THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 40, pp. 27135–27145, October 2, 2009

The Linker Region in Receptor Guanylyl Cyclases Is a Key Regulatory Module

*MUTATIONAL ANALYSIS OF GUANYLYL CYCLASE C**[S]

Received for publication, May 12, 2009, and in revised form, July 1, 2009 Published, JBC Papers in Press, July 31, 2009, DOI 10.1074/jbc.M109.020032

Sayanti Saha, Kabir Hassan Biswas[1], Chandana Kondapalli, Nishitha Isloor[2], and Sandhya S. Visweswariah[3]

From the Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India

Receptor guanylyl cyclases are multidomain proteins, and ligand binding to the extracellular domain increases the levels of intracellular cGMP. The intracellular domain of these receptors is composed of a kinase homology domain (KHD), a linker of ~70 amino acids, followed by the C-terminal guanylyl cyclase domain. Mechanisms by which these receptors are allosterically regulated by ligand binding to the extracellular domain and ATP binding to the KHD are not completely understood. Here we examine the role of the linker region in receptor guanylyl cyclases by a series of point mutations in receptor guanylyl cyclase C. The linker region is predicted to adopt a coiled coil structure and aid in dimerization, but we find that the effects of mutations neither follow a pattern predicted for a coiled coil peptide nor abrogate dimerization. Importantly, this region is critical for repressing the guanylyl cyclase activity of the receptor in the absence of ligand and permitting ligand-mediated activation of the cyclase domain. Mutant receptors with high basal guanylyl cyclase activity show no further activation in the presence of non-ionic detergents, suggesting that hydrophobic interactions in the basal and inactive conformation of the guanylyl cyclase domain are disrupted by mutation. Equivalent mutations in the linker region of guanylyl cyclase A also elevated the basal activity and abolished ligand- and detergent-mediated activation. We, therefore, have defined a key regulatory role for the linker region of receptor guanylyl cyclases which serves as a transducer of information from the extracellular domain via the KHD to the catalytic domain.

In transmembrane receptors a series of conformational changes are required to transmit the information of ligand binding (an extracellular signal) to the interior of the cell, resulting in either altered interaction with signaling intermediates or in the regulation of a catalytic activity present in the receptor. In these multidomain receptors, where the ligand binding and effector domains are present in the same polypeptide chain, the relay of conformational changes is under the exquisite control of post-translational modifications or precise structural alterations.

Receptor guanylyl cyclases (GCs)[4] have an N-terminal extracellular ligand binding domain, a single transmembrane domain, and a C-terminal intracellular domain (1). Binding of ligands to the extracellular domain elicits a conformational change that increases the guanylyl cyclase activity of the receptor, resulting in increased cGMP production. The intracellular domain of receptor GCs contains a region that shares considerable sequence similarity to protein kinases and is referred to as the kinase homology domain (KHD). Binding of ATP to the KHD induces a conformational change that regulates cGMP production by the guanylyl cyclase domain (2). Thus, receptor GCs exemplify the intricate interactions between domains in transducing the signal from an extracellular ligand to the interior of the cell.

The amino acid sequences of the extracellular domain of mammalian receptor GCs vary (less than ~15% similarity), as would be expected given the diversity in the ligands that bind to and activate these receptors. The KHD shows ~25–30% conservation in amino acid sequence across receptor GCs, and computational modeling has not only suggested that this region could adopt the overall structure of a protein kinase but also identified specific residues that could interact with ATP (2, 3). The catalytic domains of mammalian receptor GCs are more conserved (~80% sequence similarity). The gradual increase in sequence similarity across the various domains, with the extracellular domain being the most diverse and the cyclase domains sharing the maximum sequence similarity, is a reflection of the ability of these receptor GCs to converge diverse extracellular signals to a unified output of cGMP production. The guanylyl cyclase domains of receptor GCs can be classified as members of the Class III family of nucleotide cyclases (4). The recent crystal structures of a bacterial guanylyl cyclase (5) and a eukaryotic soluble guanylyl cyclase (6) show similarities in the overall three-dimensional structure of adenylyl and guanylyl cyclases and also highlight the critical residues that determine substrate utilization (either ATP or GTP) in these enzymes.

Guanylyl cyclase C (GC-C) serves as the receptor for the guanylin family of endogenous peptides as well as for the exoge-

* This work was supported by grants from the Departments of Science and Technology and Biotechnology, Government of India.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Figs. 1–3.

[1] Supported by a senior research fellowship from the Council of Scientific and Industrial Research, Government of India.

[2] Supported by a post-doctoral fellowship from the Department of Biotechnology.

[3] To whom correspondence should be addressed. Tel.: 91-80-22932542; Fax: 91-80-23600999; E-mail: sandhya@mrdg.iisc.ernet.in.

[4] The abbreviations used are: GC, guanylyl cyclase; GC-A, guanylyl cyclase A; GC-C, guanylyl cyclase C (heat-stable enterotoxin receptor); GCAP-1, GC-activating protein 1; GST, glutathione *S*-transferase; ANP, atrial natriuretic peptide; KHD, kinase homology domain; RetGC-1, retinal guanylyl cyclase; ST, heat-stable enterotoxin; HAMP, histidine kinases, adenylyl cyclases, methyl accepting chemotactic receptors, and phosphatases.

nous heat-stable enterotoxin (ST) peptides secreted by enterotoxigenic bacteria (7, 8). GC-C is predominantly expressed on the apical surface of epithelial cells in the intestine, although robust extra-intestinal expression is observed in the kidney and reproductive tissues of the rat (9–12). The extracellular domain of GC-C is glycosylated, and we have shown the importance of glycosylation in regulating receptor desensitization in colonic cells. We have also identified a critical residue (Lys-516) in the KHD of GC-C as being important for KHD-mediated modulation of the guanylyl cyclase activity (2, 3).

A sequence of ~70 amino acids is found between the KHD and the guanylyl cyclase domain of receptor GCs, which we refer to here as the linker region (13). This region is predicted to form an amphipathic α-helix and could also adopt a coiled coil conformation (14, 15). The linker region is also present in soluble (cytosolic) guanylyl cyclases where it connects the N-terminal heme binding regulatory domain to the C-terminal catalytic cyclase domain. The linker region is suggested to act as a dimerization module in receptor GCs (16–18) and has also been implicated in heterodimerization of the α and β subunits of soluble guanylyl cyclases (19, 20). However, there are several reports to the contrary that indicate that the linker does not affect the dimerization of receptor GCs (14, 15). Nevertheless, the critical importance of the linker in regulating the activity of receptor GCs is shown by the fact that mutations in this region of the retinal guanylyl cyclase (RetGC-1) are associated with autosomal dominant cone-rod dystrophy in humans (16, 21). We show here through extensive mutational and biochemical analysis that the linker regions in two receptor GCs, GC-C and guanylyl cyclase A (GC-A), play an important role in repressing the catalytic activity of the receptors in the absence of their ligands. In addition, our results provide for the first time a molecular explanation for detergent-enhanced guanylyl cyclase activity in this family of receptors and suggest a mechanism for this activation that could involve a hydrophobic interaction between the linker region and the guanylyl cyclase domain.

MATERIALS AND METHODS

Generation of Mutations in the Linker Region of GC-C and GC-A—Human GC-C cDNA (pBSK-GC-C) (22) was used as template for the generation of mutations in the linker region of GC-C using the single mutagenic oligonucleotide-based protocol described earlier (23). The mutagenic primers used are given in supplemental Table 1. pBSK-GC-$C_{L764K/L768K}$ double mutant was obtained by performing mutagenesis with the L768K primer using pBSK-GC-C_{L764K} mutant DNA as the template. After mutagenesis, a fragment encoding GC-C was excised from the respective mutant pBSK-GC-C by digestion with XhoI (or SalI in cases where the mutagenic primer introduced an XhoI site) and XbaI and ligated into XhoI-XbaI-digested pcDNA3 to allow expression in mammalian cells.

A HindIII fragment from pCMV-GC-A (24) representing the KHD and a part of the linker region was cloned into the HindIII site of pGEM-11Zf(+) (Promega Life Science) to generate the plasmid pGEM-11Zf-GC-A_{KHD-LR}. This was subsequently used as template for the generation of L812P, M816P, and Y819P mutations in the linker region of GC-A using the respective mutagenic primer as detailed in supplemental Table 1. The mutant plasmids, pGEM-11Zf-GC-$A_{KHD-LR-L812P}$, pGEM-11Zf-GC-$A_{KHD-LR-M816P}$, and pGEM-11Zf-GC-$A_{KHD-LR-Y819P}$, were digested with HindIII to excise the mutated GC-A fragment and replaced into HindIII-digested pCMV-GC-A to generate pCMV-GC-A_{L812P}, pCMV-GC-A_{M816P}, and pCMV-GC-A_{Y819P}, respectively. The presence of the desired mutation and the absence of any missense mutations were confirmed by sequencing (Macrogen).

Generation of pcDNA3-GCC-GST—A 786-bp PCR product was amplified using pcDNA3-GC-C as the template using forward primer, GC-C2496f, and reverse primer GC-C3291r HindIII, (supplemental Table 1). The reverse primer introduced a leucine in place of the stop codon. The PCR product was digested with SphI and HindIII, and the larger 559-bp fragment consisting of the C-terminal domain of GC-C with mutation at the stop codon was used to replace a similar fragment in pBSK-GC-C (22) to generate the plasmid pBSK-GC-$C_{\Delta stop}$. Full-length mutant GC-C was digested with XhoI and HindIII and cloned into pEGFP-N2 (Invitrogen) to generate the plasmid pEGFP-N2-GC-C.

The GST gene was amplified by PCR from pGEX-5X-1 plasmid with suitable primers (supplemental Table 1), and the PCR product (758 bp) was digested with HindIII-NotI. A HindIII-NotI digestion of pEGFP-N2-GC-C resulted in the release of the green fluorescent protein cDNA, which was subsequently replaced by the similarly digested PCR product of GST to generate the plasmid pGST-N2-GC-C.

pGST-N2-GC-C was digested with SphI-XbaI and the 1300-bp fragment (representing a part of GC-C along with the C-terminal GST tag) was used to replace a similarly digested fragment in pBSK-GC-C to generate pBSK-GCC-GST. The clone pBSK-GCC-GST was digested with XhoI-XbaI-PvuI, which resulted in the excision of GCC-GST as a XhoI-XbaI fragment and was subsequently cloned into a similarly digested pcDNA3 vector. This resulted in the generation of pcDNA3-GCC-GST, which would express GC-C with a GST tag at its C terminus in mammalian cells.

Measurement of Ligand-stimulated cGMP Production in HEK293T Cells—HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as described earlier (3). Wild type and mutant GC-C or GC-A were expressed in HEK293T cells by transient transfections using polyethyleneimine lipid (Polysciences, Inc.). Ligand-stimulated guanylyl cyclase activity was measured 72 h post-transfection in monolayer culture of cells in 24-well plates. The cell monolayer was incubated with serum and antibiotic containing Dulbecco's modified Eagle's medium containing 500 μM 3-isobutyl-1-methylxanthine for 30 min at 37 °C in a 5% humidified CO_2 incubator. ST (10^{-7} M, to GC-C expressing cells) or ANP (10^{-6} M to GC-A expressing cells) was then added, and incubation was continued for another 30 min (in the case of ST stimulation of GC-C) or 60 min (in the case of ANP stimulation of GC-A) after which the cells were lysed in 200 μl of 0.1 N HCl. Cyclic GMP produced was measured by radioimmunoassay as described earlier (25).

Preparation of Membrane Fraction from HEK293T Cells—Confluent cell monolayers were washed with chilled phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.2,

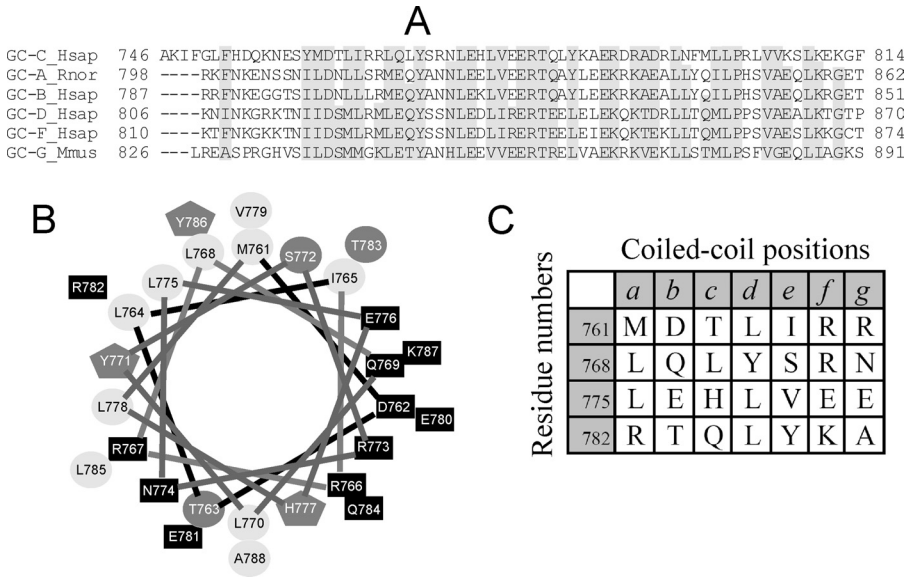

FIGURE 1. **Sequence analysis of the linker region in receptor GCs.** *A*, multiple sequence alignment of the linker region of receptor GCs generated by MUSCLE (51) and depicted using ClustalX 2.0. *Hsap*, *Homo sapiens*; *Rnor*, *Rattus norvegicus*; *Mmus*, *Mus musculus*. *Shaded boxes* indicate residues that are similar in all the sequences. The human *GC-E* gene is a pseudogene (52). *B*, helical wheel representation of the linker residues (Met-761—Ala-788) of GC-C showing the amphipathic nature of the helix with a predominance of hydrophobic residues on one face of the helix and a predominance of hydrophilic residues on the other. Hydrophobic residues are shown in *light gray*, whereas predominantly hydrophilic residues are *shaded in black*. *C*, residue positions in the predicted heptad repeats in the coiled coil structure adopted by the linker region based on the COILS program (32).

and 0.9% sodium chloride) and scraped into homogenization buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). The cell lysate was sonicated and centrifuged at 12,000 $\times$ *g* for 60 min at 4 °C. The pellet obtained was resuspended in a buffer containing 50 mM HEPES, pH 7.5, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, and 20% glycerol. The protein concentration was estimated by using a modification of the Bradford protein assay (26).

Western Blot Analysis—Cells after transfection were directly lysed in SDS sample buffer and subjected to Western blot analysis using the GCC:C8 monoclonal antibody to GC-C essentially as described earlier (2). In cells transfected with GC-A encoding plasmids, a polyclonal antibody to GC-A (a kind gift of Dr. Lincoln R. Potter, University of Minnesota) was used. In some cases membranes prepared from transfected cells were used for Western blot analysis with the respective antibodies. Blots were developed by enhanced chemiluminescence.

Receptor Binding Assays—An analogue of ST, ST_{Y72F}, was iodinated using $Na^{125}I$ as described earlier (25). Membrane protein (50 μg) was incubated in binding buffer (50 mM HEPES, pH 7.5, 4 mM $MgCl_2$, 0.1% bovine serum albumin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) with ~100,000 cpm of ^{125}I-labeled ST_{Y72F} for 1 h at 37 °C in the presence (nonspecific binding) or absence of 10^{-7} M concentrations of unlabeled ST in a total volume of 100 μl. For Scatchard analysis, membrane protein was incubated with varying concentrations (10^{-11}–10^{-9} M) of ^{125}I-labeled ST_{Y72F} for 1 h at 37 °C. After incubation, samples were filtered through GF/C filters (Whatman) and washed with 5 ml of chilled 10 mM sodium phosphate buffer, pH 7.2, containing 0.9% NaCl and 0.2% bovine serum albumin through a filtration unit (Millipore). The filters were dried, and the associated radioactivity was measured. Saturation binding and Scatchard analyses were carried out using Graph Pad Prism5 software.

In Vitro Guanylyl Cyclase Assays—Membrane prepared from mammalian cells was incubated in assay buffer (60 mM Tris-HCl, pH 7.5, containing 500 μM 3-isobutyl-1-methylxanthine, and an NTP-regenerating system consisting of 7.5 mM creatine phosphate and 10 μg of creatine phosphokinase) in the presence of 1 mM MgGTP or MnGTP with free metal concentrations maintained at 10 mM. Detergent-mediated activation was determined with membrane protein (2 μg) incubated in assay buffer in the absence or presence of 0.1% Lubrol-PX. To study the effect of ATP, assays were performed in the absence or presence of 1 mM MgATP along with 0.1% Lubrol-PX, 1 mM MgGTP, and 10 mM free $MgCl_2$. The concentrations of free magnesium and magnesium-GTP/ATP complexes present in assays were calculated using Maxchelator (Stanford University). All assays were incubated at 37 °C for 10 min for the production of cGMP. The assay was terminated by the addition of 400 μl of 50 mM sodium acetate buffer, pH 4.75, and boiling of the samples. After centrifugation, the supernatant was taken for cGMP radioimmunoassay as described earlier (25).

Modeling of the Cyclase Domain—The recently solved crystal structures of Cyg12, a soluble cyclase from *Chlamydomonas reinhardtii* (a eukaryote; PDB code 3ET6) (5) and Cya2 from *Synechocystis PCC6803* (a prokaryote; PDB code 2W01) (6) were used as templates to generate the models of the inactive and active states, respectively, of the cyclase domain of GC-C. The Cyg12 cyclase domain shows 60% similarity (43% identity), whereas the Cya2 cyclase domain shows 46% similarity (25% identity) to the cyclase domain of GC-C. Multiple cyclase domain sequences were used in generating the alignment of the templates and target sequences using ProbCons (27) and checked manually. Models were generated using MODELLER (9v6 release) (28). The stereochemical quality of the models was assessed with PROCHECK (29). Structures were visualized and analyzed using PyMol.

RESULTS

Predicted Structure of the Linker Region—The linker region of about 70 residues is conserved in all members of the receptor GC family (Fig. 1*A*) (13). Secondary structure prediction of this stretch of residues in GC-C using JPRED (30) and PSI-PRED

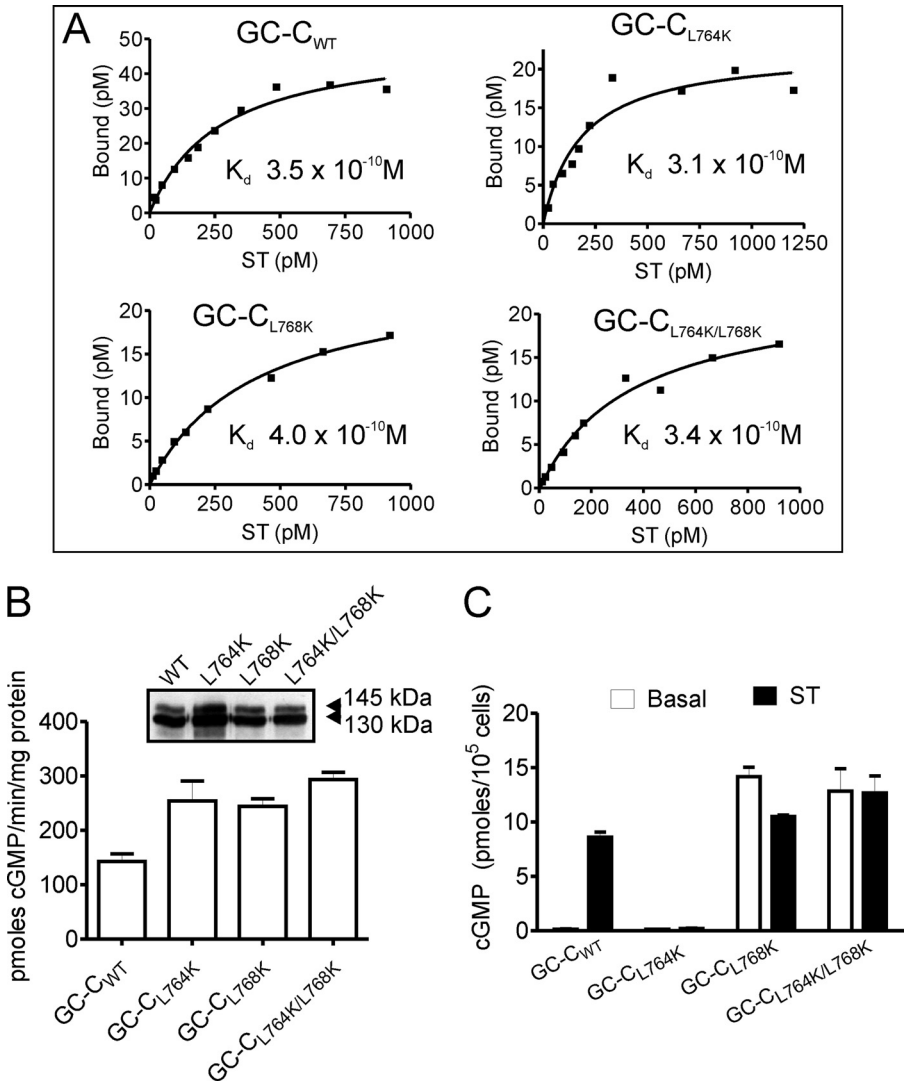

FIGURE 2. **Characterization of GC-C$_{L764K}$, GC-C$_{L768K}$, and GC-C$_{L764K/L768K}$ mutants.** *A*, membranes prepared from HEK293T cells expressing the wild type or mutant receptor were incubated with varying concentrations of ^{125}I-labeled ST$_{Y72F}$ for 1 h at 37 °C. After incubation samples were filtered, and bound radioligand was monitored. Data were analyzed using GraphPad Prism, and values shown are representative of assays repeated at least twice. *B*, membrane protein prepared from HEK293T cells expressing the wild type (*WT*) or mutant GC-C receptors were taken for *in vitro* guanylyl cyclase assays using MnGTP as the substrate. The assay was performed twice, and values represent the mean ± S.E. of duplicate determinations. *C*, HEK293T cells expressing GC-C$_{WT}$, GC-C$_{L764K}$, GC-C$_{L768K}$, or GC-C$_{L764K/L768K}$ were treated with ST (10^{-7} M) for 30 min. Cells were lysed, and intracellular cGMP levels were determined. Data shown are the mean ± S.E. of duplicate measurements of a representative experiment, with $n = 3$. The *inset* shows a Western blot of whole cell lysates prepared from HEK293T cells transfected with GC-C$_{WT}$, GC-C$_{L764K}$, GC-C$_{L768K}$, or GC-C$_{L764K/L768K}$ using GCC:C8 monoclonal antibody.

(31) suggested the presence of an amphipathic α-helix between residues 760 and 800 (Fig. 1*B*). The COILS algorithm (32) was used to compute the probability of coiled coil formation in this region, and amino acid residues between positions Met-761 to Ala-788 were predicted to form a coiled coil structure (Fig. 1*C*) with four heptad repeats identified in this region.

That this region of the receptor could adopt a helical structure was shown by using a synthetic peptide of residues 761–788, which was able to form a helix as measured by circular dichroism in the presence of 10% trifluoroethanol. The ratio of mean residual ellipticity at 222 and 208 nm was found to increase with increasing peptide concentrations, indicating that this helix had the potential to oligomerize (supplemental Fig. 1 and Table 2). We, therefore, were justified in suggesting that this region of GC-C could indeed form a coiled coil structure under favorable conditions.

The *a* and *d* positions in a coiled coil heptad repeat are known to form the interhelical hydrophobic core (the most common residues in these positions are Leu, Ile, and Val), and this hydrophobic interaction is known to be the dominant determinant of coiled coil stability. Another factor for interhelical stability in coiled coils is the ionic interaction between residues at positions *e* and *g*, which are usually occupied by Lys and Glu (33). Inspection of the predicted heptad repeats of the GC-C coiled coil region shows that the *e* and *g* positions are not always charged. Therefore, the *a* and *d* positions are likely to be important in providing the interaction energy in the coiled coil region of GC-C.

Mutational Analysis of a and d Positions in the Putative Coiled Coil Region of GC-C—The leucine residue at position 764, which occupies the hydrophobic *d* position in heptad I in GC-C, is conserved in GC-A and GC-B and is conservatively substituted to a methionine residue in GC-D, GC-E, GC-F, and GC-G (Fig. 1*A*). Leu-768 also shows a high degree of conservation and occupies the hydrophobic *a* position in heptad II of the predicted coiled coil region of these receptors (Fig. 1*A*). We, therefore, mutated leucines at positions 764 and 768 in GC-C to lysine to disrupt the hydrophobic interactions in the coiled coil interface while retaining the helical nature of this region.

GC-C$_{L764K}$, GC-C$_{L768K}$, and the double mutant (GC-C$_{L764K/L768K}$) were expressed in HEK293T cells, and membranes from untransfected cells and cells expressing wild type or mutant GC-C were tested for their ability to bind the ST peptide. Membranes prepared from untransfected cells showed no ST binding, indicating that HEK293T cells do not express GC-C (data not shown). The mutant receptors were able to bind ST peptide with affinities comparable with that of the wild type receptor (Fig. 2*A*). Western blot analysis of the corresponding membranes using a GC-C specific monoclonal antibody showed the presence of the differentially glycosylated forms of GC-C of sizes 130 and 145 kDa (Fig. 2*B*, *inset*). This suggested that the mutations in the linker region did not affect

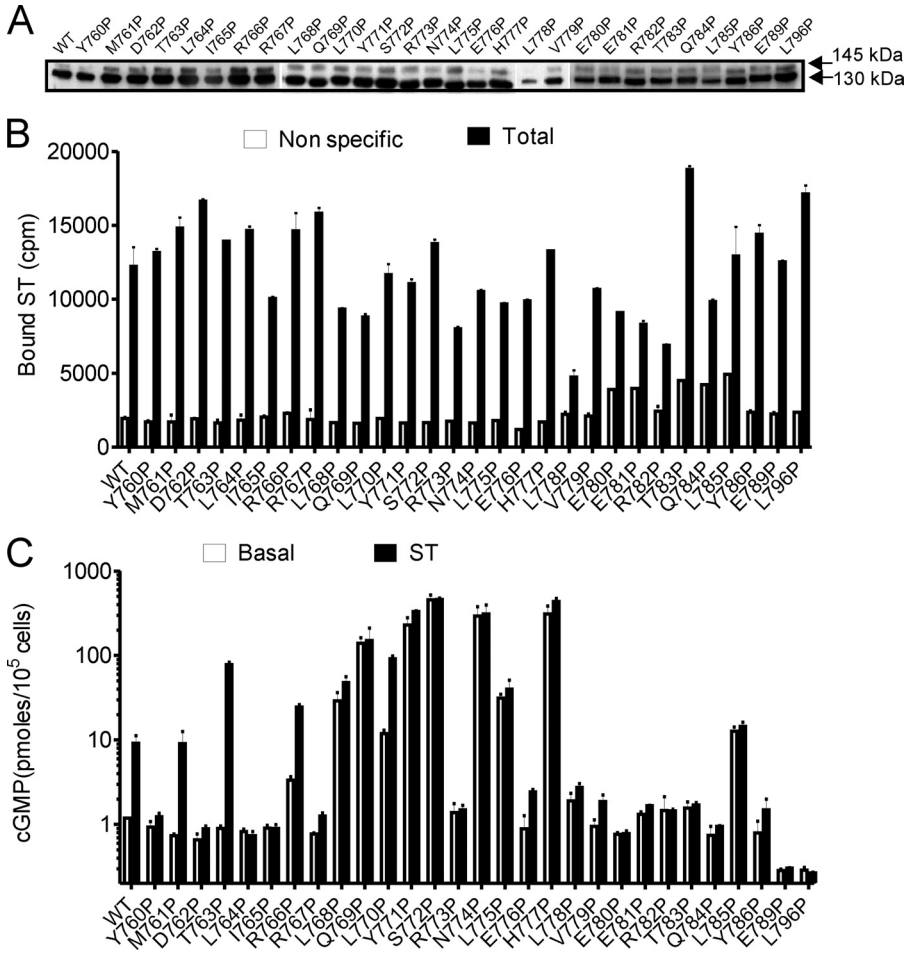

FIGURE 3. **Proline-scanning mutagenesis in the linker region of GC-C.** *A*, membrane protein prepared from HEK293T cells expressing mutant GC-C receptors was subjected to Western blot analysis using GCC:C8 monoclonal antibody. *WT*, wild type. *B*, HEK293T membrane protein expressing wild type or mutant GC-C receptors was incubated with ^{125}I-labeled ST_{Y72F} in the presence or absence of unlabeled ST (10^{-7} M). After incubation, samples were filtered, and bound radioligand was monitored. *C*, HEK293T cell monolayers expressing either wild type or mutant GC-C receptors were either untreated (basal cGMP) or treated with ST peptide, and intracellular cGMP was measured. Assays were performed on three independent sets of transfections, and values correspond to the mean ± S.E. of duplicate determinations carried out in a single representative experiment. Only the wild type, M761P, L763P, R766P, and L770P mutant receptors showed significant ST-stimulated cGMP accumulation ($p < 0.01$) across multiple experiments.

the trafficking of the mutant receptors to the cell surface, as we have shown earlier that the 145-kDa form of GC-C is found only on the plasma membrane in HEK293 cells (34). Guanylyl cyclase assays using MnGTP as substrate with membranes expressing either the wild type or mutant receptors showed efficient cGMP production (Fig. 2*B*), thus confirming the functional integrity of the guanylyl cyclase domain of the mutant receptors. No immunoreactive bands corresponding to GC-C could be detected in membranes prepared from untransfected cells, and guanylyl cyclase activity present in HEK293T membranes was <5% of the activity detected in cells transfected with GC-C.

When HEK293T monolayers expressing GC-C_{L764K} were treated with ST peptide, no increase in cGMP level was seen (Fig. 2*C*), indicating that Leu-764 is critically required for transmission of the ligand binding signal to activation of the catalytic cyclase domain. Most interestingly, HEK293T monolayers expressing GC-C_{L768K} or GC-$C_{L764K/L768K}$ mutant receptors showed elevated levels of cGMP accumulation in the basal state that could not be stimulated further with the addition of ST peptide (Fig. 2*C*). The fact that the L768K mutation overrides the properties of the L764K mutation in the double mutant suggests that the Leu-768 residue plays an important role in retaining the receptor in an inactive conformation in the basal state.

Proline-scanning Mutagenesis of the Linker Region—Given the dramatic effects of mutations of Leu-764 and Leu-768 residues, it became imperative to test the importance of other residues in this region on GC-C function. Proline residues are known to disrupt the regular helical structure by the introduction of a kink and a change in the "face" of the helix relative to other secondary structural elements in the protein. We, therefore, generated proline mutants from residue Tyr-760 (one residue upstream of the predicted coiled coil) to Tyr-786 as well as E789P and L796P mutants. Mutant proteins were expressed in mammalian cells, and membrane fractions prepared from the cells showed that all mutant proteins were present as 145- and 130-kDa forms, indicating proper folding and export to the membrane (Fig. 3*A*). *In vitro* ligand binding assays were performed, and as shown in Fig. 3*B*, all mutants bound the ST peptide, and binding correlated to the expression of the mutant receptors as judged by Western blot analysis. Furthermore, *in vitro* guanylyl cyclase assays using MnGTP as the substrate demonstrated that all mutant receptors retained catalytic activity (data not shown).

Transfected HEK293T cells were treated with ST, and intracellular cGMP levels were measured (Fig. 3*C*). Untransfected cells showed no cGMP production in response to ST addition. Interestingly, some of the mutants showed intracellular cGMP accumulation even in the absence of ST (which is a reflection of ligand-independent guanylyl cyclase activity). Q769P, Y771P, S772P, N774P, and H777P mutant receptors showed highly elevated intracellular cGMP levels in the absence of ligand. L768P, L770P, L775P, and L785P mutant receptors showed moderately high levels of cGMP in the basal state, whereas the remaining mutants showed basal levels of intracellular cGMP. Most remarkably, the constitutive activation of the guanylyl cyclase domain on introduction of single point mutations in the region from Tyr-771 to His-777 follows a periodic repeat pattern wherein mutations at every third residue in the stretch (Arg-773 and Glu-E776) failed to give a constitutively active receptor.

Of the mutants with low basal activity, M761P, T763P, and R766P showed ligand-stimulated cGMP production in response to ST treatment (Fig. 3*C*). None of the constitutively active mutant receptors except the L770P mutant showed any further increase in cGMP levels upon ST treatment. The majority of the mutants in the linker region were very poorly responsive or unable to respond to ST treatment, suggesting the importance of this region in generating the correct conformation for ligand-induced activation of GC-C.

Effects on ligand binding, ligand-stimulable activity, and *in vitro* guanylyl cyclase activity were similar with the introduction of either proline or lysine residues at 764 or 768 positions in the linker. This observation suggested that the effects seen were not due to a gross structural distortion introduced by the proline residues but because of the disruption of certain critical interactions. Conservative substitutions of leucine residues at positions 768 and 775 to isoleucine were generated, and Tyr-771 was conservatively substituted with a phenylalanine residue. These mutations restored the wild type phenotype in GC-C and showed ligand-stimulated cGMP production and low basal activity (supplemental Fig. 2). In contrast, the L764I mutant remained unresponsive to ST stimulation, indicating that either the hydrophobic nature of the residue at 764 position alone was not the critical determinant of receptor functioning or a highly specific interaction of leucine residue with the other helix of the coiled coil dimer is lost even in the L764I mutant (supplemental Fig. 2).

Role of the Coiled Coil Region in Oligomerization of GC-C—Nucleotide cyclases must dimerize in a head-to-tail fashion to be able to produce cGMP from GTP (35, 36). We were interested in determining if the mutant receptors could heterodimerize with the wild type receptor to delineate the contribution of the linker region in receptor oligomerization.

Wild type GC-C was expressed as a GST fusion (GCC-GST) to aid in distinguishing the wild type and mutant receptors on a Western blot on the basis of molecular weight (Fig. 4, *A* and *B*, *insets*). GCC-GST and GC-C_{L764P} mutant plasmids (with a 6-fold more GC-C_{L746P} plasmid than GCC-GST plasmid) were co-transfected in HEK293T cells, and ST-induced intracellular cGMP levels were monitored. As shown in Fig. 4*A*, the presence of mutant receptor in the same cell resulted in reduced ST-induced cGMP production by the wild type receptor. This indicated that the L764P mutant receptor was able to heterodimerize with the wild type receptor and that the heterodimeric receptor had similar properties as the GC-C_{L764P} homodimer (no ST stimulation).

To study the ability of GC-C_{L768P} to functionally heterodimerize with the wild type receptor, HEK293T cells were co-transfected with GCC-GST plasmid along with GC-C_{L768P} mutant plasmid (ratio of 6:1). The hypothesis was that if the two receptors were able to heterodimerize, then the higher concentration of the wild type receptor (with low basal activity) should inhibit the high basal activity of the GC-C_{L768P} mutant receptor. Indeed, inhibition of the high basal activity of GC-C_{L768P} was observed in the presence of high levels of the wild type receptor (Fig. 4*B*), indicating that the two receptors are able to heterodimerize. These data confirm that the mutation in the coiled coil region does not drastically affect the dimerization ability of the receptors, possibly because a single mutation in a coiled coil region is unable to dramatically reduce the ability of the coiled coil to form a dimer.

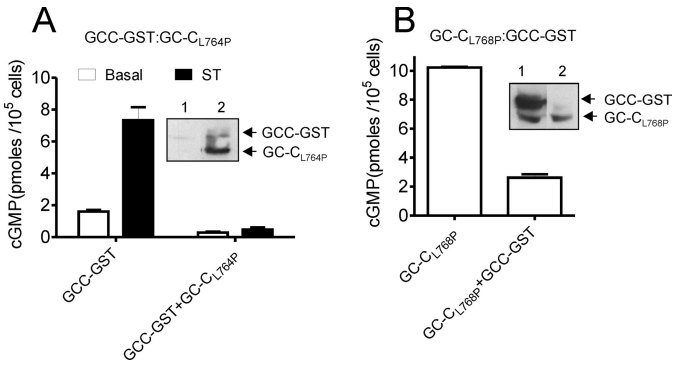

FIGURE 4. **Mutations in the linker region of GC-C do not affect oligomerization.** *A*, HEK293T cells were cotransfected with a fixed concentration of GCC-GST plasmid and a 6-fold higher concentration of GC-C_{L764P} plasmid and 72 h after transfection were treated with 10^{-7} M ST, and intracellular cGMP levels were monitored. Assays were repeated three times, with duplicate determinations. Values represent the mean ± S.E. of duplicate determination in a single experiment. *Inset*, membrane protein was prepared from parallel transfections and used for Western blot analysis using GCC:C8 monoclonal antibody. *Lane 1*, GC-C-GST alone; *lane 2*, GCC-GST and GC-C co-transfection. *B*, HEK293T cells were co-transfected with a fixed concentration of GC-C_{L768P} mutant plasmid and 6-fold higher amounts of GCC-GST plasmid, and 72 h post-transfection cells were lysed, and intracellular cGMP levels monitored. Data shown are the mean ± S.E. of duplicate measurements of a representative assay ($n = 3$). *Inset*, membrane protein prepared from parallel transfections were taken for Western blot analysis using GCC:C8 monoclonal antibody. *Lane 1*, GC-C alone; *lane 2*, GC-C and GCC-GST co-transfection.

Detergent-mediated Activation of GC-C Is Modulated by Residues in the Coiled Coil Region—Results described so far have established that the linker region is an important regulatory feature in GC-C. The activity of receptor GCs is markedly enhanced *in vitro* in the presence of non-ionic detergents, but the molecular basis for this activation is not known. Membranes prepared from cells expressing the linker region mutants were subjected to *in vitro* guanylyl cyclase assays in the absence or presence of 0.1% Lubrol-PX and high substrate concentration (10 mM MgGTP). Mutant receptors that showed robust ST-stimulated cGMP production were activated by detergents to an extent seen with the wild type receptor (supplemental Fig. 3). Among the mutant receptors with low basal activity, some (Y760P to R767P, E776P, T783P, and Q784P) showed an increase in guanylyl cyclase activity in the presence of detergent, in a manner similar to that of wild type GC-C, whereas others (R773P, L778P, V779P, E780P, E781P, R782P, Y786P, E789P, and L796P) failed to do so (supplemental Fig. 3). Interestingly, none of the constitutively active mutant receptors was further activated by the Lubrol-PX (supplemental Fig. 3). Representative examples of some mutant receptors phenotypes are shown in Fig. 5. Thus, we can suggest that in the wild type receptor, the linker region forms hydrophobic interactions that keep the receptor in a conformation that possesses low guanylyl cyclase activity. In the presence of ligand (or detergent) these hydrophobic interactions are removed, thereby allowing the cyclase domain to dimerize in such a way that optimal guanylyl cyclase activity is seen. Our mutational analysis has, therefore, suggested for the first time the molecular basis for detergent-mediated activation of GC-C.

The KHD regulates the activity of the guanylyl cyclase domain by its ability to bind ATP (2). Detergent-mediated

activity is inhibited in the presence of ATP, suggesting that the disruption of hydrophobic interactions brought about by detergents may be reversed subsequent to the ATP-induced conformational change in the KHD. Because some constitutively active mutant receptors showed no detergent-stimulated activity, it is possible that these conformations are locked in an active state. We, therefore, wished to test if the presence of ATP could "unlock" this state and restore basal levels of cGMP production. The guanylyl cyclase activity of all mutants that showed elevated basal activity could not be inhibited by ATP in the presence of detergent (supplemental Fig. 2). Representative examples are shown in Fig. 5. Therefore, it is possible that the coiled coil region in GC-C may also interact with the KHD on one face of the helix and regulate ATP interaction. Thus, in receptors with low basal activity, the linker region could be sandwiched between the KHD and the guanylyl cyclase domain, restraining the conformation of the guanylyl cyclase domain.

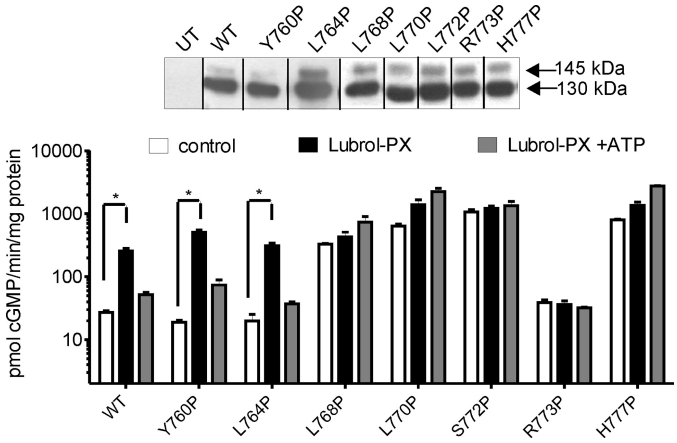

FIGURE 5. **Detergent-mediated activation and ATP-mediated regulation of wild type GC-C and representative linker region mutants.** Membrane protein prepared from HEK293T cells expressing the mutant receptor were assayed for guanylyl cyclase activity using 1 mM MgGTP as the substrate and 10 mM free $MgCl^{2+}$ in the absence or presence of 0.1% Lubrol-PX. Assays were also performed in the presence 0.1% Lubrol-PX and 1 mM MgATP using 1 mM MgGTP as substrate and 10 mM free $MgCl_2$. The amount of cGMP produced was monitored, and data shown are the mean ± S.E. of duplicate measurements of assays repeated three times. Mutant receptors from Y760P to R767P, E776P, T783P, and Q784P showed a significant detergent-stimulated activity (*, $p < 0.02$) and also at least a 2-fold reduction in guanylyl cyclase activity in the presence of ATP (supplemental Fig. 3). The inset shows a Western blot of membrane preparations prepared from cells expressing wild type (*WT*) or mutant GC-C receptors. Membranes prepared from untransfected cells (*UT*) showed no bands corresponding to GC-C on Western blot analysis. Guanylyl cyclase activity measured in these membranes was ~1 pmol cGMP/min/mg protein, and no change in activity was seen in the presence of Lubrol-PX with or without ATP.

The Coiled Coil Region Plays a Similar Regulatory Role in GC-A—Results described so far have served to establish the important role played by the linker region in regulating the activity of GC-C. The linker region shows ~45% sequence identity across all receptor GCs (13) (Fig. 1*A*), but the average sequence similarity is as high as 67% as calculated using BLOSUM62 substitution matrix implemented in MatGAT (37). To generalize the features of the linker region in another receptor guanylyl cyclase, mutations were generated in the important *a* and *d* positions in the putative coiled coil region of GC-A. Single point proline mutations were generated at Leu-812 (*d* position), Met-816 (*a* position), and Tyr-819 (*d* position) residues in rat GC-A (equivalent to Leu-764, Leu-768, and Tyr-771 in GC-C, respectively). Expression of mutant GC-A proteins in membranes prepared from HEK293T cells was checked by Western blot analysis using a polyclonal antibody to GC-A, raised to the last 17 C-terminal amino acids of GC-A (38). As shown in Fig. 6*A*, the mutant proteins were expressed to equivalent levels and showed the characteristic differentially glycosylated forms of GC-A (39). The mutant receptors were catalytically active when cGMP production was measured in the presence of MnGTP as a substrate (data not shown). Although HEK293T monolayers expressing GC-A_{L812P} showed low intracellular cGMP production, similar to wild type GC-A, they showed no ANP-stimulated cGMP production (Fig. 6*B*). Moreover, cells expressing GC-A_{M816P} and GC-A_{Y819P} showed high basal levels of intracellular cGMP that were not further stimulated by ligand treatment, as was seen in GC-C by mutations at equivalent positions. Finally, the activation of GC-A by

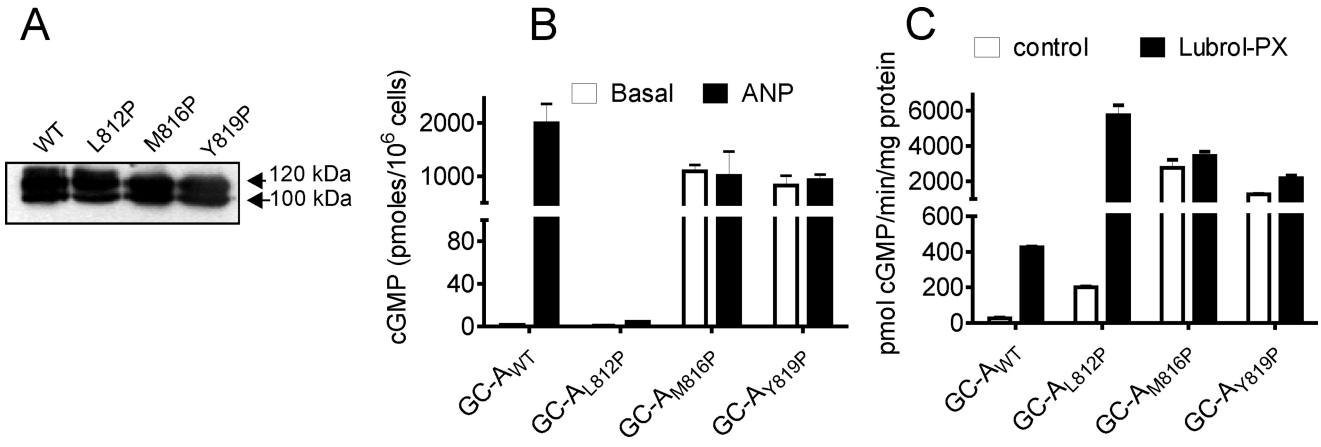

FIGURE 6. **Characterization of GC-A linker mutants.** *A*, membrane protein prepared from HEK293T cells expressing wild type (*WT*) GC-A or the linker region GC-A mutants was taken for Western blot analysis using GC-A polyclonal antibody. *B*, HEK293T monolayers transfected with wild type GC-A or mutant GC-A were incubated in the absence or presence of 10^{-6} M ANP after which the cells were lysed, and intracellular cGMP levels were measured. Data shown are the mean ± S.E. of duplicate measurements of a representative experiment, with experiments repeated three times. *C*, membrane protein (2 μg) prepared from HEK293T cells expressing wild type or mutant GC-A receptors was used for *in vitro* guanylyl cyclase assay in the presence of 0.1% Lubrol-PX using 1 mM MgGTP and 10 mM free $MgCl^{2+}$. The amount of cGMP produced was monitored, and data shown are the mean ± S.E. of duplicate measurements of a representative assay, with the assay repeated three times.

Lubrol-PX was measured. The *in vitro* guanylyl cyclase activity of the GC-A_{L812P} mutant receptor was activated by Lubrol-PX in a manner similar to the wild type receptor and, therefore, shows properties equivalent to the L764P mutation in GC-C. However, the guanylyl cyclase activities of GC-A_{M816P} and GC-A_{Y819P} were not activated by detergent, as was seen in mutant GC-C receptors (L768P and Y771P).

ATP-mediated effects are markedly different between GC-A and GC-C in that the presence of ATP increases ANP-stimulated activity of GC-A manyfold, in contrast to the more modest increase seen with GC-C and ST (40). We observed no change in the activity of wild type GC-A or mutant receptors in the presence of detergent and ATP (data not shown). Clearly, there are specific regulatory features in the interaction between the KHD and the GC domain in receptor GCs, which await further analysis. Nonetheless, our studies have revealed a common role for the linker region in receptor GCs. Because the highest homology between receptor GCs is in the catalytic cyclase domain, we suggest that specific residues in the linker region, through their interactions with the cyclase domain, assist in repressing guanylyl cyclase activity. Detergents can disrupt these interactions, leading to high cGMP production by generating the conformation of the receptor that mimics the ligand-activated state.

DISCUSSION

This is the first comprehensive study on the role of the putative coiled coil region in receptor GCs. Based on the phenotypes of the mutations that were observed and a lack of similarity in properties of mutants generated in equivalent positions in a coiled coil, we suggest that this region in guanylyl cyclases may not adopt a classical coiled coil helical structure. Instead, we suggest that a helix is formed by this linker region, where the amphipathic nature of the helix presents a hydrophobic stretch that may interact with regions on the guanylyl cyclase domain, thereby acting as a clamp to ensure low levels of cGMP production.

High intracellular cGMP accumulation would result from conversion of MgGTP to cGMP, as mM concentrations of Mg^{2+}, but only trace Mn^{2+}, are found within the eukaryotic cell (41). The fact that all the mutant receptors possessed *in vitro* guanylyl cyclase activity when measured using MnGTP as a substrate showed that the cyclase domains were able to dimerize in a functional manner when Mn^{2+} was present as the metal co-factor. Perhaps the larger size and flexible co-ordination geometry of Mn^{2+} (42) allows it to bind and form a functional catalytic site even in the presence of mutations that render the guanylyl cyclase domain poorly active when measured with MgGTP as a substrate. Nevertheless, because some linker mutant receptors showed robust guanylyl cyclase activity even when MgGTP alone was used as a substrate, we suggest that the linker region has an inhibitory role on the receptor guanylyl cyclase domain, perhaps by preventing the two catalytic domain subunits from juxtaposing themselves in a way suitable for catalysis in the absence of the ligand.

Interestingly, interspersed among the high basal mutants were residues which when mutated to proline reduced the guanylyl cyclase activity to levels seen in the wild type receptor. Based on the periodicity that is observed, it is tempting to suggest that this region of the receptor (residues Tyr-771 to His-777) could show an alteration in the regular α-helical structure and generate interactions similar to those seen in a 3_{10} helix. It is not clear, however, if this structural variation is seen in the basal conformation of the receptor or is a prerequisite structural transition mimicking the ligand-activated state of the receptor.

We show that the phenotypes of mutations at equivalent positions in GC-C and GC-A are similar, indicating conservation of the role of these residues in these receptors. Naturally occurring mutations in the linker region of RetGC-1 have been identified in patients with autosomal cone-rod dystrophy (43, 44). All mutations include a change in the Arg-838 residue, corresponding to the Arg-782 position in GC-C. These mutations showed guanylyl cyclase activity when measured using MnGTP in one study (45) or MnGTP and the non-ionic detergent Triton X-100 in other studies (16, 46). Because all the mutants in GC-C were active in the presence of MnGTP and we saw detergent-mediated effects when MgGTP was used as a substrate, we cannot comment on whether mutations in RetGC-1 abrogated detergent-stimulated activity as is seen with the R782P mutant in GC-C. However, mutant receptors showed ~3-fold reduction in basal activity in comparison to the wild type receptor when assayed in the presence of MgGTP (45). In none of these studies were intracellular levels of cGMP measured in cells overexpressing the mutant receptors, possibly because RetGC-1 is activated only in the presence of GCAPs. Mutations at Arg-838 showed an increased sensitivity to GCAP-1 stimulation of cyclase activity, and the inhibition potential of Ca^{2+} was decreased (16). In these studies it was assumed that the coiled coil region forms a parallel dimer, and mutation of the critical Arg-838 disrupts the dimerization interface leading to movement of the C-terminal parts of the two helices away from each other. Molecular dynamic simulations with the modeled coiled coil region suggested that mutation of Arg-838 led to the lengthening of the coiled coil region, thereby affecting the regulation of cyclase domain by GCAP-1 and Ca^{2+}. The distinct mechanisms of regulation of GC-C (by an extracellular ligand) and RetGC-1 (by the intracellular GCAPs) may result in different phenotypes in linker region mutants. However, we predict that the lack of activation by detergent in mutations at certain positions in the linker region could be seen in all receptor GCs including RetGC-1 when assayed in the presence of MgGTP.

A mutation in the catalytic domain of GC-A (E974A) has been reported that results in phenotypes very similar to many of the constitutively active mutants in GC-C (and GC-A) described in the current study (47). Full-length GC-A_{E974A} is constitutively active and shows no further ANP-mediated activation and no regulation by ATP. In addition, a construct (HCAT) that contains a fragment of the C-terminal region of the KHD, the entire linker region, and the guanylyl cyclase domain also shows higher *in vitro* guanylyl cyclase activity than the wild type protein (47). It is, therefore, conceivable that the Glu-974 residue may interact with residues in the linker region, and disruption of this

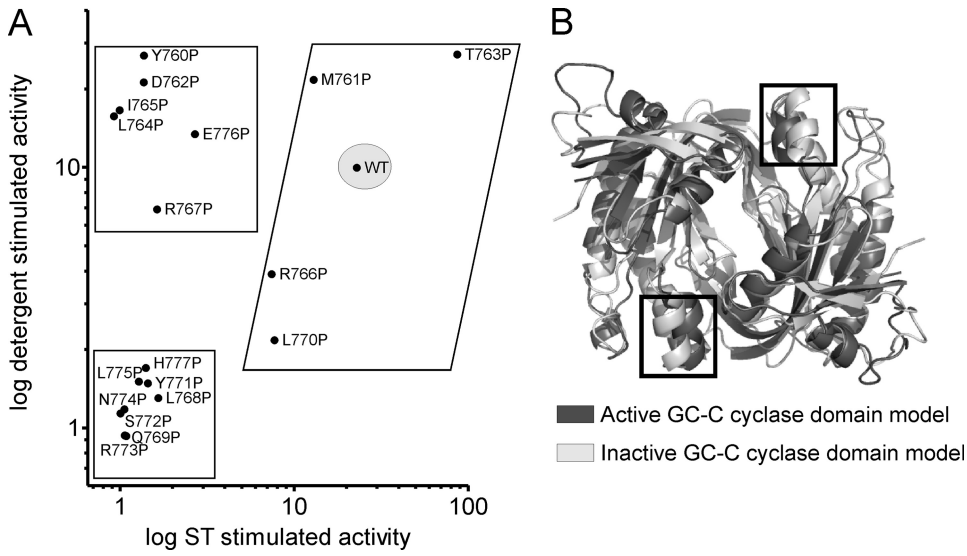

FIGURE 7. **The role of the linker region in GC-C.** *A*, a summary of the phenotypes of the linker mutations from Tyr-760 to His-777 in terms of their ligand and detergent-stimulable properties. Three groups of mutations can be defined, with the majority severely compromised in ligand-mediated activation of GC-C even though they show significant activation in the presence of detergent. *B*, superposition of the models generated using the available crystal structures of guanylyl cyclase domains (PDB codes 3ET6 and 2W01) indicating the movement of the helix α1 in the activation of the cyclase domain.

interaction either by mutation of the Glu residue or by mutation of interacting residues in the linker region (suggested by our study) could result in the same phenotype. However, it is important to note that the N-terminal residues of the catalytic domain (to which the linker sequences are attached) may lie on a face of the protein opposite to that of the Glu-974 residue (based on modeling analysis; data not shown). Therefore, an alternative explanation can be provided for the phenotype of the E974A mutant by stating that mutations in the linker region, which result in constitutive activity of receptor guanylyl cyclases, bring about a conformational change in the cyclase domain involving the conserved residue at position Glu-974 in GC-A. Clearly these hypotheses are likely to be verified when the structure of a receptor guanylyl cyclase is described or combination mutations are made in both the linker and the Glu-974 residue in GC-A.

Mutations in the linker region of GC-C caused essentially three distinct phenotypes, as summarized in Fig. 7*A*. The fact that very few mutants possessed wild type-like activity in terms of ligand and detergent stimulability indicated that this region is critical for proper functioning of receptor GCs. A number of mutants lay in the *upper left hand quadrant* of the *graph*, and these represented mutant receptors that had lost all ligand stimulated activity but retained detergent-stimulated guanylyl cyclase activity. The most interesting mutant receptors (with the exception of R773P) were those in the *lower left hand quadrant* of the *graph*, which showed highly elevated basal guanylyl cyclase activity and no further ligand- or detergent-mediated activation. This suggested to us that detergents activate the wild type receptor by disrupting hydrophobic interactions that are held in place by residues whose mutation results in high basal activity. It is important to note, however, that mutations of some charged residues as well as hydrophobic residues also resulted in high basal activity, indicating that these charged residues may play a role in correct positioning of the regulatory hydrophobic region.

The fact that ATP-mediated regulation is also lost in the mutant receptors which have high basal activity suggests that the linker region could also interact with the KHD. Because the cyclase domains need to form head to tail dimers, we suggest that the linker region could form anti-parallel helices, one face of which lies along the active site of the cyclase domain. The other face of the linker region could juxtapose to the KHD, with the KHD domains lying parallel to each other. We suggest this topology based on our mutational analysis and the effects that mutations in the linker region have on both ATP-mediated interaction with the KHD and guanylyl cyclase activity.

An example of a nucleotide cyclase whose activity is regulated by sequences N-terminal to the cyclase domain is well demonstrated both biochemically and structurally by Rv1264, a pH-sensing adenylyl cyclase from *Mycobacterium tuberculosis*. This enzyme has maximum activity at acidic pH (6.0) and has an N-terminal regulatory domain and a C-terminal cyclase domain connected by a small linker (48). The N-terminal domain is autoinhibitory to the cyclase domain of the enzyme (49), and interestingly, a mutation in this region of Rv1264 (D107A) led to an increase in V_{max} (3-fold) with little change in K_m. The full-length crystal structure of Rv1264 revealed the conformational changes that are responsible for the pH-dependent switch between the active and inactive states of the enzyme. In the inactive state the linker between the N terminus and the catalytic domain attains a helical conformation, and the same region is a random coil in the active state of the enzyme. This structural change along with others facilitates the cyclase domains from two monomers to come in an appropriate head-to-tail orientation in the active state (48). Therefore, evidence for dramatic structural changes brought about by residues N terminus to a nucleotide cyclase domain is available, strengthening our suggestion that such conformational changes can be speculated to occur in the linker region of GC-C in conjunction with the N-terminal KHD and the C-terminal guanylyl cyclase domain to attain a ligand-mediated activated state.

Some Class III nucleotide cyclases show regulation of their catalytic activity by associated HAMP (histidine kinases, adenylyl cyclases, methyl accepting chemotactic receptors, and phosphatases) domains. For example, point mutations in the HAMP domain of the mycobacterial adenylyl cyclase Rv3645 that were designed to remove hydrophobic surfaces led to a stimulation of Rv3645 adenylyl cyclase activity. In contrast, similar mutations in the HAMP domain of Rv1318c only marginally increased the activity of the associated cyclase

domain, indicating specificity in the regulation brought about by the HAMP domains (50).

To date, two guanylyl cyclase domain structures have been solved (5, 6). The Cya2 (cyanobacterial) catalytic domain structure is similar to the structure of the activated adenylyl cyclase, whereas the Cyg12 (*Synechocystis*) catalytic domain is thought to be in the inactive conformation (because of dimethylarsenic additions to cysteine residues). We modeled the cyclase domain of GC-C into these two cyclase structures to generate inactive and active states to hypothesize on the role of the linker region in activating the cyclase domain. In the model of the active (Cya2) conformation of GC-C, helix α1 is found closer to the active site, and helix α4 is in the second monomer compared with the inactive (Cyg12) structure (Fig. 7*B*). This movement as suggested by Winger *et al.* (6) is important for positioning the nucleotide correctly in the active site, allowing catalysis. It was also suggested that regulatory proteins/domains can interact with the cyclase domain by docking onto the cavity formed between the α1-α2 loop and α3-β4 loop and regulating the activity of the cyclase domain, as seen with the adenylyl cyclase. It is possible that the linker region, which can form helical structures in the circular dichroism experiment, interacts with the cyclase domain, similar to the docking of the $G_s\alpha$ switch II helix in adenylyl cyclases (36). However, given the limitations of computational methods to model loops in protein tertiary structure, it is not possible for us to conclusively say that this is the mode of regulation of the cyclase domain of receptor GCs by the associated linker region. Taken together, the mechanisms of activation of the cyclase domain remain speculations as of now and await the structural determination of a guanylyl cyclase domain with the regulator linker region and of course the entire intracellular domain. Nevertheless, we have shown in this study that the linker region of receptor GCs has a critical role to play in regulating not only the guanylyl cyclase activity of these receptors but also in the relay of conformational changes that occur from the extracellular domain and the KHD to the cyclase domain.

Acknowledgments—We thank Prof. Manju Bansal for useful discussions and Vani R. Iyer for technical assistance.

REFERENCES

1. Padayatti, P. S., Pattanaik, P., Ma, X., and van den Akker, F. (2004) *Pharmacol. Ther.* **104,** 83–99
2. Jaleel, M., Saha, S., Shenoy, A. R., and Visweswariah, S. S. (2006) *Biochemistry* **45,** 1888–1898
3. Bhandari, R., Srinivasan, N., Mahaboobi, M., Ghanekar, Y., Suguna, K., and Visweswariah, S. S. (2001) *Biochemistry* **40,** 9196–9206
4. Linder, J. U., and Schultz, J. E. (2003) *Cell. Signal.* **15,** 1081–1089
5. Rauch, A., Leipelt, M., Russwurm, M., and Steegborn, C. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 15720–15725
6. Winger, J. A., Derbyshire, E. R., Lamers, M. H., Marletta, M. A., and Kuriyan, J. (2008) *BMC Struct. Biol.* **8,** 42
7. Forte, L. R. (1999) *Regul. Pept.* **81,** 25–39
8. Vaandrager, A. B. (2002) *Mol. Cell. Biochem.* **230,** 73–83
9. Forte, L. R., Krause, W. J., and Freeman, R. H. (1989) *Am. J. Physiol.* **257,** F874–F881
10. Jaleel, M., London, R. M., Eber, S. L., Forte, L. R., and Visweswariah, S. S. (2002) *Biol. Reprod.* **67,** 1975–1980
11. Nandi, A., Bhandari, R., and Visweswariah, S. S. (1997) *J. Cell. Biochem.* **66,** 500–511
12. Qian, X., Prabhakar, S., Nandi, A., Visweswariah, S. S., and Goy, M. F. (2000) *Endocrinology* **141,** 3210–3224
13. Biswas, K. H., Shenoy, A. R., Dutta, A., and Visweswariah, S. S. (2009) *J. Mol. Evol.* **68,** 587–602
14. Hirayama, T., Wada, A., Hidaka, Y., Fujisawa, J., Takeda, Y., and Shimonishi, Y. (1993) *Microb. Pathog.* **15,** 283–291
15. van den Akker, F., Zhang, X., Miyagi, M., Huo, X., Misono, K. S., and Yee, V. C. (2000) *Nature* **406,** 101–104
16. Ramamurthy, V., Tucker, C., Wilkie, S. E., Daggett, V., Hunt, D. M., and Hurley, J. B. (2001) *J. Biol. Chem.* **276,** 26218–26229
17. Vijayachandra, K., Guruprasad, M., Bhandari, R., Manjunath, U. H., Somesh, B. P., Srinivasan, N., Suguna, K., and Visweswariah, S. S. (2000) *Biochemistry* **39,** 16075–16083
18. Wilson, E. M., and Chinkers, M. (1995) *Biochemistry* **34,** 4696–4701
19. Shiga, T., and Suzuki, N. (2005) *Zool. Sci.* **22,** 735–742
20. Zhou, Z., Gross, S., Roussos, C., Meurer, S., Müller-Esterl, W., and Papapetropoulos, A. (2004) *J. Biol. Chem.* **279,** 24935–24943
21. Smith, M., Whittock, N., Searle, A., Croft, M., Brewer, C., and Cole, M. (2007) *Eye* **21,** 1220–1225
22. Singh, S., Singh, G., Heim, J. M., and Gerzer, R. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1455–1463
23. Shenoy, A. R., and Visweswariah, S. S. (2003) *Anal. Biochem.* **319,** 335–336
24. Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H. M., Goeddel, D. V., and Schulz, S. (1989) *Nature* **338,** 78–83
25. Visweswariah, S. S., Ramachandran, V., Ramamohan, S., Das, G., and Ramachandran, J. (1994) *Eur. J. Biochem.* **219,** 727–736
26. Zor, T., and Selinger, Z. (1996) *Anal. Biochem.* **236,** 302–308
27. Do, C. B., Mahabhashyam, M. S., Brudno, M., and Batzoglou, S. (2005) *Genome Res.* **15,** 330–340
28. Eswar, N., Eramian, D., Webb, B., Shen, M. Y., and Sali, A. (2008) *Methods Mol. Biol.* **426,** 145–159
29. Laskowski, R. A., M. M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26,** 283–291
30. Cuff, J. A., Clamp, M. E., Siddiqui, A. S., Finlay, M., and Barton, G. J. (1998) *Bioinformatics* **14,** 892–893
31. McGuffin, L. J., Bryson, K., and Jones, D. T. (2000) *Bioinformatics* **16,** 404–405
32. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science* **252,** 1162–1164
33. Yu, Y. B. (2002) *Adv. Drug Deliv. Rev.* **54,** 1113–1129
34. Ghanekar, Y., Chandrashaker, A., Tatu, U., and Visweswariah, S. S. (2004) *Biochem. J.* **379,** 653–663
35. Liu, Y., Ruoho, A. E., Rao, V. D., and Hurley, J. H. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 13414–13419
36. Tesmer, J. J., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) *Science* **278,** 1907–1916
37. Campanella, J. J., Bitincka, L., and Smalley, J. (2003) *BMC Bioinformatics* **4,** 29
38. Abbey, S. E., and Potter, L. R. (2002) *J. Biol. Chem.* **277,** 42423–42430
39. Bryan, P. M., Smirnov, D., Smolenski, A., Feil, S., Feil, R., Hofmann, F., Lohmann, S., and Potter, L. R. (2006) *Biochemistry* **45,** 1295–1303
40. Antos, L. K., Abbey-Hosch, S. E., Flora, D. R., and Potter, L. R. (2005) *J. Biol. Chem.* **280,** 26928–26932
41. Finney, L. A., and O'Halloran, T. V. (2003) *Science* **300,** 931–936
42. Seebeck, B., Reulecke, I., Kämper, A., and Rarey, M. (2008) *Proteins* **71,** 1237–1254
43. Kitiratschky, V. B., Wilke, R., Renner, A. B., Kellner, U., Vadalà, M., Birch, D. G., Wissinger, B., Zrenner, E., and Kohl, S. (2008) *Invest. Ophthalmol. Vis. Sci.* **49,** 5015–5023
44. Koch, K. W., Duda, T., and Sharma, R. K. (2002) *Mol. Cell. Biochem.* **230,** 97–106
45. Duda, T., Krishnan, A., Venkataraman, V., Lange, C., Koch, K. W., and Sharma, R. K. (1999) *Biochemistry* **38,** 13912–13919
46. Tucker, C. L., Woodcock, S. C., Kelsell, R. E., Ramamurthy, V., Hunt, D. M., and Hurley, J. B. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,**

9039–9044
47. Wedel, B. J., Foster, D. C., Miller, D. E., and Garbers, D. L. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 459–462
48. Tews, I., Findeisen, F., Sinning, I., Schultz, A., Schultz, J. E., and Linder, J. U. (2005) *Science* **308,** 1020–1023
49. Linder, J. U., Schultz, A., and Schultz, J. E. (2002) *J. Biol. Chem.* **277,** 15271–15276
50. Linder, J. U., Hammer, A., and Schultz, J. E. (2004) *Eur. J. Biochem.* **271,** 2446–2451
51. Edgar, R. C. (2004) *Nucleic Acids Res.* **32,** 1792–1797
52. Young, J. M., Waters, H., Dong, C., Fülle, H. J., and Liman, E. R. (2007) *PLoS ONE* **2,** e884