Two Eye Guanylyl Cyclases Are Expressed in the Same Photoreceptor Cells and Form Homomers in Preference to Heteromers

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We recently described two eye guanylyl cyclases (GC-E and GC-F) that contain an apparent extracellular domain potentially capable of binding ligands (Yang, R.-B., Foster, D. C., Garbers, D. L., and Fülle, H.-J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 602–606). Here, Northern and Western analyses showed that both cyclases are expressed in the retina and enriched in photoreceptor outer segments. By the use of specific GC-E and GC-F antibodies coupled to different sized gold particles both cyclases were colocalized within the same photoreceptor cells raising the possibility of homomeric and/or heteromeric interactions. A point mutant of GC-E (D878A) was constructed and expressed; it contained no detectable cyclase activity but acted in a dominant negative fashion to abolish the activity of native GC-E and GC-F in coexpression studies. These results suggested that GC-E and GC-F could form either homomers or heteromers, at least when overexpressed in COS-7 cells. Immunoprecipitation with GC-E and GC-F antibody followed by Western analysis confirmed that both homomers and heteromers could be formed. However, similar experiments using retina or outer segments revealed that a vast majority of GC-E and GC-F were precipitated as homomers in the eye. Therefore, like other members of the membrane guanylyl cyclase subfamily, GC-E and GC-F appear to preferentially form homomers.

Rod and cone cells convert the energy of an absorbed photon to an electrophysiologically signal in a process called phototransduction. The photoexcitation beginning with rhodopsin leads to an enzymatic cascade resulting in the increased hydrolysis of cyclic GMP (cGMP) and closure of the cGMP-gated cation channel. Photoreceptor guanylyl cyclases have been suggested to be regulated by Ca\(^{2+}\)-sensitive guanylyl cyclase-activating proteins (1, 2); thus a decrease in cytoplasmic Ca\(^{2+}\) in response to light is suggested to relieve inhibition of the cyclases, for subsequent restoration of cGMP concentrations, and for a return to the dark state (3, 4). Two membrane forms of guanylyl cyclase have been found specifically expressed in the eye of the rat (5). The eye cyclases also contain an intracellular protein kinase homology domain and a cyclase catalytic domain potentially capable of binding ligands (Yang, R.-B., Foster, D. C., Garbers, D. L., and Fülle, H.-J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 602–606). Here, Northern and Western analyses showed that both cyclases are expressed in the retina and enriched in photoreceptor outer segments. By the use of specific GC-E and GC-F antibodies coupled to different sized gold particles both cyclases were colocalized within the same photoreceptor cells raising the possibility of homomeric and/or heteromeric interactions. A point mutant of GC-E (D878A) was constructed and expressed; it contained no detectable cyclase activity but acted in a dominant negative fashion to abolish the activity of native GC-E and GC-F in coexpression studies. These results suggested that GC-E and GC-F could form either homomers or heteromers, at least when overexpressed in COS-7 cells. Immunoprecipitation with GC-E and GC-F antibody followed by Western analysis confirmed that both homomers and heteromers could be formed. However, similar experiments using retina or outer segments revealed that a vast majority of GC-E and GC-F were precipitated as homomers in the eye. Therefore, like other members of the membrane guanylyl cyclase subfamily, GC-E and GC-F appear to preferentially form homomers.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The full-length cDNAs for GC-E and GC-F were cloned into the mammalian expression vector pCMV5 and termed pCMV5-GC-E and pCMV5-GC-F, respectively (5). The FLAG (DYKDDDDK) and HA (YPYDVPDYA) epitopes were positioned immediately after the cleavage site of the signal peptide by PCR-mediated constructions (modified sequences are underlined); FLAG-E1S, 5\‘-AAG GAC GAT GAC AAG CTC CCC TAC AAG ATA GGG GTC-3\’; FLAG-E1A, 5\‘-GAC TTG GTG TTG TTG GTA GTC GTA AGG ATA GGA GAA GGC AGA-3\’; HA-E1S, 5\‘-TAT CCT TAC GAC GTA CCT GAT TAC GCT GCT TGC CCT GGT-3\’; HA-E1A, 5\‘-ACC GTA ATC AGG TAC-3\’; FLAG-F1S, 5\‘-TCC TGG TCT AAT AGC TCG-3\’; FLAG-F1A, 5\‘-CTT ATC GTC ATC GTC CTT GTA GGA GAA GGC AGA CCG AGA TGG-3\’; HA-E1S, 5\‘-TAT CCT TAC GAC GTA CCT GAT TAC GCT GCT TGC CCT GGT-3\’; HA-E1A, 5\‘-ACC GTA ATC AGG TAC-3\’; FLAG-E1S, 5\‘-TCC TGG TCT AAT AGC TCG-3\’; FLAG-F1S, 5\‘-ACC GTA ATC AGG TAC-3\’; HA-E1S, 5\‘-TAT CCT TAC GAC GTA CCT GAT TAC GCT GCT TGC CCT GGT-3\’; HA-E1A, 5\‘-ACC GTA ATC AGG TAC-3\’; FLAG-E1S, 5\‘-TCC TGG TCT AAT AGC TCG-3\’; FLAG-F1S, 5\‘-ACC GTA ATC AGG TAC-3\’. The following primers were used in the constructions (modified sequences are underlined): FLAG-E1S, 5\‘-GAC TAC AAG GAC GAT GAC GAT GAG AAG CCT GTG TGC TTT AAA AAG GGG GTG-3\’; FLAG-E1A, 5\‘-TCC TGG TCT AAT AGC TCG-3\’; FLAG-F1S, 5\‘-ACC GTA ATC AGG TAC-3\’. The full-length cDNAs for GC-E and GC-F were cloned into the mammalian expression vector pCMV5 and termed pCMV5-GC-E and pCMV5-GC-F, respectively (5). The FLAG (DYKDDDDK) and HA (YPYDVPDYA) epitopes were positioned immediately after the cleavage site of the signal peptide by PCR-mediated mutagenesis (15). Briefly, the resulting PCR fragments containing the desired epitope insertions were used to replace 5\‘ wild-type EcoRI/XhoI or BglII/NsiI fragments in pCMV5-GC-E or pCMV5-GC-F constructs, respectively. A similar approach was used to generate a GC-E D878A point mutation. The PCR product with the D878A mutation was digested with Sall and Ndel and then ligated with additional wild-type 5\‘ EcoRI/Sall and 3\‘ Ndel/Sall fragments into the pCMV5 vector. Modified sequences in all constructs were confirmed by sequencing of both strands.

Oligonucleotide Primers—The following primers were used in the constructions (modified sequences are underlined): FLAG-E1S, 5\‘-GAC TAC AAG GAC GAT GAC GAT GAG AAG CCT GTG TGC TTT AAA AAG GGG GTG-3\’; FLAG-E1A, 5\‘-TCC TGG TCT AAT AGC TCG-3\’; FLAG-F1S, 5\‘-ACC GTA ATC AGG TAC-3\’. The full-length cDNAs for GC-E and GC-F were cloned into the mammalian expression vector pCMV5 and termed pCMV5-GC-E and pCMV5-GC-F, respectively (5). The FLAG (DYKDDDDK) and HA (YPYDVPDYA) epitopes were positioned immediately after the cleavage site of the signal peptide by PCR-mediated mutagenesis (15). Briefly, the resulting PCR fragments containing the desired epitope insertions were used to replace 5\‘ wild-type EcoRI/XhoI or BglII/NsiI fragments in pCMV5-GC-E or pCMV5-GC-F constructs, respectively. A similar approach was used to generate a GC-E D878A point mutation. The PCR product with the D878A mutation was digested with Sall and Ndel and then ligated with additional wild-type 5\‘ EcoRI/Sall and 3\‘ Ndel/Sall fragments into the pCMV5 vector. Modified sequences in all constructs were confirmed by sequencing of both strands.
FLAG-F1A, 5′-CTT ATC GTC ATC ATC GTC TGT GCC CCA CAA TAT GAG-3′; HA-F1A, 5′-TAT CTT TAC GAG GTA CCT CCG TGT CAC CCG TCT CCC TAC AAG ATA GGG GTC-3′; HA-F1A, 5′-AGG GTA ATC AGG TAC GTC AGA AAG AGT AGA TGC TGT CCC CCA CAA TAT GAG-3′; F-PCR2S, 5′-AGA GTC CCT CCA GTT TCC AC-3′; F-PCR2S, and F-5A primes were used to create PFLAG-GC-F and PHA-GC-F constructs. Other primers were designed for the point mutation E287A plasmid.

Northern Analysis—Whole eyes were collected from Sprague-Dawley rats after decapitation. The retinas were dissected from the posterior chamber of the eye cup, quickly frozen in liquid nitrogen, and stored at −80 °C until use. Total RNAs were isolated from the whole eye, retina, and retina-depleted eye by homogenization in guanidinium thiocyanate followed by phenol/chloroform extraction and precipitation with isopropyl alcohol (16). 30 μg of RNA was separated on 2.2% formaldehyde, 1% agarose gels and transferred to nylon membranes in 20 × SSC. Blots were hybridized overnight at 42 °C in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt’s, 1% SDS, and 100 μg/ml denatured salmon sperm DNA with a 1.6-kilobase pair GC-E or a 1.0-kilobase pair GC-F cDNA probe (5). Blots were washed twice at room temperature and twice at 65 °C in 0.5 × SSC, 0.1% SDS (30 min each wash). Autoradiography was performed at −80 °C for 3 days.

Antiseras—Two peptides, NH2-IPERRKLEKRARGPPTGTKOH and NH2-ARIAAFFERQKLRVNRKPKOH, corresponding to the COOH-terminal amino acid sequence of GC-E and GC-F were synthesized (3). Peptides were conjugated to tuberculin (Serumstaatsinstitut, Copenhagen, Denmark). Each conjugated complex mixed with complete Freund's adjuvant was injected subcutaneously in female New Zealand White rabbits. Booster injections were given every 3 weeks. Anti-FLAG (M2) and HA (12CA5) monoclonal antibodies were purchased from Eastman Kodak Co. and Boehringer Mannheim, respectively.

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 95% air and 5% CO2 at 37 °C. Transfection was by the DEAE-dextran method on 100-mm plates (17). Two days following transfection, cells were washed twice with cold PBS (pH 7.4) and scraped into centrifuge tubes. The cell pellet was frozen quickly in liquid nitrogen and stored at −80 °C until use.

Determination of Guanylyl Cyclase Activity and Cellular cGMP—To determine guanylyl cyclase activity, membrane proteins from transfected cells or chromatographic fractions were added to a total reaction volume of 100 μl containing 25 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 2 mM 3-isobutyl-1-methylxanthine, 4 mM MnCl2, 0.1 mM GTP, and 1 μCi of [γ-32P]GTP. Incubations were for 10 min at 37 °C and terminated by the addition of 0.5 ml of 110 mM zinc acetate and 0.5 ml of 110 mM Na2CO3. [32P]cGMP was purified by Dowex chromatography and quantitated by liquid scintillation counting as described (18). To determine cellular cGMP, transfected cells in one 100-mm dish were split into a 6-well plate. The next day transfected cells were washed with serum-free medium and then incubated in serum-free medium containing 0.25 mM 3-isobutyl-1-methylxanthine for 10 min at 37 °C. 0.5 mM perchloric acid was used to terminate the reaction. Cyclic GMP in the acidified cell extracts was purified by column chromatography and quantitated by radioimmunoassay (18).

Triton-solubilized Retinal and Outer Segment Membranes—Bovine eyes were freshly collected from the local slaughterhouse. All procedures were performed in ambient light and at 4 °C. Ten retinas were dissected and washed twice with cold PBS and homogenized in 25 ml of cold sample buffer containing 25 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 20% glycerol. The homogenates were centrifuged for 10 min at 1,000 × g. The resulting supernatant fluids were centrifuged for 30 min at 20,000 × g to obtain the crude retinal membranes, which were then washed once by the same sample buffer. The washed retinal membranes were solubilized by 5 ml of the same buffer containing 1% Triton X-100. After 30 min on ice, lysate was clarified by centrifugation for 20 min at 350,000 × g to remove insoluble material, and the supernatant fraction was stored at −20 °C until use. The outer segments were prepared by a modification of the discontinuous sucrose density gradient technique as described (19). Purity of the outer segment membranes was confirmed by the enriched presence of rhodopsin (greater than 90%).

Western Blot Analysis and Immunoprecipitation—Proteins were separated by SDS-PAGE. After electophoretic transfer, the nitrocellulose membranes were blocked with PBS (pH 7.5) containing 5% nonfat dry milk and 0.1% Tween 20 overnight at 4 °C. The membranes were incubated with anti-GC-E or GC-F antisera at 1:10,000 or 1:5,000 dilution for 1 h at room temperature. For the control, each antisera was incubated with 1 mg/ml peptide antigen in PBS (pH 7.5) overnight at 4 °C. Following two washes (15 min with PBS containing 0.1% Tween 20), the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (Tago Inc.) at a 1:10,000 dilution for 1 h. After washing the membranes, the enhanced chemiluminescence system (Amersham Corp.) was used for detection. For immunoprecipitation, transfected cells were solubilized in 0.5 ml of cold lysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 20% glycerol) for 40 min on ice. Lysates were clarified by centrifugation at 4 °C for 20 min at 350,000 × g. 2.5 μl of polyclonal antisera were added to the clarified lysates. The immunocomplexes were precipitated by adsorption to protein A-agarose (Pierce), washed four times with lysis buffer, and subjected to Western blot analysis.

Immunoelectron Microscope—Light-adapted female Sprague-Dawley rats maintained on a 12-h light/dark cycle were sacrificed. The posterior portion of each eye was dissected and immediately placed in a fixative containing 4% formaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 °C. The specimens were then rinsed three times (1 h each) in 0.1 M phosphate buffer (pH 7.4) containing 0.9% NaCl and incubated in the same rinse solution overnight at 4 °C. The next day the samples were transferred into the same rinse buffer containing 0.1 M glycine. The ultrathin sections were prepared from LR White-embedded tissue and used to perform colloidal gold labeling as described (20).

RESULTS AND DISCUSSION

Expression of GC-E and GC-F mRNA—Northern blot analysis using RNA (30 μg) isolated from the whole eye, retina (Ret−), and retina-depleted eye (Ret+) were separated on 1% denaturing formaldehyde-agarose gels and blotted to nylon membranes. The blots were hybridized with a GC-E-specific probe (left panel) or a GC-F-specific probe (right panel).

FIG. 1. Retinal expression of GC-E and GC-F by Northern analysis. Total RNAs (30 μg) from the whole eye (Whole), retina (Ret−), and retina-depleted eye (Ret+) were separated on 1% denaturing formaldehyde-agarose gels and blotted to nylon membranes. The blots were hybridized with a GC-E-specific probe (left panel) or a GC-F-specific probe (right panel).

Expression of GC-E and GC-F mRNA—Northern blot analysis using RNA (30 μg) isolated from the whole eye, retina, and retina-depleted eye demonstrates that GC-E and GC-F mRNA is enriched in the retina but not detectable in samples from the retina-depleted eye (Fig. 1). Thus, both eye guanylyl cyclase proteins (10 μg) extracted from the GC-E- or GC-F-transfected COS cells were analyzed for antibody cross-reactivity by Western blot. As shown in Fig. 2, the anti-GC-E polyclonal antibody reacts with a single 120-kDa polypeptide in COS cells expressing GC-E. COS cells expressing GC-F or vector alone show no reactivity. Likewise, the anti-GC-F antis serum specifically rec-
Enrichment of both GC-E and GC-F in the outer segments leads to an important question of whether they are colocalized in the same photoreceptors or not. To determine their subcellular localization at the ultrastructural level, the rat retinas were used for subsequent immunoelectron microscopy studies with specific anti-GC-E and GC-F antisera.

A double immunolabeling technique was used in which sections were immunolabeled on one side with 5-nm gold-labeled anti-GC-E antisera and the other with antisera against GC-F coupled to 10-nm gold particles (Fig. 5B). The data clearly showed that both cyclases are located within the same rod cells (Fig. 5B). In addition, the gold particles of either size are primarily distributed over the marginal region of both disk and plasma membranes in the outer segments. Therefore, if GC-E and GC-F are receptors, ligands apparently are present either outside the photoreceptors or in the disk itself.

No labeling was seen in the connecting cilium, inner segment, and photoreceptor cell body (not shown). In addition, the immunogold label of GC-E over the outer segments appears to be consistent with a proposed role of RetGC in phototransduction in several species (22–25). Due to the rod-rich rat retina that contains very few cones, we were not able to compare the expression differences between rods and cones. In our experiments, peptide antigens do not completely abolish the immu-

**Fig. 2. Specificity of anti-GC-E and GC-F antisera.** Triton extracts (10 μg) of COS-7 cells transfected with 5 μg of DNA of pCMV5 (−), pCMV3-GC-E (E), or pCMV3-GC-F (F) were separated by SDS-PAGE and then probed with polyclonal antibodies against GC-E (αGC-E) or GC-F (αGC-F).

**Fig. 3. Detection of GC-E and GC-F proteins in the retina.** Freshly dissected rat retinas were directly solubilized in the SDS-loading buffer. Samples equivalent to one-half of a retina were loaded on each lane and immunoblotted with polyclonal anti-GC-E (GC-E), GC-F (GC-F) (−), or peptide-blocked antiserum (+).

**Fig. 4. Enhanced immunoreactivity of GC-E and GC-F in the outer segments.** The retinas were further separated into the outer segment and outer segment-depleted fractions by step sucrose gradients (19). Detergent extracts (20 μg) from the retina (R), outer segments (OS), and outer segment-depleted portions (OS−) were separated on SDS-PAGE gels, then Western blotted with anti-GC-E (αGC-E) and GC-F specific antisera (αGC-F), respectively.

**Fig. 5. Immunogold labeling of GC-E and GC-F in the rod outer segments.** Thin sections of rat retina were immunolabeled by preimmune (A) or immune (B) serum at a final concentration of 1:50 dilution. The control experiments using preimmune serum show no significant label over any structure, and a section of outer segment is shown in panel A. In panel B, the 5- or 10-nm gold particle representing GC-E and GC-F are evident over the disk and plasma membranes within the same rod outer segment. Original magnification: × 60,000 (A) and × 112,000 (B).
Colocalization and Interactions of Two RetGCs

Homo- and hetero-oligomerization of GC-E and GC-F in transfected COS cells. pFLAG-GC-E or pHA-GC-E (1.5 μg (A), pFLAG-GC-F or pHA-GC-F (4.0 μg) (B), and pFLAG-GC-E or wild-type GC-F constructs (1.5 μg) (C) were singly or co-transfected in COS cells. Detergent lysates of each transfection were immunoprecipitated with anti-FLAG M2 antibody, and then one-fifth of each precipitate was immunoblotted with indicated antisera to determine the associated proteins. IP, immunoprecipitation; WB, Western blot.

**TABLE I**
Dominant negative effects of the GC-E D878A mutant on cellular cGMP production

| Plasmid                  | cGMP pmol/well | Inhibition |
|--------------------------|----------------|------------|
| pCMV5-GC-E + pCMV5       | 16.4 ± 1.3     |            |
| pCMV5-GC-F + pCMV5       | 24.3 ± 3.2     |            |
| pE-D878A + pCMV5         | ND*            |            |
| pCMV5-GC-E + pE-D878A    | 1.3 ± 0.1      | 92         |
| pCMV5-GC-F + pE-D878A    | 1.0 ± 0.2      | 96         |

* ND, not detectable.

nogold label, which may be due to a higher affinity of the antibody for the true antigens rather than peptide alone. Regardless, the gold labels are considered significant since the anti-GC-E and GC-F antibodies invariably give rise to a stronger immunogold signal compared with a negligible background by the preimmune serum (Fig. 5A).

**Homo- and Hetero-oligomerization in COS Cells**—Previous studies have documented that other membrane guanylyl cyclases can assemble into homo- or higher ordered homomeric complexes in the absence of ligand (10, 11). However, soluble cyclases are known to form heterodimers and therefore the existence of GC-E and GC-F within the same photoreceptors raised the important question of whether or not heteromeric forms of the retinal cyclases exist. We constructed cDNAs encoding HA or FLAG epitope-tagged GC-E and GC-F proteins and examined their association by co-immunoprecipitation assays from both singly and co-transfected COS cells.

We first examined whether GC-E or GC-F formed homomeric complexes. COS cells were transfected with cDNAs for FLAG-

**FIG. 6.** homo- and hetero-oligomerization of GC-E and GC-F in transfected COS cells. pFLAG-GC-E or pHA-GC-E (1.5 μg (A), pFLAG-GC-F or pHA-GC-F (4.0 μg) (B), and pFLAG-GC-E or wild-type GC-F constructs (1.5 μg) (C) were singly or co-transfected in COS cells. Detergent lysates of each transfection were immunoprecipitated with anti-FLAG M2 antibody, and then one-fifth of each precipitate was immunoblotted with indicated antisera to determine the associated proteins. IP, immunoprecipitation; WB, Western blot.

**FIG. 7.** Dominant negative effects of the GC-E D878A mutant on wild-type GC-E and GC-F. The effects of coexpression of the GC-E D878A mutant with wild-type GC-E (A) and GC-F (B) were performed in separate experiments. COS-7 cells were transfected with 1 μg of the indicated constructs. Approximately 15–20 μg of membrane proteins of each transfection were used to determine the guanylyl cyclase activity. Cell lysates were also immunoblotted with GC-E, GC-F, or FLAG antibodies to examine the protein expression levels. The FLAG-tagged GC-E construct neither interfered with protein expression nor diminished intrinsic guanylyl cyclase activity. Results shown are representative of three experiments in duplication.

GC-E and/or GC-F tagged with a HA epitope (HA-GC-E). Lysates of these cells were immunoprecipitated with the anti-FLAG M2 monoclonal antibody, then the precipitates were analyzed by immunoblotting with M2 or the anti-HA monoclonal antibody 12CA5, respectively. A 120-kDa immunoreactive band recognized by the anti-HA antibody was observed in the M2 immunoprecipitates from cells coexpressing FLAG-GC-E and HA-GC-E proteins, but not from cells transfected with individual tagged constructs alone (Fig. 6A). Similarly, the M2 monoclonal antibody immunoprecipitates both FLAG-GC-F and HA-GC-F in cells co-transfected with both epitope-tagged GC-F constructs (Fig. 6B). These results demonstrated that photoreceptor guanylyl cyclases are capable of forming homomeric complexes.

Whether heteromeric complexes could form was ascertained by co-transfection of FLAG-GC-E and untagged GC-F constructs in COS cells. Cells were then subjected to assay for complex formation by immunoblotting of anti-FLAG M2 immunoprecipitates with GC-F or GC-E antisera. If a stable interaction is maintained by coexpressing COS cells, it is predicted to co-immunoprecipitate the FLAG-GC-E-GC-F complexes. The anti-FLAG M2 immunoprecipitates from coexpressing cells resulted in a coprecipitation of GC-F, but not from cells expressing either FLAG-GC-E or GC-F alone (Fig. 6C). Likewise, the reciprocal immunoprecipitation of GC-F results in the coprecipitation of the overexpressed GC-E (not shown).

Furthermore, association between the expressed receptors appears to require coexpression and is not an artifact formed only after cell lysis, since a mixture of lysates containing separately expressed proteins is not sufficient for complex formation in heterologous expression systems (not shown).

**Dominant Negative Effects of the GC-E D878A Mutant**—The apparent ability to form heteromers was confirmed by introduction of an alanine (D878A) at the same relative position as Asp-893 of GC-A. The D893A mutation results in an inactive cyclase that forms dimers with wild-type GC-A, thus acting as a dominant negative protein (26).

No cGMP production could be detected in cells expressing the GC-E D878A construct (Table I). However, coexpression of
GC-E D878A and wild-type FLAG-GC-E constructs resulted in a substantial decrease (75%) in guanylyl cyclase activity as well as a 92% decrease in overall cGMP production (Fig. 7A and Table I). Likewise, expression of this mutant with wild-type GC-F also resulted in inhibition of cyclase activity and intact cell cGMP concentrations to 30 and 4% of wild-type controls, respectively (Fig. 7B). Importantly, the expression of wild-type GC-E and GC-F, as monitored by immunoblots, remained unchanged in the presence of either vector plasmid or the GC-E D878A mutant construct (Fig. 7, A and B).

Immunoprecipitation of GC-E and GC-F from the retina—That GC-E and GC-F could form heteromers and homomers in overexpressing cells leads to the question of which forms are normally present in the retina. GC-E or GC-F was immunoprecipitated from Triton-solubilized retinal membranes with either anti-GC-E or GC-F-specific antibodies. The immunoprecipitates were then analyzed for the presence of GC-E and GC-F by Western blot analyses. As shown in Fig. 8A, GC-E antibody predominantly immunoprecipitated GC-E, but a signal was only barely detectable with GC-F. Likewise, anti-GC-F antiserum also quantitatively immunoprecipitated GC-F and a limited amount of GC-E.

The same patterns of precipitation were also observed in the outer segment membranes (Fig. 8B). Examination of the unbound supernatant fluids from the immunoprecipitation assay indicates that all photoreceptor guanylyl cyclases were precipitated (Fig. 8B). These results demonstrate that individual photoreceptor guanylyl cyclases preferentially interact to form a homomeric complex in the outer segments; only a small portion of cyclases appear to assemble as heteromers.

Molecular Size of Receptor Complexes—The molecular size of receptor oligomers was further determined by gel filtration chromatography. The freshly detergent-solubilized retinal cyclase activity was eluted in a relatively broad peak within a range of estimated molecular mass of 240–480 kDa (not shown); a similar elution profile was also reported in other studies (27, 28). This apparent size is consistent with the oligomeric state of GC-E and GC-F shown by immunoprecipitation assay, suggesting an enzymatic complex of a dimer or higher ordered structure (Fig. 6). Therefore, like other membrane receptor guanylyl cyclases (10–12), photoreceptor GC-E and GC-F appear to preexist in an oligomeric state.

In summary, we have demonstrated that rat GC-E and GC-F are photoreceptor guanylyl cyclases that can be expressed within the same cells. These two photoreceptor guanylyl cyclases, therefore, appear unique compared with various other components of the phototransduction cascade that are expressed in rod- or cone-specific manners (29–35). Using immunoprecipitation followed by immunoblotting, GC-E and GC-F expressed in COS cells could assemble in homomeric or heteromorphic complexes. This is further supported by a point mutation of D878A GC-E, which exerts a dominant negative effect to block the activity of wild-type GC-E and GC-F in coexpression studies. However, similar experiments with retina and outer segments showed that both cyclases appear to preferentially form homomers.

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