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EXPRESSION OF EXTRACELLULAR LIGAND-BINDING DOMAIN OF MURINE GUANYLATE CYCLASE/ATRIAL NATRIURETIC FACTOR RECEPTOR cDNA IN *ESCHERICHIA COLI*

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Abstract

The membrane-bound form of guanylate cyclase/atrial natriuretic factor receptor (GC/ANF-R) is a 135 kDa transmembrane glycoprotein which binds ANF with high affinity. We have expressed the extracellular ligand-binding domain of murine guanylate cyclase ANF-R (GC/ANFR-LBD) cDNA in *Escherichia coli*. The cDNA encoding the extracellular ANF-binding domain (nucleotide positions covering from 432-1755 base pair) of GC/ANF-R was amplified by polymerase chain reaction, cloned into BamHI site of pGEX-3X prokaryotic expression vector and was transfected into *E. coli*, strain JM101. After isopropyl- β -D-thiogalactopyranoside (IPTG) induction of bacterial cells, the GC/ANFR-LBD was expressed as the glutathione-S-transferase (GST) fusion protein, yielding a molecular mass of 70 kDa. The expressed fusion protein was characterized for binding affinity to both full length and truncated ANF molecules. After expression in *E. coli*, the binding of ¹²⁵I-ANF to the extracellular region of GC/ANF-R was similar and corresponded to the pharmacological class of native receptor protein. The 70 kDa fusion product was purified as a predominant single protein band by glutathione-affinity chromatography. These findings establish that *E. coli* may be utilized as an effective heterologous model system to delineate the structure-function analysis of guanylate cyclase-coupled ANF receptor molecules.

Atrial natriuretic factor (ANF) is synthesized in the dense-core granules of mammalian atrial cardiocytes, secreted into plasma and elicits multiple physiological responses, largely directed towards reduction of blood pressure and blood volume (1). In addition to its natriuretic and diuretic action in kidney and vasorelaxant activity in the vascular smooth muscle cells, ANF has been found to inhibit the release of aldosterone in adrenal gland, renin in kidney and vasopressin in anterior pituitary and to stimulate the release of androgen in Leydig cells, progesterone in granulosa cells and growth hormone in posterior pituitary (2–5). The biological actions of ANF are mediated by its interaction with specific cell-surface receptors and the membrane-bound form of guanylate cyclase represents a biologically active ANF receptor (4,6). The cDNA of the guanylate cyclase-coupled ANF receptor has been cloned from human placenta (7,8), rat brain (9,10) and murine Leydig tumor cell line (11) and corresponding amino acid sequence determined.

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The binding of ANF to the extracellular amino-terminal region of GC/ANF-R elicits the generation of cGMP by its cytoplasmic catalytic domain leading to an unique mechanism of intracellular signalling by this receptor protein (6). The deduced amino acid sequence from cDNA showed that entire structure of GC/ANF-R can be divided into three major structural motifs. A 21-residue transmembrane domain which separates the extracellular ligand binding region (469 amino acid residues) from the intracellular guanylate cyclase catalytic domain (567 amino acid residues). The sequence of 300 amino acids in the catalytic region adjoining the transmembrane domain exhibits structural similarity to a protein kinase-like domain. The deletion of this protein kinase-like domain resulted in the significant elevation of the guanylate cyclase activity (12). Recently, the intracellular carboxyl-terminal portion of membrane-bound guanylate cyclase was transfected into *E. coli* and the cyclase activity was induced which provided the evidence that the carboxyl-region of GC/ANF-R contains enzymatic catalytic domain (13). In this report, we demonstrate that extracellular ligand-binding domain of GC/ANF-R is expressed in the heterologous prokaryotic system and the ANF-binding characteristics closely correlate to the pharmacological class of native guanylate cyclase-coupled ANF receptor protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli, strain JM101 was used for transfection. The pGEX-3X plasmid, a prokaryotic expression vector is designed to direct the synthesis of foreign proteins as a fusion product with the C-terminus of 26 kDa glutathione-S-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum* (14). The plasmid contains *tac* promoter, the β -lactamase-coding gene AP^R and a fragment of lac operon containing the overexpressed lac I^q allele of the lac repressor and part of lac Z. The induction of the *tac* promoter with isopropyl- β -D-thiogalactopyranoside (IPTG) in bacterial cells transfected with pGEX-3X containing the desired cDNA insert results in the translation of a fusion protein.

Media and Cell Culture

Bacterial cells were cultured in Luria broth (L-broth) containing tryptone (1%), NaCl (1%) and yeast extract (1%), pH 7.0. The L-agar medium was solidified with 1.5% bacto-agar and plated in 100-mm² Petri dishes. Both liquid and solid media contained 100 μ g/ml ampicillin. For routine cultures, 10 ml of liquid media was inoculated with a single colony and incubated at 37°C with vigorous shaking. After the cultures reached the density of 0.4 - 0.6 OD₆₅₀, the IPTG was added to a final concentration of 1 mM. The cells were allowed to grow for an additional 3-4 h at room temperature. For large scale cultures, the liquid media was inoculated with a bacterial cell suspension growing in the exponential phase.

Polymerase Chain Reaction fPCR

The extracellular ligand-binding domain of full length murine GC/ANF-R cDNA was amplified by PCR using the 5' sense oligonucleotides (5' - CTC.CAG.TGT.GGA.AAA.GTG.GTC.T-3') and the 3' antisense oligonucleotides (5' - AGC.GAC.CTG.ACC.GTG.GCT.GTG.G-3') as primers. The 5' sense primer covered the nucleotide positions from 431-453 base pairs and the 3' antisense primer covered the

nucleotides from 1733-1755 base pairs of murine GC/ANF-R cDNA. To avoid any possibility of secretion of fusion protein into the culture media, the signal peptide was excluded from the coding sequence. The PCR was carried out in 100 μ l volume containing; Tris-HCl, pH 8.3 (10 mM), KCl (50 mM), MgCl₂ (2.5 mM), cDNA template (70 ng), dNTPs (each 50 mM), primers (each 100 pmol), Taq DNA polymerase (2 units) and one drop of mineral oil. The amplification was performed with 30 cycles in a Perkin-Elmer/Cetus DNA Thermal Cycler, using a cycle of denaturation for 1.5 min at 94°C, annealing for 1.5 min at 55°C and an extension at 72°C for 3.0 min. The PCR- amplified DNA fragment was treated with chloroform and ethanol- precipitated.

Ligation of PCR-Amplified DNA Fragment to pGEX-3X Plasmid Vector

The amplified PCR product was kinased in a 40 μ l reaction mixture containing DNA (2 μ g), Tris-HCl, pH 7.6, (66 mM), MgCl₂ (10 mM), dithiothreitol (10 mM), bovine serum albumin (BSA, 0.2 mg/ml), ATP (20 mM) and T4 polynucleotide kinase (2.0 units) at 37°C for 1 h. At the end of reaction, the tubes were heated to 75°C for 10 min and cooled at 4°C. After the kinase reaction, DNA was subjected to a filling-in reaction which contained 5 mM dNTPs and one unit of the Klenow fragment of DNA polymerase I for 30 min at room temperature. After the reaction was completed, the mixture was loaded onto a low melting point agarose gel to remove the primers and dNTPs by electrophoresis. The kinased and blunt-ended DNA fragment was purified using Gene-Clean Kit (Bio 101, Inc., La Jolla, CA) and BamHI linkers (5'-GGGATCCC-3') were added. The restriction endonuclease digestion of the linker-extended DNA fragment to generate the flanking BamHI sites and the ligation of transfected pGEX-3X plasmid vector were performed according to the published protocols (15).

ANF-Binding Assays

Bacterial cells were harvested by centrifugation, washed and the pellet suspended in solution containing Tris-HCl, pH 7.2 (10 mM), NaCl (0.15 M), CaCl₂ (1 mM), phenylmethylsulfonylfluoride (PMSF, 1 mM), N-ethylmaleimide (2 mM), EDTA (2 mM), pepstatin (4 μ g/ml), bacitracin (0.5 mg/ml) and 20 μ g/ml each of aprotinin, leupeptin and phosphoramidon. The cells were disrupted by sonication and the lysate was passed through an 18-gauge needle. To this cell lysate, Triton X-100 was added to a final concentration of 1%. The mixture was stirred at 4°C for 30 min. The homogenate was centrifuged at 1000 rpm for 5 min to remove the unbroken materials. A clear supernatant was obtained by centrifugation at 100,000 $\times$ g for 1 h and was used for ¹²⁵I-ANF binding assays. The bound ¹²⁵I-ANF-receptor complex was precipitated by adding 0.25% bovine γ -globulin and 2.5 ml 10% polyethyleneglycol 8000 in 20 mM HEPES, pH 7.4 and 0.15 M NaCl as previously described (16). The mixture was filtered under vacuum through Whatman GF/B filters, treated with 0.3% (w/v) polyethyleneimine.

Purification of Fusion Protein

The IPTG-induced bacterial cells were harvested by centrifugation at 5000 $\times$ g for 20 min. The pellet was frozen at -70°C to facilitate cell lysis, resuspended in phosphate buffered saline (PBS), containing Triton X-100 (1%), glycerol (5%), aprotinin (10 μ g/ml), pepstatin (5 μ g/ml), benzamidine (20 μ M), leupeptin (2.5 μ g/ml), PMSF (1 mM) and EDTA (1 mM).

The suspension was sonicated on ice, centrifuged at 8,000 rpm for 5 min and the supernatant collected. The pellet was resuspended in homogenizing buffer and supernatant collected. Both supernatants were combined and applied onto prepacked Glutathione-Sepharose 4B column equilibrated with PBS containing Triton X-100 according to the manufacturer's protocol (Pharmacia, Piscataway, NJ). The column was washed with 15 bed volume of PBS and then eluted with 10 ml of 5 mM glutathione in 50 mM Tris-HCl, pH 8.0 and 1 ml fractions were collected. The aliquots of the fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% gel according to Laemmli (17). The fractions containing expressed fusion protein were combined and concentrated with Centricon. Protein concentrations were determined by the methods of Lowry *et al.* (18) using bovine serum albumin as standard. The purified fusion protein from the glutathione affinity column was further characterized by capillary electrophoresis using capillary column (50×75 μ ID, Beckman Instruments). The samples were applied and separated in 50 mM borate buffer, pH 8.5, containing 50 mM SDS at absorbance 280 nm.

RESULTS AND DISCUSSION

The synthetic PCR primers were designed to amplify the extracellular ANF-binding domain from the full length murine GC/ANF-R cDNA (11). A PCR product of 1.32 kilobase (kb) fragment covering the nucleotide positions from 432 to 1755 base pairs of the extracellular domain of GC/ANF-R cDNA was amplified. This product did not contain the hydrophobic signal sequence, the transmembrane domain nor the cytoplasmic guanylate cyclase catalytic domain. The cDNA fragment was subcloned into the BamHI site of pGEX-3X prokaryotic expression vector and was designated as pGEX-3X/GC-ANFR-LBD expression plasmid (Fig. 1). The resulting plasmid allowed the IPTG-inducible expression of a protein containing the glutathione-S-transferase (GST) gene product (26 kDa) fused to the amino-terminus of the extracellular ligand-binding domain (44 kDa) of GC/ANF-R cDNA, yielding a molecular mass of 70 kDa fusion protein. The transfected *E. coli* strain JM101, was able to induce the 70 kDa fusion protein in a time-dependent manner after IPTG induction (Fig. 2A, lanes a-d). This expressed fusion protein (70 kDa) was purified by glutathione-affinity chromatography and separated as a predominant single protein band after SDS-PAGE (Fig. 2, lane e). To further check the purity of the purified preparations, the samples were subjected to a capillary electrophoresis and a major predominant peak was detected at absorbance 280 nm (Fig. 3).

The binding of 125 I-ANF was determined in the solubilized preparations of IPTG-induced *E. coli* cells which were transfected with pGEX-3X/GC-ANFR-LBD. The specific binding of 125 I-ANF to IPTG-induced bacterial cell extracts was approximately 7-fold greater than the controls (not induced by IPTG) (Fig. 4). The binding of 125 I-ANF to IPTG-induced cell lysates was displaced with excess molar concentrations of unlabeled ANF in a specific manner (Fig. 5). The analysis of binding data indicated a K_d of 4×10^{-10} M and binding capacity (B_{max}) 2.5 pmol/mg protein. The 125 I-ANF binding to *E. coli* cell lysates, closely correlates with the previously reported binding characteristics of the native GC/ANF-R from mammalian intact cells and plasma membrane preparations (19–23).

The ligand-binding domain of GC/ANF-R was modified by removing the DNA segment encoding the N-terminal hydrophobic leader sequence of 28-amino acid residues. By this procedure the removal of signal peptide by *E. coli* signal peptidase was circumvented. A second reason for deletion of the signal sequence was to avoid the secretion of the expressed fusion protein into culture media. Thus by the removal of signal sequence of GC/ANFR-LBD, the expressed fusion protein was retained within the bacterial cell. These present results establish that the *E. coli* system can be utilized as an ideal expression system for the native mammalian guanylate cyclase-coupled ANF receptor protein. The expression method reported here will allow the production of a large amount of the extracellular ligand-binding domain of GC/ANF-R and a biologically active recombinant pGC/ANF-R-LBD will provide an ideal system to delineate the structure-function analysis and the determination of ligand-binding sites of this receptor protein.

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Abbreviations used

GC/ANFR-LBD	ligand binding domain of guanylate cyclase-coupled atrial natriuretic factor receptor
GST	glutathione-S-transferase
IPTG	isopropyl- β -D- thiogalactopyranoside
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1. Schematic representation of the strategy for construction of expression vector pGEX-3X/GC-ANFR-LBD

The synthetic PCR primers (filled arrows on cDNA) were designed to amplify the extracellular ANF-binding domain from the full length murine GC/ANF-R cDNA. The hydrophobic signal sequence (dotted region), the transmembrane domain (vertical hatched region) and the entire cytoplasmic catalytic portion (horizontal hatched region) of the protein were excluded from the region amplified. The BamHI linker restriction sites included in the PCR primers were used to clone the amplified product into the corresponding sites in prokaryotic expression vector pGEX-3X. The resulting expression construct (pGEX-3X/GC-ANFR-LBD) allows the IPTG-inducible expression of the fusion protein with an amino-terminal portion consisting of glutathione-S-transferase gene and the extracellular domain of GC/ANF-R cDNA which is represented by the filled box in the pGEX-3X/GC-ANFR-LBD expression vector.

Figure 2. The expression analysis of the 70 kDa fusion protein consisting of GST and extracellular ANF-binding domain of GC/ANF-R

The bacterial cell lysates were subjected to SDS-PAGE using 7.5% gel. Lane a, cell lysate of transformed *E. coli* not treated with IPTG; lanes b, c and d, cell lysates of transformed *E. coli* which were induced with IPTG for 30, 60 and 90 min, respectively, as described in the Materials and Methods. Arrow indicates the position and molecular mass of the 70 kDa fusion protein, induced in a time-dependent manner. Lane e shows the purified 70 kDa fusion protein as a single band after glutathione-affinity chromatography. Bars on the left margin indicate the position and molecular mass of the marker proteins.

Figure 3. Capillary electrophoresis of the purified 70 kDa fusion protein

The samples containing the fusion protein eluted from the glutathione-affinity column were applied on to the capillary column ($50 \times 75, \mu\text{ID}$). The electrophoresis was carried out in 50 mM borate buffer, pH 8.5, containing 50 mM SDS at 20 kilo volts. A predominant single protein peak was detected after 8 min at absorbance 280 nm.

Figure 4. Binding of ^{125}I -ANF to solubilized extracts of bacterial cells transfected with pgex-3X/GC-ANFR-LBD

The bacterial cells without and with IPTG-treatment were harvested and the cell lysates were prepared as described in the Materials and Methods section. The 50 μl solubilized cell extracts were incubated with 1 nM ^{125}I -ANF in 450 μl binding buffer with and without unlabeled ANF at room temperature for 1 h. The bound ^{125}I -ANF-receptor complex was precipitated by adding 0.25% bovine γ -globulin and 2.5 ml of 10% polyethyleneglycol 8000 as previously described in the Materials and Methods section. The mixture was filtered under vacuum through Whatman GF/B filters, treated with 0.3% (w/v) polyethyleneimine. After washing the free ligand, the radioactivity retained on the filter was counted in Beckman Gamma-5500. The data represent the mean of three separate determinations.

Figure 5. Binding competition of ^{125}I -ANF to the solubilized lysates of bacterial cells transfected with pGEX-3X/GC-ANFR-LBD

The 50 μl lysates from IPTG-induced bacterial cell were incubated with 1 nM ^{125}I -ANF in 450 μl binding buffer for 1 h with various concentrations of unlabeled hormones. The binding assay was carried out as described in the Material and Methods section. •, ANF₍₉₉₋₁₂₆₎; ▲, ANF₍₁₀₂₋₁₂₆₎; ○, ANF₍₁₀₃₋₁₂₃₎ and □, angiotensin II and vasopressin.