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## Cloning and gene expression of G protein competitive inhibitory polypeptide and its prophylactic effects on myocardial hypertrophy *in vitro*<sup>1</sup>

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**KEY WORDS** GTP-binding proteins; plasmids; genetic vectors; left ventricular hypertrophy; gene expression

### ABSTRACT

**AIM:** To clone and express G protein competitive inhibitory polypeptide (GCIP) gene and investigate the prophylactic effects of GCIP on myocardial hypertrophy *in vitro*. **METHODS:** The pIVEX2.3MCS-GCIP plasmid expressing GCIP was constructed by inserting double-stranded oligonucleotide into pIVEX2.3-MCS plasmid vector. Recombinant plasmid was expressed in Rapid Translation System 500 (RTS500). The expression of GCIP was identified by SDS-PAGE and Western blotting. The purification of GCIP with 6×His tag was carried out on a Ni-NTA agarose column. The prophylactic effects of GCIP was observed on cardiomyocytes isolated from newborn Wistar rats. Protein synthesis rate were assessed by [<sup>3</sup>H]Leu incorporation. Protein contents were measured by Lowry method. **RESULTS:** The plasmid pIVEX2.3MCS-GCIP was successfully constructed. The expression of GCIP was about 2.43 % of total protein. GCIP was purified on Ni-NTA agarose column. The purity of the purified GCIP peptide was about 98 %. The contents of total protein and the rate of [<sup>3</sup>H]Leu incorporation were significantly decreased in (100 µg/L, 1 mg/L, and 10 mg/L) GCIP-treated groups in myocardial cellular hypertrophy model ( $P < 0.01$ ). **CONCLUSION:** The pIVEX2.3MCS-GCIP expression vector has been constructed successfully. The GCIP was expressed in RTS500 system and was purified with Ni-NTA agarose. GCIP was able to inhibit myocardial hypertrophy concentration-dependently *in vitro*.

### INTRODUCTION

Myocardial hypertrophy is an adaptive response to various mechanical (for example, hemodynamic changes) and hormonal stimuli (such as endothelin I, angiotensin II, norepinephrine, and TGF) and represents an initial step in the pathogenesis of many cardiac dis-

eases that ultimately progress to ventricular failure, which leads the patients to death<sup>[1]</sup>. Hormones and neurotransmitters may mediate common responses through receptors that couple to the same class of heterotrimeric guanine nucleotide-binding protein, which is called “molecular switch” of the signal transduction<sup>[2]</sup>. It was reported that inhibition of myocardial hypertrophy was produced in the hearts of transgenic mice by targeted expression of the G protein competitive inhibitory polypeptide (GCIP). Therefore, targeting the receptor-G protein interface may point the way to the development of therapies that have the potential advantage over

<sup>1</sup> Project supported by the grants from Science Foundation of Chongqing, No 20010725.

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Received 2002-10-15

Accepted 2003-03-25

traditional receptor antagonists<sup>[3-7]</sup>.

In the present study, we cloned the GCIP gene into expression vector, expressed the GCIP polypeptide in RTS500 system, and investigated its prophylactic effects *in vitro* on myocardial hypertrophy induced by norepinephrine (NE).

## MATERIALS AND METHODS

**Materials** The GCIP gene was synthesized by Sangon Company (Shanghai, China) and purified by polyacrylamide gel electrophoresis. The restriction endonucleases were purchased from TaKaRa Company. The plasmid pEGFP-N1, *E. coli* strain DH5 $\alpha$  were granted by Dr WAN Ying and DAI Jia-Ping. The RTS500 *E. coli* Circular Template Kit was purchased from Roche Company, which included the expression plasmid pIVEX2.3-MCS and the positive control plasmid pIVEX2.3-GFP (Green Fluorescent Protein, GFP). The Ni-NTA agarose column was purchased from Pierce Company.

**Construction of the expression vector pIVEX2.3MCS-GCIP** The nucleotide fragments encoding the GCIP gene was obtained from Sangon Company (Shanghai, China). The fragments were digested with *Xho*I and *Sma*I and subcloned into the plasmid pIVEX2.3-MCS for expression in *E. coli* and RTS500 System under the control of T7 promoter. The inserted nucleotide sequences were confirmed by restriction analysis and DNA sequencing.

**Protein expression and purification** The recombinant plasmid was expressed using the RTS500 Instrument as described by the manufacturer. The pIVEX2.3MCS-GCIP plasmid (15 mg/L) was added in the RTS reaction solution. The condition of expression was at 30 °C with a stirrer speed of 120 r/min for 24 h.

A Ni-NTA agarose column was equilibrated with 5 mL binding buffer (Tris 20 mmol/L pH 7.9, NaCl 0.5 mol/L, glycerol 10 %). Then the column was loaded with reaction solution at a rate of 1 mL/h, and was washed with 3 $\times$ 5 mL washing buffer (Tris 20 mmol/L pH 7.9, NaCl 0.5 mol/L, glycerol 10 %, imidazole 20 mmol/L). The fusion protein was eluted with 1.5 mL elution buffer (Tris 20 mmol/L pH 7.9, NaCl 0.5 mol/L, glycerol 10 %, imidazole 250 mmol/L). Eluate was collected and analyzed for SDS-PAGE and Western blotting<sup>[8]</sup>.

**SDS-PAGE and Western blotting** The reaction solution was analyzed on a discontinuous SDS/polyacrylamide gel electrophoresis system according to

Kratzin and Wiltfang<sup>[9]</sup> using a concentration 16.5 % T and 3 % C as separating gels, which could separated 2.5-100 kDa protein. This corresponds to 18 % (w/v) acrylamide and 0.5 % (w/v) *bis*-acrylamide. Proteins were then transferred to PVDF membrane by electroblotting (110 V for 2 h). The lane of molecule marker of PVDF membrane was cut with Coomassie brilliant blue-stained. The other lanes of PVDF membrane were blocked at 37 °C for 1 h in 5 % (w/v) skimmed milk powder in TBS (Tris 10 mmol/L pH 8.0, NaCl 150 mmol/L). Then the membrane was incubated with 1:1000 dilution of mouse anti-His(6) antibody at 37 °C for 0.5 h, and 1:1500 dilution of goat anti-mouse IgG horse radish peroxidase conjugate was added and incubated at 37 °C for 0.5 h. Following detection was performed by DAB. The result was analyzed by SXimage98 software.

**Cell culture** Cardiomyocytes were cultured according to the method reported by Simpson P<sup>[10]</sup>. Cardiomyocytes were isolated from newborn Wistar rats. NE were added into the media to induce myocyte hypertrophy<sup>[10]</sup>.

**Prophylactic effects on myocardial hypertrophy** Cardiomyocytes were seeded at a density of 5 $\times$ 10<sup>5</sup> cells/well in a 24-well plate. The medium containing standard 10 % fetal bovine serum (FBS) was replaced by medium without FBS for 48 h. After a 24-h incubation, the different agents were added (respectively the normal control was added BSA, the NE group was added NE, the GCIP control group was added NE and elution buffer, the GCIP group was added NE and GCIP 1  $\mu$ g/L, 10  $\mu$ g/L, 100  $\mu$ g/L, 1 mg/L, or 10 mg/L). The final [<sup>3</sup>H]Leu concentration of each well was 37 MBq/L (1 mCi/L). Protein synthesis rate was assessed by [<sup>3</sup>H]Leu incorporation<sup>[11]</sup>. Protein contents were measured with Lowry method.

**Statistical analysis** Statistical analysis was performed using statistical program SPSS8.0. Data were expressed as mean $\pm$ SD and compared by *t*-test. Statistical significance was judged at *P*<0.05.

## RESULTS

**Construction and characterization of pIVEX2.3MCS-GCIP plasmid** The 2.5 % agarose gel electrophoresis of plasmid pIVEX2.3MCS-GCIP showed that the inserted fragment was about 180 bp (Fig 1). The plasmid was sent to Sangon Company for DNA sequencing. The sequence of inserted fragment was completely coincident with the GCIP gene.

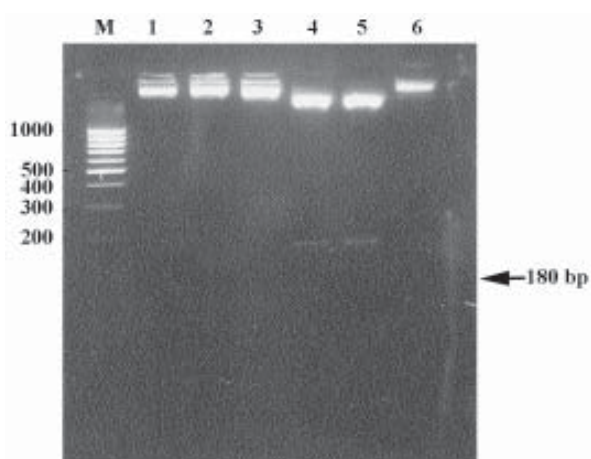


Fig 1. Restriction analysis of recombinant plasmid. Lane M: DNA Marker; Lane 1 to Lane 6: confirmed plasmid by *Xho I/Sma I*, Lane 4 and Lane 5: positive clone.

**Expression in RTS500 system and Western blotting** SDS-PAGE of total protein analysis indicated that the molecule weight of the GCIP protein was about 8.5 kDa (Fig 2, lane 1) in RTS500 System. The yield was 2.43 % of total protein in the RTS reaction solution. As a positive control [the expression of GFP was found a band of molecule weight about 30 kDa (Fig 2, lane 2)]. The yield was 10.59 % of total reaction solution.

Western blot analysis showed that a band of GCIP was about 8.5 kDa (Fig 3, lane 2), and a band of GFP was about 30 kDa (Fig 3, lane 1).

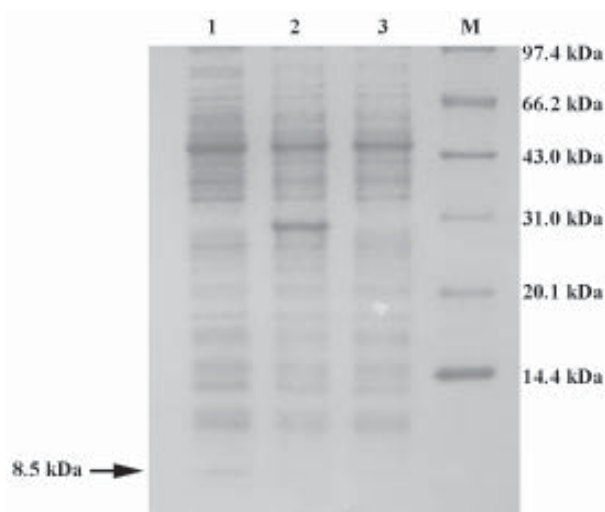


Fig 2. SDS-PAGE analysis of GCIP expression in RTS500 system. Lane 1: Expression GCIP; Lane 2: the positive control (expression GFP); Lane 3: the negative control (the RTS reaction fluid); Lane M: molecule weight marker.

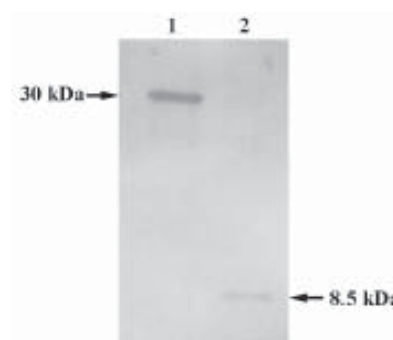


Fig 3. Western analysis of mixed protein from RTS500 reaction fluid. Lane 1: the positive control (expression GFP); Lane 2: the expression GCIP.

**Purification** Densitometry analysis of Coomassie brilliant blue-stained (Fig 4) SDS-PAGE demonstrated that the elution fragment of affinity chromatography on Ni-NTA agarose by imidazole 250 mmol/L contained about 98 % GCIP.

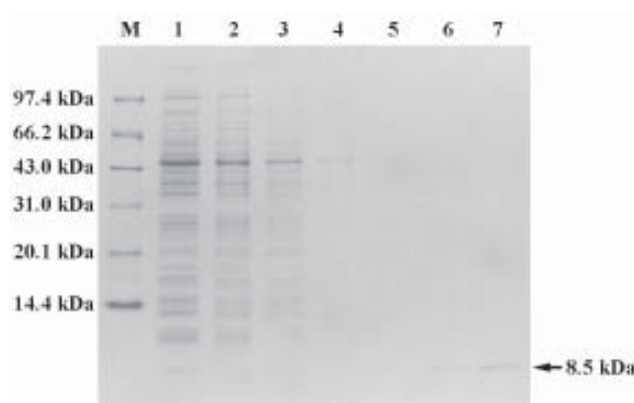


Fig 4. Purification of His-tagged GCIP peptide. Lane M: molecule mass marker; Lane 1: the control; Lane 2: penetrated fluid; Lane 3-5: washing fluid; Lane 6: the second eluted by imidazole 250 mmol/L; Lane 7: the first eluted by imidazole 250 mmol/L.

**Bioactivity of prophylactic effects on myocardial hypertrophy** In NE-stimulated group, the rate of [<sup>3</sup>H]Leu incorporation and the contents of total protein were increased obviously compared with the normal control group ( $P < 0.01$ ). The results indicated that myocyte hypertrophy model was successful. The rate of [<sup>3</sup>H]Leu incorporation and the contents of total protein in GCIP control group were higher than that of normal control group ( $P < 0.01$ ), but not different from NE group ( $P > 0.05$ ). In GCIP-treated groups, the rate of [<sup>3</sup>H]Leu incorporation and the contents of total pro-

tein were of no obvious difference in 1  $\mu\text{g/L}$  or 10  $\mu\text{g/L}$  group. however, in the 100  $\mu\text{g/L}$ , 1  $\text{mg/L}$  or 10  $\text{mg/L}$  group the rate of [ $^3\text{H}$ ]Leu incorporation (Tab 1) and the contents of total protein (Tab 2) were reduced significantly ( $P<0.01$ ), compared with the GCIP control group.

**Tab 1. Effects of GCIP on the contents of total protein.  $n=6$ . Mean $\pm$ SD.  $^cP<0.01$  vs normal control group.  $^dP>0.05$  vs NE group.  $^gP>0.05$ ,  $^iP<0.01$  vs GCIP control group.**

Group	Absorbance ( <i>A</i> ) values	Concentration of protein/ $\text{mg}\cdot\text{L}^{-1}$
Normal control	0.146 $\pm$ 0.027	81 $\pm$ 15
NE group	0.21 $\pm$ 0.04 <sup>c</sup>	116 $\pm$ 22 <sup>c</sup>
GCIP control	0.194 $\pm$ 0.013 <sup>d</sup>	108 $\pm$ 7 <sup>cd</sup>
GCIP group:		
1 $\mu\text{g/L}$	0.188 $\pm$ 0.019 <sup>g</sup>	105 $\pm$ 11 <sup>g</sup>
10 $\mu\text{g/L}$	0.174 $\pm$ 0.021 <sup>g</sup>	96 $\pm$ 12 <sup>g</sup>
100 $\mu\text{g/L}$	0.141 $\pm$ 0.020 <sup>i</sup>	78 $\pm$ 11 <sup>i</sup>
1 $\text{mg/L}$	0.146 $\pm$ 0.016 <sup>i</sup>	81 $\pm$ 9 <sup>i</sup>
10 $\text{mg/L}$	0.145 $\pm$ 0.035 <sup>i</sup>	80 $\pm$ 20 <sup>i</sup>

**Tab 2. Effects of GCIP on the rate of [ $^3\text{H}$ ]Leu incorporation.  $n=6$ . Mean $\pm$ SD.  $^cP<0.01$  vs normal control group.  $^dP>0.05$  vs NE group.  $^gP>0.05$ ,  $^iP<0.01$  vs GCIP control group.**

Group	Radioactivity/Bq
Control	25 $\pm$ 8
NE group	43 $\pm$ 9 <sup>c</sup>
GCIP control	40 $\pm$ 6 <sup>d</sup>
GCIP group:	
1 $\mu\text{g/L}$	44 $\pm$ 6 <sup>g</sup>
10 $\mu\text{g/L}$	39 $\pm$ 11 <sup>g</sup>
100 $\mu\text{g/L}$	27 $\pm$ 6 <sup>i</sup>
1 $\text{mg/L}$	23 $\pm$ 8 <sup>i</sup>
10 $\text{mg/L}$	23 $\pm$ 6 <sup>i</sup>

## DISCUSSION

In recent years, the understanding of the myocardial hypertrophy has become an important focus of biomedical researches. Myocardial hypertrophy is associated with enhanced  $G_q$  signaling. Several mechanical and hormonal stimuli signaling pathways have been implicated in the activation of the hypertrophy<sup>[12,13]</sup>. These pathways may mediate common responses leading to the myocyte hypertrophy through the stimulation

of  $G_q$ -coupled receptors. GCIP can act as the competition candidate of the activated binding sites on  $G_q$  alpha. Since with the receptors, it can inhibit enhanced  $G_q$  signaling, thus GCIP has many advantages over traditional receptor antagonists derived from only a single type of receptor<sup>[3-7]</sup>.

Rapid Translation System500 (RTS500) was invented and manufactured by Roche Company recently. It is one of the simplest system to express target protein *in vitro* and has many advantages, for example, fast, high yield, cell-free and expression of toxic proteins and so on. In our study, we chose this instrument to express GCIP. The pIVEX2.3-MCS plasmid is included in RTS500 kit. The plasmid has a MCS under the control of T7 promoter and a 6 $\times$ His-tag.

In our study, we constructed the recombinant vector. The GCIP was expressed with RTS500 as an insoluble protein, then purified on a Ni-NTA agarose column without degeneration and refold. GCIP was harvested in high quantities (about 100  $\text{mg/L}$ ) as a soluble protein. It was shown that the GCIP could block NE-mediated cardiomyocyte hypertrophy by determination of the inhibitory rate of protein synthesis.

In conclusion, the methods of GCIP expression and purification that had been established in our protocol were rapid and effective. GCIP may be a very useful peptide to further study the molecular mechanisms of  $G_q$  protein signal transduction. In addition, GCIP, as a new agent, may point the way to the potential therapies of myocardial hypertrophy.

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