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甲殼類升血糖荷爾蒙受體—鳥鳥鳥鳥鳥鳥鳥鳥鳥鳥鳥鳥析(2/3)

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CONTENT

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 first year of the project, we achieved the cloning of a cDNA encoding a GC-like protein (PcGC-M2) from muscle of the crayfish, *Procambarus clarkii*. The cDNA (*PcGC-M2*) encodes a protein with 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. The tissue distribution of the *PcGC-M2* transcript is consistent with the hypothesis that it encodes a CHH receptor. Now, in the second year of the project, we reported the cloning of a cDNA encoding a truncated GC-like protein (PcGC-M2'), which possesses only the extracellular domain, and transmembrane domain, and a partial kinase homology domain, but lacks a cyclase catalytic domain. Furthermore, antibodies against the extracellular domain of PcGC-M2 were raised, purified, and tested for immunoreactivity. Heterologous expression of PcGC-M2, PcGC-M2' was carried out in insect S2 cells or HEK293 cells. Optimal protocol for heterologous expression is being tested. The expressed proteins will provide a foundation for future studies of the signaling cascade initiated by CHH.

Key words: receptor guanylyl cyclase, crustacean hyperglycemic hormone, molt-inhibiting hormone, cGMP, X-organ-sinus gland, crustacean

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、X-器官/血竇腺、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).

Previous studies on the cellular mechanism of action of CHH (crustacean hyperglycemic hormone) 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 (Sedlmeier and Keller, '81), *in vitro* incubation of the target tissues with CHH elevates tissue cGMP levels in a dose- and time-dependent manner (Sedlmeier and Keller, '81; Goy et al., '87; Goy, '90), and the increase in intracellular cGMP precedes an increase in glucose release into incubation media (Sedlmeier and Keller, '81). The effect of CHH on cGMP levels is potentiated by phosphodiesterase inhibitors, suggesting that CHH acts primarily by stimulating guanylyl cyclase (Goy, '90). Further, CHH stimulates cyclase 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 (Goy, '90).

In the first year of the project, we achieved the cloning of a cDNA encoding a GC-like protein (PcGC-M2) from muscle of the crayfish, *Procambarus clarkii*, which was 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.

Now, in the second year of the project, we reported the cloning of a cDNA encoding a truncated GC-like protein (PcGC-M2'), which possesses only the extracellular domain, and transmembrane domain, and a partial kinase homology domain of the PcGC-M2 identified in the first year. Furthermore, antibodies against the extracellular domain of PcGC-M2 were raised, purified, and tested for immunoreactivity. Heterologous expression of PcGC-M2, PcGC-M2' was carried in insect S2 cells or HEK293 cells. The expressed proteins will provide a foundation for future studies of the signaling cascade initiated by CHH.

MATERIALS AND METHODS

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/; Hall, '99).

Production of antisera

Preimmune and anti-ECD immune sera were produced in New Zealand white rabbits. Before immunization, blood was withdrawn from ear veins for preparation of preimmune serum. Synthetic peptide designed based on the extracellular domain of PcGC-M2 was emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis), the solution was injected subcutaneously into the rabbit. Four weeks later, a booster injection of synthetic peptide emulsified with Freund's incomplete adjuvant was administered. One week after the booster injection, blood was withdrawn, allowed to clot (37℃ for 1 h, then 4℃ overnight), and centrifuged (10,000 *g*, 10 min). Blood withdrawn before immunization was similarly processed to obtain preimmune serum. Both preimmune and immune sera were divided into aliquots of 500 μL and stored at 20℃.

Heterologous expression of GCs

The pMT-DEST 48 plasmid containing *PcGC-M2* gene (previously constructed by Liu *et al.*, 2004) was amplified by a pair of specific primers (forward primer designed from the *PcGC-M2* gene and reverse primer designed from the pMT-DEST 48 vector 6XHis tag). Then the PCR product was digested with *EcoR1* and *Xbal* and ligated into the expression vector pcDNA 3.1 (Invitrogen). To ensure in-frame insertion, the *PcGC-M2* DNA sequence of the construct was determined by automated DNA sequencer (PRISM 3100, ABI). This pcDNA3.1- *PcGC-M2* construct will be used for the transfection of S2 cells.

RESULTS AND DISCUSSION

A cDNA encoding a GC-like protein (PcGC-M2) from muscle of the crayfish, *Procambarus clarkii*, was cloned in the first year of the project, which was the first report of a GC cloned from any crustacean species (Liu et al., 2004). 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. The *PcGC-M2* transcript is expressed in several CHH target tissues, including muscle, hepatopancreas, heart, ovary, testis, and gill, suggesting that PcGC-M2 may participate in the signaling cascade activated by CHH (Liu et al., 2004).

Now, we have cloned from the muscle another GC-like gene, PcGC-M2', which, due to an earlier stop codon, ends the coding region in a way so that the encoded protein constitutes only an extracellular ligand-binding domain, a transmembrane, and a partial kinase-like domain (Fig. 1). The presence of a truncated GC, which is exactly the same as PcGC-M2 up to the extracellular ligand-binding domain and transmembrane domain, has important implication. As the truncated PcGC-M2' retains the extracellular ligand-binding domain, it might have competed with PcGC-M2 with ligand binding; however, with a truncated partial kinase-like domain and lacking completely a cyclase catalytic domain, it is expected that PcGC-M2' might be able to bind ligand but without downstream cyclase activity being generated. In fact similar truncated GCs have been reported, which function as negative regulator of full-length GC by binding with the common ligands (Tamura N, Garbers, 2003). In this regard, it is interesting to note that a recent study of CHH/MIH receptors has found that the receptor number does not significantly change, but the signaling output (that is, guanylyl cyclase activity) varied significantly (Chung and Webster, 2003), an observation that would have been predicted by a target expressing, in addition to a functional full-length GC, a ligand-binding but cyclase-lacking truncated GC. Such speculation should be tested in subsequent endeavor.

In order to be able to probe for the heterologously expressed crayfish GCs, antibodies were raised against the extracellular ligand-binding domain of the crayfish GCs. A synthetic peptide was designed based on a stretch of a 13-mers, synthesized commercially as a Multiple Antigenic Peptide, and injected into rabbits for generation of antiserum (Anti-ECD). The antisera were used for probing in a Western blotting analysis of muscle proteins, the results of which showed that, in addition other muscle proteins, a band of ~ 150 kD has been labeled suggesting possible presence of the GC as predicted by the estimated molecular mass of PcGC-M2. The labeled proteins with lower MWs might represent the truncated PcGC-M2' (Fig. 2). The exact specificity of the antisera will be tested when expressed GCs are available.

In an effort to express the cloned crayfish GCs, a recombinant pcDNA3.1- *PcGC-M2* construct will be used for the transfection of S2 cells. Expression of fusion protein (*PcGC-M2*-6XHis tag) was confirmed by Westren blot analysis using an anti-6Xhistidine antibody (Fig. 3). However, the

expression was extremely low (see lane 4-6, Fig. 3A). We decided to express GCs in another cell line, HEK 293, which has been used for expression of GCs. In this regard, we subcloned the insert containing GC into an expression vector pcDNA/V5-DEST. A PCR reaction was used to test whether the insert was indeed inserted into the vector, the results of which indicated that all inserts (PcGC-M2, PcGC-M2', and ECD) were all inserted (Fig. 4). Auto sequencing analysis also indicated that the inserted are correctly inserted (data not shown).

The recombinant plasmids were used for transfection of HEK 293 cells. Western blotting analysis of cell lyaste with anti-ECD recognized, among other proteins, proteins of the expected size of PcGC-M2 (Fig. 5). Analysis of PcGC-M2' and ECD are underway. Further efforts are directed towards a optimal protocol of transfection for expression of GCs.

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.

SELF EVALUATION

In the second year of the project, we have cloned a cDNA encoding a truncated GC-like protein (PcGC-M2'), which possesses only the extracellular domain, and transmembrane domain, and a partial kinase homology domain, but lacks a cyclase catalytic domain. The significance of this truncated GC may be regulating the signal output of ligand binding to the full-length PcGC-M2, making the regulation by its ligand more diversified depending on the relative expression of the full-length and truncated GCs. Furthermore, antibodies against the extracellular domain of PcGC-M2 were raised, purified, and tested for immunoreactivity. The antibodies are expected to be useful for further probing of heterologously expressed GCs, as well as functional determination of the roles of these GCs in the CHH signaling pathway. Heterologous expression of PcGC-M2, PcGC-M2' was carried out in insect S2 cells or HEK293 cells. Although at a very low level and a optimal protocol still needed to be worked out, proteins possibly represent the crayfish were detected, especially when the insect S2 cells were used. Optimal protocol for heterologous expression is being tested. The expressed proteins will provide a foundation for future studies of the signaling cascade initiated by CHH, that is the objective of the third year of the project : to determine experimentally whether PcGC-M2 is the CHH receptor.

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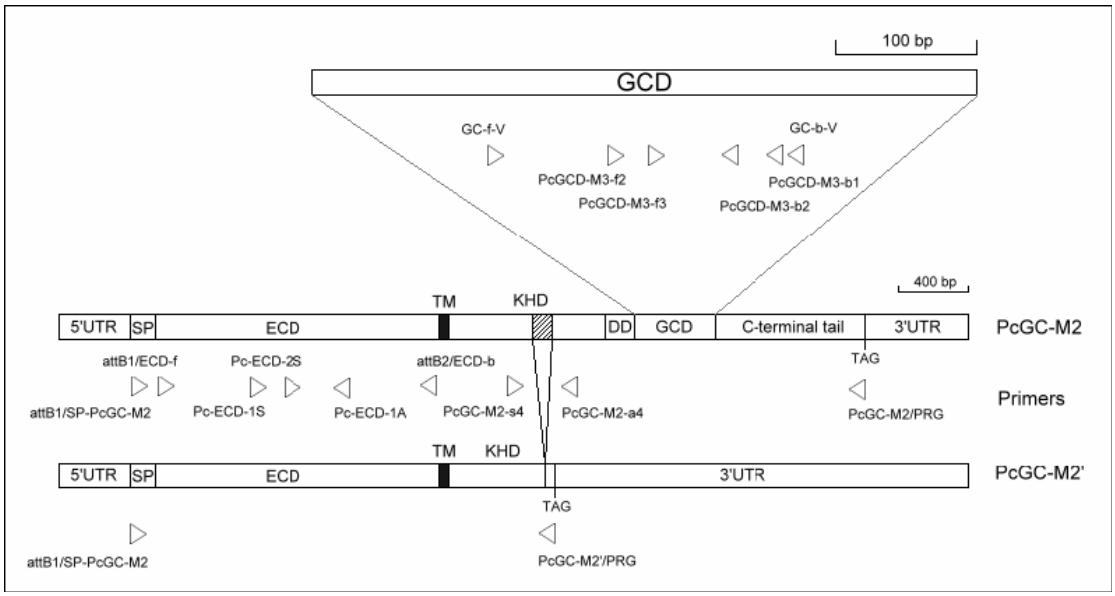

Fig. 1. Comparison of domain structures of PcGC-M2 and PcGC-M2'. SP: signal peptide; ECD: extracellular domain; TM: transmemebrane domain; KDH: kinase homologous domain; GCD: guanylyl cyclase domain. A stop codon appears in the region coding for the KDH of PcGC-M2' prematurely ends the coding region.

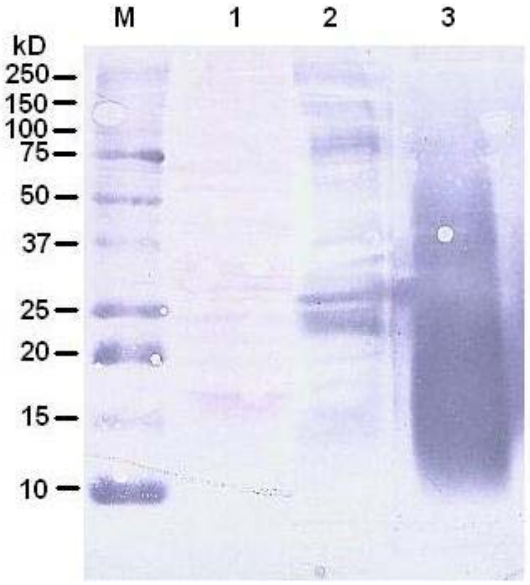

Fig. 2. Western blot analysis of muscle proteins using anti-ECD. Crayfish muscle was homogenized and centrifuged. Supernatant (lane 1) and pellets (lane 2) were separated using SDS-PAGE and probed by anti-ECD antibodies. Note the presence of proteins of approximately 150 kD, the expected size of PcGC-M2, as well as several proteins of lower molecular masses, possibly PcGC-M2', in lane 2.

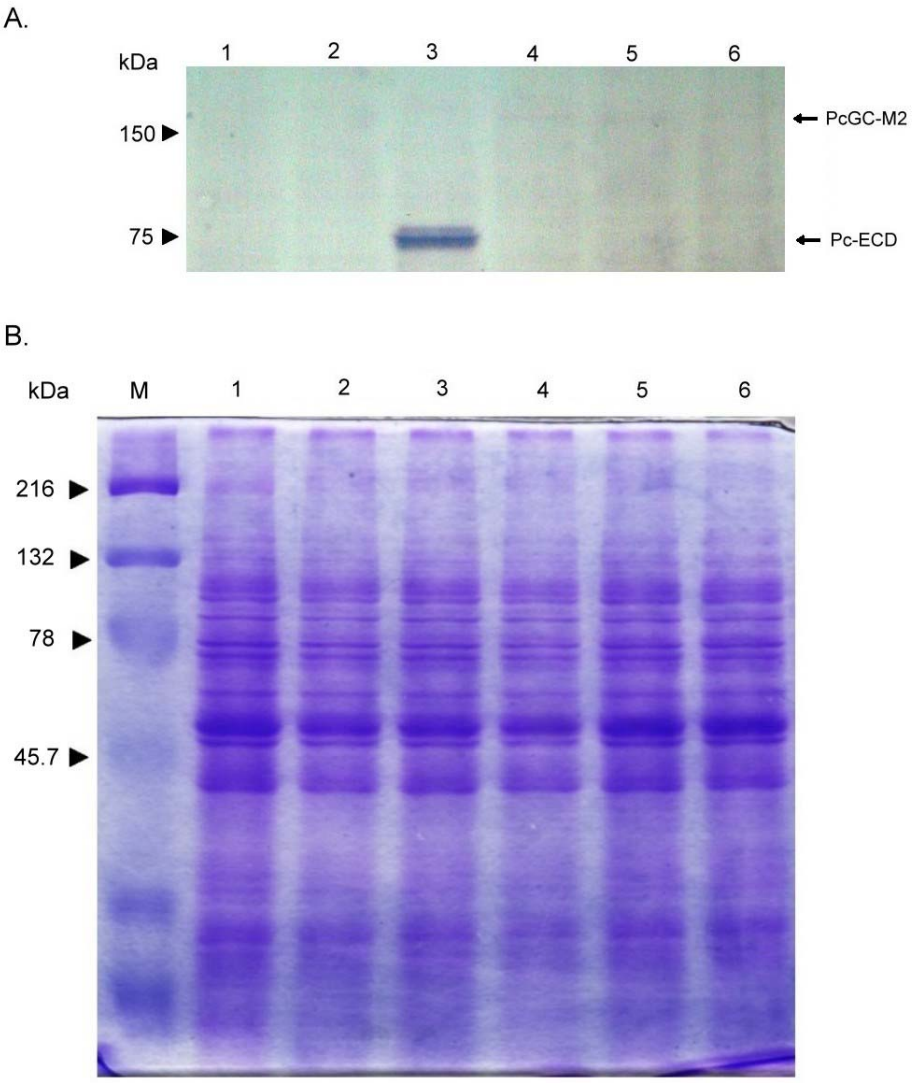

Fig. 3. Expression of recombinant GC proteins in S2 cells. Western blot (A) and SDS-PAGE (B) analysis of proteins extracted from non-transfected S2 cells (1) or S2 cells transfected with vector pMT-DEST48 only (2, mock control), or transfected with vector containing extracellular domain of PcGC-M2 (3, pMT-DEST48/Pc-ECD), or vector containing full-length PcGC-M2 (4-6, pMT-DEST48/Pc-GCM2). Note the presence of an immunoreactive band in lane 3, and immunoreactive bands in lanes 4-6 (arrowheads in A), which correspond, respectively, to the expected sizes of the extracellular domain of PcGC-M2 (75 kDa) and full-length PcGC-M2 (150 kDa).

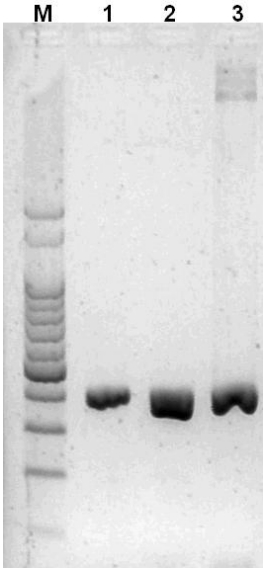

Fig. 4. Confirmation of insertion of GCs into the expression vector. The recombinant vectors were amplified with primers specific for PcGCM2、PcGCM2'、ECD (lanes 1, 2, 3, respectively). Amplicons of expected sizes are visualized for all lanes.

Fig. 5. Heterologous expression of PcGC-M2 in HEK 293 cells. Arrow ⟶ points to protein of about 150 kD, the expected size of PcGC-M2.