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A structural motif that defines the ATP-regulatory module of guanylate cyclase in atrial natriuretic factor signalling

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Atrial natriuretic factor (ANF)-dependent guanylate cyclase is a single-chain transmembrane-spanning protein, containing an ANF receptor and having catalytic activity. ANF binding to the receptor domain activates the catalytic domain, generating the second messenger cyclic GMP. Obligatory in this activation process is an intervening step regulated by ATP, but its mechanism is not known. Through a programme of site-directed and deletion mutagenesis/expression studies, we report herein the identity of a structural motif (Gly^{503}-Arg-Gly-Ser-Asn-Tyr-Gly^{509}) that binds ATP and amplifies the ANF-dependent cyclase activity; this, therefore, represents an ATP-regulatory module (ARM) of the enzyme, which plays a pivotal role in ANF signalling.

INTRODUCTION

Atrial natriuretic factor (ANF) is a member of a family of structurally related peptide hormones which is primarily released from atria in response to volume expansion and which regulates sodium excretion, water balance, blood pressure [1–3] and steroidogenesis [4–7]. One important second messenger of ANF-mediated biological processes is cyclic GMP [8]. The cyclic GMP-linked ANF receptor is also a guanylate cyclase [9–12]. One such receptor has been characterized at a molecular level, and has been termed as GC-A [13]. ANF binds to the receptor domain and activates the catalytic domain of the enzyme, causing production of the second messenger cyclic GMP. But this binding signal by itself is not sufficient to stimulate the cyclase activity; the presence of ATP is necessary [14,15]. Thus between the two domains, i.e. ligand-binding and catalytic, there appears to be a module in the guanylate cyclase molecule which is allosterically regulated by ATP, and this putative ATP-regulatory module (ARM) bridges the binding signal with the signal transduction. There are two mechanisms, direct and indirect, by which the ARM could mediate the ATP effect. In the indirect mechanism, interaction between ATP and the ARM would be through a separate ATP-binding protein; in the direct mechanism, the ARM of guanylate cyclase itself would be an ATP-binding site. There is evidence supporting both the direct [16,17] and the indirect [18,19] mechanisms. The principal support for the direct mechanism comes from two observations: (1) the homogeneous ANF receptor guanylate cyclase specifically binds ATP [14], and (2) the GC-A mutant, in which the kinase-like domain is deleted, is not stimulated by ATP (in the presence or the absence of ANF) in its cyclase activity [16]. Implicit in this interpretation is that the ATP-binding site of guanylate cyclase resides in the kinase-like domain, although the identity of such a binding site is not known. This is the subject of the present paper.

To accomplish this goal, the indicated cDNA regions of a plasma membrane guanylate cyclase, GCα, were remodelled by oligonucleotide-directed and deletion mutagenesis. GCα is a newly cloned member of the guanylate cyclase family [20]; it is not a receptor of the known natriuretic peptides, but with the exception of two amino acids it is structurally identical to GC-A and conforms to the purported topographical model of GC-A. The two amino acid changes are the substitutions $Gln^{338} \rightarrow His$ and $Leu^{364} \rightarrow Pro$, both involving single nucleotide changes, i.e. CAG→CAC and CTG→CCG respectively.

EXPERIMENTAL

Construction of GCα mutants

GCα-Dmut and GCα-Smut. These were constructed as described in [20].

ANFrec⁻. Using the primers GGTCAGCGGCAGGTTAA-CAGCCACGGTCA and TGCCCACCAAAGCGTTAACCT-CCAGTGTG, two *Hpa*I restriction sites at nucleotide positions 421 and 1721 were created in a 1.9 kb *Sal*I–*Xba*I restricted fragment of GCα cDNA subcloned into pSelect-1 vector (Promega Biotec. Mutagenesis kit). The 1.3 kb *Hpa*I fragment was excised and the remaining part was religated; the truncated *Sal*I–*Xba*I fragment from pSelect cDNA recombinant replaced the *Sal*I–*Xba*I fragment of GCα cDNA in the pBluescript vector, resulting in deletion of the extracellular domain, amino acid sequence 8–442.

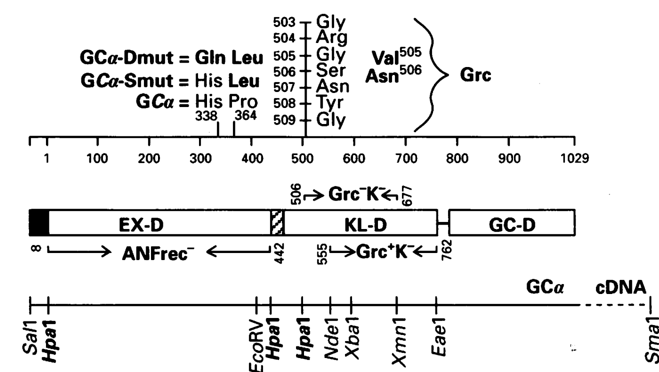

Fig. 1. Representation of GCα mutants

The theoretical topographical domains of GCα are abbreviated as in [20]: EX-D, extracellular domain; KL-D, kinase-like domain; GC-D, guanylate cyclase domain. The closed box represents the leader sequence and the shaded box represents the transmembrane domain. Mutated amino acid residues or restriction sites created by mutagenesis are in **bold**. Construction of GCα mutants is described in the Experimental section.

Abbreviations used: ANF, atrial natriuretic factor; ARM, ATP-regulatory module; Grc, glycine-rich cluster; SV40, simian virus 40.

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Table 1. Guanylate cyclase activity and ^{125}I-ANF binding in membranes of transfected COS-2A cells

COS-2A cells were transfected with appropriate GCα-mutant DNA in a pSVL expression vector, membranes were prepared as described in the Experimental section and guanylate cyclase activity was determined [14,20]. The experiments were done in triplicate and repeated at least three times, although the data depicted are from one typical experiment. ^{125}I-ANF binding was assessed as described [20]. Specific binding was calculated by subtracting the background binding from the total binding.

Transfection	Guanylate cyclase activity (pmol of cyclic GMP/min per mg of protein) Basal	+ANF (μM)	+ATP (1 mM)	Specific ^{125}I-ANF binding (c.p.m./mg of protein)
pSVL	< 0.1	< 0.1	< 0.1	1246 ± 104
GCα-Dmut	7.0 ± 1.0	17 ± 2	7 ± 1	13788 ± 925
GCα-DmutVal^{505},Asn^{506}	5.0 ± 0.4	9 ± 1	5 ± 0.6	11990 ± 800
GCα-Smut	6.1 ± 0.7	16 ± 1.5	6 ± 1	13212 ± 1100
GCα-SmutVal^{505},Asn^{506}	5.1 ± 0.6	10 ± 1	5 ± 0.4	12088 ± 1080
ANFrec$^-$	7.0 ± 0.5	7 ± 0.8	7 ± 0.8	1468 ± 115
GCα-DmutGrc$^-$K$^-$	0.8 ± 0.07	0.8 ± 0.1	0.8 ± 0.1	12400 ± 980
GCα-DmutGrc$^+$K$^-$	1.2 ± 0.1	1.5 ± 0.11	1.3 ± 0.1	12060 ± 860
GCα-SmutGrc$^-$K$^-$	0.7 ± 0.08	0.7 ± 0.10	0.7 ± 0.1	10976 ± 876
GCα-SmutGrc$^+$K$^-$	1.0 ± 0.15	1.2 ± 0.10	1.1 ± 0.15	11234 ± 902

GCα-DmutVal^{505},Asn^{506} and GCα-SmutVal^{505},Asn^{506}. The 1.9 kb *Sal*I–*Xba*I fragment of GCα cDNA was subcloned into the pSelect-1 vector. Gly^{505} was changed to Val and Ser^{506} to Asn using the mutagenic primer GGAGCCATAATTGTTAACT-CGCCCACTCAG, and *Eco*RV–*Xba*I fragments of GCα-Dmut cDNA and GCα-Smut cDNA in the pBluescript vector were replaced with a *Eco*RV–*Xba*I fragment excised from a pSelect–cDNA recombinant.

GCα-DmutGrc$^-$K$^-$ and GCα-SmutGrc$^-$K$^-$. A fragment of kinase-like domain (amino acids 506–677) was removed from GCα-DmutVal^{505},Asn^{506} and GCα-SmutVal^{505},Asn^{506} in pBluescript by digestion with *Hpa*I and *Xmn*I, and the remaining fragment was religated.

GCα-DmutGrc$^+$K$^-$ and GCα-SmutGrc$^+$K$^-$. To remove the kinase-like domain fragment (amino acid sequence 555–762), GCα-Dmut cDNA in pBluescript was linearized with *Nde*I and blunt-ended with Mung Bean nuclease. Digestion with *Xma*I resulted in the removal of a 1.97 kb fragment; the remaining part was ligated with an *Eae*I–*Xma*I 1.35 kb fragment of GCα-Dmut cDNA, whereas the *Eae*I restriction site was blunt-ended with Mung Bean nuclease. The deletion mutant GCα-SmutGrc$^+$K$^-$ was constructed by removing the *Sal*I–*Eco*RV fragment from GCα-DmutGrc$^+$K$^-$ and replacing it with the *Sal*I–*Eco*RV fragment of GCα-Smut. The mutated recombinants were sequenced to confirm their identities and correct ligations. A representation of these mutants is given in Fig. 1. The mutant GCα cDNAs were individually subcloned into the *Xho*II–*Sma*I site of pSVL to create a pSVL expression vector.

Expression studies

COS-2A cells [simian virus 40 (SV40)-transformed African Green Monkey kidney cells] maintained in Dulbecco's modified Eagle's medium with penicillin/streptomycin, supplemented with 10% fetal bovine serum, were transfected with the appropriate GCα mutant cDNA in a pSVL expression vector using calcium phosphate [21]. At 60 h after transfection, cells were washed twice with 50 mM-Tris/HCl (pH 7.5)/10 mM-$MgCl_2$ buffer, scraped into 2 ml of cold buffer, homogenized, centrifuged for 15 min at 5000 ***g*** and washed with the same buffer. The pellet represented the crude membranes. The control cells were identically treated, except that they were transfected with the pSVL vector alone.

The crude membranes were assayed for guanylate cyclase activity [14]. Briefly, membranes were preincubated with or without 1 μM-ANF for 10 min on ice. The assay system contained 10 mM-theophylline, 15 mM-phosphocreatine, 20 μg of creatine kinase, and 50 mM-Tris/HCl, pH 7.5. The total assay volume was 100 μl. The reaction was initiated by the addition of substrate solution containing 1 mM-$MgCl_2$ and 4 mM-GTP. Incubation (37 °C, 10 min) was terminated by addition of 0.9 ml of 50 mM-sodium acetate buffer, pH 6.2, followed by boiling in a water bath for 3 min. The amount of cyclic GMP was quantified by radioimmunoassay. ANF binding was assayed as in [20].

In these studies, synthetic ANF was used, consisting of the 26-amino-acid peptide 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.

RESULTS AND DISCUSSION

GCα was genetically tailored to create two mutants, GCα-Dmut and GCα-Smut [20]. GCα-Dmut is structurally and functionally identical to the cloned wild-type ANF receptor guanylate cyclase, GC-A [13]; GCα-Smut is functionally identical but structurally different in a single amino acid residue from GCα-Dmut (i.e. His^{338} instead of Gln [20]). Each of these mutants was used to create a subset of secondary mutants. Thus there was a pair of secondary mutants, one pair-member corresponding to GCα-Dmut and the other to GCα-Smut (Fig. 1). These mutants contained one of the following special features: (1) deletion of the ANF receptor (ANFrec$^-$); (2) the disrupted glycine-rich cluster (Grc) sequence (GCα-DmutVal^{505},Asn^{506} and GCα-SmutVal^{505},Asn^{506}) in which the Grc sequence was changed from Gly^{503}-Xaa-Gly-Xaa-Xaa-Xaa-Gly^{509} to Gly^{503}-Xaa-Xaa-Xaa-Xaa-Xaa-Gly^{509}; (3) deletion of the kinase-like domain, in which the amino acid sequence comprising residues 506–677 was deleted (GCα-DmutGrc$^-$K$^-$ and GCα-SmutGrc$^-$K$^-$); and (4) deletion of the kinase-like domain (amino acids 555–762) but retention of the Grc sequence (GCα-DmutGrc$^+$K$^-$ and GCα-SmutGrc$^+$K$^-$).

The coding sequence of each of the mutant proteins was introduced into an expression vector, pSVL, under the transcriptional control of the SV40 late promoter, which was then used to transfect COS-2A cells. The particulate fractions of

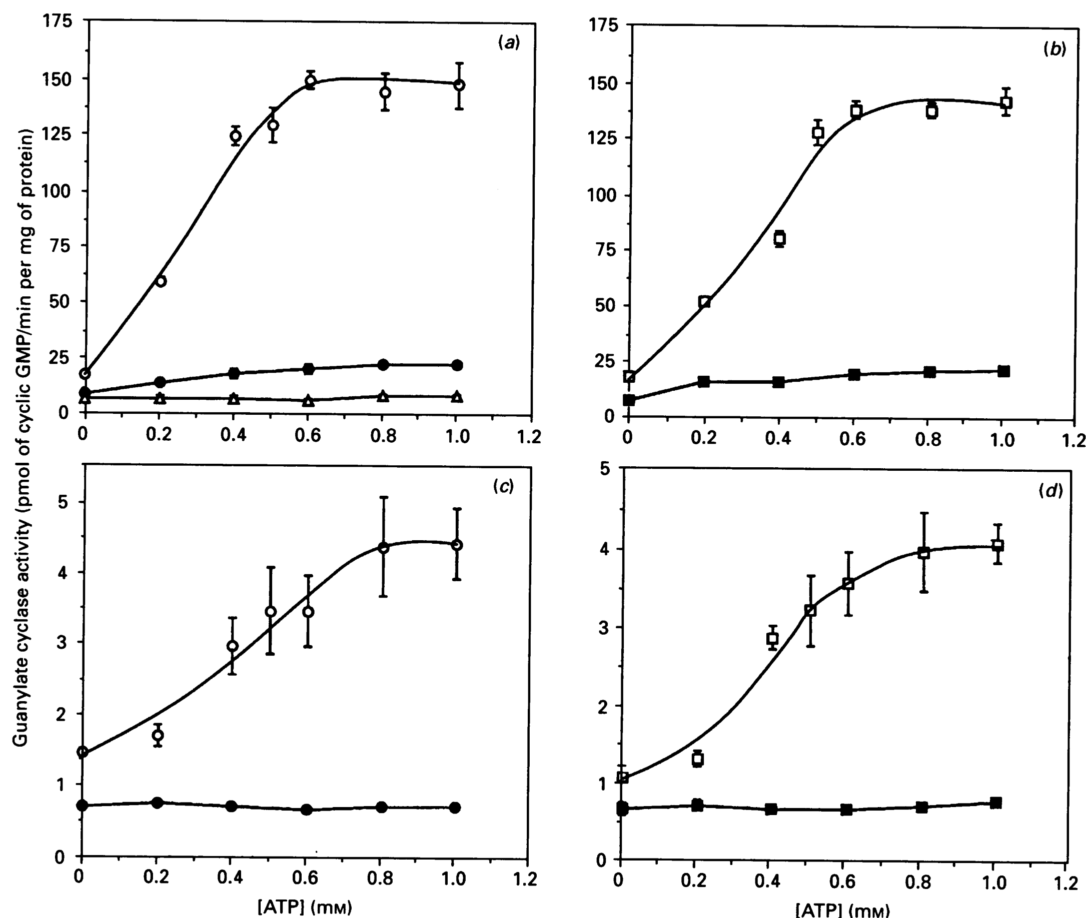

Fig. 2. Effect of ATP on ANF-dependent guanylate cyclase activity

Membranes of COS-2A cells transfected with GCα-mutant DNAs, as described in the Experimental section, were assayed for guanylate cyclase activity [14] in the presence of ANF (1 μM) with increasing concentrations of ATP. The experiments were done in triplicate and repeated at least three times, although the data depicted are from one typical experiment; means$\pm$S.E.M. are shown. (*a*) ○, GCα-Dmut; ●, GCα-DmutVal^{505},Asn^{506}; △, ANFrec$^-$. (*b*) □, GCα-Smut; ■, GCα-SmutVal^{505},Asn^{506}. (*c*) ○, GCα-DmutGrc$^+$K$^-$; ●, GCα-DmutGrc$^-$K$^-$. (*d*) □, GCα-SmutGrc$^+$K$^-$; ■, GCα-SmutGrc$^-$K$^-$.

these cells were appropriately treated and analysed for cyclase activity, and where indicated for the ANF- and ATP-binding activities.

The plasma membranes of the ANF-receptor-deleted mutant ANFrec$^-$ showed greater than 70-fold higher basal cyclase activity than the membranes of control cells transfected with pSVL alone (Table 1). The cyclase activity was not stimulated by ATP, ANF (1 μM) or ANF plus ATP. There was almost 3-fold higher ATP-binding specific activity in the ANFrec$^-$ membranes compared with the control membranes. These results indicated that the ATP-binding site was not in the extracellular domain and, therefore, must have been located in the transmembrane/intracytoplasmic domain of the protein. Because the ANF receptor guanylate cyclase contains a Grc sequence [13,20] (Gly-Xaa-Gly-Xaa-Xaa-Xaa-Gly), which is a modified form of the nucleotide-binding consensus sequence of protein kinases [22,23] (Gly-Xaa-Gly-Xaa-Xaa-Gly), we scrutinized the Grc sequence as being potentially the structural motif of the guanylate cyclase. To determine the effect of ATP on basal and ANF-dependent guanylate cyclase activities, the transfected-cell membranes were incubated with a series of increasing concentrations of ATP and Mg^{2+}, with or without ANF. Assessment of the role of the Grc sequence in the ATP effect was made by comparing the results of the two parent ANF receptor guanylate cyclases, GCα-Dmut and GCα-Smut, with those of their counterparts, GCα-DmutVal^{505},Asn^{506} and GCα-SmutVal^{505},Asn^{506}, in which the Grc sequence had been disrupted (Fig. 2). ATP alone did not alter the basal cyclase activity of any of the four mutants, and ANF by itself caused only a slight (~ 2-fold) cyclase stimulation (Table 1). These results are in general agreement with previous studies, where it was shown that ATP and ANF by themselves do not stimulate guanylate cyclase activity [14,15], although the present study demonstrates that ANF by itself has a small stimulatory cyclase effect. These results, however, are at variance with those obtained with crude membranes, where ATP alone and ANF alone stimulated the cyclase activity [17–19].

In the presence of saturating amounts of ANF, ATP stimulated the cyclase activity of both parent mutants, i.e. GCα-Dmut and GCα-Smut, in a dose-dependent fashion (Figs. 2*a* and 2*b*). Greater than 18-fold stimulation was obtained at ~ 600 μM-ATP, and the concentration causing a half-maximal response (EC_{50}) was ~ 280 μM. Similar results were obtained with adenosine 5′-[γ-thio]triphosphate, a non-hydrolysable ATP analogue, when membranes containing GCα-Dmut were used (results not shown). However, adenosine 5-[β-thio]diphosphate had no stimulatory effect on ANF signalling, emphasizing the specificity of the ATP effect (results not shown). The secondary mutants (GCα-DmutVal^{505},Asn^{506} and GCα-SmutVal^{505},Asn^{506}), however, under identical conditions responded only marginally (~ 2-fold) with respect to guanylate cyclase activation. These

Table 2. ATP binding to membranes of transfected COS-2A cells

Membranes of COS-2A cells were incubated in a total volume of 100 μl with [α-^{32}P]ATP (6×10^5 c.p.m./tube; specific radioactivity 3000 Ci/mmol) in the presence of 4 mM-Mg^{2+} at room temperature for 10 min. Membranes were filtered through GF/C filters and washed four times with ice-cold phosphate-buffered saline, pH 7.5. Non-specific binding was measured in the presence of 1 mM-ATP. Specific binding was calculated by subtracting the non-specific radioactivity from the total radioactivity bound to the filters. Values are means of duplicate determinations. Experiments were repeated twice.

Transfection	Specific ATP binding (c.p.m./mg of protein)
pSVL	37028 ± 1301
GCα-Smut	108186 ± 3405
ANFrec$^-$	101784 ± 3090
GCα-SmutVal^{505},Asn^{506}	59408 ± 2140
GCα-SmutGrc$^+$K$^-$	96768 ± 2904
GCα-SmutGrc$^-$K$^-$	45071 ± 1708

results indicate a pivotal role of the Grc sequence in ATP-mediated responses of ANF signalling. This conclusion was further consolidated by comparative studies with two subsets of two other secondary mutants: one subset (GCα-DmutGrc$^-$K$^-$ and GCα-SmutGrc$^-$K$^-$) in which the entire kinase-like domain including the Grc sequence was deleted, and the other, its counterpart (GCα-DmutGrc$^+$K$^-$ and GCα-SmutGrc$^+$K$^-$), which retained the Grc sequence but did not contain the remainder of the kinase-like domain. The guanylate cyclase activity of the GCα-DmutGrc$^-$K$^-$ and GCα-SmutGrc$^-$K$^-$ mutants was not affected by ATP in the presence or the absence of ANF (Table 1; Figs. 2*c* and 2*d*). However, when the Grc sequence was present and the kinase-like domain was absent (i.e. in GCα-DmutGrc$^+$K$^-$ and GCα-SmutGrc$^+$K$^-$), the ATP-stimulatory effect was restored (Figs. 2*c* and 2*d*). This finding establishes the obligatory role of the Grc sequence in ATP-regulated ANF signalling.

To validate that the ATP effect was a consequence of ATP binding to the Grc sequence, GCα-Smut and all of its secondary mutants were scrutinized for their ATP-binding specificity (Table 2). Comparison of [α-^{32}P]ATP binding by membranes expressing the Grc$^+$K$^-$ sequence mutant (GCα-SmutGrc$^+$K$^-$) with those expressing the Grc$^-$K$^-$ sequence (GCα-SmutGrc$^-$K$^-$) or the disrupted Grc sequence (GCα-SmutVal^{505},Asn^{506}) showed that the Grc$^+$K$^-$ sequence mutants had substantially higher ATP-binding activity than those with either the Grc$^-$K$^-$ sequence or the disrupted Grc sequence. Grc$^-$K$^-$ mutant had almost no ATP-binding activity, and the mutant with disrupted Grc sequence (GCα-SmutVal^{505},Asn^{506}) showed only very low binding activity. These results conclusively show that the Grc sequence of guanylate cyclase is an ATP-binding site. As this sequence also defines the ATP-regulatory module, we conclude that this structural motif represents the module of guanylate cyclase at which ATP binds and potentiates the ANF-binding signal, which then is transduced at the catalytic site of the guanylate cyclase.

Besides establishment of the Grc sequence as the ARM of guanylate cyclase, there are two other significant aspects of this study. First, there is an intriguing similarity between the ARM sequence Gly-Xaa-Gly-Xaa-Xaa-Xaa-Gly and the ATP-binding protein kinase consensus sequence Gly-Xaa-Gly-Xaa-Xaa-Gly. No protein kinase activity has been reported for the ANF receptor guanylate cyclase. If the guanylate cyclase is not a protein kinase, this raises the curious possibility that during the evolutionary pathway, one of the switches between the cyclase and the protein kinase families involved a single amino acid change between the two glycine residues located at the 5′-end. Secondly, our present study does not support the principal observation that forms the basis of a recently proposed ANF-signalling model [16,24], i.e. that the protein kinase deletion mutant of GC-A contained cyclase activity which was in excess of 128-fold higher than that of the wild-type GC-A, and the mutant activity was no longer dependent on ANF (with or without ATP) whereas that of the wild-type was [16,24]. This observation was interpreted to define an essential feature of the model, which was that the kinase-like domain ordinarily represses guanylate cyclase activity, and this repression is removed after ligand binding [16,24]. Our studies with four different kinase-like deletion mutants clearly indicate the opposite. In every case, the kinase-deleted mutants showed 5–10-fold suppression of basal cyclase activity. For example, compare the basal activities of 7 (GCα-Dmut) and 6.1 pmol/min per mg of protein (GCα-Smut) with those of 0.8, 1.2, 0.7 and 1.0 with the respective kinase mutants GCα-DmutGrc$^-$K$^-$, GCα-DmutGrc$^+$K$^-$, GCα-SmutGrc$^-$K$^-$ and GCα-SmutGrc$^+$K$^-$ (Table 1).

In conclusion, the discovery of a protein that is both a guanylate cyclase and an ANF receptor has opened up a new frontier of signal transduction research. In contrast to the cyclic AMP and phosphatidylinositol signal pathways, in which three membrane components (receptor, G-protein and agonist) interact to transduce the hormonal signal, cyclic GMP is a single-component transducing system, containing distinct signal modules in a single transmembrane-spanning protein chain. Two decisive modules, the ligand-binding motif [20] and the ARM, of the chain have been identified, and the cyclase catalytic module lies on the distal carboxyl portion [16]. The possibility also exists of a fourth module, the ATP–Mn^{2+} inhibitory site [14]. Based on these observations, the following four sequential events are envisaged to occur in the signal transduction process: (1) a signal is caused by the binding of the hormone to the receptor site, in which Leu^{364} plays a critical role [20]; (2) there is a transmembrane migration of the binding signal; (3) the signal is potentiated by ATP at the ARM; and (4) the amplified signal is finally transduced at the catalytic site. All of these signalling components together constitute a switch which, when turned on, generates the second messenger cyclic GMP. In this model the ARM of guanylate cyclase is analogous to the G-protein of the three-membrane signalling-component system.

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