *ngn1* injection completely abolished *1G5z* expression in the trigemi-

nal ganglion. Of interest, the lateral marginal expression of *1G5z* in the r2 is also abolished in the *ngn1* MO-injected embryos (Fig. 4C,D). However, hindbrain-specific expression of *1G5z* was relatively unaffected, except in r2, in the *ngn1* morphants (Fig. 4C,D). *ngn1* MO injection also completely eliminated *1G5z* expression in the caudal spinal cord, demonstrating restricted expression of *1G5z* in the posterior sensory neuron at the 24 hpf (Fig. 4E,F). In addition, *1G5z*-expressing interneuronal cells from the anterior to medial trunk were not sensitive to *ngn1* MO injection, further supporting that *1G5z* is exclusively expressed in the interneuron of anterior and coexpressed in both interneuron and sensory neuron of medial trunk (data not shown; Fig. 4F, arrow).

We further confirmed that the *1G5z*-expressing cells were postmitotic sensory and interneurons by incubating them with BrdU, which marks S-phase cells at 24 hpf (Fig. 5). As shown in Figure 5B–G, BrdU failed to incorporate into the cells where *1G5z* was strongly expressed. So, we verified that *1G5z* is exclusively expressed in the postmitotic sensory and interneurons but not in the neural precursors.

In conclusion, a novel zebrafish *1G5z* encodes a CaMK-like protein, and its expression pattern is dynamic and specified in the developing CNS throughout embryonic development. This article is the first report of dynamic expression of a CaMK-like gene during the early neuronal differentiation among the vertebrates.

EXPERIMENTAL PROCEDURES

Zebrafish Maintenance

Zebrafish and embryos were maintained essentially as described in the zebrafish book (Westerfield, 1995). Developmental stages were classified as hour postfertilization (hpf). Embryos were obtained after natural spawning and cultured at 28.5°C in fishwater containing 0.2 mM (PTU) to prevent pigmentation and 0.001% methylene blue to prevent fungi contamination.

cDNA Cloning and DNA Sequencing

Randomly selected cDNA clones (Clontech, catalog no. QL 40000AB) were analyzed by sequencing and in situ hybridization. Among the tissue specifically expressing cDNA clones, one encoded a novel member of the *1G5* (designated as *1G5z*). The full-length *1G5z* was subcloned using RT-PCR from 24 hpf embryos and then verified by sequencing analysis.

RT-PCR

Total RNA was isolated from the nine different stages of embryos using RNAsol B (Tel-Test. Inc.), and 3 μ*g* of total RNA was used for the RT reaction. The first strand of cDNA was synthesized using MMLV reverse transcriptase (Promega). The full-length of *1G5z* was amplified with forward (5′-GATGAATTCGCCATGCCATTCGGCTGTTTAAAACCTGG-3′) and reverse (5′-GATCCTCGAGTTAGCCGCTCTGCTCCTCCACTGTGTCC-3′) primers containing *Eco*RI and *Xho*I sites using *Pfu* DNA polymerase. The RT-PCR products were digested with *Eco*RI and *Xho*I restriction enzymes and then subcloned into the *Eco*RI and *Xho*I sites of pcDNA3 vector. This vector construct was used for further assay. For the analysis of temporal expression patterns of *1G5z*, 0.5-kb fragments were obtained by RT-PCR amplification of the first-strand cDNA (predenaturation, 94°C, 3 min; denaturation, 94°C, 30 sec; annealing, 58°C, 30 sec; elongation, 72°C, 30sec; 30 cycles). For the loading control, zebrafish β-*actin*–specific primers were used for the RT-PCR under the same condition. Two sets of primers were used to amplify *1G5z* and β-*actin* products: *1G5z*, 5′-CGTCCACAGGAACCTTAAGCTTGAG-3′ (forward) and 5′-AATTCTTCTCAATTTGAGCGCACAC-3′ (reverse); β-*actin*, 5′-GAGGAGCACCCCGTCCTGCTCAC-3′ (forward) and 5′-GATGGCTGGAACAGGGCCTCTGG-3′ (reverse). The amplified PCR products were separated on 1.5% agarose gel, and the products were visualized by ultraviolet light after ethidium bromide (0.4 μ*g*/ml) staining.

WISH

The full-length *1G5z* was PCR amplified then cloned into the multiple cloning sites of pcDNA3 (Invitrogen). Antisense digoxigenin- or fluorescence-labeled riboprobes were generated according to the instructions provided from the labeling kit (Roche). Single or double WISH was performed as previously described (Hauptmann and Gerster, 1994), with minor modification. Proteinase K treatment (10 μ*g*/ml) was performed for 3 to 20 min, depending on the stage of the embryos. The hybridized probes were detected using preabsorbed antibodies (Roche) diluted (1/2,000) in blocking solution (PBS, 0.1% Tween 20, 5% sheep serum). After staining, embryos were mounted in a 2:1 mixture of benzylbenzoate:benzylalcohol or 80% glycerol in PBS, then examined under the microscope (MZ16, Leica). Whole-mount stained embryos were subject to cryostat sectioning for detailed analysis following the protocol of Westerfield (1995).

Antisense MO Injection

Antisense MO complementary to the cDNA of *neurogenin1* (*ngn1*) was designed and synthesized by GeneTools (Corvallis, OR). The *ngn1* MO sequence is 5′-ACGGATCTCCATTGGATAACCTGG-3′. The MO was injected into the yolk of one- to four-cell stage embryos by using a pressure injection apparatus. Approximately 2 ng of MO per embryo was injected into more than 50 embryos for each experiment.

BrdU Labeling, In Situ RNA Hybridization, and Immunocytochemistry

Manually dechorionated embryos were labeled with BrdU by incubating them for 20 min on ice in a solution of 10 mM BrdU and 15% dimethyl sulfoxide in embryo medium (EM). The embryos were then placed in EM and incubated 20 min at 28.5°C and fixed using 4% paraformaldehyde in PBS. Embryos were processed for in situ RNA hybridization first to detect *1G5z* message. The stained embryos were immersed in 2 M HCl for 1 hr, and then processed for anti-BrdU im-

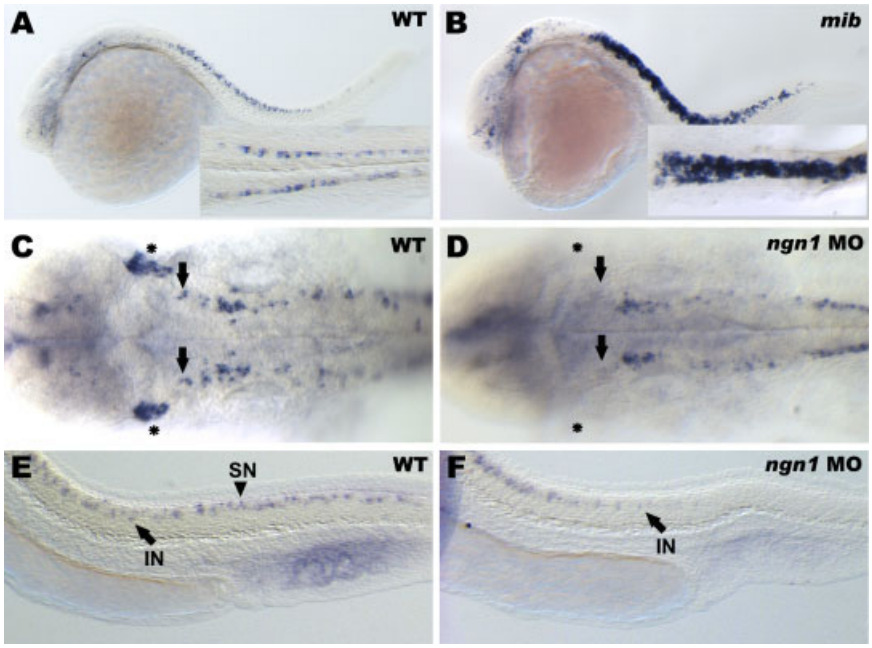

Fig. 4.

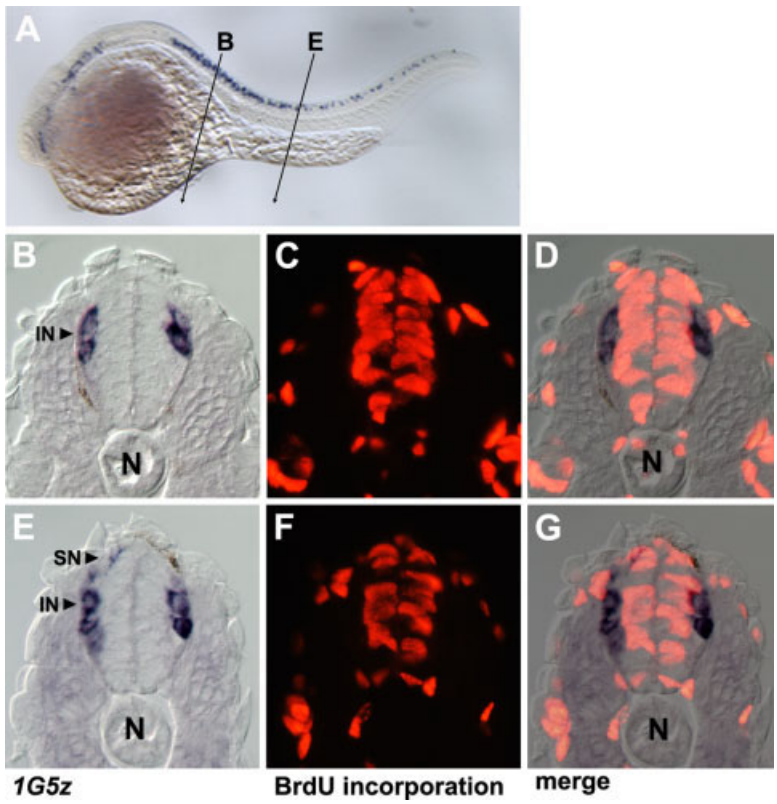

Fig. 5.

Fig. 4. Aberrant neuronal cell development in the absence of Delta-Notch signaling, or Ngn1. **A:** At 24 hours postfertilization (hpf), lateral view of wild-type embryo. Inset: High-magnification dorsal view of the spinal cord. **B:** *1G5z* expression pattern in *mib*^{ta52b} mutant. Inset: High-magnification dorsal view of the spinal cord. C–F: At the 24 hpf. **C:** Dorsal view of uninjected control embryo. *1G5z* transcripts are detected in trigeminal ganglion (asterisk) and anterior hindbrain (arrow). **D:** *ngn1* mopholino (MO) -injected embryo. Note the complete lack of *1G5z* expression in the trigeminal ganglion (asterisk) and anterior hindbrain (arrow). **E,F:** Lateral view of medial–caudal trunk of the same embryos of C and D. Note that *ngn1* MO injection abolished *1G5z* expression in the posterior sensory neuronal cells (arrowhead). Arrows indicate interneurons. IN, interneuron; SN, sensory neuron.

Fig. 5. *1G5z*-expressing cells are postmitotic neurons. **A:** Diagram showing the plane of transverse sections (10 μm) in B–D and E–G. **B:** *1G5z*-positive cells in the anterior spinal cord. **C:** Embryonic tissue in A incubated with bromodeoxyuridine (BrdU). **D:** Merged image of B and C. Note that *1G5z*-stained cells are negative for BrdU staining. **E:** *1G5z*-expressing cells in the medial trunk. **F:** Embryonic tissue in E treated with BrdU. **G:** Overlapping image of E and F. Note that *1G5z*-positive cells avoid BrdU incorporation. The embryo is fixed at 24 hpf. IN, interneuron; N, notochord; SN, sensory neuron.

munocytochemistry. For immunocytochemistry, we used mouse anti-BrdU antibody (G3G4; 1:1,000; Developmental Studies Hybridoma Bank, Iowa City, IA), and for fluorescent detection of antibody labeling, we used Alexa Fluor 568 goat anti-mouse conjugates (1:500; Molecular Probes). Embryos for sectioning were embedded in 1.5% agar/5% sucrose and frozen in 2 methyl-butane chilled by immersion in liquid nitrogen. Sections (10 μm) were obtained by using a cryostat microtome. In situ hybridization and fluorescence images were collected separately and combined using Adobe Photoshop (Adobe Systems, San Jose, CA).

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