The Cloning and Expression of a New Guanylyl Cyclase Orphan Receptor*

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*A The abbreviations used are: GC-A to -G, guanylyl cyclase-A to -G, respectively; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; and cGMP, cyclic GMP.

A novel membrane form of guanylyl cyclase (GC-G) has been identified through the isolation of a full-length cDNA clone; it is predicted to contain an extracellular ligand binding domain, a single transmembrane segment, and intracellular protein kinase-like and cyclase catalytic domains. That GC-G represents a guanylyl cyclase was confirmed by both transient expression in COS-7 cells and stable expression in H293 cells. Endogenous cyclic GMP concentrations of transfected or stable cells, however, were much higher than control cells, suggesting an inability of the cells to effectively regulate, GC-G cyclase activity. Of six Cys residues found within the extracellular domain of guanylyl cyclase-A (GC-A), the receptor for atrial natriuretic peptide, five are conserved within GC-G. Ligands for the other cyclase receptors, nevertheless, failed to stimulate GC-G expressed in transient or stable cells, suggesting that the unknown ligands possess a structure different from the natriuretic peptides or heat-stable enterotoxins. 125I-ANP was shown to be GC-G. When the unique PCR product, designated SIM3, from rat cDNA was used as a template for PCR with degenerate oligonucleotide primers based on conserved sequences within the catalytic domains of membrane and cytoplasmic forms of guanylyl cyclase (23). PCR products were cloned into M13 and sequenced. In addition to several known guanylyl cyclases, a unique sequence was amplified that was later shown to be GC-G. When the unique PCR product, designated SIM3,
was used to screen a small intestine cDNA library, a clone corresponding
to GC-C, the heat-stable enterotoxin receptor, was obtained (12).
Therefore, the sequence of SIM3 was extended using 3’-RACE (24). The
sense guanylyl cyclase primer and an oligo(dT) antisense primer were
used in the PCR, and a single product of 550 base pairs was obtained and
was used as probe. This PCR product encoded the 3’-end of GC-C and was
subsequently used as a probe to screen the same library from which
GC-C was obtained (12). Two partial length clones were obtained, both
of which contained unspliced introns. Numerous cDNA libraries were
constructed in various manners were screened, and the longest cDNA clone
obtained extended no further than about 300 base pairs of the
putative transmembrane segment; this is the beginning of exon 6 based on
other guanylyl cyclase sequences. A combination of 5’-RACE and
genomic sequence information yielded exons 1–5. Predicted exon 1 was
joined to exon 2 by splice overlap PCR and then assembled with exons
2–5 and the cDNA clone encoding exon 6 through the poly(A) tail. The
final construct was sequenced.

Northern Blots—For detection of GC-C mRNA, 5 μg of poly(A) RNA
was fractionated on a formaldehyde-1% agarose gel, then transferred to
a nylon membrane. The membrane was probed with a radioactively labeled
CDNA fragment corresponding to the 5’-end of GC-C (a part of the
extracellular coding region; see Fig. 6B) or to part of the catalytic
domain (see Fig. 6C). Following hybridization overnight at 42 °C, the
membrane was washed at 60 °C in 0.5 × SSC, and mRNA correspond-

Western blot analysis.

isolated oubain-resistant clones were tested for expression of GC-G by

31-P
cGMP was isolated and quantified as described in Ref. 25.

an assay [3H]cGMP. Shown is the similarity of the extracellular domains of guanylyl cyclases.

FIG. 1. The predicted amino acid sequence of GC-G. Shown is the deduced amino acid sequence of GC-G starting with the putative initiation methionine (single letter code). Hydrophatic analysis of the 1100 amino acid sequence predicts an amino-terminal hydrophilic peptide (underlined and in lowercase letters) with a cleavage site after residue Ala-43 (determined by the Sigseq program, The Rockefeller University). The putative transmembrane region is shown in capital letters and underlined, and five potential N-linked glycosylation sites (NXX or NXT) are marked in bold.

FIG. 2. Dendrogram comparing mammalian guanylyl cyclases. Shown is the similarity of the extracellular domains of guanylyl cyclases A-G (GC-A-G) and of the ANP-clearence (ANP-C) receptor. GC-G appears most closely related to the family of natriuretic peptide receptors (GC-A, GC-B, and ANP-C receptor). Analysis was performed using the DNAstar program Megalign, where the branch order is a function of structural similarity.

cGMP concentrations were estimated as described (25).
man GF/C filters were used for filtration (26). Membranes were incubated with 200 pM 125I-ANP in the absence or presence of 0.1 mM competing unlabeled ANP for 1 h at room temperature.

Western Blots—A synthetic peptide corresponding to the carboxyl-terminal decapeptide of GC-G (EEEAKVPEIL) was conjugated to PPD (2,5-diphenyl-1,3,4-oxadiazole, Serumstaaten Institute) using glutaraldehyde. The purified conjugate was used to raise a polyclonal antiserum in rabbits. For Western blotting, 25–50 μg of membrane protein of GC-G expressing COS-7 or H293 cells was separated on 8% sodium dodecyl sulfate-polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene fluoride membranes, and nonspecific protein-binding sites of the membranes were blocked by incubation with TBST (Tris-buffered saline with Tween-20: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% non-fat dry milk at room temperature for 1 h) overnight. Membranes were washed with TBST and incubated for 1 h at room temperature in a 1:1,000 dilution of primary antibody. Membranes were washed three times with TBST, then incubated with a 1:10,000 dilution of a horseradish-conjugated anti-rabbit antibody (Bio-Rad) in TBST. After washing the membranes three times with TBST, protein bands were detected using the enhanced chemiluminescence detection method (ECL, Amersham Corp.).

Chromosomal Localization—The chromosomal location of the gene encoding GC-G was determined by the use of a DNA panel derived from 94 progeny of an interspecific backcross (C57BL/6J x Mus spretus) of females with C57BL/6J males (Jackson BSB, Jackson Lab Backcross DNA Panel Map Service; Ref. 27). A sense primer (5’TCTCAGAACT-

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\begin{align*}
\text{FIG. 3.} & \quad \text{Cysteine residues in the GC-G extracellular domain are conserved in other guanylyl cyclases. Shown schematically is the location of conserved Cys within the extracellular domain of the ANP-C receptor and guanylyl cyclases A-G. Filled circles indicate the location of conserved Cys, and open circles indicate Cys that are conserved within the neuronal family (GC-D–F). Two amino-terminal Cys are found within all receptors shown while another pair of Cys seems to be characteristic for the neuronal family of guanylyl cyclases. All five conserved Cys of GC-G align with the Cys of GC-B.}
\end{align*}
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\begin{align*}
\text{FIG. 4.} & \quad \text{Alignment of the GC-G extracellular domain with the ANP clearance receptor (ANP-CR). The alignment was performed using the DNAstar program Megalign. Amino acids (single letter code) are colored in groups as follows: H, K, and R, light blue; D, E, N, and Q, dark blue; A, G, P, S, and T, green; F, Y, and W, magenta; I, L, M, and V, red; and C, yellow.}
\end{align*}
\]
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RESULTS AND DISCUSSION

GC-G cDNA—The cDNA and predicted amino acid sequence for GC-G are given in Fig. 1. The open reading frame predicts a protein of 1100 amino acids containing a putative signal peptide of 43 amino acids. GC-G is similar to the other membrane forms of guanylyl cyclase (Fig. 2) in that a single transmembrane domain divides the protein approximately in half, separating an extracellular region from two prominent intracellular domains (protein kinase-like and cyclase catalytic). Of seven cysteine residues within the extracellular domain of GC-G, five align with five of six found in GC-A (Fig. 3). These cysteine residues are also conserved in other guanylyl cyclases (Fig. 3; Ref. 22). The ANP clearance receptor binds natriuretic peptides and contains an extracellular domain whose primary amino acid sequence is 30–40% identical to GC-A or GC-B (2, 3, 6, 28). Recent mapping of the disulfide bonding pattern of this receptor demonstrates that the first two Cys and second two Cys residues (from the amino terminus) form internal disulfide bonds (29, 30). The site of ANP binding has been mapped just to the amino-terminal side of the second disulfide loop (31), suggesting that this region is critical for ligand binding. Limited sequence information from genomic clones of GC-G also demonstrates that intron/exon boundaries are at the same positions in the GC-G gene as in the GC-A and the ANP clearance receptor genes (32, 33). The intron/exon boundaries are different in GC-C and in the sensory guanylyl cyclases (34), and thus GC-G appears more closely related evolutionarily to the natriuretic peptide receptors than to other guanylyl cyclases (Fig. 2). A comparison of the extracellular domains of GC-G with the ANP clearance receptor, and a grouping of amino acids according to major chemical properties, shows that the two extracellular domains are more than 40% similar (Fig. 4).

Since the domain structure of GC-G is the same as that seen in guanylyl cyclases with known ligands, and Cys residues within the extracellular domain are conserved when compared
with cyclase receptors with known ligands, GC-G appears to represent a cyclase-coupled, orphan receptor. The high degree of similarity of GC-G with the natriuretic peptide receptor family within the putative ligand binding domain, as well as an apparent conservation of gene structure when compared with the GC-A or ANP clearance receptor genes, furthermore suggests that the putative ligand for GC-G may resemble a natriuretic peptide-like structure.

Expression of GC-G Activity—To confirm that GC-G encodes guanylyl cyclase activity and to facilitate searches for a putative ligand, the cDNA was expressed in either COS-7 or H293 cells. Cyclic GMP concentrations in either COS-7 or H293 cells were much higher than that seen when GC-A or GC-B are overproduced in the same cells (2, 35), suggesting that these cells do not effectively regulate GC-G activity (Fig. 5). Guanylyl cyclase activity was estimated in membranes prepared from H293 cells stably expressing GC-G (Fig. 5A). The addition of high concentrations (1 μM) of ANP, BNP, CNP, or STa had little, if any, effect on cell cGMP concentrations in COS-7 cells transiently expressing GC-G (Fig. 5B).

Natriuretic Peptide Binding—Since cGMP concentrations appeared poorly regulated by either COS-7 or H293 cells transfected with GC-G, direct binding assays were designed to determine whether GC-G might bind such a peptide with high affinity but fail to demonstrate regulation.

However, no detectable 125I-ANP binding to the GC-G overproducing cells was detected (data not shown).

Tissue Expression of GC-G—Based on Northern hybridization, mRNA for GC-G is most prominent in lung, intestine, and skeletal muscle (Fig. 6, B and C). Various cell lines have been examined by reverse transcription-PCR and also contain detectable GC-G mRNA (data not shown). Multiple sized mRNAs were observed in the various tissues using a cDNA probe encoding the extracellular domain or the catalytic domain. A cDNA probe to the apparent intracellular region of GC-G also yielded three positive-hybridizing mRNA species (36). However, when the mRNA of H293 cells stably expressing GC-G was examined, only mRNA at the size of the predicted product was detected (Fig. 6A). Therefore, the multiple sized mRNAs in the various tissues likely reflects alternative splicing. That GC-G is in various peripheral tissues raises the possibility that the ligand for GC-G circulates. Its expression in skeletal muscle is particularly provocative since a role for cyclic GMP in muscle has been largely ignored. Conceivably, however, GC-G is a component of the smooth muscle vasculature in skeletal muscle as opposed to skeletal muscle itself. If so, then possibly

Fig. 7. Chromosomal localization of the GC-G gene in mice. A, shown is the localization of the GC-G gene (gucy2g) on mouse chromosome 19 relative to various markers. The GC-G gene was mapped by backcross analysis as described under “Experimental Procedures” (Jackson Laboratories). B, haplotype figure from The Jackson BSB backcross showing the distal end of chromosome 19 with loci linked to gucy2g. Loci are listed in order with the most proximal at the top. The black boxes represent the C57BL/6J/Ei allele, and the white boxes represent the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percent recombination (R) between adjacent loci is given to the right of the figure, with the standard error (S.E.) for each R. Raw data from the Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmddata.
GC-G stimulation would lead to increased blood flow to the muscle in certain physiological states, such as in response to exercise. Our attempts to identify cells in which GC-G is expressed by in situ hybridization, however, have so far not been successful.

Chromosomal Localization—Based on these analyses, the gene for GC-G (gucy2g) maps to the distal region of mouse chromosome 19 and close to the anchor locus D19Mit1. This region is syntenic with human chromosome 10 q24–26. There do not appear to be genetic diseases in the human that have been mapped to the GC-G locus (Fig. 7).

GC-G represented a particularly difficult full-length cDNA to isolate. The recently reported sequence of a soluble guanylyl cyclase that contains only a few differences from GC-G (KsGC; Ref. 36) appears to represent a partial-length GC-G cyclase that contains a partial protein kinase homology domain. The reported open reading frame for KsGC contains only a few differences from GC-G. However, the 3 base cDNA clone. The reported open reading frame for KsGC represents a splice variant of GC-G (gucy2g) maps to the distal region of mouse chromosome 19 and close to the anchor locus D19Mit1. This region is syntenic with human chromosome 10 q24–26. There do not appear to be genetic diseases in the human that have been mapped to the GC-G locus (Fig. 7).

GC-G was not ruled out even though the proposed initiation Met diverges from the suggested initiation Met. The KsGC represents a splice variant of GC-G. However, the 3 base pairs upstream of the suggested initiation Met diverges from that of GC-G at a position identical to that of intron 12 of the GC-A gene (32). Therefore, the ksgc mRNA may contain an unspliced intron. That KsGC represents a splice variant of GC-G is not ruled out even though the proposed initiation Met does not coincide with an ideal Kozak consensus sequence (36).

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