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# A human skeletal overgrowth mutation increases maximal velocity and blocks desensitization of guanylyl cyclase-B☆

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## Abstract

C-type natriuretic peptide (CNP) increases long bone growth by stimulating guanylyl cyclase (GC)-B/NPR-B/NPR2. Recently, a Val to Met missense mutation at position 883 in the catalytic domain of GC-B was identified in humans with increased blood cGMP levels that cause abnormally long bones. Here, we determined how this mutation activates GC-B. In the absence of CNP, cGMP levels in cells expressing V883M-GC-B were increased more than 20 fold compared to cells expressing wild-type (WT)-GC-B, and the addition of CNP only further increased cGMP levels 2-fold. In the absence of CNP, maximal enzymatic activity (Vmax) of V883M-GC-B was increased 15-fold compared to WT-GC-B but the affinity of the enzymes for substrate as revealed by the Michaelis constant (Km) was unaffected. Surprisingly, CNP decreased the Km of V883M-GC-B 10-fold in a concentration dependent manner without increasing Vmax. Unlike the WT enzyme the Km reduction of V883M-GC-B did not require ATP. Unexpectedly, V883M-GC-B, but not WT-GC-B, failed to inactivate with time. Phosphorylation elevated but was not required for the activity increase associated with the mutation because the Val to Met substitution also activated a GC-B mutant lacking all known phosphorylation sites. We conclude that the V883M mutation increases maximal velocity in the absence of CNP, eliminates the requirement for ATP in the CNP-dependent Km reduction, and disrupts the normal inactivation process.

### Keywords

Natriuretic peptides; Guanylate cyclase; Bone growth; cGMP; Dwarfism; Achondroplasia

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**Conflict of interest statement**

None of the authors have a conflict of interest associated with this study.

## Introduction

C-type natriuretic peptide (CNP) stimulates long bone growth and inhibits meiotic resumption in oocytes by activating the enzyme variously known as guanylyl cyclase (GC)-B, natriuretic peptide receptor (NPR)-2 or NPR-B, which catalyzes the synthesis of the intracellular signaling molecule, cGMP[1–3]. GC-B is a homodimer containing an extracellular ligand-binding domain, a single membrane-spanning region, and an intracellular highly phosphorylated kinase homology domain, dimerization domain and C-terminal GC catalytic domain [4].

CNP binding increases GC-B activity by two mechanisms. It increases the maximal rate of cGMP production called maximal velocity (Vmax) and it also increases the affinity of the enzyme for GTP that is observed as a reduction in the Michaelis constant — the GTP concentration required to reach half the Vmax. Under non-physiologic conditions such as an enzyme assay where ATP is not present, the activity of GC-B is positive cooperative as demonstrated by a Hill coefficient of greater than 1. This means that GTP binds an allosteric site that increases the affinity of the catalytic site for GTP. However, under biological conditions where ATP concentrations are at or above 1 mM, the Hill coefficient of GC-B is 1 because the allosteric site is occupied by ATP not GTP. Recently, we demonstrated that ATP is required for the CNP-dependent reduction in the Km of GC-B [5,6]. Finally, in broken cell assays, ATP also increases GC-B activity by providing the phosphate that is added to the serine and threonine residues on the enzyme that is necessary for activation by CNP [7,8].

GC-B was identified in rat chondrocytes in 1994 [9], but the ability of natriuretic peptides to stimulate skeletal growth was first observed in transgenic mice overexpressing BNP in 1998 [10]. Subsequent bone culture studies indicated that CNP, not BNP, increased the proliferative and hypertrophic zones of the murine growth plate, which increases the length of long bones [10]. CNP also increases the earliest stage of endochondral bone development – the condensation of mesenchymal precursor cells – as well as stimulates glycosaminoglycan synthesis and extracellular matrix production [11,12]. Consistent with the requirement of CNP and GC-B in normal long bone growth in mammals, mice lacking either CNP or GC-B were dwarfs [13,14], and mice lacking the natriuretic peptide clearance receptor (NPR-C) that degrades CNP exhibited skeletal hyperplasia [15,16]. In contrast, mice lacking BNP display no skeletal abnormalities [17]. Importantly, CNP and CNP analogs were recently shown to increase long bone growth in murine models of achondroplasia [18–20].

Homozygous inactivating mutations in both alleles of GC-B were identified in humans with acromesomelic dysplasia, type Maroteaux (AMDM) dwarfism [21–23], and heterozygous mutations in GC-B were associated with non-pathological reductions in human stature [24]. Conversely, mutations associated with CNP overexpression were identified in patients with skeletal overgrowth [25,26], and a genome-wide association study identified correlations between genetic mutations that regulate CNP or NPR-C expression and height in Northwestern European populations [27].

In 2012, Miura et al. identified a conserved valine to methionine missense mutation at position 883 in the catalytic domain of human GC-B (V883M-GC-B) in three generations of a Japanese family with skeletal overgrowth, fragile bones and elevated blood cGMP concentrations [28]. Importantly, how this mutation increases GC-B activity was not determined. Here, we show that this single residue substitution increases the maximal velocity of GC-B in the absence of CNP and that CNP reduces the Km of V883M-GC-B an order of magnitude without ATP or without increasing maximal velocity. Unexpectedly, the V883M substitution blocked the normal inactivation process.

## Materials and methods

### Reagents

$^{125}$I-cGMP radioimmunoassay kits and $^{32}$P-α-GTP were from Perkin Elmer (Waltham, MA). CNP-22 was purchased from Sigma (St. Louis, MO). The plasmids encoding the N-terminally HA-tagged form of WT human GC-B (HA-WT-GC-B) [22] and HA-V883M-GC-B plasmids [28] have been described. The plasmids expressing rat GC-B-7A and GC-B-7E were also previously described [29,30]. The ATDC5 chondrocytes were from ATCC (www.atcc.org).

### Cells and transfections

293 neocells were maintained and transiently transfected by the HEPES–calcium-phosphate precipitation method as previously reported [30].

### Whole cell cGMP elevation assays

Cyclic GMP concentrations were measured by radioimmunoassay in ethanol extracts of transiently transfected 293 cells that were pre-incubated with 1 mM isobutylmethyl xanthine, a general phosphodiesterase inhibitor, for 10 min before being incubated with increasing concentrations of CNP as previously described [31].

### Guanylyl cyclase assays

Crude membranes were prepared at 4 °C in phosphatase inhibitor buffer consisting of 50 mM 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid — pH 7.4, 50 mM NaCl, 20% glycerol, 50 mM NaF, 1 mM EDTA, 0.5 μM microcystin and 1× Roche protease inhibitor cocktail. All assays were performed at 37 °C in a cocktail containing 25 mM HEPES pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM isobutylmethyl xanthine, 1 mM EDTA, 0.5 μM microcystin, 5 mM phosphocreatine, 0.1 μg/μl creatine kinase and 5 mM $MgCl_2$.

The single substrate concentration GC assays were performed using $^{32}$P-GTP as substrate in the presence of 1 mM ATP and 1 mM GTP at 37 °C for 3 min as previously described [31]. For the desensitization assays, the reaction was performed using a pool of crude membranes. The reaction was initiated by the addition of pre-warmed cocktail. At the designated times, 0.1 ml aliquots were removed and added to ice-cold tubes containing 0.5 ml zinc acetate to stop the reaction. Alumina column chromatography purified the $^{32}$P-cGMP, which was quantified by Cerenkov counting [32].

Substrate-velocity assays were performed for the indicated times with the indicated GTP concentrations. The resulting cGMP concentrations were determined by radioimmunoassay as described [33]. When included, free manganese concentrations in the assays were 2 mM. Because enzymatic activity was not completely linear with time, the kinetic parameters obtained under these conditions are considered “apparent”.

### Western blotting

293T cells were transfected with the indicated constructs, immunoprecipitated, fractionated by reducing SDS-PAGE and blotted to an Immobilon membrane for immune-detection as previously described [34]. The blot was blocked and probed with at 1/2500 dilution of rabbit serum 6328 followed by incubation with a 1/20,000 dilution of goat anti-rabbit IRDye 680 conjugated antibody and visualized on a LI-COR instrument as previously described [35].

### Statistical analysis

Statistics and graphs were generated with Prism 5 software. Student’s paired *t*-test determined significance where $p \leq 0.05$ was considered significant. The vertical bars within the symbols represent the SEM. Where not visible the bars are contained within the symbol. $EC_{50}$ values were calculated based on the nonlinear curve fitting equation $Y = Top * X/(EC_{50} + X)$. Substrate–velocity curves were analyzed using an allosteric sigmoidal model to generate Hill coefficients.

## Results

### Cyclic GMP is elevated more than twenty-fold in cells expressing GC-B-V883M

HEK293 cells were transiently transfected with human isoforms of HA-WT-GC-B or HA-V883M-GC-B. Two days later, the cells were incubated in the presence of increasing concentrations of CNP for 3 min and intracellular cGMP concentrations were determined (Fig. 1A). Basal (no CNP) cGMP concentrations were elevated 21-fold in cells expressing HA-V883M-GC-B compared to cells expressing HA-WT-GC-B. Maximal concentrations of CNP increased cGMP concentrations 29-fold in HA-WT-GC-B expressing cells but only 2-fold in cells expressing HA-V883M-GC-B. The $EC_{50}$ for CNP activation was not significantly different between the WT and mutant enzymes, consistent with the mutation not affecting the affinity of CNP for GC-B.

Plasmids expressing WT and GC-B-V883M were also transiently transfected into ATDC5 mouse chondrocytic cells that endogenously express GC-B. Since these cells express phosphodiesterases 1 and 5, we pretreated them with a general phosphodiesterase to emphasize cGMP synthesis by GC-B [36]. Overexpression of WT-GC-B slightly elevated cyclic GMP concentrations in the ATDC5 cells, but overexpression of the GC-B-V883M mutant resulted in cGMP levels that were more than four-fold higher than those observed in cells transfected with the WT enzyme (Fig. 1B). These data indicate that the increased basal activity associated with the V883M mutation occurs in a natural cellular environment for GC-B and is consistent with the increased plasma cGMP concentrations measured in patients expressing V883M-GC-B [28].

### Basal enzymatic activity of V883M-GC-B is elevated but expression is reduced

GC activity was measured in crude membranes from 293 cells expressing green fluorescent protein (GFP) as a control, WT-GC-B, HA-WT-GC-B or HA-V883M-GC-B under basal (1 mM $Mg^{2+}$GTP), hormone-stimulated (1 mM $Mg^{2+}$GTP, 1 mM ATP and 1 μM CNP), or detergent-stimulated (1 mM$Mn^{2+}$GTP and 1% Triton X-100) condition (Fig. 2). Enzyme analysis was performed in the 293 cells because they do not express detectable endogenous GC activity [6], which allows more definitive interpretation of the data because most tissues and cell lines express more than one GC.

GC activity measured in crude membranes from GFP transfected cells was insignificant under all conditions. Consistent with the whole cell cGMP analysis describe in Fig. 1, basal GC activity was low for WT-GC-B and HA-WT-GC-B but was elevated 28-fold over WT levels for HA-V883M-GC-B. Saturating concentrations of CNP and ATP stimulated WT-GC-B and HA-WT-GC-B similarly (>50-fold). However, GC activity of HA-WT-GC-B was almost double that of the WT enzyme lacking the HA tag, consistent with higher expression of the HA-tagged receptor. As in whole cells, CNP and ATP activated HA-V883M-GC-B about two-fold in enzyme assays. GC activity of HA-V883M-GC-B measured in the presence of detergent was lower than that observed for HA-WT-GC-B, which is consistent with reduced expression of HA-V883M-GC-B compared to HA-WT-GC-B. Western analysis of SDS-PAGE fractionated immunoprecipitated enzymes confirmed that the more slowly migrating, completely processed species (upper band) was expressed at lower levels than the comparably processed forms of the tagged or untagged WT version of GC-B (Fig. 2, inset). We previously demonstrated that only the upper band of GC-B is phosphorylated and that phosphorylation is required for CNP-dependent activation of GC-B [8,29].

### Maximal velocity of HA-GC-B-V883M is elevated

To determine how the mutation increased the enzymatic activity of GC-B, substrate–velocity curves were generated for HA-WT-GC-B and HA-V883M-GC-B with or without 1 μM CNP in the absence of ATP (Fig. 3A). Basal activity of the WT enzyme was low and CNP increased Vmax 12-fold without decreasing the Km. Consistent with previous observations [6], WT-GC-B was positive cooperative as indicated by a Hill slope of 1.3. In contrast, basal maximal velocity of the mutant enzyme was elevated 15-fold compared to WT-GC-B and the Km was unchanged. The Hill coefficient was 0.9, suggesting slight negative cooperativity. CNP failed to increase the maximal velocity of HA-V883M-GC-B, but reduced the Hill slope 0.4 units and the Km 10-fold. Thus, the V883M mutation increases basal maximal velocity, reduces the Hill coefficient and allows CNP to reduce the Km in the absence of ATP. In contrast, the reduction in Hill coefficient and Km for the WT enzyme was previously shown to be completely dependent on the presence of ATP [5]. These data are consistent with the V883M mutation producing a structural change in GC-B that locks it into a conformation that mimics that of the ATP-bound state. They also indicate for the first time that the CNP-dependent changes in the Vmax and Km of GC-B are separate but related processes.

### CNP reduces the Hill coefficient and Km of HA-V883M-GC-B in a concentration-dependent manner in the absence of ATP

Substrate–velocity curves were generated for HA-V883M-GC-B in the presence of increasing concentrations of CNP to evaluate the concentration-dependence of CNP on reductions in the Hill coefficient and Michaelis constant (Fig. 3B). ATP was not included in these experiments. In the absence of CNP, no cooperativity was observed, but increasing concentrations of CNP progressively increased the amount of negative cooperativity while concomitantly decreasing the Km. These data indicate that CNP converts HA-V883M-GC-B to a strongly negative cooperative enzyme. Similarly, in the absence of CNP, the Km of the mutant enzyme was high; but in the presence of increasing concentrations of CNP, the Km dropped progressively while maximal velocity was unaffected.

### ATP does not allosterically activate V883M-GC-B

We recently determined that CNP reduces the Hill coefficient and Km of WT-GC-B by a process that requires ATP binding to an allosteric site in the catalytic domain [6]. Therefore, we investigated whether the V883M mutation affected these processes as well. Substrate–velocity curves were generated for HA-WT-GC-B and HA-V883M-GC-B in the presence of 1 μM CNP with or without 0.1 mM ATP. With the WT enzyme, ATP reduced the Km 6-fold and the Hill coefficient 0.3 units without affecting the Vmax (Fig. 3C). However, ATP failed to reduce the Km or Hill coefficient or increase the Vmax of HA-V883M-GC-B. These data are consistent with a scenario where the V883M mutation causes a conformational change in GC-B that abolishes the need for ATP in the CNP-dependent reduction in Hill coefficient and Km.

### HA-V883M-GC-B is slightly negative cooperative when manganese is used as a cofactor

Substrate–velocity curves were also generated on membranes expressing HA-WT-GC-B or HA-V883M-GC-B under non-physiologic, detergent conditions using manganese-GTP as substrate (Fig. 3D). Substrate–velocity curves generated under these conditions were previously shown to be positive cooperative for GC-A [37]. Vmax was lower when measured under these conditions but the Km/S0.5 was strikingly lower compared to physiologic activation conditions. Maximal velocity was not different between the WT and mutant GC-B enzymes. The substrate–velocity curve for HA-WT-GC-B was positive cooperative as demonstrated by concave upward reciprocal plots and a Hill coefficient of 1.3 (Fig. 3D, inset). To our knowledge, this is the first demonstration of positive cooperativity for GC-B when assayed under detergent-stimulated conditions. In contrast to HA-WT-GC-B, HA-V883M-GC-B was weakly negative cooperative as indicated by a slightly concave downward curve and a Hill coefficient of 0.9 (Fig. 3D, inset), which is consistent with the slight negative cooperativity observed for V883-GC-B when assayed under basal conditions.

### HA-V883M-GC-B is resistant to desensitization

Cyclic GMP concentrations in cells expressing V883M-GC-B were highly elevated two days after transfection (Fig. 1), which suggests that the mutant enzyme was not completely desensitized or downregulated. In contrast, CNP activated WT-GC-B was shown to

desensitize in less than one hour [8]. Therefore, we examined whether the V883M mutation disrupted the inactivation of GC-B.

GC activity was measured on membranes from cells expressing HA-WT-GC-B or HA-V883M-GC-B for up to 2 h to evaluate the effect of the V883M mutation on the inactivation of GC-B as a function of time (Fig. 4, top panel). WT GC activity was determined in the presence of CNP, whereas mutant activity was determined in the presence and absence of CNP. The GC activity of the WT enzyme declined with time and was inactive after 60 min. In contrast, the GC activity of the mutant receptor was linear for the duration of the assay regardless of whether CNP was included in the assay.

We also examined the inactivation of the WT and mutant enzymes under whole cell conditions. In this experiment, intact cells were treated with 1 μM CNP for 0, 30 or 90 min then membranes were prepared from the cells and assayed for GC activity for 3 min (Fig. 4, bottom panel). The WT enzyme demonstrated a time-dependent inactivation similar to that previously reported for GC-B expressed in 3T3 cells [8]. However, exposure of the V883M-GC-B to saturating concentrations of CNP failed to inactivate the enzyme after 30 or 90 min. Together, these data indicate that the Val substitution at position 883 not only increases the maximal velocity of the enzyme, it also disrupts the normal desensitization process.

## Activation of GC-B by the V883M substitution does not require phosphorylation

CNP only activated a GC-B construct containing alanine substitutions for the first six phosphorylation sites identified in GC-B (S513, T516, S518, S523, S526, T529) two-fold as opposed to greater than 30-fold for WT-GC-B [29], whereas the analogous substitutions left GC-A completely unresponsive to NP stimulation [38]. These observations led to the idea that phosphorylation is required for NP-dependent activation of GC-A and GC-B. Here, we asked whether phosphorylation is also required for the V883M mutation to increase GC-B activity.

To do this, we mutated Val-883 to Met in the rat GC-B-7A construct that contains alanine substitutions for the first six identified sites plus Ser-522, which is not phosphorylated [29]. We also created a constitutively phosphorylated mimetic version of rat GC-B-V883M by mutating Val-883 to Met in GC-B-7E. Rat GC-B-7E contains glutamate substitutions for the first six identified sites as well as Ser-489, a newly identified putative site that reduces the Km of GC-B when phosphorylated [30].

Introducing the Val-883–Met mutation into WT-GC-B increased basal activity 39-fold, whereas the same mutation in the dephosphorylated form of the enzyme (GC-B-7A) increased activity 17-fold (Fig. 5). However, introduction of the V883M mutation into the GC-B-7E construct increased activity 68-fold. Thus, phosphorylation is not required for the elevated basal activity associated with the V883M mutation, but phosphorylation results in greater activation since the WT and phosphorylation mimetic enzymes (GC-B-7E) were activated to a greater degree than the non-phosphorylated enzyme (GC-B-7A).

## Discussion

Characterization of the missense mutant revealed several important changes in GC-B that occurred as a result of this single amino acid substitution. First, basal maximal velocity was dramatically increased. Second, the CNP-dependent reduction in Km was rendered independent of ATP, and thirdly, the normal desensitization process was inactivated. Another worthy point of discussion is that the V883M-GC-B mutant is the first example of a GC where ligand binding reduces the Km without increasing maximal velocity. Thus, the kinetic analysis of this mutant allowed the separation of the maximal velocity increasing effects of ligand binding from the Km reducing effects of ligand binding for the first time. This mutant enzyme also provides unequivocal support for the new GC-B activation model where CNP binding increases activity by reducing the Km as well as increasing maximal velocity [6].

Early studies indicated that product formation by membrane GCs in the presence of detergent is positive cooperative [39,40]. We found that GC-B is positive cooperative under basal conditions as well as when assayed in the presence of $Mn^{2+}$ GTP and Triton X-100. However, the single V883M mutation converts the enzyme from positive cooperative to slightly negative cooperative when assayed under both physiologic and detergent-activated conditions. Interestingly, CNP increased the degree of negative cooperativity of V883M-GC-B in a concentration dependent manner.

The reduction in Km and increase in negative cooperativity appear paradoxical. We hypothesize that the V883M mutant locks the enzyme into a conformation that mimics an ATP bound state. This hypothesis is supported by low or no cooperativity under basal conditions and the inability of ATP to change the activity of the mutant enzyme. In addition, CNP alone markedly decreased the Km of GC-B-V883M, a phenomenon that requires ATP with the WT enzyme. Since cooperativity is maintained, this suggests that the mutation does not destroy the ability of GTP to bind to the allosteric site but rather modifies how GTP binding to the allosteric site affects the catalytic site. However, an alternative explanation is that the reduction in the Hill coefficient results from increased inhibition resulting from GTP binding to a site independent of the allosteric site. This third GTP binding sitewould explain the appearance of negative cooperativity while also allowing for the Km reduction resulting from the previously identified allosteric site. It is also possible that the V883M mutation could increase the affinity of GC-B for the products of the reaction (pyrophosphate and cGMP), which would result in reduced GC activity and apparent negative cooperativity. Importantly, since the V883M mutation did not affect Vmax when measured in the presence of detergent and manganese, it suggests that the mutation modifies the conformation of the active site under physiologic conditions and does not directly interact with the substrate.

Near linear cGMP production with time by HA-V883M-GC-B assayed both in the presence and absence of CNP indicated that HA-V883M-GC-B is resistant to desensitization. Experiments with alanine and glutamate substituted receptors indicated that unlike CNP activation of WT-GC-B, the increased activity observed with the V883M mutation does not require phosphorylation of the kinase homology domain, although activation was greater with the phosphorylated and phosphomimetic enzymes. The lack of dependence on

phosphorylation for activity of the mutant enzyme may contribute to its resistance to desensitization.

It is surprising how much the single amino acid substitution changes the regulation of GC-B (Fig. 6). In the absence of CNP and ATP, maximal velocity of WT-GC-B was low, affinity for substrate was low (high Km), and cooperative was significant and positive. In contrast, under the same conditions, maximal velocity of V883M-GC-B was high, affinity for substrate was low, and cooperativity was low and negative. Addition of ATP in the absence of CNP abolished positive cooperativity of the WT enzyme due to ATP replacing GTP at the allosteric site [6], but had no effect on the mutant enzyme under identical conditions. CNP alone increased maximal velocity of the WT enzyme, but it did not decrease the Km in the absence of ATP. In contrast, CNP alone failed to increase maximal velocity of the mutant enzyme but decreased the Km ten-fold in the absence of ATP. Finally, CNP reduced the cooperativity of both enzymes, but the WT enzyme went from positive to no cooperativity, whereas the mutant went from slightly negative cooperative to very negative cooperative.

In conclusion, we established a molecular mechanism for how a single amino acid substitution in GC-B activates the enzyme, which results in abnormally long and fragile human bones. It will be interesting to determine the prevalence of this mutation in humans and other species.

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## Abbreviations

| | |
|---|---|
| **CNP** | C-type natriuretic peptide |
| **GC** | guanylyl cyclase |
| **NP** | natriuretic peptide |
| **WT** | wild type |

## References

1. Tamura N, Doolittle LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL. Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci U S A. 2004; 101:17300–5. [PubMed: 15572448]
2. Schmidt H, Stonkute A, Juttner R, Schaffer S, Buttgereit J, Feil R, et al. The receptor guanylyl cyclase Npr2 is essential for sensory axon bifurcation within the spinal cord. J Cell Biol. 2007; 179:331–40. [PubMed: 17954614]
3. Zhang M, Su YQ, Sugiura K, Xia G, Eppig JJ. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. Science. 2010; 330:366–9. [PubMed: 20947764]
4. Potter LR, Hunter T. Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation. J Biol Chem. 2001; 276:6057–60. [PubMed: 11152699]
5. Antos LK, Potter LR. Adenine nucleotides decrease the apparent Km of endogenous natriuretic peptide receptors for GTP. Am J Physiol Endocrinol Metab. 2007; 293:E1756–63. [PubMed: 17848634]

6. Robinson JW, Potter LR. Guanylyl cyclases A and B are asymmetric dimers that are allosterically activated by ATP binding to the catalytic domain. Sci Signal. 2012; 5:ra65. [PubMed: 22949736]
7. Duda T, Goraczniak RM, Sitaramayya A, Sharma RK. Cloning and expression of an ATP-regulated human retina C-type natriuretic factor receptor guanylate cyclase. Biochemistry. 1993; 32:1391–5. [PubMed: 7679284]
8. Potter LR. Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptor B: dephosphorylation is a mechanism of desensitization. Biochemistry. 1998; 37:2422–9. [PubMed: 9485390]
9. Hagiwara H, Sakaguchi H, Itakura M, Yoshimoto T, Furuya M, Tanaka S, et al. Autocrine regulation of rat chondrocyte proliferation by natriuretic peptide C and its receptor, natriuretic peptide receptor-B. J Biol Chem. 1994; 269:10729–33. [PubMed: 7908295]
10. Suda M, Ogawa Y, Tanaka K, Tamura N, Yasoda A, Takigawa T, et al. Skeletal overgrowth in transgenic mice that overexpress brain natriuretic peptide. Proc Natl Acad Sci U S A. 1998; 95:2337–42. [PubMed: 9482886]
11. Krejci P, Masri B, Fontaine V, Mekikian PB, Weis M, Prats H, et al. Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis. J Cell Sci. 2005; 118:5089–100. [PubMed: 16234329]
12. Woods A, Khan S, Beier F. C-type natriuretic peptide regulates cellular condensation and glycosaminoglycan synthesis during chondrogenesis. Endocrinology. 2007; 148:5030–41. [PubMed: 17640987]
13. Chusho H, Tamura N, Ogawa Y, Yasoda A, Suda M, Miyazawa T, et al. Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc Natl Acad Sci U S A. 2001; 98:4016–21. [PubMed: 11259675]
14. Tamura N, Garbers DL. Regulation of the guanylyl cyclase-B receptor by alternative splicing. J Biol Chem. 2003; 278:48880–9. [PubMed: 14514678]
15. Jaubert J, Jaubert F, Martin N, Washburn LL, Lee BK, Eicher EM, et al. Three new allelic mouse mutations that cause skeletal overgrowth involve the natriuretic peptide receptor C gene (Npr3). Proc Natl Acad Sci U S A. 1999; 96:10278–83. [PubMed: 10468599]
16. Matsukawa N, Grzesik WJ, Takahashi N, Pandey KN, Pang S, Yamauchi M, et al. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. Proc Natl Acad Sci U S A. 1999; 96:7403–8. [PubMed: 10377427]
17. Tamura N, Ogawa Y, Chusho H, Nakamura K, Nakao K, Suda M, et al. Cardiac fibrosis in mice lacking brain natriuretic peptide. Proc Natl Acad Sci U S A. 2000; 97:4239–44. [PubMed: 10737768]
18. Kake T, Kitamura H, Adachi Y, Yoshioka T, Watanabe T, Matsushita H, et al. Chronically elevated plasma C-type natriuretic peptide level stimulates skeletal growth in transgenic mice. Am J Physiol Endocrinol Metab. 2009; 297:E1339–48. [PubMed: 19808910]
19. Lorget F, Kaci N, Peng J, Benoist-Lasselin C, Mugniery E, Oppeneer T, et al. Evaluation of the therapeutic potential of a CNP analog in a Fgfr3 mouse model recapitulating achondroplasia. Am J Hum Genet. 2012; 91:1108–14. [PubMed: 23200862]
20. Yasoda A, Kitamura H, Fujii T, Kondo E, Murao N, Miura M, et al. Systemic administration of C-type natriuretic peptide as a novel therapeutic strategy for skeletal dysplasias. Endocrinology. 2009; 150:3138–44. [PubMed: 19282381]
21. Bartels CF, Bukulmez H, Padayatti P, Rhee DK, van Ravenswaaij-Arts C, Pauli RM, et al. Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. Am J Hum Genet. 2004; 75:27–34. [PubMed: 15146390]
22. Hachiya R, Ohashi Y, Kamei Y, Suganami T, Mochizuki H, Mitsui N, et al. Intact kinase homology domain of natriuretic peptide receptor-B is essential for skeletal development. J Clin Endocrinol Metab. 2007; 92:4009–14. [PubMed: 17652215]
23. Khan S, Hussain Ali R, Abbasi S, Nawaz M, Muhammad N, Ahmad W. Novel mutations in natriuretic peptide receptor-2 gene underlie acromesomelic dysplasia, type Maroteaux. BMC Med Genet. 2012; 13:44. [PubMed: 22691581]

24. Olney RC, Bukulmez H, Bartels CF, Prickett TC, Espiner EA, Potter LR, et al. Heterozygous mutations in natriuretic peptide receptor-B (NPR2) are associated with short stature. J Clin Endocrinol Metab. 2006; 91:1229–32. [PubMed: 16384845]
25. Bocciardi R, Ravazzolo R. C-type natriuretic peptide and overgrowth. Endocr Dev. 2009; 14:61–6. [PubMed: 19293575]
26. Moncla A, Missirian C, Cacciagli P, Balzamo E, Legeai-Mallet L, Jouve JL, et al. A cluster of translocation breakpoints in 2q37 is associated with overexpression of NPPC in patients with a similar overgrowth phenotype. Hum Mutat. 2007; 12:1183–8. [PubMed: 17676597]
27. Estrada K, Krawczak M, Schreiber S, van Duijn K, Stolk L, van Meurs JB, et al. A genome-wide association study of northwestern Europeans involves the C-type natriuretic peptide signaling pathway in the etiology of human height variation. Hum Mol Genet. 2009; 18:3516–24. [PubMed: 19570815]
28. Miura K, Namba N, Fujiwara M, Ohata Y, Ishida H, Kitaoka T, et al. An overgrowth disorder associated with excessive production of cGMP due to a gain-of-function mutation of the natriuretic peptide receptor 2 gene. PLoS One. 2012; 7:e42180. [PubMed: 22870295]
29. Potter LR, Hunter T. Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. J Biol Chem. 1998; 273:15533–9. [PubMed: 9624142]
30. Yoder AR, Robinson JW, Dickey DM, Andersland J, Rose BA, Stone MD, et al. A functional screen provides evidence for a conserved, regulatory, juxtamembrane phosphorylation site in guanylyl cyclase A and B. PLoS One. 2012; 7:e36747. [PubMed: 22590601]
31. Dickey DM, Burnett JC Jr, Potter LR. Novel bifunctional natriuretic peptides as potential therapeutics. J Biol Chem. 2008; 283:35003–9. [PubMed: 18940797]
32. Bryan PM, Potter LR. The atrial natriuretic peptide receptor (NPR-A/GC-A) is dephosphorylated by distinct microcystin-sensitive and magnesium-dependent protein phosphatases. J Biol Chem. 2002; 277:16041–7. [PubMed: 11821394]
33. Robinson JW, Potter LR. ATP potentiates competitive inhibition of guanylyl cyclase A and B by the staurosporine analog, Go6976: reciprocal regulation of ATP and GTP binding. J Biol Chem. 2011; 286:33841–4. [PubMed: 21828054]
34. Abbey-Hosch SE, Smirnov D, Potter LR. Differential regulation of NPR-B/GC-B by protein kinase C and calcium. Biochem Pharmacol. 2005; 70:686–94. [PubMed: 16005434]
35. Flora DR, Potter LR. Prolonged atrial natriuretic peptide exposure stimulates guanylyl cyclase-A degradation. Endocrinology. 2010; 151:2769–76. [PubMed: 20382697]
36. Fujishige K, Kotera J, Yanaka N, Akatsuka H, Omori K. Alteration of cGMP metabolism during chondrogenic differentiation of chondroprogenitor-like EC cells, ATDC5. Biochim Biophys Acta. 1999; 1452:219–27. [PubMed: 10590311]
37. Ivanova K, Heim JM, Gerzer R. Kinetic characterization of atrial natriuretic factor-sensitive particulate guanylate cyclase. Eur J Pharmacol. 1990; 189:317–26. [PubMed: 1980649]
38. Potter LR, Hunter T. Phosphorylation of the kinase homology domain is essential for activation of the A-type natriuretic peptide receptor. Mol Cell Biol. 1998; 18:2164–72. [PubMed: 9528788]
39. Chrisman TD, Garbers DL, Parks MA, Hardman JG. Characterization of particulate and soluble guanylate cyclases from rat lung. J Biol Chem. 1975; 250:374–81. [PubMed: 234425]
40. Kimura H, Murad F. Evidence for two different forms of guanylate cyclase in rat heart. J Biol Chem. 1974; 249:6910–6. [PubMed: 4153733]

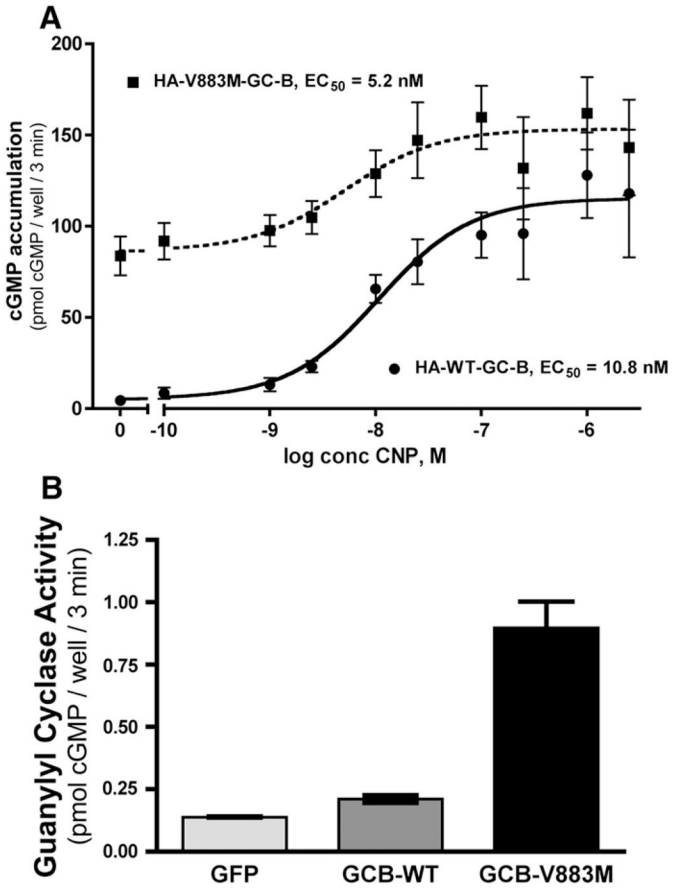


**Fig. 1.**

Basal cGMP concentrations are markedly elevated in cells expressing V883M-GC-B. A, 293 cells transiently expressing HA-WT-GC-B or HA-V883M-GC-B were incubated with the indicated concentrations of CNP for 3 min and then intracellular cGMP concentrations were determined. The $EC_{50}$s for the two enzymes were not significantly different, $p = 0.42$. B, ATDC5 cells were transiently transfected with plasmids expressing WT-GC-B or GC-B-V883M and cGMP concentrations were measured in basal (no CNP) serum-starved cells 2 days later. Cyclic GMP concentrations in cells expressing WT-GC-B were slightly higher than those observed in un-transfected cells ($p < 0.01$), but levels in cells expressing GC-B-V883M were 4.3-fold higher than those in cells expressing WT-GC-B ($p < 0.03$).

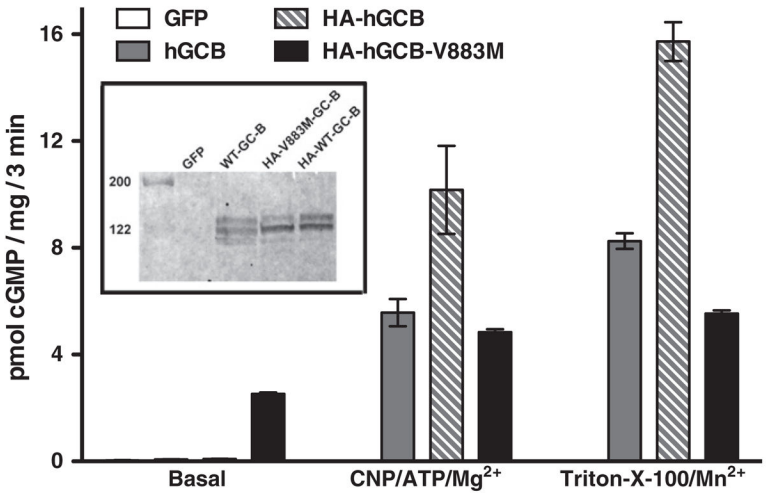


**Fig. 2.**

GC activity but not the protein level of V883M-GC-B was elevated in the absence of CNP. Crude membranes from 293 cells transfected with plasmids expressing the indicated constructs were assayed for GC activity under the conditions indicated in the figure legend and text. Bars within the symbols indicate the range of duplicate determinations. This figure is representative of two independent assays. The inset shows a Western blot of the indicated forms of GC-B purified from 293 cells transiently transfected with the indicated constructs. The numbers on the left indicate the molecular weight of standards.

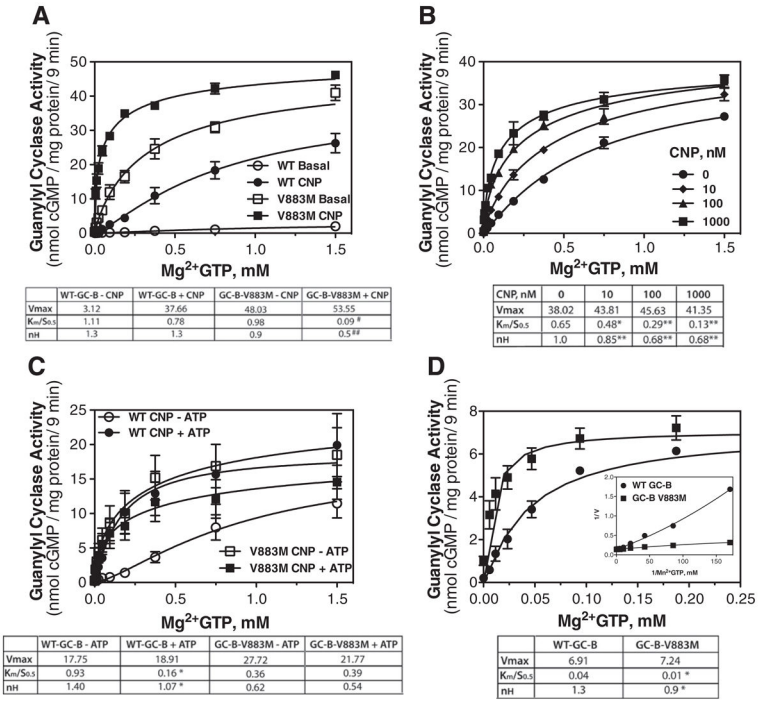


**Fig. 3.**

Kinetic characterization of V883M-GC-B. GC activity shown in panels A–D was measured for 9 min in crude membranes from 293 cells transiently expressing either HA-GC-B-WT or HA-GC-B-V883M. Bars within symbols indicate the SEM. Tables below each figure show Vmax, Km and Hill coefficient ($n_H$). A. Maximal velocity of HA-V883M-GC-B is elevated in the absence of CNP. GC activity was measured in the presence or absence of 1 μMCNP and the indicated concentrations of $Mg^{2+}$ GTP where n = 4. The # indicates a significant difference from HA-WT-GC-B-CNP at $p < 0.05$. The ## indicates a significant difference from HA-V883M-GC-B-CNP at $p < 0.03$. B. CNP decreases the Hill coefficient and Km for HA-V883M-GC-B in a concentration-dependent manner in the absence of ATP. GC activity was measured in the presence or absence of increasing concentrations of CNP and the indicated concentrations of $Mg^{2+}$GTP where n = 4. * and ** indicate a significant difference from no CNP values where $p < 0.05$ and 0.01, respectively. C. ATP does not affect the Hill coefficient or Km of HA-V883M-GC-B. GC activity was measured in the presence or absence of 0.1 mMATP, 1 μMCNP and the indicated concentrations of $Mg^{2+}$ GTP where n = 4. The * indicates a significant difference from HA-WT-GC-B (−) ATP at $p < 0.05$. D. HA-V883M-GC-B is negative cooperative. GC activity was measured with the indicated concentrations of $Mn^{2+}$GTP and 1% Triton X-100 where n = 6. The * indicates a significant difference from the corresponding value obtained for the WT enzyme at $p < 0.05$; inset. Double reciprocal plots were generated from the raw data to demonstrate a concave upward curve indicative of positive cooperativity or a slightly downward curve indicative of negative cooperativity.

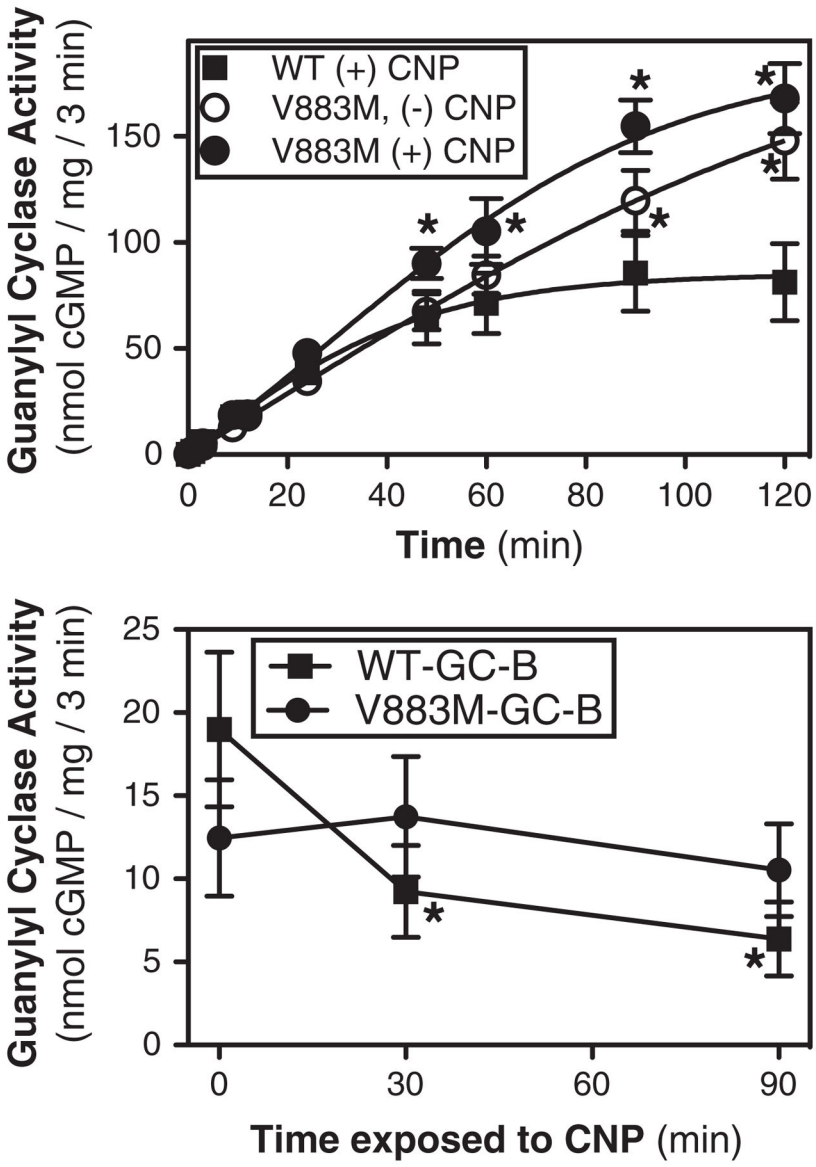


**Fig. 4.**

V883M-GC-B is resistant to desensitization. A. GC assays were conducted on crude membranes from 293 cells transfected with HA-WT-GC-B or HA-V883M-GC-B for the period of time indicated in the presence of 1 mM GTP, 1 mM ATP and 5 mM $Mg^{2+}Cl_2$ with or without 1 μM CNP. Each value represents 4 determinations. The asterisks indicate a significant difference from corresponding values obtained in membranes expressing WT-GC-B at $p < 0.02$. B. Whole 293 cells transfected with HA-WT-GC-B or HA-V883M-GC-B were incubated with 1 μM CNP for the indicated times. Membranes were then prepared and assayed for GC activity in the presence of 1 mM GTP, 1 mM ATP and 5 mM $Mg^{2+}Cl_2$. N = 4. The asterisks indicate significance from the 0 time point value where $p < 0.05$. The bars within the symbols indicate SEM in all panels.

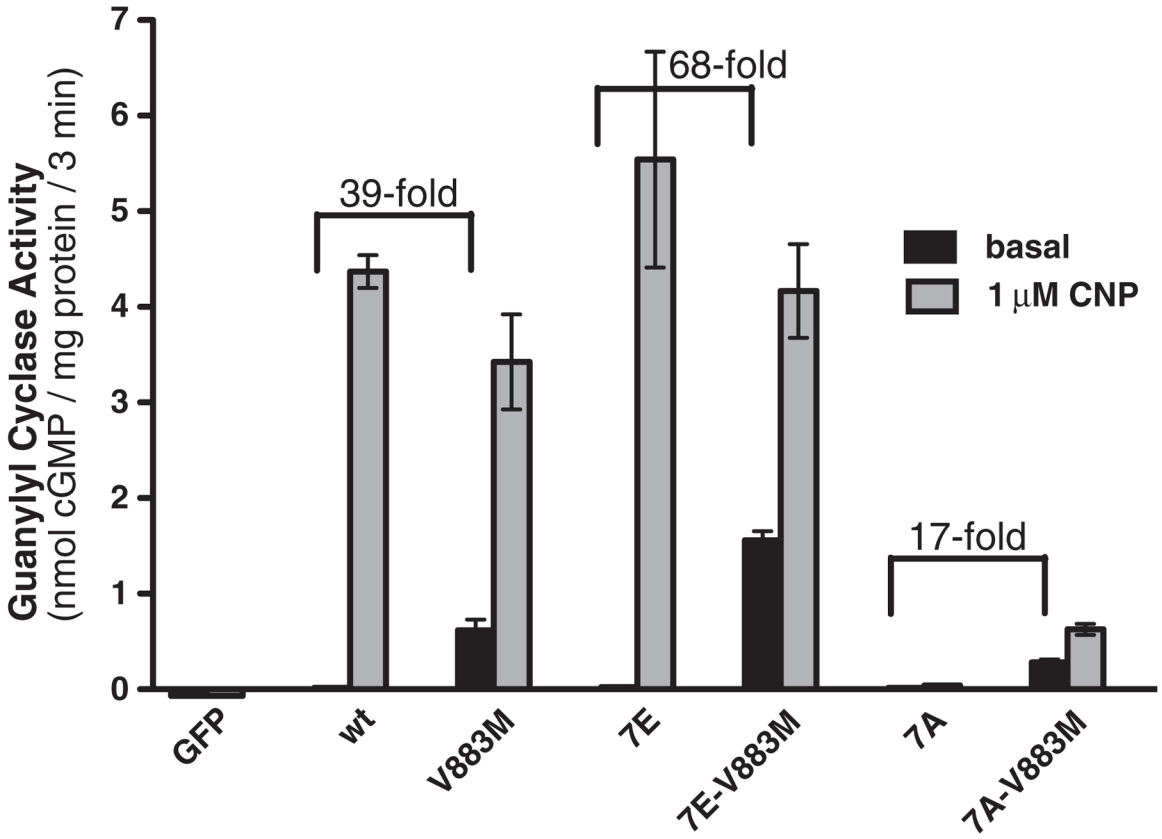


**Fig. 5.**

The GC-B-V883M mutation activates a dephosphorylated version of GC-B. The V883M mutation was introduced into WT-GC-B, constitutively phosphorylated (7E) or constitutively dephosphorylated (7A) forms of GC-B. 293 cells were transiently transfected with plasmids expressing the indicated GC-B constructs. GC assays were performed for 3 min in the presence of 0.1 mM GTP, 1 mM ATP and 5 mM $Mg^{2+}$ $Cl_2$ with or without 1 μM CNP. Each value represents 6 determinations. The bars within the symbols indicate SEM. The values above the brackets indicate the fold-difference above basal values.

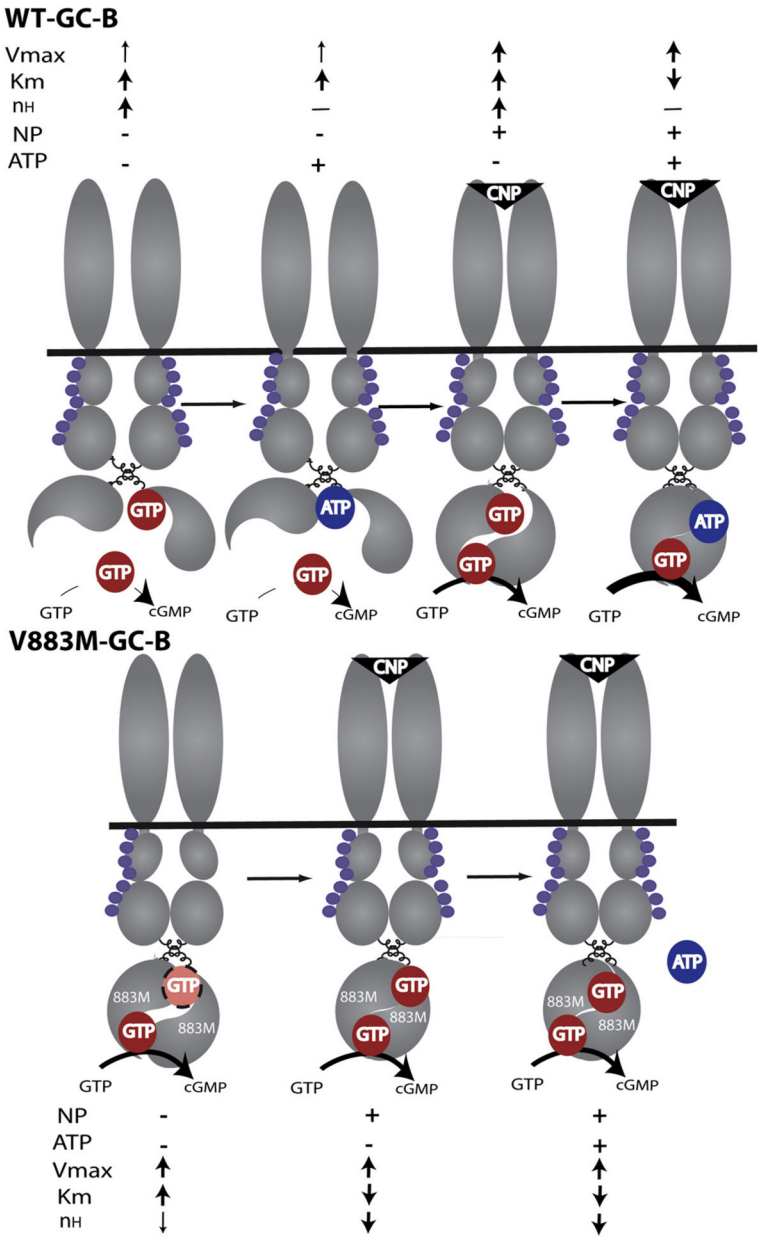


**Fig. 6.**

Activation models for wild type and mutant GC-B. The models are described in detail under the Discussion section. The blue spheres indicate known phosphorylation sites in the kinase homology domain of GC-B. The white 883M indicates that the Val to Met mutation is in the catalytic domain. The abbreviations are: CNP, C-type natriuretic peptide; Km, Michaelis constant; $n_H$, Hill coefficient; and Vmax, maximal velocity.