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甲殼類升血糖荷爾蒙受體—鳥苷酸環化酶之分子生物學研究

Crustacean hyperglycemic hormone receptor－Molecular studies of guanylate cyclase

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Abstract:

Available data indicate that crustacean hyperglycemic hormone (CHH) stimulates membrane-bound guanylyl cyclase (GC) producing cyclic guanosine 3',5'-monophosphate, which in turn mediates the effect of CHH on carbohydrate metabolism. In the present study, we report the cloning of a cDNA (*PcGC-M2*) encoding a membrane form GC from the muscle of the crayfish, *Procambarus clarkii*. Analysis of the deduced amino acid sequence shows that PcGC-M2 contains the signature domains characteristic of membrane form GCs, including an extracellular ligand-binding domain, a single transmembrane, and intracellular kinase-like and cyclase catalytic domains. In addition, a C-terminal domain of 247 residues is present following the cyclase catalytic domain. PcGC-M2 is most closely related (33% identity) to a *Drosophila* membrane form GC (DrGC-1), and an *Anopheles gambiae* membrane form GC (AgaGC); the three GCs also share a similar distribution pattern of conserved cysteine residues in the extracellular domain. The *PcGC-M2* transcript is expressed in several CHH target tissues, including muscle, hepatopancreas, heart, ovary, testis, and gill, implying that PcGC-M2 participates in the signaling cascade activated by CHH.

Background:

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) [1]. 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 [2]. 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 [2]. Among invertebrates, sea urchin (*Arbacia punctulata*) GC activated by a sperm-stimulating peptide (resact) represents the first membrane form GC to be cloned [3]. More recently, membrane form GCs were identified in various sea urchins, insects, and *Caenorhabditis elegans* [4–11].

Crustacean hyperglycemic hormone (CHH) is a peptide hormone originally identified in a crustacean neurosecretory complex, the X-organ/sinus gland [12]. Sequence analysis of CHHs isolated from various decapod crustaceans shows they are peptides of 72–73 amino acid residues with a considerable degree of sequence similarity [12]. CHH is one of several structurally related hormones that constitute the CHH family of peptides [12]. Several lines of evidence indicate CHH is pleiotropic in its regulatory functions. Thus, CHH has been

implicated in the regulation of carbohydrate metabolism [13] and several other physiological processes, including molting, osmoregulation, and reproduction [14−18]. An intriguing feature of CHH is the existence of multiple molecular variants in a given species [15,19−22]. Although it has been proposed that CHH isoforms may be specialized in different physiological functions [15,16,18], the significance of such structural polymorphism is far from clear.

Previous studies on the cellular mechanism of action of CHH suggest that cGMP plays important roles in mediating the effects of CHH on carbohydrate metabolism. Cyclic GMP levels in CHH target tissues are significantly increased shortly after injection of CHH [23], *in vitro* incubation of the target tissues with CHH elevates tissue cGMP levels in a dose- and time-dependent manner [23−25], and the increase in intracellular cGMP precedes an increase in glucose release into incubation media [23]. The effect of CHH on cGMP levels is potentiated by phosphodiesterase inhibitors, suggesting that CHH acts primarily by stimulating GC [25]. Further, CHH stimulates GC activity in membrane (but not cytosolic) preparations of muscle (a CHH target tissue), indicating that increase in cGMP levels in response to CHH is due to the activation of membrane-bound GC [25].

We describe here the cloning of a cDNA encoding a GC from muscle of the crayfish, *Procambarus clarkii*. To our knowledge, this is the first report of a GC cloned from any crustacean species. The cDNA (*PcGC-M2*) encodes a protein with sequence characteristics of a membrane form GC. The tissue distribution of the *PcGC-M2* transcript is consistent with the hypothesis that it encodes a CHH receptor. The results add to current knowledge of GC protein and gene structure. In addition, we anticipate the findings will provide a foundation for future studies of the signaling cascade initiated by CHH.

Materials and Methods

Animals. Adult animals (*Procambarus clarkii*) used in the present study were obtained and reared as described previously [26].

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Abdominal muscles 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 (GC-f-V: 5'-GTSTAYAAGGTRGARACNATHGG-3' and GC-b-V: 5'-CCRAANARRCARTANCKVGGCAT-3') 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℃), 35 cycles of denaturation (20 s, 94℃), annealing (20 s, 50℃), and extension (30 s, 72℃), followed by a final extension (7 min, 72℃).

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).

Gene-specific primers for RACE reactions were selected from the sequence of the 242-base pair (bp) PCR product amplified within the cyclase catalytic domain using degenerate primers. 3'-RACE reactions were carried out using a nested PCR approach. In the first amplification reaction, 2.5 µl of cDNA (1% of the RT reaction) was amplified with PcGCD-M3-f2 (5'-GCCTCTATGGCACTTGAACTACTGG-3'; nucleotides 3549-3573, see Fig. 1) and a universal primer (NUP) supplied by the RACE kit. In the second amplification reaction, 5 µl of the diluted (1000X dilution with 10 mM Tricine-KOH buffer, pH 8.5, containing 1.0 mM EDTA) first reaction was amplified with PcGCD-M3-f3 (5'-AATTTATCATCCGGCACCGC-3'; nucleotides 3589-3608) and NUP. Both the first and second reactions were carried out in a 50-µl reaction containing the template, 0.2 mM each of dNTPs, 0.2 µM each of the primers, 20 U of Titanium™ *Taq* DNA polymerase (Clontech), and 1X reaction buffer (40 mM Tricine-KOH, pH 8.7, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml BSA, 0.05% Tween-20, 0.005% Nonidet-P40). The PCR parameters were as follows: an initial denaturation (5 min, 94℃), 40 (first reaction) or 20 (second reaction) cycles of denaturation (30 s, 94℃), annealing [30 s, 60℃ (first reaction) or 55℃ (second reaction)], and extension (2 min, 72℃), followed by a final extension (7 min, 72℃).

5'-RACE reactions were also carried out using a nested PCR approach. In the first amplification reaction, 2.5 µl of cDNA was amplified with PcGCD-M3-b1 (5'-AGTAAGGCCAACAACCCCAGCAATC-3'; nucleotides 3680-3656) and a mixture of universal primers (UPM) supplied by the RACE kit. In the second amplification reaction, 5 µl of the diluted (50X dilution) first reaction was amplified with PcGCD-M3-b2 (5'-GTGCAGCCCGATGCGTAAC-3'; nucleotides 3644-3626) and NUP. Both the first and second reactions were carried out in a 50-µl reaction containing the template, 0.2 µM each of the primers, and other constituents as described for 3'-RACE reactions. The PCR parameters were an initial denaturation (5 min, 94℃), 35 (first reaction) or 25 (second reaction) cycles of denaturation (30 s, 94℃), annealing [30 s, 63℃ (first reaction) or 60℃ (second reaction)], and extension (3 min, 72℃), followed by a final extension (7 min, 72℃).

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/) [27].

Analysis of tissue distribution of PcGC-M2 transcript by RT-PCR. Equal amounts (1 μg) of total RNA, extracted separately from the muscle, hepatopancreas, heart, ovary, testis, and gill 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 Pc-ECD-1S (5'-AACGAGCGGGCGTCAAAG-3'; nucleotides 1053-1070) and Pc-ECD-1A (5'-CAAGCTGGAGTGAAAACCAAGACA-3'; nucleotides 1649-1626) designed based on the sequences of the putative extracellular domain of PcGC-M2 (see Fig. 1) or actin-f2 (5'-TCATCACCAACTGGGACGAC-3') and actin-r2 (5'-GAAACCTCCATAGACGGGGAC-3') selected from the published sequence of crayfish actin [28].

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

Results and discussion

A pair of degenerate primers was designed against highly conserved sequences from the cyclase catalytic domain of GCs and used to amplify cDNA derived from crayfish (*Procambarus clarkii*) abdominal total RNA. A 242-bp PCR product was thus amplified, cloned, and sequenced allowing selection of gene-specific primers for subsequent 3'- and 5'-RACE reactions to obtain a full-length cDNA sequence. The resulting 5159-bp sequence (*PcGC-M2*) contains a 4209-bp open reading frame (ORF) that predicts a protein of 1403 residues (Fig. 1). The ORF begins with an initiator methionine at nucleotides 351–353 that is flanked by conserved sequences (a G in positions –6, –3, and +4 relative to the first letter of the start codon) as described by Kozak [29] and proceeded by an in-frame stop codon (nucleotides 303–305). The ORF ends with a TAA stop codon at nucleotides 4560–4562 that is followed by a 3'-untranslated region. A polyadenylation signal AATAAA [30] is present 552 bp downstream from the stop codon and 13 bp upstream from a poly (A) tail (Fig. 1).

Analysis of the N-terminal sequence [31] of the protein encoded by *PcGC-M2* predicted a 60-residue signal peptide with the putative cleavage site located between residues 60 (G)

and 61 (D) (Fig. 1). Cleavage at this site would yield a mature protein with a relative molecular mass of 151,189. The deduced amino acid sequence of the mature PcGC-M2 is most closely related (33% identity) to a *Drosophila* membrane form GC, DrGC-1 [6,7], and an *Anopheles gambiae* membrane form GC, ENSANGP00000009147 (AgaGC) [32]; it has a range of 22% to 27% identity with mammalian GCs (Table 1). Hydropathic analysis of the mature protein [33] revealed a major hydrophobic stretch of 20-residues between residues 584 (I) and 603 (Y) (Fig. 1), suggesting the presence of a single membrane-spanning segment that divides the protein into a 523-residue extracellular domain and an 800-residue intracellular domain.

The extracellular domain exhibits the highest diversity among the members of the family of membrane form GC, which presumably confers the ligand binding specificity. The extracellular domain of PcGC-M2 has 29% sequence identity with DrGC-1, 24% with AgaGC, and a range of 14% to 17% with mammalian membrane form GCs (Table 1). In the extracellular domain of PcGC-M2, 12 potential N-linked glycosylation sites, which are critical for ligand binding [2], are present as NXT or NXS, where X is any amino acid (Fig. 1). Several cysteine residues are conserved in the extracellular domains of membrane form GCs forming intra- or inter-chain disulfide bonds that are possibly involved in ligand binding, transmembrane signal transduction, or receptor dimerization [35 −36]. In PcGC-M2, 11 cysteine residues are present in the extracellular domain. Alignment of the extracellular domain of PcGC-M2 with those of mammalian membrane form GCs, as well as DrGC-1 and AgaGC revealed that PcGC-M2 contains 4 cysteine residues (Cys_{201}, Cys_{214}, Cys_{566}, and Cys_{573}) that are conserved in all GCs (except GC-C which lacks the 2 residues located close to the transmembrane domain). In GC-A, these 4 residues have been shown to join sequentially forming 2 intra-chain disulfide bonds [36]. Two additional cysteine residues, which are found in the neuronal GCs (GC-D, GC-E, and GC-F) specifically expressed in sensory neurons [37], are also present in PcGC-M2 (Cys_{119} and Cys_{233}) and DrGC-1; AgaGC lacks the N-terminal most cysteine residue' probably because its N-terminal end is incomplete (Fig. 2). When compared with mammalian GCs, the distribution pattern of the conserved cysteine residues observed in PcGC-M2 is more similar to those found in neuronal GCs. However, PcGC-M2, as well as DrGC-1 and AgaGC, also contains 2 highly conserved cysteine residues (Cys_{386} and Cys_{534}) that are not found in mammalian GCs. It appears that PcGC-M2, DrGC-1, and AgaGC share a similar distribution pattern of cysteine residues unique to these invertebrate GCs (Fig. 2).

Analysis of protein domains using PROSITE indicated that the intracellular region of PcGC-M2 contains a kinase-like and a cyclase catalytic domain. Several sequence characteristics observed in the PcGC-M2 are typical of the kinase-like domain of membrane form GCs. In general, protein kinases contain 33 invariant amino acids that have been divided into 11 subdomains [38]. Many of these invariant amino acids are conserved in the kinase-like domain of membrane form GCs [2]. Twenty-six of the 33 invariant amino acids are present in the

kinase-like domain of PcGC-M2 (data not shown). In addition, an invariant residue D, which is found in the subdomain VI of protein kinases and mediates the transfer of a phosphate group from ATP to the substrates [2], is replaced by an N (at residue 767) in the kinase-like domain of PcGC-M2 (Fig. 1). Characteristically, this invariant D is replaced in membrane form GCs by S, R, or N [2]. Further, the glycine-rich loop (GXGXXG) that is responsible for ATP binding to protein kinases is usually altered or absent in membrane form GCs [2]. An altered glycine-rich loop is present as GXXXG at residues 655 to 659 in the kinase-like domain of PcGC-M2 (Fig. 1). Similar glycine-rich loops are also present in various membrane form GCs [3,4,7,10,39].

As expected, the cyclase catalytic domain of PcGC-M2 has high sequence identity with that of other membrane form GCs: 78% identical with DrGC-1 and AgaGC, and a range of 57% to 72% with mammalian GCs (Table 1). An 18-residue region of high similarity was found in the cyclase catalytic domains of GC-D, GC-E, GC-F, and MsGC-II (an insect neuronal GC) that are regulated by $[Ca^{2+}]_i$, possibly through guanylyl cyclase-activating proteins (GCAPs) [11,40]. This highly conserved region has been shown to be responsible for binding to GCAPs and required for activation of GC-E by GCAPs [40]. The corresponding region in PcGC-M2 (residues 1079 to 1096) does not contain a sequence characteristic of the GCAP binding site, suggesting that regulation of its activity is most likely independent of GCAPs. In several membrane form GCs, a C-terminal tail of various lengths was observed following the cyclase catalytic domain; these C-terminal tails are usually rich in uncharged polar amino acids, such as S and Q [2,7,41]. It has been proposed that the C-terminal tail is involved in ligand-dependent receptor-mediated endocytosis through an YXXZ motif (X is any amino acid and Z is L, I, V, M C, or A), or in anchoring to cytoskeletal elements [2]. Likewise, a C-terminal tail of 247 residues (residues 1157 – 1403, Fig. 1), of which 17% are S and Q, extends from the cyclase catalytic domain of PcGC-M2. However, the YXXZ motif is not present in the C-terminal tail of PcGC-M2.

The sequence characteristics presented above indicate that PcGC-M2 belongs to the family of membrane form GC. It is most closely related in several important characteristics to 2 insect membrane form GCs (DrGC-1 and AgaGC) [6,7,32]. Endogenous ligands for DrGC-1 or AgaGC are yet to be identified. Likewise, it is not known presently if CHH or other ligands bind and activate PcGC-M2. PCR amplification of muscle, hepatopancreas, heart, ovary, testis, and gill cDNAs revealed that PcGC-M2 transcript is widely expressed (Fig. 3). These tissues have been shown to be functionally regulated by CHH, contain CHH binding activity, or both [15 – 17,23,25,42]. The results are consistent with the hypothesis that PcGC-M2 participates in the signaling cascade activated by CHH. We expect that these findings will provide a foundation for future investigation of several pressing questions with respect to the cellular mechanism of action of CHH, including identification of the CHH receptor, and assessment of the receptor binding characteristics of the various CHH isoforms.

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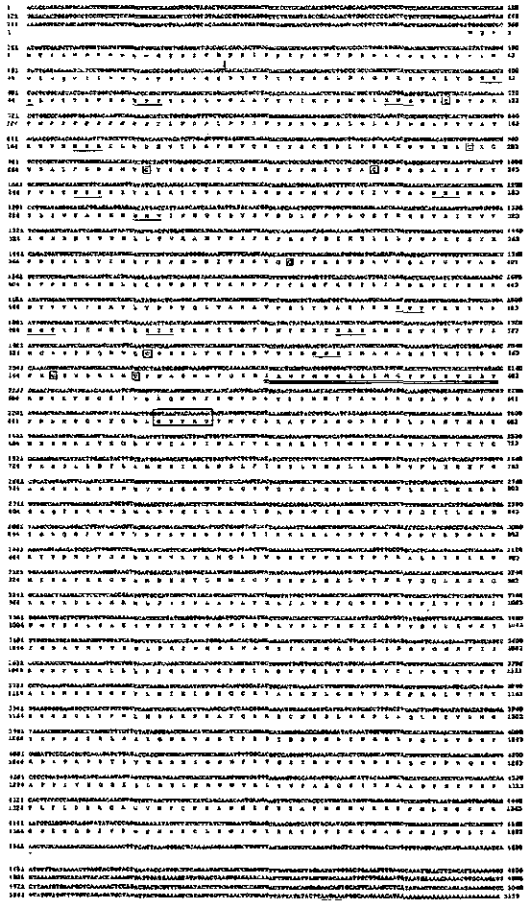

Fig. 1. The nucleotide and deduced amino acid sequences of *PcGC-M2* cDNA. Nucleotides and amino acids are numbered beginning at the first residue of the respective sequence. The deduced amino acid sequence of the open reading frame is shown in single-letter code below the nucleotide sequence. The putative signal sequence cleavage site is marked with an arrow, which is preceded by a *60-residue signal peptide shown in* lowercase letters. In the extracellular domain, 8 conserved cysteine residues are boxed by thin lines and 12 potential N-linked glycosylation sites underlined by thin lines. The putative transmembrane domain is double underlined. The glycine-rich loop is boxed by heavy lines. The stop codon is indicated with an asterisk (*). The putative polyadenylation signal is underlined by a dotted line.

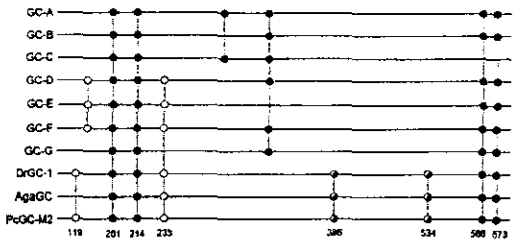

Fig. 2. Positions of conserved cysteine residues in the extracellular domain of mammalian membrane form GCs (GC-A－GC-G), and three invertebrate GCs (DrGC-1, AgaGC, and PcGC-M2). Filled circles represent the conserved cysteine residues in mammalian GCs and invertebrate GCs; open circles indicate the cysteine residues that are conserved within mammalian neuronal GCs (GC-D, GC-E, GC-F) and invertebrate GCs; semi-filled circles represent the conserved cysteine residues unique to invertebrate GCs. Positions of the conserved cysteine residues in PcGC-M2 are given below *each cysteine residue. The dashed line at the left end of* AgaGC indicates that its sequence is incomplete at the N-terminal end. Note the similar distribution pattern of cysteine residues shared by DrGC-1, AgaGC, and PcGC-M2.

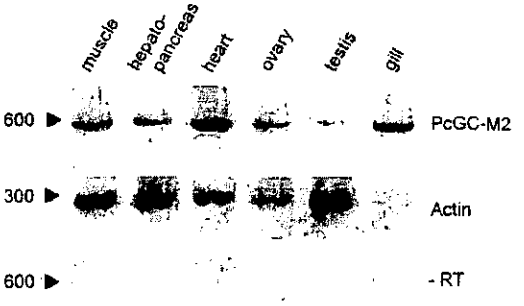

Fig. 3. Tissues distribution of the *PcGC-M2* transcript. Total RNA prepared from the crayfish tissues as indicated was reverse transcribed and amplified with primers Pc-ECD-1S and Pc-ECD-1A (PcGC-M2) or actin-f2 and actin-r2 (Actin). The expected size of the amplicon was 596 bp for PcGC-M2, and 284 bp for actin. To control for genomic contamination, tissue total RNA was amplified (without reverse transcription) with primers Pc-ECD-1S and Pc-ECD-1A (- RT). See **Materials and methods** for the design of the primers. Arrowheads indicate positions of the size markers.