Identification of an orphan guanylate cyclase receptor selectively expressed in mouse testis

Michaela KUHN*, Chi Kin Domingos NG†, Yueh-Hsing SU†, Ana KILIĆ*, Danuta MITKO*, Nga BIEN-LY†, László G. KÖMÜVES† and Ruey-Bing YANG†[1]

*Institut fuer Pharmakologie und Toxikologie, Universitaetsklinikum Muenster, Muenster, Germany, and †Institute of Biomedical Sciences, Academia Sinica, 128 Academia Road Sec. 2, Taipei 115, Taiwan, Republic of China

We have identified a novel membrane form of guanylate cyclase (GC) from a mouse testis cDNA library and termed it mGC-G (mouse GC-G) based on its high sequence homology to rat GC-G. It encodes a potential type I transmembrane receptor, with the characteristic domain structure common to all members of the family of membrane GCs, including an extracellular, putative ligand-binding domain, a single membrane-spanning segment and cytoplasmic protein kinase-like and cyclase catalytic domains. Real-time quantitative reverse transcriptase–PCR and Northern-blot analyses showed that mGC-G is highly and selectively expressed in mouse testis. Phylogenetic analysis based on the extracellular protein sequence revealed that mGC-G is closely related to members of the subfamily of natriuretic peptide receptor GCs. When overexpressed in HEK-293T cells (human embryonic kidney 293T cells) or COS-7 cells, mGC-G manifests as a membrane-bound glycoprotein, which can form either homomeric or heteromeric complexes with the natriuretic peptide receptor GC-A. It exhibits marked cGMP-generating GC activity; however, notably, all ligands known to activate other receptor GCs failed to stimulate enzymic activity. The unique testis-enriched expression of mGC-G, which is completely different from the broader tissue distribution of rat GC-G, suggests the existence of as-yet-unidentified ligands and unappreciated species-specific physiological functions mediated through mGC-G/cGMP signalling in the testis.

Key words: cGMP, guanylate cyclase, receptor, testis, signal transduction.

INTRODUCTION

Intracellular second-messenger cGMP is involved in the regulation of a broad spectrum of physiological processes, such as relaxation of smooth muscle, regulation of epithelial electrolyte and fluid transport, cellular growth and differentiation and phototransduction in the retina [1,2]. However, probably, the full range of cellular functions modulated by cGMP is still not completely understood.

In the mammal, cGMP is synthesized by two major classes of biochemically distinct enzymes: the soluble and the membrane-associated receptors, GCs (guanylate cyclases). To date, seven membrane forms of GCs have been described in mammals, termed GC-A to GC-G in the order of their discovery [3–12]. These proteins appear to form a family of cell-surface receptors [13] and are characterized by the presence of four distinct domains: an extracellular ligand-binding domain, a single membrane-spanning domain and a cytoplasmic region that contains the signature domains of receptor GCs (e.g. a protein kinase-like domain and a C-terminal cyclase catalytic domain). GC-A and GC-B function as receptors for NPs (natriuretic peptides) [3–6] and GC-C encodes an intestinal receptor for bacterial heat-stable enterotoxins [7] and for two endogenous peptides, namely guanylin and uroguanylin [14,15]. The other four membrane GCs remain as orphan receptors [8,12].

GC-G is the last member of the membrane GC forms to be identified [11]. So far, it has been identified only in the rat species, where it is expressed in the lung, small intestine, skeletal muscles and kidney [11,16,17]. However, the precise biological functions of this newly identified GC are still unknown. We aimed to facilitate physiological studies in the murine system, i.e. to take advantage of gene-targeting technologies to unravel the functions of GC-G; therefore, in the present study, we identified and characterized the apparent mouse homologue, which we have termed mGC-G (mouse GC-G). Overproduction of mGC-G in HEK-293T cells (human embryonic kidney 293T cells) or COS-7 cells resulted in the expression of GC activity, but all ligands known to activate other receptor GCs failed to stimulate enzymic activity. Real-time quantitative RT (reverse transcriptase)–PCR and Northern-blot analyses revealed that mGC-G is highly and exclusively expressed in the testis, where it might interact with a still unknown novel class of ligands that signal through cGMP. The unique testis expression of mGC-G, which is completely different from that of rGC-G, suggests that GC-G exerts different and species-specific physiological functions.

EXPERIMENTAL

Reagents

Synthetic rat ANP (atrial NP) and CNP (C-type NP) were obtained from Bachem (Heidelberg, Germany) and mouse BNP (B-type NP) from American Peptide Co. (Sunnyvale, CA, U.S.A.). Rat guanylin and uroguanylin were obtained from Peptide Institute (Osaka, Japan) and the NO (nitric oxide) donor SIN-1 (3-morpholinosydnonimine) was from Biolog (Bremen, Germany).

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECD, extracellular domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, guanylate cyclase; mGC-G and rGC-G, mouse and rat GC-G respectively; IBMX, isobutylmethylxanthine; SIN-1, 3-morpholinosydnonimine; NP, natriuretic peptide; ANP, BNP and CNP, atrial, B-type and C-type NPs respectively; RT, reverse transcriptase.

[1] To whom correspondence should be addressed (e-mail rbyang@ibms.sinica.edu.tw).

Quantitative real-time RT–PCR (TaqMan) analysis

The assays were performed using the Applied Biosystems PRISM 7700 sequence detection system with a panel of mouse tissue cDNAs (BD Clontech, Palo Alto, CA, U.S.A.). Normalization was performed using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels as controls. The following primers and fluorescent probes were used: for mGC-G, forward primer: 5′-agg cct cag gaa cat gtc gt-3′, reverse primer: 5′-cgc ctt gca tcc tcc g-3′ and the mGC-G probe: 5′-tgt cgc cag ggt cat cat cct aat ctg-3′; for GADPH, forward primer: 5′-ggc aaa ttc aac ggc aca gt-3′, reverse primer: 5′-aag atg gtg atg ggc ttc cc-3′ and the GADPH probe: 5′-aag gcc gag aat ggg aag ctt gtc atc-3′.

Full-length cDNA cloning

One pair of oligonucleotides (5′-gcggtacctgggctgggaacata-3′ and 5′-tggggatcacagcagtcacagtca-3′) was designed based on the gene prediction in a public mouse genome database (www.ensemble.org) and it was used to amplify a 920 bp PCR fragment of mGC-G (nt 534–1453) from mouse testis cDNA (BD Clontech). This PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and sequenced to confirm that it encoded a part of the ECD (extracellular domain) of mGC-G (amino acids 178–484). This fragment was then labelled with [^{32}P]dCTP and used as a probe to screen a mouse testis large-insert cDNA library (BD Clontech). Five positive cDNA clones were purified and rescued according the manufacturer's instructions. All the clones corresponded to the mGC-G sequences. The longest cDNA clone obtained lacked 293 bp in the 5′-region of exon 1. Therefore the predicted exon 1 of mGC-G deduced from rGC-G (rat GC-G) [11] was amplified from mouse genomic DNA and joined to the longest cDNA clone to obtain the physical full-length clone. The final full-length clone of mGC-G was confirmed by sequencing.

Northern-blot analyses

Mouse tissue Northern blots were purchased from BD Clontech or OriGene (Rockville, MD, U.S.A.) and hybridized with a radiolabelled 0.9 kb cDNA probe (nt 534–1453) according to the manufacturer's instructions. After hybridization overnight, the blots were washed once at room temperature (25 °C) and twice at 65 °C with 0.1 × SSC (15 mM NaCl/1.5 mM sodium citrate) and 0.1 % SDS (each wash for 1 h). Autoradiography was performed at − 80 °C for 2 days. The blot was then stripped and hybridized with a β-actin probe as a positive control.

Expression plasmids

The following epitope-tagged expression constructs of mGC-G were prepared by cloning the PCR fragment encoding the mature mGC-G (amino acids 44–1100) into the mammalian expression vectors. The FLAG tag (DYKDDDDK) was added at the N-terminus, followed by the mature mGC-G (amino acids 44–1100) into the pFLAG-CMV-1 expression vector (Sigma, St. Louis, MO, U.S.A.). The pSecTag2 vector (Invitrogen, Carlsbad, CA, U.S.A.) was used to add a Myc tag (EQKLISEEDL) at the C-terminus of mGC-G. For epitope-tagging of rGC-A, the FLAG epitope was added after the signal peptide cleavage site (thereby representing the N-terminus of the processed receptor), whereas the Myc tag was introduced at the C-terminus using Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.).

Cell culture and transfection

HEK-293T cells and monkey kidney COS-7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10 % (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded on plates overnight and then transfected with FuGENE™ 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). For stable expression, HEK-293T cells were co-transfected with the FLAG.mGC-G (FLAG-tagged GC-G) expression plasmid and pcDNA4-Myc/His vector (Invitrogen) containing a Zeocin-resistant gene. For selection of Zeocin-resistant clones, 400 μg/ml Zeocin (Invitrogen) was included in the medium. After several rounds of amplification, individual Zeocin-resistant cell lines were examined for expression of mGC-G by Western-blot and flow-cytometric analyses.

GC activity assay

Basal and Triton X-100-stimulated GC activities in membranes prepared from COS-7 cells transiently expressing mGC-G, rGC-A or both GC-G and GC-A were determined as described in [18]. Cells were harvested 48 h after transfection. For preparing membranes, cells were washed twice with ice-cold PBS and scraped into 1 ml of ice-cold homogenization buffer [10 % (v/v) glycerol, 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 10 μg/ml aprotinin]. Cells were disrupted by sonication and centrifuged at 100 000 ***g*** for 20 min at 2 °C, and the membrane pellet was then resuspended in homogenization buffer. Transfection efficiency was assessed by Western-blot analysis. To initiate cyclase activity, 20 μg of membrane protein was incubated in a assay buffer containing 25 mM Hepes, 1 mM IBMX (isobutylmethylxanthine), 2 mM ATP, 2 mM GTP, 30 mM phosphocreatine, 400 μg/ml creatine kinase (185 units/mg), 0.5 mg/ml BSA and 5 mM $MgCl_2$ or $MnCl_2$ as well as 0.1 % (v/v) Triton X-100 as indicated. In additional samples, effects of ANP (1–1000 nM) and BNP (1 μM) were tested. At the end of a 10 min incubation at 37 °C, the reaction was stopped by adding ice-cold 100 % (v/v) ethanol (final concentration, 70 %). After centrifugation (3000 ***g***, 5 min, 4 °C), the supernatants were dried in a speed-vacuum concentrator, resuspended in sodium acetate buffer (50 mM, pH 6.0) and acetylated, and the cGMP content was determined by RIA [19].

Effect of ligands known to activate other receptor GCs on cGMP formation in intact HEK-293T cells stably expressing mGC-G

The effect of known GC-activating ligands on cGMP synthesis by mGC-G was assessed in HEK-293T cells stably transfected with mGC-G (clone 5). In brief, untransfected (as controls) as well as mGC-G-transfected HEK-293T cells were cultured in DMEM, supplemented with 10 % (v/v) heat-inactivated foetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin (Gibco) and 400 μg/ml Zeocin. Experiments were performed with cells grown to confluence in 24-well plates. Before starting the experiments, the cells were washed twice with DMEM and then incubated at 37 °C for 15 min in 300 μl of DMEM with or without 1 mM of the phosphodiesterase inhibitor IBMX. Synthetic peptides (1 μM ANP, BNP, CNP, guanylin and uroguanylin) as well as the NO donor SIN-1 (100 μM) were added to the wells for 10 or 30 min, in both the presence and absence of IBMX. The incubation media were rapidly transferred to 700 μl of ice-cold 100 % ethanol (final concentration, 70 % ethanol). Simultaneously, cellular cGMP synthesis was terminated by adding

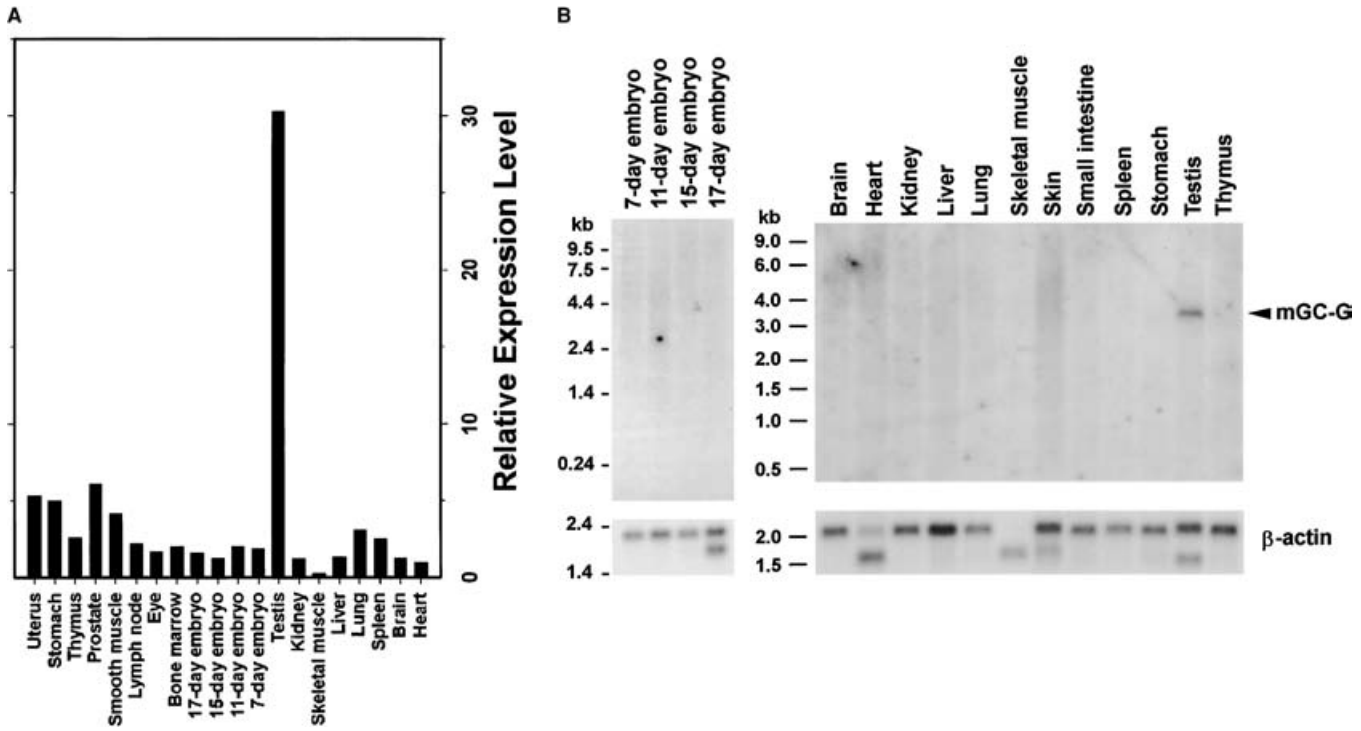

Figure 1 Selective expression of mGC-G in mouse testis

(**A**) Quantitative real-time RT–PCR (TaqMan) analysis of the mGC-G mRNA expression profile. A panel of mouse tissue cDNAs was used for the TaqMan analysis with mGC-G-specific oligonucleotide pairs and probes. Expression levels were normalized to GAPDH. (**B**) Northern-blot analysis of polyadenylated mRNA obtained from a variety of mouse tissues. Polyadenylated mRNA (2 μg) obtained from whole mouse embryos at different stages (left panel) or from different adult tissues (right panel) was hybridized with a 0.9 kb cDNA 5′-fragment of mGC-G. The mGC-G probe identified an approx. 3.5 kb transcript in adult testis, but in none of the other tissues examined. The lower panel shows the same blot hybridized with β-actin probe as a control.

0.1 ml of ice-cold 70 % ethanol to the cells, which remained completely adherent to the wells. After freeze–thawing, the samples were dried and then resuspended in 50 mM sodium acetate buffer (pH 6.0). Supernatant fractions were acetylated and both extracellular (in the incubation media) and intracellular cGMP contents were measured by RIA [19].

Immunoprecipitation and Western-blot analyses

Transfected cells were washed once with PBS and lysed for 15 min on ice using 0.5 ml of lysis buffer (20 mM Tris/HCl, pH 7.5/ 150 mM NaCl/1 mM EDTA/1 mM EGTA/1 % Triton X-100/ 25 mM sodium pyrophosphate/1 mM β-glycerophosphate/1 mM Na_3VO_4/1 μg/ml leupeptin). Lysates were clarified by centrifugation at 10000 ***g*** for 15 min at 4 °C. Cells lysates were incubated with 1 μg of the indicated antibodies and 20 μl of 50 % (v/v) Protein A–agarose (Pierce, Rockford, IL, U.S.A.) for 2 h with gentle rocking. After two washes with lysis buffer, the immunoprecipitated complexes were solubilized by boiling in Laemmli sample buffer, fractionated by SDS/PAGE and transferred on to PVDF membranes. The membranes were blocked with PBS (pH 7.5) containing 0.1 % (v/v) gelatin and 0.05 % (v/v) Tween 20 and then incubated with various antibodies indicated in the Results and discussion section. After two washes, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Groove, PA, U.S.A.) for 1 h. After washing the membranes, the immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL, U.S.A.).

In situ hybridization

The region corresponding to nucleotides 534–1453 of mGC-G was amplified by PCR and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) as a probe. [^{35}S]cRNA sense and anti-sense probes were transcribed from the linearized plasmid and hybridized on mouse testicular and epididymal sections.

RESULTS AND DISCUSSION

Identification and tissue expression of mGC-G

As a step towards elucidating the physiological function of GC-G, the goal of the present study was to identify and characterize the mouse homologue. Despite a comprehensive, large-scale sequencing of mouse expressed sequence tags [20], only two partial-length clones of mGC-G were identified (GenBank® accession nos. AK049940 and AK010727). To obtain the full-length sequence of mGC-G, we first performed gene prediction studies based on its putative homology to rGC-G [11], using a public mouse genome database (www.ensembl.org). On the basis of this gene prediction, the mGC-G-specific oligonucleotide primers and probe were designed to study the expression profile of mGC-G by real-time quantitative RT–PCR (TaqMan). Interestingly, when a panel of cDNAs derived from a number of mouse embryonic and adult tissues was assessed for mGC-G expression by TaqMan analysis, mGC-G mRNA was highly and selectively expressed in adult mouse testis (Figure 1A). To verify this testis-enriched expression, we further performed semi-quantitative RT–PCR in a panel of mouse tissue cDNAs. A 920 bp

mGC-G-specific cDNA fragment, encoding a part of the ECD, was amplified from cDNA of adult mouse testis, but not from the other tissues examined (results not shown).

This fragment was subsequently confirmed by sequencing, and used as a probe to evaluate tissue expression by Northern-blot analysis. As shown in Figure 1(B), mGC-G mRNA is indeed selectively and uniquely expressed in mouse testis (Figure 1B), in good agreement with TaqMan analysis (Figure 1A). The expression in all other adult and embryonic tissues was virtually undetectable (Figure 1B). The testis-enriched expression of mGC-G was re-examined and confirmed by an independent commercially available tissue Northern blot (results not shown). Therefore the markedly different tissue expression profiles of mGC-G and rGC-G, the latter being expressed in the lung, intestine, skeletal muscles and kidney [11,17], indicate that GC-G probably exerts its biological functions in a species-specific manner. Notably, GC-G expression was not detected in rat testis [11,16] (M. Kuhn and R.-B. Yang, unpublished work). Moreover, unlike the presence of multiple splice variants of rGC-G transcripts, a single mGC-G transcript of approx. 3.5 kb was detected, consistent with the genome-predicted and cloned full-length cDNA (see below and Figure 2A). However, we could not identify the specific cells expressing GC-G in mouse testis by *in situ* hybridization. It is possible that the mRNA expression level in individual cells is below the sensitivity limit of the *in situ* hybridization method we used. Since the GC-G mRNA is expressed in a relative low level in mouse testis, it is possible that GC-G mRNAs of other transcript sizes are present in other tissues or cell types but were not detected by the methodologies used in the present study.

Full-length cloning of mGC-G

Since the mGC-G transcripts are enriched in the testis, the mGC-G-specific cDNA fragment, identified as described above, was used to screen and isolate a full-length clone from a mouse testis library. This cDNA contains an open reading frame of 3300 nucleotides and encodes a polypeptide of 1100 amino acids, which is consistent with the gene prediction in the mouse genome database (Figure 2A). Hydropathy [21] and protein family [22] analyses predicted a 43-residue N-terminal signal peptide, followed by a 435-residue putative ligand-binding ECD, a 25-amino-acid membrane-spanning segment and a 597-amino-acid cytoplasmic region, which can be subdivided into (i) a protein kinase-like domain (amino acids 563–838), followed by (ii) a cyclase catalytic domain (amino acids 892–1078) at the C-terminus (Figure 2A). In addition, the ECD contains nine potential sites for N-linked glycosylation (Figure 2A).

Phylogenetic analysis comparing the protein sequence of mGC-G with that of other rat receptor GCs (rGC-A to rGC-G) revealed that mGC-G and rGC-G are clustered together, sharing 84% similarity in the ECD and an overall 88% sequence similarity (Figure 2B). In addition, physical localization of the mGC-G gene in the distal region of mouse chromosome 19, as described in the public mouse genome database [20], is consistent with the chromosomal mapping of the GC-G locus determined by the interspecific backcross analysis [11]. These results strongly suggest that mGC-G is the mouse orthologue of rGC-G (Figure 2B). Out of seven cysteine residues within the ECD of mGC-G, five (Cys-97, Cys-123, Cys-250, Cys-458 and Cys-465) align with five of the six residues located in the NP receptor GC-A (Figure 2A). These cysteine residues are also invariant in other receptor GCs [11,23] and have been shown to play a critical role in maintaining proper disulphide bonds and the ligand-binding function of receptor GCs

Figure 2 Deduced amino acid sequence and phylogenetic analysis of mGC-G

(**A**) Primary sequence of the 1100-amino-acid mGC-G as deduced from the full-length cDNA clone. The signal peptide, transmembrane, protein kinase-like domains and cyclase catalytic domains are marked. Five invariant cysteine residues, conserved among mGC-G and NP receptors, are indicated by arrows. Nine potential sites for N-linked glycosylation are underlined in boldface. (**B**) Dendrogram analysis of the mammalian GC receptor family. Similarity of mGC-G ECD to ECD of other rat GCs (rGC-A to rGC-G) was analysed by the Lasergene MEGALIGN program (Clustal algorithm with PAM250 weight table). Branch order indicates structural relativeness, and branch length reflects sequence identity.

[24,25]. Since mGC-G shares a high degree of similarity with the NP receptor subfamily (Figure 2B) and since five extracellular cysteine residues known to be critical for ligand-binding and receptor functions are conserved among GC receptors for NPs (GC-A and GC-B), it is tempting to speculate that the putative ligands for mGC-G, if any, may resemble an NP-like structure.

mGC-G is an N-glycosylated cell-surface protein

Since mGC-G possesses nine putative N-linked glycosylation motifs (Figure 2A), we hypothesized that mGC-G was subjected

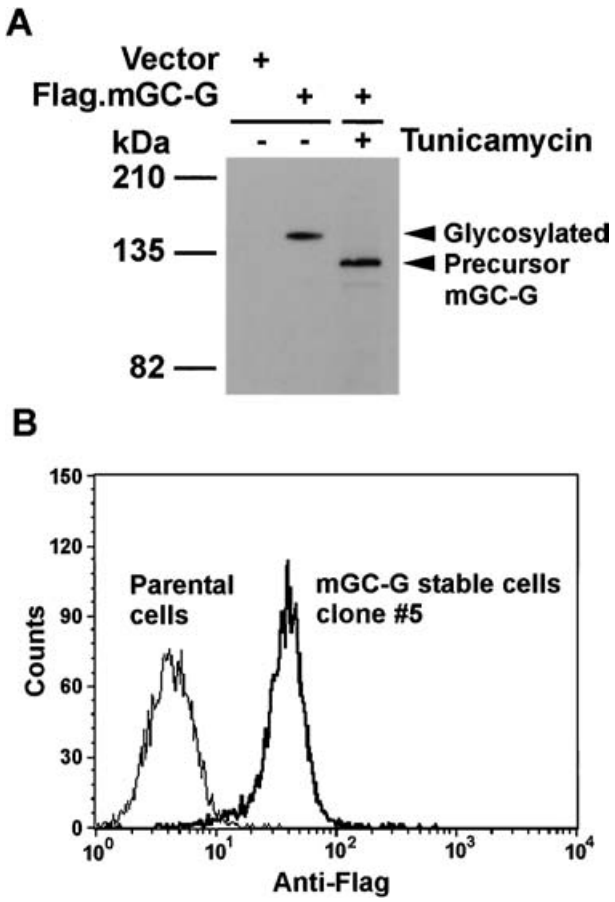

Figure 3 Glycosylation and cell-surface expression of mGC-G

(**A**) mGC-G protein is an N-glycosylated protein. HEK-293T cells were transfected with the expression vector encoding FLAG-tagged mGC-G (FLAG.mGC-G). Transfected cells were cultured in the absence (−) or presence (+) of the N-glycosylation inhibitor tunicamycin (5 μg/ml) for 24 h. Cell lysates were analysed by Western blotting with anti-FLAG M2 antibody. (**B**) mGC-G is a cell-surface protein. HEK-293T cells stably expressing FLAG.mGC-G were collected and stained with anti-FLAG M2 monoclonal antibody for FACS analysis.

to post-translational modification by glycosylation, similar to other receptor GCs [26–28]. Thus we examined whether tunicamycin, an inhibitor of N-glycosylation, affected the molecular mass of expressed mGC-G. As shown in Figure 3(A), the apparent molecular mass of expressed FLAG-tagged GC-G protein is approx. 145 kDa. Tunicamycin treatment of the cells resulted in a decrease in the mass of the precursor form of mGC-G (to approx. 120 kDa), suggesting that most of the mGC-G is glycosylated when expressed in HEK-293T cells. Next, we examined whether mGC-G is targeted to the cell surface. HEK-293T cells stably expressing FLAG.mGC-G (clone 5) were incubated with an anti-FLAG M2 antibody, followed by an FITC-conjugated goat anti-mouse IgG secondary antibody and then analysed by flow cytometry. As shown in Figure 3(B), expression of FLAG-tagged mGC-G in the stable clone 5 resulted in a shift of the entire cell population stained with an anti-FLAG antibody by FACS analysis, suggesting that mGC-G behaves like a receptor and is targeted to the cell surface. Taken together, these results demonstrate that the molecular and biochemical properties of mGC-G are in accordance with those of other receptor GCs, i.e. they all are cell-surface glycoproteins with molecular masses over the range 120–160 kDa [27,28].

Basal and Triton X-100-stimulated mGC-G activities

To examine whether mGC-G exhibits GC activity, cGMP-synthesizing activity was estimated in membranes prepared from COS-7 cells transiently expressing mGC-G. As shown in Figure 4, in the presence of Mg^{2+}, the GC activity in mGC-G-overexpressing membranes was approx. 4-fold greater when compared with mock-transfected COS-7 cells. Mn^{2+} or $Mn^{2+} + 0.1\%$ Triton X-100 increased mGC-G activity by approx. 3- and 9-fold respectively. ANP and BNP (both at 1 μM) had no effect on mGC-G activity. Therefore mGC-G indeed possesses an intrinsic GC activity that is not stimulated by NPs. For comparison, GC activity was also measured in membranes of COS-7 cells transiently expressing GC-A. As shown in Figure 4, in the presence of Mg^{2+}, the GC activities in GC-A- and mGC-G-overexpressing membranes were similar. Mn^{2+} or $Mn^{2+} + 0.1\%$ Triton X-100 markedly increased GC-A activity by approx. 14- and 27-fold respectively. ANP or BNP (both at 1 μM) also clearly increased GC-A activity by 24- and 20-fold respectively.

Effect of NPs on cGMP content of mGC-G-expressing HEK-293T cells

To facilitate searches for a putative ligand, the mGC-G cDNA was stably expressed in HEK-293T cells. As shown in Figure 5, in the presence of 1 mM IBMX, the intracellular cGMP content of mGC-G-expressing HEK-293T cells was approx. 2400-fold greater when compared with untransfected HEK-293T cells incubated under identical conditions. The addition of high concentrations (1 μM) of the peptide ligands known to stimulate other receptor GCs (ANP, BNP, CNP, guanylin or uroguanylin) did not significantly affect intracellular cGMP concentrations in mGC-G-expressing HEK-293T cells during a 10 or 30 min incubation period (Figure 5). The NO donor SIN-1 (up to 0.1 mM), an activator of soluble GC, also did not affect the cGMP content of mGC-G-expressing HEK-293T cells (results not shown). In the absence of IBMX, the intracellular cGMP contents of both mGC-G-expressing as well as untransfected HEK-293T cells were lower by approx. 30% when compared with the respective IBMX-treated cell lines. Again, the intracellular cGMP content of mGC-G-expressing cells was approx. 2300-fold greater when compared with untransfected HEK-293T cells and it was not altered by the addition of any of the above-mentioned peptides or SIN-1 ($n = 4$ per condition; results not shown). Lastly, within the same experiments and both in the presence and absence of IBMX, a very high cGMP content was also detected in the extracellular media of mGC-G-expressing cells (254 ± 22 and 397 ± 15 pmol/well respectively). Again, the extracellular cGMP content was not significantly modified by the presence of the synthetic peptides or SIN-1. Taken together, these results indicate that mGC-G has a very high basal cGMP-synthesizing activity that is not modulated by ligands known to stimulate other GCs.

Previous studies on the first membrane GC identified, namely the NP receptor GC-A, suggested that ligand binding induces a conformational change in the juxtamembrane hinge structure to mediate transmembrane signal transduction [29,30]. These studies also demonstrated that the elimination of the juxtamembrane intramolecular disulphide bond by Cys to Ser mutations resulted in a constitutively active GC-A receptor [29,30]. However, double mutations of the corresponding juxtamembrane Cys to Ser (residues 458 and 465) within mGC-G had no effect on mGC-G activity (results not shown), suggesting that, after binding a putative ligand, mGC-G may utilize a different regulatory mechanism from GC-A.

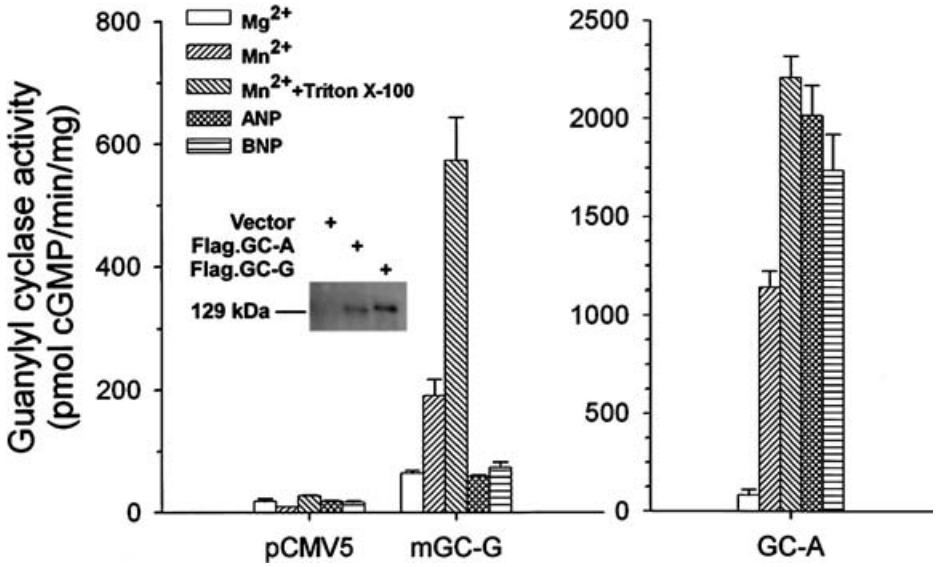

Figure 4 GC activity in membranes of COS-7 cells overexpressing mGC-G

Membranes of COS-7 cells transiently expressing FLAG.mGC-G were assayed for GC activity in the presence of 5 mM Mg^{2+} or 5 mM Mn^{2+} or 5 mM Mn^{2+} + 0.1 % Triton X-100 or 1 μM ANP or 1 μM BNP (in the presence of 5 mM Mg^{2+}). As a control, GC activity was also measured in membranes of mock- or FLAG.GC-A-transfected COS-7 cells. Results are expressed as means $\pm$ S.E.M. ($n = 4$). Cell lysates were immunoblotted with anti-FLAG antibodies to examine the protein expression levels (inset).

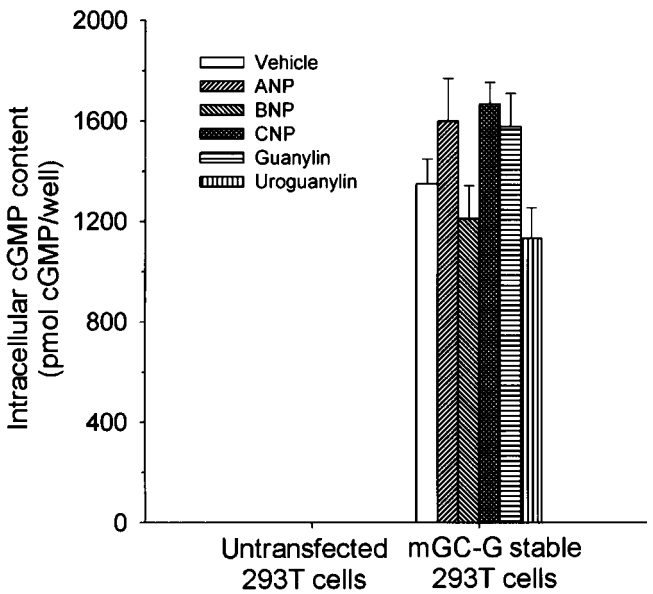

Figure 5 Effect of known stimulators of GC-A (ANP and BNP), GC-B (CNP) and GC-C (guanylin and uroguanylin) on the intracellular cGMP content of untransfected and stably mGC-G-transfected HEK-293T cells

Cells were incubated with 1 μM of all peptides for 10 min in the presence of 1 mM IBMX. After aspirating the medium, intracellular cGMP content was measured by RIA. The cGMP content of mGC-G-expressing HEK-293T cells was approx. 2400-fold greater when compared with untransfected HEK-293T cells and was not significantly affected by either peptide. Results are expressed as means $\pm$ S.E.M. ($n = 4$).

As mentioned above, the basal cGMP levels in stably mGC-G-expressing HEK-293T cells were much higher compared with those of control, untransfected cells (Figure 5). This observation might indicate that HEK-293T cells cannot effectively regulate mGC-G activity, which might be due to the lack of specific regulatory proteins. It is noteworthy that two retinal receptor GCs, GC-E and GC-F, also showed dysregulated, increased cGMP production when expressed in cells [9,12]. The activity of both GC-E and GC-F appears to be tightly regulated, in a calcium-dependent manner, by a family of intracellular Ca^{2+}-binding regulators [e.g. GCAP (GC-activating protein)], which bind physically to the intracellular domain of these receptor GCs [10,31–34]. Although the precise molecular mechanism underlying the increased basal activity of mGC-G is still unknown, whether or not a novel class of intracellular regulators, such as GCAP, exists to modulate mGC-G activity in the testis remains an intriguing issue for our future studies.

Homo- and hetero-oligomerization of mGC-G

Since previous studies have documented that other receptor GCs can assemble into heteromeric or homomeric complexes in the absence of ligands [35–39], we hypothesized that oligomeric forms of mGC-G may exist. The differential epitope-tagged versions of mGC-G, FLAG.mGC-G and mGC-G.Myc, were singly transfected or co-transfected in HEK-293T cells. We then examined their association with the anti-FLAG antibody by co-immunoprecipitation. The precipitates were subsequently analysed by immunoblotting with an anti-Myc antibody (Figure 6). An immunoreactive band of 145 kDa recognized by the anti-Myc antibody was observed in the anti-FLAG immunoprecipitates from cells co-expressing FLAG.mGC-G and mGC-G.Myc proteins (Figure 6, top panel). In addition, to ascertain the formation of heteromeric complexes, differentially epitope-tagged mGC-G and rGC-A constructs were co-transfected in HEK-293T cells and immunoprecipitations were performed. When FLAG.mGC-G was co-expressed with rGC-A.Myc, immunoprecipitation with a FLAG antibody co-precipitated the Myc–rGC-A protein (Figure 6, top panel). In addition, the reciprocal immunoprecipitation using the Myc antibody, followed by anti-FLAG Western-blot analysis, led to a similar result (Figure 6, second panel). As a control, the homomeric interaction of GC-A was verified by a similar pull-down assay, consistent with previous studies

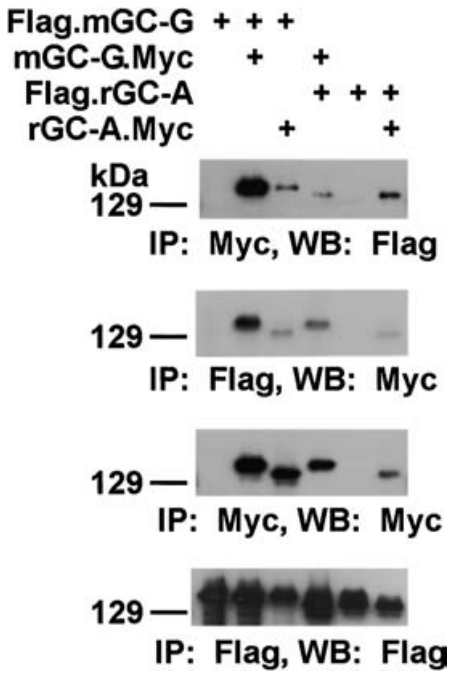

Figure 6 Homo- and hetero-oligomerization of mGC-G in transfected HEK-293T cells

HEK-293T cells were transfected with differential epitope-tagged rGC-A (1 μg) and/or mGC-G (1 μg). The total amount of DNA was kept constant in all transfections by supplementing with pCMV5 vector DNA. After 2 days, detergent lysates of each transfection were subjected to immunoprecipitation (IP) by FLAG or Myc antibodies, followed by immunoblotting (WB) using FLAG or Myc antibodies respectively to determine the amount of associated proteins. Cell lysates were also immunoblotted with anti-FLAG or Myc antibodies to examine the protein expression levels (third and fourth panels). Experiments were repeated twice with similar results.

[35,36]. We did not observe an association between mGC-G and human interleukin-1 receptor, suggesting the specificity of this assay (results not shown). Taken together, these results demonstrate that mGC-G protein is capable of forming both homomers or heteromeric complexes, at least in HEK-293T cells.

The interaction between mGC-G and rGC-A (Figure 6) suggests the possibility that a heteromeric mGC-G–GC-A complex may exist *in vivo*. If so, such a receptor may have novel ligand specificities or may modify the responsiveness of GC-A to ANP and BNP. To test this possibility, GC-A and GC-G expression plasmids (5 μg each) were singly transfected or co-transfected into COS-7 cells. Membranes of transfected COS-7 cells were harvested 2 days after transfection, and subjected to Western-blot analysis and GC activity assays in the presence of 5 mM Mg^{2+}. As shown in Figure 7, co-expression of mGC-G with rGC-A did not affect GC-A expression (inset) or ANP (1–100 nM)-stimulated GC-A activity. Thus, whether this heteromeric interaction indeed occurs *in vivo*, in the testis, as well as the physiological consequences, remain to be determined.

Interestingly, a recent study showed that BNP can stimulate a small but significant amount of GC activity in certain tissues from GC-A-deficient mice, such as testis and adrenal gland [40], strongly suggesting that there is an as-yet-unidentified (cGMP-synthesizing) BNP receptor. However, mouse BNP failed to stimulate mGC-G in our experiments (Figure 4). Therefore probably mGC-G is not the corresponding BNP-preferring receptor described in [40]. Alternatively, it remains to be determined whether heteromeric mGC-G complexes with other receptors exist *in vivo* and are responsible for this BNP-preferring receptor activity preferentially expressed in the testis.

An important and unresolved issue which we will address in future studies concerns the specific physiological functions that are modulated by mGC-G in the testis. Many published studies have shown that cyclic nucleotides, especially cGMP, modulate

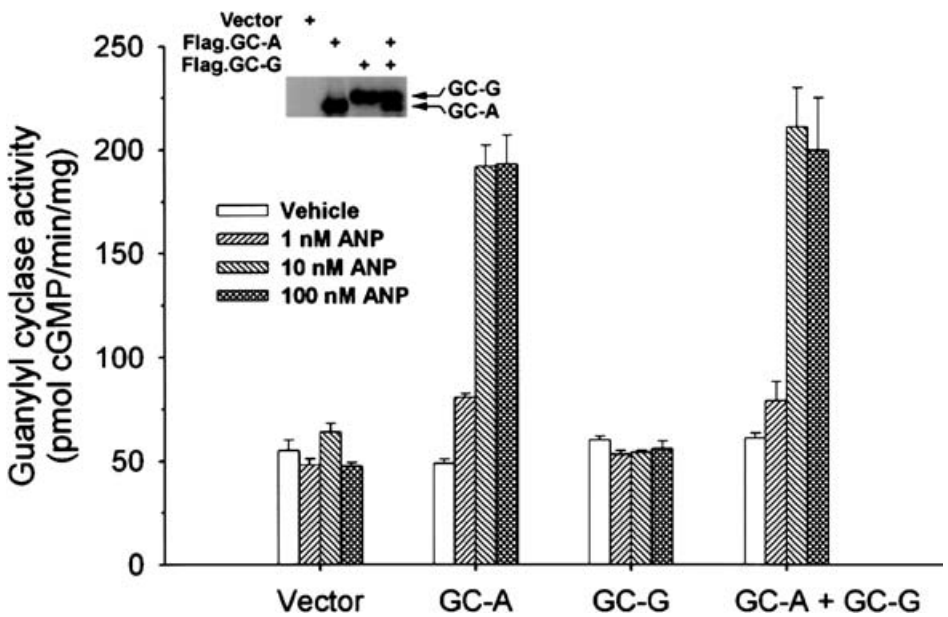

Figure 7 ANP-dependent GC activity in membranes of COS-7 cells co-expressing mGC-G and rGC-A

COS-7 cells were transfected with FLAG-tagged rGC-A (5 μg) and/or mGC-G (5 μg). The total amount of DNA was kept constant in all transfections by supplementing with pCMV5 vector DNA. After 2 days, membranes of COS-7 cells transiently expressing FLAG.mGC-G, rGC-A or both GC-G and GC-A were assayed for GC activity in the absence and presence of ANP (1–100 nM). As a control, GC activity was also measured in membranes of mock-transfected COS-7 cells. Results are expressed as means $\pm$ S.E.M. ($n = 4$). In parallel, cell lysates were immunoblotted with anti-FLAG antibodies to examine the protein expression levels (inset).

vertebrate or invertebrate spermatozoa functions (e.g. spermatozoa motility, chemoattraction or acrosomal exocytosis) and other cellular processes in the testis [41–44]. In addition, it has been shown that the α- and β-subunits of cyclic nucleotide-gated Ca^{2+} channels, which are specific targets of cyclic nucleotide signalling, are expressed in spermatozoa and precursor cells [45,46]. We are currently raising mGC-G-specific antibodies to determine whether mGC-G is expressed in germ cells and is functionally coupled with these cyclic nucleotide-gated channels.

As a candidate approach, we utilized chromosomal localization of the GC-G gene that is mapped to the distal region of mouse chromosome 19 or on human chromosome 10q25 [11], to search the disease databases. After extensive reviewing of disease databases, including the Online Mendelian Inheritance in Man (http://www.ncbi.nlm.gov/omim/) and Mouse Genome Database [47], there is no apparent testis- or reproduction-related disorder that has been ascribed to the GC-G locus. In summary, we have identified and characterized a novel receptor GC (mGC-G) that is selectively expressed in mouse testis. The molecular and biochemical properties of mGC-G are similar to those of other receptor GCs. All known GC-activating ligands failed to stimulate mGC-G activity, and therefore it remains an orphan receptor. Its unique tissue expression points to unexplored functions mediated by mGC-G signalling through cGMP in mammalian testis.

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REFERENCES

1 Warner, T. D., Mitchell, J. A., Sheng, H. and Murad, F. (1994) Effects of cyclic GMP on smooth muscle relaxation. Adv. Pharmacol. **26**, 171–194
2 Vaandrager, A. B. and de Jonge, H. R. (1994) Effect of cyclic GMP on intestinal transport. Adv. Pharmacol. **26**, 253–283
3 Chinkers, M., Garbers, D. L., Chang, M.-S., Lowe, D. G., Chin, H., Goeddel, D. V. and Schulz, S. (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. Nature (London) **338**, 78–83
4 Lowe, D. G., Chang, M. S., Hellmiss, R., Chen, E., Singh, S., Garbers, D. L. and Goeddel, D. V. (1989) Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. EMBO J. **8**, 1377–1384
5 Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H. and Garbers, D. L. (1989) The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. Cell (Cambridge, Mass.) **58**, 1155–1162
6 Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H. and Goeddel, D. V. (1991) Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). Science **252**, 120–123
7 Schulz, S., Green, C. K., Yuen, P. S. and Garbers, D. L. (1990) Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell (Cambridge, Mass.) **63**, 941–948
8 Fulle, H.-J., Vassar, R., Foster, D. C., Yang, R.-B., Axel, R. and Garbers, D. L. (1995) A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons. Proc. Natl. Acad. Sci. U.S.A. **92**, 3571–3575
9 Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V. and Lowe, D. G. (1992) Molecular cloning of a retina-specific membrane guanylyl cyclase. Neuron **9**, 727–737
10 Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L. and Hurley, J. B. (1995) Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2. Proc. Natl. Acad. Sci. U.S.A. **92**, 5535–5539
11 Schulz, S., Wedel, B. J., Matthews, A. and Garbers, D. L. (1998) The cloning and expression of a new guanylyl cyclase orphan receptor. J. Biol. Chem. **273**, 1032–1037
12 Yang, R.-B., Foster, D. C., Garbers, D. L. and Fulle, H.-J. (1995) Two membrane forms of guanylyl cyclase found in the eye. Proc. Natl. Acad. Sci. U.S.A. **92**, 602–606
13 Tamura, N., Chrisman, T. D. and Garbers, D. L. (2001) The regulation and physiological roles of the guanylyl cyclase receptors. Endocrinol. J. **48**, 611–634
14 Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L. and Smith, C. E. (1992) Guanylin: an endogenous activator of intestinal guanylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **89**, 947–951
15 Hamra, F. K., Forte, L. R., Eber, S. L., Pidhorodeckyj, N. V., Krause, W. J., Freeman, R. H., Chin, D. T., Tompkins, J. A., Fok, K. F., Smith, C. E. et al. (1993) Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **90**, 10464–10468
16 Matsuoka, I., Mori, T., Sato, T., Sakai, M. and Kurihara, K. (1995) Identification of novel guanylyl cyclases from chemosensory tissues of rat and cattle. Biochem. Biophys. Res. Commun. **216**, 242–248
17 Kojima, M., Hisaki, K., Matsuo, H. and Kangawa, K. (1995) A new type soluble guanylyl cyclase, which contains a kinase-like domain: its structure and expression. Biochem. Biophys. Res. Commun. **217**, 993–1000
18 Holtwick, R., van Eickels, M., Skryabin, B. V., Baba, H. A., Bubikat, A., Begrow, F., Schneider, M. D., Garbers, D. L. and Kuhn, M. (2003) Pressure-independent cardiac hypertrophy in mice with cardiomyocyte-restricted inactivation of the atrial natriuretic peptide receptor guanylyl cyclase-A. J. Clin. Invest. **111**, 1399–1407
19 Kuhn, M., Raida, M., Adermann, K., Schulz-Knappe, P., Gerzer, R., Heim, J. M. and Forssmann, W. G. (1993) The circulating bioactive form of human guanylin is a high molecular weight peptide (10.3 kDa). FEBS Lett. **318**, 205–209
20 The FANTOM Consortium and the RIKEN Genome Exploration Research Group Phase I and II Team (2002) Analysis of the mouse transcriptome based on functional annotation of 60 770 full-length cDNAs. Nature (London) **420**, 563–573
21 Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**, 105–132
22 Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M. and Sonnhammer, E. L. (2002) The Pfam protein families database. Nucleic Acids Res. **30**, 276–280
23 Yu, S., Avery, L., Baude, E. and Garbers, D. L. (1997) Guanylyl cyclase expression in specific sensory neurons: a new family of chemosensory receptors. Proc. Natl. Acad. Sci. U.S.A. **94**, 3384–3387
24 McNicoll, N., Gagnon, J., Rondeau, J. J., Ong, H. and de Lean, A. (1996) Localization by photoaffinity labeling of natriuretic peptide receptor-A binding domain. Biochemistry **35**, 12950–12956
25 Van den Akker, F., Zhang, X., Miyagi, M., Huo, X., Misono, K. S. and Yee, V. C. (2000) Structure of the dimerized hormone-binding domain of a guanylyl-cyclase-coupled receptor. Nature (London) **406**, 101–104
26 Lowe, D. G. and Fendly, B. M. (1992) Human natriuretic peptide receptor-A guanylyl cyclase. Hormone cross-linking and antibody reactivity distinguish receptor glycoforms. J. Biol. Chem. **267**, 21691–21697
27 Vaandrager, A. B., Schulz, S., de Jonge, H. R. and Garbers, D. L. (1993) Guanylyl cyclase C is an N-linked glycoprotein receptor that accounts for multiple heat-stable enterotoxin-binding proteins in the intestine. J. Biol. Chem. **268**, 2174–2179
28 Koch, K. W., Stecher, P. and Kellner, R. (1994) Bovine retinal rod guanyl cyclase represents a new N-glycosylated subtype of membrane-bound guanyl cyclases. Eur. J. Biochem. **222**, 589–595
29 Labrecque, J., McNicoll, N., Marquis, M. and de Lean, A. (1999) A disulfide-bridged mutant of natriuretic peptide receptor-A displays constitutive activity. Role of receptor dimerization in signal transduction. J. Biol. Chem. **274**, 9752–9759
30 Huo, X., Abe, T. and Misono, K. S. (1999) Ligand binding-dependent limited proteolysis of the atrial natriuretic peptide receptor: juxtamembrane hinge structure essential for transmembrane signal transduction. Biochemistry **38**, 16941–16951
31 Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekar, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., Johnson, R. S. et al. (1994) Molecular cloning and characterization of retinal photoreceptor guanylyl cyclase-activating protein. Neuron **13**, 395–404
32 Dizhoor, A. M., Olshevskaya, E. V., Henzel, W. J., Wong, S. C., Stults, J. T., Ankoudinova, I. and Hurley, J. B. (1995) Cloning, sequencing, and expression of a 24-kDa Ca^{2+}-binding protein activating photoreceptor guanylyl cyclase. J. Biol. Chem. **270**, 25200–25206
33 Haeseleer, F., Sokal, I., Li, N., Pettenati, M., Rao, N., Bronson, D., Wechter, R., Baehr, W. and Palczewski, K. (1999) Molecular characterization of a third member of the guanylyl cyclase-activating protein subfamily. J. Biol. Chem. **274**, 6526–6535
34 Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P. and Hurley, J. B. (1994) The human photoreceptor membrane guanylyl cyclase, RetGC, is present in outer segments and is regulated by calcium and a soluble activator. Neuron **12**, 1345–1352
35 Lowe, D. G. (1992) Human natriuretic peptide receptor-A guanylyl cyclase is self-associated prior to hormone binding. Biochemistry **31**, 10421–10425
36 Chinkers, M. and Wilson, E. M. (1992) Ligand-independent oligomerization of natriuretic peptide receptors. Identification of heteromeric receptors and a dominant negative mutant. J. Biol. Chem. **267**, 18589–18597
37 Yang, R. B. and Garbers, D. L. (1997) Two eye guanylyl cyclases are expressed in the same photoreceptor cells and form homomers in preference to heteromers. J. Biol. Chem. **272**, 13738–13742

38 Goldberg, A. F. and Molday, R. S. (1996) Subunit composition of the peripherin–rds-rom-1 disk rim complex from rod photoreceptors: hydrodynamic evidence for a tetrameric quaternary structure. Biochemistry **35**, 6144–6149
39 Vaandrager, A. B., van der Wiel, E., Hom, M. L., Luthjens, L. H. and de Jonge, H. R. (1994) Heat-stable enterotoxin receptor/guanylyl cyclase C is an oligomer consisting of functionally distinct subunits, which are non-covalently linked in the intestine. J. Biol. Chem. **269**, 16409–16415
40 Goy, M. F., Oliver, P. M., Purdy, K. E., Knowles, J. W., Fox, J. E., Mohler, P. J., Qian, X., Smithies, O. and Maeda, N. (2001) Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide. Biochem. J. **358**, 379–387
41 Darszon, A., Labarca, P., Nishigaki, T. and Espinosa, F. (1999) Ion channels in sperm physiology. Physiol. Rev. **79**, 481–510
42 Aoki, F., Sakai, S. and Kohmoto, K. (1999) Regulation of flagellar bending by cAMP and Ca^{2+} in hamster sperm. Mol. Reprod. Dev. **53**, 77–83
43 Anderson, R. A., Feathergill, K. A., Drisdel, R. C., Rawlins, R. G., Mack, S. R. and Zaneveld, L. J. (1994) Atrial natriuretic peptide (ANP) as a stimulus of the human acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with *in vitro* fertilization outcome. J. Androl. **15**, 61–70
44 Rotem, R., Zamir, N., Keynan, N., Barkan, D., Breitbart, H. and Naor, Z. (1998) Atrial natriuretic peptide induces acrosomal exocytosis of human spermatozoa. Am. J. Physiol. **274**, E218–E223
45 Weyand, I., Godde, M., Frings, S., Weiner, J., Muller, F., Altenhofen, W., Hatt, H. and Kaupp, U. B. (1994) Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. Nature (London) **368**, 859–863
46 Wiesner, B., Weiner, J., Middendorff, R., Hagen, V., Kaupp, U. B. and Weyand, I. (1998) Cyclic nucleotide-gated channels on the flagellum control Ca^{2+} entry into sperm. J. Cell Biol. **142**, 473–484
47 Blake, J. A., Richardson, J. E., Bult, C. J., Kadin, J. A. and Eppig, J. T. (2003) MGD: the Mouse Genome Database. Nucleic Acids Res. **31**, 193–195

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