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Site-directed mutational analysis of a membrane guanylate cyclase cDNA reveals the atrial natriuretic factor signaling site

(atriopeptin/peptide hormone receptor/cyclic GMP)

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ABSTRACT **Natriuretic peptides are structurally related hormones that regulate hemodynamics of the physiological processes of diuresis, water balance, and blood pressure. One of the second messengers of these hormones is cGMP, and the type of receptor that is involved in the generation of cGMP is also a guanylate cyclase. Recent genetic evidence has revealed such a receptor family; two family members, GC-A and GC-B, have been cloned. We now describe the molecular cloning, sequencing, and expression of a cDNA clone from rat adrenal gland that encodes a membrane guanylate cyclase, GCα, that, with the exception of two amino acids, is structurally identical to GC-A and conforms to the purported topographical model of GC-A. The two amino acid changes are the substitutions $Gln^{338} \rightarrow His^{338}$ and $Leu^{364} \rightarrow Pro^{364}$, involving single nucleotide changes, CA*G* → CA*C* and C*T*G → C*C*G, respectively. Expression studies indicate that GCα cyclase activity is independent of the known natriuretic peptides, and direct binding studies demonstrate that GCα is not an ANF receptor. To determine the importance of Gln^{338} and Leu^{364} in ANF signaling, the GCα cDNA regions encoding amino acid residues 338 and 364 were remodeled by oligonucleotide-directed mutagenesis. A double mutant encoding Gln^{338} and Leu^{364}, and a single-substitution mutant encoding Leu^{364} expressed both ANF binding and ANF-dependent cyclase activities, but the mutant encoding Gln^{338} and a deletion mutant lacking residue 364 did not express either of the above activities. These results define the critical role of Leu^{364} in ANF signal transduction.**

Based on studies with the model systems of isolated fasciculata cells of rat adrenal cortex and rat adrenocortical carcinoma, a hypothetical working model was proposed in which membrane guanylate cyclase was the key enzyme in the receptor-mediated cGMP signal pathway (ref. 1; reviewed in ref. 2). The validity of the model was greatly strengthened by the demonstration that atrial natriuretic factor (ANF) elevates cGMP levels by stimulating the plasma membrane guanylate cyclase (3, 4). 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 (5–7). Other known members of this family are brain natriuretic peptide (BNP; ref. 8) and cardiac natriuretic peptide (CNP; refs. 9–11), which, like ANF, stimulate membrane guanylate cyclase (12). It is therefore possible that certain biological responses of ANF and other natriuretic peptides are mediated by cGMP. The ANF receptor is also a guanylate cyclase (13–16), and hormone binding to its receptor domain stimulates the catalytic activity of guanylate cyclase. Molecular cloning studies have identified two structurally related ANF receptor-containing guanylate cyclases, GC-A and GC-B (17–19); the natural ligand for GC-A appears to be ANF (17). GC-B, although it binds ANF and its cyclase activity is stimulated by this hormone, is relatively more specific for BNP in both binding and guanylate cyclase activity (19). However, the concentrations of BNP required to elicit cyclase activation are pharmacological (19); therefore the natural ligand of GC-B is not known. We now describe GCα, a type of guanylate cyclase from rat adrenal cortex that is structurally different from GC-A in only two amino acids but is not a receptor of the known natriuretic peptides ANF, BNP, and CNP. Furthermore, GCα cDNA remodeling studies demonstrate the critical role of Leu^{364} in ANF signaling.†

MATERIALS AND METHODS

Materials. ANF (rat, residues 8–33) used in these studies was a 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. BNP (porcine) was also a 26-amino acid peptide, Asp-Ser-Gly-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Arg-Ile-Gly-Ser-Leu-Ser-Cys-Leu-Gly-Cys-Asn-Val-Leu-Arg-Arg-Tyr. The 5-kDa CNP was a 45-amino acid peptide, Ser-Gln-Asp-Ser-Ala-Phe-Arg-Ile-Gln-Glu-Arg-Leu-Arg-Asn-Ser-Lys-Met-Ala-His-Ser-Ser-Ser-Cys-Phe-Gly-Gln-Lys-Ile-Asp-Arg-Ile-Gly-Ala-Val-Ser-Arg-Leu-Gly-Cys-Asp-Gly-Leu-Arg-Leu-Phe. These peptides were purchased from Peninsula Laboratories.

Molecular Cloning. A cDNA library constructed from rat adrenal poly(A)$^+$ RNA and cloned into the *Eco*RI site of phage vector λZAP (Stratagene) was screened with four 30-mer oligonucleotide probes, corresponding to nucleotides 555–584, 843–872, 2380–2409, and 2688–2717 of the GC-A cDNA sequence (17). The library was plated at a density of 40,000 plaques per plate; plaques were transferred to nitrocellulose filters, treated by standard procedures (20), prehybridized for 1 hr at 37°C in 6× standard saline citrate (SSC)/5× Denhardt's solution/0.05% sodium pyrophosphate/0.5% SDS/0.01% boiled salmon sperm DNA, and hybridized with end-labeled oligonucleotides ($5–10 \times 10^4$ cpm/ml) at 50°C for 64 hr in 6× SSC/1× Denhardt's solution/0.05% sodium pyrophosphate/0.01% yeast tRNA (21). Filters were washed with 6× SSC/0.05% sodium pyrophosphate at room temperature (three times, 15 min each) and in the same buffer at 60°C for 30 min. The cloned cDNA was "rescued" from phage vector as a pBluescript (Stratagene) plasmid by means of the automatic excision process of λZAP (22). Nucleotide sequencing was done by the dideoxy chain-termination method (23) on denatured double-stranded plasmid templates with Sequenase 2.0 (United States Biochemical).

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Abbreviations: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, cardiac natriuretic peptide.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M74535).

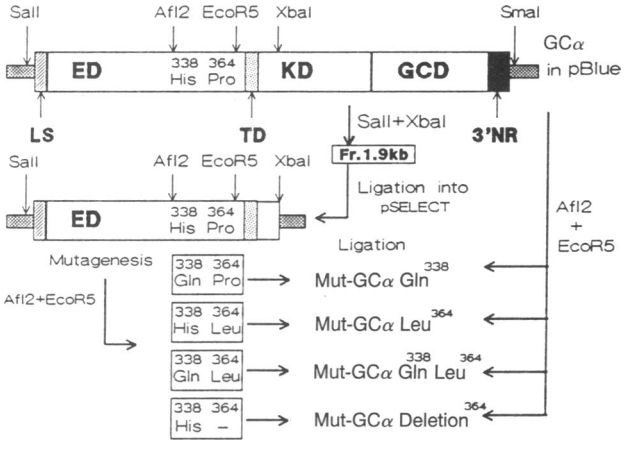

FIG. 1. Construction of GCα mutants. LS, leader sequence; ED, extracellular domain; TD, transmembrane domain; KD, kinase-like domain; GCD, guanylate cyclase domain; 3'NR, 3' noncoding region (these topographical domains are theoretical). Fr., fragment. Details of construction of these mutants are described in *Materials and Methods*.

Site-Directed Mutagenesis. The GCα cDNA in pBluescript was cleaved with *Xba* I to give two fragments, a 5' fragment of 2.16 kilobases (kb) and a 3' fragment of 1.85 kb. The 2.16-kb fragment was religated, and from it the 287 base-pair (bp) 5' noncoding fragment was removed with *Cla* I and *Nco* I; the *Cla* I/*Nco* I restriction sites were blunt-ended with the Klenow polymerase and religated. The resulting plasmid was cut with *Xba* I and ligated with the 3' (1.85-kb) *Xba* I fragment. Proper ligation and deletion were checked by double-stranded sequencing and restriction analysis. The 1.9-kb *Sal* I–*Xba* I fragment of this plasmid was inserted into the mutagenic vector pSELECT-1 (Promega) and used as the single-stranded template. "Coupled priming mutagenesis" (24) was performed using the selection-ampicillin-repair primer (Promega mutagenesis kit) and the appropriate mutagenic primers:

M1 5'-TGACAGTTCCCCCCTGTGCCAGAGTCT-3'
M2 5'-TTCTATCAATTTTCAGGTATCCTGTCACA-3'
M3 5'-TCCGTTTCTATCAATTTTGTATCTTGTCA-CACCTGT-3'.

M1 was used to convert the His^{338} codon to Gln; M2 to convert the Pro^{364} codon to Leu, and M3 to delete the Pro^{364} codon. Correct mutation sequences were validated by dideoxy sequencing of single-stranded DNA (Sequenase kit, United States Biochemical). The *Eco*RV–*Afl* II fragment, containing appropriate mutations, was used to replace the wild-type 0.5-kb *Eco*RV–*Afl* II-fragment of GCα cDNA in pBluescript vector (Stratagene). The resulting mutated recombinants were sequenced to confirm their identities and proper ligations. A graphical representation of the construction of these mutants is given in Fig. 1. The *Sal* I–*Xma* I fragment containing the GCα cDNA or mutant GCα cDNA was cloned into *Xho* I–*Xma* I site of pSVL (Pharmacia) to create the pSVL-GCα expression vector.

GCα Expression Studies. COS-2A cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with the expression vector by the calcium phosphate technique (25). Sixty hours after transfection, cells on 100-mm culture plates were washed twice with 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$, 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 (14, 26). These fractions, as indicated, were treated with a series of concentrations of ANF, BNP, or CNP for 10 min. The control cells were treated identically, except that they were transfected with the pSVL vector alone. The guanylate cyclase and the ANF binding activities were determined (14, 26).

RESULTS AND DISCUSSION

Using four 30-mer oligonucleotide probes corresponding to the various sequenced regions of GC-A cDNA (17), we characterized a cDNA clone from a rat adrenal cDNA library that encodes a protein (GCα) whose deduced amino acid sequence, except for two amino acids, is identical to that of GC-A (ref. 17 and Fig. 2). In both cloned receptor cDNAs the eukaryotic consensus sequence for translation initiation (27), AGGCCATGCC, situated at nucleotides 343–352 determines the initiation of a 1057-amino acid open reading frame, the first 28 amino acid residues represent an N-terminal hydrophobic signal peptide. If the putative signal-peptide sequence is excluded, the calculated molecular weight of the mature GCα protein is 115,824. The sequence topology of both proteins is consistent with the predicted model (18) in which the N-terminal 441 amino acids constitute the extracellular domain, followed by a 21-amino acid transmembrane domain that leads into an Arg-Lys stop transfer sequence (28) and then continues into the 567-amino acid sequence representing the cytosolic portion of the protein. The cytosolic portion in turn appears to be divided into a kinase-like domain followed by a guanylate cyclase catalytic domain. The sequence of the extracellular domain reveals six potential sites for N-linked glycosylation (based on the conserved sequence Asn-Xaa-

Table 1. Particulate guanylate cyclase activity of COS-2A cells transfected with cDNA encoding GCα or its mutants

Peptide	Conc., μm	Specific activity, pmol of cGMP per mg of protein per min					
		Control (pSVL)	GCα	$His^{338} \rightarrow Gln$, $Pro^{364} \rightarrow Leu$	$His^{338} \rightarrow Gln$	$Pro^{364} \rightarrow Leu$	Deletion 364
None	—	7 ± 1	110 ± 6	90 ± 5	78 ± 4	92 ± 4	49 ± 7
ANF	0.01	9 ± 2	112 ± 6	201 ± 7.5	90 ± 9	192 ± 10	39 ± 4
	0.1	9 ± 1	112 ± 5	236 ± 8.5	95 ± 8	228 ± 11	40 ± 4
	1	9 ± 1.5	117 ± 8	250 ± 11.5	101 ± 8.5	246 ± 12.7	47 ± 5
BNP	0.01	8.8 ± 1.2	111 ± 4	124 ± 11.3	91 ± 8	139 ± 10.4	43 ± 2
	0.1	9 ± 1.3	112 ± 6	128 ± 9.5	76 ± 5	136 ± 10	47 ± 3
	1	9.7 ± 1.5	112 ± 5	128 ± 8.5	76 ± 5	136 ± 7	38 ± 4
CNP	0.01	7 ± 1	110 ± 8	90 ± 7	52 ± 6	93 ± 6	40 ± 2
	0.1	7 ± 1	110 ± 10	102 ± 8.1	59 ± 6	110 ± 6.8	42 ± 2
	1	7 ± 1	110 ± 10	102 ± 8.1	59 ± 6	110 ± 7	44 ± 3

Crude membranes were prepared as described in *Materials and Methods*. The membrane suspension was incubated with or without indicated natriuretic peptides for 10 min on ice and assayed for guanylate cyclase activity in a total volume of 100 μl. Experiments were performed in triplicate and repeated at least three times. Results are representative for one experiment (mean ± SEM).

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1 CCCCTTTCTTCTTCCTAGGAACCAGACCGTCCTCTCCTTCCCTCGCTCTCCACCGACTCCCTTCGGTGCTGTGCTTGCTCCCACCTGCTCTGAAGCGCTCTCCGCTCTCGGACGCTCCCAATTTAGC

128 GCTCCTGCTCGACGGCCGAACCGTCGCAGCCTCCGCAGGCAGCGTGCCCTCGGGGTTGCGGCTTCAACCCACCCCAGCTTCCTCCCTCGCTACGACTCGGGCGCCCTGGACGTTCGACCCTCGCCGC

255 TGAGCCCGAGGATGGCGAGCAGACCATGGTGACAGCGCTGCCCGGTCGCTGCACTCGCTGAGGCC  ATG CCG GGC TCC CGA CGC GTC CGT CCG CGC CTA AGG GCG CTG CTG
-28                                                                  Met Pro Gly Ser Arg Arg Val Arg Pro Arg Leu Arg Ala Leu Leu
                                                                                  1
365 CTG CTG CCG CCG CTT CTG CTA CTC CGG GGC GGC CAC GCG AGC GAC CTG ACC GTG GCT GTG GTG CTG CCG CTG ACC AAC ACC TCG TAC CCG TGG TCC
-16 Leu Leu Pro Pro Leu Leu Leu Leu Arg Gly Gly His Ala Ser Asp Leu Thr Val Ala Val Val Leu Pro Leu Thr Asn Thr Ser Tyr Pro Trp Ser

461 TGG GCG CGT GTA GGG CCG GCC GTG GAA CTG GCT CTC GCG CGG GTG AAG GCT CGG CCG GAC TTG CTG CCG GGT TGG ACG GTC CGC ATG GTG CTG GGC
 20 Try Ala Arg Val Gly Pro Ala Val Glu Leu Ala Leu Ala Arg Val Lys Ala Arg Pro Asp Leu Leu Pro Gly Trp Thr Val Arg Met Val Leu Gly

557 AGC AGT GAG AAC GCG GCG GGC GTC TGC TCG GAC ACC GCC GCA CCG CTG GCC GCG GTG GAC CTC AAG TGG GAG CAC AGC CCC GCG GTG TTC CTG GGC
 52 Ser Ser Glu Asn Ala Ala Gly Val Cys Ser Asp Thr Ala Ala Pro Leu Ala Ala Val Asp Leu Lys Trp Glu His Ser Pro Ala Val Phe Leu Gly

653 CCC GGC TGC GTC TAC TCC GCT GCC CCG GTG GGG CGC TTC ACC GCG CAC TGG CGG GTG CCG CTG CTG ACC GCC GGC GCC CCG GCT CTG GGC ATC GGG
 84 Pro Gly Cys Val Tyr Ser Ala Ala Pro Val Gly Arg Phe Thr Ala His Trp Arg Val Pro Leu Leu Thr Ala Gly Ala Pro Ala Leu Gly Ile Gly

749 GTC AAG GAT GAG TAT GCG CTA ACC ACC CGC ACA GGA CCC AGC CAT GTC AAG CTG GGC GAT TTC GTG ACG GCG CTG CAT CGA CGG CTG GGC TGG GAG
116 Val Lys Asp Glu Tyr Ala Leu Thr Thr Arg Thr Gly Pro Ser His Val Lys Leu Gly Asp Phe Val Thr Ala Leu His Arg Arg Leu Gly Trp Glu

845 CAC CAG GCG CTG GTG CTC TAT GCA GAT CGG CTG GGC GAC GAC CGG CCT TGC TTC TTC ATA GTG GAG GGG CTG TAC ATG CGG GTG CGT GAA CGC CTC
148 His Gln Ala Leu Val Leu Tyr Ala Asp Arg Leu Gly Asp Asp Arg Pro Cys Phe Phe Ile Val Glu Gly Leu Tyr Met Arg Val Arg Glu Arg Leu

941 AAC ATC ACA GTG AAT CAC CAG GAG TTC GTC GAG GGC GAC CCG GAC CAC TAC CCC AAG CTA CTG CGG GCC GTG CGG CGA AAG GGC AGA GTT ATC TAC
180 Asn Ile Thr Val Asn His Gln Glu Phe Val Glu Gly Asp Pro Asp His Tyr Pro Lys Leu Leu Arg Ala Val Arg Arg Lys Gly Arg Val Ile Tyr

1037 ATC TGC AGT TCT CCG GAT GCC TTC AGG AAT CTG ATG CTT CTG GCC CTG AAC GCT GGC CTG ACT GGG GAG GAC TAT GTT TTC TTC CAC CTG GAT CTG
212 Ile Cys Ser Ser Pro Asp Ala Phe Arg Asn Leu Met Leu Leu Ala Leu Asn Ala Gly Leu Thr Gly Glu Asp Tyr Val Phe Phe His Leu Asp Val

1133 TTT GGG CAA AGC CTT AAG AGT GCT CAG GGC CTT GTT CCC CAG AAA CCC TGG GAA AGA GGA GAT GGG CAG GAC AGG AGT GCC CGC CAA GCC TTT CAG
244 Phe Gly Gln Ser Leu Lys Ser Ala Gln Gly Leu Val Pro Gln Lys Pro Trp Glu Arg Gly Asp Gly Gln Asp Arg Ser Ala Arg Gln Ala Phe Gln

1229 GCT GCC AAA ATT ATT ACT TAC AAA GAG CCT GAT AAT CCT GAG TAC TTG GAA TTC CTG AAG CAG CTG AAA CTC TTG GCT GAC AAG AAG TTC AAC TTC
276 Ala Ala Lys Ile Ile Thr Tyr Lys Glu Pro Asp Asn Pro Glu Tyr Leu Glu Phe Leu Lys Gln Leu Lys Leu Leu Ala Asp Lys Lys Phe Asn Phe

1325 ACC GTG GAG GAT GGC CTG AAG AAT ATC ATC CCA GCC TCC TTC CAC GAC GGG CTC CTG CTC TAT GTC CAG GCA GTG ACA GAG ACT CTG GCA CAC GGG
308 Thr Val Glu Asp Gly Leu Lys Asn Ile Ile Pro Ala Ser Phe His Asp Gly Leu Leu Leu Tyr Val Gln Ala Val Thr Glu Thr Leu Ala His Gly

1421 GGA ACT GTC ACA GAT GGA GAG AAC ATC ACT CAG CGG ATG TGG AAC CGA AGC TTC CAA GGT GTG ACA GGA TAC CCG AAA ATT GAT AGA AAC GGA GAT
340 Gly Thr Val Thr Asp Gly Glu Asn Ile Thr Gln Arg Met Trp Asn Arg Ser Phe Gln Gly Val Thr Gly Tyr Pro Lys Ile Asp Arg Asn Gly Asp

1517 CGG GAC ACC GAT TTC TCT CTC TGG GAT ATG GAT CCA GAG ACG GGT GCC TTC AGG GTT GTC CTG AAC TAT AAT GGT ACT TCC CAG GAG CTA ATG GCT
372 Arg Asp Thr Asp Phe Ser Leu Trp Asp Met Asp Pro Glu Thr Gly Ala Phe Arg Val Val Leu Asn Tyr Asn Gly Thr Ser Gln Glu Leu Met Ala

1613 GTG TCA GAA CAC AAA TTA TAC TGG CCT CTG GGA TAT CCA CCT CCT GAC GTC CCT AAA TGT GGC TTT GAC AAT GAG GAC CCA GCC TGC AAC CAA GAC
404 Val Ser Glu His Lys Leu Tyr Trp Pro Leu Gly Tyr Pro Pro Pro Asp Val Pro Lys Cys Gly Phe Asp Asn Glu Asp Pro Ala Cys Asn Gln Asp

1709 CAC TTT TCC ACA CTG GAG GTT CTG GCT TTG GTG GGC AGC CTC TCT CTG ATT AGC TTT CTG ATT GTG TCT TTC TTC ATA TAC AGG AAG ATG CAG CTG
436 His Phe Ser Thr Leu Glu Val Leu Ala Leu Val Gly Ser Leu Ser Leu Ile Ser Phe Leu Ile Val Ser Phe Phe Ile Tyr Arg Lys Met Gln Leu

1805 GAA AAG GAG CTG GTC TCA GAG TTG TGG CGG GTG CGC TGG GAG GAC TTG CAG CCC AGC AGC CTG GAG AGG CAC CTT CGG AGC GCT GGC AGC CGG CTG
468 Glu Lys Glu Leu Val Ser Glu Leu Trp Arg Val Arg Trp Glu Asp Leu Gln Pro Ser Ser Leu Glu Arg His Leu Arg Ser Ala Gly Ser Arg Leu

1901 ACC CTG AGT GGG CGA GGC TCC AAT TAT GGC TCC CTG CTA ACC ACC GAG GGC CAG TTC CAA GTC TTT GCC AAG ACA GCA TAC TAT AAG GGC AAC CTT
500 Thr Leu Ser Gly Arg Gly Ser Asn Tyr Gly Ser Leu Leu Thr Thr Glu Gly Gln Phe Gln Val Phe Ala Lys Thr Ala Tyr Tyr Lys Gly Asn Leu

1997 GTG GCT GTG AAA CGT GTG AAC CGG AAA CGC ATT GAG TTG ACA CGA AAA GTC CTG TTT GAA CTT AAA CAT ATG CGG GAT GTG CAG AAT GAG CAC TTG
532 Val Ala Val Lys Arg Val Asn Arg Lys Arg Ile Glu Leu Thr Arg Lys Val Leu Phe Glu Leu Lys His Met Arg Asp Val Gln Asn Glu His Leu

2093 ACA AGA TTT GTG GGT GCT TGT ACC GAC CCC CCC AAC ATC TGT ATC CTC ACA GAG TAC TGT CCC CGT GGA AGC CTA CAG GAC ATT CTA GAG AAT GAG
564 Thr Arg Phe Val Gly Ala Cys Thr Asp Pro Pro Asn Ile Cys Ile Leu Thr Glu Tyr Cys Pro Arg Gly Ser Leu Gln Asp Ile Leu Glu Asn Glu

2189 AGT ATC ACC CTG GAC TGG ATG TTT CGG TAC TCG CTC ACC AAT GAC ATT GTC AAG GGA ATG CTC TTT CTA CAC AAT GGG GCC ATT TGT TCC CAT GGG
596 Ser Ile Thr Leu Asp Trp Met Phe Arg Tyr Ser Leu Thr Asn Asp Ile Val Lys Gly Met Leu Phe Leu His Asn Gly Ala Ile Cys Ser His Gly

2285 AAC CTC AAG TCA TCC AAC TGT GTG GTA GAC GGG CGC TTC GTG TTA AAG ATC ACA GAC TAC GGT CTT GAG AGC TTC AGA GAC CCG GAG CCA GAG CAA
628 Asn Leu Lys Ser Ser Asn Cys Val Val Asp Gly Arg Phe Val Leu Lys Ile Thr Asp Tyr Gly Leu Glu Ser Phe Arg Asp Pro Glu Pro Glu Gln

2381 GGA CAC ACC CTC TTT GCC AAA AAA TTG TGG ACG GCA CCT GAG CTC CTG CGA ATG GCT TCG CCA CCT GCC CGT GGC TCC CAA GCT GGG GAT GTG TAC
660 Gly His Thr Leu Phe Ala Lys Lys Leu Trp Thr Ala Pro Glu Leu Leu Arg Met Ala Ser Pro Pro Ala Arg Gly Ser Gln Ala Gly Asp Val Tyr

2477 AGC TTT GGT ATC ATC CTG CAG GAG ATT GCC CTA AGA AGT GGG GTC TTC TAT GTG GAA GGT TTG GAC CTC AGC CCA AAA GAG ATC ATT GAG CGT GTG
692 Ser Phe Gly Ile Ile Leu Gln Glu Ile Ala Leu Arg Ser Gly Val Phe Tyr Val Glu Gly Leu Asp Leu Ser Pro Lys Glu Ile Ile Glu Arg Val

2573 ACT CGG GGT GAG CAG CCC CCA TTC CGA CCC TCC ATG GAT CTG CAG AGC CAC CTG GAG GAA CTG GGG CAG CTG ATG CAG CGG TGC TGG GCA GAG GAC
724 Thr Arg Gly Glu Gln Pro Pro Phe Arg Pro Ser Met Asp Leu Gln Ser His Leu Glu Glu Leu Gly Gln Leu Met Gln Arg Cys Trp Ala Glu Asp

2669 CCA CAG GAG CGG CCA CCC TTT CAG CAG ATC CGC CTG GCG CTG CGC AAG TTC AAC AAG GAG AAC AGC AGC AAC ATC CTG GAC AAC CTG CTG TCA CGC
756 Pro Gln Glu Arg Pro Pro Phe Gln Gln Ile Arg Leu Ala Leu Arg Lys Phe Asn Lys Glu Asn Ser Ser Asn Ile Leu Asp Asn Leu Leu Ser Arg

2765 ATG GAG CAG TAT GCT AAC AAC CTG GAG GAA CTG GTA GAG GAG AGA ACA CAA GCT TAT CTG GAG GAG AAG CGC AAA GCT GAG GCC TTG CTT TAC CAG
788 Met Glu Gln Tyr Ala Asn Asn Leu Glu Glu Leu Val Glu Glu Arg Thr Gln Ala Tyr Leu Glu Glu Lys Arg Lys Ala Glu Ala Leu Leu Tyr Gln

2861 ATT CTG CCT CAC TCC GTG GCT GAG CAG CTG AAG AGA GGC GAG ACA GTC CAG GCT GAG GCC TTT GAT AGT GTT ACC ATC TAC TTC AGT GAT ATT GTG
820 Ile Leu Pro His Ser Val Ala Glu Gln Leu Lys Arg Gly Glu Thr Val Gln Ala Glu Ala Phe Asp Ser Val Thr Ile Tyr Phe Ser Asp Ile Val
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FIG. 2. *(Figure continues on the opposite page.)*

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2957 GGC TTT ACA GCT CTT TCA GCA GAA AGC ACA CCC ATG CAG GTG GTG ACT CTG CTC AAT GAT CTG TAC ACC TGT TTT GAT GCT GTC ATA GAC AAC TTT
 852 Gly Phe Thr Ala Leu Ser Ala Glu Ser Thr Pro Met Gln Val Val Thr Leu Leu Asn Asp Leu Tyr Thr Cys Phe Asp Ala Val Ile Asp Asn Phe

3053 GAT GTG TAC AAG GTG GAG ACC ATT GGT GAT GCT TAC ATG GTG GTG TCA GGG CTC CCA GTG CGG AAT GGA CAA CTC CAC GCC CGA GAG GTG GCC CGA
 884 Asp Val Tyr Lys Val Glu Thr Ile Gly Asp Ala Tyr Met Val Val Ser Gly Leu Pro Val Arg Asn Gly Gln Leu His Ala Arg Glu Val Ala Arg

3149 ATG GCA CTT GCA CTA CTG GAT GCT GTG CGC TCC TTC CGC ATC CGC CAT AGG CCC CAG GAA CAG CTG CGC TTG CGC ATT GGC ATC CAC ACA GGT CCT
 916 Met Ala Leu Ala Leu Leu Asp Ala Val Arg Ser Phe Arg Ile Arg His Arg Pro Gln Glu Gln Leu Arg Leu Arg Ile Gly Ile His Thr Gly Pro

3245 GTG TGT GCT GGT GTG GTA GGG CTA AAG ATG CCC CGA TAC TGC CTC TTT GGA GAC ACA GTC AAC ACA GCT TCA AGA ATG GAG TCT AAT GGA GAA GCC
 948 Val Cys Ala Gly Val Val Gly Leu Lys Met Pro Arg Tyr Cys Leu Phe Gly Asp Thr Val Asn Thr Ala Ser Arg Met Glu Ser Asn Gly Glu Ala

3341 CTC AAG ATC CAC TTG TCT TCA GAG ACC AAG GCT GTG CTG GAA GAG TTC GAT GGT TTC GAG CTG GAG CTC CGA GGG GAT GTG GAA ATG AAG GGC AAA
 980 Leu Lys Ile His Leu Ser Ser Glu Thr Lys Ala Val Leu Glu Glu Phe Asp Gly Phe Glu Leu Glu Leu Arg Gly Asp Val Glu Met Lys Gly Lys

3437 GGC AAG GTT CGG ACC TAT TGG CTC CTG GGG GAG CGG GGA TGT AGC ACT CGA GGC TGACCTACTGCCCTGCTGTTCCTTGTCACCCCTCCTCCCTGTGCCAGAGGTGACA
1012 Gly Lys Val Arg Thr Tyr Trp Leu Leu Gly Glu Arg Gly Cys Ser Thr Arg Gly

3546 GAGGTGTCCAGCTTCCACCTCTCCCACAGCAGCCCAGCCACTGTGGAAGGATTAGGGACCTGACCAGCACAGTCACCAGATGTGACCTCTGAGAGAGGATGGAGATGGTGGGGACTGCAGGGGACAC

3673 CTAAGTTTGTAGGACTGACTGAAACACACAGTCCCTCCCATGGCACCCTTGTGGCACACATGCCCAGTCCCACCCTTACTCTGCTGCCTAGATTGGGACAGCGATTCCTTCTCTGCCCTCAACTTAG

3800 CTCCACTGTGACTTATAGGGAGGGAATTGCCACCTGAAGGAAACAGAAAGAGGTTAGAGTTTGCAGGAGGCAGGCAGTCCTGTGTCACAAATACTCCCCTCACTTCCAGCCCACCACCTGCCCCACA

3927 GACTTTGGACACAGCTCACTGAGGAGAAGAGAAGCTGCCGGTTACCTTGCTTCTCCTGTGAACCAAACCATTAAAGTCTTTATTCCTGTG
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FIG. 2. Nucleotide sequence and deduced amino acid sequence of the GCα rat adrenal guanylate cyclase cDNA. Nucleotides and amino acids are numbered at left. Overline shows the 23-nucleotide stretch in the 5′ noncoding region of GCα cDNA that is absent from the GC-A gene. The deduced amino acid sequence of the open reading frame is shown below the nucleotide sequence. The 28-amino acid signal-peptide sequence is doubly underlined, the transmembrane domain is boxed, the polyadenylylation signal sequence is doubly overlined, and the cysteine residues and the potential glycosylation sites are underlined. The predicted first amino acid residue of the mature protein is numbered as 1, the TGA stop codon is at nucleotides 3491–3493. The single nucleotide changes are marked by dots and two distinctive amino acids located at positions 338 and 364 are boxed; in GC-A these positions are occupied by Gln and Leu instead of His and Pro.

Ser/Thr) and six cysteine residues. There are two potential N-linked glycosylation sites and 10 cysteine residues in the cytoplasmic portion of the protein.

The two amino acid residues that distinguish GCα from GC-A are at positions 338 and 364, within the purported extracellular domain. Positions 338 and 364 are respectively occupied by Gln and Leu in GC-A and by His and Pro in GCα.

There are other minor structural differences between the GCα and GC-A cDNAs. (*i*) There is a stretch of 23 nucleotides in the 5′ noncoding region of GCα that is absent from the corresponding region of the GC-A gene (ref. 29 and Fig. 2). (*ii*) In the 5′ noncoding region there is a deletion of one nucleotide in GCα cDNA. (*iii*) In the codons for Gln^{272}, His^{491}, and Gly^{660} there are single nucleotide changes that are neutral and do not affect the nature of these amino acids in: GCα, Gln^{272} is encoded by CAA instead of CAG as in GC-A, His^{491} is encoded by CAC instead of CAT as in GC-A, and Gly^{660} is encoded by GGA instead of GGG as in GC-A.

To assess the functional consequences of the two amino acid changes in GCα, we ligated GCα DNA into a mammalian expression vector, pSVL, in which the coding region of GCα cDNA was under the transcriptional control of the simian virus 40 late promoter. COS-2A cells were transfected with the GCα expression vector and the guanylate cyclase activity was determined in the crude membrane fractions (Table 1).

Table 2. Binding of ^{125}I-labeled ANF by transfected COS-2A cells

Plasmid	Specific ^{125}I-ANF binding, cpm per 10^5 cells	
	Cells	Membranes
Control (pSVL)	2,174 ± 110	1,722 ± 120
GCα	2,286 ± 95	2,190 ± 120
$His^{338} \rightarrow$ Gln, $Pro^{364} \rightarrow$ Leu	13,821 ± 320	12,755 ± 350
$His^{338} \rightarrow$ Gln	2,432 ± 390	2,164 ± 410
$Pro^{364} \rightarrow$ Leu	14,020 ± 440	13,221 ± 480
Deletion 364	2,050 ± 140	1,962 ± 140

^{125}I-ANF binding was determined for cells and for crude membranes prepared as described in *Materials and Methods*. Values are mean ± SEM of triplicate assays from three experiments.

The cells transfected with the GCα expression vector showed a 12-fold increase in guanylate cyclase activity, indicating that the encoded protein GCα is a guanylate cyclase. However, there was no ANF-, BNP-, or CNP-dependent increment in guanylate cyclase activity (Table 1). Further, the GCα-transfected intact cells and the crude membranes of those cells showed no specific binding of ^{125}I-labeled ANF (Table 2). These results demonstrate that GCα is not a receptor of any of the three known natriuretic peptides ANF, BNP, and CNP. Another member of the natriuretic peptide receptor/guanylate cyclase family, GC-B (19), has only 44% sequence identity with the extracellular domain of GC-A and still shows binding specificity for ANF and a 3-fold stimulation in ANF-dependent guanylate cyclase activity; BNP is more specific in both binding and in its potency in stimulating the guanylate cyclase activity in GC-B-transfected cells (19).

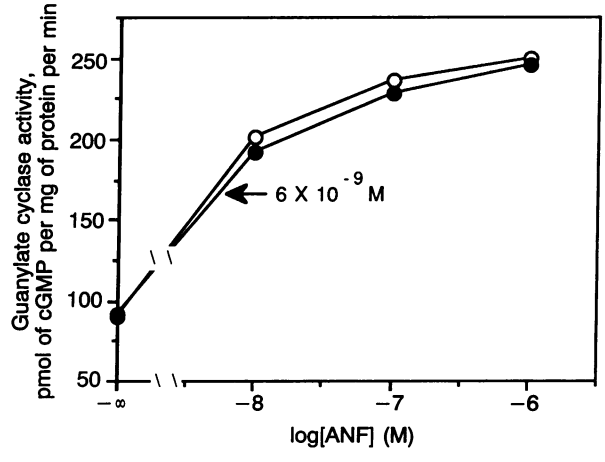

FIG. 3. ANF-dependent guanylate cyclase activity in membranes of transfected COS-2A cells. Membranes of COS-2A cells transfected with GCα double mutant ($His^{338} \rightarrow$ Gln; $Pro^{364} \rightarrow$ Leu) (○) or single mutant ($Pro^{364} \rightarrow$ Leu) (●) were incubated with the indicated concentrations of ANF as described in the legend to Table 1. The data points from Table 1 were used to compare the ability of ANF to stimulate guanylate cyclase activity in the membranes of the mutant transfected cells.

Similarly, the extracellular-domain sequence identity between GC-A and another ANF-binding protein, the ANP-C receptor (30), is only 33%; ANP-C receptor is not a guanylate cyclase.

In view of the virtual structural identity between GCα and GC-A, it is possible that GCα is a receptor of an as yet unidentified natriuretic peptide or represents a product of an allelic form of the GC-A gene.

These expression studies suggested that Gln^{338} and Leu^{364} in GC-A may be critical in the ANF-dependent generation of a cGMP signal. To test such a possibility, the GCα cDNA regions encoding His^{338} and Pro^{364} were replaced with DNA specifying Gln^{338} and Leu^{364}, by site-directed mutagenesis. This double mutant encoded a protein that bound ANF and responded to ANF in a dose-dependent fashion in the generation of cGMP (Fig. 3), establishing a critical role for both or one of these amino acids in ANF signaling.

To determine whether both, or only one, of these amino acids are critical in signaling, two single-substitution mutants were created. One contained Gln^{338} and the other Leu^{364}. The mutant containing Gln^{338} neither bound ANF nor responded to ANF in the stimulation of guanylate cyclase activity, but the mutant containing Leu^{364} bound ANF and showed ANF-dependent cyclase activity (Table 1). ANF was equipotent in stimulating the cyclase activity of the Leu^{364} mutant and the Gln^{338}, Leu^{364} double mutant; in both cases, the ANF concentration causing half-maximal activation was 6 nM (Fig. 3). A deletion mutant lacking amino acid residue 364 did not bind ANF or show ANF-dependent cyclase activity (Tables 1 and 2). These results demonstrate that Leu^{364} is a critical site of ANF signaling. We emphasize that this study does not conclude in absolute terms a direct interaction between the ligand (ANF) and Leu^{364}. It is possible that the configurational arrangement of Leu^{364} provides a three-dimensional structure for ANF binding. In the situation where it is replaced by Pro, the Pro residue may alter the binding site drastically and eliminate ANF binding.

In summary, we have identified a guanylate cyclase (GCα) that is not a receptor of the known natriuretic peptides. Because this receptor varied only in two amino acids from the wild-type ANF receptor/guanylate cyclase GC-A, it was possible to spot two amino acid residues, 338 and 364, that might be critical for ANF binding. Site-specific mutagenesis studies demonstrated that the single amino acid residue Leu^{364} is critical for ligand binding, which results in the activation of guanylate cyclase. Recognition of this signaling site represents a major step toward understanding the basic mechanism by which hormonal signal transduction occurs through the generation of the second messenger cGMP.

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