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Molecular Phylogenetic Analysis of Diverse Forms of Echinoderm Guanylyl Cyclases

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ABSTRACT—We found many isoforms of membrane-bound guanylyl cyclase (membrane GC) in the gonads of several echinoid species including *Hemicentrotus pulcherrimus* (six isoforms), *Glyptocidaris crenularis* (four isoforms), *Clypeaster japonicus* (three isoforms), *Diadema setosum* (three isoforms), and *Brissus agassizii* (three isoforms). In addition to these membrane GC isoforms, several putative soluble GC isoforms were also found, one in *H. pulcherrimus*, two in *G. crenularis*, and three in *D. setosum*. Other echinoderm species also possess many GC isoforms in their gonads: the starfish, *Asterina pectinifera*, has five membrane GC isoforms; the brittle star, *Ophioplocus japonicus*, possesses six membrane and two putative soluble GC isoforms; and the sea cucumber, *Stichopus japonicus*, contains five membrane and two putative soluble GC isoforms. We also obtained the full-length cDNA sequence of some of these isoforms, one for sperm-activating peptide (SAP) receptor-type GC, and the other for a homolog of the vertebrate natriuretic peptide receptors/membrane GCs, and analyzed their phylogenetic relationship among various invertebrate and vertebrate GC isoforms.

INTRODUCTION

For over twenty years, it has been known that cGMP concentrations can be increased by a wide variety of agents (Goldberg and Haddox, 1977). The formation of cGMP from GTP is catalyzed by guanylyl cyclase [GTP pyrophosphate-lyase (cycling), EC 4.6.1.2] (Mittal and Murad, 1982). Guanylyl cyclase is found in various cellular compartments of many organisms, in soluble and/or membrane-bound forms. The membrane-bound guanylyl cyclase (membrane GC) is a single polypeptide, the first established by cloning and sequencing of the cDNA encoding a sperm protein crosslinked to a sperm-activating peptide-IIA (SAP-IIA) and was initially obtained from a testis cDNA library of the sea urchin *Arbacia punctulata* (Singh *et al*., 1988). The soluble guanylyl cyclase (soluble GC) consists of two different subunits (α and β) (Drewett and Garbers, 1994). In mammals, seven isoforms of membrane GC (GC-A, -B, -C, -D, -E, -F, and -G) have been found (Garbers, 1992; Garbers and Lowe, 1994; Schulz *et al*., 1998), all of which are composed of an extracellular domain, a single membrane-spanning domain, and an intracellular domain that is further divided into two clearly defined domains; a protein kinase-like regulatory domain and a cyclase catalytic domain. The primary structure of the catalytic domain of both membrane and soluble GCs is highly conserved among vertebrates and invertebrates (Garbers, 1992; Garbers and Lowe, 1994).

It has been reported that the binding of sperm-activating peptides (SAPs) to sperm surface receptors causes a marked and rapid increase, then subsequent rapid decrease, in cGMP concentrations in sperm cells (Kopf *et al*., 1979; Suzuki, 1990, 1995). The transient increase in cGMP concentrations has been attributed to the transient activation and subsequent inactivation of the membrane GC, which is closely linked to the state of the enzyme (Garbers, 1989; Suzuki, 1999). A specific SAP such as SAP-IIA binds specifically to the sperm membrane GC, and causes an initial transient activation and subsequent inactivation of the enzyme (Ramarao and Garbers, 1985; Shimomura *et al*., 1986; Suzuki *et al*., 1984). SAP-I and SAP-IIB, isolated from the egg jelly of the sea urchin *Hemicentrotus pulcherrimus* or *Glyptocidaris crenularis* bind specifically to a 71 kDa and a 62 kDa sperm protein, respectively, and in either case the binding of the peptide to the protein results in activation of the respective sperm membrane GC (Harumi *et al*., 1991; Shimizu *et al*., 1994). Similarly, SAP-III isolated from the sand dollar *Clypeaster japonicus* causes increases of cGMP levels in the sperm cells after it binds to three sperm proteins, one of which appears to be a membrane GC (Yoshino and Suzuki, 1992). These facts indicate that the membrane GC is the receptor itself for SAP or for a protein associated with the receptor for SAP.

In the last decade, a number of SAP-I derivatives have been isolated from the egg jelly of several sea urchin species: two from *Strongylocentrotus purpuratus* and four from *H. pulcherrimus*. These derivatives all show the same biological

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activity on the respective spermatozoa (Suzuki, 1995, 1999), and their amino acid sequences are found in the respective precursor protein (Kinoh *et al.*, 1994; Ramarao *et al.*, 1990). Such structural diversity is also found in SAPs from other sea urchin species: five SAP-IIB derivatives in the egg jelly of *G. crenularis*; eight SAP-III derivatives in the *C. japonicus* egg jelly; and one SAP-IV derivative in the egg jelly of *Diadema setosum* (Suzuki, 1990, 1995). Therefore, it might be the case that the receptors for each group of SAP are heterogeneous with regard to the composition of their constituent proteins, and particularly in their primary structure, which allows for binding to the peptide.

The cDNA encoding the membrane GC has been isolated from three species of sea urchin testis cDNA library: *A. punctulata* (Singh *et al.*, 1989), *S. purpuratus* (Thorpe and Garbers, 1989), and *H. pulcherrimus* (Shimizu *et al.*, 1996). The primary structures of these membrane GCs are to some degree similar to those of the membrane GCs serving as the natriuretic peptide receptors. This is also the case for *Caenorhabditis elegans*, in which twenty-nine genes encoding putative GCs have been identified (Baude *et al.*, 1997; Wedel and Garbers, 1997). Vertebrates possess much smaller numbers of the membrane GC isoforms, but they can be divided into three major groups: (i) natriuretic peptide receptors, (ii) enterotoxin/guanylin receptors, and (iii) sensory organ-specific membrane GCs (Seimiya *et al.*, 1997). However, little is known about the origin of the membrane GC in each vertebrate group or about the molecular phylogenetic relationship among the membrane GC isoforms in vertebrates and invertebrates. In this study, to understand the diversity of the membrane GC isoforms in invertebrates, we began a characterization of the membrane GC isoforms in sea urchins, the spermatozoa of which contain a generous amount of the SAP receptor-type membrane GC, a condition conductive to biochemical analysis of the enzyme and mechanisms of interaction between peptide ligands such as SAPs and their receptors. The study extended to the characterization of the membrane GC isoforms in other echinoderms such as the starfish *Asterina pectinifera*, the brittle star *Ophioplocus japonicus*, and the sea cucumber *Stichopus japonicus*. In addition, we describe here in the isolation and characterization of cDNA clones for a SAP receptor-type membrane GC isoform obtained from a testis cDNA library of the sea urchin *Brissus agasssizii* and from an echinoderm homolog of the vertebrate natriuretic peptide receptor/membrane GC isoform from an ovary cDNA library of the sea cucumber *S. japonicus*, and the phylogenetic relationship among these GC isoforms and known GC isoforms.

MATERIALS AND METHODS

Materials

Individual specimens of *H. pulcherrimus*, *C. japonicus*, *B. agassizii*, *A. pectinifera*, *O. japonicus*, and *S. japonicus* were collected off the coast near Noto Marine Laboratory, Kanazawa University. Individual specimens of *G. crenularis* and *D. setosum* were collected at Aomori Bay near the Asamushi Marine Biological Station of Tohoku University and off the coast near Usa Marine Biological Institute of Kochi University, respectively. The gonads (testes and/or ovaries) were dissected out from the adult specimens and frozen with liquid nitrogen.

Preparation of RNA and cDNA library construction

Total RNA was prepared from the growing gonads of various species of echinoderms and adult medaka fishes by the LiCl method of Cathala *et al.* (1983) or by the acid/guanidinium/thiocyanate/phenol/chloroform extraction method (Chomcznski and Sacchi, 1987). Poly(A)$^+$RNA was isolated from the total RNA by chromatography on oligo(dT)-cellulose (Pharmacia Biotech.) (Davis *et al.*, 1986). Using the poly(A)$^+$RNA, a cDNA library was constructed in a λZAP II vector with a ZAP-cDNA Synthesis Kit (Stratagene).

Amplification of GC cDNA fragments by reverse transcription-polymerase chain reaction (RT-PCR)

Three degenerate oligonucleotide primers (P2: 5'-GAYATHGTN-GGNTTYAC-3'; P6: 5'-GTRTTNACNGTRTCNCC-3'; P7: 5'-ARR-CARTANCKNGGCAT-3') were synthesized based on the amino acid sequences of three conserved regions (DIVGFT, GDTVNT, and MPRYCL) in known GCs. These primers were used to amplify a cDNA fragment encoding GC in cDNA reverse-transcribed from the total RNA of the gonad as described previously (Seimiya *et al.*, 1997). The PCR product was purified and subcloned into the plasmid vector pBluescript II KS(–) (Stratagene), and used as a probe for the screening of a cDNA library as described previously (Mikami *et al.*, 1998).

Screening of a cDNA clone for a membrane GC from a *B. agassizii* or a *C. japonicus* testis cDNA library

A cDNA fragment corresponding to the nucleotides (nt) 2943 to 3283 of a full-length sequence of *BaSTGC01* was labeled with ^{32}P-dCTP using the Random Primer DNA labeling Kit, Ver.2 (Takara Shuzo Co., Ltd.), then used as a probe for the screening of a *B. agassizii* testis cDNA library. About 1.5×10^5 recombinant phages were plated, and plaques were transferred to a nylon membrane (Hybond-N$^+$, Amersham). The membrane was prehybridized for 2 hr at 37°C in 6×SSPE containing 0.5% SDS, 5×Denhardt's solution, 50 μg/ml denatured herring sperm DNA, and 50% formamide. The radioactive probe (36.7×10^6cpm/ml) was added to the prehybridization solution and incubated overnight at 37°C. The membrane was washed three times for 15 min at 50°C in 2×SSC containing 0.1% SDS. After repeated screening under the same conditions as above, the cDNA insert of a positive clone (*BaSTGC01*) was excised in vivo into pBluescript SK(–) (Stratagene) in accordance with the manufacturer's protocol.

Similarly, using a 341-bp cDNA fragment (*CjPTGC03*) as a probe, two clones designated as *CjSTGC01* and *CjSTGC02* were obtained from a *C. japonicus* testis cDNA library.

Screening of a cDNA clone for a membrane GC from a *S. japonicus* ovary cDNA library

A cDNA fragment corresponding to the nt 3215 to 3555 of a full-length sequence of *SjSOGC01* obtained by RT-PCR with total RNA of the *S. japonicus* ovary and degenerate oligonucleotide primers (P2, P6, and P7) was labeled with digoxigenin (DIG)-dUTP using the DIG High Prime (Boehringer Manheim), then used as a probe for the screening of a *S. japonicus* ovary cDNA library. About 2.2×10^5 recombinant phages were plated, and plaques were transferred to a nylon membrane (Hybond-N$^+$, Amersham). The membrane was prehybridized for 30 min at 42°C in 6×SSC containing 0.02% SDS, 0.1% N-laurorylsarcosine, 2% blocking reagent, and 50% formamide. Then, the membrane was immersed in the hybridization solution containing the labeled probe and incubated overnight at 42°C. The membrane was washed three times for 15 min at 50°C in 2×SSC containing 0.1% SDS, and one time for 10 min at 55°C in 0.1×SSC containing 0.1% SDS. After repeated screening under the same conditions

as above, the cDNA insert of a positive clone (*SjSOGC01*) was excised *in vivo* into pBluescript SK(–) (Stratagene) in accordance with the manufacture's protocol.

5'-Rapid amplification of cDNA ends (5'-RACE)

To obtain a full length cDNA sequence of *SjSOGC01*, 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 (Gibco BRL). Three μg of total RNA was reverse-transcribed with gene-specific antisense oligonucleotide primers (GSP4, GSP7). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase, and amplified by PCR with the Abridged Anchor Primer (Gibco BRL) and other gene-specific antisense oligonucleotide primers (GSP5, GSP8). One-tenth of the volume of the PCR product was amplified by PCR with the Abridged Universal Amplification Primer (Gibco BRL) and other gene-specific antisense oligonucleotide primers (GSP6, GSP9). The following PCR conditions were used: for GSP5 and GSP6, denaturation at 94°C for 5 min followed by 30 amplification cycles (94°C for 1 min, 63°C for 1 min, and 72°C for 2 min) and a final extension at 72°C for 7 min; for GSP8 and GSP9, denaturation at 94°C for 5 min followed by 30 amplification cycles (94°C for 1 min, 57°C for 1 min, and 72°C for 2 min) and a final extension at 72°C for 7 min. The PCR products were subcloned into pBluescript II KS (–) and sequenced. The gene-specific primers used were complementary to nt 2390–2415 (GSP4), 2370–2392 (GSP5), 2342–2365 (GSP6), 847–864 (GSP7), 803–821 (GSP8), and 758–776 (GSP9), respectively. The 5'-RACE products overlapped in 63–136 bp with the 5'- end of *SjSOGC01*.

DNA Sequencing

The DNA fragment in the pBluescript vector was used directly to determine the nucleotide sequence as a template by the dideoxy chain termination procedure (Sanger *et al*., 1977) with an Applied Biosystems 373A or 377 DNA sequencer, and analyzed on DNASIS software (Hitachi Software Engineering Co.).

Molecular phylogenetic analysis

DNA sequences and the deduced amino acid sequences of various echinoderms and medaka fish GC isoforms were compared with those of known vertebrate GC isoforms using the ClustalW program (Thompson *et al*., 1994) and the sequence editor SeqPup (Gilbert, Indiana University). A phylogenetic tree was constructed with the aligned sequences by the neighbor-joining (Saitou and Nei, 1987) algorithms in the PROTRAS program of PHYLIP (version 3.572; Felsenstein, 1989) and the ClustalW program (Thompson *et al*., 1994), respectively. For the neighbor-joining analysis, the evolutionary distance was estimated using Kimura's empirical method for protein distances (Kimura, 1983). GenBank/EMBL/DDBJ accession numbers for the sequences used are: rat GC-A (X14773) (Chinkers *et al*., 1989); rat GC-B (M26896) (Schulz *et al*., 1989); rat GC-C (M55636) (Schulz *et al*., 1990); rat GC-D (M26896) (Fülle *et al*., 1995); rat GC-E (L36029) (Yang *et al*., 1995); rat GC-F (L36030) (Yang *et al*., 1995); rat GC-G (AF024622) (Schulz *et al*., 1998); rat GCS-α_1 (M57405) (Nakane *et a*l., 1990); rat GCS-β_1 (M22562) (Nakane *et al*., 1988); human GC-A (X15357) (Lowe *et al*., 1989); human GC-B (113916) (Chang *et al*., 1989); human GC-C (M73489) (de Sauvage *et al*., 1991); human RetGC-1 (M92432) (Shyjan *et al*., 1992); human RetGC-2 (L37378) (Lowe *et al*., 1995); human GCS-α_1 (X66534) (Giuili *et al*.,1992); human GCS-β_1 (X66533) (Giuili *et al*., 1992); OlGC1 (AB004921) (Takeda K and Suzuki N, unpublished data); OlGC2 (AB016082) (Yamagami S, Muramatsu R, and Suzuki N, unpublished data); OlGC3 (AB000899) (Seimiya *et al*., 1997); OlGC4 (AB000900) (Seimiya *et al*., 1997); OlGC5 (AB000901) (Seimiya *et al*., 1997); OlGC6 (AB007192) (Mantoku *et al*.,1999); OlGCS-α_1 (AB000849) (Mikami *et al*., 1998); OlGCS-β_1 (AB000850) (Mikami *et al*., 1998); eel GC-A (AB012869) (Kashiwagi *et al*., 1998); eel GC-B (D25417) (Katafuchi *et al*.,1994); *D. melanogaster* DGC1 (L35598) (McNeil *et al*., 1995); *D. melanogaster* GC (X72801) (Gigliotti *et al*., 1993); *D. melanogaster* GCS-α_1 (U27117) (Shah *et al*.,1995); *D. melanogaster* GCS-β_1 (U27123) (Shah *et al*., 1995); *Manduca sexta* GC-1 (AF073342) (Simpson *et al*., 1999); *M. sexta* GCS-α_1 (AF062750) (Nighorn *et al*., 1998); *M. sexta* GCS-β_1 (AF062751) (Nighorn *et al*.,1998); sea urchin (*H. pulcherrimus*) sperm GC (HpGC) (D21101) (Shimizu *et al*., 1996); sea urchin (*S. purpuratus*) sperm GC (SpGC) (M22444) (Thorpe and Garbers, 1989); and *C. elegans* GCY-X1 (L80003) (Baude *et al*., 1998).

RESULTS

Amplification of various GC cDNA fragments by RT-PCR

In order to confirm the applicability of RT-PCR for amplification of cDNA fragments for various GC isoforms, we first

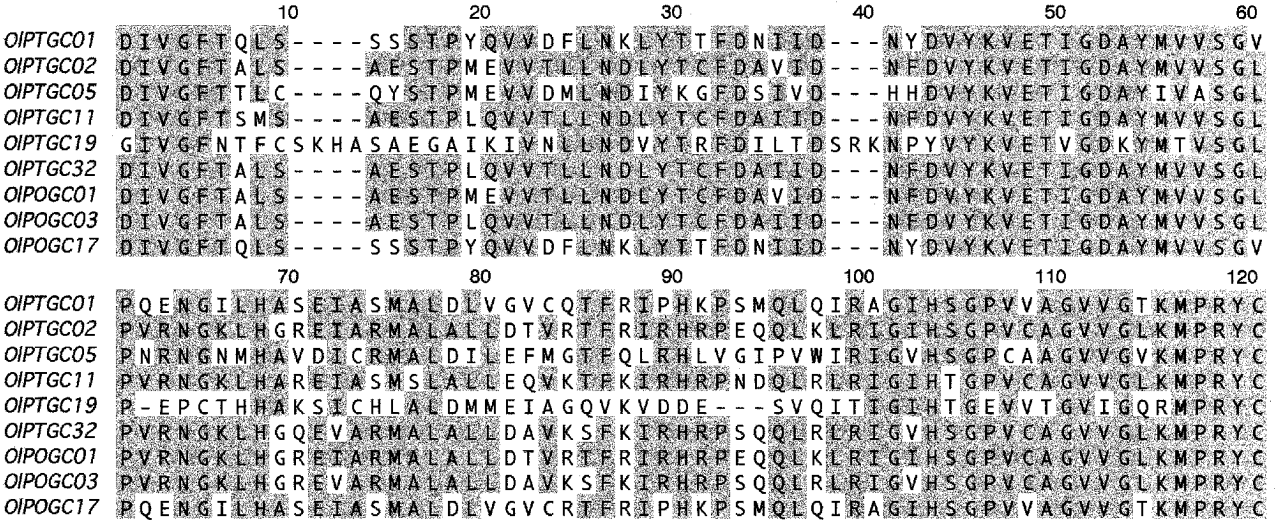

Fig. 1. Alignment of the amino acid sequences of GC isoforms identified in the testis (OlPTGC**) and ovary (OlPOGC**) of medaka fish *O. latipes*. Amino acids identical among more than five isoforms are indicated by single-letter code on gray background. Gaps in the sequences are indicated by dash (–).

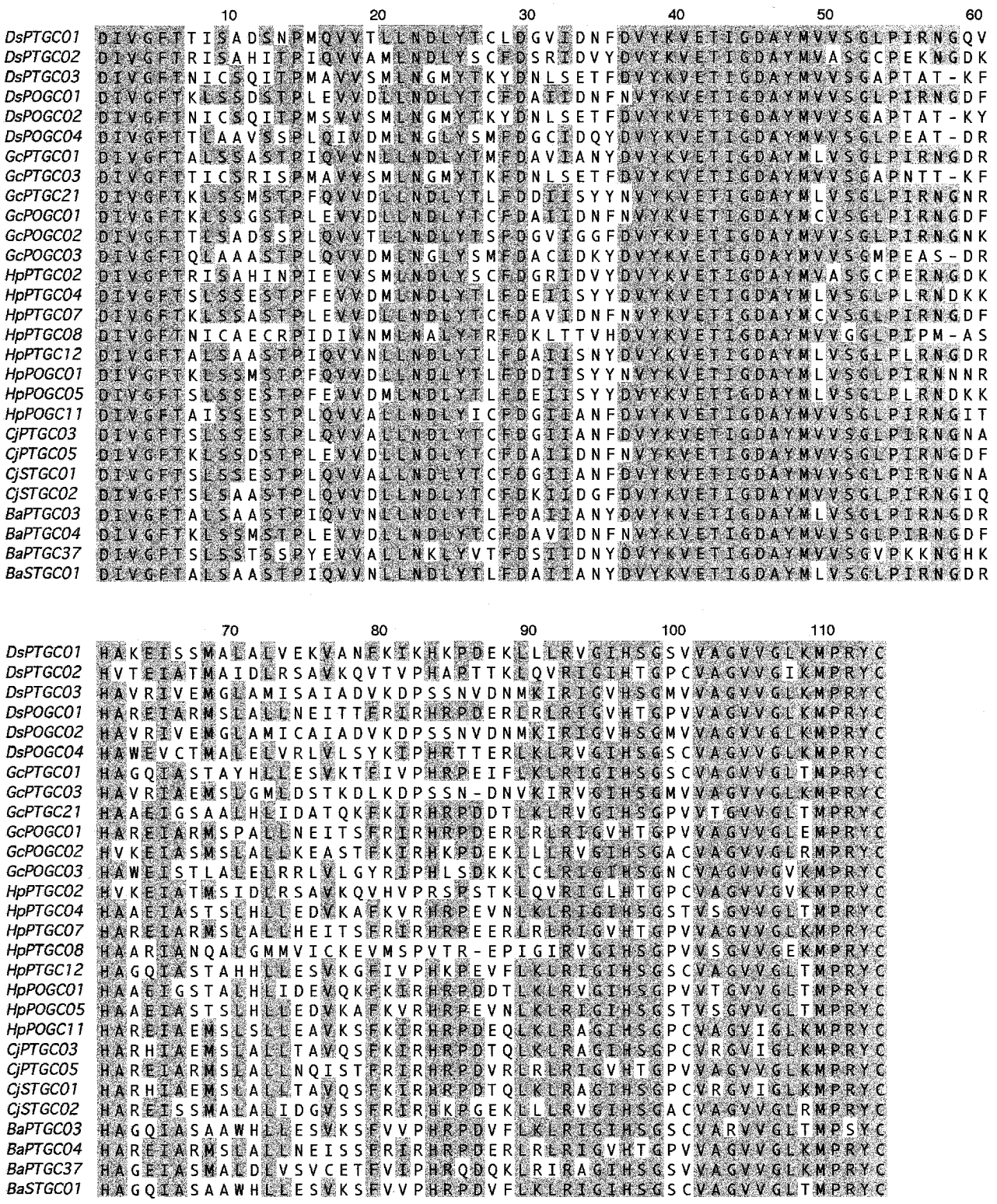

Fig. 2. Comparison of the amino acid sequences of GC isoforms identified in the gonads of various sea urchin species. DsPTGC** and DsPOGC**, and GcPTGC** and GcPOGC** denote the isoforms in the testis and ovary of *D. setosum*, and in the testis and the ovary of *G. crenularis*, respectively. Similarly, HpPTGC** and HpPOGC** denote the isoforms in the testis and the ovary of *H. pulcherrimus*, respectively. The ovary of sea urchin species *C. japonicus* and *B. agassizii* was not used for RT-PCR experiments; all isoforms of these species used herein are in the testis. Amino acids identical among more than fifteen isoforms are indicated by single-letter code on gray background. Gaps in the sequences are indicated by dash (–).

applied the method to amplification of GC isoforms in the gonads of medaka fish, which have been know to possess six membrane GC isoforms and a soluble GC (α and β subunits). By this method, the six GC isoforms (*OlPTGC01*, *OlPTGC02*, *OlPTGC05*, *OlPTGC11*, *OlPTGC19*, and *OlPTGC32*) were amplified from the testis sample and three GC isoforms (*OlPOGC01*, *OlPOGC03*, and *OlPOGC17*) were amplified from the ovary sample (Fig. 1). *OlPTGC02* and *OlPOGC01* are identical, and each corresponded to *OlGC2* which could be a member of the natriuretic peptide receptor family (Yamagami S, Muramatsu R, and Suzuki N, unpublished data). OlPTGC01 and OlPOGC17, and OlPTGC32 and OlPOGC03 were identical, respectively. OlPTGC11 corresponded to OlGC1, which could be considered another member of the natriuretic peptide receptor family (Takeda K and Suzuki N, unpublished data). OlPTGC05 corresponded to OlGC6, which

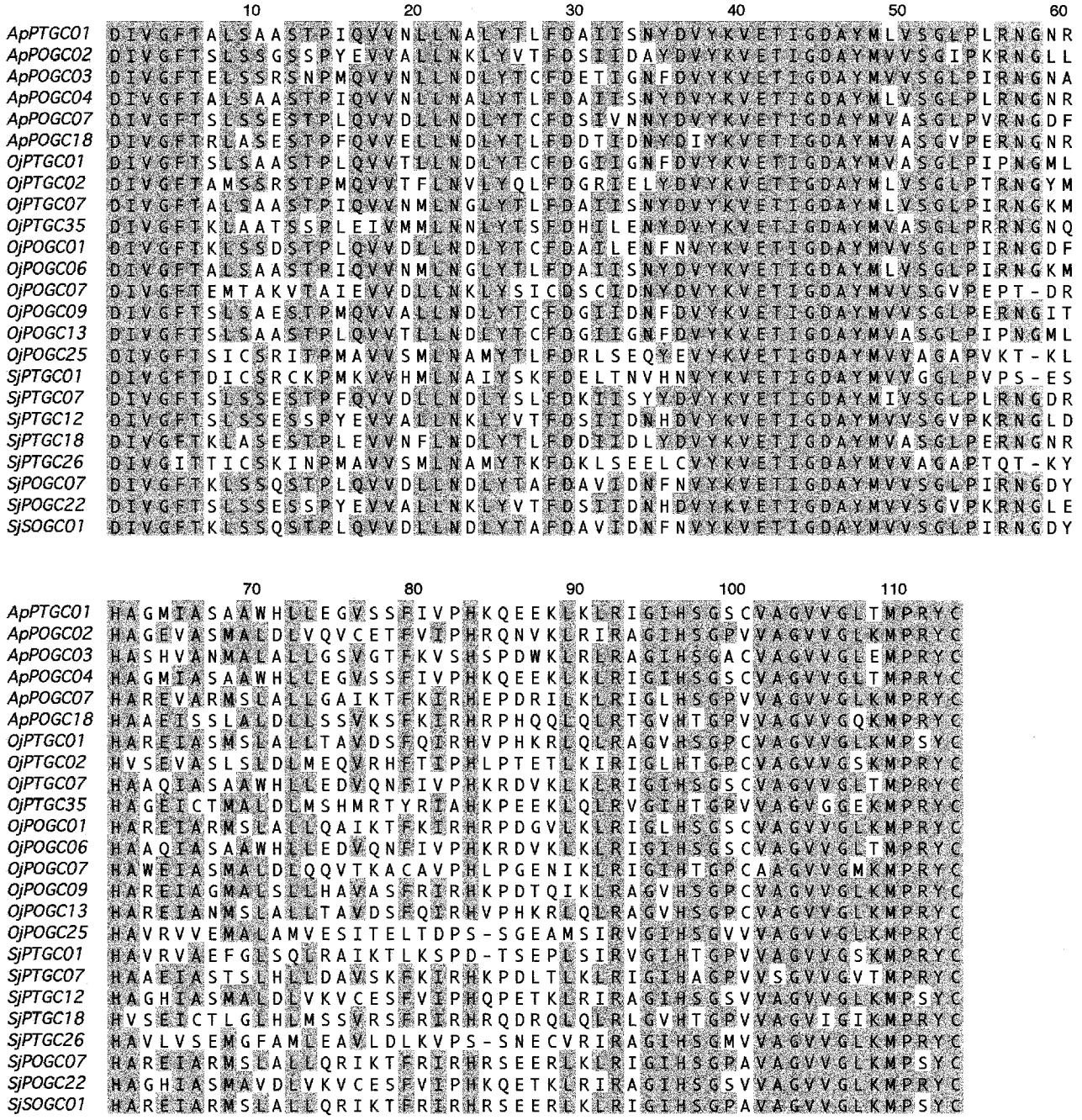

Fig. 3. GC isoforms identified in the gonads of several echinoderm species. The nomenclature for the isoforms is same as that in the legend for Fig. 2; ApPTGC** and ApPOGC** are the isoforms in the testis and ovary of the starfish *A. pectinifera*, respectively; OjPTGC** and OjPOGC** are the isoforms in the testis and ovary of the brittle star *O. japonicus*, respectively; and SjPTGC** and SjPOGC** are the isoforms in the testis and ovary of the sea cucumber *S. japonicus*, respectively. Amino acids identical among more than 13 isoforms are indicated by single-letter code on gray background. Gaps in the sequences are indicated by dash (–).

has been suggested to be a medaka fish homolog of the mammalian enterotoxin/guanylin receptor (Mantoku *et al.*, 1999). The amino acid sequence of *OlPTGC19* was identical to that of a part of *OlGCS-*β_1, which encodes the β subunit of a NO-sensitive soluble GC of medaka fish (Mikami *et al.*, 1998).

Using the same method as described above, three GC isoforms were identified in the testis sample of *D. setosum* and three GC isoforms were found in the ovary sample. Similarly, three GC isoforms were obtained from the testis and three from the ovary sample of *G. crenularis*; five isoforms were obtained from the testis of *H. pulcherrimus*, and three from the ovary. The sequence of HpPTGC04 was identical to that of HpPOGC05. HpPTGC12 corresponded to HpGC, which has been proved to be a protein associated with SAP-I receptors (Shimizu *et al.*, 1996). Thus, this isoform is referred to herein as a SAP receptor-type GC. The testis of *C. japonicus* and that of *B. agassizii* each contained three isoforms. Alignment of the deduced amino acid sequences of these GC isoforms are shown in Fig. 2. Some of these isoforms (DsPTGC03, DsPOGC02, DsPOGC04, GcPTGC03, GcPOGC03, and HpPTGC08) could be considered to be putative soluble GC isoforms. Other echinoderm species also possessed various GC isoforms in their testis and ovary (Fig. 3). ApPTGC01, the only isoform identified in the testis of *A. pectinifera*, was identical to ApPOGC04 in the ovary of the species. OjPTGC01 and OjPTGC07 in the testis of *O. japonicus* were identical to OjPOGC13 and OjPOGC06, respectively, in the ovary of the species. The ovary of the brittle star *O. japonicus* contained two putative soluble GC isoforms, and the testis of the sea cucumber *S. japonicus* possessed two putative soluble GC isoforms.

Comparison and phylogenetic analysis of the amino acid sequences of various GCs

In order to understand structural and evolutionary relationship among various GC isoforms obtained in this study and already known various membrane and soluble GC isoforms, the amino acid sequences of the catalytic domain of these GC isoforms were compared and phylogenetic analysis was carried out. An unrooted phylogenetic tree indicated that these GC isoforms appear to fall into two groups: (i) the membrane GCs, and (ii) the soluble GCs, which can be further divided into α and β subunits of soluble GCs and putative soluble GCs found in echinoderms and nematoda (Fig. 4). The membrane GCs can also be further divided, into vertebrate GCs and invertebrate GCs. The vertebrate membrane GCs can be divided into three major group GCs, as was previously reported (Seimiya *et al.*, 1997): (i) natriuretic peptide receptors, (ii) heat-stable enterotoxin/guanylin receptors, and (iii) sensory organ-specific membrane GCs. The SAP receptor-type GC was distantly related to all of the vertebrate membrane GCs (Fig. 5). Phylogenetic analysis with nucleotide sequences demonstrated similar results (data not shown).

Phylogenetic analysis with various echinoderm membrane GC isoforms and known membrane GC isoforms demonstrated that these membrane GC isoforms can be divided into several groups: (i) a group comprised of five isoforms (HpPTGC12, BaPTGC03, GcPTGC01, ApPTGC01, and OjPTGC07), and (ii) several other groups, including Group A (Fig. 6). The phylogenetic tree with nucleotide sequences of the catalytic domains of these membrane GC isoforms demonstrated similar results (data not shown).

Isolation and characterization of a cDNA clone encoding a membrane GC from a *B. agassizii* or *C. japonicus* testis cDNA library

Molecular phylogenetic analysis with the 341-bp cDNA fragment of *BaPTGC03* obtained by RT-PCR suggested that the cDNA fragment could be part of a SAP receptor-type GC of *B. agassizii* spermatozoa. Using *BaPTGC03* as a probe, a clone named *BaSTGC01*, 4110-bp in length, was obtained from a *B. agassizii* testis cDNA library. Comparison of the deduced amino acid sequence of *BaSTGC01* with those of known SAP receptor-type GCs indicated that *BaSTGC01* contains the entire coding region typical of these. *BaSTGC01* consists of a 149-bp 5'-untranslated region (5'-UTR), a 3414-bp open reading frame (ORF), and a 547-bp 3'- untranslated region (3'-UTR). Termination codons occur in all three frames upstream of the putative initiation codon of *BaSTGC01*. In addition, nucleotides around the putative initiation codon of *BaSTGC01* fit to the preferred sequence context for the initiation of protein synthesis in eukaryotic mRNAs (Kozak, 1983). The ORF of *BaSTGC01* encodes a polypeptide of 1138 amino acids. Hydropathic analysis (Kyte and Doolittle, 1982; data not shown) and comparison of the deduced amino acid sequence of *BaSTGC01* with those of known SAP receptor-type GCs suggest that the domain organization of BaSTGC01 is similar to that of known SAP receptor-type GCs (Fig. 7). BaSTGC01 contains an amino-terminal signal sequence of 22 amino acids (von Heijne, 1983). Cleavage of the signal sequence would result in a mature protein of 1116 amino acids. The mature protein is comprised of a large extracellular domain (residues 23–520), a single membrane-spanning domain (residues 521–542), a protein kinase-like domain (residues 576–859), and a cyclase catalytic domain (residues 879–1006). Comparison of the deduced amino acid sequence of BaSTGC01 with that of HpGC, a SAP receptor-type GC, in *H. pulcherrimus* spermatozoa, indicates that thirteen serine residues at positions 574, 579, 665, 679, 684, 785, 907, 910, 927, 931, 940, 943, and 998 in the intracellular domain of BaSTGC01 correspond respectively to those at positions 561, 565, 652, 666, 671, 772, 894, 897, 914, 918, 927, 930, and 985 in the intracellular domain of HpGC, all of which have been proved to be phosphorylated (Furuya *et al.*, 1998).

The clones *CjSTGC01* and *CjSTGC02* were 1922 bp and 2953 bp in length, respectively, and both lacking the 5'-portion of coding region. Portions of their deduced amino acid sequences are shown in Fig. 2.

Isolation and characterization of a cDNA clone encoding a membrane GC from a *S. japonicus* ovary cDNA library

Molecular phylogenetic analysis with a 341-bp cDNA frag-

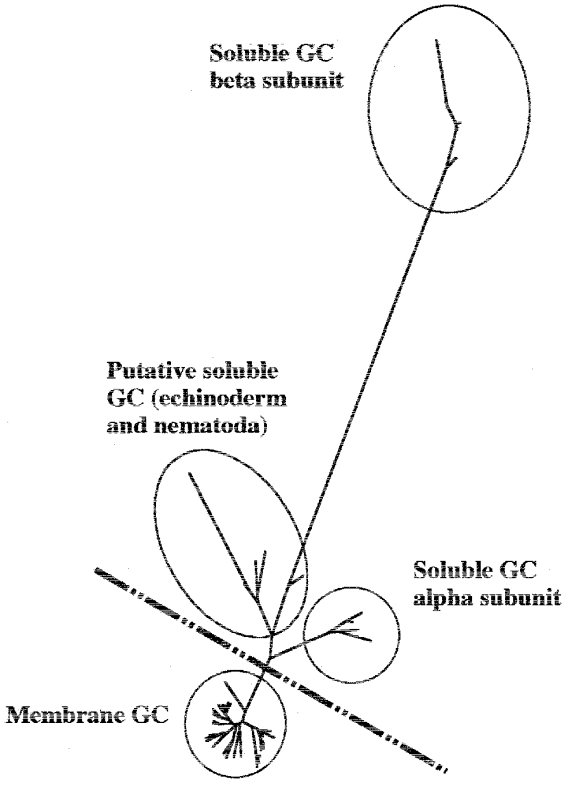

Fig. 4. Molecular phylogenetic relationship among various membrane and soluble GC isoforms. Amino acid sequences of the catalytic domain of various membrane GC isoforms and soluble GC isoforms were subjected to phylogenetic analysis. An unrooted phylogenetic tree was constructed by the neighbor-joining method. Branch lengths were proportional to evolutionary distances.

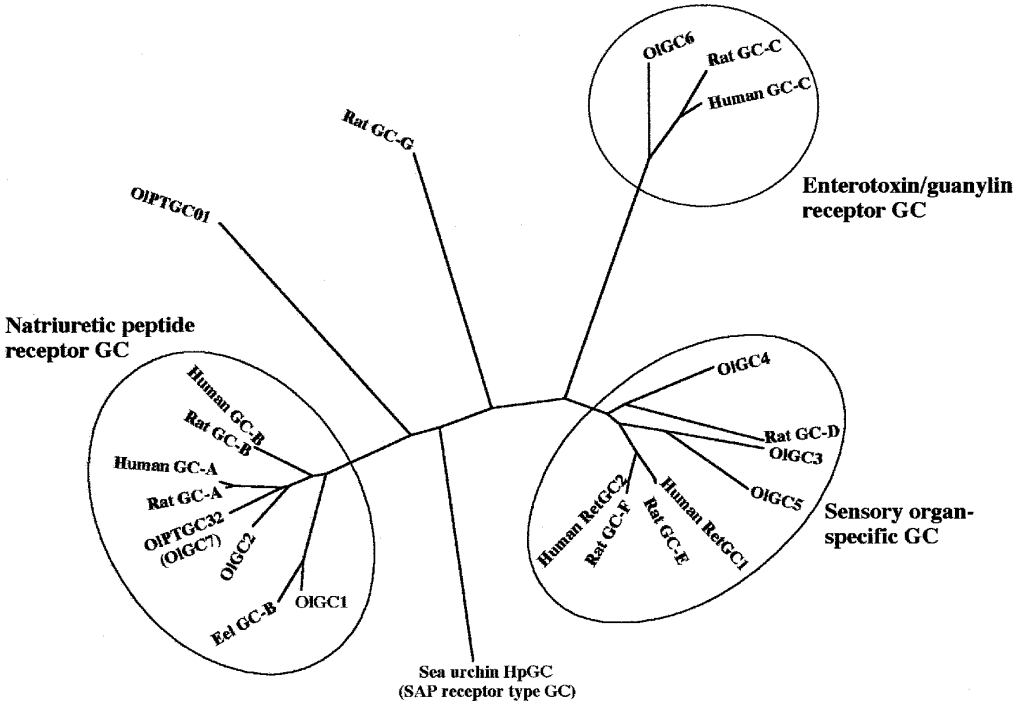

Fig. 5. Molecular phylogenetic analysis with the amino acid sequences conserved in the catalytic domain of various vertebrate membrane GC isoforms. An unrooted phylogenetic tree was constructed by the neighbor-joining method. Branch lengths were proportional to evolutionary distances.

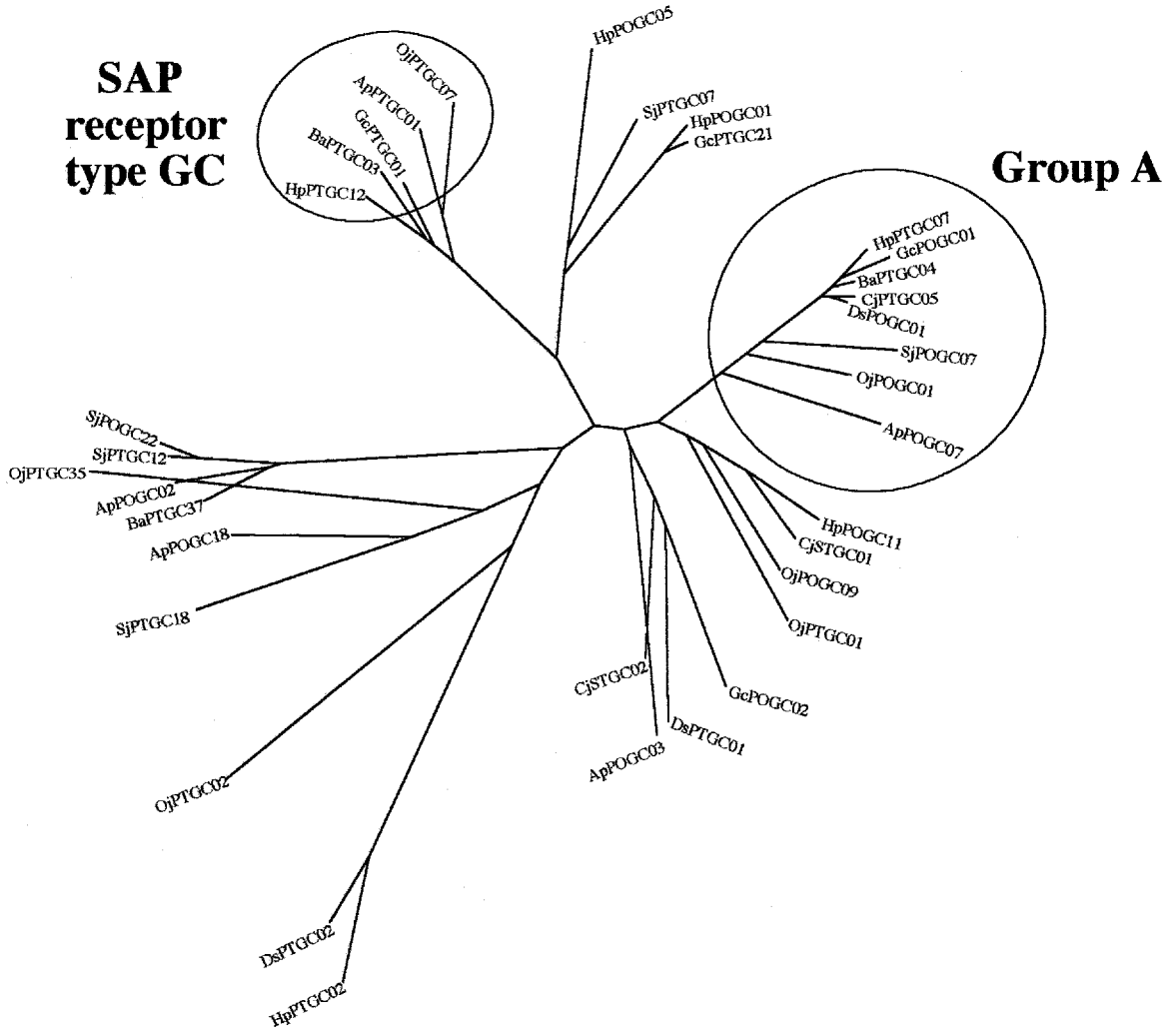

Fig. 6. Molecular phylogenetic relationship among various echinoderm membrane GC isoforms. Amino acid sequences of the catalytic domain of various echinoderm membrane GC isoforms were subjected to phylogenetic analysis. An unrooted phylogenetic tree was constructed by the neighbor-joining method. Branch lengths were proportional to evolutionary distances.

ment (*SjPOGC07*) obtained by RT-PCR indicated that *SjPOGC07* belongs to Group A and appears to be a sea cucumber homolog of the mammalian natriuretic peptide receptor/membrane GC. By screening a *S. japonicus* ovary cDNA library with the insert cDNA of *SjPOGC07* as a probe, a clone named *SjSOGC01* 1752-bp in length was obtained. Comparison of the deduced amino acid sequence of *SjSOGC01* with those of known natriuretic peptide receptors/membrane GCs suggested that *SjSOGC01* lacks the 5'-portion of the coding region. Therefore, the 5'-RACE method was adopted to obtain the 5'-portion of *SjSOGC01*. The primary 5'-RACE product for *SjSOGC01* was 1651-bp in length and did not contain an initiation codon. The secondary 5'-RACE product for *SjSOGC01* was 776-bp in length which contained a putative translation initiation codon. The full-length cDNA sequence for *SjSOGC01* consists of a 551-bp 5'-UTR, a 3225-bp ORF, and an 167-bp 3'-UTR. Termination codons occur in all three frames upstream of the putative initiation codon of *SjSOGC01*. In addition, nucleotides around the putative initiation codon of *SjSOGC01* fit to the preferred sequence context for initiation of protein synthesis in eukaryotic mRNAs (Kozak, 1983). The ORF of *SjSOGC01* encodes a polypeptide of 1075 amino acids. Hydropathic analysis of the deduced amino acid of *SjSOGC01* (Kyte and Doolittle, 1982; data not shown) predicted that SjSOGC01 contains an amino-terminal signal sequence of 28 amino acids (von Heijne, 1983). Cleavage of the signal sequence would result in a mature protein of 1047 amino acids. The mature protein is comprised of a large extracellular domain (residues 29–474), a single membrane-spanning domain (residues 475–493), a protein kinase-like domain (residues 525–811), and a cyclase catalytic domain (residues 832–1059) (Fig. 8). Five cysteine residues at positions 100, 125, 248, 455, and 463, and histidine and tryptophan at positions 138 and 139 in the extracellular domain of SjSTGC01 were conserved in corresponding positions of natriuretic peptide receptors/membrane GCs including eel GC-B,

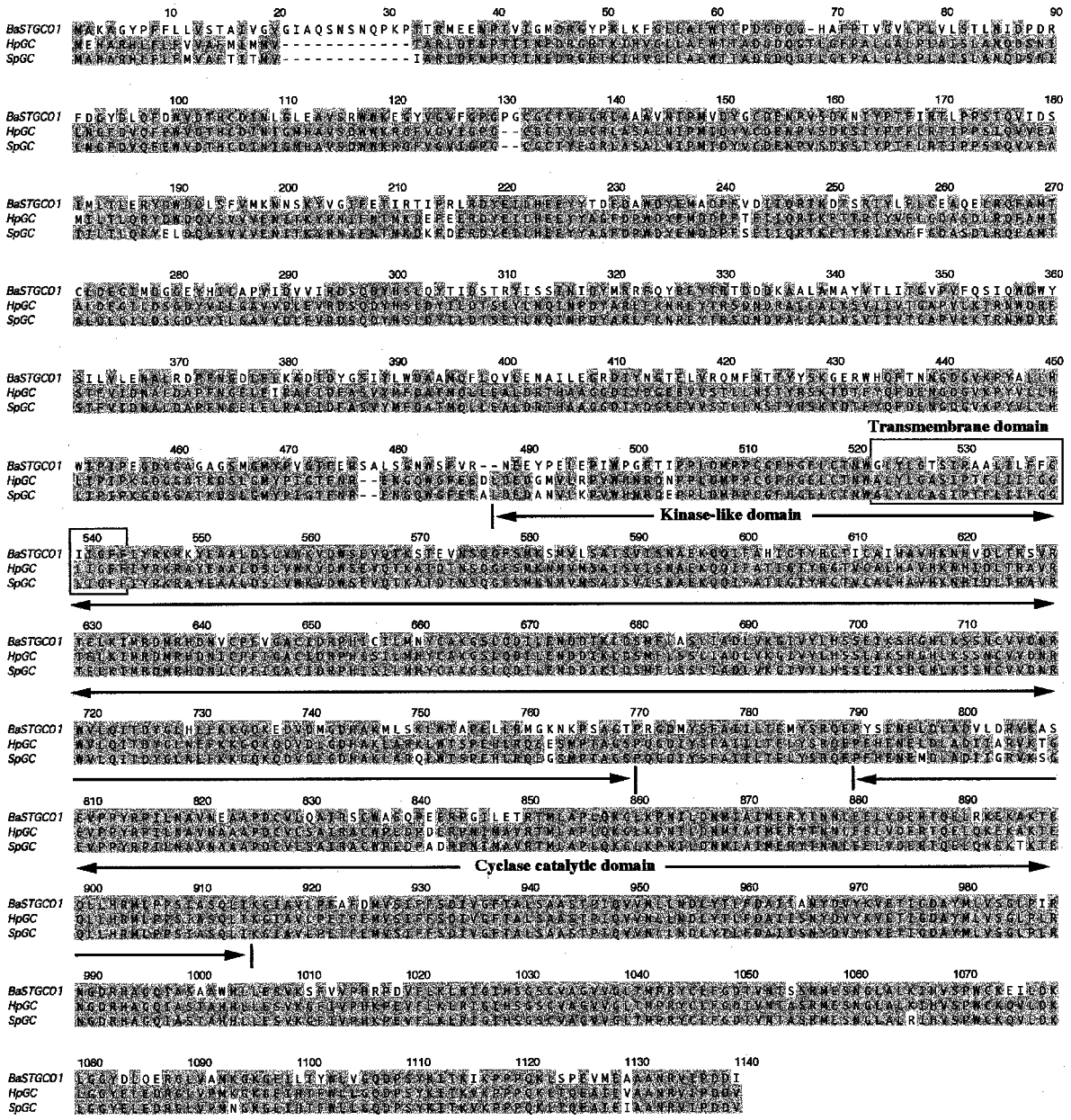

Fig. 7. Alignment of the amino acid sequences of three SAP receptor-type GCs. The deduced amino acid sequence of BaSTGC01 was compared with those of HpGC and SpGC. Transmembrane domain, kinase-like domain, and catalytic domain are indicated by an open box or lines with arrows. Amino acids identical in two isoforms are indicated by single-letter code on gray background. Gaps in the sequences are indicated by dash (–).

rat GC-A, and rat GC-B (Fig. 8).

DISCUSSION

In this paper we demonstrated that the gonads of medaka fish contain many GC isoforms including natriuretic peptide receptors/membrane GCs and a soluble GC. This is consistent with the results obtained from RT-PCR analysis on *OlGC6* and *OlGCS-β_1* transcripts in the ovary and/or testis of medaka fish (Mantoku et al., 1999; Mikami *et al.*, 1998) (Fig. 1). This is also the case for the gonads of various echinoderms including species of sea urchin (Figs. 2, 3). These facts suggest that the unfertilized eggs of these species possess messages for various GCs as stored mRNA, and the mature spermatozoa of these species may carry their translation products, although there is the possibility that somatic cells surrounding the immature eggs or spermatozoa possess such mRNAs. To our knowledge, no report has demonstrated that the gonads of echinoderms including species of sea urchin contain various GC isoforms such as the natriuretic peptide receptor/membrane GC and soluble GC excepting SAP receptor-type GC. In this regard, it should be mentioned that natriuretic pep-

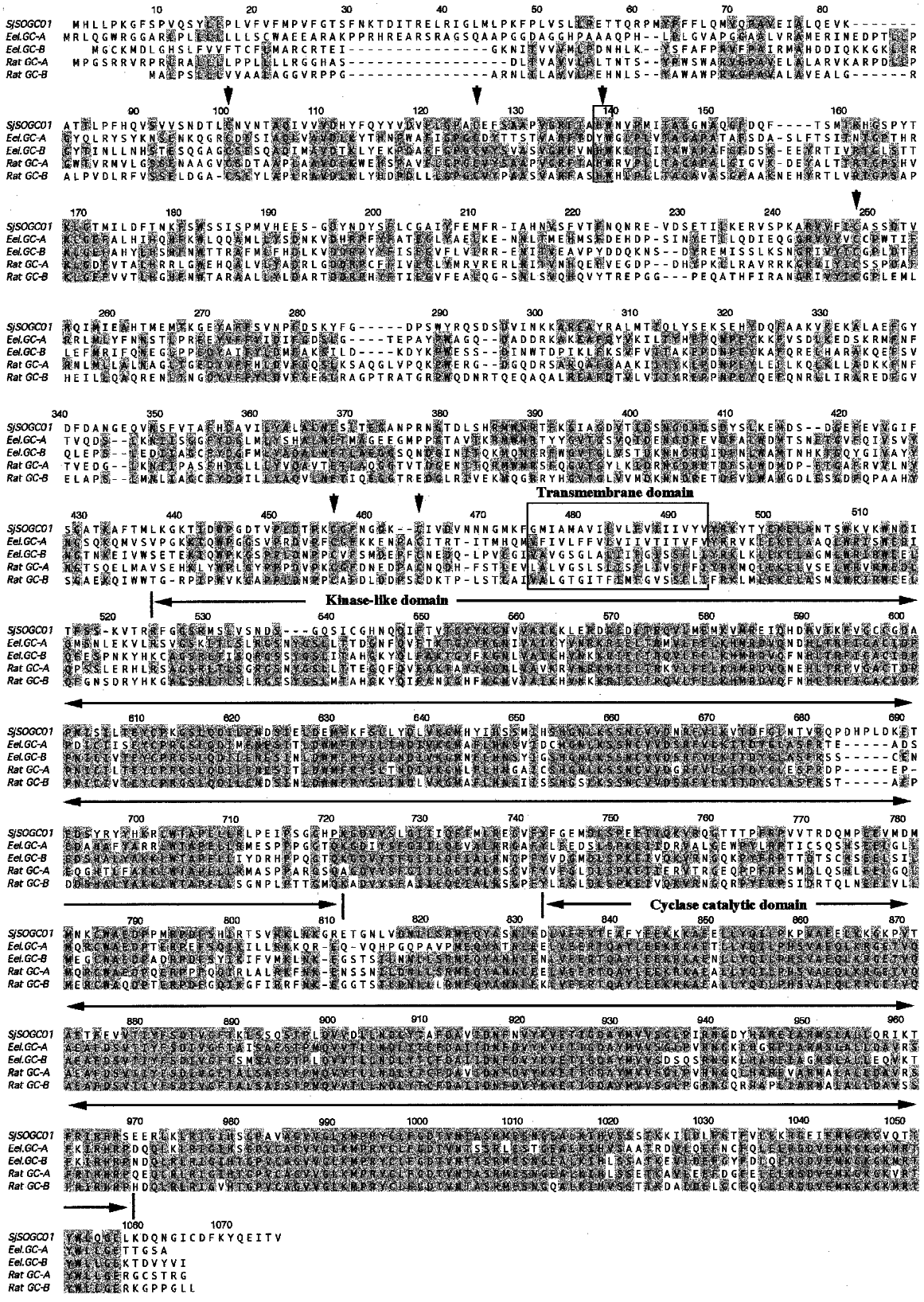

Fig. 8. Comparison of the deduced amino acid sequence of SjSOGC01 with those of natriuretic peptide receptors. The conserved cysteine residues in the extracellular domain are indicated by arrows, and the conserved histidine-tryptophan (^{138}H-^{139}W) residues in the extracellular domain, which are important for ligand-binding, are indicated by an open box with an arrow. Transmembrane domain, kinase-like domain, and cyclase catalytic domain are indicated by open box or lines with arrows. Amino acids identical among three isoforms are indicated by single-letter code on gray background. Gaps in the sequence are indicated by dash (–).

tides and NO have been shown to induce a sperm acrosome reaction in mammals (Anderson *et al*., 1994 ; Herrero *et al*., 1998). These results suggest that a natriuretic peptide receptor/membrane GC and/or a NO-sensitive soluble GC are present in the spermatozoa and involved in these processes.

Phylogenetic analysis with the amino acid sequences of medaka fish and other vertebrate membrane GC isoforms demonstrates that vertebrate membrane GC isoforms could be divided into three groups: (i) natriuretic peptide receptors, (ii) heat-stable enterotoxin/guanylin receptors, and (iii) sensory organ-specific membrane GCs (Fig. 5). The analysis also shows that neither rat GC-G or OlPTGC01 from medaka fish testis is related to any member among the three groups. It is possibile that the orthologues for rat GC-G and for OlPTGC01 are present in other vertebrates. Similarly, the phylogenetic analysis on echinoderm membrane GC isoforms indicates that echinoderm membrane GCs could be divided into several groups: (i) SAP receptor-type GC; (ii) Group A, and (iii) others. Further, the analysis indicates that every echinoderm species contains one membrane GC isoform belonging to Group A. However, the position of DsPOGC01 differs from the position expected in the phylogenetic tree from a morphological standpoint (Fig. 6; Shigei, 1974).

Though it remains to be proved that such a membrane GC exists in *B. agassizii* spermatozoa and can be activated by SAP-V, the molecular phylogenetic analysis carried out in this study suggests that BaSTGC01 (identical to BaPTGC03) is a SAP receptor-type GC isoform in *B. agassizii* spermatozoa. Similarly, our analysis demonstrated that BaPTGC03, GcPTGC01, and HpPTGC12 would be SAP receptor-type GCs in sea urchins *B. agassizii*, *G. crenularis*, and *H. pulcherrimus*, respectively; and that ApPTGC01 and OjPTGC07 would be this type of GC in the starfish and brittle star, respectively. However, despite uniform application of the RT-PCR method, we could not amplify a cDNA fragment for the SAP receptor-type GC in the sea urchin species of *C. japonicus* or *D. setosum* or the sea cucumber *S. japonicus*. This may be due to use of inadequate primers to amplify the cDNA fragment for the SAP receptor-type GC in those species. The SAP receptor-type GCs have several conserved regions other than those we used for the synthesis of primers in this study (Fig. 7). Therefore, we could perhaps amplify the cDNA fragment for the SAP receptor-type GC in *C. japonicus*, *D. setosum*, and *S. japonicus* if we used other conserved amino acid sequences such as QKGLKP, MIAIME, VSPWCK, and DKLGGY to design PCR-primers.

The extracellular domain of natriuretic peptide receptors/membrane GCs contains conserved ligand-binding sites such as cysteine residues, which form two disulfide-linked loops and histidine-tryptophan residues in the extracellular domain (Iwashina *et al*., 1994). SjSOGC01, belonging to Group A, has such cysteine residues and histidine-tryptphan residues in the extracellular domain at positions corresponding to those in vertebrate natriuretic peptide receptors/membrane GCs (Fig. 8). Therefore, these conserved residues in SjSOGC01 might serve to bind unknown ligands that have a structure similar to those ligands that bind to vertebrate natriuretic peptide receptors. In this regard, it may be valuable to note that CNP, a ligand for the natriuretic peptide receptors in mammals, has been reported to exist in a wide variety of tissues in various animals (Suga *et al*., 1992; Vollmar *et al*., 1993; Chrisman *et al*., 1993; Hagiwara *et al*., 1995) and to be involved in the regulation of cardiovascular homeostasis by means of potent natriuretic, diuretic, vasodilatory, and cell growth inhibitory activities. Thus, we presume that SjSOGC01 and the vertebrate natriuretic peptide receptors/membrane GC isoforms might be derived from an ancestral gene. The deduced amino acid sequence of *CjSTGC01* obtained from a *C. japonicus* testis cDNA library is similar to those of the vertebrate natriuretic peptide receptors/membrane GCs, although it is truncated at the 5'-portion of the coding region (data not shown).

It has been reported that a nematoda, *C. elegans*, contains a large family of GC isoforms, and some of these isoforms express at sensory neurons or interneurons (Yu *et al*., 1997). In this study, we carried out phylogenetic analysis with the deduced amino acid sequences of the nematoda GC isoforms and other GC isoforms to determine whether orthologues for such nematoda GC isoforms are present in other animals, but the results were negative. Further, phylogenetic analysis performed on soluble GCs only suggests that genes for soluble GCs can be divided into three groups: (i) soluble GC α subunit group, (ii) soluble GC β subunit group, and (iii) putative soluble GC group found in echinoderms and *C.elegans* (Fig. 4). At present, little data on soluble GCs is available for molecular phylogenetic analysis. When more data on soluble GCs fas been accumulated, we can obtain a clearer understanding of the phylogenetic relationship among diverse GC isoforms.

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