Glutamic Acid-332 Residue of the Type C Natriuretic Peptide Receptor Guanylate Cyclase Is Important for Signaling†

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ABSTRACT: The type C natriuretic peptide (CNP)-activated guanylate cyclase (CNP-RGC) is a single-chain transmembrane-spanning protein, predicted to contain both ligand binding and catalytic activities. Upon binding CNP, CNP-RGC catalyzes the formation of cyclic GMP. We now show that the Glu-332 residue residing in the extracelluar region of CNP-RGC plays an important role in signal transduction. Deletion of the CNP-RGC intracellular region resulted in the CNP receptor which lacked cyclase activity; deletion or substitution of Glu-332 with His or Lys resulted in almost total loss of both CNP binding and the CNP-dependent cyclase activity without affecting the basal cyclase activity of the mutant proteins. These observations support the general signal transduction model of the subfamily of natriuretic factor receptor cyclases where it is predicted that ligand binding to the extracellular receptor domain of the protein activates the cytosolic catalytic domain, generating the second-messenger cyclic GMP, and identify an amino acid residue of CNP-RGC that plays an important role in CNP signaling.

The natriuretic factors constitute a family of three structurally related peptide hormones—atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and type C natriuretic peptide (CNP)—that regulate the hemodynamics of the physiological processes of diuresis, water balance, and blood pressure [deBold et al., 1981; reviewed in Brenner et al. (1990), Needleman et al. (1989), and Rosenzweig and Seidman (1991)]. CNP is somewhat selectively predominant in the central nervous system (Sudoh et al., 1990; Kojima et al., 1990). It is, therefore, possible that it acts as a neurotransmitter and/or participates in the centrally-related processes of fluid secretion and blood pressure regulation. One important second messenger of these hormones is cyclic GMP [reviewed in Sharma et al. (1994)]. The receptors for ANF (ANF-RGC) (Chinkers et al., 1989; Duda et al., 1991; Kuno et al., 1986; Marala et al., 1992; Meloche et al., 1988; Paul et al., 1987; Takayanagi et al., 1987) and CNP (CNP-RGC) (Koller et al., 1991; Duda et al., 1993) have been characterized; both are guanylate cyclases and are structurally similar. The predicted topographical models of these receptor proteins indicate similarity in that both contain a single membrane-spanning helical domain which divides the protein into two roughly equal portions, the N-terminal extracellular domain and the C-terminal intracellular domain; the receptor domain lies in the extracellular portion while the intracellular portion contains the catalytic domain [reviewed in Sharma et al. (1994)].

This topographical arrangement for the ANF-RGC protein is supported by site-directed (Marala et al., 1992; Goraczniak et al., 1992) and deletion–mutagenesis studies (Chinkers & Garbers, 1989; Goraczniak et al., 1992; Thorpe & Morkin, 1990), which show that the ligand binding and catalytic cyclase activities occur at two opposite ends of the protein. The binding activity resides at the N-terminal extracellular region and the catalytic cyclase activity at the carboxyl end. Additional point mutation studies show that the Leu-364 residue, localized in the extracellular region of ANF-RGC, is critical in the ANF binding event of signal transduction (Duda et al., 1991).

The topographic validity of the CNP-RGC protein has not been clearly tested experimentally so far, and was, therefore, the subject of the present investigation. This study supports the model, and identifies an important CNP signaling site which resides in the extracellular region of CNP-RGC. This finding represents a significant advancement in our understanding of the mechanism of CNP signal transduction.

EXPERIMENTAL PROCEDURES

Materials. CNP-53 (porcine rat) and [^{125}I]CNP were purchased from Peninsula Laboratories; GTP, cyclic GMP, and bovine serum albumin were from Sigma; ATP was from Boehringer-Mannheim; Na^{125}I was from ICN; the mutagenesis kit was from Promega; the restriction enzymes were purchased from USB, BioLabs, and Promega; cell culture media were from Gibco–BRL; and CNP-RGC cDNA clone was isolated from human retina (Duda et al., 1993).

Site-Directed Mutagenesis. CNP-RGC-332 mutants were prepared by converting the GAA codon for Glu-332 to GAT for Asp, to GCA for Ala, to AAA for Lys, and to CAT for His, and by its deletion for the deletion mutant. To construct these mutants, CNP-RGC cDNA in pBluescript vector was cleaved with *Sal*I–*Xba*I. The 1.85-kb *Sal*I–*Xba*I fragment was subcloned into mutagenic vector pSelect-1, which was then used as the single-stranded template. "Coupled priming mutagenesis" was performed using the selection–ampicillin–repair primer (Promega mutagenesis kit) and the appropriate mutagenic primers:

[CNP–RGC–Asp–332]
5′-CCGGGTGCCTCCATCCTGTATTGTC-3′

[CNP-RGC-Ala-332]
5′-CGGGTGCCTCCTGCCTGTATTGTCT-3′

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[CNP-RGC-Lys-332]
5′-GGGTGCCTCCTTTCTGTATTGTCTC-3′

[CNP-RGC-His-332]
5′-CCGGGTGCCTCCATGCTGTATTGTCT-3′

[CNP-RGC-Del-332]
5′-CTCCCGGGTGCCTCCCTGTATTGTCTCATT-3′

The *Sal*I–*Eco*RV fragment, containing appropriate mutations, was used to replace the wild-type *Sal*I–*Eco*RV fragment of CNP-RGC in pBluescript vector (Figure 1). The resulting mutated recombinants were sequenced by double-stranded sequencing (Sanger et al., 1977) to confirm their identities and proper ligations.

The ID⁻ mutant, which lacks the entire intracellular domain of CNP-RGC, was constructed as follows: The coding region of CNP-RGC cDNA (*Sal*I–*Sac*I fragment) was inserted into the pSelect-1 vector, and a unique *Hpa*I restriction site was created by the conversion of Ala-466 into Val and Ser-467 into Asn. The 2-kb fragment at the 3′ end of CNP-RGC was removed by restriction with *Hpa*I–*Sac*I, and the remaining part was religated.

The mutated cDNAs were individually subcloned into the pSVL vector to create pSVL-mutated cDNA expression constructs. For the expression of the ID⁻ mutant, codon TGA from the pSVL vector sequence served as a STOP codon. The CNP-RGC mutant proteins expressed by these expression constructs are graphically represented in Figure 1.

Expression Studies. COS-7 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 constructs by the calcium phosphate coprecipitation technique (Sambrook et al., 1989). Sixty hours after transfection, cells 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 (Paul et al., 1987). These membranes were treated with increasing concentrations of CNP in the presence of 0.8 mM ATP and $MgCl_2$ as a cofactor. The guanylate cyclase and the [^{125}I]CNP binding activities were determined as described earlier (Duda et al., 1991).

RESULTS AND DISCUSSION

A CNP-RGC cDNA deletion mutant (ID⁻ mutant) lacking the intracellular domain and five other mutants related to the amino acid residue at position 332 of CNP-RGC, termed CNP-RGC-332 mutants, were constructed (Figure 1). The coding sequence of CNP-RGC and each of the mutant proteins was introduced into an expression vector, pSVL, under transcriptional control of the SV 40 late promoter, which was then used to transfect COS-7 cells. The particulate fractions of these cells were appropriately treated and analyzed for cyclase and CNP binding activities.

The results of three separate transfection experiments showed that the level of transfection for CNP-RGC and all mutants was roughly the same. This was based on the similar level of basal cyclase activity expressed by the wild-type CNP-RGC and its mutants.

The plasma membranes of CNP-RGC showed more than 10-fold CNP binding specific activity than the membranes of control cells transfected with pSVL alone (Table 1). The

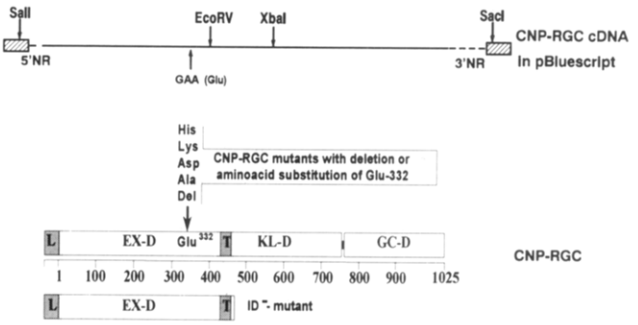

FIGURE 1: Graphical representation of CNP-RGC and its mutants. The amino acid residue Glu-332 of CNP-RGC was changed to each of the indicated amino acids. The GAA codon for Glu and the restriction sites of CNP-RGC cDNA are shown at the top of the figure. 5′NR and 3′NR represent the 5′ and 3′ noncoding regions, and the hatched boxes represent the polylinker of pBluescript vector. Theoretical topographical domains of CNP-RGC are abbreviated as follows: EX-D, extracellular domain; KL-D, kinase-like domain; GC-D, guanylate cyclase domain. The shaded boxes represent the leader sequence (L) and the transmembrane domain (T). The mutants were constructed as described under Experimental Procedures.

Table 1: Particulate Guanylate Cyclase Activity and [^{125}I]CNP Binding in Membranes of Transfected COS Cells[a]

transfection	guanylate cyclase activity [pmol of cGMP (mg of protein)$^{-1}$ min^{-1}]	specific [^{125}I]CNP binding (cpm/mg of protein)
CNP–RGC	15.80 ± 0.9	100789 ± 2800
CNP–RGC–Asp–332	16.30 ± 1.0	81293 ± 3750
CNP–RGC–Ala–332	16.70 ± 0.8	41578 ± 4100
CNP–RGC–His–332	17.40 ± 0.7	14122 ● 590
CNP–RGC–Lys–332	17.90 ± 1.0	17172 ± 450
CNP–RGC–Del–332	16.10 ± 0.9	9873 ± 450
CNP–RGC–ID⁻	0.06	91616 ± 3050

[a] COS cells were transfected with CNP–RGC or its mutant cDNAs in a pSVL expression vector, membranes were prepared as described under Experimental Procedures, and guanylate cyclase activity was detetermined (Paul et al., 1987). The experiments were done in triplicate and repeated 3 times, although the data depicted are from one experiment. [^{125}I]CNP binding was assessed as described (Duda et al., 1991). Specific binding was calculated by subtracting the background binding from the total binding. The control (pSVL) value (8914 ± 700 cpm) has been subtracted in the values shown for the expressed proteins.

CNP binding was not displaced by ANF and BNP (data not shown), indicating that CNP-RGC is a CNP-specific receptor.

This conclusion is in accord with that of the previous study conducted with the extracellular and transmembrane domains of CNP-RGC (Koller et al., 1991). The binding affinity of CNP for CNP-RGC was ~50-fold higher than that of ANF and ~500-fold higher than that of BNP. The K_d of CNP for CNP-RGC was 3×10^{-11} M (Koller et al., 1991).

The ID⁻ bound CNP with a specific activity that was comparable to the wild-type CNP-RGC, but the mutant protein had no cyclase activity (Table 1). This indicated two things. One, the CNP binding activity resides in the extracellular region of the protein. Two, because the cyclase activity was not found in the extracellular region (Table 1), the catalytic domain must have been located in the intracytoplasmic domain of CNP-RGC.

It is, therefore, concluded that the ligand binding and the catalytic cyclase domains are respectively located in the extracellular and the intracellular regions of the CNP-RGC protein.

The reasoning that Glu-332 in CNP-RGC might be important in the ligand binding signal was based on intuition derived from studies with a membrane guanylate cyclase GCα (Duda et al., 1991). With the exception of two amino acids,

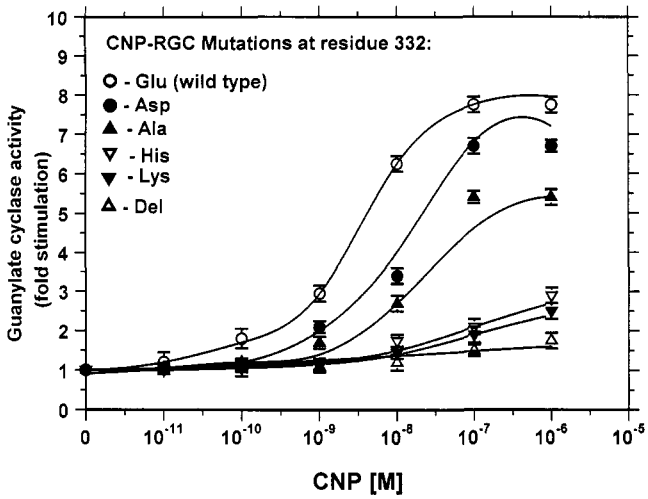

FIGURE 2: Effect of CNP on particulate guanylate cyclase activity in membranes of COS cells transfected with CNP-RGC cDNA or its mutants. Membranes, prepared as described under Experimental Procedures, were assayed for guanylate cyclase activity using Mg^{2+} as a cofactor in the presence of 0.8 mM ATP with varied additions of CNP. The experiments were done in triplicate and repeated 3 times. Results (mean ± SD) shown are from one typical experiment.

GCα is structurally identical to ANF-RGC. The two amino acid changes are the substitutions $Gln^{338} \rightarrow His^{338}$ and $Leu^{364} \rightarrow Pro^{364}$. GC$\alpha$ is not an ANF receptor and is, therefore, independent of ANF in its cyclase activity. When GCα residues 338 and 364 are changed to those corresponding to ANF-RGC residues, the resulting GCα mutant becomes the ANF receptor and also the ANF-dependent cyclase. The counterparts of the 338 and 364 amino acid positions in CNP-RGC are Glu-332 and Val-358. In pilot experiments, no role for CNP-RGC residue 358 in CNP signaling was found.

To assess the signaling role of the Glu-332 residue, five single-substitution CNP-RGC-332 mutants were created (Figure 1): Del (deletion), His, Lys, Asp, and Ala. They were individually expressed in COS cells, and consequences of these mutations on the CNP binding and CNP-dependent guanylate cyclase activities were assessed.

The CNP-specific binding activity of the wild-type CNP-RGC compared to the Del mutant was in excess of 10-fold, and it was in excess of 6-fold when compared to the Lys and His mutants (Table 1). The CNP binding activity of all these mutants was approximately 2-fold higher than the control cells, which were transfected with pSVL alone. In contrast, the CNP-RGC-transfected cells had over 11-fold CNP-specific binding activity (Table 1).

These results indicate the important role of the Glu-332 residue of CNP-RGC in the CNP binding activity, a conclusion further supported by the following results.

The lesions that impair ligand binding activity should also result in the concomitant loss of signal transduction; i.e., the CNP-dependent cyclase activity of the above CNP-RGC-332 mutants should be impaired. To determine if this was the case, the membranes of the cells transfected with the mutated cDNAs were incubated with a series of concentrations of CNP and the results compared with the membranes transfected with the wild-type CNP-RGC cDNA.

CNP stimulated the guanylate cyclase activity of CNP-RGC in a dose-dependent fashion (Figure 2); the EC_{50} concentration was 5 nM, with a significant stimulation occurring below 10^{-10} M. In contrast, the three CNP-RGC-332 mutants did not significantly respond in their cyclase activities up to 10^{-8} M CNP, and at the highest hormone concentration (10^{-6} M) tested, the Del mutant showed no significant cyclase stimulation, and the His and Lys mutants were stimulated only 2-fold (Figure 2). The basal cyclase activities of the wild-type and the mutant proteins were almost the same, however.

These results establish an important role of CNP-RGC's Glu-332 residue in the CNP binding event of signaling. The residue, however, has no effect on the basal cyclase activity.

It needs to be emphasized that these results in no way suggest that the Glu-332 residue of CNP-RGC is the direct binding site. They may merely suggest the importance of this residue in the proper folding of the CNP binding pocket. This interpretation is supported by studies conducted with two other CNP-RGC-332 mutants. In one mutant, the change involved Glu-332 to Asp, and in the other to Ala. These were the choice substituent amino acids, because the changes associated with these amino acids would cause minimum deviation in the tertiary structure of the mutant proteins; Glu and Asp have the same charge, and Ala is neutral.

Substitution of the Glu-332 residue with Asp resulted in the mutant protein which compared to the wild-type CNP-RGC had 20% less CNP binding activity (Table 1) and 2-fold less potency in the CNP-dependent cyclase activity (Figure 2). This suggested that, to some degree, the CNP binding activity associated with the 332 residue is charge-dependent. This conclusion was substantiated by studies with the CNP-RGC-Ala-332 mutant. As compared to the wild-type CNP-RGC, the Ala-332 mutant possessed only 40% CNP binding activity and one-tenth CNP-dependent cyclase activity.

It is instructive to note that it will be unrealistic to consider that modification of the Glu-332 residue with the above residues will result in the complete elimination of CNP binding to the mutant proteins. Such changes may merely result in the altered mode of binding to the ligand. This interpretation fits with the observed results, where the Asp mutant has higher CNP binding and higher CNP-dependent cyclase activity than the Ala mutant (Figure 2 and Table 1).

In conclusion, the present study identifies an amino acid residue in CNP-RGC that is important for a ligand binding event of CNP signaling, and thus provides significant support to the general natriuretic signaling model (Sharma et al., 1994). According to this model, the following sequential signaling events are envisioned to occur: A signal is initiated by binding of the hormone to the ligand binding module; (2) the signal is potentiated by ATP at the ATP regulatory module (ARM); and (3) the amplified signal is finally transduced at the catalytic site, which when turned on generates second-messenger cyclic GMP.

REFERENCES

Brenner, B. M., Ballermann, B. J., Gunning, M. E., & Zeidel, M. L. (1990) *Physiol. Rev. 70*, 665–699.

Chinkers, M ., & Garbers, D. L. (1989) *Science 245*, 1392–1394.

Chinkers, M., Garbers, D. L., Chang, M.-S., Lowe, D. G., Chin, H., Goeddel, D. V., & Schulz, S. (1989) *Nature 338*, 78–83.

deBold, A. J., Bornstein, H. B., Veress, A. T., & Sonnenberg, H. (1981) *Life Sci. 28*, 89–94.

Duda, T., Goraczniak, R., & Sharma, R. K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7882–7886.

Duda, T., Goraczniak, R. M., Sitaramayya, A., & Sharma, R. K. (1993) *Biochemistry 32*, 1391–1395.

Goraczniak, R. M., Duda, T., & Sharma, R. K. (1992) *Biochem. J. 282*, 533–537.

Kojima, M., Minamino, N., Kangawa, K., & Matsuo, H. (1990) *FEBS Lett. 226*, 209–213.

Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H., & Goeddel, D. V. (1991) *Science 252*, 120–123.
Kuno, T., Andresson, W., Kamisaki, Y., Waldman, S. A., Chang, L. Y., Saheki, S., Leitman, D. C., Nakane, M., & Murad, F. (1986) *J. Biol. Chem. 261*, 5817–5823.
Marala, R. B., Duda, T., Goraczniak, R. M., & Sharma, R. K. (1992) *FEBS Lett. 296*, 254–258.
Meloche, S., McNicoll, N., Liu, B., Ong, H., & DeLean, A. D. (1988) *Biochemistry 27*, 8151–8158.
Needleman, P., Blaine, E. H., Greenwald, J. E., Michener, M. L., Saper, C. B., Stockmann, P. T., & Toulnay, H. E. (1989) *Annu. Rev. Pharmacol. Toxicol. 29*, 23–54.
Paul, A. K., Marala, R. B., Jaiswal, R. K., & Sharma, R. K. (1987) *Science 235*, 1224–1226.
Rosenzweig, A., & Seidman, C. E. (1991) *Annu. Rev. Biochem. 60*, 229–255.
Sambrook, M. J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Sharma, R. K., Duda, T., & Sitaramayya, A. (1994) *Amino Acids* (in press).
Sudoh, T., Minamino, N., Kangawa, K., & Matsuo, H. (1990) *Biochem. Biophys. Res. Commun. 168*, 863–870.
Takayanagi, R., Inagami, T., Snajdar, R. M., Imada, T., Tamura, M., & Misono, K. S. (1987) *J. Biol. Chem. 262*, 12104–12113.
Thorpe, D. S., & Morkin, E. (1990) *J. Biol. Chem. 265*, 14717–14720.