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Expression of Sperm-Activating Peptide IV Receptor-Associated Membrane Guanylyl Cyclase in the Testis of the Sea Urchin *Diadema setosum*

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ABSTRACT—We obtained the full-length cDNA clone (*DsPTGC04*) encoding a membrane guanylyl cyclase expressed in the testis of the sea urchin *Diadema setosum*, the egg jelly of which contains sperm-activating peptide IV. The cDNA was 4305 bp long and an open reading frame predicted a protein of 1127 amino acids including an apparent signal peptide of 24 residues. The mature protein of 1103 amino acids is composed of a single transmembrane domain of 25 amino acids that divides the mature protein (Mw 123818) into an amino-terminal, extracellular domain of 484 amino acids and a carboxyl-terminal, intracellular domain of 594 amino acids, with the latter consisting of two clearly defined subdomains, a protein kinase-like and a cyclase catalytic. Four potential N-linked glycosylation sites are present in the extracellular domain and 4 presumed phosphorylatable serine residues are conserved in the cyclase catalytic domain. Northern blot analysis demonstrated that the 4.5 kb mRNA for *DsPTGC04* is expressed only in the testis. Antibodies raised against two synthetic peptides, ^{800}WVENPDERPN^{809} and ^{1080}KPPPQKLSAEVMEAAANREIPEDL^{1103}, corresponding to two carboxyl-terminal portions of DsPTGC04, reacted with a protein of about 120 kDa in *D. setosum* spermatozoa and testis, but not with any protein in the ovary, eggs, or intestine. Immunohistochemistry showed that both antibodies react with the mature spermatozoa in the testis.

INTRODUCTION

Sea urchin egg jelly contains oligopeptides called sperm-activating peptides (SAPs) which have many biological effects on sea urchin spermatozoa such as transient increases in intracellular cGMP and cAMP concentrations, activation of a Na^+/H^+ exchange system, and increases in intracellular pH (Suzuki and Yoshino, 1992). In the last two decades, 74 SAPs have been isolated from the egg jelly of 17 species of sea urchins distributed over five taxonomic orders (Echinoida, Arbacioda, Clypeasteroida, Diadematoida and Spatangoida for review, see Suzuki, 1995). These SAPs show essentially the same biological effects on sea urchin spermatozoa, although the biological effects and structures of SAPs are specific at the ordinal level. Therefore, we divided these SAPs into five groups, SAP-I from species in Echinoida, SAP-II from Arbacioda, SAP-III from Clypeasteroida, SAP-IV from Diadematoida and SAP-V from Spatangoida (Suzuki, 1990).

The transient increase in cGMP concentrations induced by SAPs has been attributed to the transient activation and subsequent inactivation of the sperm plasma membrane guanylyl cyclase (membrane GC), which is closely linked to the state of enzyme (Suzuki, 1999). SAP-IIA, a subtype of SAP-II, isolated from the egg jelly of *Arbacia punctulata* binds specifically to a 160 kDa sperm membrane GC on the plasma membrane and causes an initial transient activation and subsequent inactivation of the enzyme (Ramarao and Garbers, 1985; Shimomura *et al.*, 1986). 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 62 kDa non-enzymatic sperm protein, respectively, and activate the respective sperm membrane GC (Harumi *et al.*, 1991; Shimizu *et al.*, 1994). Similarly, SAP-III isolated from the sand dollar *Clypeaster japonicus binds* to three sperm plasma proteins, one of which is a membrane GC (Yoshino and Suzuki, 1992; Suzuki, 1999). These facts indicate that the membrane GC is itself SAP-binding protein or a protein associated with the receptor for SAP.

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In our previous studies, we isolated a cDNA clone (λ*GC4-7-1/HpPTGC12*) for a *H. pulcherrimus* sperm membrane GC associated with the receptor for SAP-I and demonstrated that the *HpPTGC12* gene is expressed only in the testis (Shimizu *et al.*, 1996). We also isolated a cDNA clone (*BaSTGC01*) encoding a membrane GC associated with the receptor for SAP-V (GCEGLFHGMGNC) in the egg jelly of the sea urchin *Brissus agassizii* (Suzuki *et al.*, 1999). In the present paper, we report the complete nucleotide sequence and the expression of a cDNA clone (*DsPTGC04*) for a membrane GC associated with the receptor for SAP-IV (GCPWGGAVC) in the egg jelly of the sea urchin *Diadema setosum*.

MATERIALS AND METHODS

Materials

D. setosum sea urchins were collected along the coast near Usa Marine Biological Institute, Kochi University in June, 2000. The gonads (testes and ovaries) and intestines were dissected out from the adult specimens. Spermatozoa were obtained from the dissected mature testis using a pipette.

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

Total RNA was prepared from the *D. setosum* growing testis and ovary 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 (sdp F1, 5'-CAGAARG-GAYTGAARCC-3'; sdp F2, 5'-ATGATYGCSATCATGGA-3'; sdp R1, 5'-TAACCWCCAAGCTTATC-3'; sdp R2, 5'-TTTRCACCATGGAC-TMAC-3') were synthesized based on the amino acid sequences of 4 conserved regions (QKGLKP, MIAIME, DKLGGY, USPWCK) in known sea urchin sperm membrane GCs (see Shimizu *et al.*, 1996 for *H. pulcherrimus*; Thorpe and Garbers, 1989 for *Strongylocentrotus purpuratus*; Suzuki *et al.*, 1999 for *B. agassizii*). These primers were used to amplify membrane GC cDNA fragments from cDNA reverse-transcribed total RNA of the *D. setosum* gonad 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 *DsPTGC04* 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). Total RNA (2 μg) isolated from testis was reverse-transcribed with gene-specific antisense oligonucleotide primers (GSP1, GSP4, GSP7, and GSP10). 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 (GSP2, GSP5, GSP8, GSP11). The following PCR conditions were applied: for GSP2, denaturation at 94°C for 5 min followed by 30 amplification cycles (96°C for 30 sec, 61.7°C for 1 min, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP 5, denaturation at 94°C for 5 min followed by 30 amplification cycles (96°C for 30 sec, 62°C for 1 min, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP 8, denaturation at 94°C for 5 min followed by 30 amplification cycles (96°C for 30 sec, 62°C for 1 min, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP 11, denaturation at 94°C for 5 min followed by 30 amplification cycles (96°C for 30 sec, 62°C for 1 min, 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) and nested primers (GSP3, GSP6, GSP9, and GSP12). Amplification was performed as follows: for GSP3, denaturation at 94°C for 5 min followed by 25 amplification cycles (96°C for 30 sec, 63.7°C for 1 min, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP6, GSP9, and GSP12, amplification was performed under the same conditions as used for GSP3 except for annealing temperature (62°C, 63.8°C, 62°C), respectively. The PCR products were cloned into pBluescript II KS (+) and sequenced. The gene-specific primers used were complementary to nucleotide positions 2945–2964 (GSP1), 2923-2943 (GSP2), 2878-2899 (GSP3), 2634-2655 (GSP4), 2571–2587 (GSP5), 2539–2560 (GSP6), 1985-2007 (GSP7), 1955-1978 (GSP8), 1922-1944 (GSP9), 1033–1054 (Gsp 10), 1008–1029 (Gsp11), and 962-983 (Gsp12). The 5'-RACE products overlapped in 46–77 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 (3 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.) and another gene-specific oligonucleotide primer (GSP13). One-fifteenth volume of the PCR product was amplified by PCR with the 3'sites Adaptor Primer (Takara Shuzo Co., Ltd.) and another gene-specific oligonucleotide primer (GSP14). The following PCR conditions were used: for GSP13, denaturation at 94°C for 5 min followed by 30 amplification cycles (96°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 GSP14, denaturation at 94°C for 5 min followed by 25 amplification cycles (96°C for 30 sec, 64°C for 1 min, and 72°C for 90 sec) 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 3304-3323 (GSP13) and 3358-3379 (GSP14). The 3'-RACE product overlapped in 88 bp with the 3' end of the clone isolated previously.

Molecular phylogenetic analysis

The nucleotide and deduced amino acid sequences of *DsPTGC04* were compared with those of known echinoderm membrane GC isoforms (see Suzuki *et al.*, 1999) using the Clustal W program (Thompson *et al.*, 1994) and the sequence editor SeqPub (Gilbert, Indiana University). The unrooted phylogenetic tree was constructed using the aligned sequences 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).

Immunological methods

The sequences, ^{800}WVENPDERPN^{809} and ^{1080}KPPPQKLSAEVM-EAAANREIPEDL^{1103} , which correspond to two parts of the carboxyl-terminal portion of *D. setosum* sperm membrane GC (DsPTGC04) were selected as the antigenic determinant according to Hopp and Woods (1981), and designed to contain a cysteine residue to the amino terminus. The peptide was chemically synthesized with a 432 Peptide Synthesizer (Applied Biosystems Inc., Foster, CA, USA) and purified by HPLC as described previously (Shimizu *et al.*, 1996). The purified peptide was conjugated with maleimide-activated keyhole limpet hemocyanin and used for immunization of a Japanese white rabbit (Jia:JW, male, 3 months) as described previously (Shimizu *et al.*, 1996).

The testis, ovary, intestine or spermatozoa was homogenized in a 2% SDS solution with a glass homogenizer. The homogenate was centrifuged at 12,000xg for 30 min at 4°C and the resultant supernatant was used for Western blotting experiments. Western blotting was carried out essentially by the method of Towbin *et al.* (1979) using the 5,000-fold diluted anti-DsPTGC04 rabbit serum obtained from above experiments.

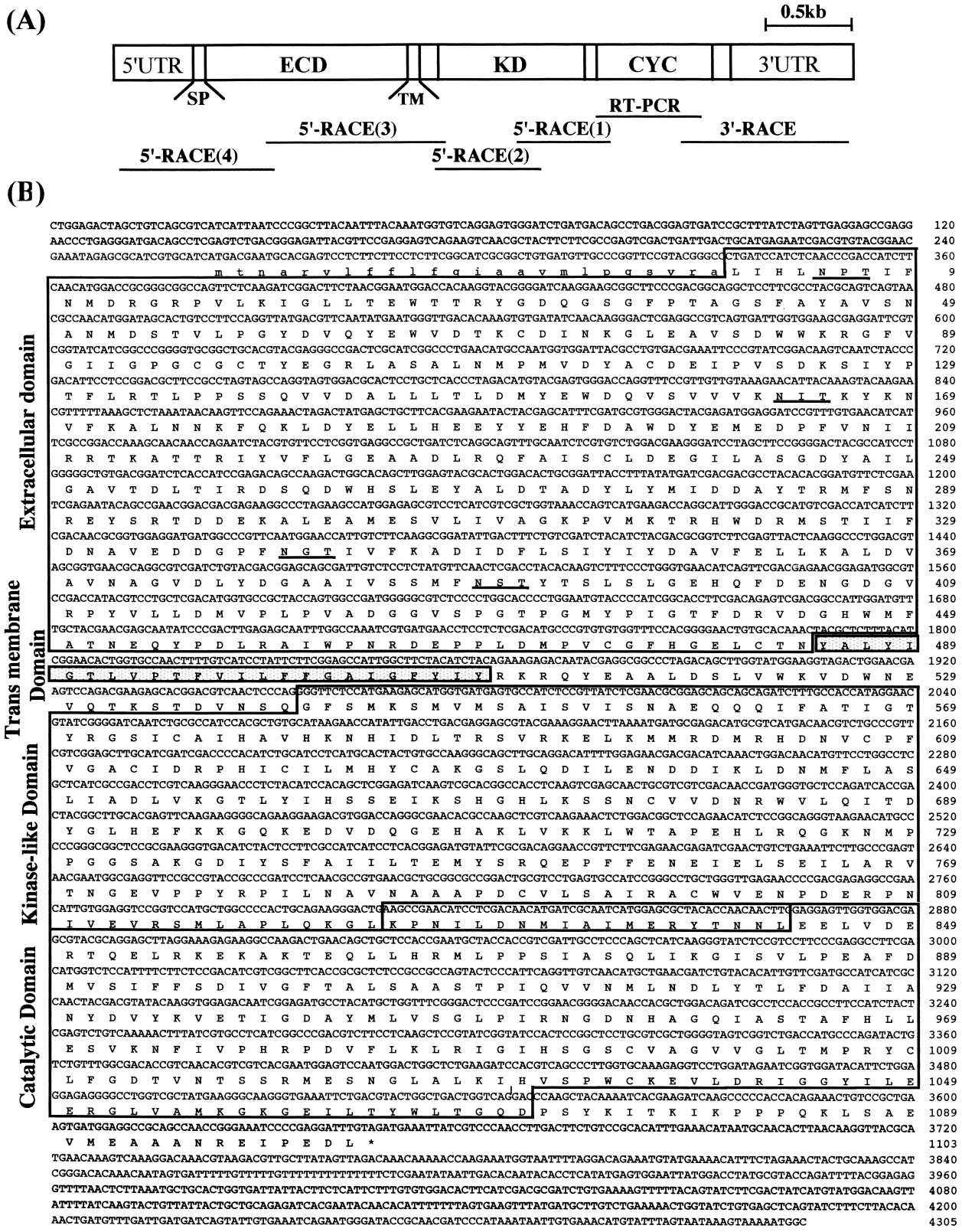

Fig. 1. Schematic diagram of cDNA clone (*DsPTGC04*) (A) and the nucleotide sequence and deduced amino acid sequences of *DsPTGC04* cDNA (B). The predicted structure for *D. setosum* membrane GC cDNA (*DsPTGC04*) is presented at the top. SP, signal peptide; ECD, extracellular domain; TM, transmembrane domain; KD, protein kinase-like domain; CYC, cyclase catalytic domain. The lower lines represent partial length cDNA clones obtained by RT-PCR, or the 5'- and 3'-RACE method. The deduced amino acid sequence is indicated by single-letter code. The signal sequence is indicated by lowercase letters. Extracellular, protein kinase-like, and cyclase catalytic domains are open-boxed. The transmembrane domain is indicated by a shaded box. The potential N-linked glycosylation sequences are underlined.

The testis was isolated, cut out to several pieces, and fixed in 8% paraformaldehyde/0.25 M PIPES, pH 7.5/0.2 M sucrose overnight at 4°C. After washing in 0.25 M PIPES, pH 7.5/0.2 M sucrose, the fixed testis sample was dehydrated in an alcohol series and embedded in TissuePrep® (FisherScientific, Pittsburgh, PA, USA). Sections (5 μm thick) were cut and deparaffinized. After washing in PBS and blocking with 10% fetal cow serum (FCS) in PBS for 1 hr at room temperature, the sections were incubated with anti-DsPTGC04 rabbit serum at 1:200 dilution in 10% FCS in PBS overnight at 4°C. Then, the sections were incubated with a secondary antibody, goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Biosource International, Inc., Camarillo, CA, USA), at 1:100 dilution for 2 hr at room temperature. The cell nuclei were stained with hoechst dye (1:3000 mg/ml) in PBS. After mounting with 50% glycerol in PBS, the sections were observed under a fluorescent microscope (BX50WI; OLYMPUS®, Japan).

Other methods

Northern blot analysis was carried out using poly(A)$^+$RNA (5 μg) and a *DsPTGC04* cDNA fragment (nucleotides 3929-4265) as a probe by the procedure described previously (Seimiya *et al.*, 1997). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out

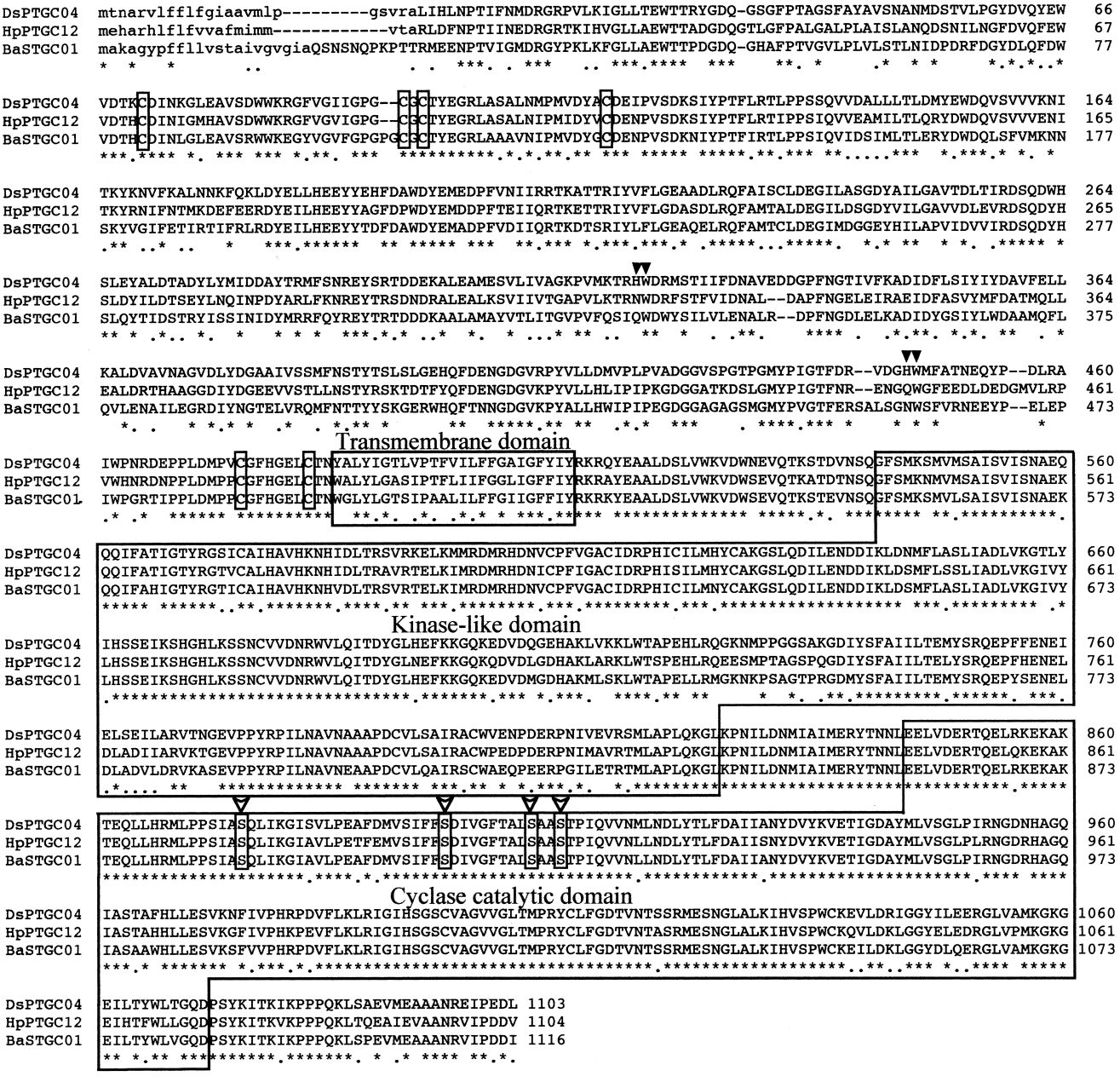

Fig. 2. Alignment of the amino acid sequences of DsPTGC04, HpPTGC12, and BaSTGC01. The deduced amino acid sequence of *DsPTGC04* was compared with those of *HpPTGC12* and *BaSTGC01*. Transmembrane, kinase-like, and catalytic domains are open-boxed. Amino acids identical among the three membrane GCs are indicated with an asterisk (*) below the residues. Gaps in the sequences are indicated by dash (–). The conserved cysteine residues and two histidine-tryptophan sequences (^{320}HW^{321}, ^{446}HW^{447}) which are presumed ligand-binding sites are indicated by open box and closed arrowhead, respectively. Presumed phosphorylatable serine residues are indicated by open box with open arrowhead.

essentially as described by Laemmli (1970). The protein concentration was determined by the method of Schacterle and Pollack (1973). The nucleotide sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) with an Applied Biosystem 377 sequencer (PE Biosystems, Foster City, CA, USA or a 3100 Genetic Analyzer, and analyzed with DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan) and GENETYX-MAX/version 7.2.0. (Software Development, Tokyo, Japan).

RESULTS AND DISCUSSION

Structural characterization of *D. setosum* membrane GC

A 618-bp cDNA fragment (*DsPTGC04*) encoding a part of a membrane GC was obtained by RT-PCR using 4 degenerate oligonucleotide primers and total RNA isolated from the testis of *D. setosum*. To obtain the full-length cDNA clone, we carried out repeated 5'-RACE (4 times) and 3'-RACE, and determined the complete nucleotide sequence. The *DsPTGC04* cDNA was 4305 bp in length, which is in good agreement with the size (4.6 kb) of DsPTGC04 mRNA obtained by Northern blot analysis (Fig. 4A). The cDNA consists of a 382-bp 5'-untranslated region (UTR), a 3348-bp open reading frame (ORF), and a 762-bp 3'-UTR. Termination codons occur in all three frames upstream of the putative initiation codon (ATG) and nucleotides around the putative initiation codon fit to the preferred sequence context for initiation of protein synthesis in eukaryotic mRNAs (Kozak, 1983). As shown in Fig. 1, the ORF of *DsPTGC04* cDNA predicts a protein of 1127 amino acids which contains a 24-amino acid signal sequence (Kyte and Doolittle, 1982; von Heijne, 1983). The mature protein (Mw 123818) with 1103 amino acids is composed of a large extracellular domain (residues 1–483), a transmembrane domain (residues 484–509), and an intracellular protein kinase-like (residues 541–824) and cyclase catalytic (residues 844–1071) domains. There are 4 putative N-linked glycosilation sites (residues 5–7, 163–165, 340–342, and 389–391) in the extracellular domain. The amino acid sequences of both the extracellular and intracellular domains among DsPTGC04, HpPTGC12, and BaSTGC01 are fairly similar (Fig. 2). Six cysteine residues which are predicted to form two disulfide-linked loops in known vertebrate natriuretic peptide receptor/membrane GC (GC-A) are conserved in the corresponding positions of DsPTGC04 (residues 71, 96, 98, 117, 475, and 482). Furthermore, there are histidine-tryptophan residues which are considered to be the ligand-binding site in the extracellular domain of vertebrate natriuretic peptide receptor/membrane GC (GC-A) (Iwashina *et al.*, 1994), while the positions (^{320}HW^{321} and ^{446}HW^{447}) are not conserved in the extracellular domain of DsPTGC04.

The amino acid identities of the catalytic domain of DsPTGC04 to those of HpPTGC12 (*H. pulcherrimus*) and BaSTGC01 (*B. agassizii*) were 90% and 94%, respectively. Phylogenetic analysis using the amino acid sequences of the catalytic domain of various echinoderm membrane GCs (Suzuki *et al.*, 1999) demonstrated that DsPTGC04 can be classfied as an SAP receptor-associated GC (Fig. 3). It has been reported that sea urchin sperm membrane GC is a highly

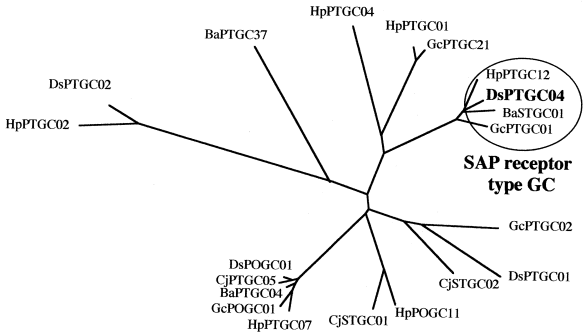

Fig. 3. Molecular phylogenetic relationship among various echinoderm GCs. Aligned amino acid sequences of the catalytic domain of various echinoderm membrane GCs were subjected to phylogenetic analysis. An unrooted phylogenetic tree was constructed by the neighbor-joining method. Branch lengths were proportional to evolutionary distances.

phosphorylated protein and that the state of phosphorylation is closely linked to activation/inactivation of the enzyme (Suzuki *et al.*, 1984; Ramarao and Garbers, 1985). The active *A. punctulata* sperm membrane GC contains up to 17 mol phosphates/mol enzyme, all on serine residues, but after treatment of the spermatozoa with SAP-IIA, the number of phosphoserines decreases to less than 2 mol phosphates/mol enzyme and most of the activity is lost (Vacquier and Moy, 1986). Similarly, the active *H. pulcherrimus* sperm membrane GC (HpPTGC12) contains a maximum of 26 mol phosphates/mol enzyme and the inactive form contains only 4 mol phosphates/mol enzyme (Harumi *et al.*, 1992; Furuya *et al.*, 1998). Furuya *et al.* (1998) identified the positions of 13 phosphoserines in the *H. pulcherrimus* sperm membrane GC by mass spectrometric analysis of isolated phosphoserine-containing peptides and reported that 4 phosphoserine residues (residues 875, 896, 905, and 908) in HpPTGC12 are conserved in the sequence of vertebrate natriuretic peptide receptor/membrane GCs. As shown in Fig. 2, DsPTGC04 possesses 4 serine residues in the corresponding positions (residues 896, 920, 929, and 932), suggesting that these serine residues would be phosphorylated and participate in the control of the enzyme activity.

Immunological characterization of *D. setosum* membrane GC

Sea urchin spermatozoa seem to possess a single molecular species of membrane GC (Radany *et al.*, 1983; Ramarao and Garbers, 1988; Harumi *et al.*, 1992), which are associated with SAP receptor (Ward *et al.*, 1985; Shimomura *et al.*, 1986; Harumi *et al.*, 1991; Yoshino and Suzuki, 1992). It has also been reported that mRNA for the membrane GC is detected only in the testis (Shimizu *et al.*, 1996) and the activity of the enzyme increases during the testis development (Harumi *et al.*, 1992). As shown in Fig. 4B, the site-directed antibody against the carboxyl-terminal portion of DsPTGC04 reacted with an approximately 120 kDa protein of *D. setosum* spermatozoa as well as with the testis, but this antibody did not react with any protein in the ovary or intestine of an adult

individual of *D. setosum*. This is consistent with the results of the Northern blot analysis, which showed that the *DsPTGC04* gene was expressed only in the testis sample (Fig. 4A). Immunohistochemical analysis of the *D. setosum* testis demonstrated that the mature spermatozoa are stained with the DsPTGC04-specific antibody (Fig. 4C).

SAP-IV (GCPWGGAVC) is only one peptide isolated from the egg jelly of the sea urchin *D. setosum* (Yoshino *et al.*, 1990). This peptide stimulates respiration rates and cGMP concentrations of as low as 10^{-9} M in *D. setosum* spermatozoa. Furthermore, it has been reported that the addition of SAP-IV to the spermatozoa results in the mobility change of a major sperm protein from 134 kDa to 128 kDa on SDS-PAGE (Yoshino *et al.*, 1990). The mobility change of the major sperm plasma protein upon addition of specific SAP is typically observed in membrane GCs that are associated with SAP receptors (Suzuki, 1999). These facts and the present results strongly suggest that DsPTGC04 is a membrane GC associated the SAP-IV receptor. To confirm this, we are currently performing cross-linking experiments using an iodinated SAP-IV analogue and *D. setosum* spermatozoa/sperm plasma membrane.

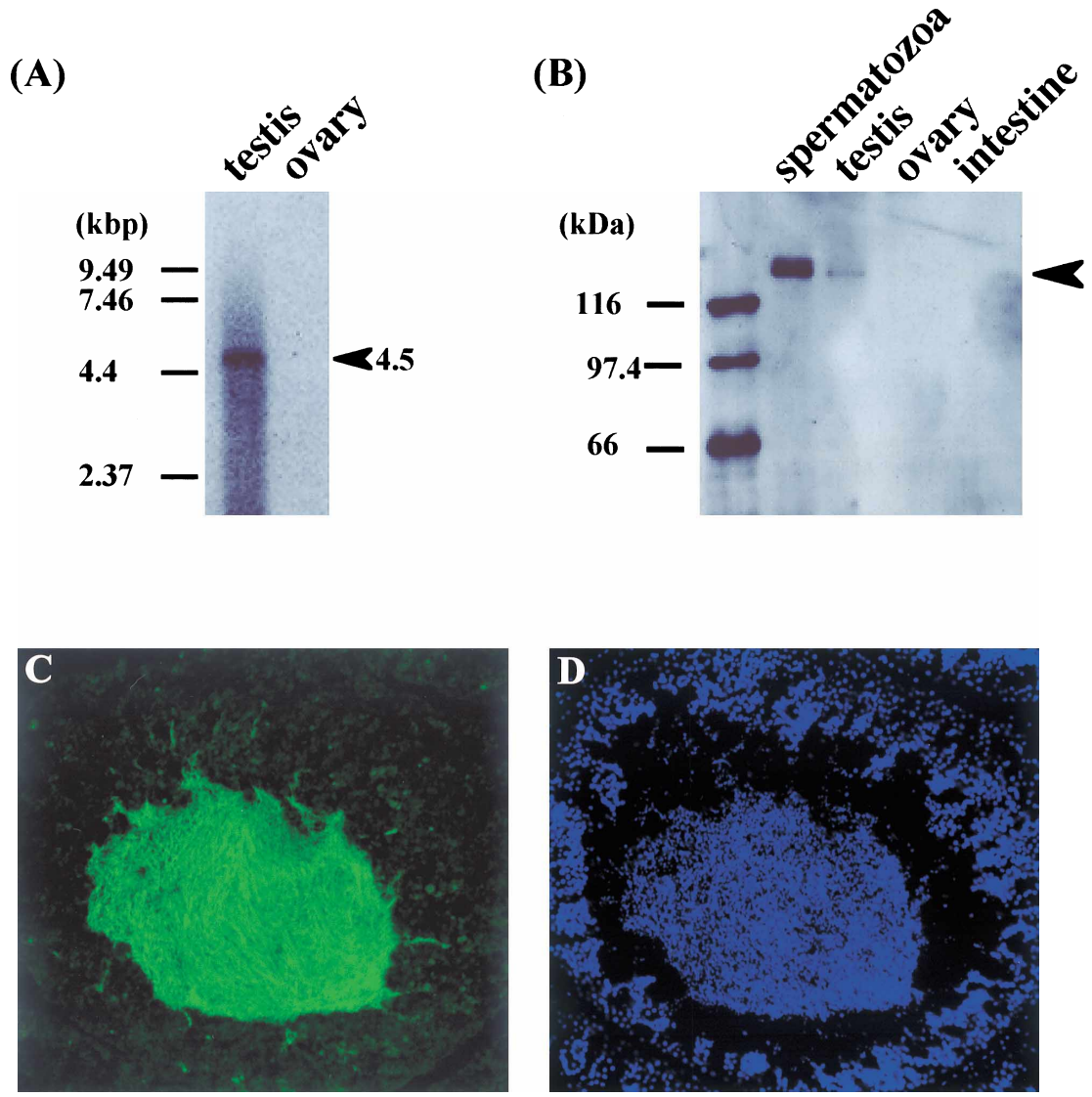

Fig. 4. Northern and Western blot analyses and immunohistochemistry. (A) Poly(A)$^+$RNA (5 μg) prepared from the *D. setosum* growing testis or growing ovary was hybridized to a part (nucleotides 3929-4265) of the 3'-UTR of *DsPTGC04* cDNA. (B) The proteins from the growing testis, growing ovary, unfertilized eggs and intestine were separated by SDS-PAGE with a 6% gel, and then transferred onto a nitrocellulose filter. The proteins on the filter were located by the method of Towbin *et al.* (1979) using a site-directed antibody against the *D. setosum membrane* GC (DsPTGC04). (C) Immunostaining of the mature spermatozoa in the testis by the site-directed anti-DsPTGC04 rabbit antiserum. (D) Hoechst dye-staining of the cell nuclei in the testis.

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