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Two membrane forms of guanylyl cyclase found in the eye

(cGMP/phototransduction/retina/pineal gland/molecular cloning)

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ABSTRACT **The cDNAs for two membrane guanylyl cyclases, designated E (GC-E) and F (GC-F), were isolated from a rat eye cDNA library. Their deduced topographic structures correspond to known members of the guanylyl cyclase receptor family, containing an extracellular domain, a single membrane-spanning domain, a protein kinase-like domain, and a cyclase catalytic domain. GC-E was expressed in the eye and the pineal gland, whereas GC-F expression was confined to the eye. Overproduction of GC-E and GC-F in COS cells resulted in expression of guanylyl cyclase activity, but ligands known to activate other guanylyl cyclase receptors failed to stimulate enzyme activity. Thus, both GC-E and GC-F remain orphan receptors. Amino acid sequence similarity between GC-E and GC-F in the extracellular region and homology with a cyclase expressed in olfactory neurons and retGC, a rod outer-segment-specific cyclase, suggest that there is another subfamily of guanylyl cyclase receptors, possibly restricted to sensory tissues.**

In phototransduction, light activates a signaling cascade that leads to the hydrolysis of cGMP and closure of a cGMP-gated cation channel (1). A retinal guanylyl cyclase that is activated by decreases in free Ca^{2+} in rod outer segments restores cGMP levels and may be responsible for light adaptation and recovery of the dark state (1–3). Guanylyl cyclases are currently classified into soluble and membrane forms, based on both their cellular distribution and general structure (4). Soluble guanylyl cyclases exist as heterodimers (based on purification and cloning of an $\alpha 1/\beta 1$ form) and contain heme; they are activated by nitric oxide and related vasodilatory agents such as nitroprusside (4). In the rat, the membrane guanylyl cyclases, termed guanylyl cyclases A–C (GC-A, GC-B, and GC-C), respectively, in order of their discovery (5–7), form a family of cell-surface receptors. The same receptors also have been cloned from human cDNA libraries (4). Recently, another guanylyl cyclase (GC-D) (H.-J.F., R. Vassar, D.C.F., R.-B.Y., R. Axel, and D.L.G., unpublished data) was described in rat olfactory neurons. The topographic structure of the membrane guanylyl cyclases suggests at least four distinct domains: an extracellular ligand-binding domain, a single membrane-spanning domain, and an intracellular region that contains the signature domains of membrane guanylyl cyclases (a protein kinase-like domain and a cyclase catalytic domain). GC-A and GC-B function as receptors for natriuretic peptides (5, 6) and GC-C encodes an intestinal receptor for bacterial heat-stable enterotoxin (7) and guanylin (8).

GC-A, GC-B, soluble guanylyl cyclases, and another guanylyl cyclase (retGC) have been identified in retina (9–13). *In situ* hybridization and immunocytochemical studies have shown that retGC is expressed in photoreceptor cells (12, 14), and although no extracellular ligands have been identified for retGC, its activity appears to be regulated *in vitro* by calcium and a 24-kDa heat-stable factor isolated from bovine rod outer segments (14).

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In this study, we report the isolation and functional characterization of two cDNAs encoding additional membrane guanylyl cyclases, designated GC-E and GC-F§, from a rat eye cDNA library. Their primary structure is similar to other known members of the guanylyl cyclase receptor family. Both guanylyl cyclases are expressed in the eye, and GC-E is also detected in the pineal gland, an organ developmentally related to the retina. Based on amino acid sequence comparisons and the failure of ligands of other known cyclase receptors to stimulate, these two guanylyl cyclases and GC-D found in olfactory neurons and retGC (12, 13) appear to define another subfamily of guanylyl cyclase receptors.

MATERIALS AND METHODS

Rat Eye cDNA Library Construction. Whole eyes were collected from Sprague–Dawley rats after decapitation. A random-primed cDNA library was constructed in λZAPII (Stratagene) by using poly(A)$^+$ RNA isolated from the whole eyes and a Superscript cDNA synthesis kit (GIBCO/BRL).

Cloning and Sequencing of GC-E and GC-F. A cDNA fragment corresponding to residues Val^{188}–Val^{446} of human retGC (kindly provided by David G. Lowe, Genentech) was generated by PCR, labeled with ^{32}P, and used as a probe to screen the rat eye cDNA library. Plaque hybridization was performed overnight at 42°C and filters were washed at 60°C in 1× SSC/0.1% SDS for 1 hr. Thirty-five positive cDNA clones were purified and rescued according to the manufacturer's suggestions (Stratagene). All clones corresponded to the membrane guanylyl cyclase that was termed GC-E, and 5 clones contained complete open reading frames. Next, degenerate primers were designed based on invariant sequences in the extracellular domain of GC-D, GC-E, and human retGC (see Fig. 3): 5′-CCG GAA TTC GGT ACC T(T/A)(C/T) A(A/C)I ITI GGI GTI ITI GGI CCI TGG G(A/C)I TG(C/T) GA-3′ and 5′-GCT CTA GAG GAT CCT T(C/T)(G/A) TAI ATI GTI CC(G/A) AAI A(G/A)I GGI (C/G)(T/A)I AC(T/C) TG-3′. These degenerate primers were used in PCR with the rat eye cDNA library as a template, and amplified products were subcloned into pBluescript II KS (Stratagene) and sequenced. From a total of 24 clones, 5 clones had sequences similar to but distinct from known membrane guanylyl cyclases. These PCR fragments were used as probes to rescreen the rat eye cDNA library as described. Twenty-six positive recombinant phages were isolated and sequenced. All of them contained the sequence of a distinct guanylyl cyclase, termed GC-F, and 3 full-length clones were found. For GC-E and GC-F, both strands of DNA sequence were determined by the dideoxynucleotide chain-termination method (15) using Sequenase (United States Biochemical) or the Prism kit (Applied Biosystems). Nucleic acid and amino acid analyses were performed with DNASTAR software.

Abbreviations: GC-A to -F, guanylyl cyclases A–F, respectively.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L36029 and L36030).

Transient Expression and Assay of Guanylyl Cyclase Activity. The cDNA fragments from clones E31 and F5, containing the complete open reading frames of GC-E and GC-F in addition to 5′ and 3′ untranslated sequences, respectively, were ligated into the mammalian expression vector pCMV5 for transient expression in COS cells (16, 17). Guanylyl cyclase activity was measured as described (18).

Northern Blot Analysis. Total RNA (30 μg) from various rat tissues was separated on 1% denaturing formaldehyde agarose gels, transferred to nylon membranes, and hybridized overnight at 42°C with a 1.6-kb cDNA fragment (nucleotides 1–1624; see Fig. 1) of GC-E or a 1.0-kb cDNA probe (nucleotides 1–984; see Fig. 2) of GC-F. Blots were washed at high stringency (65°C in 0.5× SSC/0.1% SDS for 1 hr). Autoradiography was performed at −80°C for 4–7 days.

RESULTS AND DISCUSSION

The nucleotide and deduced amino acid sequences of GC-E and GC-F are shown in Figs. 1 and 2, respectively. Both cDNAs contain open reading frames of 3324 nucleotides and thus encode polypeptides of 1108 amino acids. Hydropathic analyses predict amino-terminal signal sequences (54 and 50 amino acids, in GC-E and GC-F, respectively) and putative single transmembrane domains within the middle region of the two proteins (19, 20). Mature GC-E and GC-F are predicted to contain 1054 and 1058 amino acids with calculated molecular masses of about 115 and 119 kDa, respectively. Similar to other known members of the guanylyl cyclase receptor family, four distinct domains are apparent: a large extracellular domain, a putative ligand-binding domain, a single membrane-spanning domain, and a cytoplasmic region with the characteristic signature domains of cell-surface receptor guanylyl cyclases (a protein kinase-like domain and a cyclase catalytic domain).

The cyclase catalytic domains of GC-E and GC-F are the most conserved regions, followed by the kinase-like domains. The extracellular domains are the most divergent and are only about 13% similar to GC-A, GC-B, or GC-C. The extracellular domains of GC-E and GC-F are 32% similar to each other at the amino acid level and also resemble the olfactory-specific clone GC-D and bovine retGC (GC-D is about 38% similar to either GC-E or GC-F and bovine retGC is about 84% similar to GC-E and 30% similar to GC-F). The alignment of the extracellular domains also reveals several highly conserved stretches of amino acids (Fig. 3). Six cysteine residues are conserved within the extracellular domains of GC-D, GC-E, GC-F, and bovine retGC (Fig. 3). A frameshift in human retGC would also align these six cysteine residues (12, 13). GC-E, GC-F, GC-D, and the human and bovine retinal guanylyl cyclases, therefore, may define another subfamily of guanylyl cyclase receptors.

N-linked glycosylation was observed in a purified guanylyl cyclase from bovine rod outer segments (21) and a potential glycosylation site is also predicted in the extracellular domain of GC-E (Fig. 1). Since GC-E is most similar to human and bovine retGC, it may represent the rat homologue (12, 13).

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FIG. 1. Nucleotide and deduced amino acid sequence of GC-E. Amino acids are numbered from the predicted signal cleavage site. The putative signal sequence (residues −54 to −1) is in lowercase type. The predicted transmembrane domain and a potential N-linked glycosylation site are underlined.

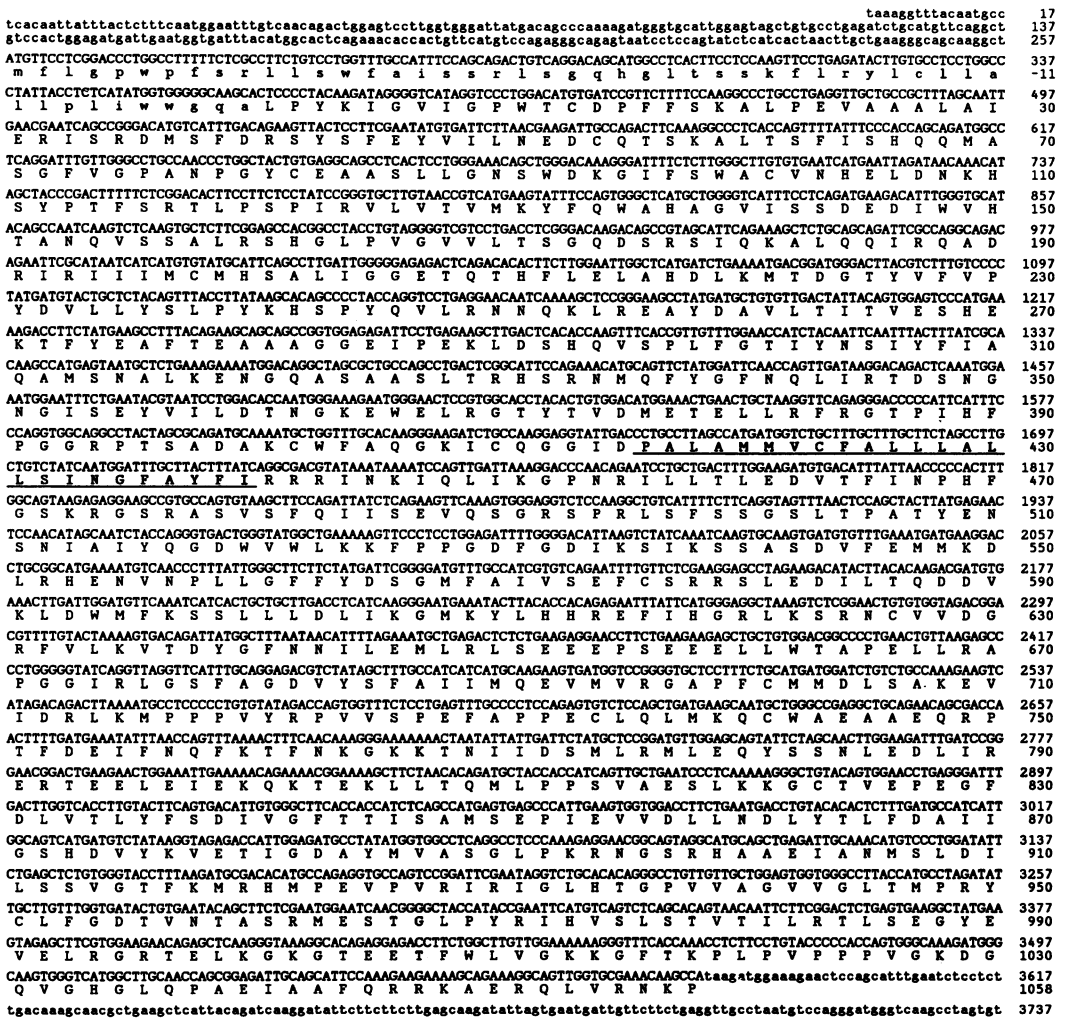

FIG. 2. Nucleotide and deduced amino acid sequence of GC-F. Symbols for the predicted signal sequence and transmembrane domain are as in Fig. 1.

Multiple forms of guanylyl cyclases from toad, frog, and bovine rods have been resolved by two-dimensional gel electrophoresis (22), and GC-E and GC-F with calculated isoelectric points of 7.1 and 6.6, respectively, therefore, could account for some or all of the different forms observed.

The size of the primary transcripts for GC-E and GC-F of 8.5 kb and 11 kb, based on RNA blots, is larger than those of other membrane guanylyl cyclases (≈4.0 kb) (5–7). The increase in size is partly attributed to extended 3′ untranslated regions since additional 1.0- and 2.2-kb sequences are present in the longest clones of GC-E and GC-F, respectively. GC-E is expressed in the pineal gland and eye, but no expression of either clone is detected in brain, heart, kidney, liver, lung, skeletal muscle, small intestine, spleen, or testes (Fig. 4).

The expression of GC-E in both rat eyes and pineal gland is consistent with the expression pattern of some proteins that are involved in the phototransduction cascade, since pinealocytes and retinal photoreceptor cells share a number of identical proteins such as arrestin, phosducin, rhodopsin, and rhodopsin kinase (23). Furthermore, a cGMP-gated cation channel has been identified in chicken pineal cells (24).

To determine whether GC-E or GC-F contained guanylyl cyclase activity, both cDNAs were inserted into the mammalian expression vector pCMV5 and transiently expressed in COS cells. Guanylyl cyclase activity, assayed in the presence of 1% Triton X-100 and Mn^{2+}, was detected in crude membrane fractions prepared from GC-E- and GC-F-transfected COS cells (Table 1). No guanylyl cyclase activity was detected in cells transfected with vector alone. Neither GC-E nor GC-F were activated by the ligands of other known guanylyl cyclase receptors (rat natriuretic peptides, bacterial heat-stable enterotoxin, or sodium nitroprusside) (data not shown).

These studies, which describe GC-E and GC-F, two additional guanylyl cyclases in rat eye, also provide evidence that the primary amino acid sequence of these cyclases defines another subfamily of guanylyl cyclase receptors. GC-D is expressed in olfactory neurons, and therefore, it is possible that the family is further characterized by its localization to sensory tissue neurons or associated structures. Based on the common structure of the natriuretic peptides, which interact with a subfamily of guanylyl cyclase receptors (4), it is reasonable to assume that this apparently distinct subfamily of receptors also defines a family of ligands or regulatory mol-

Table 1. Guanylyl cyclase activity of transfected COS cells

Transfected plasmid	Guanylyl cyclase activity, pmol of cGMP per min per mg of protein
pCMV5	ND
pCMV5-E	193 ± 1.6
pCMV5-F	120 ± 2.6

Membrane fractions were prepared from COS cells transfected with pCMV5, pCMV5-E (GC-E), or pCMV5-F (GC-F). The experiment was performed twice in triplicate with similar results. ND, not detectable.

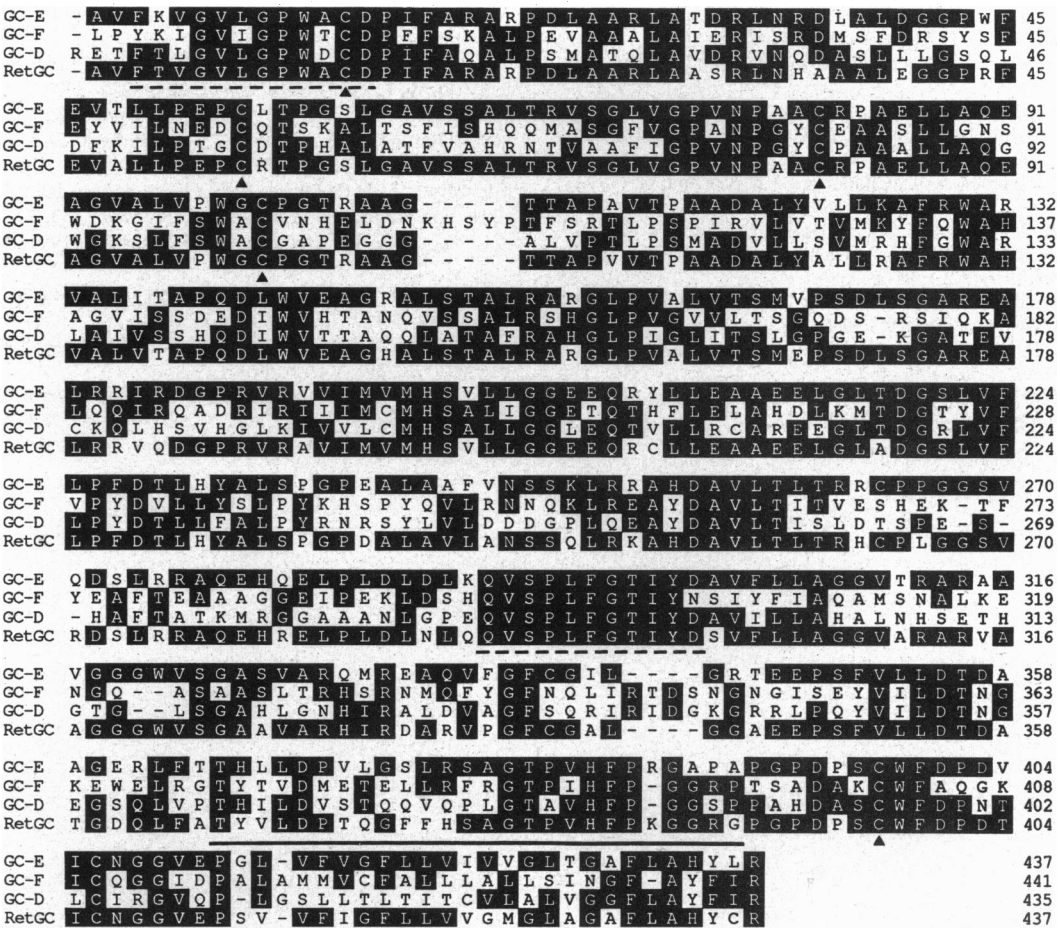

FIG. 3. Sequence alignment of extracellular and transmembrane domains. The deduced amino acid sequences of GC-E and GC-F are compared with the sequences of GC-D (H.-J.F., R. Vassar, D.C.F., R.-B.Y., R. Axel, and D.L.G., unpublished data) and bovine retGC (13). The amino acids shared with any two proteins are boxed and shaded, and conserved cysteine residues are marked with triangles. The putative transmembrane domains are overlined and invariant amino acid sequences corresponding to the degenerate primers are underlined.

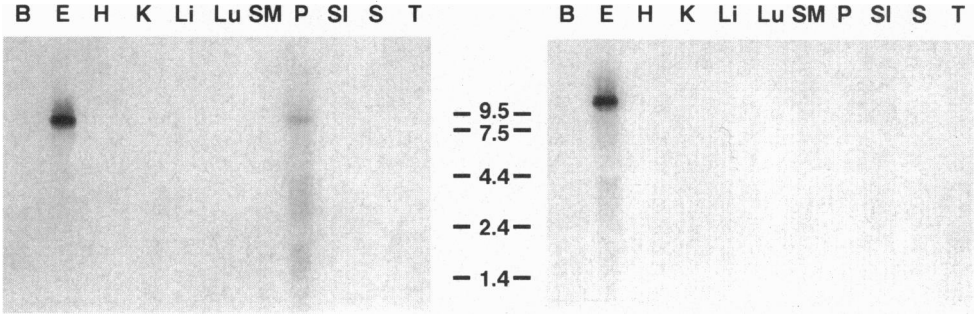

FIG. 4. Northern blot analysis of total RNA from various rat tissues for GC-E and GC-F. Total RNA (30 μg) isolated from the indicated tissues was electrophoresed and blotted to nylon membranes. The blot was probed with a 1.6-kb cDNA of GC-E (*Left*) or a 1.0-kb cDNA of GC-F (*Right*). Lanes: B, brain; E, eye; H, heart; K, kidney; Li, liver; Lu, lung; SM, skeletal muscle; P, pineal; SI, small intestine; S, spleen; T, testes. Northern blot analysis of poly(A)$^+$ mRNA gave the same results (data not shown).

ecules. The recent work showing the Ca^{2+}-dependent regulation of human retGC by a purified retinal protein (14) may point to one such family of regulatory molecules.

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