Mutations in Tyr^{808} reveal a potential auto-inhibitory mechanism of guanylate cyclase-B regulation

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Synopsis

In this study, Tyr^{808} 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 Tyr^{808} 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, Na_{3}VO_{4} and PMA. Y808A, Y808E and Y808S mutations markedly weakened S1P- and Na_{3}VO_{4}-dependent suppression of GC-B activity, whereas Y808E and Y808S mutations rather elevated cGMP production. Tyr^{808} 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

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 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; HN0BA, 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.

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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\(_2\)VO\(_4\) [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 ser/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 Tyr\(^{108}\) highlighted its importance in the regulation of GC-B activity. The characteristics of these Tyr\(^{108}\) 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 (GCBI688s) 5′-AAAGCTGATGCTGGAGAAGGA-3′ and an antisense primer for each of the mutations in Table S1, or an antisense primer (GCBmyca) 5′-GGCGCCGCTCACAGATCCTCTTCTGAGATGAGAAGGA-3′ and a sense primer for the same mutation in Table S1. The reactions were mixed, and PCR was performed again using GCBI688s 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 μg/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\(_2\)VO\(_4\) 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-Dioxiane: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\(_3\)COONa (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 μg/ml each of Na\(_2\)-p-tosyl-l-lysine chloromethyl ester, Na\(_2\)-p-tosyl-l-arginine methyl ester, Na\(_2\)-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 μg/tube. Reaction was allowed to proceed for 5 min at 37 °C in 100 μl 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 μM 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 μl 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 onto 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 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
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 Å³, 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 μM CNP, cells expressing the Y808A, Y808E and Y808S mutants produced more than 70- (750 pmol/mg protein), 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 Tyr\textsuperscript{808} mutations, the EC\textsubscript{50}s of WT and mutant GC-Bs were similar, indicating that the mutations did not alter ligand–receptor interactions. The EC\textsubscript{50} 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 Tyr\textsuperscript{808} expressed remarkably higher GC activity than WT.

**Hyperactive mutants are less susceptible to GC-B inhibitors**

I next examined whether any of the Tyr\textsuperscript{808} mutations affected the suppression of GC activity by well-characterized GC-B inhibitors, such as S1P, PMA and Na\textsubscript{3}VO\textsubscript{4}. 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 Tyr\textsuperscript{808} mutations affect GC-B inhibition by PMA and Na\textsubscript{3}VO\textsubscript{4}. PMA, an activator of PKC, is the most potent suppressor of GC-B activity currently known [21,24]. Na\textsubscript{3}VO\textsubscript{4} [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 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. Na\textsubscript{3}VO\textsubscript{4} 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 Mn\textsuperscript{2+}, they exhibit maximal cyclase activities, which are not further increased in the presence of ligands [31]. To test the effects of the Tyr\textsuperscript{808} 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/Mn\textsuperscript{2+}-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/Mn\textsuperscript{2+} activities of WT and mutant GC-Bs were similar. Thus, the Tyr\textsuperscript{808} 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.

**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 Ser\textsuperscript{523} and Ser\textsuperscript{526} to alanine or glutamate greatly reduced GC-B activity [26]. These observations have led to the widespread supposition that phosphorylation is an...
In this study I demonstrated that Tyr^{808} plays a critical role in the regulation of the enzymatic activity of GC-B. Mutations of Tyr^{808} 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 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, Tyr^{808}, with a smaller (alanine or serine) or charged (glutamic acid) amino acid, probably because these

### Table 1

| 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              |

![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](image)

**DISCUSSION**

In this study I examined the effect of S1P on the phosphorylation of the hyperactive GC-B mutants, I metabolically labelled HEK293 cells expressing myc-tagged WT or Y808E GC-B with radioactive phosphate, stimulated cells with S1P and immunoprecipitated the receptors using an anti-myc antibody. As expected, S1P treatment resulted in 41 ± 6% reduction (P < 0.01) of 32P 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).
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. (C) Tyrosine residues in the intracellular domains of GC-B can be aligned with those of HNOBA (haem nitric oxide binding associated) domain of soluble GCs. 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. (D) Partial amino acid sequence of human GC-B containing Tyr808 was aligned with those of HNOBA domain of all membrane-bound GCs, ADCY2 and ADCY4, of which have tyrosine residue at the position of Tyr808.

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.

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SUPPLEMENTARY DATA

Mutations in Tyr<sup>808</sup> reveal a potential auto-inhibitory mechanism of guanylate cyclase-B regulation

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| Mutation | Sense primer (5′ → 3′) | Antisense primer (5′ → 3′) |
|----------|-------------------------|---------------------------|
| S603A    | CCTCGTGGGGCCTTACAAGAT   | ATCTTGTAAGGCCCCACGAGG     |
| S644A    | TCTCATGGAGCCCTCAAGTCC  | GGAAGTGTAAGGCCCTCAAGTGA   |
| S667A-T671A | CTGGCTGCTTCCGATCAGCTGCTGAA | TTCAGCAGCTGATCAGGAAGACGACAG |
| S732A    | CTGACCTCGCTCCCAAGGAG  | CTCCTGAGGAAGCGAGGTCAG    |
| S751A    | TTTCCGGCCAGCCATTGACCG  | CCGGTCAGGCTGAGGCCGGAAGA  |
| S796A    | GTTGGTGACCGCCATATTGGAC | GTCCAAATGCGCAGTACCAC     |
| S841A    | CTACCCATGCTGTAGCAGAG  | CTTCGTGAGCACTGAGGATGAG   |
| S874A-S877A-T878A | GCGCTGGGCGCTGAGGCCGCCCATG | CATGGGCGCTGAGAGACGAGCGGC |
| S988A-S992A | ACTGCTGCTGAAATGGAGGGAAGAC | GCCATTGAGCAGCTGAGGACATG |
| S1004A   | GTTCCTCGGGCCACACGGAGG | GTGTGACCTGGGAGAAGGACATG |
| Y808F    | ATGGAGACAGTTTTGCTAAAC  | GCTAGGAAACTGGAAAGTGGCAT   |
| Y890F    | AATGAGACCTTGCTTTTACCTGT  | AAAGAGCGTAAAGGAGCTATT  |
| Y808A    | TGCGGCTAGGAAGCAGCTGCTGAAAC | GTTATAGCCAGCTGCTTCCATGAC |
| Y808E    | TGCGGCTAGGAAGCAGCTGCTGAAAC | GTTATAGCCAGCTGCTTCCATGAC |
| Y808S    | TGCGGCTAGGAAGCAGCTGCTGAAAC | GTTATAGCCAGCTGCTTCCATGAC |
| Y808W    | TGCGGCTAGGAAGCAGCTGCTGAAAC | GTTATAGCCAGCTGCTTCCATGAC |
| S513D-T516D-S518D | GCCGCGGCTCGCTGCTGGGTTGCCG | GCCGATCAGGATCCAGCGGATCGCTG |
| S523D-S526D-T529D | GCCGATCAGGATCCAGCGGATCGCTG | GCCGATCAGGATCCAGCGGATCGCTG |

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