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Expression and characterization of a brain-specific protein kinase BSK146 from zebrafish

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Abstract

We have previously identified a novel protein kinase, pk146, in the brain of *Tetraodon*. In the present study, we cloned the homologous protein kinase gene encoding a protein of 385 amino acid residues from zebrafish. The overall amino acid sequence and the kinase domain of zebrafish BSK146 shows 48% and 69% identity to that of rat sbk, a SH3-containing serine/threonine protein kinase. By whole-mount in situ hybridization and RT-PCR, the expression of bsk146 mRNA was mainly in the brain. To explore the in vivo function of BSK146 during zebrafish development, we used morpholino knockdown approach and found that BSK146 morphants displayed enlarged hindbrain ventricle and smaller eyes. Whole-mount in situ hybridization was further performed to analyze the brain defects in BSK146-MO-injected embryos. The expression of brain-specific markers, such as otx2, pax2.1, and krox20, was found normal in morphant embryos at 24 hpf, while expression of pax2.1 exerted changes in midbrain–hindbrain boundary and hindbrain in morphant embryos at 48 hpf. These data suggest that BSK146 may play an important role in later ventricle expansion in zebrafish brain development. Although the recombinant BSK146 protein produced in insect cells was active and could phosphorylate both histone H1 and histone 2B, the endogenous substrate of BSK146 in the embryonic brain of zebrafish is not clear at the present time and needs further investigation.

Keywords: Zebrafish; Protein kinase; Brain; Morpholino-knockdown; Brain development

Protein kinases play important roles in the regulation of several brain-specific functions, including neuronal differentiation, neuronal plasticity, long-term potentiation (LTP), long-term depression (LTD), and neurotransmitter release. In the mammalian central nervous system (CNS), neural activity is mediated by the actions of excitatory and inhibitory neurotransmitters. Glutamate is the major excitatory transmitter, while γ-aminobutyric acid (GABA) and glycine are the primary inhibitory neurotransmitters. These neurotransmitters bind to their receptors that are clustered at synapases and induce neurotransmission [1–4]. The activity-dependent changes in synaptic transmission by different neurotransmitters and their receptors have been shown to associate with long-term memory in the mature neuron [5].

The function of neurotransmitter receptor is regulated by protein phosphorylation. Several protein kinases are known to phosphorylate serine/threonine (Ser/Thr) residues of certain $GABA_A$ receptor subunits [6]. The function of synaptic GABA receptors in hippocampal CA1 pyramidal cells is regulated by cAMP-dependent kinase (PKA), whereas miniature GABAergic inhibitory postsynaptic currents (mIPSCs) in granule cells (GCs) can be augmented by Ca^{2+}/phospholipid-dependent protein kinase C (PKC)

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[7,8]. Interestingly, the brain seems to be the only tissue that expressed all 11 PKC isoforms, which can be classified into three groups: classical, novel, and atypical isoforms [9,10]. PKCγ is a member of the classical PKC group and is expressed mainly in the brain. Neural functions such as LTP and LTD require PKCγ [11]. On the other hand, Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) represents one of the most abundant Ser/Thr protein kinases in the mammalian brain, approximately 1% of the protein in brain [12]. CaMKII has broad substrate specificity and phosphorylates many neuronal proteins including receptor proteins and ion channel proteins [13]. A recent report has demonstrated that CaMKII directly binds to and phosphorylates the NMDA receptor subunits NR1 and NR2B [14].

Another protein kinase, cGMP-dependent kinase (PKG), and its substrate, G-substrate, have been shown to participate in the induction of LTD, which is an activity-dependent and long-lasting depression of synaptic transmission from parallel fibers onto Purkinje cells [15–17]. Both PKG I and PKG II, two members in the PKG family, are present in many brain regions [18]. Moreover, PKA, PKC, and CaMK II are required for the induction and the early-phase of the LTP, which is referred to a long-lasting enhancement in efficacy of synaptic transmission involved in learning and memory [19,20]. The aforementioned protein kinases are four major Ser/Thr protein kinases in the brain and were found to play important roles in the regulation of several brain-specific functions, including neuronal differentiation, neuronal plasticity, LTP, LTD, and neurotransmitter release. Therefore, novel protein kinases present in the brain have been pursued all the time.

Recently, zebrafish has become an important model organism for studies of functional genomics including neuronal plasticity and behavior. In this study, we isolated and characterized a zebrafish brain-specific protein kinase BSK146, which is homologous to a previously reported protein kinase from *Tetraodon* [21]. The kinase domain of BSK146 is also homologous to that of a rat SH3-containing protein kinase, SBK [22]. Through whole-mount in situ hybridization and RT-PCR, the expression of *bsk146* mRNA was mainly in the brain. Using morpholino approach, we showed that BSK146 morphants displayed enlarged hindbrain ventricle. Furthermore, in the absence of functional BSK146, the expression of specific markers for the midbrain–hindbrain boundary (MHB) and hindbrain, such as *pax2.1* and *krox 20*, was affected. Taken together, this study demonstrates that BSK146 is required for brain development in zebrafish embryo.

Materials and methods

The restriction enzymes were purchased from the Promega Biosciences (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA). Chemical Compounds were purchased from the Merck (Darmstadt, Germany) and Sigma (MO, USA).

Fish. Zebrafishes (*Danio rerio*) were maintained at 28 °C on a 14 h light/10 h dark cycle. Embryos were incubated at 28 °C and different developmental stages were determined according to the Zebrafish Book [23].

Total RNA isolation and first strand cDNA synthesis. Total RNA was isolated from the fertilized eggs at different stages (12-, 24-, 36-, 48-, and 144 h post-fertilization), various tissues (brain, gill, heart, intestine, liver, ovary, and testis) of zebrafish (*Danio rerio*), using the RNAzol reagent (Tel-Test, Friendswood, TX, USA) according to the instructions of the manufacturer. After treated with RQ1 RNase-Free DNase (Promega Biosciences, WI, USA), 50–100 μg of total RNA was used for the first strand cDNA synthesis in a 25 μl reaction mixture containing 10 pmol oliog(dT) primer, 30 U RNasin (Promega Biosciences, WI), 1 mM dNTP, 10 mM dithiothreitol, and 300 U Superscript II RT (Invitrogen Life Technologies, CA, USA). The reaction mixture was incubated at 42 °C for 1 h. Two microliters of the cDNA product was used for subsequent PCR amplification.

5′-RACE and 3′-RACE of zbsk146 from zebrafish. The 5′ and the 3′ ends of the zebrafish mRNA were obtained by the RACE PCR technique using the Marathon cDNA amplification kit (Clontech Lab., CA, USA). The library of adaptor ligated double-strand cDNA was prepared for PCR. The PCR program for both 5′- and 3′-RACE was 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, and the final extension at 72 °C for 15 min. The 5′-RACE was performed with an adaptor-specific sense primer, AP1: 5′-CTA ATA CGA CTC ACT ATA GGG C-3′, and a zBSK146 antisense primer, zBSK146-R1: 5′-CTC CCG CAG GAA GCT CTT CAG CTT GG-3′. The PCR product was reamplified with AP2 primer: 5′-ACT CAC TAT AGG GCT CGA GCG GC- 3′ and a nested zBSK146 antisense primer, zBSK146-R2: 5′-CTC CAG GTT CTG AGC GGT GTA GAG C-3′. For 3′-RACE, cDNA was amplified with a zBSK146 sense primer, zBSK146-F1: 5′-CAC CGC TGG ATG CTT GAT GGA ACT AGC-3′, and an oligo(dT) primer. The RACE reaction products were cloned into pGEM-T easy vector (Promega Biosciences, WI, USA) and subjected to the sequence analysis.

Isolation of full-length bsk146 cDNA from zebrafish (Danio rerio). In order to isolate the cDNA covering the complete open-reading frame (ORF) of zebrafish *bsk146* (1158 bp, 385 amino acid residues), according to the sequence of zebrafish EST (Accession No. AW281000), PCR amplification was performed in a 50 μl reaction mixture containing 2 μl of first strand cDNA, 0.5 μg of forward primer (zBSK146-F, 5′-ATG AGC TCG TCT CCG GTG GTT TCC-3′) and reverse primer (zBSK146-R, 5′-TTA GAC GCA GAT TTC TAT AGG CGT CGT-3′), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U *ExTaq* (Takara Shuzo, Shiga, Japan). The sample was incubated in a thermal cycler (Hybaid MultiBlock System, Hybaid Limited, MA) at 96 °C for 2 min, 40 cycles of 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and the final extension at 72 °C for 15 min. The PCR products were ligated into the pGEM-T easy vector (Promega Biosciences, WI, USA), and individual clone was subjected to sequence analysis.

DNA sequence analysis. DNA sequence analysis was performed by using PRISM Ready Reaction Big-Dye Termination Cycle sequencing Kit (Applied Biosystems, CA, USA) on an Applied Biosystems 310 automated DNA sequencer. Sequence analysis was performed by using the Clustal X [24] and GenDoc [25] programs.

RT-PCR analysis of zebrafish bsk146 mRNA. PCR amplifications were performed in a 50 μl reaction mixture containing 200 ng zBSK146 primers (zBSK146-RT-F, 5′-CCT GTT TGA CAT CAT TCC ACC AC-3′ and zBSK146-RT-R, 5′-CAC ACA GCG CTT CGC CAC CGG CTC-3′) or zebrafish β-actin primers (zAct-F, 5′-CCT CCG GTC GTA CCA CTG GTA T-3′ and zAct-R, 5′-CAA CGG AAG GTC TCA TTG CCG ATC GTG-3′), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U *ExTaq* (Takara Shuzo, Shiga, Japan). The samples were incubated in a thermal cycler (Hybaid MultiBlock System, Hybaid Limited, MA, USA) at 96 °C for 3 min; 30–45 cycles of 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and the final extension at 72 °C for 5 min. All the PCR products were separated on 1% agarose gel.

Construction of transfer vectors for the baculovirus-mediated expression of his-tagged BSK and its mutant. Fragments of *bsk146* cDNA were amplified with primer *Eco*RI-zBSK146-F, 5′-TCA GAA TTC ATG AGC

TCG TCT CCG GTG GTT-3′ and *Not*I-zBSK146R, 5′-GGG CGG CCG CTT AGA CGC AGA TTT CTA TAG G-3′, and ligated with a pAcSG-His-NTA vector (BD Biosciences Pharmingen, CA, USA) at the *Eco*RI/*Not*I site. The resultant plasmid, termed pAcSG-zBSK146, expresses a his-tagged BSK146 protein. Site-directed mutagenesis for an inactive form of BSK146, BSK146-K61R, was performed by PCR as follows. The oligonucleotide zBSK146-KR-F 5′-GCA AAA TGG CTC TGA GGT TTC TGA AGA AG-3′ was used to introduce a single A to G nucleotide change (underlined) altering the Lys61 codon to a Arg codon in zebrafish *bsk146* cDNA. The reverse primer was zBSK146-KR-R, 5′-CTT CTT CAG AAA CCT CAG AGC CAT TTT GC-3′. The final PCR product was digested with *Eco*RI and *Not*I, and replaced the corresponding part of the wild-type sequence in pAcSG-bsk146. The nucleotide sequences of all cloned cDNAs were verified by sequencing.

Expression and partial purification of his-tagged BSK146-FL and the BSK146-K61R mutant. Sf9 cells were cultured at 28 °C in SFM-900II medium (Invitrogen Life technologies, CA, USA). Recombinant baculovirus was prepared using a Bacvector-1000 Baculovirus Expression System (Novagen, EMD Biosciences, Darmstadt, Germany) according to the manufacturer's instructions. Exponentially growing Sf9 cells ($<2\times10^8$) were infected with the recombinant baculovirus and cultivated at 27 °C for 2 days. The cells were harvested and lysed in 20 mL lysis buffer [50 mM Tris/HCl, pH 8.2, 5 mM 2-mercaptoethanol, 0.5 M NaCl, 1% Nonidet P-40, 10 mM imidazole, and protease inhibitor cocktail (Boehringer–Mannheim, GmbH, Germany)]. The lysate was homogenized on ice by sonication twice for 60 s and centrifuged at 10,000*g* for 15 min. The supernatant was applied to 2 mL Ni^{2+}-chelating Agarose (Qiagen, GmbH, Germany) pre-equilibrated with binding buffer [50 mM Tris/HCl, pH 8.2, 5 mM 2-mercaptoethanol, 0.5 M NaCl, 10% (v/v) glycerol, and 10 mM imidazole]. The bound protein was washed with washing buffer [50 mM Tris/HCl, pH 8.2, 5 mM 2-mercaptoethanol, 0.5 M NaCl, 10% (v/v) glycerol, and 40 mM imidazole]. The recombinant BSK proteins were identified by Western blotting.

In vitro kinase assay. Partially purified BSK was incubated at 30 °C for 30 min in 50 mM Tris/HCl, pH 9.0, containing 10 mM $MgCl_2$, 1 mM ATP, 50 mM NaF, 1 mM sodium orthovanadate, 2 μg histone H1, H2A, H2B, H3 or H4 (Boehringer–Mannheim GmbH, Germany), and 1 μCi [γ-^{32}P]ATP (3000 Ci/mmol; NEN Life Science Products, MA, USA). The reaction was stopped by the addition of 30 μl of electrophoresis sample buffer and the reaction mixtures were then subjected to SDS–PAGE. Detection of the radioactivity was performed using autoradiography.

Production of anti-zBSK146 antibodies and Western blot analysis. To produce anti-BSK146 antibodies, the BSK146 C-terminal of cDNA was amplified with primer *Sph*I-zBSK146-EC-F: 5′-CGG CAT GCA TGC CGT CTG ATA CTT TCT ACG AG-3′ and *Hin*dIII-zBSK146-EC-R: 5′-GAA AGC TTG ACG CAG ATT TCT ATA GGC GTC-3′ and ligated into *Sph*I/*Hin*dIII digested pQE30 vector. The his-tagged Bsk146 fusion protein was purified from a Ni–NTA agarose and used to immunize rabbit. The purified protein was used to immunize New Zealand White rabbits by the intrasplenic immunization method [26]. Immunoblot analyses were performed by separating the recombinant BSK proteins on 10% SDS–PAGE, followed by transferring to a PVDF membrane (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 3% skimmed milk in PBS and then incubated with anti-zBSK146 antibody (1:1000 dilutions) at 4 °C, overnight. After washing with PBS-T (0.2% Tween 20 in PBS) for three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000, Jackson ImmunoResearch Laboratories, Pennsylvania, USA) at room temperature for 2 h. The membranes were washed as described as above, and signals were detected using enhanced chemiluminescence (ECL) (NEN Life Science Products, MA, USA).

Morpholino injection. Antisense morpholinos (MOs) were obtained from Gene Tools (Philomath, OR, USA). The MO sequence of zebrafish *bsk146* was as follows: 5′-GGA AAC CAC CGG AGA CGA GCT CAT C-3′. The MO was dissolved in 1× Danieau solution containing 0.5% phenol red to 0.3 mM and 1.5 nl per embryo was injected into embryos at the 1–2 cell stage. Embryos were observed under an Olympus IX70-FLA inverted fluorescence microscope. Images were taken by using the SPOT digital camera system (Diagnostic Instruments, Sterling Heights, Michigan, USA) and assembled by PhotoShop program (Adobe System, CA, USA).

Whole-mount in situ hybridization. Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes. Riboprobes against *bsk*146, *pax2.1* [27], *krox20* [28], and *otx2* [29] were made from DNA templates, which were linearized and transcribed with either SP6 or T7 RNA polymerases. Hybridizations were performed following the standard protocol [30]. Embryos were observed under an Zeiss Axioplan 2 imaging universal microscope (Carl Zeiss Meditec, Jena, Germany). Images were taken by using the SPOT digital camera system (Diagnostic Instruments, Sterling Heights, Michigan, USA) and assembled by PhotoShop program (Adobe System, CA, USA).

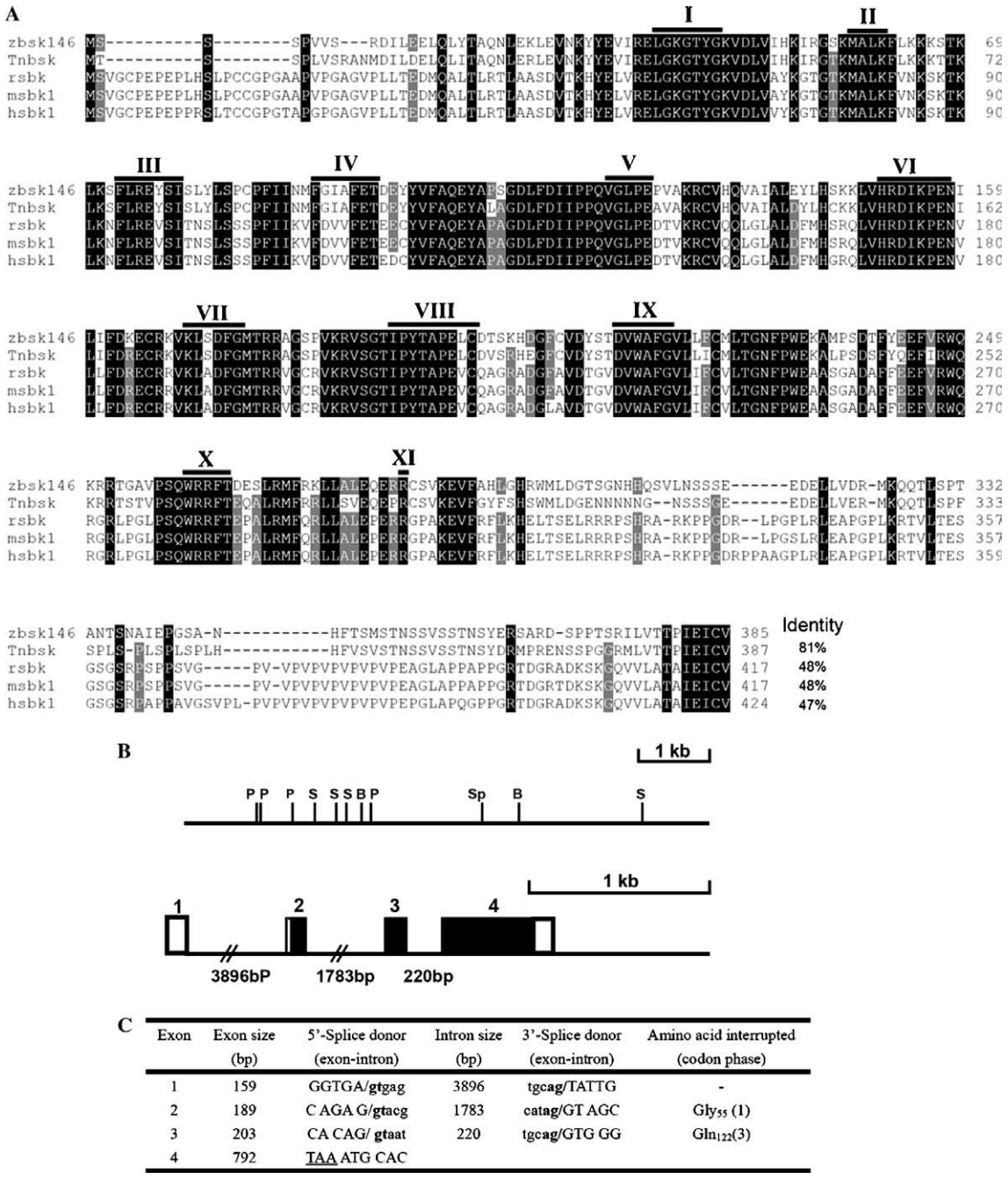
Result

Isolation of zebrafish BSK146 cDNA

In a previous study, we have isolated a brain-specific protein kinase *bsk*146 from *Tetraodon* [21]. To identify zebrafish cDNA related to pufferfish *bsk*146, we used the coding region of the pufferfish *bsk*146 to search the GenBank database for related expression sequence tag (EST) sequences by using the program tBLAST. We found a zebrafish EST clone (Accession No. AW281000) related to pufferfish *bsk*146. By using 5′- and 3′-RACE to obtain the 5′- and 3′-untranslated regions (UTR), we assembled all sequences to obtain a 2428 bp cDNA with an open-reading frame of 1158 bp encoding a protein of 385 amino acid residues. The complete sequence was deposited in GenBank with an accession number of AF265347. We searched the currently available protein and nucleic acid database using the BLAST program [31] and found no sequences with significant similarity except rat SH3-binding kinase (sbk) [22], *Tetraodon* BSK (Accession No. CAF91891), mouse sbk1 (Accession No. BC031759), and human sbk1 (Accession No. AY874862). The overall deduced amino acid sequence of zebrafish BSK146 shows 81%, 48%, 48%, and 47% identity to that of *Tetraodon* BSK, rat sbk, mouse sbk1, and human sbk1, respectively (Fig. 1A). It is worth to note that the protein kinase domain of zBSK146 displays 87% identity (93% similarity) and 69% identity (83% similarity) to those of *Tetraodon* BSK and all three mammalian sbks, respectively. Therefore, all these proteins form a new protein kinase family with distinct C-terminal region.

Genomic structure of zebrafish BSK146 gene

We then used the 2428 bp of zebrafish *bsk*146 cDNA (Accession No. AF265347) to perform an online BLAST search of the GenBank database. The zebrafish *bsk146* cDNA matched 4 non-contiguous regions in the Plus/Minus strand orientation of a 158,636 bp zebrafish BAC clone CH211-246B11 (GenBank Accession No. CR450843) at a level of 98–100% identity. Subsequently, a BLAST 2 sequence comparison of BAC CH211-246B11 with the zebrafish *bsk*146 cDNA indicated that the *bsk146* cDNA is contained within 4 putative exons and 3 introns spanning



C

Exon	Exon size (bp)	5'-Splice donor (exon-intron)	Intron size (bp)	3'-Splice donor (exon-intron)	Amino acid interrupted (codon phase)
1	159	GGTGA/gtgag	3896	tgcag/TATTG	-
2	189	C AGA G/gtacg	1783	catag/GT AGC	Gly_{55} (1)
3	203	CA CAG/ gtaat	220	tgcag/GTG GG	Gln_{122}(3)
4	792	TAA ATG CAC			

Fig. 1. Alignment of amino acid sequences of zebrafish BSK146 with other sbk proteins and genomic organization of zebrafish *bsk*146 gene. (A) Multiple alignment of orthologous zebrafish BSK146 sequences using CLUSTAL X: The deduced amino acid sequence of the zebrafish BSK146 (zbsk146; GenBank Accession No. AF265347) was compared with the sequences of sbks from rat sbk (rsbk; AB010145), mouse sbk1 (msbk; BC031759), human sbk1 (hsbk1; AY874862), and *Tetraodon* BSK (Tnbsk; CAF91891). Identical residues in all five proteins are highlighted. Dashes are introduced to maximize alignment. The percentage amino acid sequence identities between zebrafish BSK146, Tetraodon BSK, and other SBKs are shown at the end of the sequences. Conserved kinase domains are overlined and subdomains are numbered as described previously [40]. (B) Genomic organization of zebrafish *BSK*146 gene. Exons are indicated by boxes numbered from 1 to 4. Solid boxes indicate the *BSK*146 coding region whereas open boxes represent the 5′- and 3′-untranslated regions. Introns and the 3′-flanking regions are indicated by the solid lines. The restriction map was shown above the genomic structure. The entire gene spans at least 8 kb in length and contains 4 exons. (C) Exon–intron organization of zeberafish *bsk*146 gene. The amino acid interrupted (codon phase) also indicated. P, *Pst*I; S, *Sal*I; B, *Bam*HI; Sp, *Sph*I.

at least 8 kb (Fig. 1B). Using these putative exons as a model, a sequence alignment was produced such that each intron concurred with the GT/AG intron donor/acceptor site rule. Exon 1 contains the 5′-UTR, while exon 2 contains the putative translation initiation site. Exon 2 contains 26 bp of 5′-UTR and 159 bp of the first coding sequences of the *bsk*146 cDNA. Exon 3 contains the next 203 bp of the coding sequences while exon 4 contains the last 792 bp of the coding sequences and 1084 bp of 3′-UTR. The size of introns varied considerably, ranging

from 220 bp (intron 3) to 3896 bp (intron 1) (Fig. 1C). The zebrafish *bsk*146 gene spans approximately 8 kb (Fig. 1B).

Expression profiles of bsk146 mRNA in adult tissues and developing embryos

In order to determine the expression pattern of zebrafish *bsk*146 mRNA by RT-PCR, zebrafish embryos at different developmental stages and various tissues from adult zebrafish were collected and used to isolate total RNA for cDNA preparation. Our data showed that the *zbsk*146 mRNA was readily detected in embryos at 24 hpf and gradually increased from 36 to 144 hpf (Fig. 2A). In adult zebrafish, *bsk*146 mRNA is mainly expressed in brain (Fig. 2B). The expression of β-actin transcript was also determined as well and served as internal control (Fig. 2).

Differential expression of zbsk146 mRNA during embryogenesis

In order to elucidate the role of BSK146 in the development of the nervous system, we examined the temporal and spatial patterns of *zbsk146* expression using whole-mount in situ hybridization. In 24, 36, 48, 72, 96, and 120 hpf embryos (Fig. 3A–F), *bsk146* mRNA was expressed predominantly in the developing neural structures including eye, forebrain, midbrain, and hindbrain, which is consistent with our previous data that zebrafish *bsk*146 expressed in adult brain (Fig. 2).

Effects of BSK146 knockdown on MHB structure and hindbrain integrity during zebrafish development

Morpholino (MO)-mediated knockdown of genes in zebrafish embryos has become a routine and efficient method to provide information about gene function in vivo [32]. To examine the function of *bsk146* in vivo, we injected BSK146 MO into zebrafish embryos at 2–4 cell stage. Embryos injected with BSK146 MO, designated as morphants, showed no early developmental defects at 24 hpf (Fig. 4A and A′). However, the morphants at 48 hpf displayed smaller eyes and enlarged hindbrain ventricle (Figs. 4B′ and D′).

To further analyze the brain defects seen in morphants, in situ hybridization was performed with gene markers specific for the forebrain and midbrain (*otx2*; [29]), hindbrain (*krox20*, [28]), and midbrain and MHB (*pax2.1*; [27]). The expression of these genes was identical in wild-type and morphant embryos at 24 hpf, indicating that at early stage of development the entire brain is not affected by knockdown of BSK146 (data not shown). However, at 48 hpf, expression of *pax*2.1 was changed in MHB in morphant embryos (Figs. 4E′ and F′), while in the hindbrain *pax*2.1 expression signal was diffused and broadly distributed (Fig. 4F′) compared with compact expression in wild-type embryos (Fig. 4F). Furthermore, although the expression level of *krox*20 remained normal with two parallel bands of expression in rhombomers 3 and 5 (Figs. 4H and H′), the pattern of r3 was changed, such that *krox*20 expression signal in r3 on both sides of the midline became closer than that in r5 (Figs. 4H and H′). These data indicated that both MHB shape and hindbrain integrity were affected by the enlarged hindbrain ventricle. The alternation of the expression pattern observed in the brain-marker genes indicates that BSK146 may play an important role in the later stage of ventricle expansion during zebrafish brain development [33].

Expression and biochemical characterization of the zebrafish BSK146 protein

To verify the kinase activity of the *BSK*146 gene product, recombinant BSK146 protein was produced by using a baculovirus system. Expression vectors for recombinant His-tagged BSK146 protein (z146FL) and an inactive form of the BSK146 protein (K61R, z146KR) were constructed and used to infect SF9 insect cells under the control of the polyhedrin promoter [34]. The z146FL and z146KR proteins were purified from soluble fraction of insect cell extracts using Ni^{2+}–NTA agarose beads[34]. Both z146FL and z146KR proteins were expressed as 50 kDa as shown by SDS–PAGE analysis (Fig. 5A). The purified proteins were subjected to in vitro kinase assay using

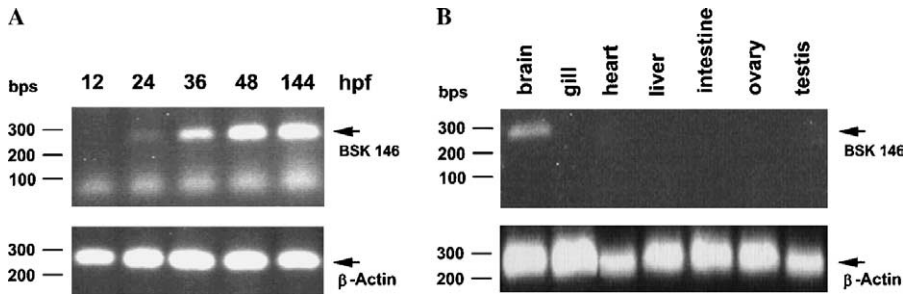


Fig. 2. Expression profile of zebrafish *bsk*146 mRNA in embryos during development (A) and adult tissues (B). RT-PCR was performed with a pair of primers to produce a DNA fragment of 290 bp. Positions of *bsk*146 transcript are indicated by arrowheads on the right. The developmental profile of *bsk*146 expression was examined in embryos from 12 to 144 hpf. β-Actin bands were used to normalize the amount of cDNA prepared from different tissues and different developmental stages.

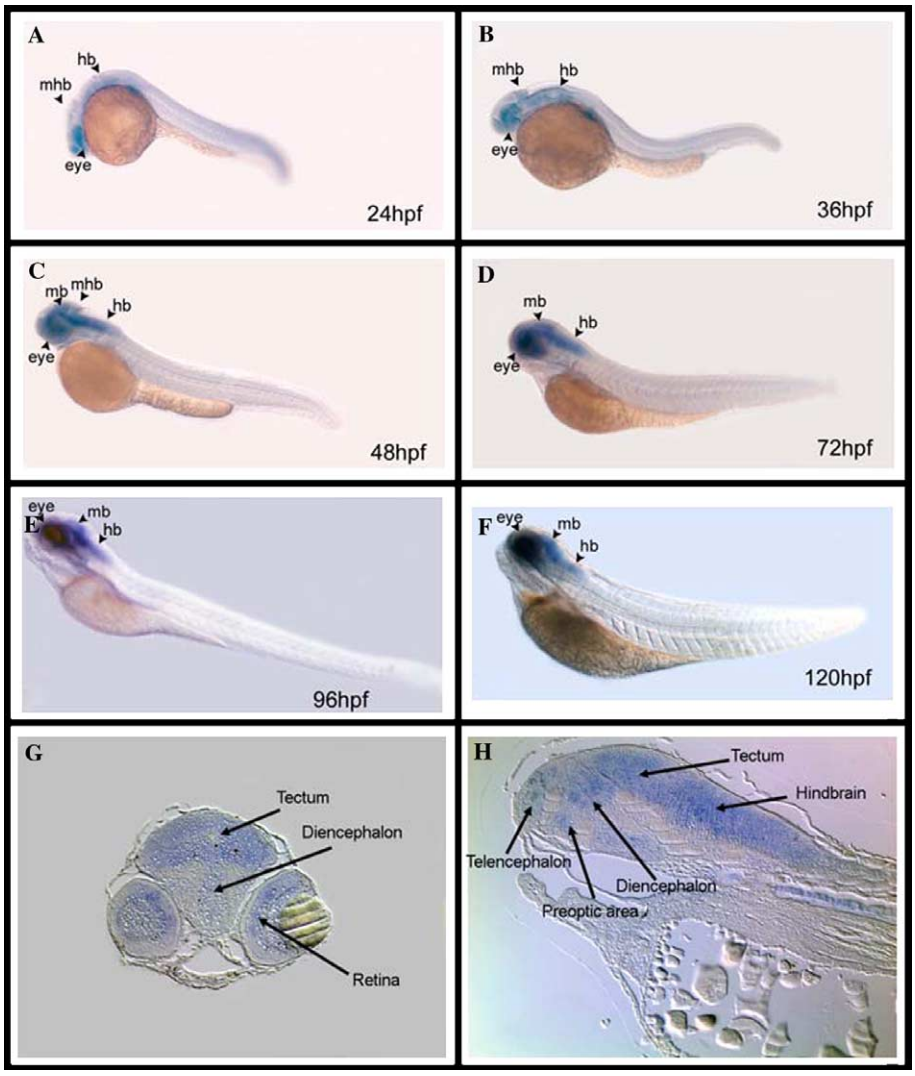


Fig. 3. Developmental expression pattern of zebrafish *bsk*146 mRNA. The *zbsk*146 transcripts were observed in the eye, midbrain, and hindbrain. Whole-mount in situ hybridization with antisense *zbsk*146 at the following developmental stages: (A) 24 hpf; (B) 36 hpf; (C) 48 hpf (D) 72 hpf; (E) 96 hpf; (F) 120 hpf. The images were taken from lateral view, with anterior to the left and dorsal to the top. Image analysis of cross (G) and sagittal sections (H) of the head confirmed that *z146bsk* mRNA is expressed in many parts of the CNS, including retina, telencephalon, preoptic area, diencephalon, tectum, and hindbrain in the embryos at 72 hpf (see arrows in G and H). mb, midbrain; mhb, midbrain–hindbrain boundary; hb, hindbrain.

artificial substrates. The recombinant z146FL protein was active by possible autophosphorylation and could phosphorylate histone H1 and histone 2B (Fig. 5B). The recombinant z146KR protein was used as a negative control and had no kinase activity.

Discussion

In this study, we cloned a brain-specific protein kinase gene *bsk146* from zebrafish, which is highly homologous to a previously reported protein kinase from *Tetraodon* [21]. Its kinase domain shows 69% identity (83% similarity) to those of mammalian sbks (Fig. 1). Rat sbk is a serine/threonine protein kinase containing a C-terminal proline-rich region that is proposed to bind to a protein bearing an SH3 domain[22]. However, the kinase domain of BSK146 displays low identity ranging from 15% to 30% to those of four major Ser/Thr protein kinases present in the brain including mammalian and zebrafish PKA, PKC, PKG, and CaMKII. Therefore, these data suggest that fish BSK146 and mammalian sbks may form a new protein kinase family with distinct C-terminal region.

*Bsk*146 mRNA is predominantly expressed in the developing brain as shown in Figs. 2 and 3. During development, *bsk146* mRNA was readily detected in embryos at 24 hpf and gradually increased after 36 hpf (Fig. 2A), suggesting that BSK146 protein may be involved in the control of neuronal proliferation or migration in the brain of zebrafish embryos. We further provided evidence about the function of BSK146 by MO-knockdown experiment in zebrafish embryos. Our data showed that there was no early developmental defect at 24 hpf (Figs. 4A and A′), but the morphants at 48 hpf displayed enlarged hindbrain ventricle (Fig. 4 B′). Analysis of brain-specific gene markers

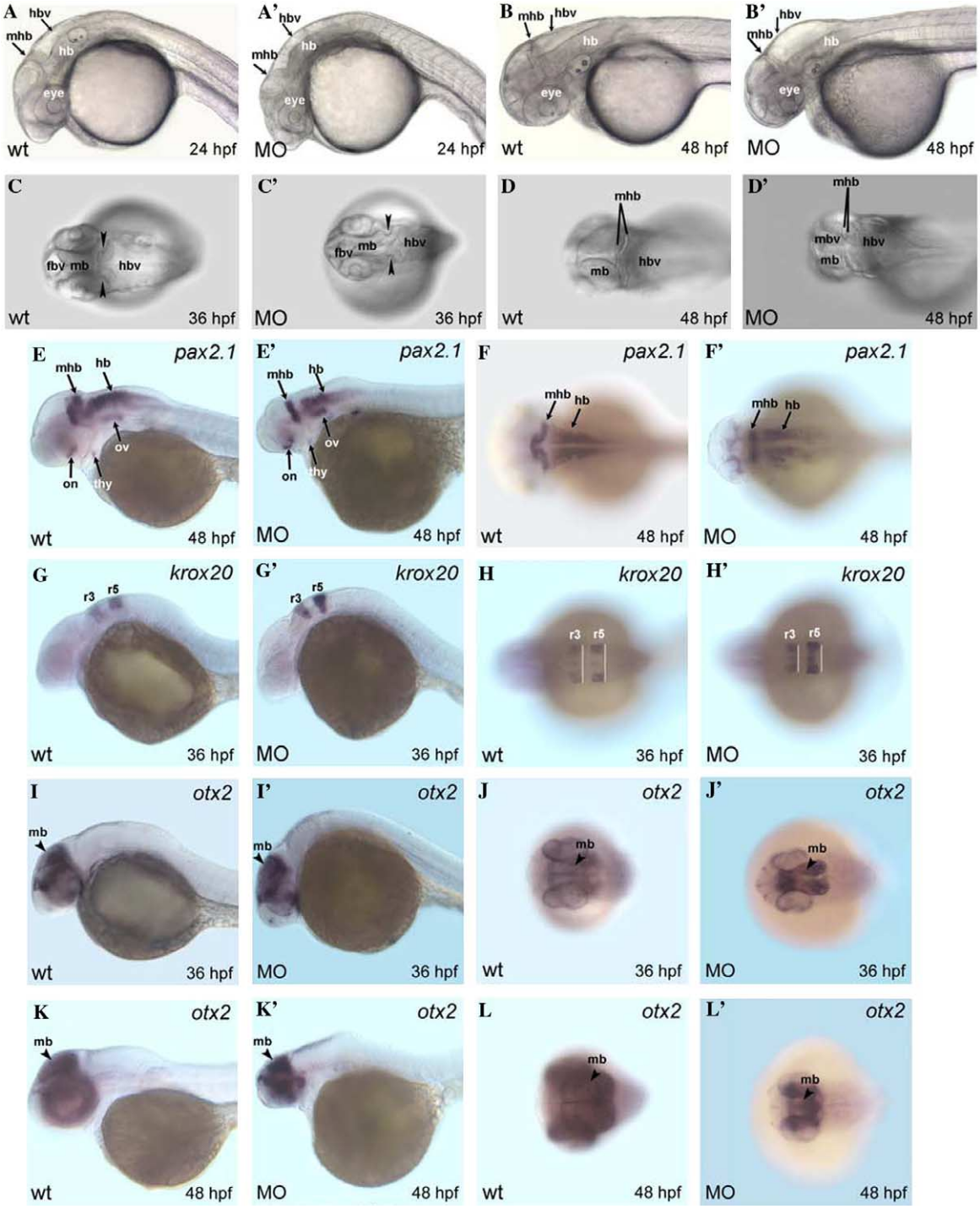


Fig. 4. Characterization of zebrafish *bsk146* morphants. Embryos injected with a dose of 6 pg zebrafish *bsk146* morpholino (MO) were allowed to develop until 24 hpf (A′), 36 hpf (C′, G′, H′, I′, and J′) or 48 hpf (B′, D′, E′, F′, K′, and L′). The images were taken as either lateral view with anterior to the left and dorsal to the top or dorsal views with anterior to the left. The morphants appeared normal at 24 hpf (A, A′, C, and C′, lateral view). Arrowheads in (C and C′) indicate mhb. At 48 hpf, the morphants exhibited morphological defects including smaller eyes and enlarged hindbrain ventricle (hbv) (B and B′, lateral view) as well as phenotypic change of midbrain–hindbrain boundary (mhb) as shown by forked arrows (D and D′, dorsal view). The expression levels of the following marker genes were examined by whole-mount in situ hybridization: *pax2.1* (E and E′, lateral view; F and F′, dorsal view), *krox20* (G and G′, lateral view; H and H′, dorsal view), and *otx2* (I, I′, K, and K′, lateral view; J, J′, L, and L′, dorsal view). Expression of *pax*2.1 was affected in mhb and hindbrain (hb, F and F′) in morphants at 48 hpf. In morphants at 36 hpf, the *krox*20 expression remained normal with two parallel bands of expression in rhombomers 3 and 5 (G and G′, lateral view; H and H′, dorsal view), but whiteline length in r3 was shorter than that in r5 (H and H′). Expression of *otx2* in morphants at 36 hpf (I and I′, lateral view, J, and J′, dorsal view) and 48 hpf (K, K′, lateral view, L and L′, dorsal view) appeared normal. Other abbreviations are used as follows: mb, midbrain; mbv, midbrain ventricle; on, optic nerve; thy, thymus; ov, otic vesicle.

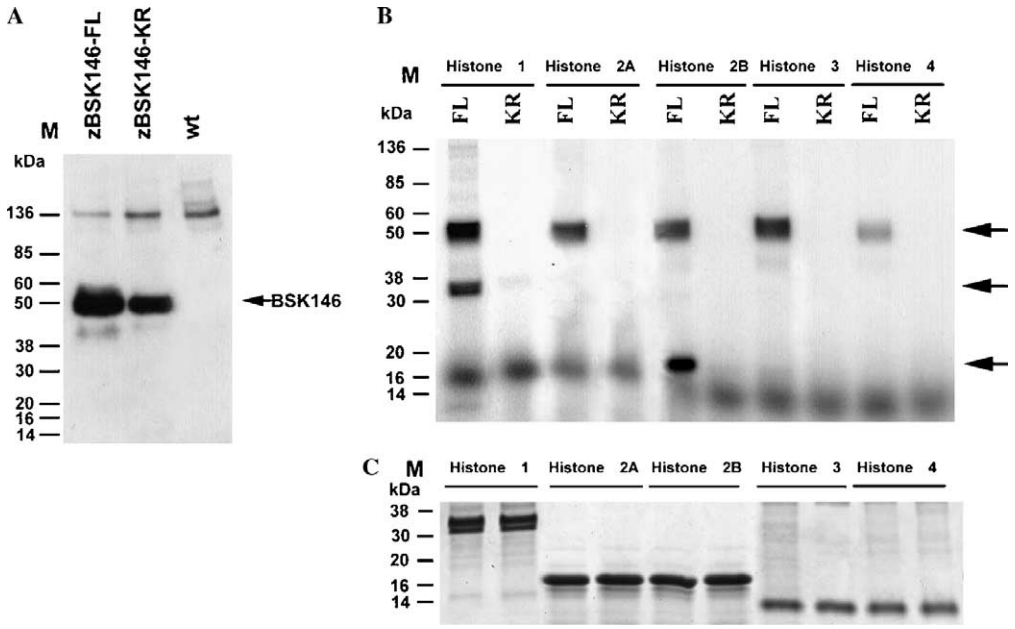


Fig. 5. Expression and kinase activity of zebrafish BSK146. (A) Expression of wild-type and kinase-dead of BSK146 in insect cells by recombinant baculovirus. Sf9 cells were infected with two different recombinant baculoviruses (lanes 1 and 2) and wild-type baculovirus, AcMNPV (lane 3), respectively. After 72 h, cells were harvested, lysed, and separated into Triton-soluble fractions. These fractions were further bound to Ni^{2+}–agarose gels and equivalent amounts of bound proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with polyclonal antibodies specific for kinase domain of BSK146. Positions of prestained molecular mass standards and their apparent sizes, in kilodaltons, are indicated on the left. (B) In vitro kinase activity of BSK146. Approximately 1 μg of recombinant zBSK146-FL or zBSK146-KR proteins was used in each kinase reaction with 2 μg histone 1, 2 A, 2B, 3 or 4 as substrates. The kinase reactions were stopped by adding SDS sample buffer and subjected to SDS–PAGE analysis. Substrate phosphorylation was detected by autoradiography and phosphorylated products are indicated by arrows. (C) The reaction mixtures were resolved by SDS–PAGE and stained with Coomassie blue to indicate the amount of each substrate.

indicated that expression of *pax*2.1 was changed in MHB and hindbrain in morphant embryos at 48 hpf (Figs. 4E′ and F′), suggesting that BSK146 possibly play important roles in the MHB formation/maintenance and hindbrain integrity. Mutants with similar enlarged hindbrain ventricles have been reported in a large-scale mutagenesis screen in zebrafish [35]. Different brain-specific gene markers, such as *hlx1* [36] and *sonic hedgehog* [37], have been used to demonstrate the abnormal architecture of mutant brains and the expansion of MHB. However, they have not been studied further. Whether BSK146 morphants are related to these mutant zebrafish, further experiments are needed to perform including injection of *bsk146* mRNA into individual mutant zebrafish embryos to rescue the phenotype.

In zebrafish, the embryonic brain begins as a single tube before 17 hpf and expands into primary forebrain, midbrain, and hindbrain ventricles in the following 4 h. Three steps independent of circulation have been proposed within this period including epithelial morphogenesis, lumen inflation, and regional cell proliferation [33]. The Na^+K^+-ATPase (Atp1a1a.1) protein has been postulated to play important roles in the initial brain ventricle lumen inflation to create osmotic gradient that drives movement of water into ventricles. A later ventricle expansion requires circulation at 36 hpf. In this study, the expression pattern of pax2.1 in MHB is correlated to the phenotype in BSK146 MO-injected embryos at 48 hpf (Figs. 4E′ and F′). Therefore, it appears that the MHB shape in morphants has been changed by the pressure from the enlarged hindbrain ventricle, suggesting that BSK146 may be required for later ventricle expansion in the development of zebrafish brain.

Although the artificial substrates, histone H1 and histone 2B, can be phosphorylated by recombinant BSK146 protein (Fig. 5B), the endogenous substrate of BSK146 in the embryonic brain remains unknown. It is possible to identify the target protein by examining the protein expression profile in the brain between the wild-type and BSK146 MO-injected embryos at 48 hpf through proteomic approach [38]. Alternatively, we can use BSK146 protein as a bait to fish out the interacting protein from an embryonic brain cDNA library by yeast two-hybrid screening system [39]. Taken together, we cloned a brain-specific protein kinase from zebrafish and found that it may play an important role in later ventricle expansion in zebrafish brain development.

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