Comparative Biochemistry and Physiology, Part B 200 (2016) 36–43

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Native recombinant kisspeptin can induce *gnrh1* and *kissr2* expression in *Paralichthys olivaceus in vitro*☆

Huayu Song, Mengxun Wang, Jie Qi, Zhigang Wang, Quanqi Zhang *

Key Laboratory of Marine Genetics and Breeding, Ministry of Education, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

ARTICLE INFO

Article history:
Received 23 November 2015
Received in revised form 19 May 2016
Accepted 26 May 2016
Available online 1 June 2016

Keywords:
Kisspeptin
GnRH
Prokaryotic expression
Cultured brain cells
Paralichthys olivaceus

ABSTRACT

Kisspeptins have been described as one of the most potent activators of the hypothalamic–pituitary–gonadal axis. Kisspeptins control the onset of reproductive functions during puberty by directly stimulating the neuronal activity and release of gonadotropin-releasing hormone (GnRH). The function of kisspeptins has been investigated *in vivo* and *in vitro*. In our study, three kinds of recombinant kisspeptin proteins were expressed in *Escherichia coli*. Kisspeptin fragments Kp54, Kp44, and Kp10 translated from *Paralichthys olivaceus kiss2* gene were then obtained. Kp44 contained 44 amide acids (aa) which are the same as the N-terminal of Kp54; Kp10 shares the same 10 aa with the C-terminal of Kp54 but Kp10 also contains some other amide acids. In the dose course of treatments with prokaryotically expressed peptides, Kp54 and Kp10 could induce the expression of *kissr2* and *gnrh1*; by contrast, Kp44 could not induce a similar expression. These results provided direct evidence that the core decapeptide of kisspeptin is necessary to ensure its biological functions. In the time course of the Kp54 treatments on two kinds of cultured brain cells, different patterns of *kissr2* and *gnrh1* mRNA suggested that the responses of these cells to kisspeptins depends on cell type and treatment duration. Thus, our research provided alternative methods to investigate the functions of kisspeptin *in vitro* and to detect biological activities; this research also established basis for kisspeptin applications in production processes.

1. Introduction

Puberty is a developmental process that culminates in the acquisition of reproductive capability. In vertebrates, such as teleosts, reproductive processes are tightly regulated by signals from the hypothalamic–pituitary–gonadal axis (Vadakkadath Meethal and Atwood, 2005). This process is initiated by the release of gonadotropin-releasing hormone (GnRH) from specialized hypothalamic neurons to stimulate hormonal cascades and to activate gonads developments. Kisspeptin and its receptor G-protein-coupled receptor 54 (Kissr, GPR54) have emerged as key elements in the regulation of GnRH secretion. In particular, kisspeptin binds to GPR54 expressed in GnRH neurons and then stimulates release of GnRH and activation of the reproductive axis (Meza-Herrera et al., 2010). Indeed, kisspeptin controls the onset of the reproductive function during puberty. However, the relationship on how kisspeptin, kissr and gnrh expressed in cell still need more investigation.

Encoded by kiss gene, kisspeptins are members of the RFamide peptide family, which is named on the basis of the C-terminal motif of kisspeptin peptide, arginine (R) and phenylalanine (F) coupled to an amide functional group, which is shared by most peptides of the superfamily (Vaudry et al., 2012). Mature kisspeptins in mammals can be cleaved into endogenous fragments, namely, Kp54, Kp16, Kp14, Kp13, and Kp10 (Beltramo et al., 2014). These peptides also share a common 10-amino acid sequence core and exhibit the same binding capacity to GPR54 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Since the discovery of the single Kiss1 gene in eutherian mammals, investigations on various vertebrates have revealed the existence of multiple paralogous Kiss genes. In most mammals, there is only Kiss1 gene, but it is common that two paralogous kisspeptin genes, namely kiss1 and kiss2 genes, or only kiss2 gene are present in several species of teleosts (Pasquier et al., 2012). Previous report on the kiss gene of *Paralichthys olivaceus* only found one kiss2 paralogous gene (Song et al., 2016).

Studies on vertebrates, such as humans and rodents, have demonstrated that the administration of kisspeptin induces GnRH release (Gottsch et al., 2004; Thompson et al., 2006; Dhillo et al., 2007; Ramzan et al., 2014). Furthermore, kisspeptin treatment of immortalized mouse hypothalamic GT1–7 cells natively expressing the receptor increases the mRNA and protein levels of GnRH (Sukhbaatar et al., 2013; Terasaka et al., 2013). In fish, the application of kisspeptin also could modulate the gonad maturation (Felip et al., 2009; Beck et al., 2012; Zmora et al., 2012; Nocillado et al., 2013). In goldfish, Kp10 induces the secretion of luteinizing hormone in goldfish pituitary cells (Yang et al., 2010). In pre-pubertal male yellowtail kingfish, the chronic

☆ These authors contributed equally to the study.

* Corresponding author at: Yushan Road 5#, Qingdao, Shandong Province, China.
E-mail address: qzhang@ouc.edu.cn (Q. Zhang).

http://dx.doi.org/10.1016/j.cbpb.2016.05.002
1096-4959/

administration of Kp10 stimulates gonadal development (Thompson et al., 2006). However, these peptides used in previous studies were purchased from specific corporations; as such, the possible uses of these peptides in animal breeding are limited in some degree considering the cost.

The Japanese flounder *P. olivaceus* (Pleuronectiformes, Paralichthyidae) is an important seawater aquaculture fish. This fish requires two years to three years to accomplish sexual maturity. Therefore, effective approaches should be developed to control the onset of puberty and maturation in artificial breeding, and such approaches are of scientific and economic significance. In our study, three kinds of kisspeptin peptides in *P. olivaceus* were prokaryotically expressed and subjected to on-column digestion; the biological functions of these peptides in cultured *P. olivaceus* brain cells were then evaluated by the expression change of *kissr2* and *gnrh1* mRNA. Our study threw light on the relation between the kisspeptin administration and the expression patterns of related gene; provided a trial to investigate the function of peptides. Meanwhile, the expression system established in the study also provided the basis of future *in vivo* experiment and extensive agricultural applications.

2. Materials and methods

2.1. Construction of the three variants of kisspeptin recombinant plasmids

Primers pairs were designed on the basis of the cDNA encoding sequences of *kiss2* in *P. olivaceus* (GenBank Accession No. KP347689) to amplify the targeted sequences. For the recombinant proteins Kp54 and Kp44 (rKp54 and rPok44, respectively), the restriction site of *Kpn I* and the encoding sequence of an enterokinase site were included at the 5′-end of the same upstream primer kp54-expF (Table 1), and the restriction site of *Sac I* and the encoding sequence of the termination codon were included at the 5′-end of the downstream primers kp54-expR and kp44-expR (Table 1). Compared with rKp54, rKp44 lacks the core decapeptide sequence. The two fragments were amplified from the brain cDNA of *P. olivaceus* through polymerase chain reaction (PCR) by using the primers described in Table 1. The PCR temperature profiles were programmed as follows: pre-denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s; and a final step at 72 °C for 7 min. The PCR products were purified, inserted into pMD-19T and sequenced to screen the clones without any mutations. After the sequences were digested with *Kpn I* and *Sac I* and gel-purified, the fragments of Kp54 and Kp44 were inserted into the expression vector pET-32a plasmid, which was stored in our laboratory and digested with the same enzyme to respectively obtain pET32a-kp54 and pET32a-kp44 plasmids. For the recombinant protein Kp10 (rKp10), which encodes only the core decapeptide sequence of kisspeptin, the restriction sites of *BamH I* and *Xho I* were respectively designed at the 5′-and 3′-ends of the primers kp10-expF and kp10-expR (Table 1). The fragment with sticky ends was obtained under the following thermal cycling conditions: denaturation at 94 °C for 40 s and renaturation at 72 °C for 40 s with 10 μL of each primer (10 μM). The product was then inserted into pET-32a plasmid that was digested with *BamH I* and *Xho I* to obtain the pET32a-kp10 plasmid. The primers are listed in Table 1. All of the plasmids were checked by sequencing. The restriction endonucleases, T4 DNA ligase, and cloning vector pMD-19T were purchased from Takara Company (Dalian, China) and used according to the manufacturer's instructions. The structures of the three recombinant plasmids are shown in Fig. 1A.

2.2. Prokaryotic expressions of recombinant kisspeptin proteins

Escherichia coli Transsetta (TransGenBiotech, Beijing, China) was transformed with the three kinds of the corresponding recombinant plasmids and cultured at 37 °C Luria-Bertani medium with 100 μg/mL ampicillin at 250 rpm/min. The transformed cells were induced by 1 mM isopropyl-b-D-1-thiogalactopyranoside (IPTG) for different durations to express the recombinant proteins when the optical density of the cultures at 600 nm reached 0.4–0.9. The cells were then harvested through centrifugation at 10,000 rpm for 10 min at 4 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl pH 8.0) and ultrasonicated for 4 s on and 10 s off in an ice bath for 15 min. The total lysate induced with IPTG was divided into soluble and insoluble fractions through centrifugation at 10,000 rpm for 10 min at 4 °C. The expression status and solubility of the recombinant proteins were then assessed through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining. In order to detect if the induced proteins were the recombinant ones, Western blot analysis were conducted to test the total lysates. Each kinds of lysate were resolved by 12% SDS-PAGE, and then transferred to a PVDF membrane. After sealed with 5% of skimmed milk powder, the membranes were immunoblotted with rabbit anti-His-tag primary antibody (Beijing CoWin Biotech) at a 1:3000 dilution and then with horseradish peroxide (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Beijing CoWin Biotech) at a 1:3000 dilution. Protein bands were developed with DAB detection method. The physiochemical properties of proteins were predicted using EditSeq.

2.3. Fusion protein purification and enterokinase digestion

Batch purification was performed by using a Ni-agarose His-tagged protein purification kit (Beijing CoWin Biotech) in accordance with the manufacturer's protocols with minor modifications. During purification, the recombinant proteins were initially bound to Ni-agarose and washed with soluble binding buffer to prevent the non-specific combination of the other proteins. Then the Ni-agarose

Table 1
Nucleotide sequences of the primers used for qPCR and plasmids construction.

Name	Sequence (5′–3′)	Restriction site
kp54–expF	GGTACCGACGACGACGACAAGGCAACAGGATCTGTCCTCTCTGC	*Kpn I*
kp54–expR	GAGCTCTCAAAAGCGAAGGGTTAACGGGTTGTAGTTG	*Sac I*
kp44–expR	GAGCTCTCATTTACTCCTGCGGTCGTTGCAC	*Sac I*
kp10–expF	GATCCTTCAACTACAACCCGTTAACCCTTCGCTTTC	*BamH I* and *Xho I*
kp10 –expR	TCGAGAAAGCGAAGGGTTAACGGGTTGTAGTTGAAG	*Xho I and BamH I*
KissR–QFw	TTCTGCTCCTCGTTCTCCCTC	
KissR–QRv	TATGGCTCGGCACAAAGGAC	
Ubce–QFw	TTACTGTCCATTTCCCCACTGAC	
Ubce–QRv	GACCACTGCGACCTCAAGATG	
gnrh1–QFw	GAAGACCTTGGCAGTGTGGCT	
gnrh1–QRv	TCCAGTTCCCTCTTCCCTCCT	

The respective restriction sites were underlined. The encoding sequence for enterokinase site was boxed and the encoding sequence for termination codon was shaded.