Skeletal overgrowth-causing mutations mimic an allosterically activated conformation of guanylyl cyclase-B that is inhibited by 2,4,6,-trinitrophenyl ATP

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Activating mutations in the receptor for C-type natriuretic peptide (CNP), guanylyl cyclase B (GC-B, also known as Npr2 or NPR-B), increase cellular cGMP and cause skeletal overgrowth, but how these mutations affect GTP catalysis is poorly understood. The A488P and R655C mutations were compared with the known mutation V883M. Neither mutation affected GC-B concentrations. The A488P mutation decreased the EC_{50} 5-fold, increased V_{max} 2.6-fold, and decreased the K_m 13-fold, whereas the R655C mutation decreased the EC_{50} 5-fold, increased the V_{max} 2.1-fold, and decreased the K_m 4.7-fold. Neither mutation affected maximum activity at saturating CNP concentrations. Activation by R655C did not require disulfide bond formation. Surprisingly, the A488P mutant only activated the receptor when it was phosphorylated. In contrast, the R655C mutation converted GC-B-7A from CNP-unresponsive to CNP-responsive. Interestingly, neither mutant was activated by ATP, and the K_m and Hill coefficient of each mutant assayed in the absence of ATP were similar to those of wild-type GC-B assayed in the presence of ATP. Finally, 1 mM 2,4,6,-trinitrophenyl ATP inhibited all three mutants by as much as 80% but failed to inhibit WT-GC-B. We conclude that 1) the A488P and R655C missense mutations result in a GC-B conformation that mimics the allosterically activated conformation, 2) GC-B phosphorylation is required for CNP-dependent activation by the A488P mutation, 3) the R655C mutation abrogates the need for phosphorylation in receptor activation, and 4) an ATP analog selectively inhibits the GC-B mutants, indicating that a pharmacologic approach could reduce GC-B dependent human skeletal overgrowth.

C-type natriuretic peptide (CNP)[2] activation of guanylyl cyclase (GC)-B stimulates endochondral ossification leading to long bone growth, neuronal bifurcation, and maintenance of oocytes in the diploid state before ovulation (1–3). C-type natriuretic peptide binding increases the maximal velocity and reduces the Michaelis constant of GC-B, which dramatically increases the synthesis of cGMP in chondrocytes (4, 5). GC-B is predicted to be a dimer (6), and each monomer contains a glycosylated, extracellular ligand binding domain, a single membrane-spanning region, and a multidomain intracellular region consisting of a phosphorylated kinase homology regulatory domain, coiled-coil dimerization domain, and C-terminal catalytic domain (7).

How CNP activates GC-B is incompletely understood, but transmission of the NP-binding signal from the extracellular domain to the catalytic domain of the homologous receptor, GC-A, requires a 30° rotation between the juxtamembrane regions of the two monomer partners in the dimer (8). Phosphorylation of multiple conserved serines and threonines at the beginning and leading into the kinase homology domains of both GC-A and GC-B is also required for transmission of the NP-binding signal from the extracellular domain to the catalytic domain (9–14).

More than 17 unique genetic mutations that inactivate both alleles of GC-B have been identified that cause acromesomelic dysplasia, type Maroteaux dwarfism (15–17). The majority of these mutations inactivate the enzyme by disrupting its normal secondary structure, which leads to improper folding and incomplete processing but normal plasma membrane targeting (18). Inactivation of single alleles was associated with reduced stature without disproportionality in one study (19), but the ability of an inactive allele to suppress a wild type (WT) allele through a dominant negative interaction raises the possibility that mutations in single alleles may cause disproportionate bone growth as well (20).

In contrast to dwarfism, mutations that increase GC-B-dependent cGMP elevations in chondrocytes cause skeletal overgrowth. Balanced chromosomal translocations that increased serum CNP levels result in skeletal overgrowth and bone abnormalities (21, 22). Regarding the receptor, three activating missense mutations of GC-B have been identified in humans (23–25), but how these mutations increase GC-B activity has only been determined for one mutant (26). The V883M mutation in GC-B increases maximal velocity and inhibits receptor desensitization. Importantly, this mutation increases the activity of both the phosphorylated and dephosphorylated forms of the enzyme. Here, we characterized two recently identified intracellular activating mutations in GC-B and find that the A488P

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[2] The abbreviations used are: CNP, C-type natriuretic peptide; GC-B, guanylyl cyclase B; NP, natriuretic peptide; TNP, 2,4,6,-trinitrophenyl; XTP, xanthosine 5′-triphosphate; PIB, phosphatase inhibitor buffer.

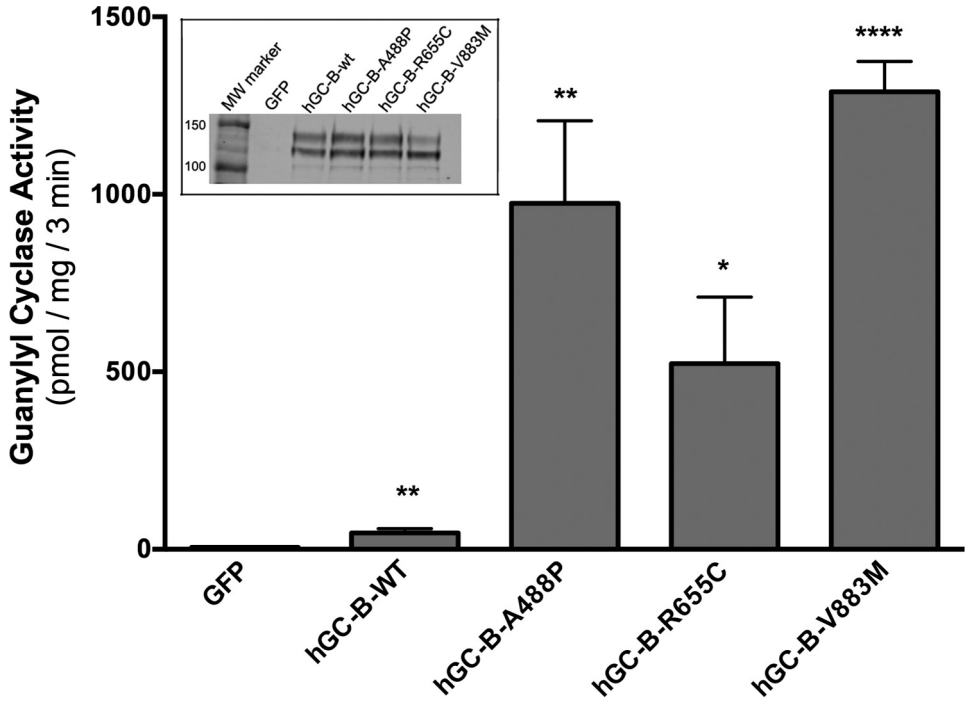


Figure 1. Activating mutations dramatically increased basal enzymatic activity of GC-B. Basal GC assays were conducted on membranes from 293T cells transiently transfected with plasmids expressing human (*h*-) versions of WT-GC-B, GC-B-A488P, GC-B-R655C, or GC-B-V883M. *Inset*, Western analysis of same constructs. 30-μg membranes were loaded/lane. Data are represented as the mean $\pm$ S.E. In all figures S.E. is indicated by *vertical bars* in the center of the columns. The graph represents three individual experiments where $n = 6$. Statistical significance was determined as compared with WT, except for WT, which was compared with GFP. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

mutation only activates the phosphorylated form of the enzyme, whereas the R655C mutation abrogates the requirement for phosphorylation in the ligand-dependent activation process. Importantly, we report that both missense mutations activate the CNP free form of GC-B by the common mechanism of mimicking the allosterically activated conformation of the enzyme and that 2,4,6,-trinitrophenyl ATP (TNP-ATP) selectively inhibits the mutant activated forms of GC-B.

Results

The three reported GC-B activating mutations are located in different intracellular regions of the receptor (23–25). Here, we determined how each mutation affects the activity of the WT human and rat enzymes as well as the effect that receptor phosphorylation has on the ability of each missense mutation to modulate enzyme activity.

Initially, each missense mutation was introduced into a plasmid containing WT human GC-B. Importantly, each mutant was expressed at similar levels to the WT receptor when transiently expressed in 293T cells, indicating that the increased activity is not explained by increased GC-B concentrations (Fig. 1, *inset*). Furthermore, each mutant was completely processed to a hormonally active terminally glycosylated and phosphorylated protein represented by the upper, most slowly migrating diffuse band when purified by SDS-PAGE (18). Guanylyl cyclase assays conducted under basal conditions (without NP) indicated that the basal enzymatic activity of each mutant was markedly elevated compared with basal activity of the WT enzyme (Fig. 1).

The effect that each mutation had on intracellular cGMP concentrations was determined by transiently expressing the WT or each mutant receptor in 293T cells. In the absence of CNP, intracellular cGMP concentrations were elevated 11-, 28-, and 21-fold over WT levels in cells expressing the R655C, V883M, and A488P mutants, respectively (Fig. 2). In addition, the potency of CNP for each mutant was slightly, but significantly, increased compared with the WT receptor (Fig. 2). As a consequence of the increased basal activity, the total -fold activation by CNP was dramatically reduced to 2–4-fold for each mutant compared with 55-fold for the WT receptor.

To determine if the increased activity of the R655C GC-B mutant is due to the formation of an intermolecular disulfide bond, Arg-655 was mutated to Ser. We found that all the GC-B receptors were expressed at similar concentrations and that the GC-B-R655S mutant had even more basal guanylyl cyclase activity than the R655C mutant (Fig. 3*A*). Again, increased protein concentrations did not explain the increased CNP-dependent activities because Western blots (Fig. 3*A*, *inset*) and GC activity measured in the presence of detergent and manganese were similar for the WT and mutant forms of GC-B. These data indicate that the increased guanylyl cyclase activity associated with mutation of Arg-655 does not require disulfide bond formation.

The effect that each mutation had on the V_{max} and K_m of GC-B was also determined by performing substrate-velocity assays on membranes from 293T cells transiently transfected with WT-GC-B or each mutant (Fig. 4). Again, the active form (*upper band*) of each mutant was expressed at similar levels as the WT enzyme (Fig. 4, *inset*). When guanylyl cyclase activities were measured under basal conditions (*panel A*), the K_m of each mutant was dramatically reduced compared with WT-GC-B.

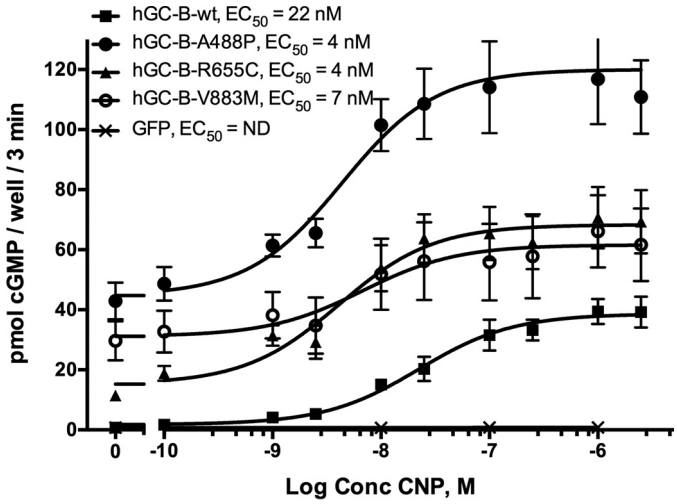


Figure 2. Activating mutations in human GC-B result in constitutively elevated intracellular cGMP concentrations. 293T cells transiently expressing human versions of WT-GC-B, GC-B-V883M, GC-B-A488P, or GC-B-R655C were incubated in the absence or presence of increasing concentrations of CNP for 3 min. Cells were lysed, and cellular cGMP concentrations were measured by radioimmunoassay. Data are represented as the mean ± S.E. The graph represents a summary of three individual experiments.

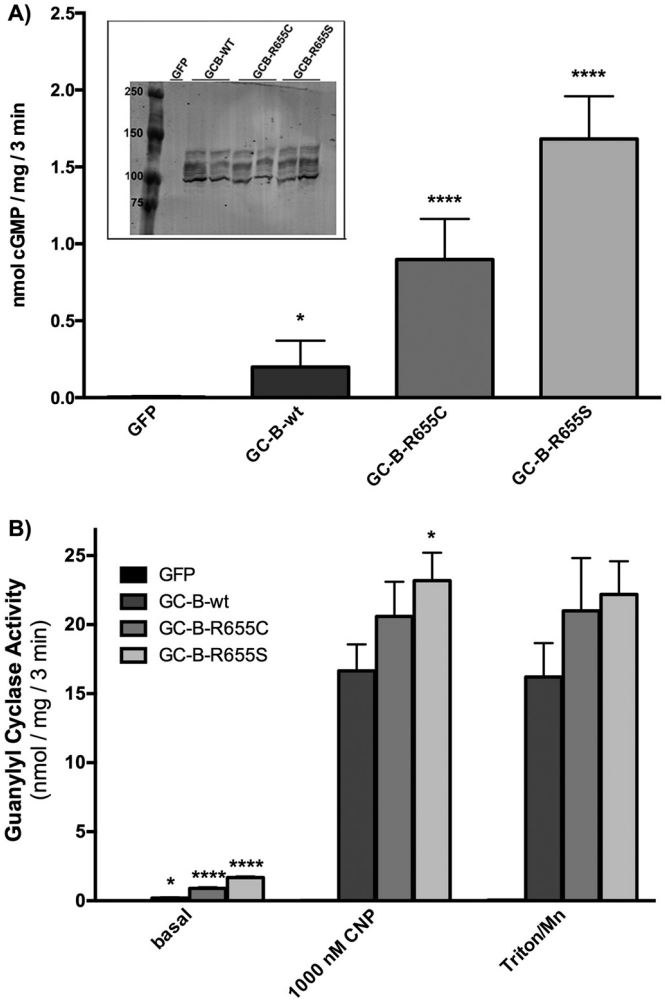


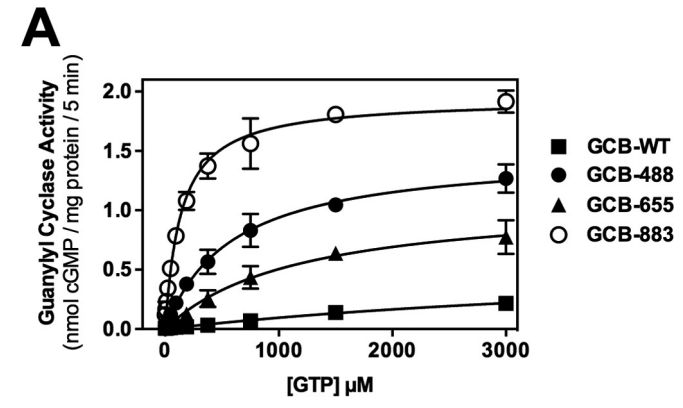
Figure 3. Activation by the R655C mutation did not require disulfide bond formation. *A*, basal GC assays conducted on membranes of 293T cells transiently transfected with WT-GC-B, GC-B-R655C, and GC-B-R655S. *Inset*, Western analysis of GC-B using 30 μg of protein/lane. *B*, guanylyl cyclase assay on membranes of transiently transfected cells were assayed in the absence of CNP (*basal*), maximal CNP (1000 nM), or Triton/Mn^{2+}. Data are represented as the mean ± S.E. The graph represents a summary of three individual experiments where $n = 6$. Statistical significance was determined as compared with WT levels except for the WT sample, which was compare with GFP levels. *, $p < 0.05$; ****, $p < 0.0001$.

The reductions ranged from 4.7-fold for R655C to 41-fold for V883M. Maximal velocities were increased but to a much lesser extent, ranging from 2.1-fold for R655C to 3.4-fold for V883M, but these differences were not significantly different. In contrast, when guanylyl cyclase activity was measured in the presence of saturating CNP concentrations, the K_m value of the WT enzyme was more similar to K_m values observed for the mutant enzymes with the mutant values only being reduced to about half that of the WT value. Furthermore, in the presence of CNP there was no difference between the maximal velocity of the WT enzyme and the maximal velocity of the mutants (Fig. 4*B*).

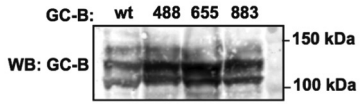
We previously determined that the V883M mutant had increased basal guanylyl cyclase activity in a GC-B mutant where all known phosphorylation sites were mutated to alanine and concluded that phosphorylation was not required for activation of the mutated enzyme (26). However, a greater increase in activity was observed when the V883M mutation was introduced into the phosphorylated WT enzyme or a phosphomimetic mutant called GC-B-7E containing glutamates for all known sites and one putative phosphorylation site, which indicated that phosphorylation increases the activating effect of the V883M mutation. The same approach was used to investigate the effect of phosphorylation on the ability of the A488P and R655C mutations to activate GC-B. In the WT enzyme, the R655C mutation increased activity 6.4-fold (Fig. 5*A*). However, activity was increased 9-fold when expressed in the constitutively dephosphorylated GC-B-7A enzyme and 19.5-fold when expressed GC-B-7E, which mimics a constitutively phosphorylated enzyme. Thus, phosphorylation is not required for the R655C mutation to activate GC-B. However, the absolute activity of the GC-B-7A-R655C enzyme was much lower than the activity of WT-GC-B-R655C or GC-B-7E-R655C mutants. In contrast, the A488P mutation increased activity 35-fold and 14-fold in the WT and GC-B-7E enzymes, respectively, but only increased activity 1.8-fold of the dephosphorylated GC-B-7A enzyme, which indicates that the vast majority of the increase in activity resulting from the A488P mutation requires a phosphorylated enzyme (Fig. 5*B*).

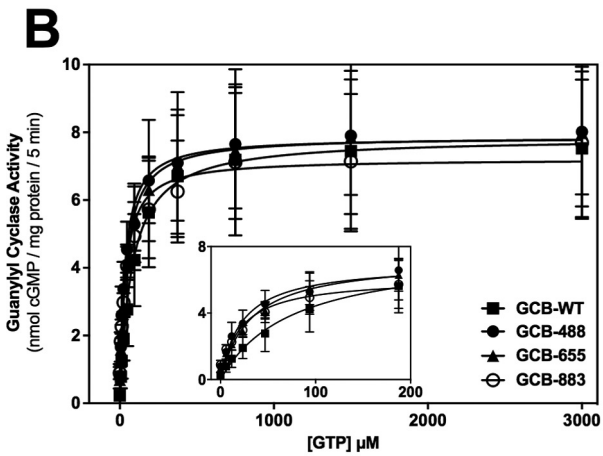
Previous studies demonstrated that CNP does not activate the dephosphorylated GC-B-7A mutant (13). However, when the R655C was incorporated into GC-B-7A, CNP stimulation of the enzyme was restored (Fig. 6*A*). In contrast, the A488P mutation did not increase the ability of CNP to increase the guanylyl cyclase activity of the dephosphorylated (7A) form of the enzyme (Fig. 6*B*). Thus, activation by the A488P mutation requires receptor phosphorylation.

The previous experiments were performed in broken membrane preparations. To better approximate the effects of these mutations *in vivo*, we measured cGMP elevations in whole cells. Again, incorporation of the R655C mutation into GC-B-7A produced an enzyme that was stimulated by CNP in a concentration-dependent manner (Fig. 7*A*). In whole cell assays, the



	GCB-WT	GCB-488	GCB-655	GCB-883
Vmax	0.6191	1.490	1.096	1.939
Km	5495	587.6 *	1170*	138.8*





	GCB-WT	GCB-488	GCB-655	GCB-883
Vmax	7.843	7.854	7.886	7.217
Km	75.63	31.76 *	38.98 *	33.51*

Figure 4. GC-B activating mutation A488P, R655C, and V883M reduced the K_m and increased V_{max} in the absence of CNP. 293T cells were transiently transfected with the indicated form of GC-B, and membranes from these cells were assayed for GC activity for 5 min in the presence of 1 mM ATP with increasing concentrations of GTP and the presence or absence of 1 μM CNP. *A*, comparison between GC-B-WT, GC-B-A488P, GC-B-R655C, and GC-B-V883M, where $n = 6$ from three experiments. Western blots (*WB*) indicate the amount of protein for each form of GC-B from one experiment. *B*, comparison between GC-B-WT, GC-B-A488P, GC-B-R655C, and GC-B-V883M, in the presence of 1 μM CNP, where $n = 4$ from two experiments. The *inset* shows lower concentration values at expanded scale. *, $p < 0.05$ compared to WT.

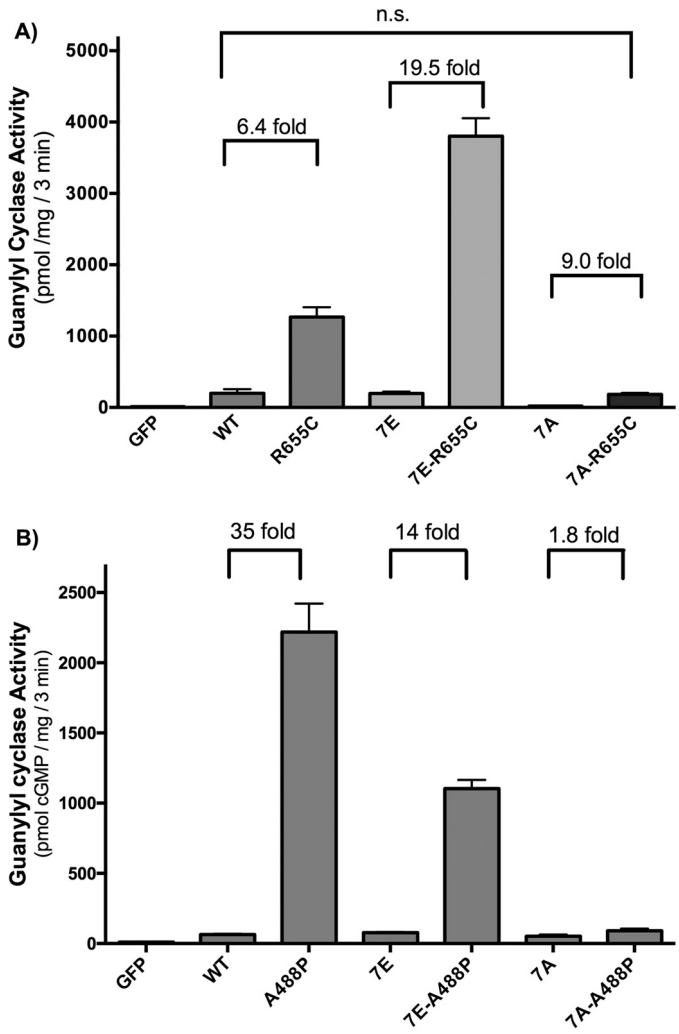


Figure 5. Basal activation by GC-B-A488P is phosphorylation-dependent. Basal GC assays were conducted on membranes from 293T cells transiently transfected with plasmids expressing rat WT or mutant GC-B in the WT, constitutively phosphorylated (*7E*), or constitutively dephosphorylated (*7A*) receptor backbones. *n.s.*, not significant. *A*, GC-B-R655C mutants *versus* WT-GC-B. *B*, GC-B-A488P mutants *versus* WT-GC-B. Data are represented as the mean ± S.E. and are the sum of two and three experiments, respectively.

A488P mutation in the GC-B-7A backbone also demonstrated a slight increase in cGMP production in response to CNP, but the sensitivity of the response was less than the GC-B-7A-R655C mutant (Fig. 7*B*). For GC-B-7A-R655C, the EC_{50} was shifted compared with WT-GC-B and had a potency similar to R655C mutation in the WT backbone. In contrast, the EC_{50} for GC-B-7A-A488P was much higher than for WT-GC-B.

We also investigated the effect of the activating mutations on the allosteric regulation of GC-B by ATP, which is known to reduce the K_m of the enzyme in the presence of CNP (4, 5). GC activity as a function of time was measured on membranes from 293T cells transfected with the WT and one of the two mutant enzymes in the presence and absence of ATP (Fig. 8). In the absence of CNP (basal conditions), ATP failed to increase the activity of WT-GC-B or either mutant. In contrast, ATP increased the linearity and activity of WT-GC-B 3.5-fold in the presence of CNP. Surprisingly, in the absence of ATP, product formation for both mutants was linear and at the same level as observed for ATP-stimulated WT-GC-B, which is consistent with the mutations producing a GC-B conformation that mimics the ATP bound activated conformation.

CNP-stimulated substrate-velocity experiments were performed on WT and mutant forms of GC-B in the presence (Fig. 9, *bottom panel*) or absence (*upper panel*) of ATP to directly determine the effect of each mutation on the K_m and Hill slope of GC-B. In the absence of ATP, the Hill slope and K_m of WT-GC-B was much higher than the value observed for either mutant, indicating that product formation is more linear in the absence of ATP for the mutants compared with WT-GC-B (Fig.

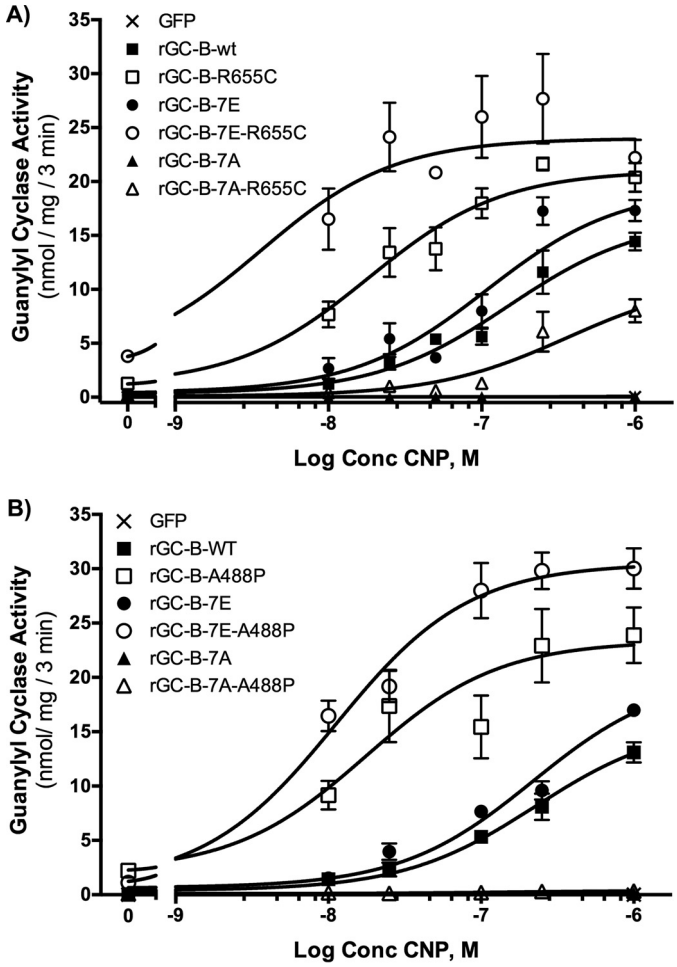


Figure 6. The GC-B-R655C mutation restores CNP responsiveness in a dephosphorylated receptor. GC assays performed in the absence or presence of increasing concentrations of CNP on membranes from transiently transfected 293T cells. *A*, dose-response curves for WT-GC-B, WT-GC-B-R655C, GC-B-7A-R655C, and GC-B-7E-R655C. *B*, dose-response curves for similar constructs with GC-B-A488P. Data are represented as the mean ± S.E.

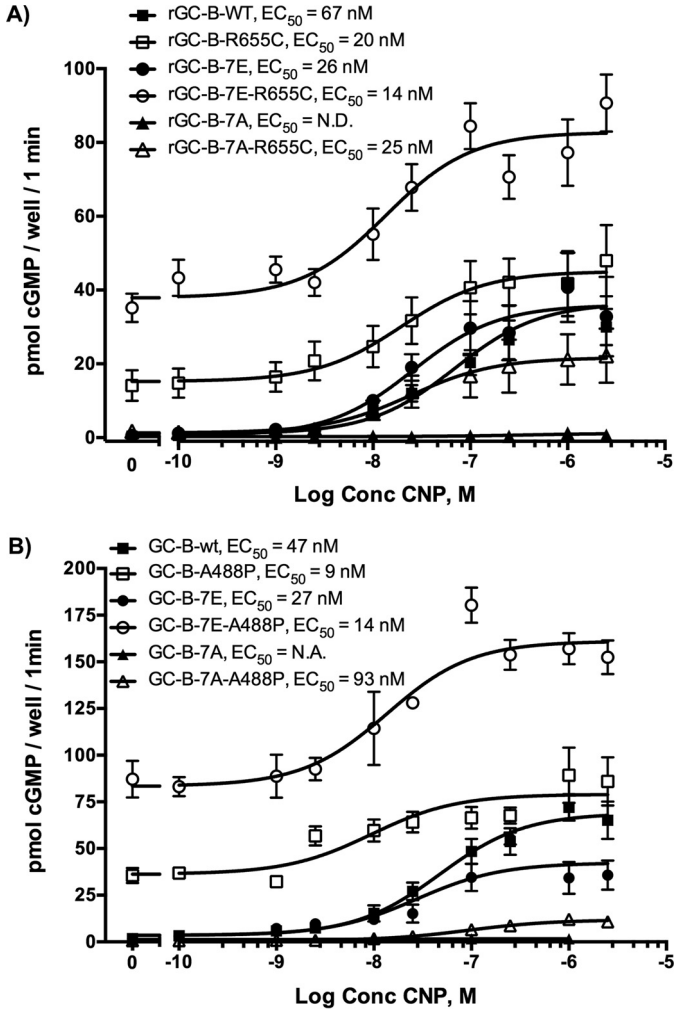


Figure 7. Phosphorylation state of GC-B had different effects on activating GC-B mutations. 293T cells transiently expressing rat WT or mutant GC-B constructs in the WT, 7E, or 7A backbone were incubated in the absence (0 min) or presence of increasing concentrations of ligand for 3 min. Cells were lysed, and cellular cGMP concentrations were measured by radioimmunoassay. Data are represented as the mean ± S.E. The graph represents the average of three individual experiments.

9*A*). In contrast, in the presence of ATP, the K_m and V_{max} values are comparable for the mutants and WT-GC-B (Fig. 9*B*). Together, these data indicate that these two single amino acid substitutions activate GC-B by producing a conformation that mimics the conformation of wild type GC-B when bound by both CNP and ATP.

Finally, because it was recently reported that TNP-ATP and xanthosine triphosphate (XTP) inhibit soluble GC and GC-A (27, 28), respectively, we investigated whether either molecule would preferentially inhibit the activated mutant forms of GC-B (Table 1). Although the addition of 1 mM XTP inhibited basal activity of GC-A in the presence of 1 mM ATP and excess magnesium, the inhibition was no better than that observed by increasing the ATP concentration from 1 mM to 2 mM. However, the addition of 1 mM TNP-ATP selectively inhibited the mutants from 72% to 85% while only slightly activating WT-GC-B. These initial data suggest that ATP analogs or other structurally similar small molecules could be developed that specifically inhibit the activated mutant forms of GC-B as a potential therapeutic approach for the treatment of skeletal overgrowth disease resulting from chronic activation of GC-B.

Discussion

Previous examination of the GC-B-V883M mutant revealed several changes in the activity of the enzyme that explained its increased production of cGMP and associated long bone growth (26). Since that study, several other activating mutations of GC-B have been identified in humans (23–25). Three properties are known to lead to maximum physiologic activation of GC-B: 1) receptor phosphorylation, 2) ligand binding to the extracellular domain, and 3) allosteric activation by ATP binding to an intracellular site or sites. Here, we examined the role of each of these components in the most recently identified GC-B-activating mutations to determine their mode of activation and whether it was similar to the previously characterized GC-B-V883M-activating mutation.

Each of the GC-B-activating mutations increased cGMP levels in the absence of ligand (CNP). For each mutant, this was primarily observed as a decrease in the K_m in the absence of CNP. In the presence of CNP, the K_m of the mutants was lower than the WT enzyme but only by about 2-fold. Interestingly,

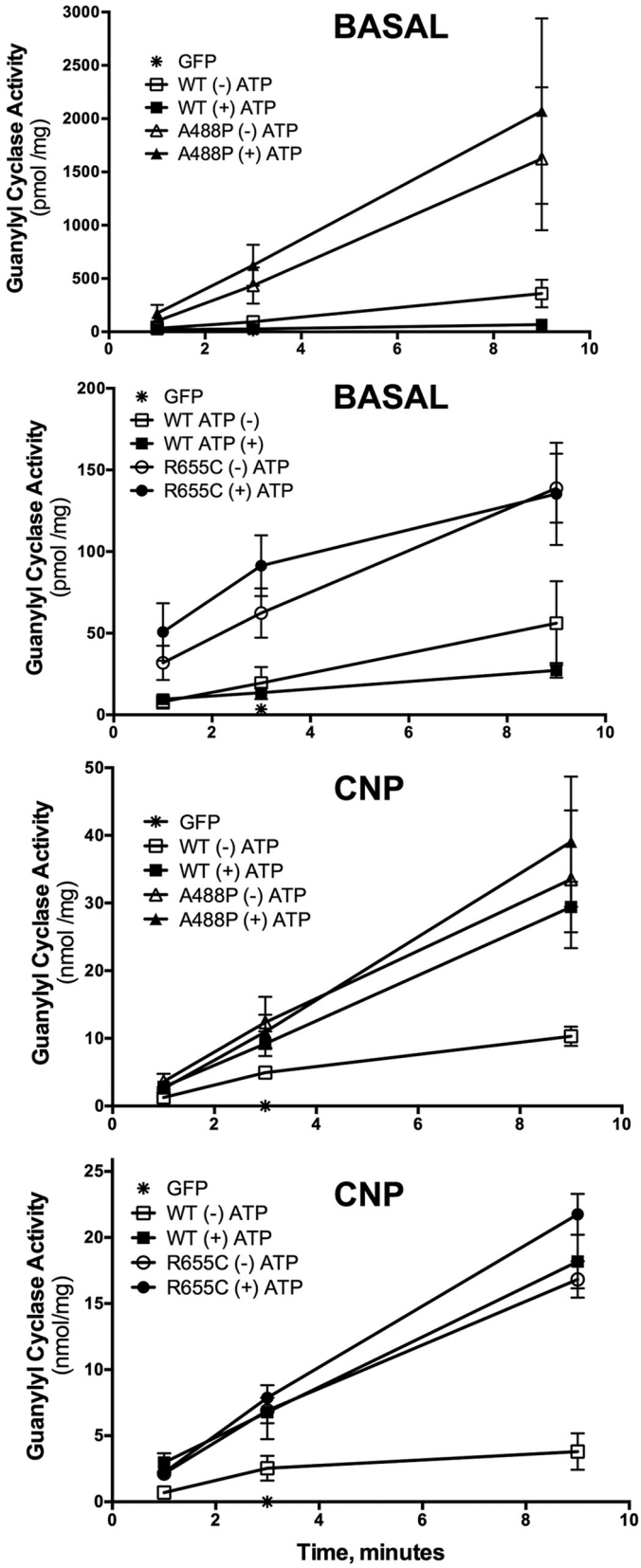
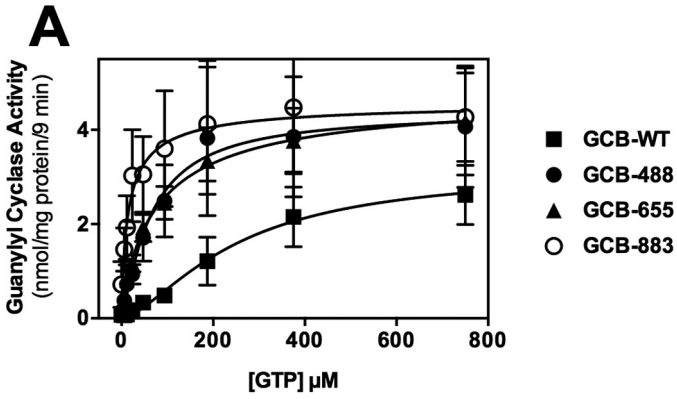
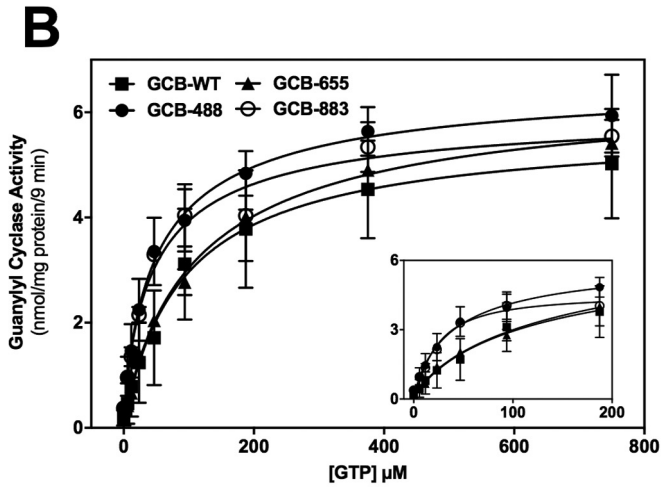


Figure 8. The A488P and R655C mutations mimicked an ATP-bound activated state of GC-B. Membranes from 293T cells transiently expressing WT or mutant GC-B constructs were incubated for 1, 3, or 9 min in the presence (*filled symbols*) or absence (*open symbols*) of 1 mM ATP under basal or 1 μM CNP. Data represent the mean ± S.E. when $n = 6$ from three experiments.



	GCB-WT	GCB-488	GCB-655	GCB-883
Vmax	3.149	4.384	4.821	4.603
h	1.534	1.207	0.8491	0.7898
Khalf	247.9	64.05	81.02	15.62



	GCB-WT	GCB-488	GCB-655	GCB-883
Vmax	5.752	6.688	6.509	6.096
h	0.9448	0.803	0.89	0.8051
Khalf	93.62	53.6	115.8	47.49

Figure 9. The A488P, R655C, and V883M missense mutations mimicked an ATP-bound and allosterically activated GC-B conformation. 293T cells were transiently transfected with the indicated forms of GC-B, and membranes from these cells were assayed for GC activity for 9 min in the presence or absence of 1 mM ATP with increasing concentrations of GTP and the presence of 1 μM CNP. *A*, comparison between GC-B-WT, GC-B-A488P, GC-B-R655C, and GC-B-V883M, where $n = 4$ from four experiments. *B*, comparison between GC-B-WT, GC-B-A488P, GC-B-R655C, and GC-B-V883M in the presence of 1 mM ATP, where $n = 4$ from two experiments. The *inset* shows lower concentration values at expanded scale.

although the mutations are located in different regions of the receptor, the V883M mutation is located in the guanylyl cyclase domain of GC-B whereas the A488P and R655C mutations are located in the juxta-membrane and in the kinase homology domain regions, respectively, the enzymatic changes induced by each mutation are similar. In the absence of ATP, each of the GC-B activating mutations had much lower K_m and Hill coefficient values than WT-GC-B. The addition of ATP dramatically lowered the K_m of WT-GC-B but did little to affect the K_m and Hill coefficient of the mutants, indicating that V883M, A488P, and R655C mutations mimic the NP- and ATP-bound conformation of the enzyme.

Table 1

Inhibition of basal guanylyl cyclase activity in GC-B mutants with activating mutations

Basal guanylyl cyclase activity was determined in the presence of 5 mM $MgCl_2$, 1 mM ATP, and 0.1 mM GTP for 3 min. The effect of inhibitors was measured under basal conditions with or without (Basal) the indicated additional nucleotide. Values are represented as the percent of basal activity for the indicated individual receptor. Basal activities (pmol of cGMP/mg/3 min) for each receptor are: WT, 9.3; A488P, 404.8; R655C, 226.5; V883M, 1345.9. Determinations were from two experiments assayed in duplicate, where $n = 4$.

Assay conditions	WT	A488P	R655C	V883M
	Mean ± S.E.	*Mean ± S.E.*	*Mean ± S.E.*	*Mean ± S.E.*
Basal	100.0 ± 6.9	100.0 ± 4.1	100.0 ± 5.7	100.0 ± 5.3
Plus 1 mM Mg^{2+}/ATP	89.3 ± 29.3	60.1 ± 10.0[a]	67.6 ± 12.5	65.9 ± 12.4[a]
Plus 1 mM Mg^{2+}/XTP	111.0 ± 16.4	66.1 ± 5.4[b]	63.2 ± 7.5[b]	84.7 ± 11.0
Plus 1 mM Mg^{2+}/TNP-ATP	242.0 ± 13.9[c]	28.0 ± 5.1[c]	20.6 ± 2.3[c]	15.3 ± 1.8[c]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.0001$.

Differences in the activities of the three mutant receptors did emerge, however, when the role of receptor phosphorylation was examined. Introduction of the R655C mutation to the constitutively dephosphorylated backbone increased basal GC activity, whereas the addition of the A488P mutation had little effect in the dephosphorylated enzyme (Fig. 5). Furthermore, the addition of the R655C mutation restored the ability of the dephosphorylated GC-B-7A mutant to respond to CNP binding. Although the amount of cGMP produced was not restored to WT levels, the affinity of the receptor as indicated by the EC_{50} was fully restored (Figs. 6 and 7). These data are consistent with the original report showing that dephosphorylated GC-B (GC-B-7A) binds ligand but is unable to transduce the binding signal to the guanylyl cyclase domain (13). However, the A488P mutation did not exhibit the same response. These differences may be due to the location of the mutations in the kinase homology domain. The A488P mutation is located approximately nine amino acids C terminus to the transmembrane region and is relatively near the phosphorylation sites of GC-B, which are critical for CNP-dependent GC-B activity, whereas the R655C mutation is in the middle of the kinase homology domain. Although all GC-B-activating mutations had lower Hill coefficients than the WT enzyme in the absence of ATP, the A488P mutation still displayed positive cooperative production formation, which is similar to the WT enzyme.

Based on these data, we propose a model where the activating mutations A488P, R655C, and V883M yield a GC-B molecule that is structurally similar to the physiological-activated enzyme that is bound by both CNP and ATP (Fig. 10). Physiological activation of GC-B requires CNP binding to the extracellular domain, which increases V_{max} in the absence of ATP. However, if ATP is bound to the allosteric site, then a large decrease in the K_m in also observed in response to CNP binding. The reduction in the K_m likely has the greatest effect on enzyme activity as physiologic GTP concentrations are well below the K_m in the absence of ATP. Significantly, all three activating mutations dramatically increased the basal activity of GC-B primarily through a reduction in the K_m. For the WT enzyme, a low K_m requires both CNP and ATP binding. Therefore, activating mutations must adapt a conformation similar to the CNP- and ATP-bound enzyme. Unlike R655C and V883M, A488P requires phosphorylation to elicit its activating effects on the enzyme. This may be due to the fact that A488P is upstream of the phosphorylation sites and, thus, requires phosphorylation to transmit the signal to the catalytic domain.

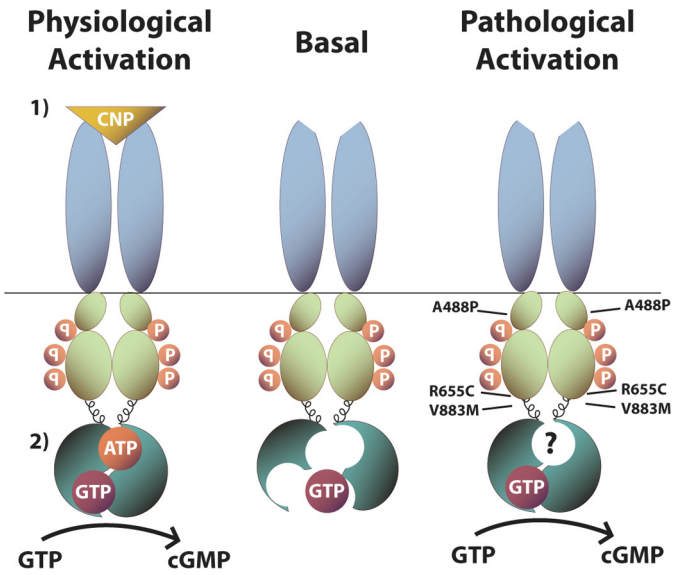


Figure 10. Pathological activating mutations in GC-B mimic the effect of physiological activation by CNP and ATP. Under basal conditions, GTP affinity toward the cyclase domain is low, resulting in a high K_m and low V_{max}. Physiological activation of GC-B involves CNP binding to the extracellular domain (1). This results in an ~10-fold increase in V_{max} that is independent of ATP. However, if CNP and ATP are present, then ATP can bind to the allosteric site in the catalytic domain, which allosterically increases the affinity of the catalytic domain to GTP (2). Pathological activation of GC-B by the missense mutations A488P, R655C, or V883M results in a confirmation similar to that adopted by GC-B when bound by CNP and ATP.

In the original published report of the R655C mutation by Hannema *et al.* (25), the authors hypothesized that one explanation for the increased activity could be the formation of an intermolecular disulfide bond stabilizing the receptor, but the hypothesis was not tested (25). Here, we mutated the Cys residue to Ser and found that the new mutant receptor, GC-B-R655S, retained the elevated basal activity. Thus, it is unlikely that the R655C mutation activates GC-B by forming a stabilizing disulfide bond.

Finally, we investigated whether known inhibitors of GCs would preferentially reduce the basal GC activity of the activated but not WT forms of GC-B. Neither XTP nor TNP-ATP inhibited WT-GC-B, but the modest ability of XTP to inhibit GC-B was no better than the inhibition observed with similar concentrations of ATP. In contrast, 1 mM TNP-ATP markedly reduced the activity of all three mutants to 20% or 30% that of the activity observed in the absence of the inhibitor. These data provide an initial foundation for the development of unique small molecule ATP-like molecules that could be used to spe-

cifically inhibit the mutant activated forms of GC-B as a potential anti-skeletal overgrowth drug.

In conclusion, GC-B is critical for long-bone growth, and single amino acid mutations in the enzyme result in aberrant skeletal growth. Identification of how these mutations change receptor function and activity is vital to increase understanding of how they affect downstream signaling and skeletal development. Ultimately, increased understanding of CNP- and GC-B-dependent bone growth has important clinical implications because activating mutations in GC-B result in skeletal overgrowth, and small molecule inhibitors of these mutants, as initially described here, have the potential to treat patients with these mutations. Regarding skeletal undergrowth, overexpression of CNP rescues the most common form of human dwarfism in mouse models (29), and a degradation-resistant form of CNP is currently in clinical trials for the treatment of adolescent achondroplasia (30).

Experimental procedures

Reagents

^{125}I-cGMP radioimmunoassay kits were from PerkinElmer Life Sciences, and protease inhibitor mixture tablets were from Roche Diagnostics. Xanthosine 5′-ATP was from TriLink Biotechnologies, Inc. (San Diego, CA). TNP-ATP, creatine kinase, phosphocreatine, and NPs were from Millipore-Sigma.

Mutagenesis

Site-directed mutagenesis using QuikChange II (Stratagene, Cedar Creek, TX) was used to introduce the A488P and R655C mutations in both human and rat GC-B constructs. GC-B-7A- and GCB-7E-A488P constructs were also made by introducing the A488P mutation into the GC-B-7A and GCB-7E constructs using site-directed mutagenesis (9). The 7E and 7A versions of GC-B-R655C were made by subcloning the regions encoding the phosphorylation sites substitutions from the pRK5-GC-B-7E and pRK5-GC-B-7A plasmids into the rat pRK5-GC-B-R655C plasmid. The R655S mutation was also introduced into the rat pRK5-GC-B plasmid by site-directed mutagenesis.

Transient transfection

Human embryonic kidney 293T cells were transiently transfected with 1 μg of the various plasmids by the HEPES-calcium-phosphate precipitation method as described (9).

Membrane preparation

Cells were placed in serum-free media for 4 h then scraped at 4 °C in phosphatase inhibitor buffer (PIB) described by Antos *et al.* (31). The cells were lysed by sonication, and lysates were centrifuged at 20,000 $\times$ *g* for 15 min at 4 °C. The resulting membranes were washed with 0.7 ml of PIB by centrifugation at 20,000 $\times$ *g* for 15 min at 4 °C before final resuspension in PIB.

Guanylyl cyclase assays

The single substrate concentration GC assays were performed at 37 °C in a buffer containing 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM isobutylmethylxanthine, 1 mM EDTA, 0.5 μM microcystin, 5 mM $MgCl_2$, 0.1 μg/ml creatine kinase, 5 mM creatine phosphate, 1 mM ATP, and 0.1 mM GTP in the absence or presence of 1 μM CNP for a duration of 3 min. The substrate velocity assays were performed for the indicated times with increasing GTP concentrations. The Mg^{2+}GTP concentrations assayed for the substrate-velocity assays were: 3000, 1500, 750, 375, 187.5, 93.7, 46.9, 23.4, 11.7, and 5.86 μM. All reactions were initiated by the addition of reaction mixture to 20 μl of crude membranes for a total volume of 0.1 ml and stopped with 0.4 ml of ice-cold 50 mM sodium acetate buffer containing 5 mM EDTA. In EC_{50} experiments, cells were assayed for GC activity for 3 min in the presence of 100 μM GTP, 1 mM ATP, and increasing concentrations of CNP. Cyclic GMP concentrations were determined by radioimmunoassay as previously described (32). Because enzymatic activity was not completely linear with time, the kinetic parameters are considered "apparent."

Immunoblot analysis

0.01 g of membrane protein prepared in PIB from transiently transfected 293T cells was fractionated on an 8% gel by SDS-PAGE and blotted to a polyvinylidene difluoride membrane. The membrane was blocked with Odyssey LI-COR blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 h at room temperature and then incubated overnight at 4 °C with rabbit polyclonal antiserum #6327 against the C terminus of rat GC-B at a dilution of 1/10,000 in 1:1 blocking buffer/PBS with 0.1% Tween. The specificity of this antiserum for human and rat GC-B has been previously demonstrated (18, 26, 32, 33). The membrane was washed 4 $\times$ 5 min with TBST and then incubated with IRDye 680 goat anti-rabbit antibody (Li-Cor Biosciences) with a dilution of 1/10,000 for 1 h at room temp in 1:1 blocking buffer/PBS with 0.1% Tween and 0.01% SDS. The membrane was washed 4 times for 5 min with Tris-buffered saline with 0.1% Tween and once with phosphate-buffered saline before imaging on a Li-Cor infrared imaging device. The relative amount of GC-B protein was determined using NIH ImageJ software for the membrane preparations from each experiment.

Whole cell cGMP elevation

24 h post-transfection equal numbers of transfected 293T cells were plated in 48-well plates coated with poly-D-lysine. The next day the cells were incubated in serum-free medium for 4 h and then pretreated for 10 min at 37 °C in DMEM containing 25 mM HEPES, pH 7.4, and 0.5 mM 1-methyl-3-isobutylxanthine. The medium was replaced with the same medium containing increasing concentrations of CNP. After 1 or 3 min, the reaction was stopped by aspiration of the medium and the addition of 0.2 ml if ice-cold 80% ethanol. Cyclic GMP concentrations were estimated from an aliquot of the ethanol extract by RIA as described (32).

Statistical analysis

Substrate-velocity curves were analyzed by nonlinear regression using a Michaelis-Menten model to determine V_{max} and K_m using Graph Pad Prism 6 software. Significant differences between nonlinear regression curves used to determine K_m and V_{max} values were determined using the extra sum of squares F

test to generate p values. Dose-response curves were analyzed by nonlinear regression using a sigmoidal dose-response curve to determine significant differences in the EC_{50}. Significant differences in all tests were $p \geq 0.05$.

Author contributions—D. M. D. conducted the experiments, analyzed the results, and wrote the paper. N. M. O. conducted and analyzed the substrate velocity experiments, created the model, and edited the paper. L. R. P. conceived and coordinated the study and wrote the paper.

References

1. Kuhn, M. (2016) Molecular physiology of membrane guanylyl cyclase receptors. *Physiol. Rev.* **96,** 751–804
2. Potter, L. R. (2011) Guanylyl cyclase structure, function, and regulation. *Cell. Signal.* **23,** 1921–1926
3. Jaffe, L. A., and Egbert, J. R. (2017) Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. *Annu. Rev. Physiol.* **79,** 237–260
4. Robinson, J. W., and Potter, L. R. (2012) Guanylyl cyclases a and B are asymmetric dimers that are allosterically activated by ATP binding to the catalytic domain. *Sci. Signal.* **5,** ra65
5. Antos, L. K., and Potter, L. R. (2007) Adenine nucleotides decrease the apparent Km of endogenous natriuretic peptide receptors for GTP. *Am. J. Physiol. Endocrinol. Metab.* **293,** E1756–E1763
6. Yoder, A. R., Kruse, A. C., Earhart, C. A., Ohlendorf, D. H., and Potter, L. R. (2008) Reduced ability of C-type natriuretic peptide (CNP) to activate natriuretic peptide receptor B (NPR-B) causes dwarfism in lbab$^{-/-}$ mice. *Peptides* **29,** 1575–1581
7. Potter, L. R., and Hunter, T. (2001) Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation. *J. Biol. Chem.* **276,** 6057–6060
8. 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
9. Yoder, A. R., Robinson, J. W., Dickey, D. M., Andersland, J., Rose, B. A., Stone, M. D., Griffin, T. J., and Potter, L. R. (2012) A functional screen provides evidence for a conserved, regulatory, juxtamembrane phosphorylation site in guanylyl cyclase A and B. *PLoS ONE* **7,** e36747
10. 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
11. Shuhaibar, L. C., Egbert, J. R., Edmund, A. B., Uliasz, T. F., Dickey, D. M., Yee, S. P., Potter, L. R., and Jaffe, L. A. (2016) Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase is required for rapid resumption of oocyte meiosis in response to luteinizing hormone. *Dev. Biol.* **409,** 194–201
12. Potter, L. R. (1998) Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptor B: dephosphorylation is a mechanism of desensitization. *Biochemistry* **37,** 2422–2429
13. 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
14. Egbert, J. R., Shuhaibar, L. C., Edmund, A. B., Van Helden, D. A., Robinson, J. W., Uliasz, T. F., Baena, V., Geerts, A., Wunder, F., Potter, L. R., and Jaffe, L. A. (2014) Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes. *Development* **141,** 3594–3604
15. Bartels, C. F., Bükülmez, H., Padayatti, P., Rhee, D. K., van Ravenswaaij-Arts, C., Pauli, R. M., Mundlos, S., Chitayat, D., Shih, L. Y., Al-Gazali, L. I., Kant, S., Cole, T., Morton, J., Cormier-Daire, V., Faivre, L., *et al.* (2004) Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. *Am. J. Hum. Genet.* **75,** 27–34
16. Wang, S. R., Jacobsen, C. M., Carmichael, H., Edmund, A. B., Robinson, J. W., Olney, R. C., Miller, T. C., Moon, J. E., Mericq, V., Potter, L. R., Warman, M. L., Hirschhorn, J. N., and Dauber, A. (2015) Heterozygous Mutations in Natriuretic Peptide Receptor-B (NPR2) Gene as a cause of short stature. *Hum. Mutat.* **36,** 474–481
17. Wang, W., Song, M. H., Miura, K., Fujiwara, M., Nawa, N., Ohata, Y., Kitaoka, T., Kubota, T., Namba, N., Jin, D. K., Kim, O. H., Ozono, K., and Cho, T. J. (2016) Acromesomelic dysplasia, type maroteaux caused by novel loss-of-function mutations of the NPR2 gene: three case reports. *Am. J. Med. Genet. A* **170,** 426–434
18. Dickey, D. M., Edmund, A. B., Otto, N. M., Chaffee, T. S., Robinson, J. W., and Potter, L. R. (2016) Catalytically active guanylyl cyclase B requires endoplasmic reticulum-mediated glycosylation, and mutations that inhibit this process cause dwarfism. *J. Biol. Chem.* **291,** 11385–11393
19. Olney, R. C., Bükülmez, H., Bartels, C. F., Prickett, T. C., Espiner, E. A., Potter, L. R., and Warman, M. L. (2006) Heterozygous mutations in natriuretic peptide receptor-B (NPR2) are associated with short stature. *J. Clin. Endocrinol. Metab.* **91,** 1229–1232
20. Hachiya, R., Ohashi, Y., Kamei, Y., Suganami, T., Mochizuki, H., Mitsui, N., Saitoh, M., Sakuragi, M., Nishimura, G., Ohashi, H., Hasegawa, T., and Ogawa, Y. (2007) Intact kinase homology domain of natriuretic peptide receptor-B is essential for skeletal development. *J. Clin. Endocrinol. Metab.* **92,** 4009–4014
21. Bocciardi, R., Giorda, R., Buttgereit, J., Gimelli, S., Divizia, M. T., Beri, S., Garofalo, S., Tavella, S., Lerone, M., Zuffardi, O., Bader, M., Ravazzolo, R., and Gimelli, G. (2007) Overexpression of the C-type natriuretic peptide (CNP) is associated with overgrowth and bone anomalies in an individual with balanced t(2;7) translocation. *Hum. Mutat.* **28,** 724–731
22. Moncla, A., Missirian, C., Cacciagli, P., Balzamo, E., Legeai-Mallet, L., Jouve, J. L., Chabrol, B., Le Merrer, M., Plessis, G., Villard, L., and Philip, N. (2007) A cluster of translocation breakpoints in 2q37 is associated with overexpression of NPPC in patients with a similar overgrowth phenotype. *Hum. Mutat.* **12,** 1183–1188
23. Miura, K., Namba, N., Fujiwara, M., Ohata, Y., Ishida, H., Kitaoka, T., Kubota, T., Hirai, H., Higuchi, C., Tsumaki, N., Yoshikawa, H., Sakai, N., Michigami, T., and Ozono, K. (2012) 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* **7,** e42180
24. Miura, K., Kim, O. H., Lee, H. R., Namba, N., Michigami, T., Yoo, W. J., Choi, I. H., Ozono, K., and Cho, T. J. (2014) Overgrowth syndrome associated with a gain-of-function mutation of the natriuretic peptide receptor 2 (NPR2) gene. *Am. J. Med. Genet. A* **164,** 156–163
25. Hannema, S. E., van Duyvenvoorde, H. A., Premsler, T., Yang, R. B., Mueller, T. D., Gassner, B., Oberwinkler, H., Roelfsema, F., Santen, G. W., Prickett, T., Kant, S. G., Verkerk, A. J., Uitterlinden, A. G., Espiner, E., Ruivenkamp, C. A., *et al.* (2013) An activating mutation in the kinase homology domain of the natriuretic peptide receptor-2 causes extremely tall stature without skeletal deformities. *J. Clin. Endocrinol. Metab.* **98,** E1988–E1998
26. Robinson, J. W., Dickey, D. M., Miura, K., Michigami, T., Ozono, K., and Potter, L. R. (2013) A human skeletal overgrowth mutation increases maximal velocity and blocks desensitization of guanylyl cyclase-B. *Bone* **56,** 375–382
27. Dove, S., Danker, K. Y., Stasch, J. P., Kaever, V., and Seifert, R. (2014) Structure/activity relationships of (M)ANT- and TNP-nucleotides for inhibition of rat soluble guanylyl cyclase $\alpha1\beta1$. *Mol. Pharmacol.* **85,** 598–607
28. Beste, K. Y., Spangler, C. M., Burhenne, H., Koch, K. W., Shen, Y., Tang, W. J., Kaever, V., and Seifert, R. (2013) Nucleotidyl cyclase activity of particulate guanylyl cyclase A: comparison with particulate guanylyl cyclases E and F, soluble guanylyl cyclase and bacterial adenylyl cyclases CyaA and edema factor. *PLoS ONE* **8,** e70223
29. Yasoda, A., Komatsu, Y., Chusho, H., Miyazawa, T., Ozasa, A., Miura, M., Kurihara, T., Rogi, T., Tanaka, S., Suda, M., Tamura, N., Ogawa, Y., and Nakao, K. (2004) Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.* **10,** 80–86
30. Lorget, F., Kaci, N., Peng, J., Benoist-Lasselin, C., Mugniery, E., Oppeneer, T., Wendt, D. J., Bell, S. M., Bullens, S., Bunting, S., Tsuruda, L. S., O'Neill,

C. A., Di Rocco, F., Munnich, A., and Legeai-Mallet, L. (2012) Evaluation of the therapeutic potential of a CNP analog in a Fgfr3 mouse model recapitulating achondroplasia. *Am. J. Hum. Genet.* **91,** 1108–1114

31. 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
32. 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
33. Abbey, S. E., and Potter, L. R. (2003) Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. *Endocrinology* **144,** 240–246