# Cytoplasmic Domains Mediate the Ligand-Induced Affinity Shift of Guanylyl Cyclase C

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ABSTRACT: Guanylyl cyclase C (GCC), the receptor for the *Escherichia coli* heat-stable enterotoxin (ST), exhibits multiple binding affinities, including high ($R_H$) and low ($R_L$) affinity sites and a ligand-induced conversion of low-affinity sites from a higher ($R_{L1}$) to a lower ($R_{L2}$) affinity state. Occupancy of the lowest affinity state of low-affinity sites is coupled to ligand-induced catalytic activation. In the present studies, ligand binding and catalytic activation properties of a series of intracellular deletion mutants of GCC were examined to identify the structural domains underlying expression of high- and low-affinity sites and the ligand-induced shift in low-affinity sites. These studies demonstrated that the cytoplasmic domains of GCC are not required, but extracellular and transmembrane domains are sufficient, for expression of high-affinity binding sites. In addition, the cytoplasmic juxtamembrane and kinase homology domains are required for expression of the ligand-induced affinity shift in low-affinity sites. Of significance, this shift in affinity was insensitive to adenine nucleotides, in contrast to other members of the receptor guanylyl cyclase family, such as guanylyl cyclase A (GCA). Also, the juxtamembrane and kinase homology domains are critical for coupling ST−receptor binding and guanylyl cyclase catalytic activation. Indeed, deletion of those domains from GCC results in a constitutively inhibited enzyme, suggesting that they function as positive effectors of ligand activation, in contrast to GCA and GCB, in which the kinase homology domain represses basal catalytic activity. These data suggest that the mechanisms regulating different members of the receptor guanylyl cyclase family are overlapping but not identical.

Guanylyl cyclase C (GCC),[1] a receptor for *Escherichia coli* heat-stable enterotoxin (ST) and an endogenous peptide produced in the intestine, guanylin, is expressed in apical membranes of mammalian intestinal cells (*1*−*4*). GCC is a member of the receptor guanylyl cyclase family of proteins, which exhibit ligand binding and guanylyl cyclase catalytic activities (*3*). Receptor guanylyl cyclases possess cognate domains characteristic of the family, including extracellular ligand binding (ECD), transmembrane (TMD), regulatory kinase homology (KHD), and catalytic domains (CD) (*3*, *5*−*9*). In addition, GCC has a cytoplasmic carboxy-terminal tail of 70 amino acids (TD), which may mediate association of the protein with the cytoskeleton (*3*, *9*). Ligand−receptor interaction activates the catalytic domain of GCC, resulting in accumulation of cyclic GMP (cGMP), which leads to activation of cyclic nucleotide-dependent protein kinase and phosphorylation of the cystic fibrosis transmembrane conductance regulator, increasing epithelial cell chloride current and intestinal secretion (*10*).

Receptor guanylyl cyclases exhibit complex ligand binding characteristics which are central to receptor-effector coupling. GCC and guanylyl cyclase A (GCA), the receptor for atrial natriuretic peptide (ANP), exhibit high (picomolar $K_D$; $R_H$) and low (nanomolar $K_D$; $R_L$) affinity ligand binding sites under equilibrium conditions (*7*, *11*−*15*). Occupancy of $R_L$ appears to be coupled to catalytic activation, while the role of $R_H$ remains unclear (*7*, *12*, *15*). Although the structural determinants underlying expression of $R_H$ and $R_L$ have not been defined, GCA and GCC undergo significant posttranslational modifications, and distinct affinities could reflect different isoforms of the same protein (*1*, *3*, *16*−*20*). In addition, GCC $R_L$ exhibits biphasic association kinetics, demonstrating that these sites undergo a ligand-induced shift from higher ($R_{L1}$) to lower ($R_{L2}$) affinity (*7*, *12*, *15*). Comparison of the concentration dependence of ST binding to, and activation of, GCC revealed that occupancy of $R_{L2}$ is coupled to activation of the guanylyl cyclase catalytic domain (*12*, *15*). Similarly, rat intestinal membranes have been isolated that display low-affinity receptors "locked" in $R_{L1}$and guanylyl cyclase activity insensitive to ST (*12*). These

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[1] Abbreviations: ANP, atrial natriuretic peptide; $B_{max}$, maximum number of binding sites; CD, catalytic domain; cGMP, cyclic guanosine monophosphate; ECD, extracellular domain; EGFR, epidermal growth factor receptor; GCA, guanylyl cyclase A; GCB, guanylyl cyclase B; GCC, guanylyl cyclase C; HGFR, hepatocyte growth factor receptor; IBMX, isobutylmethylxanthine; ID, intracellular domain; JD, juxtamembrane domain; $k_1$, association rate constant; $k_{-1}$, dissociation rate constant; $K_D$, equilibrium dissociation (affinity) constant; KHD, kinase homology domain; $k_{obs}$, observed association rate constant; *L*, ligand concentration; PI3, phosphatidylinositol 1,4-diphosphate; $R_H$, high-affinity receptor; RIA, radioimmunoassay; $R_L$, low-affinity receptor; $R_{L1}$, higher affinity state of the low-affinity receptor; $R_{L2}$, lowest affinity state of the low-affinity receptor; ST, *Escherichia coli* heat-stable enterotoxin; TD, tail domain; TED buffer, 50 mM Tris-HCl, pH 7.6, 1 mM ethylenediaminetetraacetic and (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT).

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data support the suggestion that the ST-induced affinity shift plays an important role in ligand activation of GCC catalysis (*12*, *15*). Interestingly, ANP induces a shift in the binding characteristics of GCA from higher to lower affinity that is associated with catalytic activation (*7*). The ANP-induced affinity shift of GCA is enhanced by ATP and inhibited by the ATP antagonist amiloride, and the effects of that nucleotide may be mediated by the KHD (*7*). Thus, although the ligand-induced shift of $R_L$ from higher to lower affinity appears to be critical for receptor−effector coupling of receptor guanylyl cyclases, the precise molecular mechanisms and structural determinants mediating the differential expression of $R_H$ and $R_L$, and the ligand-induced shift in affinity, remain incompletely defined.

In the present studies, the binding characteristics of intracellular deletion mutants of GCC expressed in COS-7 cells were examined to define the structural determinants underlying expression of $R_H$ and $R_L$ and the ligand-induced affinity shift important in catalytic activation. These studies demonstrated that cytoplasmic domains of GCC are not required for expression of high- and low-affinity binding sites. Rather, the transmembrane and extracellular domains are sufficient for expression of $R_H$ and $R_L$. In contrast, the juxtamembrane and kinase homology domains mediate the ligand-induced affinity shift of $R_{L1}$ to $R_{L2}$, important for catalytic activation, in an ATP-independent fashion.

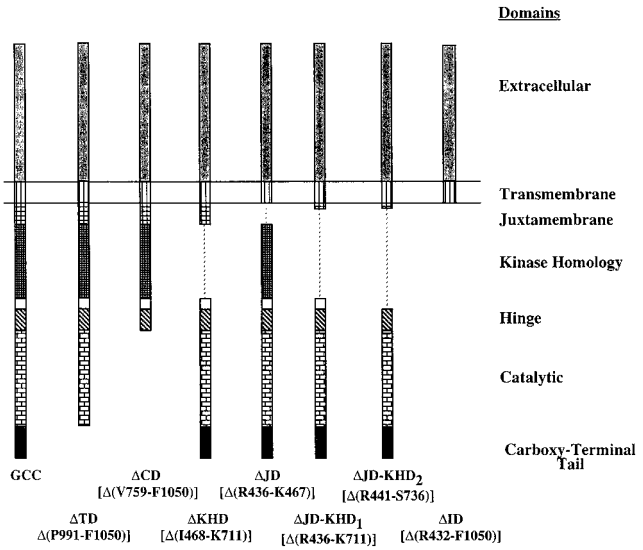


FIGURE 1: Schematic of wild-type and mutant GCCs. Constructs employed in the present studies included full-length, wild-type GCC; ΔTD, lacking the carboxy-terminal tail; ΔCD, lacking the catalytic domain and the carboxy-terminal tail; ΔKHD, lacking the kinase homology domain; ΔJD lacking the juxtamembrane domain; $\Delta$JD-KHD$_1$, lacking juxtamembrane and kinase homology domains; $\Delta$JD-KHD$_2$, lacking juxtamembrane and kinase homology domains and the hinge region; and ΔID, lacking the entire intracellular region. Construction and expression of these mutants were performed as described in Materials and Methods.

## MATERIALS AND METHODS

*Deletion Mutants.* Rat GCC cDNA subcloned into the vector pMT2 was employed for construction of deletion mutants (*15*). This vector permits high levels of transient gene expression in mammalian cells, reflecting its strong adenovirus late promoter, SV 40 origin of replication, and SV 40 T antigen-encoding sequence (*21*). The deletion mutants generated included [deleted residues]: ΔTD [P991−F1050], ΔJD [R436−K467], ΔKHD [I468−K711], $\Delta$JD-KHD$_1$ [R436−K711], $\Delta$JD-KHD$_2$ [R441−S736], ΔCD [V759−F1050], and ΔID [R432−F1050], lacking all intracellular domains (Figure 1; *3*). In general, these deletions were made by the method of overlap extension employing the polymerase chain reaction (*22*). ΔCD [V759−F1050] was made by digesting rat GCC cDNA in pMT2 with restriction enzymes *Dra*III and *Spe*I, blunting using T4 DNA polymerase, and religation using T4 DNA ligase. The nucleotide sequence of deletion mutants was verified employing the dideoxy method and Applied Biosystems Models 373A and 377 DNA sequencing systems (Kimmel Cancer Center, Thomas Jefferson University).

*Cell Culture.* COS-7 cells were maintained in DMEM/Ham's F12 medium (1:1) with 10% fetal bovine serum and penicillin and streptomycin (*15*; 10 units/mL and 10 $\mu$g/mL, respectively). Rat GCC deletion mutant constructs subcloned in pMT2 were transfected into COS-7 cells using DEAE-dextran and chloroquin (*23*). Cells were transfected with 10 $\mu$g of DNA/75 cm$^2$ flask, grown to 80% confluence, trypsinized, split 1:3, and harvested after 72 h. Transfection was confirmed employing ST binding, guanylyl cyclase assays, and Western blot analysis.

*Membrane Preparation.* Membranes were prepared from cells expressing wild-type and mutant GCCs as described previously (*15*). Cells washed with ice-cold buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM PMSF, and 1 mM dithiothreitol; TED buffer) were scraped from flasks, resuspended in TED buffer, and pelleted at 10000*g* for 30 min. Cells resuspended in TED buffer were homogenized with 15 strokes in a Teflon-on-glass homogenizer. Homogenates were employed directly or centrifuged at 105000*g* for 30 min and the membrane pellet was resuspended and stored in TED buffer at −70 °C.

*Western Blot Analysis.* The primary polyclonal antibody used for Western analysis of GCC mutants was obtained as a gift from Dr. David L. Garbers (University of Texas, Southwestern Medical Center, Dallas, TX) and was raised in rabbits against the epitope KDVLRRSEQFQEILMGRNRK, a 20 amino acid peptide in the receptor region of GCC spanning amino acids $K_{200}$−$K_{219}$. COS-7 cell homogenates (50 $\mu$g) were fractionated by SDS−PAGE and transferred to nitrocellulose membrane. Rainbow molecular weight markers (Amersham Corp.) were used as size standards. Detection was carried out as described in the protocol supplied with the Amersham ECL detection kit.

*ST Receptor Binding Assay: (A) Equilibrium Binding.* Equilibrium binding studies were performed by incubating 30 $\mu$g of membrane or homogenate protein with concentrations of $^{125}$I-ST ranging from 0.122 pM to 20 nM in binding buffer containing 50 mM Tris-HCl, pH 7.6, 0.66 mM cystamine, 0.1% bacitracin, 450 mM NaCl, and 1 mM EDTA (final volume = 100 $\mu$L) at 37 °C for 120 min (*15*). In all binding studies, parallel incubations contained a 1000-fold excess of unlabeled ST to quantify nonspecific binding. Free and bound ligand were separated by vacuum filtration employing Whatman GF/B glass fiber filters (Hillsboro, OR) presoaked in 0.3% poly(ethylenimine). Radioactivity bound to filters was quantified in a Packard $\gamma$ counter (Downers Grove, PA). Specific binding was calculated as the arithmetic difference between total (in the absence of excess unlabeled ligand) and nonspecific binding (*15*).

*(B) Association*. Association kinetics were examined employing 3 nM $^{125}$I-ST (final volume = 100 $\mu$L; *15*). The relative occupancy of high ($R_H$) and low ($R_L$) affinity ST binding sites at equilibrium at this ligand concentration may be calculated by

$$B = (B_{max}\, L)/(K_D + L) \quad (1)$$

where $B$ represents receptor sites bound at equilibrium, $B_{max}$ is the maximum number of binding sites available, $L$ is the ligand concentration, and $K_D$ is the dissociation constant of the ligand-binding site complex (*24*). In the presence of 3 nM labeled ligand, >95% of the observed binding is contributed by $R_L$ compared to $R_H$. Over the time course of these experiments, the concentration of free $^{125}$I-ST varied less than 10%, and association performed under these conditions fulfilled the criteria for being pseudo first order, since ligand concentration remained essentially constant and only a single class of receptors was visualized (*24*, *25*).

*(C) Dissociation.* Studies of dissociation were conducted under conditions identical to those of association (3 nM $^{125}$I-ST; 100 $\mu$L final volume). Dissociation of ligand−receptor complexes was initiated by the addition of excess unlabeled ST (5 $\mu$M). Under these conditions, observed dissociation reflected the activity of low-affinity binding sites, $R_L$. $^{125}$I-ST bound to $R_L$ of wild-type and mutant GCCs did not completely dissociate, as described previously (*11*, *12*, *14*, *15*). Indeed, about 25% of the associated ST remained bound to membranes after maximum dissociation was achieved. Rate constants for dissociation from $R_L$ were determined only for the dissociable fraction of the binding to those sites (*12*). The high concentration of unlabeled ST (5 $\mu$M) in dissociation incubations effectively prevented reassociation of labeled ST with receptors. Consequently, dissociation of labeled ST from low-affinity sites of GCC and deletion mutants was first-order.

*(D) Second-Order Association Rate Constants*. Second-order rate constants of association ($k_1$) of labeled ST with the different affinity states of $R_L$ of wild-type and mutant GCCs were estimated by (*15*):

$$k_1 = (k_{obs} - k_{-1})/[L] \quad (2)$$

where $k_{obs}$ represents the observed association rate constant and $k_{-1}$ is the dissociation rate constant.

*Guanylyl Cyclase Assay*. Guanylyl cyclase activity was measured as described previously (*26*). Briefly, incubations contained 30 $\mu$g of homogenate protein, 1.2 mM isobutylmethylxanthine (IBMX), 50 mM Tris-HCl, pH 7.6, a regenerating system composed of 15 mM creatine phosphate and 2.7 units of creatine phosphokinase, 4 mM $Mg^{2+}$ or $Mn^{2+}$, and 1 mM GTP. Where indicated, incubations contained 1 $\mu$M ST and/or 0.5 mM ATP$\gamma$S. Reactions were initiated by the addition of substrate, incubated for 5 min at 37 °C, and terminated with ice-cold 50 mM sodium acetate, pH 4.0, followed by immersion in boiling water for 3 min. Cyclic GMP produced was quantified by radioimmunoassay (RIA).

*Accumulation of Intracellular cGMP*. ST-induced accumulation of intracellular cGMP was quantified as described previously (*27*). COS-7 cells transfected with pMT2 alone or pMT2 containing rat GCC or deletion mutants were washed with OPTIMEM (Gibco−BRL) containing 100 $\mu$M IBMX and equilibrated in that medium for 15 min at 37 °C. Subsequently, unlabeled ST (1 $\mu$M final concentration) or vehicle (ST dilution buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% bacitracin) was added to wells and incubations were continued for 15 min at 37 °C (final volume per well = 200 $\mu$L). Incubations were terminated with 200 $\mu$L of ice-cold 6% trichloroacetic acid (TCA) and cells were collected and centrifuged at 1500*g* at 4 °C for 20 min. Resulting pellets were resuspended in 200 $\mu$L of 1 M NaOH and used for protein determination (*28*). Supernates were extracted with ether to remove TCA and cGMP quantified by RIA.

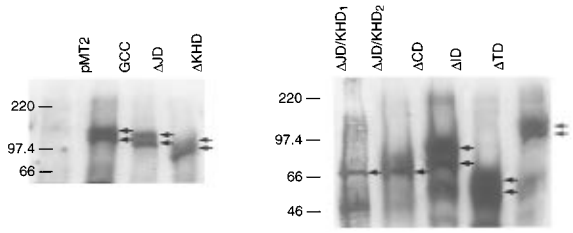


FIGURE 2: Western blot analysis of GCC deletion mutants expressed in COS-7 cells. Cell homogenates (50 $\mu$g) were fractionated by SDS−PAGE and subjected to Western blotting employing a primary antibody directed against an extracellular region of GCC, as described in Materials and Methods. Homogenates from vector-transfected cells and cells transfected with wild-type GCC were employed as negative and positive controls, respectively. Results are representative of three experiments.

*Miscellaneous*. Protein concentration was quantified by the method of Bradford employing bovine serum albumin as a standard (Bio-Rad, Richmond, CA; *28*). ST was purified from culture broth and monoiodinated to a specific activity of 2000 Ci/mmol (*29*). Equilibrium binding curves were fitted, and constants were obtained, employing *Cigale* on a Macintosh IIsi. *Cigale* was written by M. Bordes (Institute de Pharmacologie Cellulaire et Moleculaire, Universite de Nice, Sophia Antipolis, France; *11*). Linear regression to obtain association and dissociation constants was performed using Cricket Graph (Cricket Software, Malvern, PA) on a Macintosh IIci. All assays were performed in duplicate or triplicate, data presented are representative of at least three experiments, values presented are means + SEM, and $n$ = number of observations. Statistical significance was quantified employing Student's $t$ test.

## RESULTS

*Western Blot Analysis.* The expression of GCC deletion mutants was characterized by Western blotting using an antibody directed against a synthetic dodecapeptide based on an extracellular sequence of GCC (Figure 2). COS-7 cells transfected with wild-type GCC or the expression vector pMT2 were employed as controls. Wild-type GCC yielded proteins specifically recognized by the antibody of 150 and 160 kDa, reflecting the differentially glycosylated forms of this receptor described previously (Figure 2; *20*). The mutants ΔTD, ΔJD, ΔKHD, ΔJD/$KHD_1$, ΔJD/$KHD_2$, ΔCD, and ΔID yielded proteins specifically recognized by the antibody that exhibited sizes predicted by their amino acid sequence (Figure 2).

*Equilibrium Binding*. Radiolabeled ST specifically bound to membranes prepared from COS-7 cells transfected with wild-type GCC or the deletion mutants ΔTD, ΔJD, ΔKHD, ΔJD/$KHD_1$, ΔCD, or ΔID in a concentration-dependent and

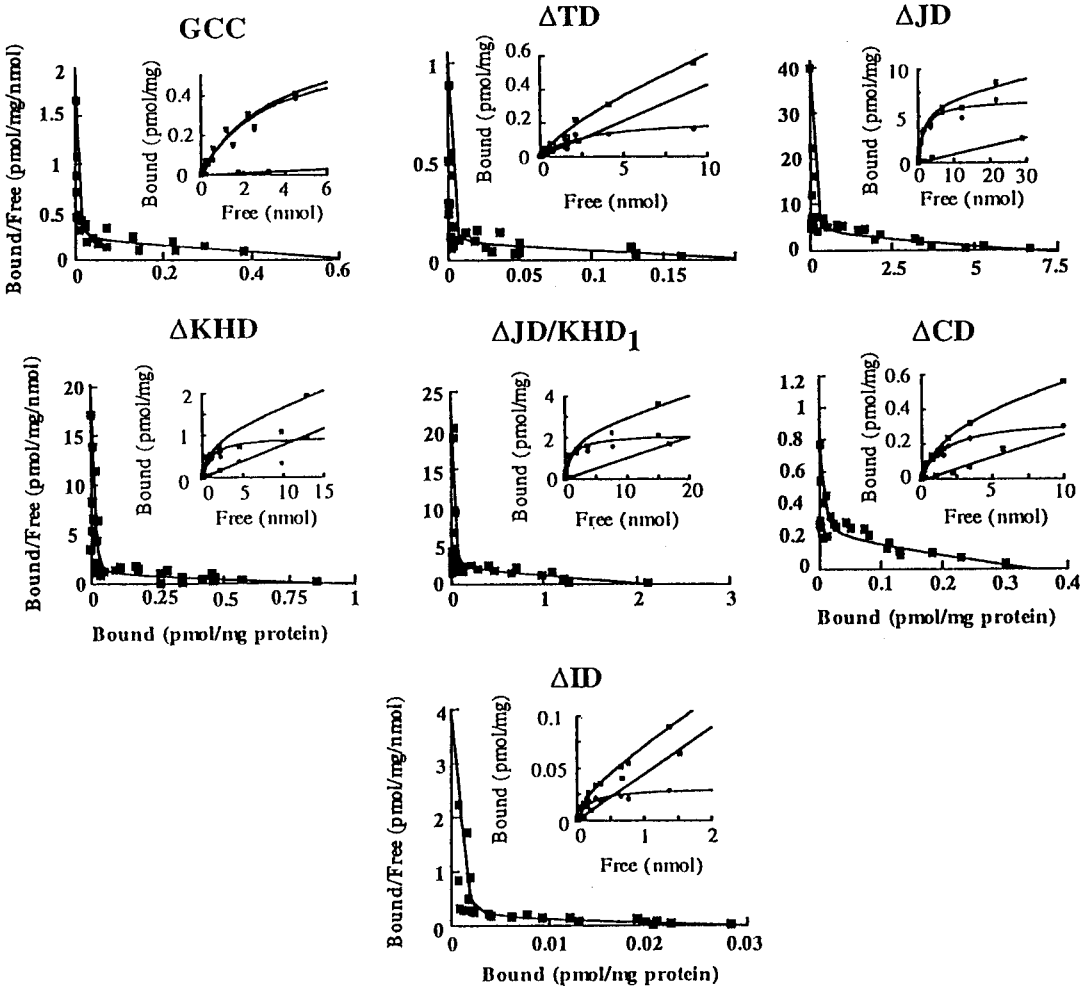


FIGURE 3: Scatchard analysis of direct equilibrium binding of $^{125}$I-ST to wild-type and mutant GCCs expressed in COS-7 cells. Cell membranes or homogenates were incubated with increasing concentrations of $^{125}$I-ST and specific binding was quantified as described in Materials and Methods. Insets: Direct equilibrium binding. (□) Total binding; (○) specific binding; (■) nonspecific binding.

Table 1: Equilibrium Binding Characteristics of Wild-Type and Mutant GCCs Expressed in COS-7 Cells[a]

| | construct | | | | | | |
|---|---|---|---|---|---|---|---|
| | GCC | ΔTD | ΔJD | ΔKHD | ΔJD/KHD$_1$ | ΔCD | ΔID |
| $K_{DH}$[b] | 3.1 ± 1.2 | 3.6 ± 2.1 | 1.4 ± 1.5 | 2.7 ± 1.6 | 0.8 ± 0.4 | 6.1 ± 1.2 | 3.4 ± 2.3 |
| $K_{DL}$[b] | 2.2 ± 1.1 | 6.7 ± 2.6 | 0.8 ± 0.3 | 2.0 ± 0.8 | 2.4 ± 1.3 | 4.0 ± 3.1 | 1.4 ± 1.2 |
| $B_{maxH}$[b] | 48.1 ± 27.0 | 6.0 ± 2.0 | 52.8 ± 57.9 | 20.2 ± 7.7 | 26.2 ± 11.0 | 4.2 ± 1.6 | 3.3 ± 3.0 |
| $B_{maxL}$[b] | 3.9 ± 2.7 | 0.5 ± 0.2 | 3.9 ± 1.6 | 0.9 ± 0.1 | 2.5 ± 1.1 | 0.3 ± 0.2 | 1.4 ± 0.8 |

[a] Data represent the means of at least three experiments ± SEM. [b] $K_{DH}$ and $K_{DL}$, dissociation constants for high (picomolar) and low (nanomolar) affinity receptors, respectively; $B_{maxH}$ and $B_{maxL}$, maximum number of high (femtomoles per milligram of protein) and low (picomoles per milligram of protein) affinity binding sites, respectively.

saturable fashion (Figure 3, insets). Scatchard analyses of these data yielded curvilinear isotherms, suggesting the presence of two populations of binding sites with high (picomolar; $R_H$) and low (nanomolar; $R_L$) affinities for ST (Table 1). Variability of $B_{max}$ values for $R_H$ and $R_L$ likely reflects variability of expression efficiencies between transfections (Figure 2, Table 1). Equilibrium binding affinities of $R_H$ and $R_L$ exhibited by GCC deletion mutants expressed in COS-7 cells are not significantly different from those of wild-type GCC (Table 1; *15*). In addition, they are similar to those reported for native ST receptors in rat intestinal cells (*11*, *12*), suckling mouse intestine (*14*), and human colorectal tumors and cell lines (*13*).

*Binding Kinetics of $R_L$: (A) Association.* The kinetics of ST binding to $R_L$ of the deletion mutants ΔTD, ΔJD, ΔKHD, ΔJD/KHD$_1$, ΔCD, and ΔID were compared to those of wild-type GCC to examine the role of specific cognate domains in ligand induction of the affinity shift important for receptor-effector coupling (*12*, *15*). $^{125}$I-ST specifically bound to $R_L$ in a time-dependent fashion in membranes prepared from COS-7 cells expressing wild-type or deletion mutants of GCC (Figure 4). Semilogarithmic transformation of these data obtained with wild-type GCC, ΔTD, and ΔCD yielded curvilinear isotherms, demonstrating the presence of two association events with different rates (*12*, *15*). Identical results were obtained when association studies were performed employing GCC expressed in human embryonic kidney 293 cells in the presence of 1 mM ATP, 1 mM ATPγS, or 1 mM amiloride, demonstrating that the affinity shift of $R_L$ was not adenine nucleotide-dependent (data not shown). The slopes of the semilogarithmic transformations of association yielded $k_{obs}$ for GCC, ΔTD, and ΔCD (Table

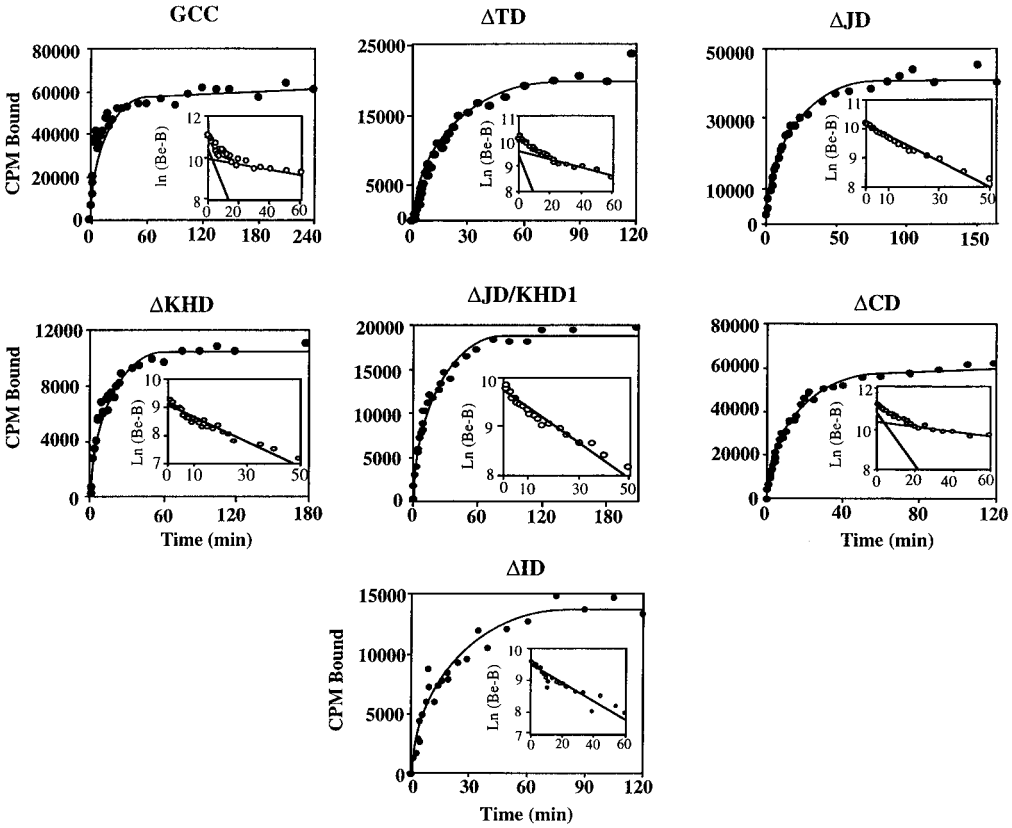


FIGURE 4: Time course of association of $^{125}$I-ST with wild-type or mutant GCCs expressed in COS-7 cells. Reactions were initiated by the addition of membranes to reactions containing 3 nM $^{125}$I-ST and aliquots were removed at the indicated times, as described in Materials and Methods. Insets: Semilogarithmic transformations of $y = (B_e - B)$, where $B_e$ and $B$ represent bound ligand at equilibrium and at time $x$, respectively. Isotherms of these transformations were resolved into components, where indicated, as described in Materials and Methods.

Table 2: Kinetic Binding Characteristics of Wild-Type and Mutant GCCs Expressed in COS-7 Cells[a]

| | construct | | | | | | |
|---|---|---|---|---|---|---|---|
| | GCC | ΔTD | ΔJD | ΔKHD | ΔJD/KHD$_1$ | ΔCD | ΔID |
| $k_{obsL_1}$[b,c] | 1.8 ± 0.4 | 1.1 ± 0.9 | | | | 0.8 ± 0.2 | |
| $k_{obsL_i}$[b,c] | | | 0.4 + 0.0 | 0.5 + 0.0 | 0.4 ± 0.1 | | 0.3 ± 0.1 |
| $k_{obsL_2}$[b,c] | 0.1 ± 0.1 | 0.1 ± 0.1 | | | | 0.2 ± 0.1 | |
| $k_{-1}$[d] | 1.8 ± 1.0 | 0.6 ± 0.1 | 1.0 ± 0.2 | 0.7 ± 0.6 | 1.2 ± 0.1 | 1.2 ± 0.3 | 0.8 ± 0.6 |
| $k_{1L_1}$[c,e] | 4.4 ± 1.0 | 3.4 ± 0.3 | | | | 2.1 ± 0.8 | |
| $k_{1L_i}$[c,e] | | | 1.0 ± 0.1 | 1.5 ± 0.2 | 0.9 ± 0.1 | | 1.7 ± 0.9 |
| $k_{1L_2}$[c,e] | 0.2 ± 0.1 | 0.2 ± 0.0 | | | | 0.2 ± 0.1 | |

[a] The data represent the means of at least three experiments ± SEM. [b] $k_{obs}$ (per minute × $10^{-1}$) was obtained by semilogarithmic analysis of association kinetics. [c] Association experiments for the mutants ΔJD, ΔKHD, ΔJD/KHD$_1$, and ΔID yielded linear isotherms, with $k_{obsL_i}$ and resultant $k_{1L_i}$ values that were intermediate between $k_{obsL_1}$ and $k_{obsL_2}$, and $k_{1L_1}$ and $k_{1L_2}$, respectively, of wild-type GCC, ΔTD, and ΔCD ($p < 0.05$). [d] $k_{-1}$ (per minute × $10^{-2}$) was obtained by semilogarithmic analysis of dissociation kinetics. [e] $k_1$ (liters per mole per minute × $10^7$) was obtained using the equation $k_1 = (k_{obs} - k_{-1})/[L]$.

2). Individual rate constants were determined by resolving curvilinear semilogarithmic transformations into two linear components (Figure 4, insets; *12*, *15*). The rate constants of the lowest affinity states of the low-affinity site, $k_{obsL2}$, were determined from the slopes of linear regression of semilogarithmic plots of time points greater than 15 min (Table 2). At those time points, there was virtually no contribution from the more rapidly associating component, $R_{L1}$, to the overall rate of association. The rate constant for the more rapidly associating component, $k_{obsL1}$, was calculated by subtracting the contribution of binding to $R_{L2}$ from total specific binding, at each time point, before semilogarithmic transformation (Table 2; 15). Inclusion of adenine nucleotides in association reactions did not affect $k_{obsL1}$ and $k_{obsL2}$ (data not shown). Ligand-induced conversion of $R_{L1}$ to $R_{L2}$ observed herein for GCC, ΔTD, and ΔCD resembles that obtained previously in studies of rat intestinal receptors and wild-type rat GCC expressed in COS-7 cells (*12*, *15*). Semilogarithmic transformation of data obtained by analysis of ST association with ΔJD, ΔKHD, ΔJD/KHD$_1$, and ΔID yielded isotherms that were best fit by a single line (correlation coefficients > 0.9; Figure 4, insets). The slopes of these isotherms yielded $k_{obs}$ values that were intermediate when compared to $k_{obsL1}$ and $k_{obsL2}$ for wild-type GCC, ΔTD, and ΔCD ($p < 0.05$; Table 2).

*(B) Dissociation.* $^{125}$I-ST dissociated from $R_L$ in a time-dependent fashion in membranes prepared from COS-7 cells expressing wild-type or deletion mutants of GCC (Figure 5). Semilogarithmic transformation of the time courses of dissociation yielded linear isotherms whose slopes represent the dissociation rate constants, $k_{-1}$, of wild-type GCC and deletion mutants (Figure 5, insets; Table 2). Dissociation

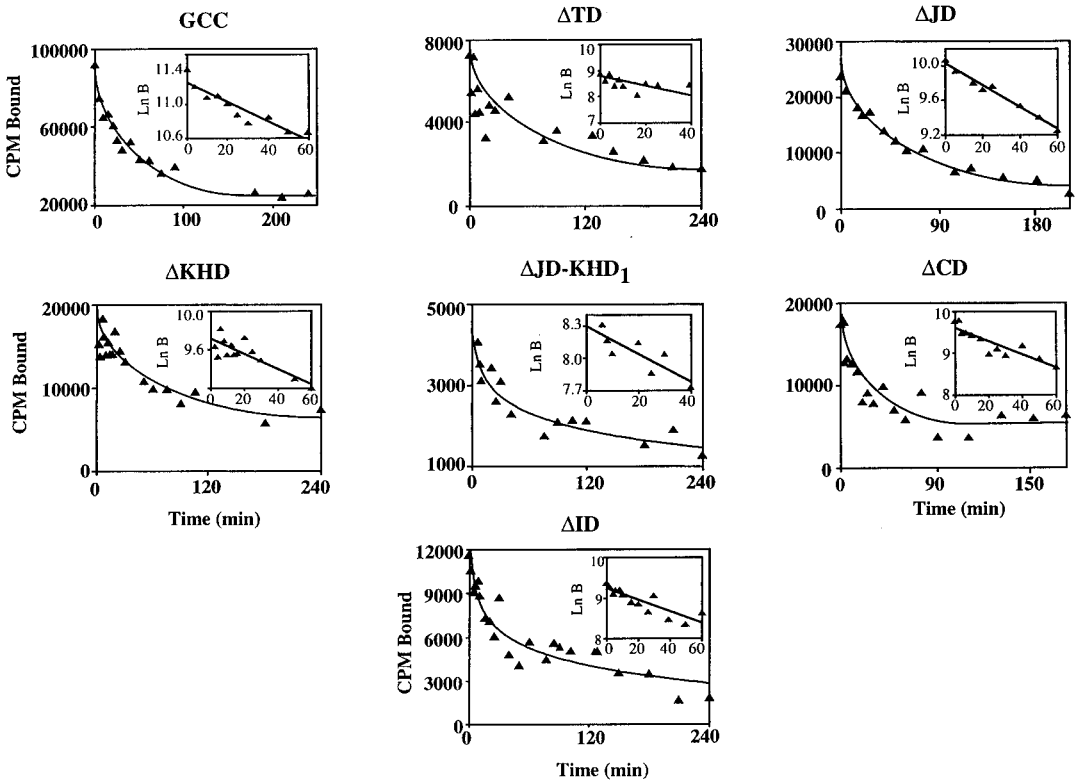


FIGURE 5: Time course of dissociation of labeled ST from wild-type and mutant GCCs expressed in COS-7 cells. Dissociation was initiated by the addition of excess (5 $\mu$M) unlabeled ST to association reactions after equilibrium had been achieved. Aliquots were removed and binding was quantified at the time points indicated as described in Materials and Methods. Insets: Semilogarithmic transformations of $B$ = the amount of ligand bound at the indicated time.

rate constants for wild-type and mutant proteins did not differ significantly (Table 2).

*(C) Second-Order Association Rate Constants of Wild-Type and Mutant GCCs.* Once the observed association ($k_{obs}$) and dissociation ($k_{-1}$) rate constants were defined, the second-order rate constants of association ($k_1$) of labeled ST with the different affinity states of $R_L$ of wild-type and mutant GCCs were estimated. Association rate constants $k_{1L1}$ and $k_{1L2}$ for GCC, ΔTD, and ΔCD were comparable to those reported previously for rat GCC expressed in COS-7 cells and ST binding sites in intestine (*11*, *12*, *15*). However, the rate constants of association for ΔJD, ΔKHD, ΔJD/KHD$_1$, and ΔID were intermediate when compared to $k_{1L1}$ and $k_{1L2}$ of wild-type GCC, ΔTD, and ΔCD ($p < 0.05$; Table 2).

*Ligand Stimulation of Guanylyl Cyclase and Accumulation of Intracellular cGMP in Cells Expressing Mutant GCCs.* Stimulation of GCC activity in broken cell preparations, and cGMP accumulation in intact cells, was examined in COS-7 cells expressing wild-type GCC, which is competent to undergo the affinity shift, and mutant GCCs, which are unable to undergo that shift. ST stimulation of guanylyl cyclase activity in broken cell preparations was measured in the presence of ATP$\gamma$S, the most potent nucleotide favoring receptor-effector coupling (*26*). ST stimulated GCC approximately 5-fold in cell-free preparations (Figure 6A) and increased cGMP accumulation about 15-fold in intact cells (Figure 6B), which compares closely with previous studies of GCC expressed in heterologous systems (*3*, *8*). ΔCD and ΔID, which lack catalytic domains, were not activated by ST (Figure 6A), and cells expressing these mutants did not accumulate cGMP in response to ST (Figure 6B), as expected. Of significance, ΔJD, ΔKHD, and ΔJD/KHD were not activated by ST (Figure 6A).

*Kinase Homology Domain and Regulation of GCC.* Previous studies suggested that deletion of the KHD from GCA, GCB, and GCC resulted in constitutive activation of those enzymes in a ligand- and adenine nucleotide-independent fashion (*5*−*8*). This hypothesis was reexamined employing the deletion mutants described herein, and transfections that exhibited relatively comparable levels of expression were quantified by ligand binding and catalytic activity (Table 3). In these experiments, expression of wild-type GCC and the JD/KHD deletion mutants was closely comparable (no statistically significant difference) by quantification of ST binding and of guanylyl cyclase catalytic activity employing $Mg^{2+}$ as the cation cofactor (basal activity). Catalytic activity employing $Mn^{2+}$ as the cation cofactor, which activates the enzyme in a ligand-independent fashion, was somewhat lower in the JD/KHD$_1$ mutant compared to the wild-type enzyme ($p < 0.05$). However, there were no significant differences between the JD/KHD$_2$ mutant and either the wild-type enzyme or the JD/KHD$_1$ mutant. Thus, in cells expressing relatively comparable levels of wild-type GCC or the JD/KHD deletion mutants, those mutants did not exhibit constitutive elevations of basal cGMP production in the absence of the JD/KHD. These data correlate closely with those demonstrating that deletion of the JD and/or KHD resulted in comparable levels of basal cGMP accumulation in intact cells (Figure 6B).

## DISCUSSION

*Contribution of Cytoplasmic Domains of GCC to Expression of $R_H$ and $R_L$.* High- and low-affinity ligand binding sites appear to be a general characteristic of the receptor guanylyl cyclase family (*15*). High- and low-affinity ANP binding sites have been identified in 293 cells stably

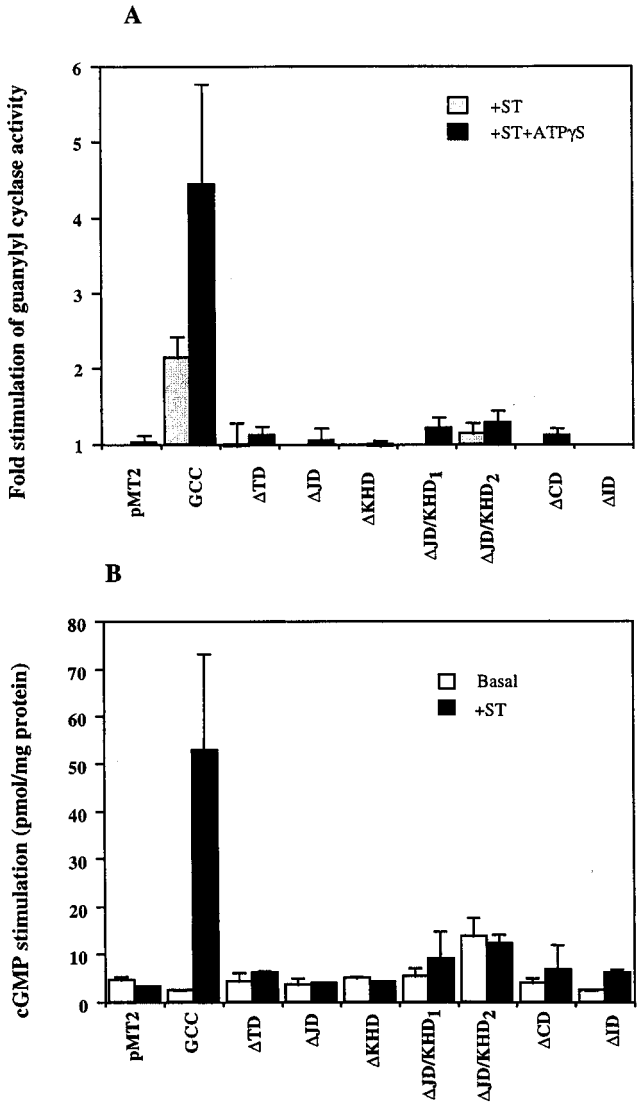


FIGURE 6: Guanylyl cyclase activity of wild-type and mutant GCCs expressed in COS-7 cells. **(**A) Guanylyl cyclase activity of wild-type and mutant GCCs in COS-7 cell homogenates was quantified as described in Materials and Methods. Some assays included ST (1 $\mu$M) and/or ATP$\gamma$S (0.5 mM) where indicated. Results are expressed as *x*-fold stimulation of guanylyl cyclase activity by ST in the absence (open bars) or presence (solid bars) of ATP$\gamma$S. (B) Intracellular accumulation of cGMP in COS-7 cells expressing wild-type and mutant GCCs. COS-7 cells transfected with vector pMT2 were used as control. Cyclic GMP accumulation was determined in the presence or absence of 1 $\mu$M ST, as described in Materials and Methods. Data represent the mean ± SEM of three experiments.

expressing the natriuretic peptide receptor GCA (*7*). Similarly, high- and low-affinity ST binding sites have been identified in rat, mouse, and human intestine *in vivo*, and in T84 human colon carcinoma cells *in vitro* (*11*−*14*). Cells heterologously expressing GCC or GCA exhibit $R_H$ and $R_L$, demonstrating that these sites are the products of a single gene, rather than of multiple genes (*7*, *15*). However, the structural and functional basis by which expression of GCA or GCC results in ligand binding sites with different affinities remains unclear. In native intestinal cells and heterologous expression systems, GCC undergoes significant posttranslational modification, yielding multiple ST binding proteins identified by Western blot analysis and affinity labeling (*1*, *13*, *16*−*20*). In part, this reflects differential proteolysis of intracellular domains resulting in membrane-bound truncated receptors which retain the ability to specifically bind ST (*1*, *13*, *16*−*20*). Thus, expression of $R_H$ and $R_L$ from a single gene may reflect distinct activities of proteolytic products of holoGCC possessing different complements of intracellular domains.

Table 3: Effect of the Kinase Homology Domain Deletion on Basal GCC Catalytic Activity[a]

| | | guanylyl cyclase activity[b] | |
|---|---|---|---|
| construct[c] | ST binding[d] | $Mn^{2+}$ | $Mg^{2+}$ |
| pMT2 | nd[e] | 5.4 ± 0.7 | 3.5 ± 0.2 |
| GCC | 44 ± 12 | 62.6 ± 9.4[f] | 3.0 ± 0.6 |
| ΔJD/$KHD_1$ | 36 ± 10 | 19.0 ± 3.0[f] | 3.3 ± 0.5 |
| ΔJD/$KHD_2$ | 39 ± 10 | 37.8 ± 10.5 | 4.5 ± 0.9 |

[a] Results represent the means of at least three determinations ± SEM of experiments with a single tranfection. [b] Guanylyl cyclase activity, quantified employing either $Mn^{2+}$ or $Mg^{2+}$ as the cation cofactor, is expressed as picomoles of cGMP produced per minute per milligram of protein. [c] COS-7 cells transfected with the indicated constructs were used to measure binding and guanylyl cyclase activities. ΔJD-$KHD_1$ is the homologue of the kinase-deficient mutant of rat GCA reported previously (*5*). ΔJD-$KHD_2$ is the homologue of human GCC reported previously (*8*). [d] ST binding is expressed as femtomoles of $^{125}$I-ST bound per milligram of protein. [e] Not detected. [f] Significantly different ($p < 0.05$).

In the present studies, deletion mutagenesis was employed to model truncation of the cytoplasmic domains of GCC by proteolysis. These studies demonstrated that cytoplasmic domains did not contribute to the expression of $R_H$ and $R_L$, since mutants lacking intracellular domains exhibited equilibrium binding characteristics that were similar to those of wild-type GCC. Rather, these data suggest that the extracellular and transmembrane domains of GCC are sufficient for expression of high- and low-affinity ST binding sites and that $R_H$ and $R_L$ do not reflect proteolysis of cytoplasmic domains of guanylyl cyclases. High- and low-affinity binding sites might reflect differential glycosylation at multiple consensus sites in the extracellular domain of guanylyl cyclases (*16*−*20*). Indeed, differential glycosylation has a profound effect on the binding characteristics of GCA (*18*). Alternatively, GCA and GCC undergo ligand-independent oligomerization, forming dimeric, trimeric and higher-order oligomers (*8*, *17*, *30*, *31*). $R_H$ and $R_L$ might reflect the binding characteristics of different oligomeric states of these proteins (*7*).

*The Ligand-Induced Affinity Shift of GCC $R_L$ Is Regulated by the JD/KHD Region: (A) The Shift in Receptor Affinity Plays a Central Role in Ligand Activation of Receptor Guanylyl Cyclases.* GCC $R_L$ undergoes an ST-induced conversion from $R_{L1}$ to $R_{L2}$, and the concentration-dependent occupancy of $R_{L2}$ correlates closely with that of ST activation of the GCC catalytic domain (*12*, *15*). In addition, membranes have been prepared from rat intestine in which GCC $R_L$ is "locked" in $R_{L1}$, and guanylyl cyclase catalytic activity is insensitive to ST (*15*). These data suggest that the mechanism by which ST activates the catalytic domain of GCC involves ligand binding to $R_{L1}$, the resultant conversion of $R_{L1}$ to $R_{L2}$, and ligand occupancy of $R_{L2}$. Similarly, GCA undergoes an ANP-induced affinity shift from $R_{L1}$ to $R_{L2}$. Of significance, ATP potentiates the ANP-induced conversion of $R_{L1}$ to $R_{L2}$, and the activation of the catalytic domain by ANP (*7*, *32*). Amiloride, an antagonist of ATP binding sites, "locks" $R_L$ in $R_{L1}$ and inhibits ANP activation of the catalytic domain (*7*, *32*). Thus, ligand-induced conversion of $R_{L1}$ to $R_{L2}$ is important for activation of the catalytic

domain of GCA by ANP, and this mechanism appears to be regulated by adenine nucleotides (*7*, *32*). Taken together, these data suggest that the ligand-induced shift of $R_{L1}$ to $R_{L2}$ is a central mechanism underlying receptor-effector coupling for receptor guanylyl cyclases (*12*, *15*).

*(B) Regulation of the Ligand-Induced Affinity Shift of GCC.* The present studies examined the contribution of intracellular domains of GCC to the ST-induced shift from higher to lower affinity. Wild-type GCC exhibited a ligand-induced shift from $R_{L1}$ to $R_{L2}$ and a concomitant stimulation of catalytic activity. ΔCD and ΔTD also exhibited an ST-induced shift in affinity, but in the absence of catalytic activation, since ΔCD lacked a catalytic domain while that domain in ΔTD was inactive (Figure 6). That ΔTD and ΔCD exhibit the affinity shift suggests that merely deleting a cytoplasmic domain of GCC does not alter the kinetics of ligand binding of the extracellular domain in a nonspecific fashion. Interestingly, ΔJD, ΔKHD, $\Delta JD/KHD_1$, and ΔID appeared to be "locked" in an intermediate affinity state, insensitive to ligand activation (see Figure 4). These data suggest that the JD and KHD play a role in mediating the transition of $R_{L1}$ to $R_{L2}$ induced by ST. Deletion of the JD and KHD does not result in misfolding and inactivation of the protein since these mutants express high- and low-affinity binding sites for ST and $Mn^{2+}$-dependent catalytic activity (Table 3). These studies are the first direct demonstration of a role for the JD and KHD in mediating the ligand-induced shift in affinity of receptor guanylyl cyclases. They are in close agreement with studies suggesting a role for the JD/KHD region in the regulation of the ANP-induced shift in affinity of GCA (*7*).

*Role of the JD/KHD Region in Regulating GCC.* Deletion of the JD/KHD region from human GCA, GCB and GCC yielded constitutively activated enzymes insensitive to ligand or adenine nucleotides (*6*, *8*). In contrast, studies described herein demonstrate that deleting homologous portions of rat GCC produced an enzyme that was constitutively suppressed and insensitive to ST and adenine nucleotides (see Figure 6 and Table 3). In earlier studies with GCA and GCB, levels of expression of mutant and wild-type proteins were quantified by $Mn^{2+}$-dependent guanylyl cyclase activities, while constitutive activation of catalysis was quantified as intracellular accumulation of cGMP (*6*). Thus, in those studies, increased accumulation of cGMP in cells expressing JD/KHD deletion mutants could be ascribed to constitutive activation and not overexpression of those mutants (*6*). However, in studies of human GCC, neither enzyme activity nor receptor binding was quantified (*8*). Rather, only cGMP levels in intact cells expressing wild-type and mutant GCCs were measured. Thus, it remains unclear whether increased concentrations of cGMP in cells expressing JD/KHD deletion mutants of GCC reflected constitutive activation or overexpression of protein (*8*). Indeed, deletion of the JD/KHD region from GCA results in a 10−20-fold increase in expression of the mutant compared to wild-type protein (*5*). By contrast, the present studies, conducted with a rat GCC mutant ($\Delta JD/KHD_2$) that is the precise homologue of the human mutant described previously, suggest that deletion of the JD/KHD region does not result in constitutive activation, as quantified by enzyme activity, ligand binding, and cGMP accumulation in intact cells (*8*). Indeed, these data suggest that the integrity of the cytoplasmic domain may be required for receptor−effector coupling of GCC.

*Receptor Guanylyl Cyclase Regulation.* The family of receptor guanylyl cyclases have been subdivided on the basis of homology of structure and function (*33*). GCA and GCB share substantial structural homology and exhibit overlapping ligand binding specificities (*33*). GCC shares less than 10% identity with these natriuretic peptide receptors in the extracellular ligand binding domain and exhibits unique ligand specificities that do not overlap with GCA or GCB (*3*). The present studies suggest that these different subtypes of the receptor guanylyl cyclase family may be regulated by overlapping, but not identical, molecular mechanisms. In both, ligand association with the extracellular domain induces a shift from higher to lower affinity that is mediated by the cytoplasmic JD/KHD region and is required for catalytic activation and second messenger production. However, the regulatory role of adenine nucleotides and the JD/KHD region appear to be dissimilar between these two subfamilies. Thus, the JD/KHD region of GCA and GCB appears to serve as a repressor, maintaining catalytic activity in the basal state, since deletion of this domain results in constitutive activation (*6*). Ligand binding and the shift in receptor affinity is associated with derepression of the catalytic domain by the JD/KHD region and production of cGMP (*7*). The affinity shift and derepression of activity are dependent on the association of adenine nucleotides with the JD/KHD region (*6*, *7*, *33*). By contrast, the JD/KHD region of GCC does not appear to be autoinhibitory, since deletion of this region results not in constitutive activation but, rather, in constitutive inhibition of catalytic activity. Indeed, these data suggest that the integrity of the cytoplasmic domain of GCC is required for receptor effector−coupling and activation of the catalytic domain, in contrast to GCA and GCB. Taken together, these observations suggest the working hypothesis that ST binding and the shift in receptor affinity appear to be associated with activation, rather than derepression, of the catalytic domain and cGMP production.

The data presented herein also suggest that the JD/KHD region of receptor guanylyl cyclases subserves separate and distinct functions, coupling ligand binding to catalytic activation and mediating regulation of that process by adenine nucleotides. Both functions are intact in the natriuretic peptide receptors, but only the coupling function appears intact in GCC. In addition, these data support the suggestion that adenine nucleotides regulate different receptor guanylyl cyclases in an overlapping, but not identical, fashion. These nucleotides are required positive effectors of GCA activation, and their effects are mediated through the JD/KHD region (*5*−*7*). In contrast, adenine nucleotides potentiate ST stimulation of GCC by preventing desensitization of catalytic activity (*20*, *30*). The precise mechanisms by which the JD/KHD region regulates guanylyl cyclase receptor affinity and catalytic activity and the role of adenine nucleotides in those processes are currently being examined in this laboratory.

*Role of the Carboxy-Terminal Tail Domain in Regulation of Catalytic Function.* The present studies demonstrated that deletion of the carboxy-terminal tail from rat GCC yielded a mutant that was catalytically inactive (see Figure 6), although sequence and Western analyses indicated that the protein was intact when expressed in COS-7 cells (see Figure 2). According to previous suggestions, the concentration of polar amino acids in this domain may contribute to an association of GCC with the cytoskeleton (*3*, *9*). Studies presented herein suggest that the carboxy-terminal tail

domain may also contribute to the regulation of catalytic activity of GCC. This regulation appears to be ligand-independent, as the $Mn^{2+}$-stimulated activity associated with ΔTD was negligible. Alternatively, these data may suggest that deletion of the tail domain results in misfolding of the mutant and loss of catalytic activity. These observations are particularly interesting since this domain is absent in some members of the receptor guanylyl cyclase family, such as GCA and GCB, whereas in other members possessing this domain, such as GCD, GCE, and GCF, it is not highly conserved (*3*, *34*, *35*). The precise contribution of the tail domain to the structure and catalytic activity of GCC is currently being examined.

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