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Genetically tailored atrial natriuretic factor-dependent guanylate cyclase

Immunological and functional identity with 180 kDa membrane guanylate cyclase and ATP signaling site

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Biochemical and immunological studies have established that one of the signal transducers of atrial natriuretic factor (ANF) is a 180 kDa membrane guanylate cyclase (180 kDa mGC), which is also an ANF receptor; obligatory in the transduction process is an intervening ATP-regulated step, but its mechanism is not known. GCα is a newly discovered member of the guanylate cyclase family whose activity is independent of the known natriuretic peptides, and the enzyme is not an ANF receptor. The genetically tailored GCα, GCα-DmutGln^{338}Leu^{364}, however, is not only a guanylate cyclase but also an ANF receptor and is structurally and functionally identical to the cloned wild-type ANF receptor guanylate cyclase, GC-A. We now report that the ANF-dependent guanylate cyclase activity in the particulate fractions of cells transfected with GCα-DmutGln^{338}Leu^{364} was inhibited by the 180 kDa mGC polyclonal antibody, and with this antibody probe it was possible to purify the 130 kDa expressed receptor; the hormone-dependent cyclase activity of this receptor was exclusively dependent upon ATP; and through site-directed mutational studies with GCα mutants, the signaling sequence that defines ATP binding site was identified. We thus conclude that 180 kDa mGC and the mutant protein are immunologically similar; both proteins are linked to the ANF signal in the generation of cyclic GMP synthesis; and in both the ligand binding and catalytic activities are bridged through a defined ATP binding module.

Atrial natriuretic factor; Guanylate cyclase; Cyclic GMP; ATP-binding protein; Atrial natriuretic factor receptor guanylate cyclase

1. INTRODUCTION

Atrial natriuretic factor (ANF) is one of the family of structurally related natriuretic peptides that regulate hemodynamics of the physiological processes of diuresis, water balance, and blood pressure [1–3]. Other known members of this family are brain natriuretic peptide (BNP; [4]) and cardiac natriuretic peptide (CNP; [5–7]) which, like ANF, stimulate membrane guanylate cyclase activity [8]. It is, therefore, possible that the second messenger of certain biological responses of ANF and other natriuretic peptides is cyclic GMP. Consistent with this notion, original biochemical studies identified an unusual bifunctional characteristic of guanylate cyclase: it is both an ANF receptor and a cyclase [9–12]. Two such monomeric proteins, one 120–140 kDa [9,11,12] and the other 180 kDa (termed 180 kDa mGC) [10], were described. From these findings a new concept of transmembrane signaling evolved, which was drastically different from the one (at the time) well-established for the adenylate cyclase system: in hormone-dependent adenylate cyclase, there is an assemblage of individual components – receptor, GTP-binding protein and catalytic moiety – for signal transduction. In contrast, the presence of dual activities – receptor binding and enzymic – on a single peptide chain indicated that this transmembrane protein contained both the information for signal recognition and its transduction into a second messenger [10]. This mechanism of transmembrane signaling involving mediation by second messenger, cyclic GMP, is now validated and extended by molecular cloning studies; the existence of two natriuretic peptide receptors – GC-A and GC-B (both of which are also guanylate cyclases) – has been documented [13–15]; GC-A appears to be a receptor for ANF [13], GC-B for CNP [16], and the receptor for BNP is unknown. Thus the existence of a natriuretic peptide receptor guanylate cyclase family has been established. The family members show a striking similarity in their structures, and the predicted topographical model indicates that all family members contain a single membrane-spanning helical domain which divides the protein in roughly two equal portions, N-terminal extracellular and the C-terminal intracellular. The receptor domain lies in the extracellular portion and the intracellular portion contains two domains:

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the one adjacent to the transmembrane is termed a kinase-like domain due to its sequence similarity to the tyrosine kinase family, and the C-terminal region contains the catalytic domain [13]. Receptor site Leu^{364} in GC-A is critical for ANF binding [18]. Recent direct biochemical-immunological studies with rat adrenal gland and testes complement the molecular cloning studies in establishing the multimember nature of ANF receptor guanylate cyclase family: both rat adrenal gland and testes in addition to the well-characterized 180 kDa mGC contain an additional 130 kDa ANF receptor guanylate cyclase which is immunologically identical to the 180 kDa mGC, but the two cyclases are separable by GTP-affinity chromatography [17]; most probably this 130 kDa ANF receptor guanylate cyclase is the counterpart of the originally purified bovine adrenal ANF receptor cyclase [11,12].

Heterogeneity of natriuretic peptides and their receptors indicates that the natriuretic peptides signaling network is complex; each member of the guanylate cyclase family may uniquely sense and transduce each natriuretic peptide biological signal. To determine the precise mechanism of the whole signaling network, it is important to resolve the structural and functional relationship between the individual guanylate cyclase receptors identified on a biochemical and molecular cloning basis. The present study addresses this issue in determining the biochemical and functional relationship between the two atrial natriuretic factor receptor guanylate cyclases – 180 kDa mGC characterized on a biochemical basis, and the genetically tailored ANF receptor guanylate cyclase (GCα-$DmutGln^{338}Leu^{364}$) [18], which is structurally and functionally identical to the cloned GC-A [13]. The results indicate that the two guanylate cyclases are immunologically similar and transduce the ANF signal in an identical fashion; ATP plays an obligatory role in signaling [19,20]. Immunological similarity between the two cyclases made it possible to use the 180 kDa mGC antibody probe to completely purify the genetically tailored enzyme from the transfected cells and show its linkage to the ANF-dependent cyclase activity.

2. MATERIALS AND METHODS

ANF (rat 8-33) used in these studies was a 26-amino acid peptide H-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH, purchased from Peninsula Laboratories; GTP, 3-[(3-cholaimidopropyl)dimethyl ammonio]-1-propane sulfonate (CHAPS), cyclic GMP, ATP and bovine serum albumin were from Sigma; ATPγS and AMP-PNP were from Boehringer-Mannheim, [α-^{32}P]ATP, and [^{125}I]NaI from Amersham.

GCα-$DmutGln^{338}Leu^{364}$ was constructed as in [18]. To build GCα-$DmutVal^{505}Asn^{506}$, conversions of Gly^{505} to Val, and Ser^{506} to Asn, were performed on 1.9 kb *Sal*I-*Xba*I fragment of GCαcDNA subcloned into pSelect-1 mutagenic vector using primers: selection-ampicillin-repair (Promega Biotec Mutagenesis Kit) and mutagenic 5′-GGAGCCATAATTGTTAACTCGCCCACTCAG-3′. *Eco*RV–*Xba*I fragment of GCαDmut cDNA in pBluescript vector was replaced with *Eco*RV–*Xba*I fragment excised from pSelect-cDNA recombinant. Mutation and ligation were verified by double-stranded sequencing (Sequenase 2.0, U.S.B.). GCαDmut$Gln^{338}Leu^{364}K^-Cyc^-$ was represented by the 1.65 kb *Sal*I–*Hpa*I fragment exised from GCα-$DmutVal^{505}Asn^{506}$ (Fig. 1) For expression studies, mutants were subcloned into *Xho*I-*Sma*I sites of pSVL expression vector. COS-2A and COS-7 cells (simian virus 40-transformed African green monkey kidney cells) were transfected with the expression vector by calcium phosphate technique [28]; for expression of GCα-$DmutGln^{338}Leu^{364}K^-$-$Cyc^-$, codon TGA from pSVL vector sequence located 27 nucleotides from the 3′ end of the mutant cDNA served as a STOP codon. Membranes were prepared as in [18], and guanylate cyclase activity was determined as in [14].

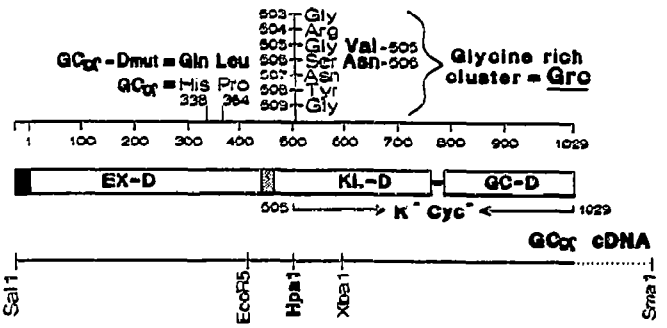

Fig. 1. Graphical representation of GCα mutants. The theoretical topographical domains of GCα are abbreviated as in [18]: EX-D, extracellular domain; KL-D, kinase-like domain; GC-D, guanylate cyclase domain. The closed box represents the leader sequence; the shaded box, the transmembrane domain; mutated amino acid residues and restriction site created by mutagenesis are bold-lettered. Construction of GCα mutants is described in Materials and Methods.

GCα-Dmut was purified by immunoaffinity chromatography in which the affinity ligand was rabbit anti-180 kDa mGC polyclonal antibody raised against rat adrenocortical carcinoma 180 kDa mGC. The purification protocol was identical to the one previously described for the purification of 180 kDa mGC [21], except that the GTP-affinity purification step was omitted; consequently the solubilized membranes were directly subjected to the immunoaffinity purification step. Eluted fractions were immediately neutralized with 0.1 M Tris, pH 8.5, and subjected to SDS-PAGE.

3. RESULTS AND DISCUSSION

In this study, the combined tools of biochemistry, immunology and recombinant DNA have been utilized to determine the structural and functional relationships of two ANF receptor guanylate cyclases, the biochemically characterized 180 kDa mGC [10] and the cloned GC-A [13], and to identify the ATP-binding structural motif of guanylate cyclase that participates in ANF signaling. A plasma membrane guanylate cyclase, GCα, which is not an ANF receptor guanylate cyclase, was genetically tailored to construct GCα-$DmutGln^{338}Leu^{364}$ involving two amino acid residue substitutions: His^{338} to Gln and Pro^{364} to Leu [18]. This GCα-Dmut is structurally and functionally identical to the cloned wild-type GC-A [13], and was used as a prototype of GC-A in the present studies. To determine the immunological similarity between GCα-

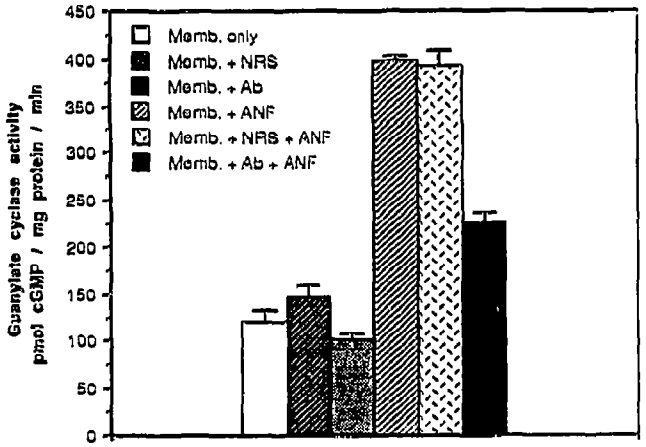

Fig. 2. Stimulation of particulate guanylate cyclase activity by ANF and its inhibition by 180 kDa membrane guanylate cyclase polyclonal antibody. COS-2A cell membranes transfected with GC-A were pre-incubated with or without antibody (IgG fraction) for 1 h on ice, followed by assay for guanylate cyclase activity in the absence or presence of ANF (10^{-7} M). Pre-immune serum was used for control. The experiments were done in triplicate and repeated two times, although the data depicted is from one typical experiment. The mean ± SEM is shown. Memb., COS-2A cell membranes transfected with GC-A; NRS, normal rabbit serum; AB, 180 kDa mGC antibody.

DmutGln^{338}Leu^{364} and 180 kDa mGC, the 180 kDa mGC polyclonal antibody probe was used. This probe is highly specific to the structural determinants of 180 kDa mGC, including the ANF-dependent cyclase epitope (see [21] for details of the antibody specificity).

The coding sequence of GCα-DmutGln^{338}Leu^{364} was introduced into an expression vector, pSVL, under the transcriptional control of the SV40 late promoter, which was then used to transfect COS cells. The particulate fractions of these cells were appropriately treated and analyzed for cyclase activity, and where indicated for the ATP binding activity.

ANF stimulated by approximately 3-fold the particulate guanylate cyclase activity of the expressed protein, and nearly 70% of the stimulated activity was blocked by the 180 kDa mGC polyclonal antibody (Fig. 2); non-immune rabbit serum had no influence on the stimulated activity. Thus the 180 kDa mGC and the mutant protein are immunologically similar, and both proteins are linked to the ANF-dependent cyclic GMP synthesis. Less than complete blockage of the hormone-dependent cyclase activity of the recombinant protein suggests minor structural differences between this and the 180 kDa protein.

The shared feature of immunological identity between 180 kDa mGC and GCα-DmutGln^{338}Leu^{364} proteins formed the basis of purification of the mutant receptor guanylate cyclase by the 180 kDa mGC antibody probe. The immunopurified enzyme showed a single Coomassie blue-stained 130 kDa protein band, although the non-immunopurified fraction showed multiple protein-stained bands (Fig. 3A) To determine the biochemical authenticity of the immunopurified 130 kDa protein as the ANF receptor, the expressed protein in plasma membranes was affinity-crosslinked with [^{125}I]ANF through disuccinamide suberate. SDS-PAGE under reducing conditions revealed a 130 kDa labeled band (Fig. 3B, lanes 3 and 4). Because the labeling was abolished upon inclusion of the non-radioactive ANF (1 μM) in the reaction mixture (Fig. 3B: compare lane 3 with 4), the 130 kDa protein represented as true ANF receptor. Therefore, we conclude that the immunopurified 130 kDa guanylate cyclase is an authentic ANF receptor, is biologically linked to the ANF signal, and is immunologically similar to the 180 kDa mGC.

In previous studies an assignment of the molecular weight of 130 kDa to the GC-A-expressed protein was based solely on the [^{125}I]ANF crosslinking studies [15,20]; the protein was not purified and thus no guanylate cyclase activity could be demonstrated to co-exist in the 130 kDa protein. With the availability of the cyclase antibody probe, the present results establish that the cloned 130 kDa expressed protein is indeed both a guanylate cyclase and an ANF receptor, as is the case with the 180 kDa mGC. It is anticipated that this antibody probe will now prove invaluable in direct characterization, quantifying and purifying GC-A in cells of diverse nature, the task so far hampered due to the extremely low occurrence of these receptors in cellular plasma membranes.

Previous studies have shown that depending upon the

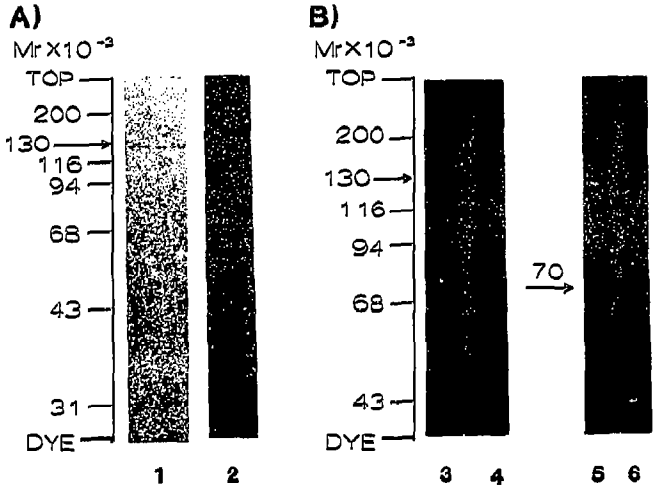

Fig. 3. (A) SDS-8.75% polyacrylamide gel electrophoresis (SDS-PAGE) analysis of immunoaffinity purified 130 kDa membrane guanylate cyclase from COS-2A cell membranes transfected with GC-A (lane 1); (lane 2) solubilized membranes. 1 μg of the immunoaffinity purified fraction was subjected to 8.75% SDS-PAGE and visualized with Coomassie blue. (B) GCα mutants crosslinking with [^{125}I]ANF. Membranes of COS-2A cells transfected with GCα-DmutGln^{338}Leu^{364} (lanes 3 and 4) and GCα-DmutGln^{338}Leu^{364}K^{-}Cyc^{-} (lanes 5 and 6) were incubated in a total volume of 100 μl with [^{125}I]ANF without (lanes 3 and 5) or with (lanes 4 and 6) 1 μM ANF at room temperature for 2 h, followed by incubation with disuccinamide suberate (DSS) for 15 min on ice. Membranes were subjected to 7.5% SDS-PAGE, and the gel was autoradiographed for 7 days at −70°C.

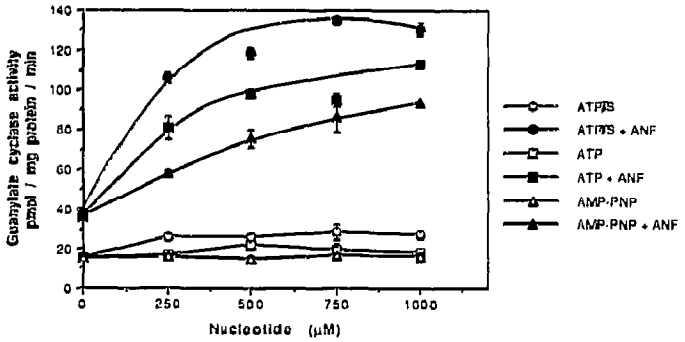

Fig. 4. Effect of ATP and its analogs on basal and ANF-dependent membrane guanylate cyclase activities in the presence of Mg^{2+} as cofactor. COS-2A cell membranes transfected with GC-A were assayed for guanylate cyclase activity in the absence or presence of ANF (10^{-7} M) and increasing concentrations of ATP, ATPγS and AMP-PNP. The experiment was done in triplicate and repeated three times, although the data depicted is from one typical experiment. The mean ± SEM is shown.

cofactors Mg^{2+} or Mn^{2+}, ATP enhances [19,20,22] or diminishes the ANF-dependent cyclase activity [19]. The ATP-stimulatory effect was assessed on the genetically tailored guanylate cyclase signaling. The membranes of GCα-DmutGln^{338}Leu^{364}-transfected cells were incubated with a series of increasing concentrations of ATP or its non-hydrolyzable analogs with or without ANF. With Mg^{2+} as cofactor, ATP and its analogs – ATPγS and AMP-PNP – alone did not alter the basal cyclase activity, and ANF by itself caused only a modest (~1.5–2-fold) cyclase stimulation (Fig. 4). These studies are in general agreement with previous studies, showing that ATP and ANF by themselves do not stimulate the cyclase activity [19,20], although the present study demonstrates that ANF by itself has a small stimulatory cyclase effect.

In the presence of saturating amounts of ANF (10^{-7} M), ATP, and its analogs, stimulated the cyclase activity in a dose-dependent fashion (Fig. 4); the order of maximal cyclase activation and potency was ATPγS > ATP > AMP-PNP – their respective EC_{50} values being 0.16, 0.25 and 0.38 mM.

Thus these ATP results showed an almost exclusive requirement of ATP in ANF signaling, and are in general agreement with those obtained with the crude testicular plasma membranes containing 180 kDa mGC [19] and those expressing GC-A [20].

To account for the ATP stimulatory effect, two mechanisms have been proposed – direct and indirect. Both mechanisms predict that the guanylate cyclase molecule contains a structural module which is allosterically regulated by ATP, and this putative ATP-regulatory module bridges the binding-signal with the signal transduction. In the indirect mechanism [22,24], interaction between ATP and the putative ATP-regulatory module is through a separate ATP-binding protein, whereas in the direct mechanism the regulatory module is the direct ATP binding site [25]. The direct mechanism is supported by two observations: (i) the homogeneous 180 kDa mGC specifically binds ATP [19]; (ii) the GC-A mutant lacking a kinase-like domain is not stimulated by ATP [25], indicating that the ATP binding site resides in the kinase-like domain of the guanylate cyclase. However, the identity of ATP regulatory module is missing.

To address the missing-identity issue, direct ATP-binding studies were conducted with GCα-DmutGln^{338}Leu^{364} and two of its secondary mutants, one in which the entire kinase-like and cyclase domains are deleted (GCα-DmutGln^{338}Leu^{364}K$^-$Cyc$^-$) and the other (GCα-DmutVal^{505}Asn^{506} in which the glycine-rich cluster (Grc)-sequence is disrupted by a sequence change from Gly^{503}-X-Gly-X-X-X-Gly^{509} to Gly^{503}-X-X-X-X-X-Gly^{509} (Table I). This sequence was chosen because it is the modified form of the nucleotide-binding consensus sequence of protein kinases [26,27], although no protein kinase activity has been demonstrated in guanylate cyclase. There was no significant difference in the ATP-binding specific activity between the membranes of GCα-DmutGln^{338}Leu^{364}K$^-$Cyc$^-$ mutant and the membranes of control cells transfected with pSVL alone (Table I). The expressed GCα-DmutGln^{338}Leu^{364}K$^-$Cyc$^-$ mutant contained the ANF binding activity as was shown by [^{125}I]ANF crosslinking (Fig. 3B, lanes 5 and 6); the M_r of the radioactive band, ~70 kDa, agrees with the calculated value of the receptor portion that is in the glycosylated state. In contrast there was almost 3.5-fold higher ATP-binding specific activity in the GCα-DmutGln^{338}Leu^{364} membranes over the control membranes, indicating that the ATP binding site is located somewhere in the intracellular domain of guanylate cyclase. When the ATP-binding results were compared between the membranes expressing GCα-DmutGln^{338}Leu^{364} and those expressing the disrupted Grc-sequence (GCα-DmutVal^{505}Asn^{506}), the disrupted Grc-sequence mutant showed only marginal ATP-binding activity. These results establish that the Grc-sequence Gly^{503}-X-Gly-X-X-X-Gly^{509} is the ATP binding site of guanylate cyclase. Our ongoing studies (manuscript in preparation) using a variety of GCα mutants show that this Grc-sequence motif also is pivotal in ANF-dependent cyclase activation. It is, therefore, concluded that this structural motif represents the module of guanylate cyclase where ATP binds and potentiates the ANF-binding signal, which then is transduced at the catalytic site of the guanylate cyclase.

These results supporting the direct mechanism of ATP action in ANF signaling raise an important question: is this signal transduction mechanism shared by all members of the natriuretic peptide receptor guanylate cyclase family? The question cannot be answered at this time. There are three known natriuretic peptides, ANF, BNP and CNP. Receptors for only two, ANF and CNF, have been identified, and to date the only ATP

Table I

ATP binding to membranes of transfected COS-7 cells

Transfection	Specific [γ-^{32}P]ATP binding (cpm/mg protein)
pSVL (control)	38,871
GCα-DmutGln^{338}Leu^{361}	106,930
GCα-DmutVal^{505}Asn^{506}	60,053
GCα-DmutGln^{338}Leu^{361}K^{-}Cyc^{-}	32,137

Membranes of COS-7 cells were incubated in a total volume of 100 μl with [γ-^{32}P]ATP (~10^6 cpm/tube; specific activity 4,500 Ci/mmol) in the presence of 4 mM Mg^{2+}, at room temperature for 5 min. Membranes were filtered through GF/C filters and washed 4-times with ice-cold phosphate buffered saline, pH 7.5. Non-specific binding was measured in the presence of 10^{-3} M ATP. Specific binding was calculated by subtracting the non-specific radioactivity from the total radioactivity bound to the filters. Values are average of duplicate determinations from a representative experiment.

study conducted with a homogeneous receptor is that of ANF [20]. Studies with the crude rat lung membranes and liver indicate that ATP and its nonhydrolyzable analogs by themselves activate basal particulate guanylate cyclase [22–24]. Because the activation is lost upon partial purification of the enzyme, however, it is possible that in certain signaling processes the indirect mechanism operates, as predicted [23,24].

In conclusion, this study demonstrates immunological identity between two ANF receptor guanylate cyclases, one characterized on a biochemical basis and the other on the basis of molecular cloning. Availability of the antibody probe for the first time has established the direct coupling of ANF signal with the molecularly cloned receptor and, in this study, has made it possible to purify this receptor from the crude tissues. In addition, the antibody probe has identified the sequence of the ATP-binding site of guanylate cyclase that may be important in bridging the ANF-binding signal with signal transduction, the events occurring at the extracellular receptor site and at the intracellular catalytic site of guanylate cyclase.

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