A mRNA for Membrane Form of Guanylyl Cyclase Is Expressed Exclusively in the Testis of the Sea Urchin Hemicentrotus pulcherrimus

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A mRNA for Membrane Form of Guanylyl Cyclase Is Expressed Exclusively in the Testis of the Sea Urchin *Hemicentrotus pulcherrimus*

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ABSTRACT—A cDNA clone encoding the membrane form of guanylyl cyclase was isolated from a *Hemicentrotus pulcherrimus* testis cDNA library and its nucleotide sequence was determined. The cDNA was 4123 bp long and an open reading frame predicted a protein of 1125 amino acids including an apparent signal peptide of 21 residues; a single transmembrane domain of 25 amino acids which divides the mature protein into an amino-terminal, extracellular domain of 485 amino acids and a carboxyl-terminal, intracellular domain of 594 amino acids. Three potential N-linked glycosylation sites were present in the extracellular domain. Northern blot analysis of poly(A)$^+$RNA from testes, ovaries, eggs and embryos at various developmental stages showed that the cDNA encoding guanylyl cyclase hybridized to a mRNA of 4.4 kb from the testes.

We developed a large scale purification method of the phosphorylated (131 kDa) and dephosphorylated (128 kDa) forms of the membrane-bound guanylyl cyclase from *H. pulcherrimus* spermatozoa. The purified 131 kDa and 128 kDa forms of the guanylyl cyclase contained 26.0 ± 1.3 and 4.3 ± 0.7 moles of phosphate per mol protein (mean ± S.D.; n=6), respectively. The amino-terminal amino acids of both the 131 kDa and 128 kDa forms of the guanylyl cyclase could not be detected, suggesting that they were blocked.

INTRODUCTION

Guanylyl cyclase [GTP pyrophosphate-lyase (cycling), EC 4.6.1.2] is found in various cellular compartments as soluble and/or particulate forms and catalyzes the formation of cGMP and inorganic pyrophosphate from GTP (Mittal and Murad, 1982). cGMP concentrations in cells have long been known to increase in response to a wide variety of agents (Goldberg and Haddox, 1977). Critical functions for cGMP have been described in phototransduction (Stryer, 1986) and in mediating the actions of several peptide factors (Hamet *et al.*, 1984; Waldman *et al.*, 1984; Winquist *et al.*, 1984). The binding of sperm-activating peptides, which were originally isolated from sea urchin egg jelly by measuring the respiration-stimulating activity toward sea urchin spermatozoa (Garbers *et al.*, 1982; Suzuki *et al.*, 1981, 1984), to the sperm surface receptor causes a marked and rapid increase and subsequent rapid decrease in cGMP concentrations in sperm cells. The transient increases in cGMP concentrations have been explained by transient activation and susequent inactivation of the guanylyl cyclase, which is closely linked to the state of phosphorylation of the enzyme (Garbers, 1989). It has been reported that in sea urchin spermatozoa most or all of the guanylyl cyclase activity were recovered in particulate fractions (Garbers *et al.*, 1974; Radany *et al.*, 1983). This suggests that sea urchin sperm guanylyl cyclase is bound to the membrane. The membrane form of guanylyl cyclases contains an extracellular domain, a single transmembrane domain, and an intracellular protein kinase-like regulatory and cyclase catalytic domains which are highly conserved among invertebrates and vertebrates (Garbers, 1992; Garbers and Lowe, 1994).

In the previous study, we purified both the phosphorylated and dephosphorylated forms of guanylyl cyclase from spermatozoa of the sea urchin *Hemicentrotus pulcherrimus* and showed that the enzyme was bound to sperm membranes and the phosphorylated form of the enzyme had higher activity than the dephosphorylated form (Harumi *et al.*, 1992). In the study, we suggested that the phosphorylated form of *H. pulcherrimus* sperm guanylyl cyclase might be associated with a 71 kDa sperm-activating peptide-I (SAP-I)-binding protein which was localized in *H. pulcherrimus* sperm tails. Recently, we reported that a mRNA encoding the 71 kDa SAP-I-binding protein was ex-

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pressed exclusively in the testis of *H. pulcherrimus* (Shimizu *et al.*, 1994). In this study, we purified the membrane form of guanylyl cyclase in large amounts from *H. pulcherrimus* spermatozoa and isolated a cDNA clone encoding the guanylyl cyclase from a *H. pulcherrimus* testis cDNA library. We also show that the mRNA for the guanylyl cyclase was expressed in the testis but not in the ovary and eggs nor developing embryos.

MATERIALS AND METHODS

Materials

The sea urchins, *H. pulcherrimus* were collected at the coast near Noto Marine Laboratory, Kanazawa University. Spermatozoa and eggs were obtained by intracoelomic injection of 0.5 M KCl. Spermatozoa were collected as "dry sperm" at room temperature and stored on ice or at –70°C until use. The cDNA Synthesis Kit, Hybond-N membrane, [γ-^{32}P]ATP (111 TBq/mmol) and [α-^{32}P]dCTP (110 TBq/mmol) were products of Amersham International plc. (Amersham, UK). 7-DEAZA Sequencing Kit ver. 2.0 was from Takara Biomedicals (Kyoto, Japan). Sequenase ver. 2.0 DNA Sequencing Kit was from United States Biochemical Co. (Cleveland, OH, USA). Restriction enzymes, T4 DNA ligase, and other enzymes were purchased from Takara Biomedicals Co. or Toyobo Co. (Osaka, Japan). The Random-Primed DNA Labeling Kit was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). The plasmid pBluescript II KS(+), pBluescript II KS(–) and M13KO7 helper phage were generously provided by Professor Yoshitaka Nagahama at the National Institute for Basic Biology, Okazaki, Japan. Rabbit anti-serum against a synthetic peptide (KPPPQKLTQEAIEIAANRVIPDDV) which corresponds to the carboxyl-terminal portion of *Strongylocentrotus purpuratus* sperm guanylyl cyclase was a generous gift of Dr. Tim Quill in Professor David L. Garbers laboratory at University of Texas Southwestern Medical Center, Dallas, Texas, and rabbit anti-serum against a synthetic peptide (KPPPQKLTQEAIEVAANRVIPDDV) corresponding to the carboxyl-terminal portion (residue numbers from 1102 to 1125) of *H. pulcherrimus* sperm guanylyl cyclase was made in our laboratory. Other chemicals of analytical grade were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan), Nacalai Tesque Inc. (Kyoto, Japan) or Sigma Chemical Co. (St. Louis, MO, USA).

Fertilization and embryo culture

The collected and washed eggs were fertilized and cultured at a population density of about 2×10^4 embryos/ml of Millipore-filtered (0.45 μm) seawater at 20°C. A 10 ml-aliquot of the egg suspension was transferred to a centrifuge tube at 0, 3, 6, 8, 10, 12, 14, and 16 hr after fertilization, and then centrifuged at 2,000xg for 10 min at room temperature. The resultant precipitate was frozen in liquid nitrogen and kept at –70°C until use.

Preparation of RNA

The testes and ovaries were dissected out from the adult *H. pulcherrimus* as described previously (Suzuki *et al.*, 1982). Total RNA was prepared from samples of *H. pulcherrimus* ovaries, testes, eggs and embryos at various developmental stages by the LiCl method (Cathala *et al.*, 1983). Poly(A)$^+$RNA was then purified by two passage of the total RNA over a column of oligo(dT)-cellulose (Pharmacia) (Davis *et al.*, 1986).

Cloning and sequencing of cDNAs

A cDNA library (4.9×10^5 pfu) from poly(A)$^+$RNA isolated from *H. pulcherrimus* growing testes was constructed in λgt10 using the cDNA Synthesis System and the cDNA Cloning System λgt10 (Amersham). Approximately 7.1×10^4 plaques were screened on replicate Hybond-N membranes with ^{32}P-end-labeled, mixed antisense synthetic oligonucleotide probes which correspond to a part of the extracellular domain (nucleotide numbers from 760 to 803) (probe II, 44 mer; 3'-TCTAAGACGTGCTCCTCATGATGCGCCCTAAGCTA-GGTACCCTG-5') and the intracellular domain (nucleotide numbers from 2689 to 2731) (probe I, 45 mer; 3'-TTGTACTAGCGGTAGT-ACCTCGCGATGTGGTTGTTAGACCTCCTC-5') of the membrane form of guanylyl cyclase of *Arbacia punctulata* spermatozoa (Singh *et al.*, 1988). Finally 6 positive clones were obtained and the phage DNA was purified. Digestion of the DNA with *Kpn*I showed that four of them contained inserts of 4.0 kbp and two of them contained inserts of 4.3 kbp. Restriction mapping showed that two clones with inserts of 4.3 kbp were identical. The 4.3 kbp cDNA insert from an isolated clone λGC4-7-1 was subcloned into the plasmid vector pBluescript II KS(+) (Stratagene). Serial deletion mutants of subclones were made according to the method described by Yanisch-Perron *et al.* (1985). Nucleotide sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using the 7-DEAZA Sequencing Kit ver. 2.0 and the Sequenase ver. 2.0 DNA Sequencing Kit using [α-^{32}P]dCTP and analyzed on DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan).

Northern blot analysis

Northern blot analysis was carried out as follows: A 1.6 μg of poly(A)$^+$RNA was denatured with 2.1 M formaldehyde, electrophoresed on a 1% agarose gel in the presence of 2.2 M formaldehyde, and transferred onto a Hybond-N membrane. The RNA on the membrane was then hybridized to the random-primed, [α-^{32}P]dCTP-labeled 2249 bp cDNA insert (nucleotide numbers from 1 to 2249 of the λGC4-7-1 cDNA insert) at 65°C for 18 hr. The membrane was washed with 6 x SSC and 0.1% SDS at room temperature for 30 min, followed by final wash with 0.5 x SSC and 0.1% SDS at 65°C for 30 min. The size of RNA was estimated using 0.24–9.5 kb RNA Ladder (GIBCO BRL, Gaithersburg, MD, USA) as markers.

Purification and proteolytic digestion of the membrane form of guanylyl cyclase from H. pulcherrimus *spermatozoa*

Three test tubes, each of which contains 10 g wet weight of dry sperm were placed in a boiling water bath at 100°C for 10 min and then the test tubes were cooled down by placing in an ice bath for 10 min. The boiled dry sperm (total 30 g wet weight) were pooled, suspended in 35 ml of distilled water and kept in a freezer until use. Two ml of the suspension were mixed with 2 ml of 20% SDS and vortexed with heating. The suspension was centrifuged, and the resulting supernatant, after being mixed with an equal volume of the SDS-PAGE sample buffer without SDS, was applied on a preparative SDS-PAGE system model 491 Prep-Cell (BioRad Laboratories, Richmond, CA, USA) using a 6% polyacrylamide gel. Five hundred microlitter of fractions were collected. Every three fractions were analyzed for presence of guanylyl cyclase by Western blotting using anti-*S. purpuratus* guanylyl cyclase antiserum. Fractions containing guanylyl cyclase were pooled and used for further experiments. For purification of the dephosphorylated form of *H. pulcherrimus* guanylyl cyclase, dry sperm were suspended in seawater and then 2 μM of sperm-activating peptide I (SAP-I) was added to the sperm suspension. After 1 min incubation at 20°C, the mixture was centrifuged at 10,000xg for 30 min at 4°C. The resulting sperm pellet was treated as described above.

The purified guanylyl cyclase was digested for 8 hr at 37°C with lysyl endoprotease (Achromobacter Protease I) in 50 mM Tris-HCl (pH 9.0) containing 0.1% CHAPS at enzyme to substrate ratio of 1:100 (w/w). The peptides generated were separated by HPLC using a Shimadzu model LC6A chromatography system on a reverse-phase column (Unisil QC-18, 5 μm, 6.0×250 mm), which was developed with a linear gradient of 5–60% acetonitrile (ACN) in 0.1% trifluoroacetic acid and then 0–60% ACN in 5 mM sodium phosphate (pH 5.7) at a flow rate of 1 ml/min at 40°C. The column efflu-

ent was monitored for an absorbance at 225 nm.

Analyses of amino acid composition and amino acid sequence

Peptide samples were hydrolyzed with constant-boiling HCl at 110°C for 20 hr. The hydrolysate was dried and dissolved in 100 μl of coupling solution [ethanol:0.1 M boric acid buffer, pH 9.0:phenylisothiocyanate (PITC), 79:20:1, v/v/v]. The mixture incubated at room temperature (20–25°C) for 15 min. After being dried, 100 μl of sample buffer (3% ACN in 50 mM sodium phosphate buffer, pH 6.5 containing 50 mM sodium perchlorate) was added and submitted to HPLC on a reverse-phase column (TSKgel ODS 80 TM, 5 μm, 4.6×150 mm), which was developed with a linear gradient of 3–38.25% ACN in 50 mM sodium phosphate buffer (pH 6.5) containing 50 mM sodium perchlorate for 20 min at a flow rate of 1 ml/min at 40°C. The column effluent was monitored for an absorbance at 254 nm.

Amino acid sequence analysis was performed on an Applied Biosystem model 476A pulsed-liquid sequencer with an on-line model 120A phenylthiohydantoin amino acid analyzer. To analyze the amino-terminal amino acid sequence of both the phosphorylated and dephosphorylated forms of guanylyl cyclase, both forms of the enzyme purified by the preparative SDS-PAGE system were subjected to slab gel electrophoresis separately and then the protein in the gel was transferred to a PVDF membrane using a Multiphor II NovaBlot Electrophoresis Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) at room temperature for 1 hr at 0.8 mA/cm^2 constant current (Towbin *et al.*, 1979). The membrane was rinsed three times with distilled water for 5 min each. The protein was visualized by staining briefly with Coomassie brilliant blue R250. A Coomassie-stained protein band corresponding to each form of guanylyl cyclase (131 kDa for the phosphorylated form and 128 kDa for the dephosphorylated form) was cut out and submitted to automated Edman degradation on an Applied Biosystems model 476A pulse-liquid sequencer.

Immunological methods

The sequence KPPPQKLTQEAIEVAANRVIPDDV which corresponds to the carboxyl-terminal portion (residue numbers from 1102 to 1125) of *H. pulcherrimus* sperm guanylyl cyclase was 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 432A Peptide Synthesizer (Applied Biosystems Inc.) and purified by reversed-phase HPLC on a Unisil QC-18 column (5 μm, 4.6×250 mm). HPLC was carried out with Shimadzu Model LC-6A chromatography system. The column effluent was monitored by absorbance at 225 nm with use of a Shimadzu SPD-6AV spectrophotometer. We used the following program for purification of the peptide. The sample was applied to the column equilibrated with 5% ACN in 0.1% TFA and unabsorbed materials were washed out with the equilibration solvent. Then, peptides were eluted with a linear gradient of ACN from 5 to 60% in 0.1% TFA over a 55-min time period at a flow rate of 1 ml/min. The peptide collected in a major fraction was rechromatographed with the same program.

The peptide (210 nmol) was conjugated to 0.7 nmol of maleimide-activated keyhole limpet hemocyanin according to the manufacturer's instruction. The protein (100 μg) coupled to the peptide was emulsified in complete Freund's adjuvant (1/1, v/v) and injected intracutaneously into the back of a Japanese white rabbit. Subsequently the same amount of antigen in incomplete adjuvant was administered at 2 weeks and 4 weeks after the first injection. At 12 days after the last injection, titer of the antiserum was measured by an enzyme-linked immunosorbant assay (ELISA) using the peptide as an antigen according to the procedures of Voller *et al.* (1976). Then, the rabbit was bled from vein on the ear, and the antiserum was stored at 4°C until use.

Immunoblotting experiments were carried out essentially by the method of Towbin *et al.* (1979) using rabbit anti-*H. pulcherrimus* or anti-*S. purpuratus* guanylyl cyclase antiserum.

Other methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (1970). The gel was stained with silver by the method of Morrisey (1981). Phosphate content of the purified guanylyl cyclases was determined by the method described in a paper (Buss and Stull, 1983). To avoid unfavorable influence of SDS derived from the guanylyl cyclase preparation on color development in determination of phosphate , fractions containing low concentration of the guanylyl cyclase were dialyzed exhaustively against distilled water and then concentrated with an Amicon Diaflo Cell RK 52 using a YM100 membrane which passes through substances with molecular weight less than 100,000. This procedure was useful to avoid unneccessary accumulation of SDS in the sample. The concentration of protein was determined by the Lowry method (Lowry *et al.*, 1951) modified by Schacterle and Pollack (1973) or by the method of Bradford (1976) using BSA as a standard.

RESULTS

Isolation and sequence analysis of cDNA clone encoding H. pulcherrimus guanylyl cyclase

A cDNA library representing the mRNA from *H. pulcherrimus* testis was screened with mixed antisense oligonucleotide probes which were synthesized based on the amino acid sequences of the extracellular and intracellular

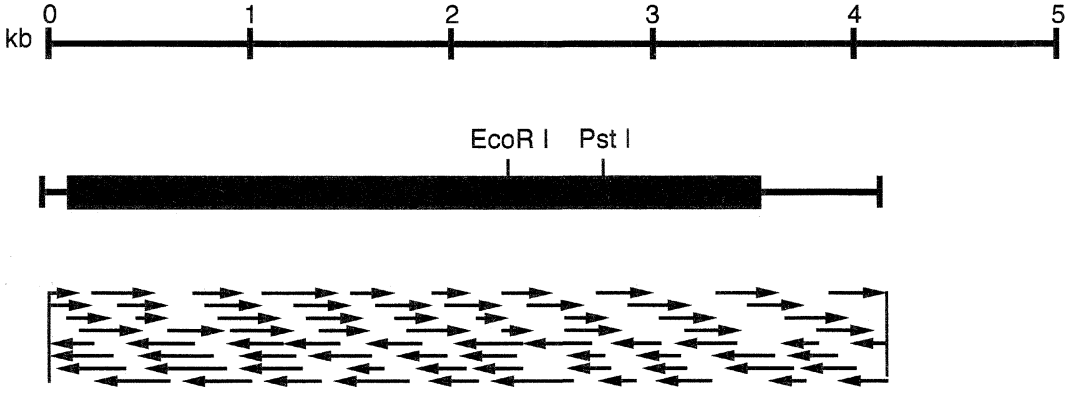

Fig.1. The restriction endonuclease map and sequencing strategy for λGC4-7-1 cDNA insert. The map only shows the relevant restriction sites. The direction and extent of the sequence determination are indicated by arrows. The deduced open reading frame is shown by a solid box.

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GCCGGGATAAACATTTAGGGTAACTGCGGGATCAAAATAAAAAGAAAGACATTTCGTGCATCTATTATTAAACGAAATTGTCCAACGCGCGCGTGCATTATGGAGCATGCACGACACCTG  120
                                                                                                  M  E  H  A  R  H  L    7
TTCCTATTTGTGGTCGCCTTTATGATCATGATGGTGACTGCGAGATTGGACTTTAATCCAACCATCATCAACGAAGATCGTGGAAGAACCAAGATTCATGTTGGATTACTGGCAGAATGG  240
F  L  F  V  V  A  F  M  I  M  M  V  T  A  R  L  D  F  N  P  T  I  I  N  E  D  R  G  R  T  K  I  H  V  G  L  L  A  E  W   47
ACAACCGCAGACGGAGACCAAGGAACACTTGGCTTTCCCGCTCTTGGTGCTTTACCTTTAGCAATATCACTGGCCAACCAAGATTCTAACATTCTAAATGGATTTGACGTTCAGTTTGAA  360
 T  T  A  D  G  D  Q  G  T  L  G  F  P  A  L  G  A  L  P  L  A  I  S  L  A  N  Q  D  S  N  I  L  N  G  F  D  V  Q  F  E   87
TGGGTCGACACCCACTGTGATATTAATATCGGAATGCATGCTGTAAGTGATTGGTGGAAACGAGGTTTTGTTGGTGTCATTGGACCGGGTTGTGGTTGTACTTATGAGGGTCGTCTTGCC  480
 W  V  D  T  H  C  D  I  N  I  G  M  H  A  V  S  D  W  W  K  R  G  F  V  G  V  I  G  P  G  C  G  C  T  Y  E  G  R  L  A  127
TCTGCTCTCAACATCCCCATGATCGACTATGTTTGTGATGAAAACCCAGTATCGGATAAATCCATCTATCCAACTTTCCTTCGTACCATTCCACCAAGCATCCAAGTTGTCGAAGCCATG  600
 S  A  L  N  I  P  M  I  D  Y  V  C  D  E  N  P  V  S  D  K  S  I  Y  P  T  F  L  R  T  I  P  P  S  I  Q  V  V  E  A  M  167
ATCCTTACACTACAAAGATACGACTGGGATCAGGTGTCAGTAGTTGTTGAAAATATCACGAAGTACCGGAACATCTTTAACACAATGAAGGACGAATTTGAAGAGCGAGATTATGAGATT  720
 I  L  T  L  Q  R  Y  D  W  D  Q  V  S  V  V  V  E  N  I  T  K  Y  R  N  I  F  N  T  M  K  D  E  F  E  E  R  D  Y  E  I  207
CTGCACGAGGAGTATTATGCAGGATTCGATCCATGGGACTACGAAATGGATGATCCTTTCACTGAAATTATCCAACGGACCAAAGAAACAACGAGAATTTATGTATTCCTTGGTGATGCT  840
 L  H  E  E  Y  Y  A  G  F  D  P  W  D  Y  E  M  D  D  P  F  T  E  I  I  Q  R  T  K  E  T  T  R  I  Y  V  F  L  G  D  A  247
AGCGACCTTCGTCAGTTTGCTATGACAGCCTTAGATGAGGGAATCTTAGACTCGGGTGATTATGTGATTCTTGGAGCCGTCGTTGATTTAGAAGTCAGAGACAGTCAAGATTATCATAGT  960
 S  D  L  R  Q  F  A  M  T  A  L  D  E  G  I  L  D  S  G  D  Y  V  I  L  G  A  V  V  D  L  E  V  R  D  S  Q  D  Y  H  S  287
CTCGATTATATCCTTGATACATCTGAATACTTGAATCAGATAAATCCTGATTATGCACGACTCTTTAAGAATCGGGAATATACTAGAAGTGACAACGACCGTGCGCTTGAAGCTTTGAAG 1080
 L  D  Y  I  L  D  T  S  E  Y  L  N  Q  I  N  P  D  Y  A  R  L  F  K  N  R  E  Y  T  R  S  D  N  D  R  A  L  E  A  L  K  327
AGTGTTATCATTGTTACCGGAGCACCTGTACTTAAAACAAGAAACTGGGATCGATTTTCAACCTTTGTGATCGACAACGCACTTGATGCGCCTTTCAATGGTGAATTAGAAATTAGAGCT 1200
 S  V  I  I  V  T  G  A  P  V  L  K  T  R  N  W  D  R  F  S  T  F  V  I  D  N  A  L  D  A  P  F  N  G  E  L  E  I  R  A  367
GAAATTGACTTTGCATCGGTTTATATGTTTGATGCCACAATGCAGCTTTTGGAAGCCTTAGATCGCACACACGCAGCTGGGGGTGATATATATGATGGAGAAGAAGTGGTTTCGACCCTC 1320
 E  I  D  F  A  S  V  Y  M  F  D  A  T  M  Q  L  L  E  A  L  D  R  T  H  A  A  G  G  D  I  Y  D  G  E  E  V  V  S  T  L  407
TTAAACTCGACCTATCGAAGTAAGACCGACACCTTCTATCAGTTCGATGAGAATGGAGACGGTGTAAAGCCTTATGTTCTACTGCATCTTATACCAATACCTAAAGGAGATGGAGGAGCA 1440
 L  N  S  T  Y  R  S  K  T  D  T  F  Y  Q  F  D  E  N  G  D  G  V  K  P  Y  V  L  L  H  L  I  P  I  P  K  G  D  G  G  A  447
ACTAAAGATTCACTCGGCATGTATCCTATTGGAACATTTAATAGAGAAAACGGCCAATGGGGTTTTGAAGAGGATTTGGATGAGGATGGAATGGTTTTGAGACCTGTTTGGCATAACCGA 1560
 T  K  D  S  L  G  M  Y  P  I  G  T  F  N  R  E  N  G  Q  W  G  F  E  E  D  L  D  E  D  G  M  V  L  R  P  V  W  H  N  R  487
GATAATCCTCCTCTGGACATGCCTCCTTGTGGATTCCATGGCGAACTTTGCACAAATTGGGCACTTTATCTTGGAGCTTCAATACCGACCTTCCTCATTATCTTTGGAGGACTTATTGGT 1680
 D  N  P  P  L  D  M  P  P  C  G  F  H  G  E  L  C  T  N  W  A  L  Y  L  G  A  S  I  P  T  F  L  I  I  F  G  G  L  I  G  527
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 F  F  I  Y  R  K  R  A  Y  E  A  A  L  D  S  L  V  W  K  V  D  W  S  E  V  Q  T  K  A  T  D  T  N  S  Q  G  F  S  M  K  567
AACATGGTTATGAGTGCTATCTCGGTCATATCGAATGCTGAAAAACAACAGATCTTTGCTACCATTGGAACATACAGGGGTACAGTGTGTGCTCTTCATGCTGTTCATAAGAACCACATT 1920
 N  M  V  M  S  A  I  S  V  I  S  N  A  E  K  Q  Q  I  F  A  T  I  G  T  Y  R  G  T  V  C  A  L  H  A  V  H  K  N  H  I  607
GATCTGACAAGGGCTGTAAGAACTGAACTGAAAATAATGCGTGACATGAGACATGATAACATTTGTCCTTTCATCGGAGCTTGTATTGATCGTCCCCACATCAGTATCCTGATGCACTAC 2040
 D  L  T  R  A  V  R  T  E  L  K  I  M  R  D  M  R  H  D  N  I  C  P  F  I  G  A  C  I  D  R  P  H  I  S  I  L  M  H  Y  647
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 C  A  K  G  S  L  Q  D  I  L  E  N  D  D  I  K  L  D  S  M  F  L  S  S  L  I  A  D  L  V  K  G  I  V  Y  L  H  S  S  E  687
ATCAAATCACATGGGCATCTGAAATCAAGCAACTGTGTGGTCGATAACCGTTGGGTTCTTCAGATCACCGACTATGGCCTGAATGAATTCAAGAAAGGACAGAAACAAGATGTCGACCTC 2280
 I  K  S  H  G  H  L  K  S  S  N  C  V  V  D  N  R  W  V  L  Q  I  T  D  Y  G  L  N  E  F  K  K  G  Q  K  Q  D  V  D  L  727
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 T  E  L  Y  S  R  Q  E  P  F  H  E  N  E  L  D  L  A  D  I  I  A  R  V  K  T  G  E  V  P  P  Y  R  P  I  L  N  A  V  N  807
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 P  N  I  L  D  N  M  I  A  I  M  E  R  Y  T  N  N  L  E  E  L  V  D  E  R  T  Q  E  L  Q  K  E  K  A  K  T  E  Q  L  L  887
CATCGTATGCTTCCACCATCCATTGCATCTCAGCTGATCAAGGGTATTGCTGTCTTACCTGAAACCTTTGAAATGGTTTCCATCTTCTTCTCTGACATCGTTGGTTTCACTGCCCTCTCT 2880
 H  R  M  L  P  P  S  I  A  S  Q  L  I  K  G  I  A  V  L  P  E  T  F  E  M  V  S  I  F  F  S  D  I  V  G  F  T  A  L  S  927
GCGGCTAGTACACCAATTCAGGTCGTGAACCTGCTGAATGATTTGTACACTCTTTTCGATGCCATCATTTCTAACTATGACGTGTATAAGGTTGAAACCATTGGAGATGCATACATGCTT 3000
 A  A  S  T  P  I  Q  V  V  N  L  L  N  D  L  Y  T  L  F  D  A  I  I  S  N  Y  D  V  Y  K  V  E  T  I  G  D  A  Y  M  L  967
GTATCCGGTTTACCTCTCCGTAATGGAGATCGTCATGCTGGTCAGATCGCATCTACTGCTCATCATCTCCTAGAATCTGTCAAAGGATTCATTGTACCTCATAAACCTGAGGTCTTCCTT 3120
 V  S  G  L  P  L  R  N  G  D  R  H  A  G  Q  I  A  S  T  A  H  H  L  L  E  S  V  K  G  F  I  V  P  H  K  P  E  V  F  L  1007
AAACTCCGTATTGGTATCCATTCGGGTTCATGTGTCGCTGGCGTAGTTGGTCTAACGATGCCTCGGTATTGTCTCTTTGGAGATACCGTCAACACCGCTTCCCGTATGGAATCAAATGGA 3240
 K  L  R  I  G  I  H  S  G  S  C  V  A  G  V  V  G  L  T  M  P  R  Y  C  L  F  G  D  T  V  N  T  A  S  R  M  E  S  N  G  1047
CTTGCTCTGAAAATCCACGTTAGTCCATGGTGCAAACAGGTTCTGGATAAGCTTGGTGGTTATGAACTTGAAGATCGAGGCCTTGTTCCAATGAAGGGTAAAGGAGAAATCCATACCTTC 3360
 L  A  L  K  I  H  V  S  P  W  C  K  Q  V  L  D  K  L  G  G  Y  E  L  E  D  R  G  L  V  P  M  K  G  K  G  E  I  H  T  F  1087
TGGTTGCTAGGACAAGATCCAAGCTACAAGATCACCAAGGTTAAGCCACCACCACAGAAGCTCACCCAAGAGGCCATAGAGGTCGCTGCTAATCGTGTCATACCTGATGACGTCTAAATA 3480
 W  L  L  G  Q  D  P  S  Y  K  I  T  K  V  K  P  P  P  Q  K  L  T  Q  E  A  I  E  V  A  A  N  R  V  I  P  D  D  V        1125
TACAACCCACTTGTACAATTAGATTTAACACAAAATTAAGTGCCATATACCAGATACTTTTTGTCCAATGCGAGGTATGCTATTCCAAACCCTTCACGTTAAATATAACGTCGGGTAAAC 3600
TTATTGGTCTTAAAACTTACATAATTCAGTAAGCATCGTAGGAAACATATAAAATGTTTGATAATATTTCAAACGTTTATAGAACTAATAAATGTTACTTTGGTCTGTTGATGAAATGAG 3720
TTAGAGCAAATGTTTAGCAGTTGCTACTCCCCTATAAGCAGAGGTTTGATGAAATTAAGTATACAAGAAAGCTCCTGTTTCGAATTAGAGTTGTCTTATCGAAATAAGAATGAATTTAAT 3840
ACCTCCCTTACTAGCAATGAAAAAATGAACGTAAAACGTTTGTATAATCGCATTGACATTATTTTGCTTCATCATGGCGTATAAATTACGTGTGCCTCAATTTCATCTCGACTAAAAGCT 3960
ACTTGGTAATGTTTATTTACGTGCAGTGTTAAAATCAAACATTGCATGGTAAAGTTACATTTAGAAAAATCCCAGGTAGTTTGCATCTAATCTATGTAACTTGAATTGTTCACGAGTAGC 4080
CAAAAAAAGAATGGAATAAATTATTCGTCAATTAAAAAAAAAA                                                                                4123
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Fig.2. Complete nucleotide sequence and deduced amino acid sequence of the λGC4-7-1 cDNA insert. The predicted amino acid sequence of the open reading frame is shown below the nucleotide sequence. Nucleotide and amino acid number are listed on the right hand side. The signal sequence and the putative transmembrane sequence are indicated by shaded-boxes. The potential N-linked carbohydrate binding sites are indicated by open-boxes. Polyadenylation signal sequences at the 3'-untranslated region are underlined.

domains of *A. punctulata* guanylyl cyclase. The analysis of 7.1×10^4 recombinants from an amplified cDNA library gave rise to finally 6 positively hybridizing clones. The size of the inserts was determined by agarose gel electrophoresis. Four clones contained approximately 4.0 kbp cDNA insert and two clones (λGC4-7-1 and λGC4-7-2) contained almost the same size cDNA insert (approximately 4.3 kbp). One clone, λGC4-7-1, was used for nucleotide sequence determination. The sequencing strategy for λGC4-7-1 is shown in Fig. 1. The complete nucleotide sequence and the deduced amino acid sequence are presented in Fig. 2. The λGC4-7-1 cDNA insert was 4123 bp in length. The oligonucleotide sequences used for screening the clone were found in the sequence at nucleotide positions 708-751 for probe II and 2656-2700 for probe I, respectively. We have assigned the initiation codon to the ATG at nucleotide position 100 because (1) there is an upstream in-frame stop codon, (2) this ATG is flanked by sequences that fit Kozak's criteria for translation initiation codon (Kozak, 1981) and (3) the 21-amino acid sequence following this ATG possesses the features characteristic of signal sequences (Watson, 1984). The initiation codon is followed by an open reading frame of 3378 bp. An in-frame stop codon occurs at nucleotide position 3475 and the 3'-untranslated region composed of 646 bp includes polyadenylation sites (AATAAA). The deduced amino acid sequence suggests that cleavage of signal peptide would yield a protein of 1104 amino acids with a calculated molecular weight of 124,061. The protein contains three potential N-linked glycosylation sites (NXT) at residues 5-7, 164-166 and 409-411, respectively. The protein also contains a hydrophobic region composed of 25 amino acids at residues 486-510 that is flanked on the carboxyl-terminal side by RKR (Fig. 3). These features are typical of membrane-spanning domain of many membrane proteins.

Purification and characterization of H. pulcherrimus sperm guanylyl cyclase

In the previous study, we purified the phosphorylated and dephosphorylated forms of *H. pulcherrimus* sperm guanylyl cyclase, which retained enzyme activity (Harumi *et al.*, 1992). However, the method used in the study was not adequate for large scale purification because the guanylyl cyclase purified by the method, which comprised several initial steps using low pH-solutions containing 100 mM NaF that are known to inhibit the activity of protein phosphatases, lost enzyme-bound phosphates gradually during the storage without phosphatase inhibitors. This might be due to action of a protein phosphatase(s) which is associated tightly with the guanylyl cyclase or is contaminated in the enzyme preparation, and under the conditions without inhibitors the protein phosphatase became active to release phosphates from the enzyme. Therefore, we used the boiled spermatozoa, in which almost all enzymes should be inactive, for purification of the guanylyl cyclase. By this improved method, we could obtain 2.6 mg (about 20 nmol) of the guanylyl cyclase from 10 g wet weight of spermatozoa. The phosphorylated

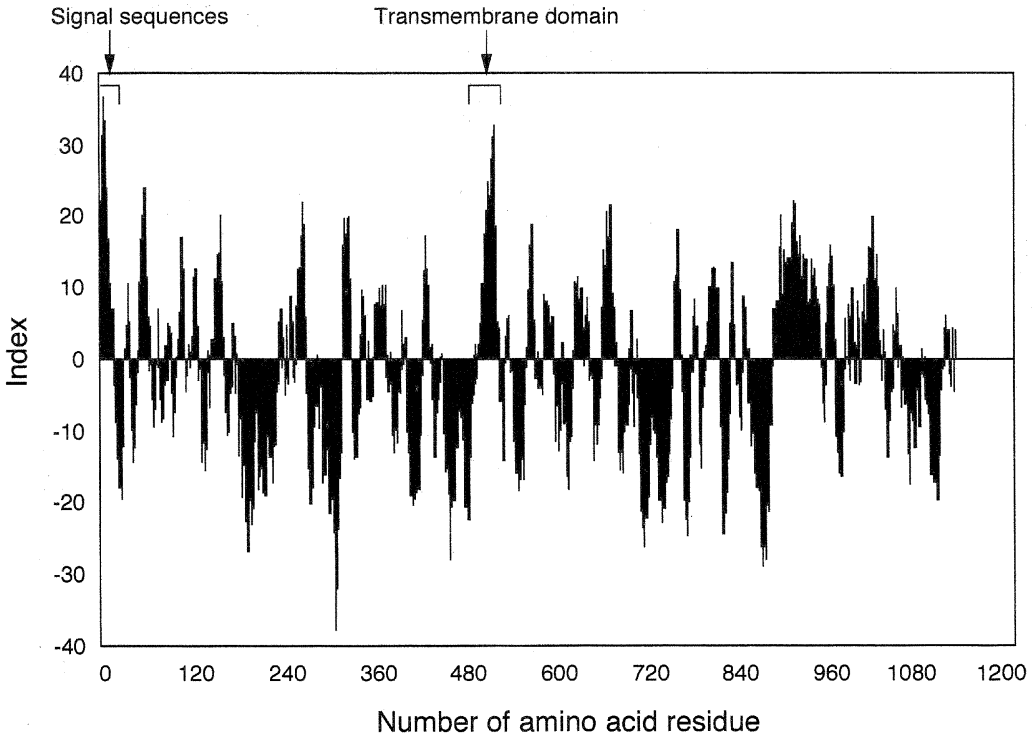

Fig.3. Hydropathy profile of the protein with 1125 amino acids. Hydropathic index is plotted as a function of amino acid number according to Kyte and Doolittle (1982) based upon a window of 12 residues. Region with values below the midpoint line is hydrophilic.

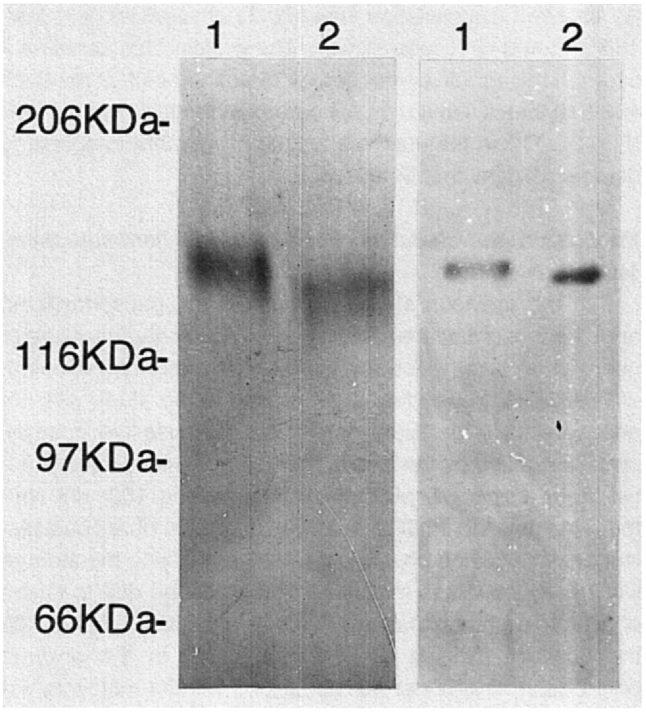

Fig.4. SDS-PAGE and Western blotting analysis of *H. pulcherrimus* sperm guanylyl cyclase. The phosphorylated (1) and dephosphorylated (2) forms of the guanylyl cyclase were analyzed by SDS-PAGE using a 6% gel. The proteins in the gel were silver-stained (left panel) or transferred onto a nitrocellulose filter. The proteins on the filter were located by the method of Towbin *et al.* (1979) using site-directed antibody against the carboxyl-terminal portion of *S. purpuratus* sperm guanylyl cyclase (right panel). The dephosphorylated form of the guanylyl cyclase was purified from *H. pulcherrimus* spermatozoa which were incubated in seawater containing 2 μM SAP-I for 1 min at 20°C.

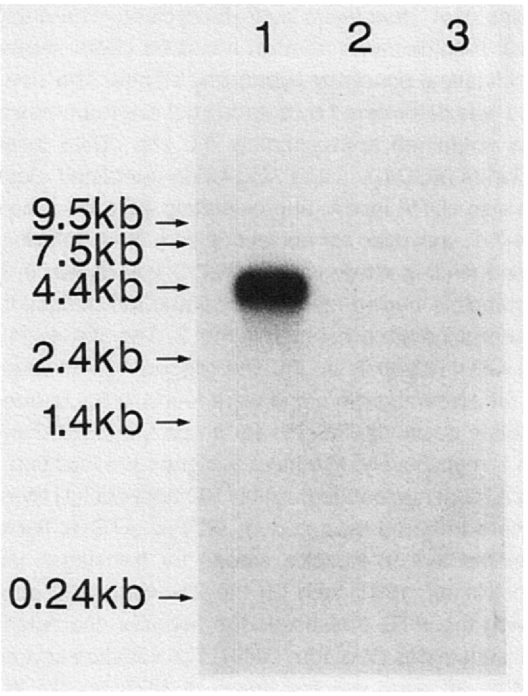

Fig.5. Northern blot analysis. Approximately 1.6 μg of poly(A)$^+$RNA prepared from *H. pulcherrimus* growing testes (1), ovaries (2) or unfertilized eggs (3) was hybridized to a part (nucleotides 1-2248) of the λGC4-7-1 cDNA insert.

and dephosphorylated forms of the guanylyl cyclase thus purified contained 26.0 ± 1.3 and 4.3 ± 0.7 moles of phosphate/mol protein (mean ± S.D., n=6), repectively. These values were comparable to those reported previously (Harumi *et al.*, 1992). Both forms of the guanylyl cyclase reacted with site-directed anti-*H. pulcherrimus* sperm guanylyl cyclase antibody as well as site-directed anti-*S. purpuratus* sperm guanylyl cyclase antibody (Fig. 4). Seven peptides were isolated from lysyl endoprotease-digests of the phosphorylated and dephosphorylated forms of guanylyl cyclase and their amino acid sequences were determined as follows: RAYEAALDSLVWK (peptide 1), VDWSEVQTK (peptide 2), GSLQDILENDDIK (peptide 3), GIVYLHSSEIK (peptide 4), PNILDNMIAIMERYTNNLEELVDERTQELQK (peptide 5), IHVSPWXK (peptide 6) and GEIHTFWLLGQDPSYK (peptide 7). These sequences were found in the deduced amino acid sequence for *H. pulcherrimus* guanylyl cyclase as follows: peptide 1, residues 534 - 546; peptide 2, residues 547 - 555; peptide 3, residues 651 - 663; peptide 4, residues 679 - 689; peptide 5, residues 848 - 878; peptide 6, residues 1052 - 1059; and peptide 7, residues 1082 - 1097. The amino-terminal amino acids of both forms of the guanylyl cyclase could not be detectecd in the sequencing.

Northern blot analysis

To determine the size of the mRNA for the λGC4-7-1 cDNA insert and to see whether the mRNA exists in testes, ovaries, eggs or developing embryos, poly(A)$^+$RNA prepared from these tissues and embryos was analyzed by Northern blot hybridization using a part (nucleotide numbers from 1 to 2248 of the λGC4-7-1) of the λGC4-7-1 cDNA insert as a probe. A strong hybridization signal at the position corresponding to 4.4 kb was detected only with poly(A)$^+$RNA from a testis sample (Fig. 5).

DISCUSSION

It has been reported that SAP-I caused electrophoretic mobility change of *H. pulcherrimus* sperm guanylyl cyclase from 131 kDa to 128 kDa and this mobility change was due to dephosphorylation of the enzyme (Harumi *et al.*, 1992). In this study, site-directed antibody against *S. purpuratus* sperm guanylyl cyclase reacted with both the phosphorylated (131 kDa) and dephosphorylated (128 kDa) forms of *H.*

pulcherrimus sperm guanylyl cyclase (Fig. 4). This suggests that apparent molecular weight difference between the 131 kDa and 128 kDa forms of *H. pulcherrimus* sperm guanylyl cyclase is not due to proteolytic degradation of the carboxyl-terminal portion of the guanylyl cyclase because the antibody was made against the synthetic peptide (KPPPQKLTQEAIEIAANRVIPDDV) which corresponds to the residues 1102 to 1125 of *S. purpuratus* sperm guanylyl cyclase (Thorpe and Garbers, 1989) and identical to the carboxyl-terminal sequence (KPPPQKLTQEAIEVAANRVIPDDV) of *H. pulcherrimus* sperm guanylyl cyclase except underlined valine residue. On the other hand, the amino-terminal amino acids of both the 131 kDa and 128 kDa forms of *H. pulcherrimus* sperm guanylyl cyclase could not be detected, suggesting that the amino-terminal amino acid is blocked. Although there is possibility that after proteolytic degradation of the amino-terminal portion of the enzyme, the resulting new amino-terminal amino acid was blocked again, we presume that the amino-terminal amino acid of *H. pulcherrimus* sperm guanylyl cyclase was post-translationally modified. Therefore, apparent molecular weight change of *H. pulcherrimus* sperm guanylyl cyclase from 131 kDa and 128 kDa upon SAP-I treatment of spermatozoa does not seem to be due to proteolytic degradation of the amino-terminal portion of the enzyme. As with membrane forms of guanylyl cyclases of *H. pulcherrimus, S. purpuratus* and *A. punctulata* spermatozoa, the loss of phosphates from the enzymes which is induced at fertilization by a specific sperm-activating peptide is correlated with a decrease in the enzymatic activity (Harumi *et al.*, 1992; Ramarao and Garbers, 1985; Vacquier and Moy, 1986). These facts lead to a model for the sea urchin sperm guanylyl cyclase: the binding of a ligand (specific sperm-activating peptide) to the receptors activates the cyclase and the activated cyclase is dephosphorylated by a protein phosphatase activated upon the ligand-binding or already active protein phosphatases which became accessible to the cyclase due to its conformational change induced upon the ligand-binding, and the cyclase is subsequently desensitized.

The predicted molecular weight of mature *H. pulcherrimus* guanylyl cyclase (residues 22-1125) was 124,061 which is comparable to the molecular weight of 128,000 for the dephosphorylated form. The apparent small difference between the values may be attributable to glycosylation. We presume that at least one of three potential N-linked glycosylation sites is glycosylated since the *H. pulcherrimus* guanylyl cyclase binds to Concanavalin A-Sepharose. A homology search using NBRF-PIR and SWISS-PROT databases demonstrated that the deduced amino acid sequence of cDNA for *H. pulcherrimus* sperm guanylyl cyclase showed 98% identity over 1125 amino acids with that of *S. purpuratus* spermatozoa (Thorpe and Garbers, 1989) and 77% identity over 926 amino acids with that of *A. punctulata* spermatozoa (Singh *et al.*, 1988). Less identity with *A. punctulata* guanylyl cyclase is due to dissimilarity in the extracellular domain because the intracellular domain of *H. pulcherrimus* guanylyl cyclase has 98% identity with the intracellular domain of *A. punctulata* guanylyl cyclase (Fig. 6). The predicted primary structure of *H. pulcherrimus* guanylyl cyclase bears virtually no resemblance to mammalian receptor/guanylyl cyclase in the extracellular domain (Garbers, 1992). However, the intracellular domain of *H. pulcherrimus* guanylyl cyclase had relatively high similarity to those of mammalian membrane forms of guanylyl cyclase (30-50% identical) (Fig. 6). As shown in Fig. 7, a predicted secondary structure of the intracellular cellular domain of *H. pulcherrimus* guanylyl cyclase is similar to that of *A. punctulata* guanylyl cyclase, although the catalytic domain of *A. punctulata* is smaller than that of *H. pulcherrimus.* Apparent similarity in the predicticted secondary structure of the catalytic domain is seen between *H. pulcherrimus* guanylyl cyclase and mammalian receptor/guanylyl cyclases (Fig. 7). All of the membrane forms of guanylyl cyclases studied so far possess both a protein kinase-like domain and a cyclase catalytic domain (Garbers and Low, 1994; Yang *et al.*, 1995). The protein kinase-like domain contains a majority of the conserved amino acids identified by Hanks *et al.* (1988) as conserved or invariant within the catalytic domain of protein kinases. Although the protein kinase-like domain shows no protein kinase activity, it is suggested that the protein kinase-like domain is involved in regulation of the guanylyl cyclase activity. In mammalian receptor/guanylyl cyclase (GC-A), ATP-binding to the protein kinase-like domain has been reported to be a key step for transduction of the ligand binding signal to activate the cyclase catalytic domain (Chinkers *et al.*, 1991). Recently, it has been reported that a novel protein phosphatase binds to the protein kinase-like domain in mammalian receptor/guanylyl cyclase, GC-A (Chinkers, 1994). In theory, single-transmembrane receptors must form dimers, either between themselves or with other transmembrane proteins, in order to transduce a signal across the membrane. Both intracellular and extracellular interactions between receptor subunits are necessary for this process. In GC-A, a membrane guanylyl cyclase which is a receptor for atrial natriuretic peptide (Chinkers *et al.*, 1989; Lowe *et al.*, 1989), only a region composed of 43 amino acids located between the protein kinase-like domain and the cyclase catalytic domain is necessary for dimerization and it is required for guanylyl cyclase-catalytic activity.

Northern blot analysis demonstrated that the gene encoding *H. pulcherrimus* guanylyl cyclase was expressed only in the testis. This was also the case for the expression of the gene for the sperm-activating peptide I (SAP-I)-crosslinked 71 kDa protein (Shimizu *et al.*, 1994). The exclusive expression of both genes in the testis suggests that apparent co-expression of both genes in *H. pulcherrimus* testis may be due to the necessity of resultant physiological response to SAP-I and/or its derivatives.

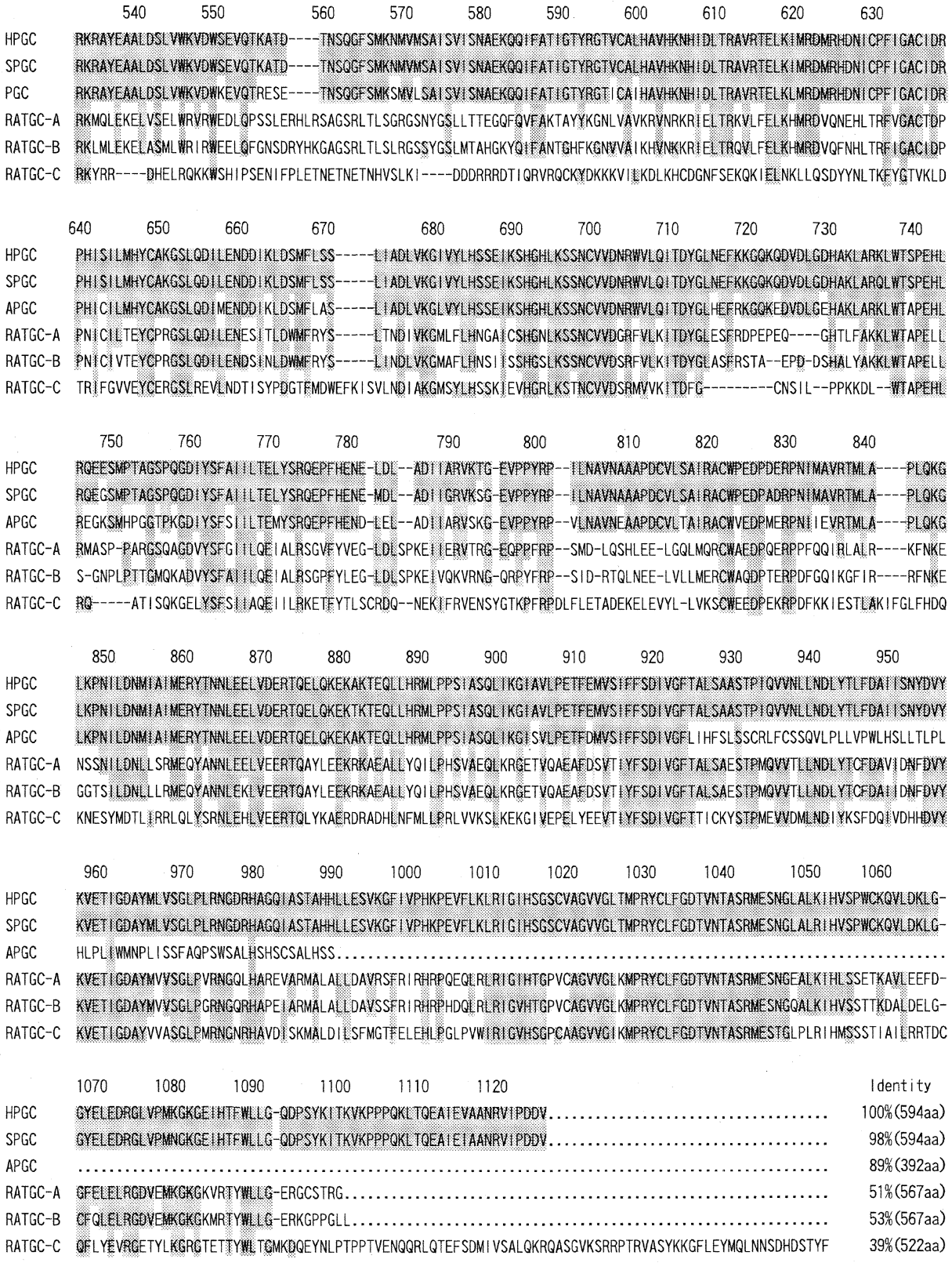

Fig.6. Sequence comparisons of the intracellular domains of *H. pulcherrimus* guanylyl cyclase (HPGC) and the other membrane forms of guanylyl cyclases. The deduced amino acid sequence of the predicted intracelular domain of *H. pulcherrimus* guanylyl cyclase is compared with the sequences of *S. purpuratus* (Thorpe and Garbers, 1989) and *A. punctulata* (Singh *et al.*, 1988) guanylyl cyclases, rat GC-A (Chinkers *et al.*, 1989), GC-B (Schulz *et al.*, 1989), and GC-C (Schulz *et al.*, 1990). Amino acid identities are shaded, and gaps are represented by dashes.

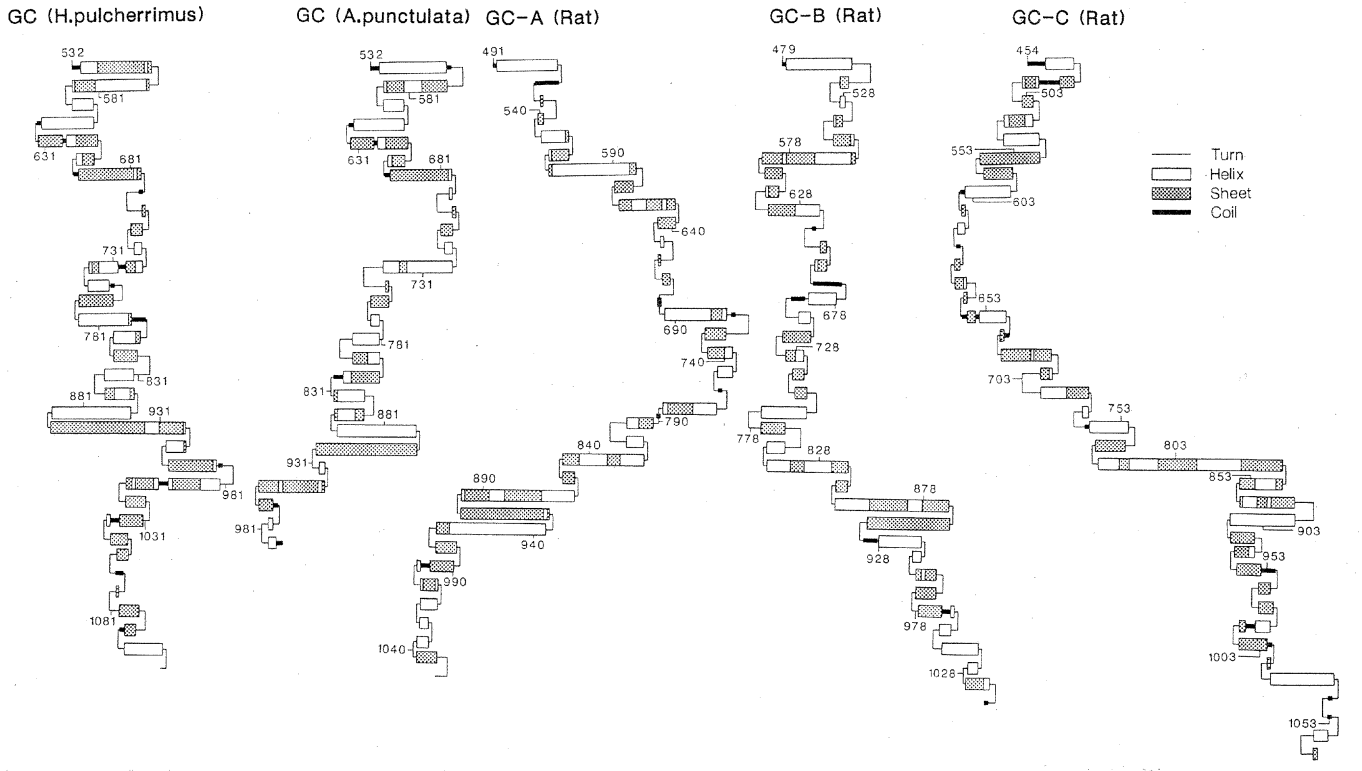

Fig.7. Comparison of the predicted secondary structure of the intracellular domain of *H. pulcherrimus* sperm guanylyl cyclase with those of *A. punctulata* sperm guanylyl cyclase and mammalian receptor/guanylyl cyclases. The secondary structure was estimated according to the method of Chou and Fasman (1978). The secondary structure of the kinase-like domain (residues from 600 to 720)and the catalytic domain (residues 860 to 1090) predicted for *H. pulcherrimus* sperm guanylyl cyclase were similar to the corresponding domains for other membrane form of guanylyl cyclases.

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REFERENCES

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248–254

Buss JE, Stull JT (1983) Measurement of chemical phosphate in proteins. Methods in Enzymol 99:7–14

Cathala G, Savouret JF, Mendez B, West BL, Karin M, Martial JA, Baxter JD (1983) A method for isolation of intact, translationally active ribonucleic acid. DNA 2:329–335

Chinkers M, Garbers DL, Chang M-S, Lowe DG, Chin H, Goeddel DV, Schulz S (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. Nature 338:78–83

Chinkers M, Singh S, Garbers DL (1991) Adenine nucleotides are required for activation of rat atrial natriuretic peptide receptor/guanylate cyclase expressed in a baculovirus system. J Biol Chem 266:4088–4093

Chinkers M (1994) Targeting of a distinctive protein-serine phosphatase to the protein kinase-like domain of the atrial natriuretic peptide receptor. Proc Natl Acad Sci USA 91:11075–11079

Chou PY, Fasman GD (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol 47:45–148

Davis LG, Dibner MD, Battey JF (1986) Basic Methods in Molecular Biology. Elsevier, New York

Garbers DL, Hardman JG, Rudolph FG (1974) Kinetic analysis of sea urchin sperm guanylate cyclase. Biochemistry 13:4166–4171

Garbers DL, Watkins HD, Hansbrough JR, Misono KS (1982) The amino acid sequence and chemical synthesis of speract and of speract analogues. J Biol Chem 257:2734–2737

Garbers DL (1989) Guanylate cyclase, a cell surface receptor. J Biol Chem 264:9103–9106

Garbers DL (1992) Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. Cell 71:1–4

Garbers DL, Lowe DG (1994) Guanylyl cyclase receptors. J Biol Chem 269:30741–30744

Goldberg ND, Haddox MK (1977) Cyclic GMP metabolism and involvement in biological regulation. Annu Rev Biochem 46:823–896

Hamet P, Tremblay J, Pang SC, Garcia R, Thibault G, Gutkowski J, Cantin M, Genest J (1984) Effect of native and synthetic atrial natriuretic factor on cyclic GMP. Biochem Biophys Res Commun 123:515–527

Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science 241:42–52

Harumi T, Kurita M, Suzuki N (1992) Purification and characterization of sperm creatine kinase and guanylate cyclase of the sea

urchin *Hemicentrotus pulcherrimus*. Dev Growth Differ 34:151–162

Hopp TP, Woods KR (1981) Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 78:3824–3828

Kozak M (1981) Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res 9:5233–5262

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 257:680–685

Lowe DG, Chang M-S, Hellmiss R, Chen E, Singh S, Garbers DL, Goeddel DV (1989) Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. EMBO J 8:1377–1384

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Mittal CK, Murad F (1982) In "Handbook of Experimental Pharmacology" Ed by Nathanson JA, Kebabian JW, Vol 58-1, pp 225–260, Springer-Verlag, Berlin

Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Analyt Biochem 117:307–310

Radany EW, Gerzer R, Garbers DL (1983) Purification and characterization of particulate guanylate cyclase from sea urchin spermatozoa. J Biol Chem 258:8346–8351

Ramarao CA, Garbers DL (1985) Receptor-mediated regulation of guanylate cyclase activity in spermatozoa. J Biol Chem 260:8390–8396

Sanger R, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schacterle GR, Pollack RL (1973) A simplified method for the quantitative assay of small amount of protein in biologic material. Analyt Biochem 51:654–655

Schulz S, Singh S, Bellet RA, Singh G, Tubb DJ, Chin H, Garbers DL (1989) The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. Cell 58:1155–1162

Schulz S, Green CK, Yuen PST, Garbers DL (1990) Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell 63:941–948

Shimizu T, Yoshino K, Suzuki N (1994) Identification and characterization of putative receptors for sperm-activating peptide I (SAP-I) in spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*. Dev Growth Differ 36:209–221

Singh S, Lowe DG, Thrope DS, Rodriguez H, Kuang W, Dangott LJ, Chinkers M, Goeddel DV, Garbers DL (1988) Membrane guanylate cyclase is a cell-surface receptor with homology to protein kinases. Nature 334:708–712

Stryer L (1986) Cyclic GMP cascades of vision. Annu Rev Neurosci 9:87–119

Suzuki N, Nomura K, Ohtake H, Isaka S (1981) Purification and the primary structure of sperm-activating peptides from the jelly coat of sea urchin eggs. Biochem Biophys Res Commun 99:1238–1244

Suzuki N, Kobayashi K, Isaka S (1982) Appearance of sperm activation factors in the ovary of the sea urchin *Hemicentrotus pulcherrimus* with maturation. Experientia 38:1245–1246

Suzuki N, Shimomura H, Radany EW, Ramarao CS, Ward GE, Bentley JK, Garbers DL (1984) A peptide associated with egg causes a mobility shift in a major plasma membrane protein of spermatozoa. J Biol Chem 259:14874–14879

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Vacquier VD, Moy GW (1986) Stoichiometry of phosphate loss from sea urchin sperm guanylate cyclase during fertilization. Biochem Biophys Res Commun 137:1148–1152

Voller A, Bidwell D Bartless A (1976) Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In" Manual of Clinical Immunology" Ed by Rose NR, Friedman H, pp 506–512, American Society for Microbiology, Washington DC

Waldman SA, Rapoport RM, Murad F (1984) Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. J Biol Chem 259:14332–14334

Watson MEE (1984) Compilation of published signal sequences. Nucleic Acids Res 13:5145–5164

Winquist RJ, Faison EP, Waldman SA, Schwartz K, Murad F, Rapoport RM (1984) Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. Proc Natl Acad Sci USA 81:7661–7664

Yang R-B, Foster DC, Garbers DL, Fülle H-J (1995) Two membrane forms of guanylyl cyclase found in the eye. Proc Natl Acad Sci USA 92:602–606

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

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