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甲殼類升血糖激素家族分子與其受體之研究

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ABSTRACT

In studies reported here, we used a PCR-based cloning strategy (RT-PCR followed by 5'- and 3'-RACE) to clone from Y-organs a cDNA (*CsGC-YO1*) encoding a putative rGC. DNA sequence analysis revealed a 3807 base pair open reading frame encoding a 56 residue signal peptide and a 1213 residue rGC. Analysis of the deduced amino acid sequence showed that CsGC-YO1 contains the signature domains characteristic of rGCs, including an extracellular ligand-binding domain, a single transmembrane domain, a kinase-like domain, a dimerization domain, and a cyclase catalytic domain. CsGC-YO1 is most closely related to an rGC from the crayfish, *Procambarus claikii* (PcGC-M2, 58.4% identity), and rGCs from three insect species (33.1 - 37.5% identity). Conserved cysteine residues are similarly distributed in the extracellular domains of CsGC-YO1, PcGC-M2, and the three insect rGCs. RT-PCR revealed the CsGC-YO1 transcript is strongly expressed in Y-organs and several other tissues.

Key words: receptor guanylyl cyclase, crustacean hyperglycemic hormone, molt-inhibiting hormone, cGMP, crustacean

現有的證據顯示甲殼類升血糖激素 (CHH) 活化細胞膜上之鳥甘酸環化胺，產生 cGMP (cyclic guanosine 3’,5’-monophosphate) 為二級傳訊者，來傳達 CHH 對於碳水化合物代謝之訊息。本計畫之成果自 Y-器官選殖一膜型鳥甘酸環化胺基因(PcGC-M2)。分析其推估之胺基酸序列顯示具有膜型鳥甘酸環化胺所有特有之區域，包含胞外配體結合區、穿膜區、以及胞內激胺同源區與環化胺催化區、及一段 274 殘基之 C-端段落。PcGC-M2 和螯蝦 (*P. clarkii*)、果蠅之膜型鳥甘酸環化胺最為相似；此三種膜型鳥甘酸環化胺其胞外配體結合區有相似之保守性 cysteine 殘基之分佈。CsGC-YO1 在數個組織均有表現。

關鍵詞：鳥苷酸環化酶受體、甲殼類升血糖荷爾蒙、蛻殼抑制荷爾蒙、環鳥苷酸、甲殼類動物

INTRODUCTION

In both invertebrates and vertebrates, cyclic guanosine 3’,5’-monophosphate (cGMP) acts as an intracellular second messenger mediating the actions of hormones and neurotransmitters. Synthesis of cGMP is catalyzed by guanylyl cyclases (GCs) (Wedel and Garbers, ’01). Based on their cellular distribution and structure, GCs are classified as either soluble form or membrane form. Studies of peptide ligands acting via the cGMP signaling cascade indicate that receptors for these ligands are membrane form GCs containing, from amino to carboxyl terminus, an extracellular ligand-binding

domain, a transmembrane domain, and intracellular kinase-like and cyclase catalytic domains. These membrane form GCs function primarily as homodimers that are activated by the binding of an extracellular ligand (Lucas et al., ’00). Presently, 7 isoforms of mammalian membrane form GCs have been identified. GC-A and GC-B are natriuretic peptide receptors that bind and are activated by atrial, brain, and C-type natriuretic peptides; GC-C is the receptor for the bacterial heat-stable enterotoxins and the endogenous intestinal peptides guanylin and uroguanylin; GC-D, GC-E, GC-F and GC-G are orphan receptors whose ligands remain to be identified (Lucas et al., ’00).

Crustacean hyperglycemic hormone (CHH) family is consisted of several peptides produced by a neuroendocrine system (the X-organ/sinus gland, XOSG). CHH family peptides exist in multiple isoforms. For example, XOSG-CHH isolated from the crayfish (*Procambarus clarkii*) exists as L-CHH and D-CHH, which differ from each other only at the configuration of the third residue. In addition to its classic hyperglycemic activity, XOSG-CHH also inhibits secretion of molting hormone ecdysteriods by Y-organs, and is hence considered exhibiting molt-inhibiting activity, a biological activity shared by another CHH family peptide, molt-inhibiting hormone (MIH). The hyperglycemic and molt-inhibitiing activity of D-CHH is considerably more potent than L-CHH. It is intriguing to determine, on the one hand, whether the potency difference between L-CHH and D-CHH is due to their differential activation efficacy of a common CHH receptor or due to the presence of separate receptors for each CHH isoforms, and on the other hand, whether CHH (both L-CHH and D-CHH) and MIH share a common receptor (but with differential activation efficacy) or bind and activate separate receptors on Y-organs.

Combined results from several laboratories indicated that the regulatory effects of CHH or MIH are mediated by cGMP (3',5'-cyclic guanosine monophosphate) and suggested that their receptor belongs to the type of receptors that contain intrinsic guanylyl cyclase activity (i.e., membrane form guanylyl cyclases, GC) (Sedlmeier and Keller, ’81; Goy et al., ’87; Goy, ’90; Spaziani et al., ‘99, ,01). We have cloned a full-length cDNAs encoding membrane form GCs from the muscle of the crayfish, *Procambarus clarkii* (Liu et al., 2004). Their transcripts are expressed in several CHH or MIH target tissues, implying that these GCs participate in the signaling cascade activated by CHH or MIH. In addition to these typical GCs, partial cDNA sequences, resembling in sequence to the typical GCs, were also identified in the muscle; these GC sequences, however, are truncated at the kinase-homology domain, suggesting the GC proteins they encode may form heterodimers with typical GCs and modulate the levels of the signal (cGMP production) initiated by peptide ligands.

We report here the cloning of a cDNA encoding an rGC-like protein from Y-organs, a target tissues of CHH.

MATERIALS AND METHODS

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Tissues were dissected from ice-anesthetized animals and promptly placed in wells containing RNA*later*™ (Ambion). Approximately 100 mg of tissues were extracted for total RNA using TRIZOL® reagent (Life Technologies) and the extracted RNA samples were treated with RQ1 RNase-free DNase (Promega) according to the supplier’s procedures. For first-strand cDNA synthesis, 4 μg of total RNA

were reverse transcribed with 1 μg of random primers using 200 U of M-MLV reverse transcriptase in a 25-μl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 500 μM dNTP, 40 U RNasin, and 10 mM DTT (Promega).

Degenerate primers were designed against highly conserved sequences (VYKVETIG and MPRYCLFG) from cyclase catalytic domains of membrane form GCs. PCR reactions contained 2.5 μl of the RT reaction, 0.5 mM each of dNTPs, 10 μM each of primers, 5U of *Taq* DNA polymerase, and 1X reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$). The final volume was adjusted to 20 μl with sterile distilled water. Amplification was carried out in a PTC-200 thermal cycler (MJ Research) with the following parameters: an initial denaturation (5 min, 94°C), 35 cycles of denaturation (20 s, 94°C), annealing (20 s, 50°C), and extension (30 s, 72°C), followed by a final extension (7 min, 72°C).

Rapid amplification of 3'- and 5'-cDNA ends (RACE)

Poly(A)$^+$ RNA was isolated from abdominal muscles using oligo (dT) cellulose (Micro-FastTrack™, Invitrogen). For the synthesis of first-strand cDNA for 3'- and 5'-RACE reactions, 1 μg of Poly(A)$^+$ RNA was reverse transcribed using reagents and a protocol provided by the Smart™ RACE cDNA Amplification Kit (Clontech).

RACE reactions were performed as described previously (Liu et al., 2004) with gene-specific primers selected from the sequence of the PCR product amplified within the cyclase catalytic domain using degenerate primers.

Gel purification, cloning, sequencing and sequence analysis

After PCR amplification, an aliquot of the reaction was separated on a 1.2% or 2% agarose gel and visualized with GelStar® (Cambrex). PCR products of expected sizes were excised from gels, purified (QIAquick® Gel Extraction Kit, QIAGEN), and cloned (TOPO TA Cloning® Kit for sequencing, Invitrogen). Recombinant plasmids were extracted (Mini-M™ Plasma DNA Extraction System, Viogene) and sequenced using an autosequencer (PRISM 3100, ABI). Analysis of nucleotide and amino acid sequences was performed using software provided by the ExPASy Molecular Biology Server (http://us.expasy.org/) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/; Hall, '99).

Analysis of tissue distribution of PcGC-M2 transcript by RT-PCR

Equal amounts (1 μg) of total RNA, extracted separately from the tested tissues were DNase-treated and reverse transcribed as described above. Each 50 μl-PCR reaction contained 2.5 μl of the RT reaction, 0.2 mM each of dNTPs, 0.2 μM each of primers, 2.5 U of *Taq* DNA polymerase, and 1X reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$). To control for genomic contamination, total RNA samples (0.2 μg for each of the tissues), instead of cDNA samples, were used as templates in the PCR reactions. The primers used were designed based on the sequences of the putative extracellular domain

All PCR reactions were carried out with the following parameters: an initial denaturation (5 min, 94°C), 35 cycles of denaturation (1 min, 94°C), annealing (1 min, 50°C), and extension (1 min, 72°C), followed by a final extension (7 min, 72°C).

RESULTS AND DISCUSSION

A PCR-based cloning strategy (RT-PCR followed by 3'- and 5'-RACE) was used to clone a cDNA (*CsGC-YO1*) encoding a putative receptor guanylyl cyclase (rGC) from Y-organs. The 4010 base pair (bp) cDNA contains a 3807 bp open reading frame that predicts a protein of 1269 residues (Fig.1). The open reading frame begins with an initiator codon (ATG) at nucleotides 133-135 and ends with a stop codon (TAA) at nucleotides 3936-3939. A short 3'-untranslated region contains a polyadenylation signal (AATTAAA) (Birnstiel et al., 1985) located 13 bp upstream from a poly(A) tail (Fig.1).

Analysis of the N-terminal sequence of the protein encoded by *CsGC-YO1* predicts a 56 residue signal peptide and a 1213 residue mature protein, with the cleavage site located between amino acids 56 (f) and 57 (E) (http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html) (Fig. 1). Cleavage at this site would yield a mature protein with a relative molecular mass of 136,797. Analysis using PROSITE (http://us.expasy.org/prosite/) indicated the mature protein contains all signature domains characteristic of an rGC, including an extracellular ligand-binding domain, a single transmembrane domain, a kinase-like domain, a dimerization domain, and an (a) cyclase catalytic domain.

The 461-residue extracellular domain has 11 potential N-linked glycosylation sites, (predicted by NXT or NXS, where X is any amino acid) (Fig. 1); such sites appear to be critical for ligand binding to rGCs (Lucas et al., 2000). Alignment of the extracellular domains of rGCs from vertebrate and invertebrate sources shows four cysteine residues (C148, C161, C503, C510) present in 13 of the 14 rGCs (GC-C lacks two cysteines adjacent to the transmembrane domain) (Fig. 2). C180 is conserved among rat sensory receptors (GC-D, GC-E, GC-F) and all invertebrate rGCs. Residues C368 and C471 are conserved among arthropod rGCs. The pattern of cysteine residues is remarkably similar between CsGC-YO1 (from the blue crab) and PcGC-M2 (from the crayfish). A single transmembrane domain (23 amino acids) links the extracellular domain to the intracellular portion of the protein.

The intracellular domains of CsGC-YO1 include a kinase-like domain, a dimerization domain, and a cyclase catalytic domain. The kinase-like domain of CsGC-YO1 (residues 541-842) is similar in several respects to the kinase like domain of known rGCs. In general, protein kinases contain 33 invariant amino acids present in 11 subdomains (Hanks et al., 1988); twenty-four of the invariant residues, comprising 8 of 11 subdomains, are present in CsGC-YO1. An invariant D present in subdomain VI of protein kinases is typically replaced by S,R, or N in rGCs (Koller st al., 1992); in CsGC-YO1, the relevant D is replaced by N. A conserved glycine-rich loop (GXGXXG) found in subdomain I and responsible for ATP binding to protein kinases is typically absent or modified in rGCs (Lucas et al., 2000; Koller et al., 1992); the conserved glycine-rich loop is modified in CsGC-YO1 (GXXGXXXG, see residues 679-686).

Receptors with a single transmembrane domain typically form homodimers or heterodimers in order to transduce a signal across the plasma membrane (Wilson and Chinkers, 1995). Among rGCs, a region between the kinase-like domain and the cyclase catalytic domain is predicted to form an amphipathic α-helix hypothesized to function as a dimerization domain (Wilson and Chinkers, 1995).

Data from several sources suggests the dimerization domain of rGCs forms a coiled-coil structure (Wilson and Chinkers,1995; Lupas et al., 1991; Morton and Nighorn, 2003). Sequence analysis (http://paircoil.1cs.mit.edu/cgi-bin/paircoil) revealed the 88 amino acid region between the kinase-like domain and the cyclase catalytic domain of CsGC-YO1 is likewise predicted to form a coiled-coil.

The predicted cyclase catalytic domain of CsGC-YO1 extends from amino acid 929 (T) to amino acid 1060 (E). Previous results indicate the cyclase catalytic domain of several Ca^{++}-sensitive rGCs contains an 18-residue region thought to serve as a binding site for guanylyl cyclase activating proteins (GCAPs) (Sokal et al., 1999; Morton and Nighorn, 2003). The 18-residue region was not detected in the cyclase catalytic domain of CsGC-YO1, suggesting CsGC-YO1 is not likely to be regulated by GCAPs.

CsGC-YO1 has a C-terminal tail of 208 residues following the catalytic domain. In several rGCs, the C-terminal tail is rich in uncharged polar amino acids such as S and Q (Schulz et al., 1990; McNeil et al., 1995). In the 208 residue C-terminus of CsGC-YO1, 11% of the amino acids are S or Q. The precise functions of such tails in rGC proteins are not known. The motif YXXZ (where X is any amino acid and Z is one of the following hydrophobic amino acids: L, I, V, M, C, or A), present in the C-terminus of a number of rGCs, has been suggested to mediate the internalization of rGCs by interacting with the endocytotic apparatus (Johnson et. al., 1990; Canfield et. al., 1991; Thomas and Roth, 1994). The YXXZ motif is not present in CsGC-YO1.

A comparison of the amino acid sequence of CsGC-YO1 to the amino acid sequences of known rGCs is shown in Table 1. The overall sequence of CsGC-YO1 is most similar to the overall sequence of PcGC-M2 (58.4% identity), a putative rGC from crayfish (*Procambarus clarkii*) muscle. Regarding the sequence of individual domains, the primary structure of the cyclase catalytic domain is highly conserved across species (61.4-93.2% identity), while the primary structure of the extracellular domain shows the lowest percent identity (16.9-51.3% identity) (Table 1).

A determination of the phylogenetic relationships among rGCs showed the proteins cluster mainly according to major phylogenetic groups (Fig. 3). CsGC-YO1 appears most closely related to PcGC-M2 (from crayfish), and also clusters with rGCs from insects. Regarding the phylogenetic relationship between CsGC-YO1 and mammalian rGCs, the crustacean protein is more similar to natriuretic peptide receptors (GC-A and GC-B) than sensory receptors (GC-D, GC-E, and GC-F), an entertoxin receptor (GC-C), and the orphan receptor GC-G.

The tissue distribution of CsGC-YO1 mRNA was assessed by RT-PCR using primers designed from the sequence of the extracellular domain. In general, the CsGC-YO1 transcript was widely expressed: An amplicon of the predicted size (578 bp) was detected in all tissues tested (Fig. 4). The CsGC-YO1 transcript appeared to be strongly expressed in Y-organs, neural tissue, and hepatopancreas; the level of expression was lower in ovary, muscle, and gill.

Data presented here strongly support the hypothesis that *CsGC-YO1*, a cDNA cloned from Y-organs, encodes a receptor guanylyl cyclase (rGC). The predicted protein contains all signature domains of an rGC, and is structurally similar in several additional respects to known members of the

rGC family of proteins. Proof that *CsGC-YO1* encodes an authentic rGC will require expression of the protein and determination of whether the recombinant protein has guanylyl cyclase activity.

SELF EVALUATION

The main results of the project were to successfully clone a membrane form guanylyl cyclase. The part of molecular cloning has been published (Zheng et al., 2006). While CsGC-YO1 appears to be strongly expressed in Y-organs, the transcript also appears to be abundant in neural tissue and hepatopancreas, and is detectable at lower levels in all tissues tested. This pattern of expression was not predicted by current understanding of MIH action (i.e., Y-organs are the only known target tissues for MIH). One explanation is that CsGC-YO1 is not an MIH receptor. It is possible, for example, that CSGC-YO1 is a receptor for crustacean hyperglycemic hormone (CHH). Previous studies indicate that Y-organs possess distinct CHH receptors, and addition of CHH (in high dose) to Y-organ incubations suppresses ecdysteroid production (Webster, 1993). Liu et al. (2004) have recently cloned a cDNA encoding a putative CHH receptor (*PcGC-M2*) from muscle of the crayfish, *Procambarus clarkii*. Like the CsGC-YO1 transcript, the PcGC-M2 transcript shows wide tissue distribution. However, the tissue distribution pattern of CsGC-YO1 does not precisely track the tissue distribution pattern the PcGC-M2. Another interpretation of the observed tissue distribution pattern of the CsGC-YO1 transcript is that MIH affects target tissues other than Y-organs. Pleiotropic effects of polypeptide hormones are widely observed in both invertebrate and vertebrate systems. Conclusive identification of the activating ligand for CsGC-YO1 will require studies designed to test the ability of MIH (and other candidate ligands) to bind and activate the receptor.

LITERATURE CITED

Birnstiel MJ, Busslinger M, Strub K. 1985. Transcription termination and 3' processing: the end is in site! Cell 41:349-359.

Chang ES, Prestwich GD, Bruce MJ. 1990. Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster *Homarus americanus*. Biochem Biophys Res Commun 171:818-826.

Chinker M, Garbers DL, Chang MS, 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.

Dircksen H, Böcking D, Heyn U, Mandel C, Chung JS, Baggerman G, Verhaert P, Daufeldt S, Plösch T, Jaros PP, Waelkens E, Keller R, Webster SG. 2001. Crustacean hyperglycemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochem J 356:159-170.

Fülle HJ, Vassar R, Foster DC, Yang RB, Axel R, Garbers DL. 1995. A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons. Proc Natl Acad Sci USA 92:3571-3575.

Gigliotti S, Cavaliere V, Manzi A, Tino A, Graziani F, Malva C. 1993. A membrane guanylate cyclase *Drosophila* homolog gene exhibits maternal and zygotic expression. Develop Biol 159:450-61.

Goy MF. 1990. Activation of membrane guanylate cyclase by an invertebrate peptide hormone. J Biol Chem 265:20220-20227.

Goy MF, Mandelbrot DA, York CM. 1987. Identification and characterization of a polypeptide from a lobster neurosecretory gland that induces cyclic GMP accumulation in lobster neuromuscular preparations. J Neurochem 48:954-966.

Gu PL, Chan SM. 1998. The shrimp hyperglycemic hormone-like neuropeptides is encoded by multiple copies of genes arranged in a cluster. FEBS Lett 441:397-403.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.

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

Huo X, Abe T, Misono KS. 1999. Ligand binding-dependent limited proteolysis of the atrial natriuretic peptide receptor: juxtamembrane hinge structure essential for transmembrane signal transduction. Biochemistry 38:16941-16951.

Itakura M, Iwashina M, Mizuno T, Ito T, Hagiwara H, Hirose S. 1994. Mutational analysis of disulfide bridges in the type C atrial natriuretic peptide receptor. J Biol Chem 269:8314-8318.

Kang WK, Naya Y. 1993. Sequence of the cDNA encoding an actin homolog in the crayfish *Procambarus clarkii*. Gene 133:303-304.

Khayat M, Yang W, Aida K, Nagasawa H, Tietz A, Funkenstein B, Lubzens E. 1998. Hyperglycemic hormones inhibit protein and mRNA synthesis in *in vitro*-incubated ovarian fragments of the

marine shrimp *Penaeus semisulcatus*. Gen Comp Endocrinol 110:307-318.

Kozak M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15:8125-8148.

Kummer G, Keller R. 1993. High-affinity binding of crustacean hyperglycemic hormone (CHH) to hepatopancreatic plasma membranes of the crab *Carcinus maenas* and the crayfish *Orconectes limosus*. Peptides 14:103-108.

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

Lee CY, Yang PF, Zou HS. 2001. Serotonergic regulation of crustacean hyperglycemic hormone secretion in the crayfish, *Procambarus clarkii*. Physiol Biochem Zool 74:376-382.

Liu W, Yoon J, Burg M, Chen L, Pak WL. 1995. Molecular characterization of two *Drosophila* guanylate cyclases expressed in the nervous system. J Biol Chem 270:12418-12427.

Liu, HF, Lai, CY, Watson, RD, and Lee, CY. (2004) Molecular cloning of a putative membrane form guanylyl cyclase from the crayfish, *Procambarus clarkii* Journal of Experimental Zoology 301A (6): 512-520.

Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. 2000. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev 52:375-413.

McNeil L, Chinkers M, Forte M. 1995. Identification, characterization, and developmental regulation of a receptor guanylyl cyclase expressed during early stages of *Drosophila* development. J Biol Chem 270:7189-7196.

Miyagi M, Misono KS. 2000. Disulfide bond structure of the atrial natriuretic peptide receptor extracellular domain: conserved disulfide bonds among guanylate cyclase-coupled receptors. Biochim Biophys Acta 1478:30-38.

Morton D, Nighorn A. 2003. MsGC-II, a receptor guanylyl cyclase isolated from the CNS of *Manduca sexta* that is inhibited by calcium. J Neurochem 84:363-372.

Santos EA, Keller R. 1993. Crustacean hyperglycemic hormone (CHH) and the regulation of carbohydrate metabolism: current perspectives. Comp Biochem Physiol 106A:405-411.

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 PS, Garbers DL. 1990. Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell 63:941-948.

Schulz S, Wedel BJ, Mattews A, Garbers DL. 1998. The cloning and expression of a new guanylyl cyclase. J Biol Chem 273:1032-1037.

Sedlmeier D, Keller R.1981. The mode of action of the crustacean neurosecretory hyperglycemic hormone. I. Involvement of cyclic nucleotides. Gen Comp Endocrinol 45:82-90.

Serrano L, Blanvillain G, Soyez D, Charmantier G, Grousset E, Aujoulat F, Spanings-Pierrot C. 2003. Putative involvement of crustacean hyperglycemic hormone isoforms in the neuroendocrine

mediation of osmoregulation in the crayfish *Astacus leptodactylus*. J Exp Biol 206:979-988.

Shimizu T, Takeda K, Furuya H, Hoshino K, Nomura K, Suzuki N. 1996. A mRNA for membrane form of guanylyl cyclase is expressed exclusively in the testis of the sea urchin *Hemicentrotus pulcherrimus*. Zool Sci 13:285-294.

Singh S, Lowe DG, Thorpe DS, Rodriguez H, Kuang WJ, 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.

Sokal I, Haeseleer F, Arendt A, Adman ET, Hargrave PA, Palczewski K. 1999. Identification of a guanylyl cyclase-activating protein-binding site within the catalytic domain of retinal guanylyl cyclase1. Biochemistry 38:1387-1393.

Soyez D. 1997. Occurrence and diversity of neuropeptides from the crustacean hyperglycemic hormone family in arthropods. Ann N Y Acad Sci 814:319-323.

Soyez D, Van Herp F, Rossier J, Le Caer JP, Tensen CP, Lafont R. 1994. Evidence for a conformational polymorphism of invertebrate neurohormones. J Biol Chem 269:18295-18298.

Spanings-Pierrot C, Soyez D, Van Herp F, Gompel M, Skaret G, Grousset E, Charmantier G. 2000. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. Gen Comp Endocrinol 119:340-350.

Tanoue S, Sumida S, Suetsugu T, Endo Y, Nishioka T. 2001. Identification of a receptor type guanylyl cyclase in the antennal lobe and antennal sensory neurons of the silkmoth, *Bombyx mori*. Insect Biochem Molec Biol 31:971-979.

Thorpe DS, Garbers DL. 1989. The membrane form of guanylate cyclases: Homology with a subunit of the cytoplasmic form of the enzyme. J Biol Chem 264:6545-6549.

Von Heijne G. 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem 133:17-21.

Wedel BJ, Garbers DL. 2001. The guanylyl cyclase family at Y2K. Ann Rev Physiol 63:215-233.

Yang RB, Foster DC, Garbers DL, Fülle HJ. 1995. Two membrane forms of guanylyl cyclases found in the eye. Proc Natl Acad Sci USA 92:602-606.

Yang WJ, Aida K, Nagasawa H. 1997. Amino acid sequences and activities of multiple hyperglycemic hormones from the kuruma prawn, *Penaeus japonicus*. Peptides 18:479-485.

Yasuda A, Yasuda Y, Fujita T, Naya Y. 1994. Characterization of crustacean hyperglycemic hormone from the crayfish (*Procambarus clarkii*): Multiplicity of molecular forms by stereoinversion and diverse functions. Gen Comp Endocrinol 95:387-398.

Yu S, Avery L, Baude E, Garbers DL. 1997. Guanylyl cyclases expression in specific sensory neurons: A new family of chemosensory receptors. Proc Natl Acad Sci USA 94:3384-3387.

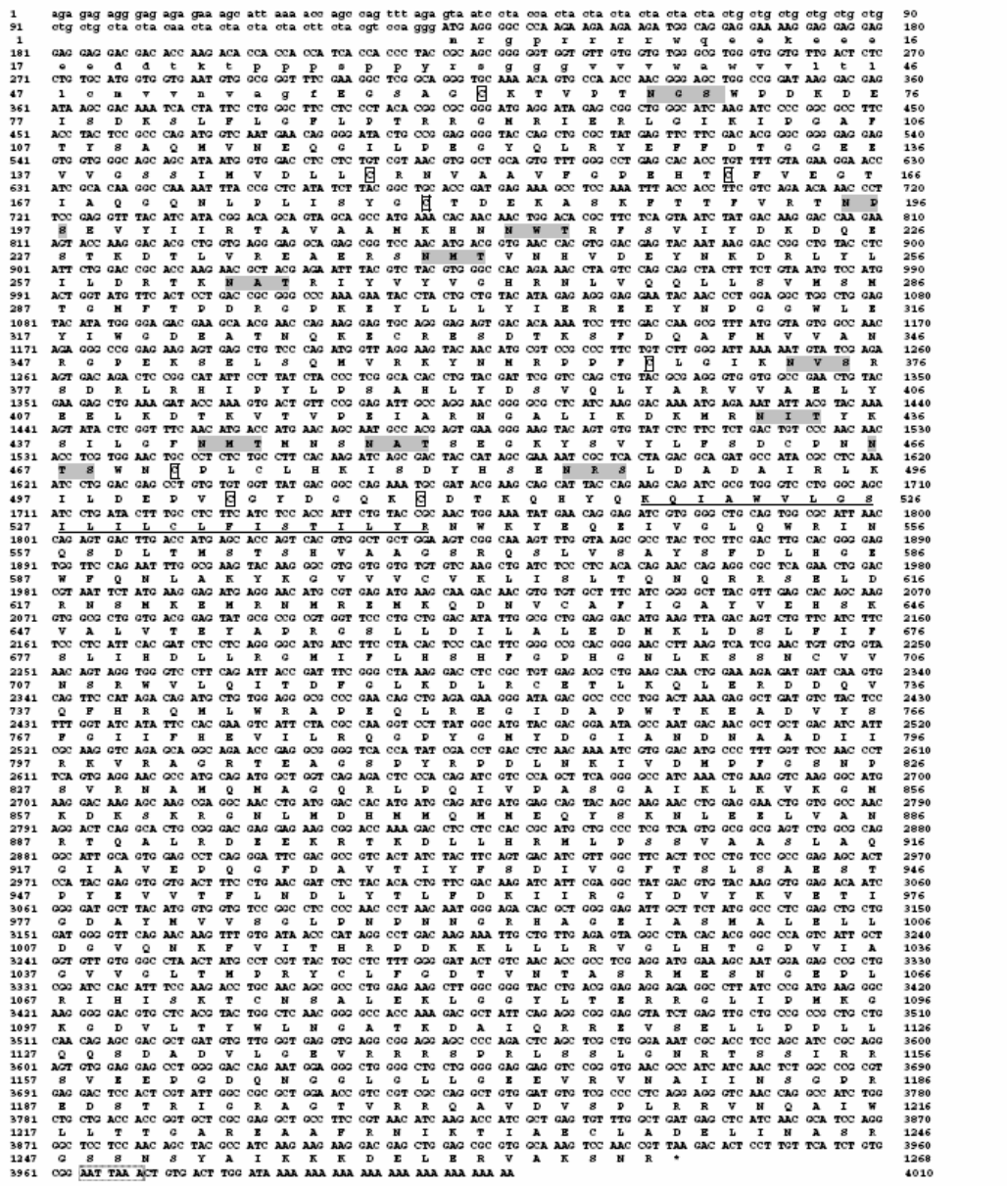

Fig. 1. The nucleotide and deduced amino acid sequences of a receptor guanylyl cyclase cDNA (*CsGC-YO1*) from Y-organs of the blue crab, *C. sapidus*. The deduced amino acid sequence of the open reading frame is shown in single letter code below the nucleotide sequence. The putative sequence cleavage site is marked with an arrow, which is preceded by a 56-residue signal peptide shown in lowercase letters. In the extracellular domain, 8 conserved cysteines are boxed and 11 N-linked glycosylation sites are shaded. The putative transmembrane domain is underlined with a solid line. The stop codon is indicated with an asterisk (*). The putative poly(A) signal is boxed. GenBank Accession No. AY785292.

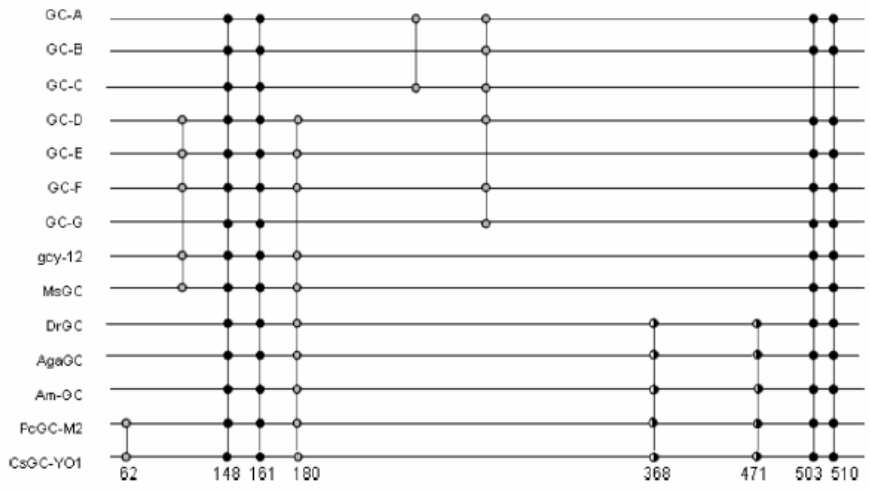

Fig. 2. Positions of conserved cysteine residues in the extracellular domain of receptor guanylyl cyclases. Cysteine residues are indicated by circles. Filled circles indicate cysteine residues conserved among all GCs. Open and semi-filled circles indicate cysteine residues that are differentially conserved among species. Note the similar position of cysteines conserved between the crayfish (PcGC-M2) and blue crab (CsGC-YO1) rGCs.

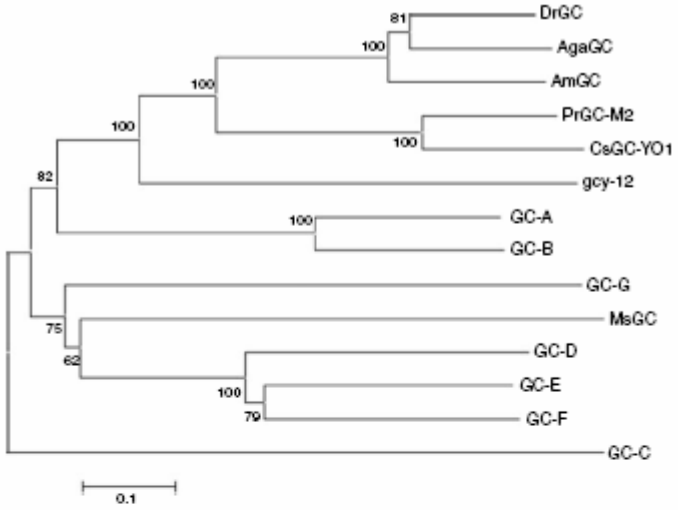

Fig. 3. Phylogenetic relationships among receptor guanylyl cyclase proteins. The deduced amino acid sequences of known rGCs were analyzed using ClustalW. The phylogenetic tree was generated using Mega 2.1. GC-A–GC-G are receptor guanylyl cyclases from the rat (*Rattus norvegicus*). GC-A and GC-B are atrial natriuretic peptide receptors (Chinkers et al., 1989; Schulz et al., 1989). GC-C is a heat-stable enterotoxin receptor (Schulz et al., 1990). GC-D is a sensory receptor expressed in olfactory neurons (Fülle et al., 1995). GC-E and GC-F are sensory receptors expressed in retinal tissue (Yang et al., 1995). GC-G is an orphan receptor expressed in lung, intestine, and skeletal muscle (Schulz et al., 1998). gcy-12 is an rGC from *C. elegans* (Accession No.: NP_494995). MsGC is an rGC from the tobacco hornworm, *Manduca sexta* (Morton and Nighorn, 2003). DrGC is a GC widely expressed embryonic and adult tissues of *Drosophila melanogaster* (McNeil et al., 1995). AgaGC is a GC from the mosquito, *Anopheles gambiae* (ENSANGP00000009147; Accession No.: XP320371). AmGC is a GC from the honeybee, *Apis mellifera* (Accession NO.: XP_623361). PeGC-M2 is an rGC from the crayfish, *Procambarus clarkii* (Liu et al., 2004). Scale bar represents 0.1 substitutions/site.

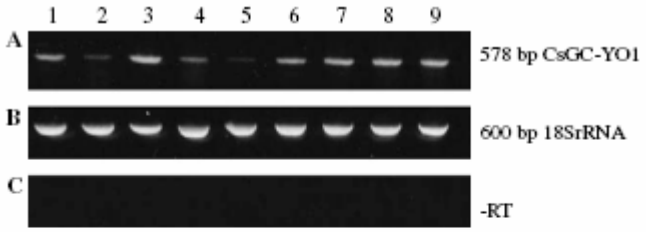

Fig. 4. Distribution of the CsGC-YO1 transcript in tissues of the blue crab. (A) With total RNA as template, RT-PCR was carried out using primers designed from the extracellular domain of CsGC-YO1. The expected size of the amplicon was 578 bp. (B) With total RNA as template, RT-PCR was carried out using primers designed from the sequence of 18S rRNA. The expected size of the amplicon was 600 bp. (C) With total RNA as template, and primers as in A, above, PCR was carried out without prior reverse transcription. Lane 1, thoracic ganglion; lane 2, ovary; lane 3, ventral nerve cord; lane 4, muscle; lane 5, gill; lane 6: hepatopancreas; lane 7, eyestalk; lane 8, brain; lane 9, Y-organ.

Table 1
Comparison of the deduced amino acid sequence of CsGC-YO1 with other membrane form GCs

	Sequence identity to CsGC-YO1 (%)				
	Extracellular domain (57–517)	Transmembrane domain (518–540)	Kinase-like domain (541–842)	Cyclase catalytic domain (929–1060)	Overall sequence
GC-A	18.6	13.0	34.0	75.0	30.1
GC-B	19.7	20.5	38.2	77.3	31.1
GC-C	17.6	4.3	27.2	61.4	26.5
GC-D	20.2	13.0	35.4	70.5	30.9
GC-E	18.9	21.7	30.3	68.9	28.5
GC-F	20.0	8.7	28.8	70.5	29.1
GC-G	20.6	8.7	28.9	68.2	27.1
gcy-12	24.9	16.0	33.1	69.9	32.4
MsGC	16.9	27.1	30.3	67.4	28.5
DrGC	29.0	30.0	38.4	80.3	33.1
AgaGC	26.4	20.8	37.7	78.8	37.5
AmGC	28.8	28.0	35.0	80.3	35.6
PcGC-M2	51.3	65.2	65.4	93.2	58.4

GC-A–GC-G are receptor guanylyl cyclases from the rat (*Rattus norvegicus*). GC-A and GC-B are atrial natriuretic peptide receptors (Chinkers et al., 1989; Schulz et al., 1989). GC-C is an heat-stable enterotoxin receptor (Schulz et al., 1990). GC-D is a sensory receptor expressed in olfactory neurons (Fülle et al., 1995). GC-E and GC-F are sensory receptors expressed in retinal tissue (Yang et al., 1995). GC-G is an orphan receptor expressed in lung, intestine, and skeletal muscle (Schulz et al., 1998). gcy-12 is an rGC from *C. elegans* (Accession No.: NP_494995). MsGC is an rGC from the tobacco hornworm, *Manduca sexta* (Morton and Nighorn, 2003). DrGC is a GC widely expressed embryonic and adult tissues of *Drosophila melanogaster* (McNeil et al., 1995). AgaGC is a GC from the mosquito, *Anopheles gambiae* (ENSANGP00000009147; Accession No.: XP320371). AmGC is a GC from the honeybee, *Apis mellifera* (Accession No.: XP_393152). PcGC-M2 is an rGC from the crayfish, *Procambarus clarkii* (Liu et al., 2004).