Regulation of the Guanylyl Cyclase-B Receptor by Alternative Splicing*

Received for publication, August 6, 2003, and in revised form, September 23, 2003
Published, JBC Papers in Press, September 26, 2003, DOI 10.1074/jbc.M308680200

Naohisa Tamura§ and David L. Garbers§§¶¶

From the §Cecil H. and Ida Green Center for Reproductive Biology Sciences, the ¶Department of Pharmacology, and the ¶¶Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041

Guanylyl cyclase-B (GC-B) is a single transmembrane receptor that binds C-type natriuretic peptide (CNP). The ligand/receptor appears critical in the regulation of cell proliferation and differentiation where it acts as an adversary of mitogenic signaling pathways. We have isolated three guanylyl cyclase-B isoforms generated from a single gene by alternative splicing and termed them GC-B1, GC-B2, and GC-B3. GC-B1 is full-length and responds maximally to CNP, GC-B2 contains a 25-amino acid deletion in the protein kinase homology domain, and GC-B3 only retains a part of the extracellular ligand-binding domain. GC-B2 binds CNP, but the ligand fails to activate the cyclase, while GC-B3 fails to bind ligand. When GC-B2 or GC-B3 is expressed coincident with GC-B1, they act as dominant negative isoforms by virtue of blocking formation of active GC-B1 homodimers. Relative expression levels of GC-B1, GC-B2, and GC-B3 vary across tissues and as a function of in vitro culture; the relative amount of GC-B2 to GC-B1 is repressed in cultured smooth muscle cells relative to endogenous ratios in the medial layer cells of the aorta. Thus, GC-B isoform levels can be independently regulated. Given that the splice variants serve as dominant negative forms, these will serve as regulators of the full-length GC-B.

The guanylyl cyclase (GC) family of enzymes synthesize cGMP from Mg-GTP (1). Two subfamilies of the enzyme are found in mammals: single transmembrane GCs and soluble GCs (2). Single transmembrane GCs (seven in mammals labeled GC-A through GC-G) exist as homodimers, are located on the plasma membrane, and share a common domain structure: an extracellular ligand-binding domain (ECD), a single transmembrane segment (TM), a protein kinase homology domain (KHD), and a cyclase catalytic (CYC) domain (2). The KHD is homologous with the catalytic domain of protein kinases, and although no protein kinase activity has been reported, the KHD acts as a critical regulatory domain (3). The single transmembrane GCs are receptors for extracellular signaling molecules, although ligands have been found for only three. GC-A is a receptor for atrial natriuretic peptide and brain natriuretic peptide, GC-B is a receptor for C-type natriuretic peptide (CNP), and GC-C is a receptor for heat-stable enterotoxins and endogenous peptides of the guanylin family (4–11). The soluble GC family exists as heterodimers that bind heme, the mediator of nitric oxide stimulation (2, 12).

CNP/GC-B has been suggested as a strong adversary of various mitogens in that elevations of intracellular cGMP induced by CNP block proliferation of cell types such as fibroblasts and myofibroblastic hepatic stellate cells and the brain-derived neuronal growth factor- or nerve growth factor-induced proliferation of neuronal precursors (13–15). Many of these effects seem to be mediated by inhibition of the mitogen-activated protein kinase cascade. CNP may also inhibit proliferation through the suppression of arginine vasopressin-induced increases in intracellular Ca²⁺ levels (16). Additionally CNP at high concentrations inhibits progression of angioplasty-induced balloon injuries (17) and induces cultured aortic smooth muscle cells to change from the synthetic to the contractile phenotype (18). CNP/GC-B also stimulates the differentiation of proliferating chondrocytes into hypertrophic chondrocytes through the activation of GC-B signaling, which is critical for endochondral ossification and longitudinal bone growth (19–21). On the other side of the adversarial relationship, arginine vasopressin, platelet-derived growth factor, basic fibroblast growth factor, serum, phorbol 12-myristate 13-acetate, and lysophosphatidic acid rapidly and effectively desensitize GC-B to CNP in fibroblasts and other cells (13, 16, 22, 23).

In this report, we isolated and characterized the murine GC-B (Npr2) gene and found that three GC-B isoforms (GC-B1, GC-B2, and GC-B3) are generated from a single Npr2 gene and that GC-B2 and GC-B3 function as dominant negative isoforms.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dCTP (3,000 Ci/mmol), Na⁺[125I]−, nylon membranes (Hybond-N), penicillin, streptomycin, and amphotericin B were purchased from Amersham Biosciences. Salmon sperm DNA, TRIzol reagent, Dulbecco’s modified Eagle’s medium, penicillin, streptomycin, and amphotericin B were purchased from Invitrogen. FuGENE 6 transfection reagent was purchased from Roche Applied Science. Anti-Cypriate natriuretic peptide, CNP, and [Tyr31]CNP were purchased from Peninsula Laboratories. Anti-α-smooth muscle actin mouse monoclonal antibody (clone 1A4), mouse monoclonal anti-FLAG antibody (M2), and FLAG peptide were purchased from Sigma. Anti- 

Von Willebrand factor rabbit polyclonal antibody (H-300) and mouse...
monoclonal anti-c-Myc antibody were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-HA antibody was obtained from Clontech. Rabbit polyclonal anti-GC-B ECD serum (no. 282) was a gift from Dr. Sharon Milgarm, University of North Carolina.

Animals—Mouse F1 hybrids between 129/SvEvTac and C57BL/6N strains were obtained from Taconic and used for tissue collection to isolate genomic DNA, RNA, protein, and cultured aortic smooth muscle cells (SMCs). All experiments with the mice were conducted as approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas.

Isolation and Characterization of Murine Guanylyl Cyclase-B Gene—Approximately 1 × 10^6 clones of a 129/SvEvTac mouse genomic library (Stratagene) were screened with a rat GC-B cDNA probe fragment (EcoRI-XbaI, 2.0 kb) (5). Phage plaques were transferred to Hybond-N as recommended by the supplier and were then used for screening. The probe was labeled with [α-32P]dCTP by the random prime method (RediPrime II random prime labeling kit, Amersham Biosciences) to give a specific activity of about 1 × 10^8 dpm/μg of DNA. Hybridization was with a Rapid-hyb hybridization solution supplemented with 0.1 μg/ml salmon sperm DNA (sonicated) at 65 °C for 16 h. The membranes were washed once in 2× SSC, 0.1% SDS at ambient temperature for 10 min, once in 2× SSC, 0.1% SDS at 65 °C for 30 min, and twice in 0.1× SSC, 0.1% SDS at 65 °C for 30 min. The membranes were then exposed to Hyperfilm at ambient temperature for 24 h. Five positive clones were isolated, and three clones (nos. 7, 11, and 12) (Fig. 1A) were characterized. Fragments of the phage clones were subcloned into pBluescript II KS(+) (Stratagene) and characterized by restriction enzyme mapping, Southern blot analyses, and DNA sequencing. Exon-intron and intron-exon boundaries were determined by a comparison of nucleotide sequences of cDNA and the genomic DNA. The copy number was determined by Southern blot analysis of genomic DNA isolated from murine tails. DNA was digested with KpnI, SpeI, SacI, or XbaI, electrophoresed on a 0.8% agarose, Tris-acetate-EDTA gel; transferred to Hybond-N+; and then probed with the 0.3-kb cDNA fragment encompassing exons 4–7 (from the KpnI site to the location of primer RT-AS, Fig. 1A). The probe labeling, hybridization, and washing were as described for the filtering screen. The membranes were then exposed to Hyperfilm at −80 °C with intensifying screens for 48 h.

RNA Isolation—RNA was extracted from various organs and primary cultures of aortic SMCs with TRIzol reagent as recommended by the supplier. Pituitary glands, adrenal glands, aortas, and ovaries were pooled (n = 3–5) prior to RNA isolation.

Reverse Transcription and Polymerase Chain Reaction—Five micrograms of total RNA was reverse-transcribed by a Superscript II reverse transcriptase with a random primer (Invitrogen) following the supplier’s instruction. PCR was with a recombinant Taq DNA polymerase (Invitrogen) using 2 μl each of the 20-μl reverse transcription (RT) reactions as the template unless otherwise specified. To isolate cDNA fragments containing full-length coding sequences, PCR of 40 cycles was accomplished with the cerebellar RT reaction with an Ex-Taq DNA polymerase (Takara) and primers listed in Table I (first set). Amplified cDNA fragments were subcloned into a pGEM-Teasy TA cloning vector (Promega) and used for further analyses. The expression of GC-B1 and GC-B2 mRNA and the expression of GC-B1 and GC-B3 mRNA were detected by PCR with primers listed in Table I (second and third sets). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected by PCR using 1 μl of the RT reaction and a mouse glyceraldehyde-3-phosphate dehydrogenase Control Amplimer Set (Clontech) as internal control. The cycle number in each PCR was determined as amplification is in the exponential phase: 35 cycles for GC-B1 and GC-B2, 27 cycles for GC-B1 and GC-B3, and 25 cycles for Gapdh. Transcripts of smooth muscle myosin heavy chain isoforms (SM1 and SM2) (24) were detected by PCR with the primers listed in Table I (fourth set); the cycle number of the PCR was 35. The PCR products were electrophoresed on 1.2% agarose, Tris-acetate-EDTA gels, detected by ethidium bromide staining, and quantified by a Multi Image Light Cabinet (Alpha Innotech). For some experiments, the gels were transferred to Hybond-N and detected by 32P-labeled oligonucleotide probes located between the primers without containing the primer sequences: 5′-TGAGCTTGTGGCACATCTGAGCCGC-3′ for GC-B1 and GC-B2, 5′-GGAGAAGACAGTTGTGACATCAGCC-3′ for GC-B1 and GC-B2, and 5′-GGGCTTGGCTGAGTTGGCAG-3′ for GC-B1 and GC-B3, and 5′-GGGCTTGGCTGAGTTGGCAG-3′ for Gapdh; the hybridization and wash were at 42 °C. The 0.7-kb cDNA fragment corresponding to the C-terminal half of the ECD and the TM of GC-B, which was used as the probe for Northern blot analysis, was amplified for 40 cycles with the primers for GC-B1 and GC-B3 (Table I) and subcloned in a pGEM-Teasy vector.

Northern Blot Analysis—Total RNA (20 μg unless otherwise specified) was denatured by the formamide method; separated on a 1.0% agarose, MOPS gel; and transferred to Hybond-N. The 0.7-kb murine GC-B cDNA probe was labeled, and the hybridization and wash were as described for the filtering screen. The membranes were then exposed to Hyperfilm at −80 °C with intensifying screens for 48 h.

Expression Vector Construction—Complementary DNA clones of each murine GC-B isoform (GC-B1, GC-B2, and GC-B3) were distinguished by restriction enzyme mapping and DNA sequencing. The 3.4-kb insert was isolated from pGEM/mGc-B3 after EcoRI digestion and ligated into the EcoRI site of a pCMV5 expression vector, generated from a pCMV1 vector through deletion of the HpaI-EcoRI fragment from the SV40 origin region (25), to make pCMV5/mGc-B3. About 2.2-kb SmaI-EcoRI fragments of the inserts of pGEM/mGc-B1 and pGEM/mGc-B2, in which three GC-B isoforms are different from each other, were ligated between the SmaI and EcoRI sites of pBluescript II SK(−) vectors (Stratagene). The SmaI-EcoRV fragments of resultant plasmids were used to replace the 2.4-kb SmaI-EcoRV fragment of the pCMV5/mGc-B3, which generated pCMV5/mGc-B1 and pCMV5/mGc-B2; the EcoRI site existed outside the EcoRI cloning site. To generate expression vectors with epitope tags, the SacII and HpaI sites were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) at nucleotides +55 to +80 and +68 to +73, respectively, on pCMV5/mGc-B1, pCMV5/mGc-B2, or pCMV5/mGc-B3; nucleotide +66 is the last nucleotide of the signal peptide sequence (Fig. 2a). Linkers encoding the FLAG epitope (DYKDDDDK; sense, 5′-CGGGGGCGGACTTACCAAGGACGAGTACACAGGGGG-3′; antisense, 5′-CGGGGGCGGACTTACCAAGGACGAGTACACAGGGGG-3′) and the Myc epitope (EQLISEELDSL; sense, 5′-CGGGGGGACAACAAAATCTATCCTAAGAAGGATAGTCCCGG-3′; antisense, 5′-CGGGGGGACAACAAAATCTATCCTAAGAAGGATAGTCCCGG-3′) were inserted between the SacII and HpaI sites of the mutated expression vectors to generate pCMV5/FLAG-mGc-B1, pCMV5/FLAG-mGc-B2, pCMV5/FLAG-mGc-B3, pCMV5/Myc-mGc-B2, and pCMV5/HA-mGc-B3.

Cell Culture—COS-7 cells were grown to 30–50% confluence in 100-mm plates in Dulbecco’s modified Eagle’s medium (high glucose)
**Fig. 1. Structure of Npr2 and murine GC-B isoforms.** a, the structure and alternative splicing of Npr2. Regions of the gene covered by phage clones are shown at the top, the restriction enzyme map and the exon-intron organization of Npr2 are shown in the middle, and the three splicing patterns that result in generation of GC-B1, GC-B2, and GC-B3 mRNA are shown at the bottom. Restriction enzyme sites of SacI (Sc), KpnI (K), SpeI (Sp), and XbaI (X) are as indicated. Exons are shown as open boxes, and exon 7 encoding the TM is shown as a closed box. Groups of exons encoding the ECD, the KHD, and the CYC domain are indicated above the map of the gene. Locations of primers used to isolate cDNA fragments are shown below the map by open arrowheads with names indicated. Intron 5, the optional intron retained in GC-B3 mRNA, is shown as a shaded box, and the location of the in-frame termination codon for intron 5 (TAA) is indicated. b, Southern blot analysis of murine genomic DNA. The DNA
supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B. The cells were then transfected with one or a combination of pCMV5 (mock), pCMV5/mGC-B1, pCMV5/mGC-B2, pCMV5/mGC-B3, pCMV5/FLAG-mGC-B1, pCMV5/FLAG-mGC-B2, pCMV5/FLAG-mGC-B3, pCMV5/Mye-mGC-B2, or pCMV5/HA-mGC-B3 by FuGENE 6 transfection reagent following the supplier’s instruction; plasmids of 5 μg in total were used for a 100-mm plate. The cells were transfected for 24 h, grown in the growth medium for another 24 h, and then used for the experiments. For some experiments, the cells were split into 12-well plates after transfection.

Aortic SMCs were isolated from the thoracic and abdominal portion of the aorta of a male mouse at 5 weeks of age as described previously (28). After 3 d of culture, the cells were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer (pH 7.4) and permeabilized with Dulbecco’s phosphate-buffered saline (Ca2+- and Mg2+-free) containing 0.5% Triton X-100 and 1% normal horse serum. Immunocytochemistry was with the anti-a-smooth muscle actin mouse monoclonal antibody (1:400) and anti-von Willebrand factor rabbit polyclonal antibody (1:200) using a Vector M.O.M. immunodetection kit (Vector Laboratories) and a VectASTAIN Elite ABC kit (Vector Laboratories), respectively. Greater than 95% of the cells were positive for a-smooth muscle actin, and no cells positive for von Willebrand factor could be detected, indicating that the cells were predominantly SMCs with little contamination by endothelial cells.

Estimation of Gaunyl Cyclase Activity—COS-7 cells transfected with GC-B isoform expression vectors were incubated with CTP at various concentrations or vehicle, and intracellular cGMP concentrations were estimated as described previously (13).

Brains and lungs were isolated from mice at 5 weeks of age and homogenized with a Teflon homogenizer in 9 volumes (g wet weight/ml) of homogenization buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM Na2EDTA, 1 mM MgCl2, 1% aprotinin, 10% glycerol). The membrane fraction was collected as a pellet after centrifugation at 100,000 × g for 10 min, and then subjected to Western blot analysis as described above.

Results

Structure of Npr2 Gene—The murine GC-B (Npr2) gene is greater than 20 kb and consists of 22 exons and 21 introns (Fig. 1a). All exon-exon and intron-exon boundaries follow the GT/AG rule (Table II) (28), and only one gene exists based on Southern blot analysis (Fig. 1b). The gene structure is well conserved between murine and human (29) in that genes from both species consist of the same number of exons with introns located at the same positions (Table III). Both species also have a long second intron of about 6.5 kb (Table III). The organization of the closely related GC-A receptor gene of mice, rats, and humans is also highly conserved in structural organization (29–31) and contains introns at the same locations as in the Npr2 gene.

The coding sequence of the GC-B cDNA is 3,141 bp in length, and the deduced protein contains 1,047 amino acids. The sequence around the putative initiation codon “CCCCATGG” is consistent with a consensus initiation sequence (32); there also is an in-frame termination codon 126 nucleotides upstream from the initiation codon (Fig. 2a). The deduced amino acid sequences of murine, rat, and human GC-B show high identity across the species (e.g. murine and human GC-B (29) are 92 and 98% identical at the nucleotide or amino acid levels, respectively). Hydrophobic analysis suggests that cleavage of the signal peptide occurs after amino acid 22 (Fig. 2a) yielding a mature protein of 115 kDa. The ECDs, TM, KHD, and the CYC (domain are predicted to be encoded by exons 1–6, 7, 8–15, and 98% identical at the nucleotide or amino acid levels, respectively).

For immunoprecipitation, the cells were solubilized in 50 μl of 0.5% SDS, 1% IGEPAL CA-630 with or without pepstatin, 10% glycerol. The immuno precipitated antibody. The immunoprecipitated antibodies were detected by rabbit polyclonal anti-GC-B ECD serum (1:2,000), mouse monoclonal anti-FLAG antibody (1:2,000), mouse monoclonal anti-c-Myc antibody (1:2,000), or rabbit polyclonal anti-HA antibody (1:3,000) using an ECL plus detection system (Amersham Biosciences) with horseradish peroxide-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies (Biosource, 1:100,000); Hyperfilm was exposed for 15 s to 10 min. For immunoprecipitation, the cells were solubilized in 150 μl of solubilization buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS) by passing the solution 20 times through a 25-gauge needle followed by incubation on ice for 1 h. The solution was then cleared by centrifugation at 16,000 × g for 30 min at 4 °C.
Xaa-Xaa-Xaa-Gly\textsuperscript{525} (34) are identical between murine and rat GC-B (Fig. 1c). A polyadenylation signal-like sequence “TATAAA” is found at nucleotides +3388 through +3393 (Fig. 2b).

**Three GC-B Isoforms Generated by Alternative Splicing—**

Three different GC-B mRNA species are detected in brain. A comparison of the cDNA sequence with the genomic sequence reveals that all three mRNA species are generated by alternative splicing of a single Npr2 gene; one transcript contains all 22 exons and retains no intron sequences (GC-B1), a second (GC-B2) splices out exon 9 (75 bp) but remains in-frame with GC-B1, and a third (GC-B3) retains the 180 bp of intron 5 (Fig. 1, a and c). GC-B2 (112 kDa) lacks three of the six potential phosphorylation sites (Ser\textsuperscript{203}, Ser\textsuperscript{206}, and Thr\textsuperscript{209}) and the putative ATP-binding motif (Fig. 1c). GC-B3 is predicted to be 44 kDa and contain only a portion of the ECD due to an in-frame termination codon within intron 5 (Fig. 1, a and c). The use of primers over the entire coding region failed to detect other alternatively spliced transcripts.

GC-B1 and GC-B2 are approximately the same size (120 kDa), while GC-B3 is about 60 kDa based on Western blot analysis (Fig. 3). Digestion with peptide-N\textsuperscript{-}glycosidase F suggested N-glycosylation of all three GC-B isoforms.

**CNP Binding to GC-B Isoforms—**

Equal expression of the GC-B isoforms (Western blot analysis, not shown) allowed a determination of relative CNP binding for each isoform. GC-B1 and GC-B2 bound CNP with similar apparent affinity (one-half maximal binding at about 0.4 and 0.5 nM, respectively), while GC-B3 bound CNP with much lower apparent affinity (one-half maximal binding at about 4–5 nM). Amino acid sequence data (not shown) suggest that the low-affinity binding of GC-B3 is due to a shorter ECD.

**TABLE II**

Locations and lengths of exons and introns of the Npr2 gene

| Exon number | Position in cDNA\textsuperscript{a} | Exon size | Intron number | Codon(s) interrupted\textsuperscript{b} | Amino acid(s) interrupted\textsuperscript{c} | Intron size |
|-------------|------------------------------------|-----------|---------------|----------------------------------------|-----------------------------------|-------------|
| 1           | 1–667                              | 667       | 1             | ATT                                   | Ile\textsuperscript{231}            | 1           |
| 2           | 668–875                            | 206       | 2             | CAG/ACT                                | Glu\textsuperscript{291}, Thr\textsuperscript{292} | 6.5\textsuperscript{f} |
| 3           | 874–987                            | 113       | 3             | CTG/ATG                                | Leu\textsuperscript{292}, Met\textsuperscript{330} | 0.282       |
| 4           | 988–1123                           | 136       | 4             | G/GT                                   | Gly\textsuperscript{375}            | 0.247       |
| 5           | 1124–1218                          | 95        | 5             | CAG/CCG                                | Gln\textsuperscript{395}, Pro\textsuperscript{407} | 0.180       |
| 6           | 1219–1351                          | 133       | 6             | A/CT                                   | Thr\textsuperscript{421}            | 0.222       |
| 7           | 1352–1436                          | 85        | 7             | CCG/GG                                 | Arg\textsuperscript{479}            | 1.421       |
| 8           | 1437–1557                          | 121       | 8             | CTG/CGG                                | Leu\textsuperscript{419}, Arg\textsuperscript{530} | 0.160       |
| 9           | 1558–1632                          | 75        | 9             | AAG/GGA                                | Lys\textsuperscript{494}, Gly\textsuperscript{495} | 0.197       |
| 10          | 1633–1710                          | 78        | 10            | CAC/ATG                                | His\textsuperscript{557}, Met\textsuperscript{571} | 0.399       |
| 11          | 1711–1815                          | 105       | 11            | CAA/GAT                                | Gln\textsuperscript{605}, Arg\textsuperscript{606} | 0.120       |
| 12          | 1816–1887                          | 72        | 12            | A/AG                                   | Lys\textsuperscript{682}, Gly\textsuperscript{683} | 2.163       |
| 13          | 1888–2047                          | 160       | 13            | A/AG                                   | Lys\textsuperscript{683}            | 0.215       |
| 14          | 2048–2203                          | 156       | 14            | G/GG                                   | Glu\textsuperscript{705}            | 0.077       |
| 15          | 2204–2372                          | 169       | 15            | AAG                                   | Lys\textsuperscript{791}            | 0.153       |
| 16          | 2372–2519                          | 147       | 16            | CAT                                   | His\textsuperscript{840}            | 0.459       |
| 17          | 2520–2623                          | 104       | 17            | CAG/GTG                                | Gln\textsuperscript{881}, Val\textsuperscript{882} | 0.146       |
| 18          | 2624–2712                          | 89        | 18            | AAG/GTG                                | Lys\textsuperscript{894}, Val\textsuperscript{905} | 1.733       |
| 19          | 2713–2887                          | 175       | 19            | G/GG                                   | Gly\textsuperscript{983}            | 0.070       |
| 20          | 2888–2986                          | 99        | 20            | G/GT                                   | Ala\textsuperscript{1096}           | 0.215       |
| 21          | 2987–3078                          | 92        | 21            | AAG/GGA                                | Lys\textsuperscript{1026}, Gly\textsuperscript{1027} | 0.113       |
| 22          | 3079–...                            | >111      |               |                                        |                                   |             |

\textsuperscript{a} Nucleotides of cDNA are numbered as the adenine of the ATG translational initiation codon is 1.

\textsuperscript{b} slashes indicate locations of introns.

\textsuperscript{c} Amino acids are numbered as Met of the translational initiation codon is 1.

\textsuperscript{d} Approximate length based on the restriction enzyme mapping.
GC-B Isoform Activities—CNP-independent and CNP-activated activities of GC-B isoforms were examined using COS-7 cells. Western blot analysis confirmed equal expression of the various isoforms. Intracellular cGMP levels were more than 50-fold higher in cells expressing GC-B1 than in mock-transfected cells and were increased by CNP in a dose-dependent manner (Fig. 5). Almost a 300-fold increase in cGMP occurred with 100 nM CNP. Basal cGMP levels of cells expressing GC-B2 were similar to those of cells expressing GC-B1, but CNP failed to elevate cyclic nucleotide concentrations (Fig. 5). There were no significant differences in cGMP levels between cells expressing GC-B3 and mock-transfected cells and no effects of CNP (Fig. 5).

Interactions among GC-B Isoforms—Compared with intracellular cGMP levels of COS-7 cells transfected with pCMV5/

**FIG. 2.** Nucleotide and amino acid sequences of Npr2. The nucleotide sequence around the translational initiation codon (a) and the nucleotide sequence around the termination codon (b) are shown along with the deduced amino acid sequence. Nucleotide sequences of coding regions and the 3′-untranslated region are written in *uppercase* letters; nucleotide sequences of other regions are written in *lowercase* letters. DEDUCED AMINO ACIDS are written below nucleotide sequences in *one-letter codes*; the *asterisk* indicates the termination codon. The number to the right is the number of the nucleotides (without parentheses) or amino acids (with parentheses) at the end of the cDNA or amino acid sequences in each line. Nucleotide numbers of cDNA are determined as the adenine of the translational initiation codon as 1. The *downward arrow* indicates the expected cleavage site of the signal sequence. The initiation codon, the in-frame termination codon preceding the initiation codon, the "ag" sequence at the 3′ end of intron 21, and a putative polyadenylation signal (tataaa) are underlined.

**FIG. 3.** Detection of GC-B isoform proteins. GC-B isoform proteins expressed in COS-7 cells are detected by Western blot analysis with anti-rat GC-B extracellular domain antibody (no. 282) (a) or anti-FLAG antibody (b). a, extracts from the cells transfected with pCMV5 (M), pCMV5/mGC-B1 (1), pCMV5/mGC-B2 (2), or pCMV5/mGC-B3 (3) treated with (+) or without (−) peptide-N-glycosidase F (PNGaseF) and then subjected to Western blot analysis. Closed and open triangles indicate glycosylated and deglycosylated molecules, respectively. b, extracts from the cells transfected with pCMV5 (M), pCMV5/FLAG-mGC-B1 (1), pCMV5/FLAG-mGC-B2 (2), and pCMV5/FLAG-mGC-B3 (3) are analyzed.

**FIG. 4.** C-type natriuretic peptide binding to GC-B isoforms. Binding of 125I-[Tyr0]CNP to COS-7 cells expressing each GC-B isoform was analyzed by a saturation binding experiment. The data of GC-B1, GC-B2, and GC-B3 are shown as triangles, squares, and inverted triangles, respectively. Regression curves of GC-B1 and GC-B2 are shown by solid and dashed lines, respectively.
muscle, eyes, and the growth plate cartilage; and were low in glands, and ovaries; were moderate in lungs, heart, skeletal the cerebrum, cerebellum, brain stem, pituitary gland, adrenal
of murine tissues (Fig. 8). The expression levels were high in
Npr2
respectively. Then measured. The data from the cells transfected with pCMV5 (the
at 1, 10, or 100 nM for 10 min, and intracellular cGMP contents were
expressing each GC-B isoform were incubated with the vehicle or CNP
for CNP-induced cGMP elevations was similar between cells
48886
mGC-B1 and Myc-mGC-B2, or cells expressing FLAG-mGC-B1
transfected with pCMV5/FLAG-mGC-B1 and pCMV5/Myc-
mGC-B2. The magnitude of the suppression was increased up
cellular cGMP levels were decreased by co-expression of Myc-
mGC-B2. The magnitude of the suppression was increased up
to 75% as the molar ratio of pCMV5/Myc-mGC-B2 to pCMV5/
FLAG-mGC-B1 alone, cells expressing FLAG-mGC-B1 and Myc-mGC-B2, or cells expressing FLAG-mGC-B1 and HA-mGC-B3 (~30 nM) (Fig. 6, a and b).

When FLAG-mGC-B1 was stimulated by 100 nM CNP, intracellular cGMP levels were decreased by co-expression of Myc-
mGC-B2. The magnitude of the suppression was increased up to 75% as the molar ratio of pCMV5/Myc-mGC-B2 to pCMV5/
FLAG-mGC-B1 was increased as high as 2 (Fig. 6c). HA-
mGC-B3 had much smaller effects when co-expressed with
FLAG-mGC-B1 (Fig. 6c).

Heterodimer formation between GC-B1 and GC-B2 or be-
tween GC-B1 and GC-B3 was assessed in co-immunoprecipita-
experiments. Myc-mGC-B2 was co-immunoprecipitated with
FLAG-mGC-B1 by anti-FLAG-agarose when both FLAG-
mGC-B1 and Myc-mGC-B2 were co-expressed, and this co-
immunoprecipitation was completely blocked by the FLAG pep-
tide (Fig. 7a). HA-mGC-B3 was also co-immunoprecipitated
with FLAG-mGC-B1 when both FLAG-mGC-B1 and HA-
mGC-B3 were co-expressed, and again the co-immunoprecipita-
tion was completely blocked by the FLAG peptide (Fig. 7b).
Thus, GC-B2 or GC-B3 is able to form heterodimers with GC-B1.

Expression of GC-B Isoform mRNAs in Murine Organs—
Npr2 transcripts of about 4 kb were detected in a wide variety of murine tissues (Fig. 8a). The expression levels were high in the cerebrum, cerebellum, brain stem, pituitary gland, adrenal glands, and ovaries; were moderate in lungs, heart, skeletal muscle, eyes, and the growth plate cartilage; and were low in

Fig. 5. Guanylyl cyclase activities of GC-B isoforms. COS-7 cells
expressing each GC-B isoform were incubated with the vehicle or CNP
at 1, 10, or 100 nM for 10 min, and intracellular cGMP contents were
then measured. The data from the cells transfected with pCMV5 (the
mock construct), pCMV5/mGC-B1, pCMV5/mGC-B2, and pCMV5/
mGC-B3 are shown as open, closed, hatched, and shaded bars, respectively.
induced activation of GC-B, although this region is not critical for either basal guanylyl cyclase activity or ligand binding. GC-B3 does not possess either guanylyl cyclase activity or the ability to bind CNP. The failure of CNP to bind to GC-B3 could have been due to secretion of the protein into the medium as a soluble receptor; however, we detected GC-B3 in the membrane preparation (Fig. 3a) but not in the medium by Western blot analysis. GC-B3 might attach to the plasma membrane mGC-B1 and HA-mGC-B3 (indicated by an arrow).

Fig. 6. Dominant negative effects of GC-B2 and GC-B3 on CNP-induced increases in guanylyl cyclase activity of GC-B1. a, the effect of GC-B2 co-transfection on GC-B1 activity. COS-7 cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5 (the mock construct) and the cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5/Myc-mGC-B2 were incubated with the vehicle (shown as –) and CNP at 1 x 10^-10, 1 x 10^-9, 1 x 10^-8, and 1 x 10^-7 M for 10 min, and intracellular cGMP contents were then measured. Cyclic GMP contents of cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5 are shown as squares (n = 3, bars are S.E.), and the dose dependence curve is drawn with a solid line. Data of the cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5/Myc-mGC-B2 are shown as triangles and a dashed line (n = 3). Insets are results of immunoblot (IB) analyses of extracts from cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5 (lane 1) and cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5/Myc-mGC-B2 (lane 2); anti-FLAG antibody (FLAG) and anti-c-Myc antibody (Myc) monitor the expression levels of FLAG-mGC-B1 (indicated as closed triangle) and Myc-mGC-B2 (indicated as open triangle). b, the effect of GC-B3 co-transfection on GC-B1 activity. Co-transfections with pCMV5/FLAG-mGC-B1 and pCMV5/Myc-mGC-B2 are shown as circles, and the dose-response curve is drawn with a dotted line. The inset is the result of immunoblot analysis with anti-FLAG antibody (FLAG) and anti-c-Myc antibody (Myc) (no. 282). Extracts of the cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5 (lane 1) and cells co-transfected with pCMV5/FLAG-mGC-B1 and pCMV5/HA-mGC-B3 (lane 3) are analyzed for protein expression levels of FLAG-mGC-B1 and HA-mGC-B3 (indicated by an arrow), c, dose-dependent effects of GC-B2 and GC-B3 on GC-B1 activity. Intracellular cGMP contents of cells expressing FLAG-mGC-B1 alone (closed bars); FLAG-mGC-B1 to Myc-mGC-B2 at ratios of 1:0.5 (dotted bars); FLAG-mGC-B1 to HA-mGC-B3 at ratios of 1:0.5, 1:1 (hatched bars), and 1:2 (horizontally striped bars); and FLAG-mGC-B1 to HA-mGC-B3 at ratios of 1:0.5 (cross-hatched bars), 1:1 (vertically striped bars), and 1:2 (open bars) are shown upon stimulation by the vehicle or 100 nM CNP (n = 3, means ± S.E.). *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with cells expressing FLAG-mGC-B1 alone at each CNP dose by two-way analysis of variance with Bonferroni’s posttest (a and b) or by one-way analysis of variance within each CNP dose (c).
after secretion into the extracellular space, or there may be failure of GC-B3 trafficking from the endoplasmic reticulum to the plasma membrane, resulting in no CNP binding.

In addition to these isoforms, previous work in the human has suggested the presence of a fourth isoform (NPR-Bi); it contains a 71-bp insertion (intron 19) resulting in a frameshift that would disrupt the CYC domain (38). This isoform, nevertheless, has been suggested as a signaling receptor for CNP; in this case activation of a protein tyrosine kinase pathway is suggested to inhibit a K+ conductance in the apical membrane of human proximal tubule cells (39). We did not detect this isoform in the mouse. It should also be noted that human expressed sequence tags encoding GC-B transcripts lacking exon 9 have been reported. The possibility that GC-B2 or GC-B3 utilizes a signaling pathway other than cGMP, as seen with NPR-Bi, remains to be determined.

Npr2 mRNA expression was detected in a wide variety of tissues by Northern blot analysis, and RT-PCR suggests that GC-B isoform expression levels and the ratios among the isoforms vary from tissue to tissue (Fig. 8b). Thus, an analysis of the expression level of only GC-B1 is probably not sufficient information for estimation of CNP-sensitive guanylyl cyclase activity in tissues or cells. Npr2 mRNA expression, estimated by Northern blot analysis, was high in brain tissues (Fig. 8a), but GC-B2 or GC-B3 ratios relative to GC-B1 are also relatively high in the cerebrum and cerebellum. Thus, GC-B2 and GC-B3, given the dominant negative effects of the isoforms, would be expected to play an important role in adjusting the magnitude of a CNP signal in brain, and in fact, CNP marginally increased guanylyl cyclase activity in homogenates of brain while significantly increasing cyclase activity in homogenates of lungs where GC-B2 mRNA was barely detectable (Fig. 8b).

It has been shown that GC-B mRNA expression is increased in cultured aortic SMCs relative to normal arterial walls (40). CNP/GC-B signaling in SMCs of the synthetic phenotype serves as a counter-regulatory system to inhibit the migration and proliferation and to induce a phenotypic reversion of cultured aortic SMCs (41, 42). GC-B1 mRNA expression is increased, and GC-B2 mRNA expression is almost completely repressed in cultured aortic SMCs compared with aortic tissue, and thus GC-B2 may serve as a new molecular marker for SMCs of the contractile phenotype. We also suggest that CNP/GC-B signaling is activated in cultured aortic SMCs not only because of increased expression of GC-B1 but also because of repression of the dominant negative isoform, GC-B2.

CNP/GC-B signaling is therefore regulated at two levels with respect to expression: transcription and post-translational modification. GC-B mRNA expression in cultured rat aortic SMCs is attenuated by transforming growth factor-β1 (43). Signaling through GC-B also can be regulated by changes in the mRNA expression of natriuretic peptide receptor-C, which plays an important role in the clearance of natriuretic peptides. Stimulation of the β1-adrenoreceptor attenuates natriuretic peptide receptor-C mRNA expression in cultured rat aortic SMCs (44), thus potentially affecting GC-B signaling as well. The dephosphorylation of GC-B desensitizes GC-B to CNP as a post-translational modification (22, 45). Serum, platelet-derived growth factor, basic fibroblast growth factor, and phorbol

![Fig. 8. Expression of Npr2 mRNA in murine tissues.](Image)

**Fig. 8.** Expression of Npr2 mRNA in murine tissues. a, Npr2 mRNA expression in murine tissues detected by Northern blot analysis. Twenty micrograms each of total RNA isolated from a series of murine tissues was analyzed unless otherwise specified. Signals of Gapdh are shown at the bottom as internal controls. b, messenger RNA expression of GC-B isoforms in murine tissues detected by RT-PCR. Five micrograms each of total RNA was reverse-transcribed and one-tenth of the RT reaction was subjected to PCR to detect transcripts of GC-B isoforms (GC-B1, GC-B2, and GC-B3). One fiftieth of the RT reaction was used to detect the Gapdh transcript. *, growth plate cartilages of knee and ankle joints from mice at 8 days of age.

![Fig. 9. Difference in mRNA expression of GC-B isoforms between medial layer of aorta and cultured aortic smooth muscle cells.](Image)

**Fig. 9.** Difference in mRNA expression of GC-B isoforms between medial layer of aorta and cultured aortic smooth muscle cells. Five micrograms each of total RNA isolated from the medial layer of the aorta and cultured aortic SMCs at passages 1, 2, and 3 (p1, p2, and p3) were reverse-transcribed, and one-tenth of the RT reaction was subjected to PCR to detect mRNA of smooth muscle myosin heavy chain isoforms (SM1 and SM2 at the top row) and GC-B isoforms (GC-B1 and GC-B2 at the second row and GC-B1 and GC-B3 at the third row). One-fiftieth of the RT reaction was used to detect the Gapdh transcript. RT reactions of each sample were with (+) or without (−) the reverse transcriptase to rule out signals from contaminating genomic DNA, and PCR primers were designed so the size of amplification products would be different among the isoform mRNA or genomic DNA. In the lane indicated as 0.5x, the RT reaction was diluted 2-fold with the RT reaction and then used in PCR.
12-myristate 13-acetate cause dephosphorylation and decrease CNP-induced activation of GC-B in NIH-3T3 fibroblasts over-expressing rat GC-B1 (13, 22, 45). Here we show yet another means of regulating CNP/GC-B signaling: the regulation of relative levels of the various isoforms. This type of regulation has been previously suggested for soluble GC where α2 and β2 isoforms have been proposed as dominant negative isoforms (46, 47), and thus the guanylyl cyclase family, in general, may utilize such dominant negative isoforms as regulators.

Acknowledgments—We thank Lynda K. Doolittle for technical assistance and Dr. Ted D. Chrisman for valuable discussions.

REFERENCES
1. Garbers, D. L. (1992) Cell 71, 1–4
2. Garbers, D. L., and Lowe, D. G. (1994) J. Biol. Chem. 269, 30741–30744
3. Chinkers, M., and Garbers, D. L. (1989) Science 245, 1392–1394
4. Waldman, S. A., Rapoport, R. M., and Murad, F. (1984) J. Biol. Chem. 259, 14332–14334
5. Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., and Garbers, D. L. (1989) Cell 59, 1155–1162
6. Schulz, S., Green, C. K., Yuen, P. S., and Garbers, D. L. (1990) Cell 63, 941–948
7. Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H. M., Goeddel, D. V., and Schulz, S. (1989) Nature 339, 74–83
8. Koller, K. J., Love, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H., and Goeddel, D. V. (1991) Science 252, 120–123
9. Hamra, F. K., Forte, L. R., Eber, S. L., Pidhorodecky, N. V., Krause, W. J., Freeman, R. H., Chin, D. T., Tompkins, J. A., Fok, K. F., Smith, C. E., Duffin, K. L., Siegel, N. R., and Currie, M. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10464–10468
10. Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L., and Smith, C. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 947–951
11. Forte, L. R., Eber, S. L., Fan, X., London, R. M., Wang, Y., Rowland, L. M., Chin, D. T., Freeman, R. H., and Krause, W. J. (1999) Endocrinology 140, 1800–1806
12. Keeling, M. (1999) Methods 19, 485–493
13. Chrimson, T. D., and Garbers, D. L. (1999) J. Biol. Chem. 274, 4292–4299
14. Tao, J., Mallat, A., Gallois, C., Belmadani, S., Mery, P. F., N'hieu, J. T., Pavoine, C., and Lotersztajn, S. (1999) J. Biol. Chem. 274, 23761–23769
15. Simpson, P. J., Miller, J., Noon, C., Hannan, A. L., Lief, D. J., and Honnett, G. V. (2002) J. Neurosci. 22, 5536–5551
16. Abbey, S. E., and Potter, L. R. (2002) J. Biol. Chem. 277, 42423–42430
17. Furuya, M., Issaka, K., Miyazaki, T., Horibe, N., Kawashima, K., Ohno, T., Tanaka, S., Minamino, N., Kangawa, K., and Matsuo, H. (1993) Biochem. Biophys. Res. Commun. 193, 248–253
18. Dui, K., Ikeda, T., Itoh, H., Ueyama, K., Hosoda, K., Ogawa, Y., Yamashita, J., Chun, T. H., Inoue, M., Matsuoka, K., Doi, K., Chun, T. H., Inoue, M., Matsuoka, K., Yashima, T., Ueda, M., Ban, T., and Nakao, K. (1997) Annu. N. Y. Acad. Sci. 811, 534–541
19. Dui, K., Itoh, H., Ikeda, T., Hosoda, K., Ogawa, Y., Ijaki, T., Yamashita, J., Chun, T. H., Inoue, M., Matsuoka, K., Matsuoka, K., Ohnori, K., and Nakao, K. (1997) Biochem. Biophys. Res. Commun. 239, 889–894
20. Fujio, N., Gassard, F., Bayard, F., and Tremblay, J. (1994) Hypertension 23, 908–913
21. Kishimoto, I., Yoshimasa, T., Suga, S., Ogawa, Y., Komatsu, Y., Nakagawa, O., Hama, N., and Inuma, H. (1995) Circ. Res. 76, 34–39
22. Ijaki, T., Suga, S., Ogawa, Y., Komatsu, Y., Nakagawa, O., Hama, N., and Inuma, H. (1992) Circ. Res. 71, 34–39
23. Itoh, H., Suga, S., Ogawa, Y., Komatsu, Y., Tanaka, N., Ijaki, T., Yamashita, J., Ikeda, T., Doi, K., Chun, T. H., Inoue, M., Matsuoka, K., Yashima, T., Ueda, M., Ban, T., and Nakao, K. (1997) Ann. N. Y. Acad. Sci. 811, 534–541
24. Dui, K., Itoh, H., Ikeda, T., Hosoda, K., Ogawa, Y., Ijaki, T., Yamashita, J., Chun, T. H., Inoue, M., Matsuoka, K., Matsuoka, K., Ohnori, K., and Nakao, K. (1997) Biochem. Biophys. Res. Commun. 239, 889–894
25. Fujio, N., Gassard, F., Bayard, F., and Tremblay, J. (1994) Hypertension 23, 908–913
26. Kishimoto, I., Yoshimasa, T., Suga, S., Ogawa, Y., Komatsu, Y., Nakagawa, O., Itoh, H., and Nakao, K. (1994) J. Biol. Chem. 269, 28300–28308
27. Peter, L. R. (1998) Biochemistry 37, 2422–2429
28. Behrends, S., Harteneck, C., Schultz, G., and Keeling, M. (1995) J. Biol. Chem. 270, 21109–21113
29. Gupta, G., Auyeung, M., Yang, L., and Danziger, R. S. (1997) J. Clin. Invest. 100, 1488–1492
