



OPEN ACCESS

Mutations in Tyr⁸⁰⁸ reveal a potential auto-inhibitory mechanism of guanylate cyclase-B regulation

Takeshi KATAFUCHI¹

Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, TX, U.S.A.

Synopsis

In this study, Tyr⁸⁰⁸ in GC-B (guanylate cyclase-B), a receptor of the CNP (C-type natriuretic peptide), has been shown to be a critical regulator of GC-B activity. In searching for phosphorylation sites that could account for suppression of GC-B activity by S1P (sphingosine-1-phosphate), mutations were introduced into several candidate serine/threonine and tyrosine residues. Although no novel phosphorylation sites that influenced the suppression of GC-B were identified, experiments revealed that mutations in Tyr⁸⁰⁸ markedly enhanced GC-B activity. CNP-stimulated activities of the Y808F and Y808A mutants were greater than 30-fold and 70-fold higher, respectively, than that of WT (wild-type) GC-B. The Y808E and Y808S mutants were constitutively active, expressing 270-fold higher activity without CNP stimulation than WT GC-B. Those mutations also influenced the sensitivity of GC-B to a variety of inhibitors, including S1P, Na₃VO₄ and PMA. Y808A, Y808E and Y808S mutations markedly weakened S1P- and Na₃VO₄-dependent suppression of GC-B activity, whereas Y808E and Y808S mutations rather elevated cGMP production. Tyr⁸⁰⁸ is conserved in all membrane-bound GCs and located in the niche domain showing sequence similarity to a partial fragment of the HNOBA (haem nitric oxide binding associated) domain, which is found in soluble GC and in bacterial haem-binding kinases. This finding provides new insight into the activation mechanism of GCs.

Key words: cGMP, C-type natriuretic peptide, guanylate cyclase-B, hyperactive mutation

Cite this article as: Katafuchi, T. (2013) Mutations in Tyr⁸⁰⁸ reveal a potential auto-inhibitory mechanism of guanylate cyclase-B regulation. *Biosci. Rep.* **33**(3), art:e00039.doi:10.1042/BSR20130025

INTRODUCTION

The membrane-bound GC (guanylate cyclase) family consists of five (human) and seven (rodent) members [1]. Among the human cyclases, GC-A is a receptor for ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptides), GC-B is a receptor for CNP (C-type natriuretic peptide) and GC-C is a receptor for guanylin (guanylin and uroguanylin). GC-A is expressed primarily in the cardiovascular system, where it regulates vascular tone and body fluid level [1]. GC-B is expressed in a variety of tissues, including chondrocytes, female reproductive organs, the CNS (central nervous system) and fibroblasts, and is believed to regulate morphogenesis [2–4]. GC-C is mainly expressed in the digestive system and regulates ion transport and crypt growth [5]. Little is known about the extracellular activating mechanisms of GC-E and GC-F, which are specifically expressed in retinal

and olfactory cells, respectively, and regulate cGMP levels that control sensory processes [6,7].

Membrane-bound GCs consist of three major functional domains: an extracellular LBD (ligand-binding domain), an intracellular KHD (kinase-homology domain) and a GC catalytic domain [1]. Several lines of biochemical evidence [8–11], including the elucidation of the three-dimensional structure of its LBD [12,13], indicate that GC-A forms at least a homodimer. Upon binding to ligand, the two subunits of the LBD dimer re-orient their positions with respect to each other, giving rise to rotation of each of the juxtamembrane domains, presumably causing structural alteration(s) of the catalytic domains that elicit GC activity [12]. As other GCs show high sequence similarity to GC-A, they are likely to be activated by a similar mechanism. The KHD has slight but significant amino acid sequence identity to the tyrosine kinase domain of PDGF (platelet-derived growth factor) [14]. However, the KHD is apparently unable to catalyse protein

Abbreviations used: ANP atrial natriuretic peptide; CNP C-type natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGFR 3, fibroblast growth factor receptor 3; GC, guanylate cyclase; HNOBA, haem nitric oxide binding associated; IBMX, isobutylmethylxanthine; KHD, kinase-homology domain; LBD, ligand-binding domain; Myc-GC-B, Myc-tagged GC-B; PKC, protein kinase C; S1P sphingosine-1-phosphate; VASP vasodilator-stimulated phosphoprotein; WT, wild-type.

¹ email Takeshi.Katafuchi@UTSouthwestern.edu

phosphorylation, but binds to ATP in the presence of Mg^{2+} to stabilize GC activity [15].

CNP-induced GC activity is suppressed in cells on treatment of cells with a wide variety of agents, including S1P (sphingosine-1-phosphate) [16,17], LPA (lysophosphatidic acid) [18], growth factors [19], peptides [20,21], the Ca^{2+} ionophore, ionomycin [22], the PKC (protein kinase C) activator, PMA [23,24] and the tyrosine phosphatase inhibitor, Na_3VO_4 [19]. Potter's group have identified six potential phosphorylation sites clustered between residues 513 and 529 in the juxtamembrane region of the KHD [25], and demonstrated that GC-B undergoes PKC- and Ca^{2+} -dependent dephosphorylation upon treatment with S1P, PMA and ionomycin [16–18,26]. However, mutation of the phosphorylatable residues with phosphomimetic residues such as glutamic acid had insignificant effects on reduction of activity by S1P and ionomycin treatment [24], pointing to the existence of inhibitory mechanisms that do not involve dephosphorylation of these six residues. Based on these observations, I hypothesized that GC-B contains previously unidentified regulatory phosphorylation sites. A database search revealed 14 potential serine/threonine and two tyrosine phosphorylation sites, which I mutated to alanine and phenylalanine, respectively. Although the majority of these mutations had negligible effects on GC-B, the Y808F mutant expressed remarkably higher activity than WT (wild-type) GC-B. Further analysis of this and other mutations of residue Tyr⁸⁰⁸ highlighted its importance in the regulation of GC-B activity. The characteristics of these Tyr⁸⁰⁸ mutants, which may prove useful in future structural, mechanistic and pharmacological investigations, are examined in this study.

EXPERIMENTAL

Construction of GC-B mutants

Mutations were introduced into WT Myc-GC-B (Myc-tagged GC-B) cDNA by PCR using primers shown in Supplementary Table S1 (at <http://www.bioscirep.org/bsr/033/bsr033e039add.htm>). PCR was carried out in a reaction containing 0.2 mM dNTP, 1× reaction buffer, 50 units/ml Pfu polymerase and Myc-GC-B cDNA (1 ng/reaction) with a sense primer (GCB1688s) 5'-AAGCTGATGCTGGAGAAGGA-3' and an antisense primer for each of the mutations in Table S1, or an antisense primer (GCBmyca) 5'-GCGGCCGCTCACAGATCCTCTTCTGAGATGAGT-3' and a sense primer for the same mutation in Table S1. The reactions were mixed, and PCR was performed again using GCB1688s and GCBmyca primers. The amplified fragment was digested with NotI and NheI, ligated with N-terminal HindIII–NheI fragment of GC-B, and introduced into HindIII–NotI-digested pcDNA3.1 mammalian expression vector.

Measurement of intracellular cGMP level

HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated FBS

(fetal bovine serum) and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). Cells were seeded onto poly-D-lysine-coated 12- or 24-well plates 8–24 h prior to transfection. WT or mutant Myc-GC-B cDNAs were transfected into HeLa cells using Lipofectamine™ 2000 according to the manufacturer's protocol. The medium was replaced with DMEM containing 0.5% FBS 6–12 h after transfection, and cells were further cultured for 24 h. The medium was replaced again with serum-free DMEM containing 20 mM Hepes (pH 7.3) and incubated for 2 h. Following addition of IBMX (isobutylmethylxanthine) and incubation for 15 min at 37°C, CNP was added to each well, and cells were incubated for 5 min at 37°C to measure maximal intracellular cGMP accumulation [27]. To evaluate the effect of S1P, PMA and Na_3VO_4 on GC-B activity, each reagent was added 30 min prior to addition of IBMX. The incubation medium was then removed by aspiration, and 95% (v/v) ethanol was added to each well. Following one freeze-thaw cycle, the solution in each well was transferred into a test tube, and the solvent was removed using a Speedvac model SC200. The resulting pellet was dissolved in serum-free DMEM containing 20 mM Hepes (pH 7.3) and succinic solution (1.4-Dioxane:triethylamine:acetic anhydride = 20:5:1) was added to the dissolved samples. Following a 30 min incubation at room temperature (22°C), the solvent was evaporated using a Speedvac, and the resulting pellet was dissolved in 90 mM CH_3COONa (pH 6.2). An aliquot of each sample was used for measurement of cGMP by RIA as described previously [19].

Measurement of GC activity in the plasma membrane

WT or mutant GC-B was expressed in HeLa cells as described above. Medium was replaced with DMEM containing 0.5% FBS 26 h prior to harvesting and again with serum-free DMEM 2 h prior to harvesting. The cells were washed twice with ice-cold PBS, and harvested with a homogenization buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA and 10 μ g/ml each of *N* α -*p*-tosyl-L-lysine chloromethyl ester, *N* α -*p*-tosyl-L-arginine methyl ester, *N* α -*p*-tosyl-L-lysine chloromethyl ketone, leupeptin, pepstatin A, 1 mM PMSF and 1× concentration of phosphatase inhibitor cocktail 1 and 2 (Sigma). Harvested cells were then centrifuged for 5 min at 21 000 g, sonicated with the homogenization buffer, and centrifuged again for 15 min at 21 000 g. The pellet was washed once with homogenization buffer and sonicated again. Following measurement of protein concentration using the BCA (bicinchoninic acid) protein assay kit (Pierce), the homogenate was aliquoted to obtain a protein amount of 50 μ g/tube. Reaction was allowed to proceed for 5 min at 37°C in 100 μ l of reaction buffer (50 mM Tris/HCl, pH 7.5, containing 0.5 mM IBMX, 0.1% BSA, 15 mM creatine phosphate, 3.5 units of creatine kinase, 1 mM GTP, 1 mM ATP, 4 mM $MgCl_2$ and 1 μ M CNP). To measure Triton X-100/ Mn^{2+} -dependent GC activity, ATP and $MgCl_2$ were replaced by 1% (v/v) Triton X-100 and 4 mM $MnCl_2$. Reactions were terminated by addition of 25 μ l of 1 N $HClO_4$. Following centrifugation for 5 min at 21 000 g, each supernatant was used to determine cGMP concentration as described above.

Immunoblotting of Myc-GC-B mutants

HeLa cells expressing Myc-GC-B or its mutants were homogenized in buffer containing 62.5 mM Tris (pH 6.8), 5% (w/v) SDS, 0.025% Bromophenol Blue and 2% (v/v) 2-mercaptoethanol. The homogenates were boiled for 3 min, then vortex-mixed vigorously to reduce viscosity. Aliquots of the homogenates were resolved on an SDS/6% PAGE, and then blotted onto a nitrocellulose membrane. WT and mutant GC-Bs were recognized using monoclonal anti-myc antibody 9E10 (National Cell Culture Center).

Quantification of GC-B phosphorylation

Myc-GC-B or Y808E mutant cDNA was transfected into HeLa cells grown in 6-well plates, and the cells were cultured in the growth medium overnight. After 24 h culture in DMEM containing 0.5% FBS, cells were labelled with serum- and phosphate-free DMEM containing 0.1 mCi/ml [³²P]orthophosphate for 4 h. HeLa cells expressing Myc-GC-B were homogenized on ice with RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% (v/v) NP-40, 0.5% deoxycholate, 0.05% SDS and protease and protein phosphatase inhibitors as described above], and the homogenates were centrifuged for 10 min at 21 000 g at 4°C. The resulting supernatants were transferred to a tube containing mouse anti-myc antibody (5 µg/ml) and mixed for 4 h at 4°C. Protein G-Sepharose was then added to each tube and further mixed for 2 h at 4°C. The Protein-G-Sepharose-anti-myc antibody complex was washed five times with RIPA buffer, and the final pellet was mixed with 2× SDS/PAGE sample buffer (Bio-Rad) containing 5% (v/v) 2-mercaptoethanol. Samples were boiled for 3 min, aliquots of the homogenate were separated by SDS/PAGE (6% polyacrylamide gel), and then blotted on to a nitrocellulose membrane. Phosphorylated WT Myc-GC-B and Y808E bands were detected by autoradiography, and WT Myc-GC-B and Y808E proteins were detected using anti-myc antibody and the ECL system (GE).

RESULTS

Mutations of Tyr⁸⁰⁸ enhance GC-B activity

As stated, there are six phosphorylation sites in the juxtamembrane portion of the KHD (designated MPS in Figure 1), but dephosphorylation of these sites cannot fully account for the suppression of GC-B activity by S1P or other inhibitors. Therefore I postulated the existence of hitherto unidentified phosphorylation sites that could be susceptible to regulation by GC-B inhibitors. To identify these hypothetical phosphorylation sites in GC-B, I employed the NetPhos 2.0 phosphorylation site prediction program [28] (<http://www.cbs.dtu.dk/services/NetPhos/>), and selected residues having scores greater than 0.800 for mutation. Based on this search, I identified 14 potential serine/threonine phosphorylation sites and two potential tyrosine phosphorylation sites which had not previously been examined (Figure 1). I replaced

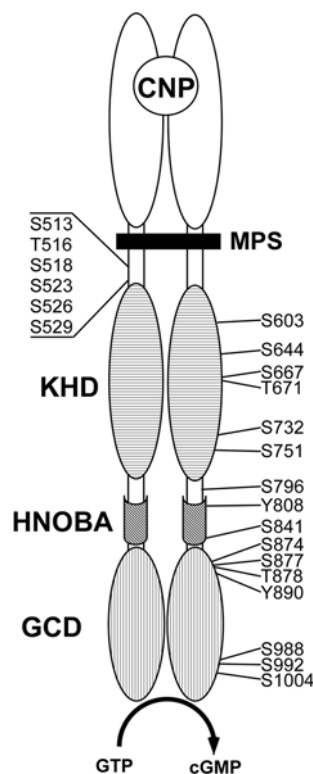


Figure 1 Schematic representation of the structure of GC-B and location of potential phosphorylation sites

All verified juxtamembrane phosphorylation sites are shown on the left, and potential serine/threonine and tyrosine phosphorylation sites predicted by the NetPhos 2.0 program are shown on the right. Each potential phosphorylation site was mutated to alanine (serine/threonine) or phenylalanine (tyrosine) using primers shown in Table S1. Blank ovals and filled bar show LBD and plasma membrane, respectively. CNP, C-type natriuretic peptide; MPS, multiple phosphorylation site; KHD, kinase-homology domain; HNOBA, the domain highly homologous to haem nitric oxide binding associated domain; and GCD, guanylyl cyclase (catalytic domain).

these serine/threonine and tyrosine residues with alanine and phenylalanine, respectively, expressed the mutants in HeLa cells, and measured CNP-stimulated cGMP production. As shown in Figure 2A, none of the mutations eliminated S1P-dependent inhibition of cyclase activity, though several of the mutants were less susceptible to inhibition than WT GC-B.

Although my mutational analysis indicated that S1P-mediated suppression of GC-B activity cannot be explained by a change in phosphorylation of any of the candidate phosphorylation sites (at least individually), I found that cells expressing the Y808F mutant produced more than 30-fold higher levels of cGMP than cells expressing WT GC-B upon stimulation with 0.1 µM CNP (Figure 2B), despite lower levels of expression of the mutant cyclase (Figure 2C). Phosphorylation of Tyr⁸⁰⁸ did not contribute to this effect, as neither WT GC-B nor the Y808F mutant was recognized by the 4G10 anti-phosphotyrosine antibody (not shown). To further analyse the significance of Tyr⁸⁰⁸ for GC-B activity, I substituted this residue with amino acids having different chemical

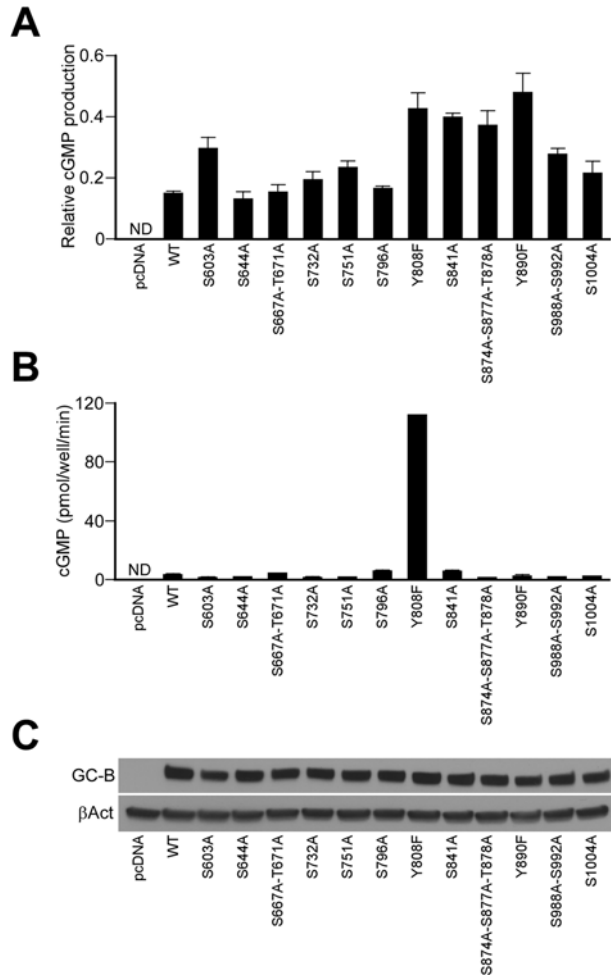


Figure 2 cGMP production in HeLa cells expressing WT Myc-GC-B and its mutants

(A) Effect of mutations on the inhibition of GC-B activity by S1P. WT and mutant forms of Myc-GC-B were expressed in HeLa cells preincubated in medium with or without 100 nM S1P for 30 min, and then stimulated with 20 nM CNP for 5 min. Bars represent the ratios of amount of cGMP produced in the presence against the absence of S1P. (B) CNP-stimulated cGMP production in HeLa cells expressing WT and mutant forms of Myc-GC-B. Cells were treated with 0.1 μ M CNP for 5 min prior to measurement of cGMP production. (C) Protein levels of WT and mutant Myc-GC-B in HeLa cell extract detected by immunoblotting with anti-myc antibody. β Act (β -actin) was used as a loading and transfer control. Each bar in panels (A) and (B) represents the means \pm S.E.M., $n = 3$.

characteristics. The effect of residue volume was examined by substituting Tyr⁸⁰⁸ with smaller (alanine) or larger (tryptophan) residues (residue volumes of alanine, phenylalanine, tyrosine and tryptophan are 67, 135, 141 and 186 \AA^3 , respectively [29]). The importance of hydrophilicity was examined by replacing Tyr⁸⁰⁸ with glutamic acid and serine. I expressed these mutants in HeLa cells to measure CNP-dependent cGMP production. Like the Y808F mutant, the Y808A, Y808E and Y808S mutants exhibited markedly higher GC activity than did WT GC-B (Figure 3). Upon stimulation with 1 μ M CNP, cells expressing the Y808A, Y808E and Y808S mutants produced more than 70- (750 pmol/mg pro-

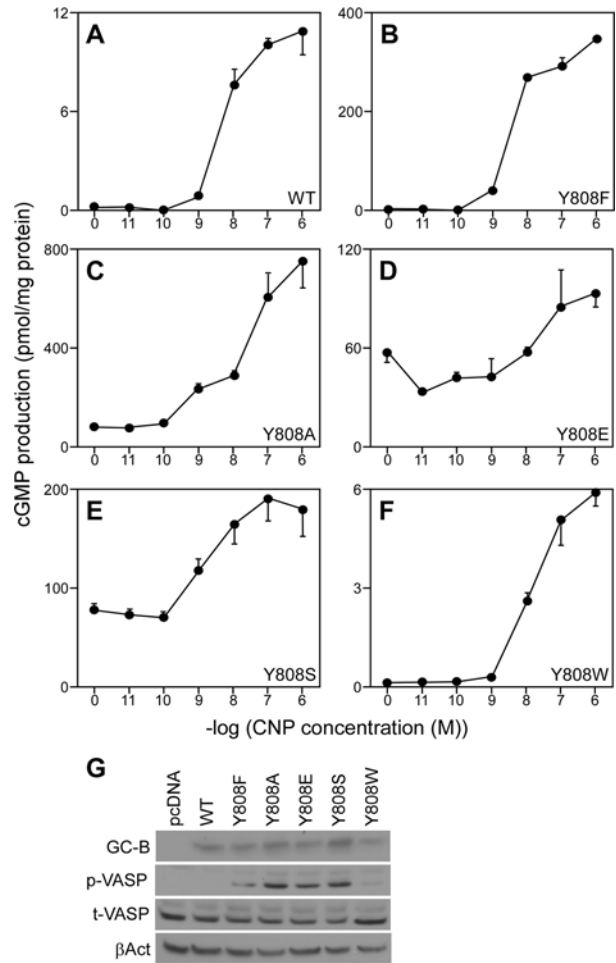


Figure 3 Dose-response elevation of cGMP production by WT Myc-GC-B and Tyr⁸⁰⁸ mutants

HeLa cells expressing WT Myc-GC-B (A) or Tyr⁸⁰⁸ mutants, such as Y808F (B), Y808A (C), Y808E (D), Y808S (E) and Y808W (F) were stimulated with indicated concentrations of CNP for 5 min, and cGMP production was measured. Data represent means \pm S.E.M., $n = 3$. (G) Levels of Myc-tagged WT GC-B and its Tyr⁸⁰⁸ mutants (GC-B), P-VASP (phosphorylated VASP), t-VASP (total VASP) and β Act (β -Actin) in HeLa cells determined by immunoblotting.

tein), 8- (92.9 pmol/mg protein) and 16-fold (178.9 pmol/mg protein) higher levels of cGMP than WT (10.7 pmol/mg protein), respectively (Figures 3A, 3C, 3D and 3E). Moreover, these three mutants were constitutively active, generating cGMP in cells 370-fold (78.3 pmol/mg protein, Y808A), 270-fold (56.8 pmol/mg protein, Y808E) and 370-fold (78.1 pmol/mg protein, Y808S) higher than WT (0.2 pmol/mg protein) (Figures 3A, 3C, 3D and 3E), even without CNP stimulation. Interestingly, the level of VASP (phosphorylated vasodilator-stimulated phosphoprotein), a well-characterized substrate of cGMP-dependent kinase [30], is higher in the cells expressing Y808A, Y808E and Y808S mutants than in cells expressing WT-GC-B even without CNP stimulation (Figure 3G). These data indicate that the cGMP production is elevated in intact cells expressing the mutants. Despite the

enhancement of catalytic potential by these Tyr⁸⁰⁸ mutations, the EC₅₀s of WT and mutant GC-Bs were similar, indicating that the mutations did not alter ligand–receptor interactions. The EC₅₀ of the single mutant that displayed lower than WT activity, Y808W, was also unchanged. In summary, GC-B mutants containing smaller and more hydrophilic residues than Tyr⁸⁰⁸ expressed remarkably higher GC activity than WT.

Hyperactive mutants are less susceptible to GC-B inhibitors

I next examined whether any of the Tyr⁸⁰⁸ mutations affected the suppression of GC activity by well-characterized GC-B inhibitors, such as S1P, PMA and Na₃VO₄. As demonstrated above, S1P-dependent suppression of GC-B activity was unaffected by the Y808F mutation, nor was it affected by the Y808W mutation (Figure 4A). However, the Y808A, Y808E and Y808S mutants were far less sensitive to S1P treatment, with the Y808E mutant showing the most dramatic resistance to inhibition (Figure 4A).

Figure 4(B) shows how the Tyr⁸⁰⁸ mutations affect GC-B inhibition by PMA and Na₃VO₄. PMA, an activator of PKC, is the most potent suppressor of GC-B activity currently known [21,24]. Na₃VO₄ [19], a non-specific tyrosine phosphatase inhibitor, also potently suppresses GC-B activity, by a mechanism believed to be similar to that of growth factor-mediated suppression of activity [19]. cGMP production by WT GC-B and the Y808F and Y808W mutants was suppressed to near-basal levels by PMA, whereas suppression of the Y808A mutant was greatly attenuated. Surprisingly, PMA treatment enhanced cGMP production in cells expressing the Y808E and Y808S mutants. Na₃VO₄ inhibited the activities of WT GC-B, as well as those of the Y808F, Y808A and Y808W mutants, but failed to significantly alter the activities of the Y808E and Y808S mutants.

Hyperactive mutants exhibit near-maximum GC-B activity

In intact cells and membranes, the activities of membrane-bound GCs are tightly regulated by their extracellular ligands. However, once these GCs are solubilized with detergents (e.g., Triton X-100) in the presence of Mn²⁺, they exhibit maximal cyclase activities, which are not further increased in the presence of ligands [31]. To test the effects of the Tyr⁸⁰⁸ mutations on this ligand-independent activity of GC-B, I expressed the mutants in HeLa cells, prepared membrane fractions and measured CNP-dependent and Triton X-100/Mn²⁺-dependent GC activities of these fractions (Table 1). Consistent with the data shown above, membranes expressing WT GC-B and the Y808W mutant displayed similar CNP-stimulated cGMP production, whereas CNP-stimulated activities of membranes expressing the Y808F, Y808A, Y808E and Y808S mutants were more than 10-fold higher than WT. In contrast, Triton X-100/Mn²⁺ activities of WT and mutant GC-Bs were similar. Thus, the Tyr⁸⁰⁸ mutations elicited striking elevations of GC-B activity in the context of intact cells and membranes, without markedly altering the maximal catalytic potential of the cyclase.

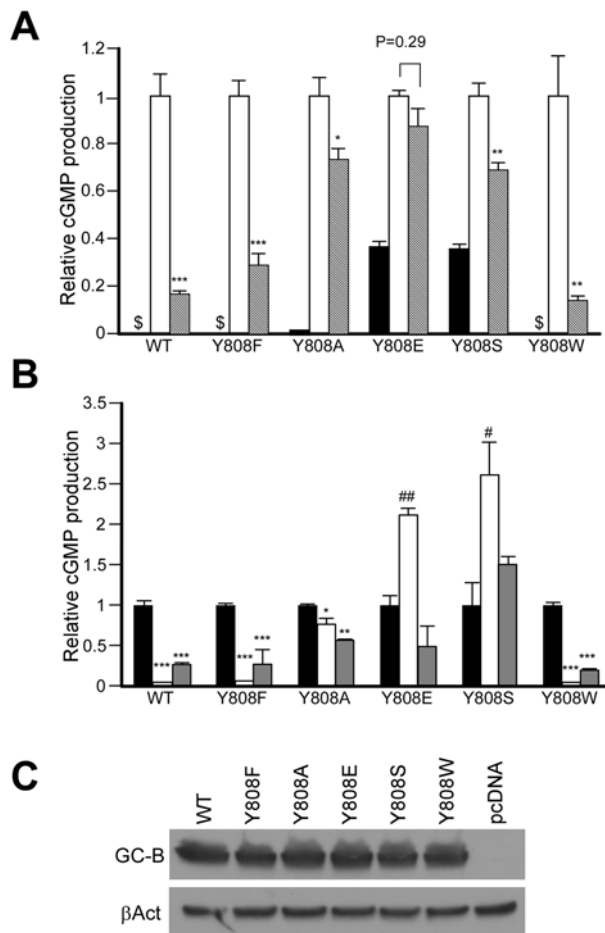


Figure 4 Effect of S1P, PMA and orthovanadate on cGMP production in WT Myc-GC-B and Tyr⁸⁰⁸ mutants

(A) HeLa cells transiently expressing Myc-tagged WT GC-B (WT) or Tyr⁸⁰⁸ mutants (Y808F, Y808A, Y808E, Y808S and Y808W) were treated with 100 nM S1P stimulated with 100 nM CNP for 5 min, and their cGMP production was measured. Hatched bars, blank bars and filled bars indicate cGMP production in HeLa cells treated without S1P and stimulated without CNP, treated without S1P and stimulated with CNP, and treated with S1P and stimulated with CNP, respectively. \$, cGMP production was less than detectable level. (B) HeLa cells transiently expressing Myc-tagged WT GC-B or its Tyr⁸⁰⁸ mutants were preincubated with vehicle (filled bars), 100 ng/ml PMA (blank bars) or 0.5 mM Na₃VO₄ (grey bars) for 30 min, and then stimulated with CNP as described above. (C) Expression levels of Myc-tagged WT GC-B and its mutants (GC-B) and βAct (β-actin) in HeLa cells estimated by immunoblotting. Data represents means ± S.E.M. *n* = 3. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; when the drug treatment reduced the value of cGMP production and #, *P* < 0.05; ##, *P* < 0.01, when the drug treatment elevated the value of cGMP production.

Phosphorylation is not essential for GC activity of Y808E

As discussed above, GC-B is constitutively phosphorylated and both its homologous and heterologous desensitization is accompanied by its dephosphorylation [25]. Moreover, mutation of phosphorylated serines Ser⁵²³ and Ser⁵²⁶ to alanine or glutamine greatly reduced GC-B activity [26]. These observations have led to the widespread supposition that phosphorylation is an

Table 1 CNP- and TX-100/Mn²⁺-dependent cGMP production in WT GC-B and Tyr⁸⁰⁸ mutants
Units are pmol/mg protein.

Type of GC-B	Basal (A)	CNP-dependent (B)	TX-100/Mn ²⁺ -dependent (C)	Ratio (B/C×100)
WT	1.4 ± 0.3	6.0 ± 0.2	310.1 ± 0.5	1.9
Y808F	20.1 ± 0.2	120.3 ± 7.9	668.2 ± 37.5	18.0
Y808A	25.4 ± 3.4	171.4 ± 33.9	552.1 ± 64.4	31.0
Y808E	54.5 ± 11.0	63.2 ± 8.8	221.1 ± 6.4	28.5
Y808S	31.6 ± 8.3	71.5 ± 8.0	117.5 ± 7.7	60.8
Y808W	8.5 ± 0.5	15.8 ± 0.2	290.4 ± 8.1	5.4

absolute requirement for GC-B activation. To examine the effect of SIP on the phosphorylation of the hyperactive GC-B mutants, I metabolically labelled HeLa cells expressing myc-tagged WT or Y808E GC-B with radioactive phosphate, stimulated cells with SIP and immunoprecipitated the receptors using an anti-myc antibody. As expected, SIP treatment resulted in $41 \pm 6\%$ reduction ($P < 0.01$) of ³²P_i incorporation level into WT GC-B (Figure 5A, left), which was consistent with a previous report [26]. In contrast, I was unable to detect phosphorylation of the Y808E mutant (Figure 5A, right).

I next introduced aspartic acid mutations (6D mutation) into all six potential phosphorylation sites in the juxtamembrane portion of both WT GC-B (WT GC-B-6D) and the Y808E mutant (Y808E-6D), and measured cGMP production in cells expressing these constructs, in the absence or presence of CNP (Figure 5B). Consistent with the previous report about GC-B having six glutamic acid mutations in the juxtamembrane phosphorylation sites [22], WT-GC-B-6D mutant exhibited greatly reduced cGMP production, which was slightly elevated upon CNP stimulation (Figure 5B, inset). Unexpectedly, Y808E-6D mutant produced remarkably higher amounts of cGMP in the absence of CNP, than did Y808E, although no significant elevation of CNP-induced cGMP production was observed (Figure 5B).

DISCUSSION

In this study I demonstrated that Tyr⁸⁰⁸ plays a critical role in the regulation of the enzymatic activity of GC-B. Mutations of Tyr⁸⁰⁸ resulted in a wide range of effects on cyclase activity, depending on the nature of the substituting residue (Figure 3). In addition, the fact that mutation of a single amino acid residue in GC-B results in catalytic hyperactivation points to an auto-inhibitory mechanism that regulates the activity of WT GC-B. Although the intracellular crystal structure of membrane-bound GC has not been solved, the extracellular crystal structure of hormone-bound ANP receptor [12] gives us clues to understand the auto-inhibition mechanism of GC-B. One probable auto-inhibition mechanism is that one of intracellular domain of GC-B dimer occludes another GC catalytic site, thereby limiting access of GTP to the catalytic site (Figure 6). As mentioned above, the two subunits of the LBD dimer undergo twisted motion, which

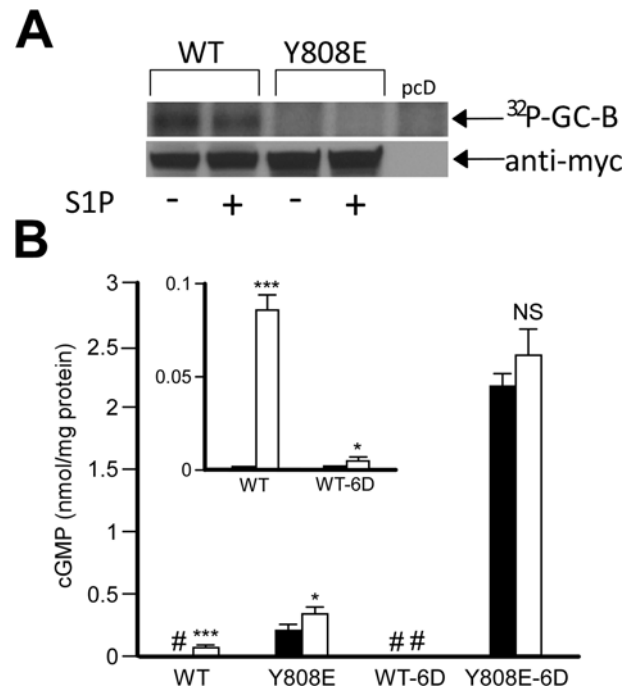


Figure 5 Phosphorylation levels (A), and effect of phosphomimetic mutation in the hyperphosphorylation sites on GC activity (B) in WT GC-B and its Y808E mutant

(A) HeLa cells transiently expressing Myc-tagged WT GC-B (WT) or Y808E mutant (Y808E), or empty vector (pcD) were cultured with the medium containing ³²P-orthophosphate and stimulated with 1 μM SIP for 30 min. The cells were then lysed with RIPA buffer, and immunoprecipitation was performed as described in 'Experimental Procedures'. Upper panel shows phosphorylated-GC bands detected by autoradiography, and panel shows WT Myc-GC-B and its Y808E mutant bands in HeLa cells probed with anti-myc antibody, and visualized with HRP (horseradish peroxidase)-conjugated secondary antibody and ECL system. The data represent one of three independent experiments. (B) HeLa cells transiently expressing Myc-tagged WT GC-B (WT), and Y808E, WT-6D and Y808E-6D mutants were cultured in the absence (filled bars) or presence (blank bars) of 100 nM CNP for 5 min, and their cGMP production was measured. #, see inset. Data represent means ± S.E.M, $n = 3$. *, $P < 0.05$; ***, $P < 0.001$.

also alters the orientation between the two intracellular domains to open their catalytic sites upon CNP binding (Figure 6B). This auto-inhibitory mechanism can be circumvented by replacement of the native residue, Tyr⁸⁰⁸, with a smaller (alanine or serine) or charged (glutamic acid) amino acid, probably because these

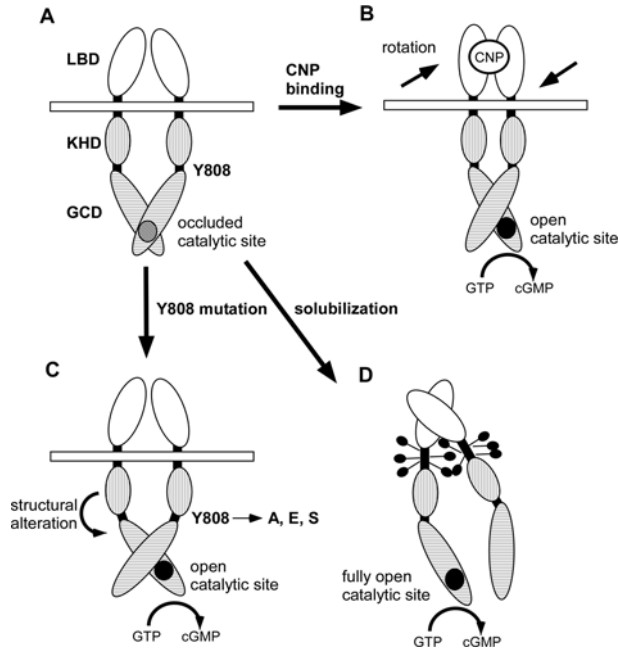


Figure 6 Hypothetical mechanism of (A) resting state, (B) ligand-dependent, (C) detergent-dependent and (D) mutation-dependent activation of GC-B

(A) One of two catalytic sites of GC-B is occluded by another subunit of GC-B dimers. GTP is prohibited access to the catalytic sites. (B) Two subunits of the LBD dimer undergo re-orientation upon CNP binding, which is followed by further re-orientation of the intracellular domains to open the catalytic sites. GTP is then allowed to access the open catalytic sites to be converted to cGMP. (C) Tyr⁸⁰⁸ mutations induce structural alteration of the intracellular domain, which results in opening the catalytic site. (D) Treatment of GC-B with detergent perturbs orientation of two dimers and renders the catalytic sites open fully. LBD, ligand binding domain; CNP, C-type natriuretic peptide; KHD, kinase homology domain and GCD, guanylyl cyclase (catalytic) domain. Inactive catalytic site is shown with a diagonally striped circle, and active catalytic sites are shown with filled circles.

mutations alter the orientation of GC domain to KHD, allowing GTP to access the catalytic site with less steric hindrance (Figure 6C). This auto-inhibitory model is likely to be relevant to the well-established observation that GC activity reaches to maximum upon solubilization with a detergent. Perhaps the proper arrangement of GC-B dimer is perturbed by the solubilization, and this perturbation may render the catalytic site open fully and producing cGMP with maximum activity (Figure 6D).

An unexpected consequence of the Y808A, Y808E and Y808S mutations was the markedly reduced suppression of GC-B activity by SIP, PMA and orthovanadate (Figure 4B). Indeed, GC activity was actually enhanced in the Y808E and Y808S mutants upon treatment with PMA, suggesting that PKC (or another target of PMA) regulates GC-B in a complex fashion, exerting both inhibitory and excitatory influences. Based on my model shown in Figure 6, I assume that the PKC or another target of PMA may alter the intracellular structure of GC-B, resulting in an unfavourable for WT GC-B to open the catalytic site but favourable for the Y808E and Y808S mutants.

(A)			
Ho GC-B	DNLLLRMEQYANNLEKLV	VEERTQAYLEEKRAEALL	YQILPHSVAEQL 846
Dr sGcb	YKLTKNLEMLTDKLGQT	FRDLESEKQKTDRLLY	YSVLPKSVANEL 516
Xe sGcb3	YKLTQGELEILTDLRGHT	LRALEDEKKTDTLLYS	VLPSPVANEL 406
Or sGca2	DGLKKRMDKLTALERT	HQALEEEKRRTVDLLYS	IFPGDVAQKL 575
Ra sGca2	DGLKKRMDKLTALERT	HQALEEEKKTVDLLYS	IFPGDVAQQL 504
Ho GCYA3	DGLKKRLGKLTALTEQA	HQALEEEKKTVDLLCS	IFPCVAEQAQL 466
Da sGca3	DGLKKRLGKAKAALTEQA	HQAGGRKEANSRAALHN	LPRKRCASAL 469
Ca GCY37	MELNKKLEETMKMKMKM	TEELVKKSGTDRLLFF	FFVPPVIAEL 419
Ca GCY36	VEMNLQLEANNEQLNM	AKDLEVEKGTDALLRE	MLPPSVAQQL 435
	--*	*-*****-*****-*	
(B)			
GC-B	LLLRMEQYANNLEKLV	VEERTQAYLEEKRAEALL	YQILPHSVAEQL 846
GC-A	LLSRMEQYANNLEEL	VEERTQAYLEEKRAEALL	YQILPHSVAEQL 861
GC-C	LIRRLQLYSRNLEHL	VEERTQYKAERDRADRL	NFMFLPRLVVKSL 809
GC-E	MLRMLEQYSSNLEDL	IRERTEELEEKQKTDRL	LTMQLPPSVAEAL 865
GC-F	MLRMLEQYSSNLEDL	IRERTEELEIEKQKTEKL	LTMQLPPSVAESL 869
ADCY2	VLGRONEYICRLDFLW	KNKFKKEREETETMEN	NRVLLLENVLP
ADCY4	VLARONEYICRLDFLW	KNKFKKEREETETMEN	TRLLLENVLP

Figure 7 Alignment of amino acid sequences of human GC-B with HNOBA domain of soluble GCs (A), and with membrane bound GCs and ADCYs (B)

(A) GC-B (amino acids 799–846) was aligned with a partial fragment of the HNOBA domain of soluble GCs of various species. Asterisks show amino acid residues of GC-B having significant similarity to soluble GCs. Ho, *Homo sapiens*; Dr, *Drosophila melanogaster*; Xe, *Xenopus laevis*, Or, *Oryzias latipes*; Ra, *Rattus norvegicus*; Da, *Danio rerio*; Ca, *Caenorhabditis elegans*. (B) Partial amino acid sequence of human GC-B including Tyr⁸⁰⁸ was aligned with those of HNOBA domain of all membrane-bound GCs, ADCY2 and ADCY4, all of which have tyrosine residue at the position equivalent to Tyr⁸⁰⁸ of GC-B. The alignment was constructed using the NCBI BLASTP amino acid sequence homology search engine. Highlighted residues show Tyr⁸⁰⁸ of GC-B and its equivalent tyrosine residues in other cyclases. Italic shows Arg⁸³⁸ of GC-E, and mutation of which has been linked to autosomal dominant inherited cone degeneration and cone-rod degeneration.

Another unexpected outcome of this study was the finding that phosphorylation is apparently not necessary for expression of GC activity by the Y808E mutant (Figure 5). This hyperactive mutant failed to incorporate radioactive phosphate in cells over a 6 h labelling period (Figure 5A), indicating that the Y808E mutant exhibits strong GC activity in cells without phosphorylation. Furthermore, the Y808E mutant carrying six aspartic acid mutations at the juxtamembrane phosphorylation motif exhibited much higher GC activity than that of Y808E, even though WT-GC-B containing the same six aspartic acid mutations exhibited only trace level of GC activity. Future structural analyses will elucidate the mechanism to clarify these results.

Based on NCBI (National Center for Biotechnology Information) Conserved Domain Database [32], Tyr⁸⁰⁸ is located in a niche domain between the KHD and GC domains and thus would not be expected to affect the structure of these domains directly. Interestingly, the niche domain containing Tyr⁸⁰⁸ shows sequence similarity to a partial fragment of HNOBA (haem nitric oxide binding associated) domain, which is found in soluble GCs and bacterial haem-binding protein kinases [33,34] (Figure 7A). HNOBAs consist of an N-terminal core subdomain having interspersed α -helices and β -strands, followed by an extended C-terminal α -helix, termed the helical linker region, which has the potential to form coiled-coils and, hence, may be involved in dimerization. Based on the sequence alignment (Figure 6A) and on secondary structure predictions of Iyer et al. [34], Tyr⁸⁰⁸ is located within the helical linker region. Mutations of Tyr⁸⁰⁸ may alter

the dimerization state of GC-B and thereby control its catalytic activity, which fits our hypothesis shown in Figure 6.

All membrane-bound GCs, as well as adenylyl cyclases 2 and 4, have tyrosine at the position corresponding to residue 808 of GC-B (Figure 6B). Therefore it may be possible to generate a variety of cyclases with enhanced activity by substituting the Tyr⁸⁰⁸-equivalent tyrosine residue with alanine or phenylalanine, and of obtaining constitutively active cyclases by replacing that residue with serine or glutamic acid. Interestingly, mutations in Glu⁸³⁷, Arg⁸³⁸ and Thr⁸³⁹ of GC-E (shown in bold italics in Figure 7B), which are within 12 amino acids of the tyrosine residue equivalent to Tyr⁸⁰⁸ in GC-B, have been linked to dominant cone-rod dystrophy [35–38]. These three residues of GC-E are also conserved among all membrane-bound GCs. My constitutively active mutants may eventually prove to be valuable therapeutic tools, as GC-B activity has been shown to be protective against several disorders. For example, Yasoda et al. [39] showed that tissue-specific overexpression of CNP and activation of GC-B counteracts dwarfism in a mouse model of achondroplasia containing a hyperactive mutation of FGFR 3 (fibroblast growth factor receptor 3). Introduction of those constitutively active GC-B mutants may mimic the overexpressing CNP-induced GC-B activation in chondrocytes and is likely to suppress FGFR3-dependent achondroplasia.

Due to its location between the KHD and the GC domain, scant attention has been paid to the potential biochemical significance of Tyr⁸⁰⁸ or its neighbouring residues. Current models suggest that membrane-bound GCs are present in an auto-inhibited conformation in unstimulated cells, and that binding of natriuretic peptides to their extracellular domains releases this inhibitory constraint. Mechanisms that underlie GC auto-inhibition have remained elusive, primarily because three-dimensional structures of the intracellular domains of membrane-bound GCs have not yet been solved. My results suggest that Tyr⁸⁰⁸ contributes to the auto-inhibition of GC-B, and raises the possibility that binding of CNP to the receptor induces a conformational change that displaces this residue from its inhibitory orientation.

ACKNOWLEDGMENT

I thank Joseph P Albanesi, Melanie H. Cobb and Barbara Barylko for helpful discussion.

FUNDING

This work was supported by American Heart Association [Grant-in-Aid 10BGIA4180105].

REFERENCES

- Garbers, D. L., Chrisman, T. D., Wiegand, P., Katafuchi, T., Albanesi, J. P., Bielinski, V., Barylko, B., Redfield, M. M. and Burnett, Jr, J. C. (2006) Membrane guanylyl cyclase receptors: an update. *Trends Endocrinol. Metab.* **17**, 251–258
- Walther, T. and Stepan, H. (2004) C-type natriuretic peptide in reproduction, pregnancy and fetal development. *J. Endocrinol.* **180**, 17–22
- Take, T., Kitamura, H., Adachi, Y., Yoshioka, T., Watanabe, T., Matsushita, H., Fujii, T., Kondo, E., Tachibe, T., Kawase, Y. et al. (2009) Chronically elevated plasma C-type natriuretic peptide level stimulates skeletal growth in transgenic mice. *Am. J. Physiol. Endocrinol. Metab.* **297**, E1339–E1348
- Herman, J. P., Dolgas, C. M., Rucker, D. and Langub, Jr, M. C. (1996) Localization of natriuretic peptide-activated guanylate cyclase mRNAs in the rat brain. *J. Comp. Neurol.* **369**, 165–187
- Vaandrager, A. B. (2002) Structure and function of the heat-stable enterotoxin receptor/guanylyl cyclase C. *Mol. Cell. Biochem.* **230**, 73–83
- Karan, S., Frederick, J. M. and Baehr, W. (2010) Novel functions of photoreceptor guanylate cyclases revealed by targeted deletion. *Mol. Cell. Biochem.* **334**, 141–155
- Zufall, F. and Munger, S. D. (2010) Receptor guanylyl cyclases in mammalian olfactory function. *Mol. Cell. Biochem.* **334**, 191–197
- Lowe, D. G. (1992) Human natriuretic peptide receptor-A guanylyl cyclase is self-associated prior to hormone binding. *Biochemistry* **31**, 10421–10425
- Thompson, D. K. and Garbers, D. L. (1995) Dominant negative mutations of the guanylyl cyclase-A receptor. Extracellular domain deletion and catalytic domain point mutations. *J. Biol. Chem.* **270**, 425–430
- Labrecque, J., Mc Nicoll, 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
- Iwata, T., Uchida-Mizuno, K., Katafuchi, T., Ito, T., Hagiwara, H. and Hirose, S. (1991) Bifunctional atrial natriuretic peptide receptor (type A) exists as a disulfide-linked tetramer in plasma membranes of bovine adrenal cortex. *J. Biochem.* **110**, 35–39
- Ogawa, H., Qiu, Y., Ogata, C. M. and Misono, K. S. (2004) Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain: rotation mechanism for transmembrane signal transduction. *J. Biol. Chem.* **279**, 28625–28631
- 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* **406**, 101–104
- Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H. M., Goeddel, D. V. and Schulz, S. (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* **338**, 78–83
- Antos, L. K., Abbey-Hosch, S. E., Flora, D. R. and Potter, L. R. (2005) ATP-independent activation of natriuretic peptide receptors. *J. Biol. Chem.* **280**, 26928–26932
- Abbey-Hosch, S. E., Cody, A. N. and Potter, L. R. (2004) Sphingosine-1-phosphate inhibits C-type natriuretic peptide activation of guanylyl cyclase B (GC-B/NPR-B). *Hypertension* **43**, 1103–1109
- Chrisman, T. D., Perkins, D. T. and Garbers, D. L. (2003) Identification of a potent serum factor that causes desensitization of the receptor for C-Type natriuretic peptide. *Cell Commun. Signal.* **1**, 4
- Abbey, S. E. and Potter, L. R. (2003) Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. *Endocrinology* **144**, 240–246
- Chrisman, T. D. and Garbers, D. L. (1999) Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts. *J. Biol. Chem.* **274**, 4293–4299

- 20 Abbey, S. E. and Potter, L. R. (2002) Vasopressin-dependent inhibition of the C-type natriuretic peptide receptor, NPR-B/GC-B, requires elevated intracellular calcium concentrations. *J. Biol. Chem.* **277**, 42423–42430
- 21 Tsang, D., Tung, C. S., Yeung, V. T. and Cockram, C. S. (1997) Endothelin-3 reduces C-type natriuretic peptide-induced cyclic GMP formation in C6 glioma cells. *Regul. Pept.* **70**, 91–96
- 22 Potthast, R., Abbey-Hosch, S. E., Antos, L. K., Marchant, J. S., Kuhn, M. and Potter, L. R. (2004) Calcium-dependent dephosphorylation mediates the hyperosmotic and lysophosphatidic acid-dependent inhibition of natriuretic peptide receptor-B/guanylyl cyclase-B. *J. Biol. Chem.* **279**, 48513–48519
- 23 Potter, L. R. and Hunter, T. (2000) Activation of protein kinase C stimulates the dephosphorylation of natriuretic peptide receptor-B at a single serine residue: a possible mechanism of heterologous desensitization. *J. Biol. Chem.* **275**, 31099–31106
- 24 Abbey-Hosch, S. E., Smirnov, D. and Potter, L. R. (2005) Differential regulation of NPR-B/GC-B by protein kinase C and calcium. *Biochem. Pharmacol.* **70**, 686–694
- 25 Yoder, A. R., Stone, M. D., Griffin, T. J. and Potter, L. R. (2010) Mass spectrometric identification of phosphorylation sites in guanylyl cyclase A and B. *Biochemistry* **49**, 10137–10145
- 26 Potter, L. R. and Hunter, T. (1998) Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. *J. Biol. Chem.* **273**, 15533–15539
- 27 Leitman, D. C., Andresen, J. W., Catalano, R. M., Waldman, S. A., Tuan, J. J. and Murad, F. (1988) Atrial natriuretic peptide binding, cross-linking, and stimulation of cyclic GMP accumulation and particulate guanylate cyclase activity in cultured cells. *J. Biol. Chem.* **263**, 3720–3728
- 28 Blom, N., Gammeltoft, S. and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294**, 1351–1362
- 29 Creighton, T. E. (1993) *Proteins: Structure and Molecular Properties*, 2 edn., Freeman, New York
- 30 Krause, M., Dent, E. W., Bear, J. E., Loureiro, J. J. and Gertler, F. B. (2003) Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Ann. Rev. Cell Dev. Biol.* **19**, 541–564
- 31 Katafuchi, T., Mizuno, T., Hagiwara, H., Itakura, M., Ito, T. and Hirose, S. (1992) Modulation by NaCl of atrial natriuretic peptide receptor levels and cyclic GMP responsiveness to atrial natriuretic peptide of cultured vascular endothelial cells. *J. Biol. Chem.* **267**, 7624–7629
- 32 Marchler-Bauer, A., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., Gonzales, N. R. and Gwadz, M. (2009) CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res.* **37**, D205–D210
- 33 Gilles-Gonzalez, M. A. and Gonzalez, G. (2005) Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *J. Inorg. Biochem.* **99**, 1–22
- 34 Iyer, L. M., Anantharaman, V. and Aravind, L. (2003) Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins. *BMC Genomics* **4**, 5
- 35 Kelsell, R. E., Gregory-Evans, K., Payne, A. M., Perrault, I., Kaplan, J., Yang, R. B., Garbers, D. L., Bird, A. C., Moore, A. T. and Hunt, D. M. (1998) Mutations in the retinal guanylate cyclase (RETGC-1) gene in dominant cone-rod dystrophy. *Hum. Mol. Genet.* **7**, 1179–1184
- 36 Perrault, I., Rozet, J. M., Gerber, S., Kelsell, R. E., Souied, E., Cabot, A., Hunt, D. M., Munnich, A. and Kaplan, J. (1998) A retGC-1 mutation in autosomal dominant cone-rod dystrophy. *Am. J. Hum. Genet.* **63**, 651–654
- 37 Kitiratschky, V. B., Wilke, R., Renner, A. B., Kellner, U., Vadala, M., Birch, D. G., Wissinger, B., Zrenner, E. and Kohl, S. (2008) Mutation analysis identifies GUCY2D as the major gene responsible for autosomal dominant progressive cone degeneration. *Invest. Ophthalmol. Vis. Sci.* **49**, 5015–5023
- 38 Downes, S. M., Payne, A. M., Kelsell, R. E., Fitzke, F. W., Holder, G. E., Hunt, D. M., Moore, A. T. and Bird, A. C. (2001) Autosomal dominant cone-rod dystrophy with mutations in the guanylate cyclase 2D gene encoding retinal guanylate cyclase-1. *Arch. Ophthalmol.* **119**, 1667–1673
- 39 Yasoda, A., Komatsu, Y., Chusho, H., Miyazawa, T., Ozasa, A., Miura, M., Kurihara, T., Rogi, T., Tanaka, S., Suda, M. et al. (2004) Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.* **10**, 80–86

Received 15 February 2013/20 March 2013; accepted 15 April 2013

Published as Immediate Publication 15 April 2013, doi 10.1042/BSR20130025



OPEN ACCESS

SUPPLEMENTARY DATA

Mutations in Tyr⁸⁰⁸ reveal a potential auto-inhibitory mechanism of guanylate cyclase-B regulationTakeshi KATAFUCHI¹

Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, TX, U.S.A.

Table S1 PCR primers used in the present study

Mutation	Sense primer (5'→3')	Antisense primer (5'→3')
S603A	CCTCGTGGGGCCTTACAAGAT	ATCTTGTAAGGCCCCACGAGG
S644A	TTCATGGAGCCCTCAAGTCC	GGACTTGAGGGCTCCATGAGA
S667A-T671A	CTGGCTGCTTCCGATCAGCTGCTGAA	TTCAGCAGCTGATCGGAAAGCAGCCAG
S732A	CTGGACCTCGTCCCAAGGAG	CTCCTTGGGAGCGAGGTCCAG
S751A	TTCCGGCCAGCCATTGACCGG	CCGGTCAATGGCTGGCCGGAA
S796A	GGTGGTACCGCCATATTGGAC	GTCCAATATGGCGTACCACC
S841A	CTACCCCATGCTGTAGCAGAG	CTCTGCTACAGCATGGGGTAG
S874A-S877A-T878A	GCGCTGGCAGCTGAGGCCGCCCATG	CATGGGGCGGCCTCAGCTGCCAGCGC
S988A-S992A	ACTGCTGCTCGAATGGAGGCGAATGGC	GCCATTCGCCTCCATTGAGCAGCAGT
S1004A	GTCTCCTCGGCCACCAAGGAC	GTCCTTGGTGGCCGAGGAGAC
Y808F	ATGGAACAGTTTGCTAATAAC	GTTATTAGCAAAGTGTCCAT
Y890F	AATGACCTTTTACCTGCTTT	AAAGCAGGTAAAAAGGTCATT
Y808A	TGCGCATGGAACAGGCTGCTAATAAC	GTTATTAGCAGCCTGTTCCATGCGCA
Y808E	TGCGCATGGAACAGGAGGCTAATAAC	GTTATTAGCCTCCTGTTCCATGCGCA
Y808S	TGCGCATGGAACAGTCTGCTAATAAC	GTTATTAGCAGACTGTTCCATGCGCA
Y808W	TGCGCATGGAACAGTGGGCTAATAAC	GTTATTAGCCCACTGTTCCATGCGCA
S513D-T516D-S518D	GCAGGCGATCGCTGGATCTGGATCTGCGG	CCGCAGATCCAGATCCAGGCGATCGCCTGC
S523D-S526D-T529D	GGATCCGATTACGGCGATCTCATGGAT	ATCCATGAGATCGCCGTAATCGGATCC

Received 15 February 2013/20 March 2013; accepted 15 April 2013

Published as Immediate Publication 15 April 2013, doi 10.1042/BSR20130025

¹ email Takeshi.Katafuchi@UTSouthwestern.edu