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<td>Author(s)</td>
<td>Tanabe, Yasunori; Suzuki, Norio</td>
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Genomic Structure and Expression of the Sea Urchin
Soluble Guanylyl Cyclase β Subunit Gene

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Hokkaido University, Sapporo, Japan

ABSTRACT—We obtained the full-length cDNA and genomic DNA clones of HpGCS-β, encoding the β subunit of soluble guanylyl cyclase (soluble GC), which is expressed in the testis and ovary of the sea urchin Hemicentrotus pulcherrimus. Reverse transcription-polymerase chain reaction analysis demonstrated that the HpGCS-β transcript was detected in unfertilized eggs as well as in the testis and ovary. The open reading frame predicted a protein of 604 amino acids with a putative cyclase catalytic domain conserved in membrane GCs and adenylyl cyclases. 105His, 78Cys, and 122Cys, important amino acids for heme-binding in the β subunit of soluble GCs in vertebrates, were conserved in the corresponding positions of HpGCS-β. The HpGCS-β gene consisted of 8 exons and had a span of 26 kbp. A comparison of genomic structure of the HpGCS-β gene with that of the soluble GC β subunit gene (OlGCS-β) of the medaka fish indicated that exon 4 in the HpGCS-β gene corresponds to exons 4 to 9 in the OlGCS-β gene.

Key words: soluble guanylyl cyclase, sea urchin, genome, cDNA, *Hemicentrotus pulcherrimus*

INTRODUCTION

During animal fertilizations, an egg must produce a proper intracellular calcium signal in order to allow for development to proceed normally. Calcium release mechanisms have been extensively studied in sea urchins (Stricker, 1999). Willmott et al. (1996) demonstrated that nitric oxide (NO) mobilizes calcium from intracellular stores in the sea urchin egg via a pathway involving guanosine 3',5'-cyclic monophosphate (cGMP) and cyclic ADP-ribose (cADPR). It has been reported that NO synthase (NOS) is active in spermatozoa and eggs in the sea urchin Strongylocentrotus purpuratus and endogenously activated NO-associated bioactivities are necessary for successful fertilization (Kuo et al., 2000). Soluble guanylyl cyclase (soluble GC) is a heme-containing heterodimer composed of α and β subunits and is activated by binding of NO to a single heme associated with the β subunit (Wedel et al., 1994). To date, many cDNA clones and several genomic DNA clones for both subunits have been obtained from various sources, e.g., human (Giuli et al., 1992; Zabel et al., 1998; Behrends et al., 2000), bovine (Koesling et al., 1988, 1990), rat (Nakane et al., 1988, 1990; Yuen et al., 1990; Harteneck et al., 1991; Koglin et al., 2000), mouse (Sharina et al., 2000), medaka fish (Mikami et al., 1998, 1999), Drosophila (Shah et al., 1995), the tobacco hornworm moth Manduca sexta (Nighoron et al., 1998), nematode (Yu et al., 1997), and yeast (Kuo et al., 1998); the chemical and biological natures of these clones have been characterized. However, little is known about the soluble GC/cGMP-mediated biochemical pathway in marine invertebrates including the sea urchins. Here, we report that the sea urchin Hemicentrotus pulcherrimus, a very common species in Japan that is very closely related to S. purpuratus, possesses a soluble GC β subunit of the highly conserved primary structure and the gene is expressed in the testis and ovary, as well as in unfertilized eggs. We also report that exon 4 in the β subunit gene (HpGCS-β) of H. pulcherrimus soluble GC corresponds to exons 4 to 9 of the medaka fish β subunit gene (OlGCS-β).

MATERIALS AND METHODS

Materials

H. pulcherrimus sea urchins were collected along the coast near Noto Marine Laboratory, Kanazawa University. The gonads and gametes were obtained from mature adult animals as described previously (Xu et al., 2001).

Preparation of RNA and amplification of cDNA fragments by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the H. pulcherrimus tissues, eggs, and embryos according to the method described by Chomczynski and Sacchi (1987). Poly(A)+RNA was isolated using Oligotex-dT30-<Super>(Roche Diagnostics, Japan), according to the manufacturer’s protocol.

Four degenerate oligonucleotide primers (F1, 5'-CTIMGNCTNAARGGNCAAATGATCTAC-3'; F2, 5'-AAGACIGACAGGYTNC-TNTAYTNGT-3'; R1, 5’-ATTICCRANAGACARTANCNGGACAT-3'; R2, 5’-SAGRTTSACWGTATTNCCRAANAGACA-3’) were synthesized based on the amino acid sequences of 4 conserved regions (LRLKGQMIY, KTDRLLYSV, CLFGNTVNL, MPRYCLFGN) in known...
soluble GCs. A set of the primers was used to amplify the cDNA fragments of *H. pulcherrimus* soluble GC from cDNA reverse-transcribed total RNA or poly(A)^+ RNA of the *H. pulcherrimus* testis, as described previously (Seimiya et al., 1997). The PCR products were purified, subcloned into the plasmid vector pBluescript II KS(−) (Stratagene, La Jolla, CA, USA), and sequenced.
5' - and 3' -Rapid amplification of cDNA ends (5' - and 3' -RACE)

To obtain the full-length sequence of HpGCS-β cDNA, the 5'-portion of the cDNA was amplified by the 5'-RACE method (Frohman et al., 1988) using the 5'-RACE System for Rapid Amplification of cDNA Ends, ver 2.0 (Life Technologies Inc.). Total RNA (1 µg) isolated from the tissues was reverse-transcribed with gene-specific antisense oligonucleotide primers (GSP1-1, GSP2-1, and GSP3-1). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase, and amplified by PCR with the Abridged Anchor Primer (Life Technologies) and other gene-specific antisense oligonucleotide primers (GSP1-2, GSP2-2, and GSP3-2). The following PCR conditions were applied: for GSP1-2, denaturation at 95°C for 5 min followed by 35 amplification cycles (95°C for 30 sec, 66°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP2-2, denaturation at 95°C for 5 min followed by 35 amplification cycles (95°C for 30 sec, 66°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP3-2, denaturation at 95°C for 5 min followed by 35 amplification cycles (95°C for 30 sec, 67°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min. To enrich the 5'-RACE products, one-fifteenth volume of the primary 5'-RACE products was reamplified by 25 additional cycles using the Abridged Universal Amplification Primer (AUAP; Life Technologies Inc.) and nested primers (GSP1-3, GSP2-3, and GSP3-3). Amplification was performed as follows: for GSP1-3, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 63°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP2-3 and GSP3-3, amplification was performed under the same conditions as used for GSP1-3. The PCR products were cloned into pBluescript II KS (+) and sequenced. The gene-specific primers used were complementary to nucleotide positions of HpGCS-β cDNA, 1649-1668 (GSP1-1), 1529-1555 (GSP1-2), 1483-1508 (GSP1-3), 1438-1459 (GSP2-1), 1354-1381 (GSP2-2), 1327-1352 (GSP2-3), 542-561 (GSP3-1), 510-538 (GSP3-2), and 480-505 (GSP3-3). The 5'-RACE products overlapped in 50–200 bp with the 5' end of the clone that had been isolated.

The 3' -portion of the cDNA was amplified by the 3'-RACE method (Frohman et al., 1988) using the 3'-Full RACE Core Set (Takara Shuzo Co., Ltd., Osaka, Japan). Total RNA (1 µg) was reverse-transcribed with an Oligo dT-3'sites Adaptor Primer (Takara Shuzo Co., Ltd., Osaka, Japan). The cDNA was amplified by PCR with the 3'sites Adaptor Primer (Takara Shuzo Co., Ltd., Osaka, Japan) and another gene-specific oligonucleotide primer (GSP4-1). One-fifteenth volume of the PCR product was amplified by PCR with the 3'sites Adaptor Primer (Takara Shuzo Co., Ltd., Osaka, Japan) and another gene-specific oligonucleotide primer (GSP4-2). The following PCR conditions were used: for GSP4-1, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 61.5°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min; for GSP4-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 61.5°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min. The PCR products were cloned into pBluescript II KS (+) and sequenced. The gene-specific primers used for 3'-RACE were nucleotide positions of HpGCS-β cDNA, 1670-1691 (GSP4-1) and 1780-1801 (GSP4-2). The 3'-RACE product overlapped in 50 bp with the 3' end of the clone previously isolated.

Molecular phylogenetic analysis

The nucleotide and deduced amino acid sequences of HpGCS-β were compared with those of known soluble GC subunits using the Clustal W program (Thompson et al., 1994) and the sequence editor SeqPub (Gilbert, Indiana University, Indianapolis, USA). The unrooted phylogenetic tree was constructed using the aligned amino acid sequences of the catalytic domain of various soluble GCs by the neighbor-joining algorithms in the PROTRAS program of PHYLIP (version 3.572) (Felsenstein, 1989) and the Clustal W program (Saitou and Nei, 1987).

Isolation and sequencing of genomic DNA encoding HpGCS-β

Genomic DNA was isolated from the H. pulcherrimus testes by the procedure described previously (Mikami et al., 1998). In order to obtain a genomic DNA fragment containing an intron and a portion of

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Table 1. Exon and intron sizes in the β, subunit genes of H. pulcherrimus (HpGCS-β), medaka fish (OlGCS-β), and human soluble GCs.

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<td>&gt;45063</td>
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* Mikami et al., 1998.
** from Celera Human Genome Sequence Data.

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** from Celera Human Genome Sequence Data.
exons, we performed PCR using *H. pulcherrimus* testis genomic DNA (10 µg) as a template and various gene-specific primers (EX1, EX2-1, EX2-2, EX3-1, EX3-2, EX4-1, EX4-2, EX5, EX6, EX7, EX8, GSP3-1, GSP4-1, and GSP 4-2). These primers were synthesized based on the nucleotide sequence of HpGCS-β cDNA. The following PCR conditions were applied: for EX1 and EX2-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (98°C for 20 sec and 61.5°C for 5 min) and a final extension at 72°C for 10 min; for EX2-1 and EX3-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (98°C for 20 sec and 61.5°C for 5 min) and a final extension at 72°C for 10 min; for EX3-1 and EX3-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (98°C for 20 sec and 64°C for 8 min) and a final extension at 72°C for 10 min; for EX4-1 and GSP3-1, and EX5 and EX6, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 63°C for 30 sec, and 72°C for 2 min) and a final extension at 72°C for 10 min; for GSP4-1 and EX7, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 61.5°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min; for GSP4-2 and EX8, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 5 min, 62°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min. The PCR products were subjected to sequencing directly or after subcloning into pBluescript II KS(+) . The gene-specific primers used were complementary to the nucleotide positions of HpGCS-β cDNA; 37-60 (EX1), 257-285 (EX2-1), 294-320 (EX2-2), 338-361 (EX3-1), 397-420 (EX3-2), 889-911 (EX4-1), 935-961 (EX4-2), 1440-1466 (EX5), 1728-1754 (EX6), 1882-1907 (EX7), and 2465-2493 (EX8).

Other methods

The nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977) with an Applied Biosystems 373 sequencer or a 3100 Genetic Analyzer. Data was analyzed with the nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977) with an Applied Biosystems 373 sequencer or a 3100 Genetic Analyzer. Data was analyzed with the DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan) and GENETYX-MAX/Ver. 7.2.0. (Software Development, Tokyo, Japan).

RESULTS AND DISCUSSION

As shown in Fig. 1 and Table 1, the HpGCS-β cDNA consists of a 250-bp 5′ untranslated region (UTR), a 2012-bp open reading frame (ORF), and a 531-bp 3′-UTR. The HpGCS-β gene spans over 26 kbp and consists of 8 exons. The GT-AG rule was conserved for all splice sites. The sizes of all introns in the HpGCS-β gene are larger than those in the medaka fish soluble GC β subunit gene OIGCS-β, and some are larger than those in the human soluble GC β subunit gene. Exons 1 to 3 in the HpGCS-β gene are the same size as the corresponding exon in the OIGCS-β gene. However, exon 4 in the HpGCS-β gene is much larger than that of the OIGCS-β gene and the human soluble GC β subunit gene and corresponds to the nucleotides together with exons 4 to 9 in the OIGCS-β gene, suggesting that intron insertion occurred during gene evolution from invertebrates to vertebrates. Although there is little difference according to size, exons 5 to 8 of the HpGCS-β gene seem to correspond to exons 10 to 13 in the OIGCS-β gene, respectively.

The ORF of HpGCS-β cDNA encodes 604 amino acids (Fig. 2). Comparison of the deduced amino acid sequence of HpGCS-β with that of each subunit of known soluble GCs indicated that the entire amino acid sequence of HpGCS-β was 57.1, 57.9, and 58.8% identical to that of human, medaka fish, and *Manduca sexta* GCS-β, respectively. Considering that the entire amino acid sequence of the soluble GC β subunit is highly conserved among vertebrates (i.e., the identity throughout the entire protein between humans and rats is 98.5%, and that between humans and the medaka fish is 89.8%). The identity in the amino acid sequence between the sea urchin and other animals was rather low. However, the amino acid sequence identity in the catalytic domain of the α1, α2, β1, or β2 subunit between sea urchin and other animals was about 46, 45, 63, and 42%, respectively. Furthermore, phylogenetic analysis demonstrated that HpGCS-β phylogenetically separates from the α1, α2, or β2 subunit cluster and was closer to the β subunit cluster of vertebrate soluble GC than to soluble GC of the insects, *Drosophila* and *Manduca* (Fig. 2B). In the β subunit of vertebrate soluble GC, 160His, 76Cys, and 123Cys have been demonstrated to be important amino acids for heme-binding (Stone and Marletta, 1994; Wedel et al., 1994; Foerster et al., 1996). These amino acid residues are conserved in the corresponding positions of HpGCS-β, respectively (Fig. 2A). All of these studies suggest that HpGCS-β is a heme-containing *H. pulcherrimus* soluble GC β subunit and can be activated by NO after association with its proper counterpart α1 subunit. In this regard, it should be mentioned that sea urchin gametes contain a NO synthase and NO; sea urchin gamete NO-related bioactivity appears to be critical for egg activation, yet its mechanism remains unknown (Kuo et al., 2000).

Our results demonstrated that the HpGCS-β gene was expressed in the testis and ovary of *H. pulcherrimus* and its transcript was also detected in the unfertilized egg as well as in the embryo (Fig. 3). This finding is consistent with our previous results demonstrating 1) that the gene transcripts of both subunits of medaka fish soluble GC were present in unfertilized eggs, and 2) that these transcripts were temporarily reduced immediately after fertilization and then subsequently increased to previous levels (Mikami et al., 1998). However, at present the point at which the HpGCS-β gene is transcribed during oogenesis remains unknown. It also remains unclear how the transcript is stored in the unfertilized egg and whether active soluble GC is present in the unfertilized egg. We believe that the pathway involving cGMP and cADPR in calcium
papers have been published on the role of a cGMP-signaling pathway in animal fertilization (Ciapa and Epel, 1996; Lee et al., 1996). The present study of NO-activatable soluble GC in sea urchins is important for understanding the mechanisms of action during the early phase of animal fertilization. Further study on H. pulcherrimus soluble GC is currently being conducted in our laboratory.

ACKNOWLEDGMENTS

We are grateful to Mr. Masahiro Matada, Noto Marine Laboratory (Professor Yuichi Sasayama, Director), Kanazawa University for collecting and culturing the sea urchins. The present study was supported by a Grant-in-Aid for Scientific Research (No. 11236202) from the Ministry of Education, Science, Sports, and Culture of Japan. The nucleotide sequence reported in this paper appears in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under the accession numbers, AB062386, AB062387, AB062388, AB062389, and AB062390.

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Fig. 3. RT-PCR analysis of HpGCS-β, transcripts in the H. pulcherrimus adult tissues, eggs, and embryos. The HpGCS-β, transcript was detected by RT-PCR in the testis (1), ovary (2), unfertilized eggs (3), embryos directly after fertilization (4), 2-cell stage embryos (5), 4-cell stage embryos (6), and 8-cell stage embryos (7). The 16S ribosomal RNA was amplified as an internal control. RT + and RT – represent amplification with and without reverse transcriptase, respectively.
guanylyl cyclase genes. Proc Natl Acad Sci USA 97: 10878–10883

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