General and Comparative Endocrinology 162 (2009) 329–339

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Expression of the gene for ancestral glycoprotein hormone β subunit in the nerve cord of amphioxus

Yukiko Tando, Kaoru Kubokawa *

Center for Advance Marine Research, Ocean Research Institute, The University of Tokyo, 1-15-1, Minamidai, Nakano, Tokyo 164-8639, Japan

ARTICLE INFO

Article history:
Received 22 November 2008
Revised 16 April 2009
Accepted 17 April 2009
Available online 23 April 2009

Keywords:
Glycoprotein hormone
Gene expression
Neuroendocrinology
Evolution
Branchiostoma belcheri

ABSTRACT

Amphioxus belongs to the subphylum cephalochordata, a clade of chordates phylogenetically placed at the most basal position. Despite many studies on the endocrine system of amphioxus, there were no confident lines of evidence on the presence of pituitary hormones, whereas recent amphioxus genome analysis reported that amphioxus has no pituitary hormone except for thyrostimulin, which is a glycoprotein hormone in the pituitary, brain, and other organs of vertebrates. In the present study, we cloned cDNA for one glycoprotein hormone β subunit (GPB) from amphioxus, AmpGPB5, and phylogenetically indicated that AmpGPB5 is the ancestral molecule of glycoprotein hormone β subunits of vertebrates including pituitary glycoprotein hormones. Synteny analyses showed conservation of chromosomal location of genes near GPB genes from amphioxus through human. The AmpGPB5 gene was expressed in a restricted region of the dorsal part of the nerve cord, glandular atrial cells of gills, and pre-vitellogenic oocytes in amphioxus. However, expression was not detected in the Hatschek's pit which is considered to be a primitive pituitary gland. On the basis of present results, we hypothesize that a portion of vertebrate pituitary hormones might be derived from an ancestral glycoprotein hormone of amphioxus that functions as a neuroendocrine hormone.

Grants: KAKENHI (Grants-in-Aid for Scientific Research) on Priority Areas "Comparative Genomics", (B) and JSPS fellow from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The Sasakawa Scientific Research Grant from the Japan Science Society.

Disclosure statement: The authors have nothing to declare.

Data deposition footnotes: The sequence data have been submitted to the DDBJ/EMBL/GenBank database under Accession No. AB468061.

1. Introduction

The cephalochordate amphioxus, a member of the phylum Chordata, is an invertebrate phylogenetically related to vertebrates, and is considered to be in an evolutionary lineage between vertebrates and invertebrates. As of their endocrine system, some reports described similarities of reproduction in amphioxus to that in vertebrates (Chang et al., 1985; Fang et al., 1994; Fang, 1997). Recently, Mizuta and Kubokawa (2007) demonstrated the presence of homologues of sex steroid metabolizing enzymes in the amphioxus *Branchiostoma belcheri*, and showed that the levels of sex steroids were higher in the gonads during the breeding than the non-breeding season. The gonad of amphioxus thus appears to have sex steroidogenesis pathways similar to those of vertebrates. Therefore, it would be phylogenetically important to investigate the regulation of steroidogenesis in amphioxus gonads. Furthermore, amphioxus type I GnRH (BlGnRH) was identified from *B. lanceolatum* using HPLC and LC/MS/MS methods (Chambery et al., 2009). BlGnRH stimulated LH release from rat pituitary glands *in vitro*. These reports indicate that the amphioxus possesses an endocrine mechanism for the regulation of reproduction similar to that in vertebrates, although the presence of gonadotropins (GTH) is ambiguous in the amphioxus.

Glycoprotein hormone β (GPB), highly homologous to thyrostimulin of vertebrate, is the only pituitary glycoprotein hormone found in the genome database of *B. floridae* (Putnam et al., 2008; Holland et al., 2008). Thyrostimulin is a pituitary glycoprotein hormone which was recently discovered and found to be synthesized in other organs including the brain and pancreas in the human (Nakabayashi et al., 2002). Sequences for pituitary glycoprotein hormones (follicle-stimulating hormone, FSH; luteinizing hormone, LH; and thyroid-stimulating hormone, TSH) and chorionic gonadotropin (CG) were not found in the amphioxus genome database using a BLAST search with known sequences of vertebrate hormones.

The glycoprotein hormones commonly have cystine knot motifs with strong and specific non-covalent dimerization of two subunits, α and β (Hearn and Gomme, 2000). From an evolutionary viewpoint, the ancestral GPB gene evolved before the emergence of vertebrates,

* Corresponding author. Fax: +81 3 5351 6820.
E-mail address: kubokawa@ori.u-tokyo.ac.jp (K. Kubokawa).

0016-6480/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2009.04.015

because GPB-like genes are present in invertebrates, although endocrinological functions of this gene are not known (Hsu et al., 2002; Sudo et al., 2005). The cDNA for thyrostimulin was cloned and analyzed in the fly (Sudo et al., 2005), and found in the genome databases of the nematode (Park et al., 2005) and the sea urchin (Dos Santos et al., 2009). On the basis of our phylogenetic analysis of the GPB, we considered that thyrostimulin β (hereafter referred to as glycoprotein hormone β5, GPB5), which also has plausible cystine knots, is an ancestral glycoprotein hormone in the chordata.

As is abovementioned, the analysis report of genome assembly release v1.0 of *B. floridae*, showed two genes for glycoprotein hormone α subunit and one gene for β subunit from which thyrostimulin may be composed (Holland et al., 2008). However, information was quite limited whether they are actually expressed in reproductive amphioxus, if so then where their transcripts are localized, and so on. In the present study, we attempted to clarify these questions which are critical to realize amphioxus reproductive endocrinology. Prior to start this research, we reconfirmed the previous result of genome analysis by using newly released v2.0, which is a non-redundant representation presented in May, 2008. We first cloned cDNA encoding glycoprotein hormone β (AmpGPB5) from amphioxus *B. belcheri*, since neither its sequence nor evidence for expression was provided by EST and cDNA in *B. floridae*, and then characterized AmpGPB5 by use of structure and expression analyses.

2. Materials and methods

2.1. Animals

Mature adult amphioxus, *B. belcheri*, were collected at the coastal waters of the Enshu Nada Sea, Japan, during the breeding season, and taken to the Ocean Research Institute, University of Tokyo. They were maintained in seawater tanks at about 25 °C until use. The body weights of animals used in the present study were about 100 mg.

2.2. Total RNA isolation and cDNA construction

Total RNA was isolated from single whole body as described by Mizuta and Kubokawa (2007), eluted in 50 μl of DEPC water, and stored at −80 °C. First-strand cDNAs were constructed from the total RNA extracted from tissues using PrimeScript™ RNA PCR kit (TaKaRa, Japan) with Oligo-dT primer. One microgram of the total RNA was used as a template for 20 μl of RT reaction. The RT reaction was performed at 45 °C for 30 min, 55 °C for 10 min, 65 °C for 10 min, and 70 °C for 10 min, and finally PrimeScript reverse transcriptase was denaturated at 95 °C for 5 min. The RT products were stored at −30 °C until use. RT-PCR was performed using 2 μl of first-strand cDNA as a template in 20 μl of PCR mixture.

2.3. Cloning of AmpGPB5 by PCR and rapid amplification of cDNA ends (RACE)

A partial cDNA fragment of GPB5 was obtained from whole body cDNA of amphioxus using primers of 5′-CTGGGCTGCGACGT CTGGAGAG-3′ and 5′-ATACTAACGTTTCGCAGGAC-3′ under following conditions: initial denaturation at 95 °C for 1 min, 40 cycles at 95 °C for 10 s, 60 °C for 10 s, 72 °C for 30 s, and additional extension at 72 °C for 3 min. The PCR product was applied to an agarose gel electrophoresis, and an expected fragment size of approximately 300 bp was obtained. This fragment was subcloned into a pCR4 TOPO plasmid vector (Invitrogen, Carlsbad, CA) and sequenced.

For full length cloning of AmpGPB5, the cDNAs was synthesized using a GeneRacer kit (Invitrogen) with attached GeneRacer Oligo-dT Primer. Reactions of RT with Superscript III transcriptase (Invitrogen) were serially performed at 50 °C for 30 min, 55 °C for 10 min, 60 °C for 10 min, and 65 °C for 10 min followed by inactivation of the reaction at 70 °C for 15 min. The RACE with GeneRacer kit (Invitrogen) amplified both the 5′ and 3′ ends of GPB5 cDNA. The specific primer was synthesized using the GPB5 fragment obtained by the RT-PCR described just above and GeneRacer primers contained in the kit. The primer sequences were GPB5GSP-R 5′-GGGTCGACCCCTGGCTGGCAGTTCT-3′ and GeneRacer 5′ primer for 5′-RACE, and GBP5GSP-F 5′-CCGAGAAGGAGGGATGCGAGC GGCTACA-3′ and GeneRacer 3′ primer for 3′-RACE. The PCR product was applied for nested PCR as a template with primers GPB5GSP-R_Nest 5′-TTGTCCTGGTCTCCACCCGGTCGTACGT-3′ and GeneRacer 5′ nest primer for 5′-RACE, and GPB5GSP-F_Nest 5′-GCGTAGACG CTTGCAAGGGCCGCTGTGA-3′ and GeneRacer 3′ nest primer for 3′-RACE. Both 5′-and 3′-RACE were performed by touchdown PCR as follows: initial denaturation at 95 °C for 2 min, five cycles at 95 °C for 30 s and 72 °C for 2 min; five cycles at 95 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min; 25 cycles at 95 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min; and additional extension at 72 °C for 10 min. The amplified fragments were subcloned into a pCR4 vector and sequenced. The partial sequences obtained from 5′- and 3′-RACE were assembled with ATGC software (Genetyx Co., Ltd., Tokyo, Japan). The full-length coding region of GPB5 was amplified, and confirmed that the cDNA sequence was derived from a single gene (Fig. 1D).

2.4. Comparison of the genomic structure of GPB genes

Genomic DNA from single amphioxus was amplified by PCR with the specific set of primers that was used for amplification of full-length nucleotides of GPB5 cDNA including an untranslated region, and the sequence was analyzed. It was compared with the sequences of human GPB genes available from the Entrez gene in the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

2.5. RT-PCR for detection of tissue specific gene expression

The head (anterior part of animal from velum), skin, gills (including endostyle), muscle, testes, and ovaries of amphioxus were dissected out from mature adults with forceps under a dissecting microscope, immediately frozen in liquid nitrogen, and were stored at −80 °C. The head was defined as the region from the tip of the anterior end to the velum. PCR amplification of cDNA from dissected tissues was carried out under the following conditions: initial denaturation at 95 °C for 1 min, 30 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and additional extension at 72 °C for 2 min. The amphioxus GPB5 specific primers (492 bp) are: 5′-GGTGTCTTGTGACCATATGCATCTG-3′ and 5′-TACCATCCAC zAATCGTTTTCCAATG-3′. The PCR products were analyzed by electrophoresis in 2% agarose gels with ethidium bromide.

2.6. Phylogenetic analysis of AmpGPB5

Amino acid sequences of GPBs from both invertebrates and vertebrates were aligned by use of Clustal W program (Thompson et al., 1994). Phylogenetic trees were then constructed by the neighbor-joining method along with calculation of the evolutionary distances by *p*-distance using MEGA version 3.1 software (Kumar et al., 2004). The out-group used in the tree was human TGFβ (Li and Ford, 1998) and nematode TGFβ homologue (Ren et al., 1996).

2.7. Synteny comparisons

Phylogenetic analysis of genes neighboring to GPBs was carried out as described by Larsson et al. (2008) with several modifications. Briefly, synteny relationships were investigated using the Joint Gen-