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Molecular Cloning of a Regulatory Protein for Membrane-Bound Guanylate Cyclase GC-A

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Activation of membrane-bound guanylate cyclase GC-A by atrial natriuretic factor (ANF) may require the involvement of accessory proteins. To identify these postulated proteins, we isolated a 1.0-kb cDNA clone from a rat brain expression library using a polyclonal antiserum against mastoparan. The 1.0-kb cDNA encodes a protein of 111 amino acids. Expression of this cDNA in COS-7 cells potentiated ANF-stimulated GC-A activity. Therefore, the 1.0-kb gene encodes a guanylate cyclase regulatory protein (*GCRP*). Fluorescence microscopy studies using the fusion protein of GCRP with green fluorescence protein (GFP) indicated that GCRP was present in the cytosol in PC12 cells, but translocated toward the plasma membrane in the presence of ANF. Coimmunoprecipitation experiments indicate that GCRP associates with GC-A in the presence of ANF. These results suggest that ANF induces the association of GCRP with GC-A and this association contributes to the activation of GC-A.

Key Words: **mastoparan; guanylate cyclase regulatory protein; GC-A; ANF; cGMP.**

Atrial natriuretic factor (ANF), a peptide hormone synthesized primarily by the heart and brain, exerts potent effects on the regulation of intravascular volume, electrolyte balance and blood pressure (for review, see 1–5). One type of ANF receptor has been shown to be membrane-bound guanylate cyclase GC-A (or NPR-A) (6–8). The binding of ANF to GC-A stimulates enzyme activity leading to the elevation of cGMP levels, which mediates most of the biological effects of ANF. GC-A contains an extracellular ANF-binding domain, and an intracellular kinase-like domain and catalytic domain. The catalytic activity of GC-A has been suggested to be suppressed by its kinase-like domain because deletion of the kinase-like domain leads to an elevation of basal enzyme activity (9). However, the mechanism by which ANF binding releases the inhibitory constraint imposed on the active site of GC-A remains unknown.

It has been shown that ATP potentiates the effect of ANF on GC-A activation (10–20). Nucleotide binding may mediate the effect of ATP on the activation of GC-A, because AMPPNP and AMPPCP, the hydrolysis-resistant analogs of ATP, also increase ANF-stimulated GC-A activity (10–12). The ATP binding site has been suggested to be on the kinase-like domain (19).

In addition to direct binding of ATP to GC-A, accessory proteins may also be required for GC-A activation by ANF since ANF and ATP have no effect on the activity of partially purified GC-A from rat lungs (17). Furthermore, removal of the soluble proteins by intense washing leads to a decrease in GC-A activation by ANF and ATP in rat lung membranes (11). Accumulated evidence also indicates that the Ser/Thr phosphorylation of GC-A is required for ANF-mediated enzyme activation (20–22).

We have previously found that mastoparan and melittin, peptide toxins from bee venom, potentiate ANF- and ATP-stimulated GC-A activity in rat lung membranes (14, 15). It is possible that a mammalian counterpart of these bee peptides may modulate ANF-mediated GC-A activation. To identify the mastoparan/melittin-like protein, we used the mastoparan antibody as a probe and identified a cDNA with a size of around 1.0 kb from a rat brain expression library. Expression of this cDNA in COS-7 and CHO cells potentiated ANF-stimulated GC-A activity, suggesting that the 1.0-kb cDNA encodes a guanylate cyclase regulatory protein (*GCRP*). Fluorescence microscopy and co-immunoprecipitation studies indicated that addi-

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tion of ANF induced the association of GCRP with GC-A. These results demonstrate that activation of GC-A by ANF requires the participation of GCRP.

MATERIALS AND METHODS

cDNA cloning. Anti-mastoparan antibody (2000–3000 dilution) was used to screen a λgt 11 library from rat brain (Clontech, Palo Alto, CA). A positive clone of about 1.0 kb was identified and isolated from about 150,000 phage plaques. The positive clone was subcloned into the pBluescript plasmid and sequenced with the dideoxy chain termination method (Sequenase 2.0; U.S. Biochemical, Cleveland, OH). T3 and T7 primers were used to sequence the 5′ and 3′ end of positive clones, and synthetic primers were used for subsequent sequencing from both strands.

Tissue distribution of GCRP by Western blot analysis. Liver, prostate, spleen, kidney, brain, lung, and heart tissues from Sprague Dawley rats (male, 200–250 g) were homogenized at 4°C with a Polytron homogenizer in 50 mM Tris buffer, pH 7.6, containing 1 mM EDTA, 0.1% phenylmethysulfonyl fluoride, phosphoramidon (2 μg/ml), leupetin (2 μg/ml), pepstatin (2 μg/ml) and 250 mM sucrose. Homogenates were then lysed by 1% Triton X-100 in 50 mM Tris–HCl buffer, pH 7.6, containing 5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, phosphoramidon (10 μg/ml) and leupetin (10 μg/ml). The lysates (20 μg) were subjected to 17% SDS–polyacrylamide gel electrophoresis and transferred to a PVDF transfer membrane. The PVDF membrane was blocked with 3% bovine serum albumin in 50 mM Tris, pH 8.5, 0.1% sodium azide and 150 mM NaCl. The PVDF membrane was then incubated with GCRP antibodies (recognizing the 15 C-terminal amino acids of GCRP) (1000 dilution) at 4°C for 3 h. The PVDF membrane was then washed three times with saline (50 mM Tris buffer, pH 8.5, containing 150 mM NaCl) plus 0.1% Tween 20. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (4000 dilution) for 1 h at room temperature. The immunoreactive proteins were visualized using enhanced chemiluminescence.

Expression of GCRP in COS-7 cells. The cloned cDNA was subcloned to a eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA) at the *Eco*R1 restriction site. Twelve micrograms of *GCRP* cDNA in pcDNA3 were transfected into 60–80% confluent COS-7 cells in 6-well plates (35 mm) by the lipofectAMINE (Gibco BRL, Gaithersburg, MD) method according to manufacture's instructions. Two days later, transfected cells were assayed for cGMP determination.

cGMP determination. COS-7 cells were grown to confluence in 6-well plates (35 mm). The cells were washed with 2 ml of serum-free DMEM containing 10 mM Hepes, pH 7.3, and then preincubated at 37°C for 10 min with 900 μl of DMEM containing 0.5 mM isobutylmethylxanthine. Various concentrations of ANF (100 μl) were added to the cells and incubated for 10 min, or indicated time, at 37°C. After incubation, the medium was aspirated and 1 ml cold 10% trichloroacetic acid was added to the plates. The cell extracts were scraped, then centrifuged for 15 min at 2000*g*, and the supernatant fractions were extracted with water-saturated ether to remove trichloroacetic acid. The cGMP levels in the supernatants were determined by radioimmunoassay (11–15, 23).

Fluorescence microscopy. *GCRP* was in-frame inserted to the 5′ end of a *GFP* (green fluorescence protein) plasmid (GFP-N2, Clontech, Palo Alto, CA) and transfected by the lipofectAMINE method into PC12 and CHO cells that were grown on glass coverslips. Two days after transfection, the cells on the coverslips were incubated either in the absence or presence of 0.1 μM ANF for 10 min, and then fixed with 4% paraformaldehyde in phosphate saline buffer (PBS, pH 7.4) for 2 h. The fixed cells were then examined by a Nikon fluorescence microscope (Nikon Inc., Garden City, NY) equipped with the FITC filter system to view the green fluorescence proteins.

Immunoprecipitation of GC-A with GCRP antibodies. The immunoprecipitation was carried out as previously described (24). In brief, PC12 cells were washed with serum-free RPMI medium and then challenged with or without 0.1 μM ANF for 10 min. The cells were lysed (50 mM Tris, pH 7.6. containing 150 mM NaCl, 20 mM $MgCl_2$, 1% Triton, 1 mM PMSF, 1 μg/ml leupetin and 1 μg/ml pepstatin) and GCRP antibodies (500 dilution) were added to cell lysates at 4°C for 60 min with gentle agitation. Immunocomplexes were collected with protein A Sepharose 4B at 4°C for 30 min, washed gently in cold lysis buffer, subjected to SDS–polyacrylamide gel electrophoresis and Western blot analysis using rabbit polyclonal GC-A antibodies (2000 dilution) (23).

RESULTS

Molecular Cloning of a Guanylate Cyclase Regulatory Protein

We previously found that mastoparan potentiates GC-A activity stimulated by ANF and ATP (14, 15). Since mastoparan is a peptide toxin from bee venom, we reason that a mastoparan-structurally related protein may be present in mammalian cells and function as a regulatory protein for ANF-mediated GC-A activation. To clone this mastoparan-like protein, we screened an expression library from the rat brain using the anti-mastoparan antibody as a probe. An approximate 1.0-kb cDNA clone was isolated from about 150,000 recombinants. The complete nucleotide sequence of the cloned cDNA and the deduced amino acid sequence are shown in Fig. 1. The cDNA clone contains the whole coding region for GCRP and portions of the 5′ and 3′ untranslated regions. A putative polyadenylation signal AATAAA (nucleotides 580–585) is identified in the 3′ untranslated region followed by a poly(A) tail. The sequence predicts a protein of 111 amino acids with a calculated mass of 11.6 kDa.

The nucleotide sequence of the cloned gene (*GCRP*) has a significant similarity to that of a Homo Sapiens cDNA clone 71479 5′. Clone 71479 5′ is a 293-base pair cDNA fragment with an unknown function. The overall similarity between *GCRP* and clone 71479 5′ is 71%, however, with much higher identity (87%) between nucleotides 1–105 of clone 71479 5′ and 485–585 of *GCRP*. Amino acid comparison shows that GCRP has no significant homology to mastoparan. Since mastoparan is known to form a α-helical structure upon binding to phospholipid membranes (25), it is possible that the mastoparan antibodies may recognize the secondary structure, rather than the primary structure of GCRP. GCRP does not have significant homology to the known proteins in the GenBank database, indicating that it is a novel protein. GCRP does not contain the consensus sequence for the known functional motifs or domains such as the nucleotide binding site, SH2 domain, SH3 domain, tyrosine phosphatase and calmodulin-binding domain. Hydrophobicity plot analysis of GCRP reveals no long stretches of hydrophobic residues that could serve as a signal sequence for pro-

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                                                C GGATTGTCTA CGCTCAGAAG GGATA    -301

CCTGGAGGAG CTGGTGCGTC TGCGGGAGTC GCAGCTGAAG GACTGGAGGC GGAGAACCGG CGGCTGCAGC TGCAG    -226

CTGGAGGAGG CGGCGGCACC AAATCAGCGT GAGAAGCGGG AGCTGGAAGG AGTGATCCTG GAGCTGCAGG AGCAG    -151

CTGACAGGTC TGATCCCCGG TGACCATGCC CCCCTGGCCC AGGGTTCCAA GGAGCACACC ACAGCCCTGG TCAAC     -76

CAGTGGCCCT CCCTGAGCAC ACTCAGTAGA CCGGAGGGTG CCAGCAACTC CAAGCTATTT CGGAGACACA GCTTC      -1

ATGAGTACGG AGCCCCTGTC TGCAGAGGCC AGCCTGAGCT CAGACTCCCA GCGCCTGGGG AAGGACCCCA CGCCC      75
M S T E P L S A E A S L S S D S Q R L G K D P T P
TCCATGCTGG GCCTCTGCGG CTCCCTGGCC TCCATCCCCA GCTGCAAGTC CCTGGCGAGC TTCAAATCCA ACGAA    150
S M L G L C G S L A S I P S C K S L A S F K S N E
TGCCTGGTGA GCGACAGCCT GAGGGCAGCC CAGCACTCAG CCCCAGCTGA GGAGCAGCAA GGGCAATGCC AGCCC    225
C L V S D T L R A A Q H S A P A E E Q Q G Q C Q P
CACCTGCCAG GGGCCATGGA CAACTGCCAC CTTTTGAGTC CCCCACCCCA GGCCACCCTT CAAGAACAAT ACCCA    300
H L P G A M D N C H L L S P P P Q A T L Q E Q Y P
GCTAGCCTCA GGTCCCAGCT AGCCTCGGGT CTCTAGGGAA AGACAACCTG AGCCTCTTTC CTGCTTCAGC TTCCT    375
A S L R S Q L A S G L
GCCAAAGAGG CAGGAGCTAC ATGGGGAAGT GGTGGGGCCA GGAGGGACGC CCAGGTTACA GGTCGTCAGT CCCCT    450

GGGGAAGCCA CTCCATTCCG TGGTCCTGAA GGCTGCCTGG TTCCTTCTGT TCATCTTCCA CACTTGCCTC AGAAG    525

CAGGTGGTCC AGCCCTGGCA TTCCTGCTGC CCTGCCTCTG GTCTAACCCT GTGTACCCTC TGAAGTCACC CTTCC    600

TCGGTACCTA TGTGGGGAGA GATTAGGCAA ATAAAAACCA GAGGACTGGA AAAA                          654
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FIG. 1. DNA and predicted amino acid sequence from the 1.0-kb cDNA clone. The amino acid residues are numbered beginning with the initiation codon. Nucleotide 659 is followed by a poly-A^+ tail. The GenBank Accession Number is AF288611.

tein secretion or a membrane-spanning domain. In addition, GCRP does not contain potential *N*-linked glycosylation sites. These structural features indicate that GCRP is a cytosolic protein.

Tissue Expression of GCRP

The tissue expression of *GCRP* was examined by Western blot analysis of proteins from rat liver, prostate, spleen, kidney, brain, lung and heart using GCRP antibodies that were raised against the 15 N-terminal amino acids in rabbit. GCRP antibodies recognized a protein with apparent molecular mass around 16 kDa in all tested tissues except prostate (Fig. 2). The expression level of GCRP was high in rat brain, liver and lung, moderate in kidney and heart, and low in spleen. It should be noted that the apparent molecular mass of GCRP (16 kDa) is larger than its calculated molecular mass (11.5 kDa). GCRP contains many more serine residues (Fig. 1) than average proteins (17.1 vs 7.7%, ref. 26), and contains several possible phosphorylation sites for serine/threonine/tyrosine protein kinases. Therefore, GCRP may be subjected to a post-translational regulation by protein phosphorylation.

FIG. 2. Tissue distribution of PDRP. The distribution of PDRP was examined in rat liver, prostate, spleen, kidney, brain, lung, and heart using GCRP antibodies. GCRP was detected in all tested tissues except prostate. Levels of GCRP were high in brain, liver, and lung, but low in spleen.

Functional Expression of GCRP

To determine the function of GCRP, we transiently expressed the *GCRP* cDNA in COS-7 cells. The results (Fig. 3) showed that expression of GCRP significantly potentiated the effect of ANF on cGMP formation in a dose-dependent manner in COS-7 cells. GCRP mainly affected the V_{max} of ANF-mediated cGMP formation, with little effect on the EC_{50} of ANF. Similar results were also observed in a CHO cell line stably expressing GCRP (data not shown). Thus, GCRP functions as a regulatory protein for ANF-mediated GC-A activation.

Translocation of GCRP Induced by ANF

Green Fluorescence Protein (GFP) from the jellyfish *Aequorea victoria* emits green light when exposed to UV or blue light. GFP absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green

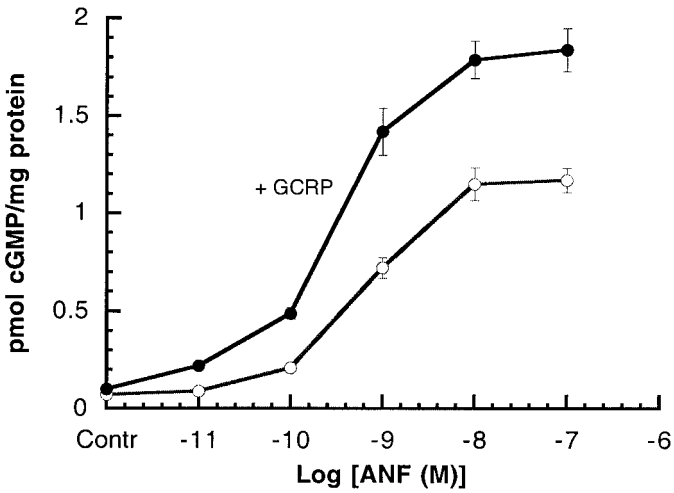

FIG. 3. Formation of cGMP by ANF in COS-7 cells and in transfected COS-7 cells. Confluent cells were exposed to increasing concentrations of ANF in the presence of 0.5 mM isobutylmethylxanthine at 37°C for 10 min. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates.

light (peak emission at 509 nm with a shoulder at 540 nm). It thus can be used as a reporter molecule for monitoring protein localization.

We in-frame inserted *GCRP* to the 5′ end of *GFP* (*GCRP-GFP*). To analyze the fluorescence of the fusion protein of GCRP with GFP, we cultured PC12 cells on glass coverslips, and then transfected the *GCRP-GFP* plasmids into PC12 cells. Two days after transfection, the cells on the cover-slips were incubated either in the absence or presence of 0.1 μM ANF for 10 min. Figure 4A shows that in the absence of ANF, GCRP-GFP was expressed evenly in the entire cytosolic region. Interestingly, in the presence of ANF, cytosolic GCRP translocated toward the plasma membrane (Fig. 4B).

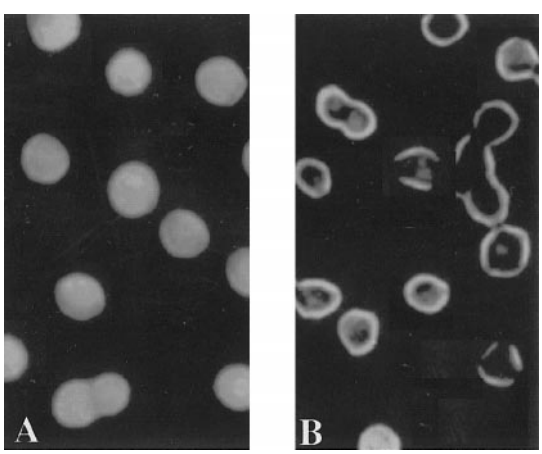

FIG. 4. Translocation of GCRP induced by ANF in PC12 cells. GCRP was subcloned into the GFP-N2 plasmid, and then transfected into PC12 cells grown on glass coverslips. In the absence of ANF, the fluorescence of GCRP was detected in the cytosol (A). However, in the presence of 0.1 μM ANF, most of the GCRP fluorescence was located around the plasma membrane (B), indicating that GCRP was translocated toward the plasma membrane by ANF.

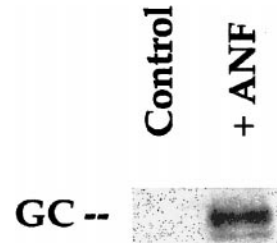

FIG. 5. Immunoprecipitation of GC-A with GCRP antibodies in PC12 cells. PC12 cells were stimulated with or without 0.1 μM ANF for 10 min. The cell lysates were immunoprecipitated with GCRP antibodies. The immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotted with GC-A antibodies. GCRP immunoprecipitated GC-A only in the presence of ANF.

Immunoprecipitation of GC-A with GCRP Antibodies in the Presence of ANF in PC12 Cells

To determine whether GC-A is the target of GCRP in the plasma membrane, we grew PC12 cells in culture flasks until confluence and challenged the cells with or without 0.1 μM ANF for 10 min. The cells were then lysed and immunoprecipitated with GCRP antibodies. The immunocomplexes were analyzed by Western blotting using GC-A antibodies (23). Figure 5 shows that in the presence of 0.1 μM ANF, GC-A was immunoprecipitated by GCRP antibodies. In contrast, in the absence of ANF, GCRP did not form immunocomplexes with GC-A.

DISCUSSION

Accessory proteins have been implicated or suggested to be involved in the activation of GC-A by ANF (3, 11, 14, 15, 17, 19). We have previously found that peptide toxins such as mastoparan and melittin potentiate ANF-stimulated GC-A activity (14, 15). To identify the mastoparan-like proteins, we raised the antibodies against mastoparan. Using this mastoparan antiserum as a probe to screen a rat brain expression library, we isolated a 1.0-kb cDNA. Expression of this gene in COS-7 cells enhances ANF-mediated GC-A activation. Since the expression of GC-A in COS-7 cells is not altered (our unpublished results), these results indicate that GCRP regulates the activity, but not expression, of GC-A. Therefore, GCRP encodes a guanylate cyclase regulatory protein rather than a transcription factor.

It should be noted that expression of GCRP potentiates ANF-stimulated GC-A activity only about 45–100% in both COS-7 and CHO cells. This effect is

slightly less than that of mastoparan (100%) on ANF-stimulated GC-A activity in rat lung membranes (15). The moderate effect of GCRP is likely due to that endogenous GCRP is present in COS-7 and CHO cells, or that other regulatory proteins are also required for the maximal activation of guanylate cyclase by ANF. Indeed, protein serine/threonine kinase and phosphatase is shown to sensitize GC-A for ANF activation (21–22).

With fluorescence microscopy and the fusion protein of GCRP-GFP, we found that GCRP is expressed in the cytosol in PC12 cells, but translocates toward the plasma membrane by the addition of ANF. This result is also confirmed by the immunofluorescence studies using GCRP antibodies and a confocal microscope (our unpublished results). Coimmunoprecipitation experiments using GCRP antibodies followed by Western blotting using GC-A antibodies (23) indicate that GCRP associates with GC-A in the presence of ANF. Thus, the association of GCRP with GC-A may be required for ANF to activate the enzyme.

Membrane-bound guanylate cyclase activity has been shown to be subjected to an inhibitory constraint (27). Subsequent studies further suggested that the catalytic activity of GC-A is suppressed by its kinase-like domain (9). Therefore, it is likely that the binding of ANF and ATP induces a conformational change on GC-A and generates a binding site for GCRP. The association of GCRP with GC-A triggers a further conformational change on GC-A leading to the release of the inhibitory constraint imposed on its active site. However, it should be noted that GCRP is probably not the sole regulatory protein for GC-A activation. Recent studies have indicated that the serine/threonine phosphorylation of GC-A is also required for ANF-stimulated enzyme activation (21, 22). It may be possible that protein phosphorylation is involved in some step of GC-A activation such as in facilitating the association of GCRP with GC-A. We are in the process of examining this possibility.

In summary, we have identified a regulatory protein, GCRP, involved in the activation of GC-A by ANF. GCRP is mostly present in the cytosol, but associates with GC-A triggered by ANF. Further study on GCRP will help to uncover the mechanism by which ANF and GCRP regulates the activation of GC-A.

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