Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 767–775
BBRC
www.elsevier.com/locate/ybbrc

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

Chih-Ming Chou [a,*], Yi-Chung Chen [b], Ming-Ting Lee [b], Gen-Der Chen [c], I-Ching Lu [b], Shui-Tsung Chen [b], Chang-Jen Huang [b,d,*]

[a] *Department of Biochemistry, Taipei Medical University, Taipei, Taiwan*
[b] *Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*
[c] *Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan*
[d] *Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan*

Received 12 December 2005
Available online 27 December 2005

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)

* Corresponding authors. Fax: +886 2 2788 9759.
E-mail addresses: cmchou@tmu.edu.tw (C.-M. Chou), cjibc@gate.sinica.edu.tw (C.-J. Huang).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.12.090

[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