The Kinase Homology Domain of Receptor Guanylyl Cyclase C: ATP Binding and Identification of an Adenine Nucleotide Sensitive Site†

Mahaboobi Jaleel,‡ Sayanti Saha,‡ Avinash R. Shenoy, and Sandhya S. Visweswariah*

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

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ABSTRACT: The role of the kinase homology domain (KHD) in receptor guanylyl cyclases is to regulate the activity of the catalytic guanylyl cyclase domain. The KHD lacks many of the amino acids required for phosphotransfer activity and, therefore, is not expected to possess kinase activity. Guanylyl cyclase activity of the receptor guanylyl cyclase C (GC-C) is modulated by ATP, and computational modeling showed that the KHD can adopt a structure similar to protein kinases, suggesting that the KHD is the site for ATP interaction. A monoclonal antibody, GCC:4D7, raised to the KHD of GC-C, fails to react with GC-C in the presence of ATP and ATP analogues that regulate GC-C catalytic activity, indicating that a conformational change occurs in the KHD on ATP binding. Mapping of the epitope of the antibody through the use of recombinant protein constructs and phage display showed that the epitope for GC-C:4D7 lies immediately C-terminal to a critical lysine residue (Lys516 in GC-C), required for ATP interaction in protein kinases. By employing a novel approach utilizing ATP-agarose affinity chromatography, we demonstrate that the intracellular domain of GC-C and the KHD bind ATP. Mutation of Lys516 to Ala abolishes ATP binding. Thus, this report is the first to show direct ATP binding to the pseudokinase domain of receptor guanylyl cyclase C, as well as to identify dramatic conformational changes that occur in this domain on ATP binding, akin to those seen in catalytically active protein kinases.

The presence of kinase homology or pseudokinase domains (KHD)[1] as fusions to other effector domains in a number of proteins suggests that these domains are integral for the activity of the full-length protein (*1*−*3*). The KHDs possess some of the residues that are present in active protein kinases, but residues that are required for mediating the phosphotransfer reaction are either missing or mutated (*3*, *4*). Given the sequence similarity between the KHDs and the protein kinases, it has been possible to computationally model the KHDs of a number of proteins, and these results suggest that KHDs can indeed adopt a structure similar to that of classical active kinases (*5*−*7*). However, given the diversity in the means by which kinases are regulated as is evident from a number of crystal structures (*8*), it is to be expected that the KHDs could also have fine differences in structural detail that are not apparent from modeling studies. To date, there is no reported crystal structure of any KHD that is found to naturally occur in a protein.

A family of proteins that contain KHDs are the Janus kinases (Jaks). Deletion of or point mutations in these pseudokinase (JH2) domains cause a dramatic increase in the catalytic activity of the C-terminal functional kinase (JH1) domain (*9*, *10*). A profound regulatory role for KHDs has also been shown in the case of the receptor guanylyl cyclases (*11*). These receptors serve as ligands for diverse polypeptides, and ligand binding to the extracellular domain results in activation of the C-terminal guanylyl cyclase domain, increasing intracellular levels of cGMP (*12*). The receptors have a single transmembrane spanning domain and exist as preformed dimers prior to ligand binding (*2*, *13*, *14*). In the intracellular juxtamembrane region, a KHD has been identified that regulates the catalytic activity of the receptor (*15*, *16*). The KHDs across different members of the receptor guanylyl cyclase family are approximately 20−25% identical in terms of primary sequence, and ATP has been shown to regulate the activity of these receptors, presumably by binding to the KHD (*7*, *17*−*19*).

Guanylyl cyclase C (GC-C) is the receptor for the guanylin family of gastrointestinal peptides (*20*), as well as the family of bacterial heat-stable enterotoxins (ST) that are one of the major causes of watery diarrhea (*21*). The receptor is predominantly expressed in intestinal cells (*22*−*24*), though robust extra-intestinal expression of the receptor is observed in the kidney and reproductive tissues of the rat (*25*, *26*). Deletion of the KHD in GC-C has been reported to have contrasting effects, in that two studies show constitutive activation of the receptor (*27*, *28*), while another indicates that the mutant receptor is unresponsive to ligand addition in vivo (*28*). Ligand-mediated activation of GC-C in vitro, resulting in cGMP accumulation, is observed even in the absence of ATP (*29*, *30*), as has recently been shown for

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* Corresponding author: Dr. Sandhya S. Visweswariah, Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India. Tel: (91) 80 22932542. Fax: (91) 80 23600999. E mail: sandhya@mrdg.iisc.ernet.in.

‡ These authors contributed equally to this study.

[1] Abbreviations: β-ME, 2-mercaptoethanol; AMP PNP, 5′-adenylyl imidodiphosphate; DTT, dithiothreitol; GC-C, receptor guanylyl cyclase C; IPTG, isopropyl-β-D-thiogalactopyranoside; IBMX, isobutylmethyl xanthine; JH1, JAK kinase domain; JH2, JAK pseudokinase domain; KHD, kinase homology domain; PMSF, phenylmethylsulfonyl fluoride; ScFv, single chain antibody; ST, stable toxin.

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GC-A (*31*). ATP inhibits guanylyl cyclase activity measured in vitro in the presence of a nonionic detergent and MgGTP as substrate, or when using MnGTP as substrate (*32*). These observations suggest that binding of ATP to the receptor must induce a profound conformational change in the receptor that regulates the guanylyl cyclase activity.

We have earlier modeled the KHD of GC-C, based on the structure of a tyrosine kinase (*32*). Our results indicated that Lys516 of human GC-C corresponds to the conserved lysine in tyrosine kinases that is involved in coordinating the α and β phosphates of ATP. Mutation of the conserved Lys516 to alanine in full-length GC-C dramatically reduced ligand-stimulated activity both in vitro and in vivo (*32*), while guanylyl cyclase activity measured using MnGTP was unimpaired. Interestingly, ATP-mediated potentiation of ligand-stimulated activity was abolished in a mutant GC-C, where Lys516 was mutated to Ala, as was the inhibition of detergent-stimulated activity in the presence of ATP. These results suggest that ATP can allosterically regulate the activity of GC-C, presumably by interacting with the KHD.

Earlier, we generated a monoclonal antibody, GCC:4D7, raised to a bacterially expressed protein encompassing the KHD of GC-C. The binding of this antibody appeared to be sensitive to the presence of ATP, as it failed to react with GC-C when ATP was present during the immunoprecipitation reaction (*32*). This suggests that GCC:4D7 is sensitive to the conformational change that occurs in the KHD of GC-C, and also that ATP binding occurs in this domain of GC-C. In this study, we have identified the region that appears to alter its conformation on ATP interaction by mapping the epitope to which GCC:4D7 is directed. Interestingly, this region is immediately adjacent to the Lys516 residue. We also show direct binding of ATP to the KHD of GC-C and demonstrate that this interaction is dependent on Lys516. Therefore, conformational changes that occur in protein kinases on ATP binding and involve repositioning of this critical lysine residue could be observed in the KHD. Our results therefore provide evidence for ATP binding to and ATP-mediated alterations in a KHD. This antibody may therefore serve to monitor subtle conformation changes in GC-C that occur under a variety of physiological states.

MATERIALS AND METHODS

Culture and Maintenance of Cell Lines. Stable cell line HEK293:hGCC has been described earlier (*32*) and was cultured in DMEM:F12 containing 10% fetal calf serum and 100 μg/mL G418 at 37 °C in a 5% CO_2 humidified incubator. Sf21 insect cells were maintained in Grace's insect cell medium containing 10% fetal calf serum in a humidified incubator at 27 °C.

Preparation of Membrane Fraction from HEK293:hGC-C Cells. Cells were washed with chilled phosphate buffered saline (10 mM sodium phosphate buffer, pH 7.5, 0.9% sodium chloride) and scraped into homogenization buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 5 μg/mL soybean trypsin inhibitor (SBTI), 5 μg/mL leupeptin, 5 μg/mL aprotinin, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate). The cell lysate was sonicated and centrifuged at 12 000*g* for 1 h at 4 °C. The pellet obtained was resuspended in a buffer containing 50 mM Hepes, pH 7.5, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 1 mM sodium orthovanadate, and 20% glycerol. The protein was estimated by using a modification of the Bradford protein assay (*33*).

Expression of Recombinant GC-C Proteins in Sf21 Cells. The generation of a clone representing the entire intracellular domain of GC-C (from residues 455−1073) was obtained by amplifying this region of GC-C by PCR and cloning the PCR product into pFastBacHTb (Invitrogen) at the *Sal*I−*Not*I sites to generate plasmid pHTb-ICD. For ease of subsequent manipulation, a unique *Bam*HI site in GC-C present in the KHD was mutated by site-directed mutagenesis using the primer 5′ GAA TAC TGT GAG AGA GGA TCT CTC CGG G 3′ and the pHTb-ICD plasmid as template, utilizing a *Dpn*I-based approach described earlier (*34*). This generated the plasmid pHTb-$ICD_{\Delta Bam}$, which was used subsequently for generation of the recombinant bacmid by procedures described earlier (*35*).

pHTb-$ICD_{\Delta Bam}$ was used as template for the generation of the K516A and K519A mutants in the KHD of GC-C, using the K516A mutagenic primer (5′-GTG CTT GAG ATC AGC GAG AAT CAC TCG-3′) and the K519A mutagenic primer (5′-GAT TCT CAA AGA CCT CGC GCA CAA TGA TG-3′), respectively (*34*). This resulted in the generation of the pHTb-$ICD_{\Delta BamK516A}$ and pHTb-$ICD_{\Delta BamK519A}$ plasmids.

Primers (1401f BamHI, 5′-CCG CGG ATC CAG AAA ATA TAG AAA AG-3′, and 2463r HindIII, 5′-CCA TTT CAG AGA CTT TTC GAA CCC G-3′) were designed in order to amplify the KHD and putative coiled-coil region of GC-C (KHD-CC) spanning residues 455−810 from pHTb-$ICD_{\Delta Bam}$ (*35*). The PCR product (∼1027 bp) was cloned as a *Bam*HI−*Hin*dIII fragment into similarly digested pFastBacHTb to generate the plasmid pHTb-KHD-CC that would express the KHD-CC of GC-C fused to a hexahistidine tag at the N-terminus encoded by the vector.

pHTb-KHD-CC was used as template for the generation of the K516A mutation in the KHD-CC construct using the K516A mutagenic primer to generate the plasmid pHTb-KHD-CC_{K516A} (*34*).

The region of KHD spanning residues 474−751 of GC-C was amplified by PCR using the forward primer, GCC:1447 5′-AAATGGATCCACATTCCTCTT-3′, and reverse primer, GCC:2315 5′-TTTTGGGAATTCTAAAGTCCAAATATC-3′. The PCR product was cloned into a pCRII TA cloning vector (Invitrogen). The KHD fragment from pCRII-GCC-$KHD_{474-751}$was cloned as a *Xho*I−*Hin*dIII fragment into the pRSET C vector to generate pRSETC-GCC-$KHD_{474-751}$ plasmid. Again, *Xho*I−*Hin*dIII fragment was then cloned into similarly digested pFastBacHTb to generate a plasmid (pHTb-KHD) that would express the GCC-KHD protein in insect cells along with an N-terminal hexahistidine tag.

All clones generated were sequenced to ascertain the absence of missense mutations (Macrogen, S. Korea).

Expression of proteins in Sf21 cells was achieved using the Bac-to-Bac baculovirus expression system (Invitrogen, USA) as described earlier (*35*), following the generation of recombinant bacmids. Extracts from Sf21 cells expressing the proteins (as detected by Western Blot using the GCC:C8 monoclonal antibody or GCC-CTD antibody) were prepared by harvesting cells in lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol (β-ME), 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2 mM PMSF, and 10%

glycerol). Cells were subjected briefly to sonication, centrifuged at 12 000*g* for 60 min at 4 °C, and the supernatant was used for in vitro guanylyl cyclase assays or interaction with ATP-agarose after protein estimation using a modified Bradford method (*33*).

In Vitro Guanylyl Cyclase Assays. Crude lysates prepared from infected insect cells or membranes from HEK293:hGC-C cells were taken for guanylyl cyclase assay. Protein (5 μg) was incubated in assay buffer (60 mM Tris-HCl, pH 7.5, containing 500 μM isobutylmethyl xanthine (IBMX), and an NTP regenerating system consisting of 7.5 mM creatine phosphate and 10 μg of creatine phosphokinase) in the absence or presence of 200 μM ATP for 20 min at 4 °C. The assay was initiated by the addition of metal-GTP, with free metal concentrations maintained at 10 mM. The concentrations of free Mg and Mg−GTP/ATP complexes present in assays were calculated using WinmaxC (http://www.stanford.edu/~cpatton/maxc.html). Assays were incubated at 37 °C for 10 min; 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 (*32*).

Immunoreactivity of Monoclonal Antibody GCC:4D7 with GC-C. Membrane protein (300 μg) prepared from HEK293:hGCC cells was solubilized in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, containing 1% Triton X100, 150 mM NaCl, 2 mM $MgCl_2$, and 5 μg/mL each of aprotinin and leupeptin) in the presence of 1 mM concentration of either ATP, ATPγS, ADP, AMP PNP, AMP, adenosine, or GTP for 1 h at 4 °C. The fraction was centrifuged for 20 min at 12 000*g*, and to the supernatant was added GCC:4D7 (2 μg/mL), raised against the KHD of GC-C (residues Arg 454 to Leu 785), and incubation was continued for 10 h at 4 °C, following which the immune complex was recovered by incubating with protein A agarose beads for 2 h at 4 °C. The protein A agarose beads were subsequently washed three times with detergent containing wash buffer (20 mM Tris-HCl, pH 7.5, containing 0.1% Triton X100, 150 mM NaCl, and 2 mM $MgCl_2$) followed by two washes with wash buffer without Triton X100. The immune complex bound to protein A agarose beads was subjected to 7.5% SDS−PAGE followed by Western Blot analysis using monoclonal antibody GCC:C8 as described earlier (*32*).

Generation of ScFv of GCC:4D7 Monoclonal Antibody. Total RNA was isolated from an actively secreting GCC:4D7 hybridoma cell line and RNA prepared. Random primed cDNAs were synthesized from 5 μg total RNA using reverse transcriptase (MBI Fermentas). The Recombinant Phage Antibody System−ScFv Module (GE Healthcare) was used to construct the ScFv according to protocols supplied by the manufacturer. The assembled PCR product was digested with *Sfi*I and *Not*I and ligated into the phagemid expression vector, pCANTAB5E.

A recombinant phage antibody library for GCC:4D7 was generated in *E. coli* TG1, and the antigen-reactive phages that display ScFv were enriched through solid phase panning. Microtiter wells (Nunc) coated with bacterially expressed and purified $KHD_{474-751}$ (*32*) (200 ng/well) were incubated with phages for 2 h at 37 °C. Unbound phages were removed by washing three times with 10 mM sodium phosphate, pH 7.5, containing 0.9% NaCl (PBS). To the bound phages were added TG1 cells for the infection to occur. Infected TG1 cells were plated on selection medium containing ampicillin (20 μg/mL). Clones that interacted with the $KHD_{474-751}$ were rescued by helper phage infection and were subsequently screened for binding to the KHD protein by ELISA. Individual clones were induced with IPTG (1 mM) at log phase, and the cells were pelleted. The phages present in the culture supernatant were incubated with the KHD protein coated on microtiter wells for 2 h at 37 °C, followed by incubation with anti-E-tag monoclonal antibody for 2 h. Positive clones were detected with horseradish peroxidase conjugated anti-mouse antibody. The phages that bound to KHD were used to infect HB2151 *E. coli* cells for production of soluble ScFv.

Two independent ScFv clones were sequenced, and the immunoglobulin sequence was analyzed using the Kabat database (http://www.bioinf.org.uk/abs/) to define framework and complementary determining regions (CDRs).

Expression and Purification of Soluble GCC:4D7 ScFv. HB2151 *E. coli* cells were infected with the recombinant antigen-positive phages for production of soluble ScFv. Cells were grown at 30 °C until $A_{600} = 0.6$ and were induced with IPTG (1 mM) for 11 h at 30 °C. Cells were pelleted by centrifugation at 1500*g*, and the soluble ScFv was concentrated and enriched from the culture supernatant by 50% ammonium sulfate precipitation. The precipitate was solubilized in and desalted into 10 mM sodium phosphate buffer, pH 7.5, containing 0.9% (w/v) NaCl using an Akta FPLC system (GE Healthcare).

Immunodetection of GC-C by GCC:4D7 ScFv. Membrane protein (300 μg) prepared from HEK293:hGC-C expressing GC-C was solubilized in immunoprecipitation buffer in either the absence or presence of ATP (1 mM). Solubilized protein was incubated with GCC:4D7 ScFv (50 μg/mL total protein) for 10 h at 4 °C, after which the immune complex was recovered by incubation with anti-E-tag antibody bound to protein A agarose beads. The recovered immune complexes were washed and subjected to 7.5% SDS−PAGE followed by Western Blot analysis using the GCC:C8 antibody (*32*).

Generation of Plasmids Expressing Various Domains of GC-C and Expression of Recombinant Proteins. The plasmids pGEX-GCC-ED2, coding for residues 16−516 of the extracellular ligand binding domain, pRSET-GC-C-ID2, coding for residues 567−1073 of the intracellular domain, and pRSET-GCD, coding for residues 732−1073 of the catalytic domain and C-terminal domain, have been described earlier (*32*). The KHD fragment from pCRII-GCC-$KHD_{474-751}$ was expressed from the pRSET C vector as described above and expressed a 39 kDa protein with an N-terminal hexahistidine tag.

Plasmids were transformed into the *E. coli* strain BL21 (DE3), and protein expression was induced using IPTG (1 mM) for 3 h at 37 °C. Cells were collected by centrifugation, resuspended in sonication buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 5 mM β-ME, 1 mM PMSF, 1 mM benzamidine HCl, and 1% Triton X100), and lysed by sonication. The cell lysate was centrifuged at 1500*g* to obtain the inclusion body pellet. The protein bands corresponding to various domains of GC-C were purified by electroelution as described earlier (*32*), and proteins (200 ng) were subjected to

12% SDS−PAGE followed by Western Blot analysis with GCC:C8 or GCC:4D7 antibodies.

Epitope Mapping of GCC:4D7 Using Random Hexamer Peptide Library. The random hexamer library constructed in fuse5 vector for phage display was a kind gift provided by Dr. G. P. Smith, University of Missouri, Columbia. Biopanning and sequencing of the clones was carried out as described earlier (*22*), with GCC:4D7 monoclonal antibody as ligate. After three rounds of biopanning, the phages were prepared from individual clones, and 10^{10} phages were subjected to 10% SDS−PAGE followed by Western Blot analysis with GCC:4D7. Normalization of phages was done by Western Blot analysis using a gpIII monoclonal antibody (1:50 000 dilution), which was a kind gift from Prof. Vijay K. Chaudary, University of Delhi South Campus. Phage DNA was prepared from GCC:4D7 reactive phages, and DNA sequencing was carried out by employing a modified Sanger's dideoxy chain termination method as described earlier (*22*).

Interaction of ATP-Agarose Beads with GC-C. Crude lysate protein prepared from Sf21 cells was diluted in interaction buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.1% Triton X100). Kinase-Bind γ-phosphate-linked high substitution ATP resin (8−12 μmol/mL, Innova Biosciences, UK) was washed three times with wash buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1mM DTT, and 0.1% Triton X100), and 10 μL of beads (representing 200 μM ATP concentration on the beads) was added to the solubilized membrane or the insect cell lysates in a total volume of 500 μL. Incubation was continued for 1 h at 4 °C. The ATP-agarose beads were subsequently washed three times with detergent containing wash buffer followed by two washes with wash buffer without Triton X100. Beads were boiled in Laemmli buffer and eluted proteins then subjected to SDS−PAGE followed by Western Blot analysis using monoclonal antibody GCC:C8 as described earlier (*32*).

RESULTS

Modulation of Guanylyl Cyclase Activity by Adenine Nucleotides. Adenine nucleotides have been shown to regulate the activity of GC-C, presumably by binding to the KHD. Ligand-mediated enhancement of the guanylyl cyclase activity of GC-C is seen in the presence of ATP with MgGTP as substrate, but *ligand-independent* guanylyl cyclase activity (i.e., assays performed in the presence of detergent and MgGTP, or MnGTP alone as substrate) is inhibited in the presence of ATP (*7*). Membranes were prepared from HEK293 cells expressing GC-C and subjected to in vitro guanylyl cyclase assays using MgGTP as substrate in the presence of detergent and adenine analogues. A significant inhibition of guanylyl cyclase activity was seen in the presence of ATP and ATPγS, whereas adenosine had little effect on GC-C activity (Figure 1A). A similar inhibition of guanylyl cyclase activity was also seen in the presence of AMP PNP (a nonhydrolyzable ATP analogue), thus suggesting that the effects of ATP on GC-C activity are due to a binding event, and do not require ATP hydrolysis and phosphorylation (Figure 1A).

Earlier studies in our laboratory have shown that the intracellular domain of GC-C expressed in insect cells has

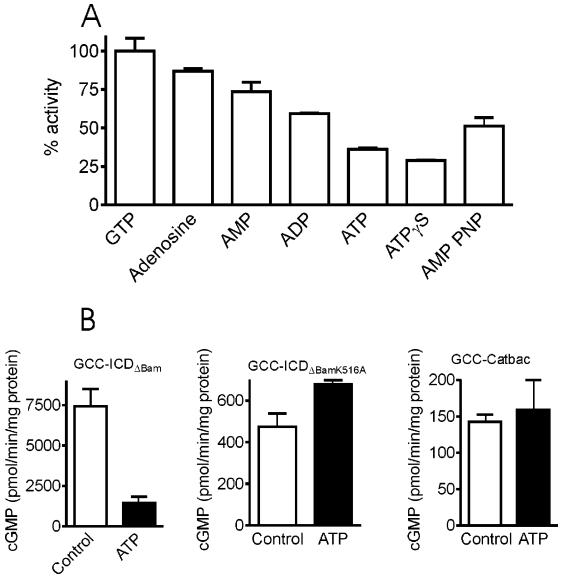

FIGURE 1: Effect of ATP and its analogues on the guanylyl cyclase activity of GC-C. (A) In vitro guanylyl cyclase assays were performed with 5 μg of membrane protein in the presence of MgGTP (1 mM) and 0.1% Lubrol-PX, in the absence or presence (1 mM) of the indicated adenine nucleotides. Values show the mean ± S.E.M. of triplicate determinations of a representative assay, with assays performed twice. Activity in the absence of ATP analogues was ~1000 pmol/min/mg protein. (B) Crude lysates prepared from infected Sf21 cells (5 μg) were taken for in vitro guanylyl cyclase assays in the absence or presence of ATP (200 μM) using MgGTP (1 mM) as substrate, in the presence of 10 mM free metal. Values shown are of a representative assay, with assays performed at least three times, with each determination in duplicate. The activity observed in untransfected lysates is 1−3 pmol/min/mg protein. GCC-ICD$_{\Delta Bam}$ represents the entire intracellular domain of GC-C with the wild-type sequence or with a mutation of a lysine residue to an alanine residue (GCC-ICD$_{\Delta BamK516A}$). GCC-Catbac refers to a protein containing only the catalytic domain of GC-C, without the KHD.

constitutive guanylyl cyclase activity (*35*). We have also shown that a protein comprising only the guanylyl cyclase domain of GC-C (residues 732−1073; GCC-Catbac) is also expressed in a catalytically active form (*36*). We generated a mutant GCC-ICD protein (GCC-ICD$_{\Delta BamK516A}$), wherein the lysine residue (Lys516) shown to be important for ATP-mediated effects in the full-length receptor was mutated to an Ala residue. We monitored the activity of all these recombinant proteins (GCC-ICD$_{\Delta Bam}$, GCC-ICD$_{\Delta BamK516A}$, and GCC-Catbac) in the presence of ATP, with Mg as the metal cofactor during guanylyl cyclase assays.

As shown in Figure 1B, ATP inhibited the activity of GCC-ICD$_{\Delta Bam}$ by ~80%, whereas GCC-ICD$_{\Delta BamK516A}$ showed no inhibition with ATP in the presence of Mg. This is in agreement with the results that we had observed with the full-length receptor, wherein the K516A mutation compromised the inhibition of guanylyl cyclase activity mediated by ATP (*32*). To see if this inhibitory effect was due to ATP interaction with the KHD, we also performed similar assays with GCC-Catbac. No inhibition of cyclase activity was seen with GCC-Catbac (Figure 1B), suggesting that the ATP effects in the presence of Mg may be mediated via binding to the KHD alone.

Conformational Change Induced upon ATP Binding. Earlier studies have shown that ATP could modulate GC-C activity by altering its oligomeric state, which could be

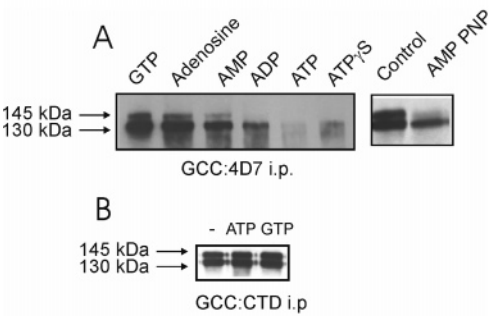

FIGURE 2: GCC:4D7 immunoreactivity is inhibited in the presence of adenine nucleotides. (A) GC-C was solubilized in the presence of various adenine analogues or GTP (1 mM each), followed by immunoprecipitation using GCC:4D7 antibody. The immune complex was subjected to SDS−PAGE, followed by Western Blot analysis using GCC:C8 antibody. Data shown are representative of experiments performed three times. (B) Membrane was solubilized in the absence or presence of ATP or GTP (1 mM each) and immunoprecipitated with a polyclonal antibody directed to the C-terminal domain of GC-C (GCC-CTD). Immune complexes were collected and subjected to SDS−PAGE and Western Blot analysis using GCC:C8 antibody. Data shown are representative of experiments performed three times.

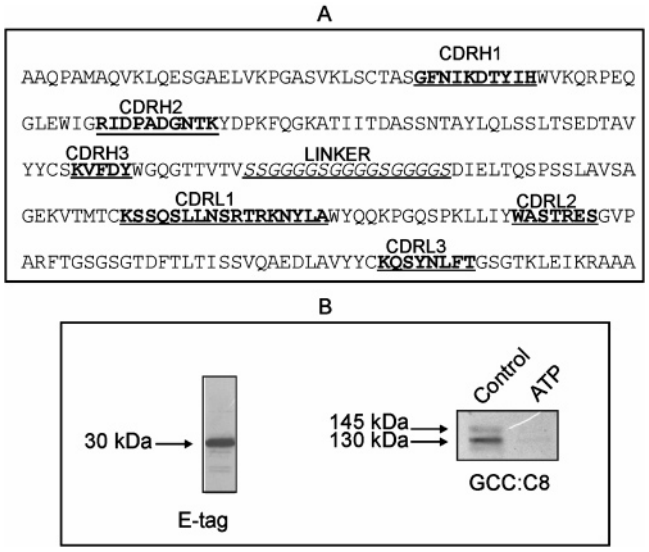

FIGURE 3: GCC:4D7 ScFv does not interact with GC-C in the presence of ATP. (A) The predicted amino acid sequence of the ScFv clone obtained from the GCC:4D7 hybridoma cells. Regions corresponding to variable domains were predicted from the Kabat database (*60*). Complementary determining regions (CDR) are indicated in bold and underlined letters, and the linker is italicized and underlined. (B) Partially purified ScFv was detected by Western Blot analysis using E-tag antibody (left panel). GC-C was solubilized from membranes prepared from HEK293:hGCC cells in either the absence of presence of ATP (1 mM) followed by interaction with ScFv and collection of the immune complex on immobilized anti-E-tag antibody. The immune complex was subjected to Western Blot analysis using GCC:C8 antibody (right panel). Data shown are representative of experiments performed twice.

associated with a dramatic alteration in the conformation of the receptor (*29*, *30*, *32*). A monoclonal antibody, GCC:4D7, is unable to recognize GC-C in the presence of ATP, suggesting that this antibody is a sensitive tool to detect conformational changes in GC-C on interaction with ATP (*32*). Since other adenine nucleotides, such as ATPγS and AMP PNP, were able to mimic the actions of ATP and modulate GC-C activity, it was of interest to see whether these and other ATP analogues had any effect on GCC:4D7 reactivity. Membranes prepared from HEK293 cells expressing GC-C were solubilized in the presence of either AMP PNP, ATPγS, ATP, ADP, AMP, adenosine, or GTP followed by immunoprecipitation of GC-C using GCC:4D7. As shown in Figure 2A, there was a significant loss of reactivity in the presence of ATP, ATPγS, and AMP PNP, which was correlated with the effect of these nucleotides on GC-C activity (Figure 1A). This suggested that a specific interaction of the adenine nucleotide with GC-C induced a conformational change that resulted in an alteration of the presentation of the epitope to which GCC:4D7 was directed.

To check whether the effect of ATP is specific to GCC:4D7 monoclonal antibody or a general effect on interaction of GC-C with any antibody, GC-C was solubilized in either the absence or presence of ATP or GTP, followed by immunoprecipitation using GCC:CTD, a polyclonal antibody raised against the C-terminal domain of GC-C (*32*, *37*, *38*). The polyclonal antibody GCC:CTD immunoprecipitated the receptor in the presence of ATP or GTP (Figure 2B), indicating that loss of immunoreactivity was specific to the GCC:4D7 antibody.

Cloning and Expression of ScFv. To test that the loss of immunoreactivity in the presence of adenine nucleotides was a property of the paratope of GCC:4D7 and not due to steric hindrance contributed by the constant regions of the intact antibody, a single chain antibody (ScFv) was generated using the recombinant phage antibody system. The complimentarity determining region (CDR) of variable regions of GCC:4D7 ScFv in both heavy and light chains were delineated using the Kabat database (Figure 3A). The predicted amino acid sequence confirmed the expected CDR1, CDR2, and CDR3 regions, which were characterized by the positions of conserved amino acids adjacent to these domains.

Soluble ScFv was expressed in HB2151 *E. coli* cells, and since a high amount of protein was detected in the supernatant of cells (data not shown), the culture supernatant was fractionated by ammonium sulfate precipitation to enrich and concentrate the soluble ScFv. Partially purified soluble GCC:4D7 ScFv was recognized by the E-tag antibody (Figure 3B). Membranes prepared from HEK293:hGC-C cells were solubilized in either the absence or presence of ATP, followed by immunoprecipitation of GC-C using the partially purified GCC:4D7 ScFv. The immune complex recovered was subjected to SDS−PAGE followed by Western Blot analysis using GCC:C8 monoclonal antibody. Recombinant GCC:4D7 ScFv did not bind to GC-C in the presence of ATP in a manner similar to that of the full-length antibody molecule (Figure 3B). These results indicate that the sensitivity of the antibody to conformation change in GC-C on ATP binding was a property of the antibody and not due to steric hindrance from other regions in the intact IgG.

Epitope Delineation of the GCC:4D7 Monoclonal Antibody. To identify the region in GC-C to which GCC:4D7 binds, that is, the antibody epitope, and thereby the region in GC-C that alters its conformation on ATP interaction, recombinant proteins encompassing various domains of human GC-C were expressed and purified. Most of the proteins were present within inclusion bodies and were electroeluted from the gel. The eluted proteins were subjected to SDS−PAGE to check for purity (Figure 4, top panel). Western Blot analysis was performed with GCC:4D7, and

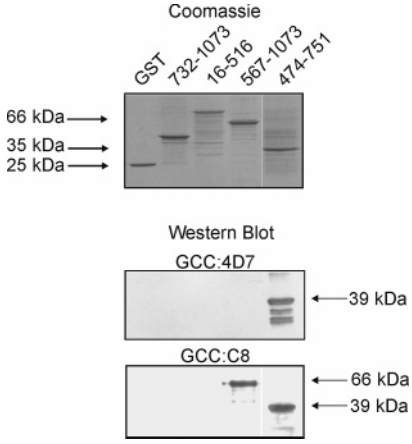

FIGURE 4: Epitope mapping of GCC:4D7 antibody. Various recombinant proteins expressing regions of GC-C were expressed and purified as described in Materials and Methods. The top panel shows the Coomassie staining of purified proteins, and numbers represent the range of amino acids expressed in the recombinant protein. Western Blot analysis of purified proteins using GCC:4D7 and GCC:C8 monoclonal antibodies is shown in the lower panels.

only fragments 474−751 and not regions from 16 to 516, 732 to 1073, or 567 to 1073 were recognized by the antibody. These results led to the conclusion that the epitope lies in the region of residues 517−568 in GC-C. Interestingly, this sequence is immediately carboxyl-terminal to Lys516, which we have shown to be essential for mediating adenine nucleotide effects (*32*). Therefore, GCC:4D7 is sensitive to an epitope that is close to the region where adenine nucleotides interact with GC-C and where a conformational change presumably occurs consequent to ATP interaction. What is striking is that this is also a region which undergoes a dramatic conformational change on ATP binding in many active protein kinases (*39*, *40*).

The GST fusion proteins were not expressed in a soluble form, and hence immunoprecipitation experiments in either the absence or presence of ATP could not be performed (data not shown).

Fine Epitope Mapping by Phage Display. Fine epitope mapping was achieved by the phage display method, and selection of specific phages interacting with GCC:4D7 was achieved after three rounds of biopanning. Immunoreactivity of the phages with GCC:4D7 antibody was confirmed by Western Blot analysis with the phage suspension and GCC:4D7 mAb, and the number of phages loaded on the gel was normalized using anti-gpIII mAb (Figure 5A). Positive phages were sequenced, and the peptides encoded as fusions with gpIII were deduced from the DNA sequence. Deduced amino acid sequences are shown (Figure 5B), and most of the phages have a conserved sequence, DXKFXD. However, differential immunoreactivity was observed, with phage 14 showing lowest recognition to GCC:4D7. This indicates that perhaps the presence of a hydrophobic residue in position 4 of the peptide sequences (Phe in all phage sequences except 14) and a basic residue (Lys or Arg) in position 3 confer a higher affinity of interaction with GCC:4D7.

The sequence alignment of the conserved phage sequence with human GC-C protein sequence in the 517−568 region suggests that a stretch corresponding to the 517DLKHND522 residues, immediately carboxyl-terminal to the Lys516, is the probable epitope for the antibody. Interestingly, this sequence is highly conserved in the GC-C of several species and is absent in other receptor GCs (Figure 5C). The *Anguilla anguilla* ortholog of GC-C also appears to contain a similar sequence with conserved amino acid substitutions, but it is not known at this time whether GCC:4D7 antibody cross-reacts with the eel protein.

Effect of a Mutation in the Epitope of GCC:4D7 on ATP-Mediated Effects. Inspection of the phage sequences indicated that only the first Asp residue was conserved in all phages as well as in the GC-C sequence. In addition, it appeared that the basic residue at the third position in the epitope (Lys or Arg) was also present in the GC-C sequence. Interestingly, a change in this basic residue to an Ala in phage 14 markedly reduces interaction of the phage with GCC:4D7 antibody (Figure 5B). We therefore generated a mutant protein (GCC-ICD$_{\Delta BamK519A}$) where a mutation in Lys519 to an alanine residue was generated, to test both the requirement for this residue in GCC:4D7 immunoreactivity as well as the role of this residue in bringing about ATP-mediated effects. As shown in Figure 6A, no immunoreactivity with GCC:4D7 was seen with either the mutant GCC-ICD$_{\Delta BamK519A}$ protein or the GCC-ICD$_{\Delta BamK516A}$ proteins, indicating that the Lys519 was also a critical residue in the epitope of GCC:4D7. However, high immunoreactivity was observed with the GCC:C8 monoclonal antibody. Interestingly, the guanylyl cyclase activity of GCC-ICD$_{\Delta BamK519A}$ in the presence of MgATP was inhibited to a similar extent as that of the wild-type GCC-ICD$_{\Delta Bam}$ (Figure 6B), in contrast to the results seen with the K516A mutant. Therefore, Lys519 may not directly interact with ATP, and more importantly, the critical role of Lys516 in modulating the guanylyl cyclase activity in the presence of ATP has been shown since a mutation at three amino acids C-terminal to this residue did not alter ATP-mediated effects.

In summary, the results described until now indicate that a dramatic conformational change occurs in GC-C in the presence of ATP, in a region C-terminal to Lys516 that appears to be critical for ATP interaction.

Binding of ATP to GC-C. The results thus far described have identified the critical role of Lys516 in bringing about ATP-mediated effects on guanylyl cyclase activity, strongly suggesting that there may be a direct interaction of ATP with the KHD of GC-C. To date, only one report has indicated binding of ATP to the KHD of GC-A, but these assays were carried out with a radiolabeled analogue of ATP which may cross-link nonspecifically to a protein (*41*). To show direct ATP interaction with the receptor, in a manner similar to that seen in protein kinases, we utilized ATP-agarose beads, which have been used earlier for the purification of protein kinases (*42*). This affinity matrix covalently couples ATP via its modified γ-phosphate to agarose beads. In crystal structures of protein kinases, ATP is positioned such that the adenosine ring is buried in the protein and the γ-phosphate projects into the solvent (*8*). On the basis of our homology modeling studies, we anticipated that the adenosine ring of ATP would also be positioned between the two lobes of the KHD in GC-C, in a manner similar to that seen in active protein kinases (*32*).

We expressed the wild-type and mutant GCC-ICD proteins in insect cells and interacted lysates with ATP-agarose beads, in the absence or presence of ATP or GTP to test for specificity of binding. The beads were washed, and protein

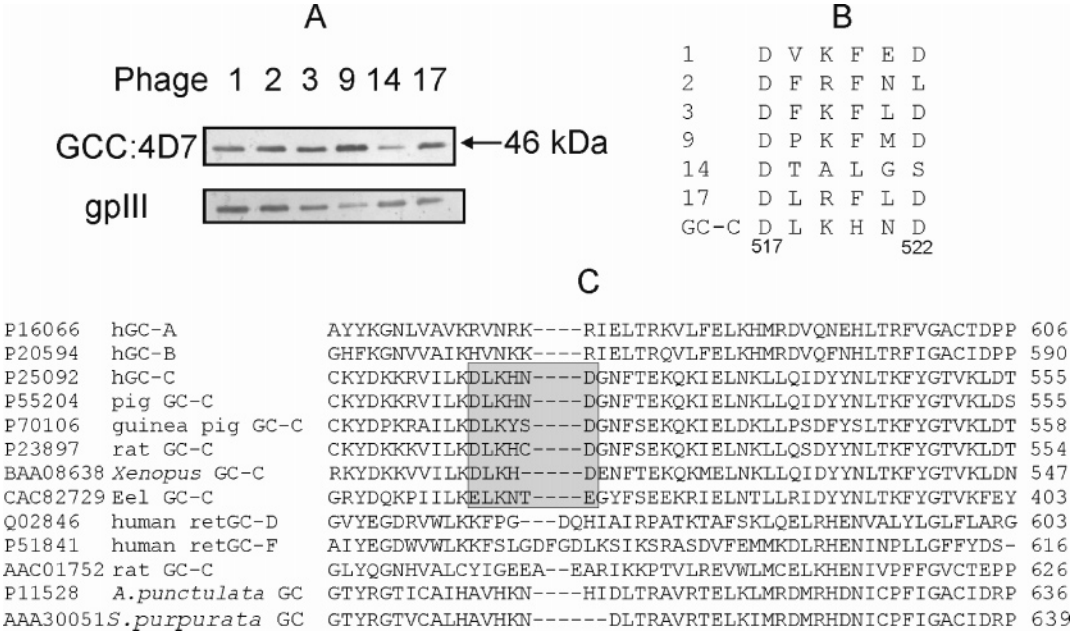

FIGURE 5: Fine epitope mapping of GCC:4D7 antibody by phage display. Phage display was performed using immobilized GCC:4D7 antibody. (A) Specific phages ($\sim 10^{11}$), isolated from GCC:4D7 reacting clones, were resolved by SDS−PAGE followed by Western Blot analysis using GCC:4D7 antibody (upper panel) or anti-gpIII monoclonal antibody (lower panel) to normalize for phage loading. (B) Deduced amino acid sequences of the hexapeptide fused to the gpIII coated protein in reactive phages, aligned with the epitope in GC-C. (C) Alignment of the intracellular domains of membrane guanylyl cyclases generated by Clustal W. The proteins are indicated by their SWISS−PROT accession numbers. The amino acids are numbered with the first residue including the signal sequence.

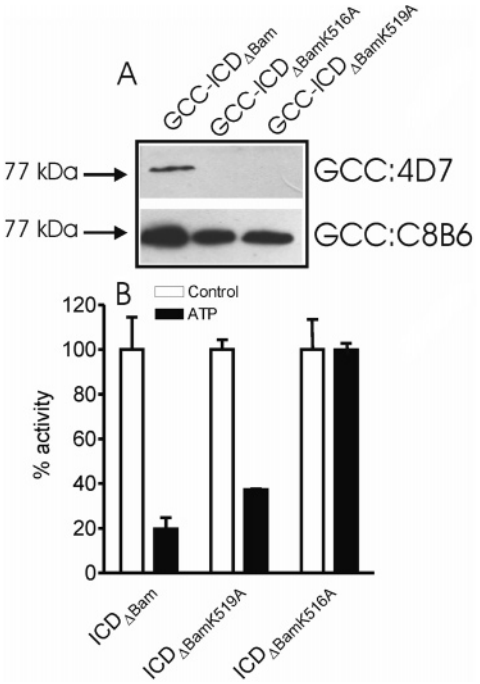

FIGURE 6: Mutation of Lys519A does not alter ATP-mediated effects, but abolishes GCC:4D7 reactivity. (A) Western Blot analysis of Sf21 lysates ($\sim 20\ \mu$g protein) prepared after infection with virus encoding wild-type or mutant GCC-ICD proteins. (B) Guanylyl cyclase activity in crude lysates prepared from Sf21 cells expressing the indicated proteins. Assays were performed in the absence or presence of 200 μM ATP, 1 mM MgGTP, and 10 mM free $MgCl_2$. Values shown represent the mean ± S.E.M. of an assay repeated at least three times, with triplicate determinations.

bound to the beads were checked by Western Blot analysis using GCC:C8 monoclonal antibody. As shown in Figure 7A (left panel), GCC-ICD$_{\Delta Bam}$ bound efficiently to the beads, indicating that the protein could interact with ATP. Significantly, binding could be completely abolished in the presence of ATP, but not GTP. We then used mutant proteins to test for interaction with the ATP-agarose beads. Most interestingly, GCC-ICD$_{\Delta BamK516A}$ was unable to bind to the ATP affinity matrix, while GCC-ICD$_{\Delta BamK519A}$ showed binding equivalent to the wild-type protein (Figure 7A, right panel). These results are in agreement with the earlier biochemical data, indicating that mutation of Lys516 abolished ATP-mediated effects (Figure 1B and ref *32*). Thus, the loss of ATP binding is clearly the cause for the absence of ATP-mediated effects on GC-C. The mutant protein containing Lys519Ala, however, continues to show ATP-mediated effects because it retains the ability to interact with ATP. These results also validate the use of these ATP-agarose beads for identifying the region in the intracellular domain of GC-C that binds ATP.

To identify the domain in GC-C to which ATP binds, a series of proteins representing various domains of GC-C were expressed in insect cells and lysates and were then used for studying ATP interaction. Thus, GCC-KHD-CC, GCC-KHD, and GCC−Catbac were interacted with ATP-agarose beads, and as shown in Figure 7B, only GCC-KHD-CC showed binding to the beads. This is the first demonstration to our knowledge of a kinase homology domain binding to ATP in a manner similar to that seen in protein kinases. Interestingly, GCC-KHD, a shorter protein construct comprising only the sequences that align with protein kinases (*32*), did not bind to the beads, indicating that perhaps the coiled-coil region C-terminus to the KHD, as well as a longer N-terminus, is required for achieving a conformation that is competent to bind ATP. GCC-Catbac showed no binding to the ATP-agarose beads. Therefore, ATP effects on the catalytic activity of GC-C in the presence of Mg as a cofactor

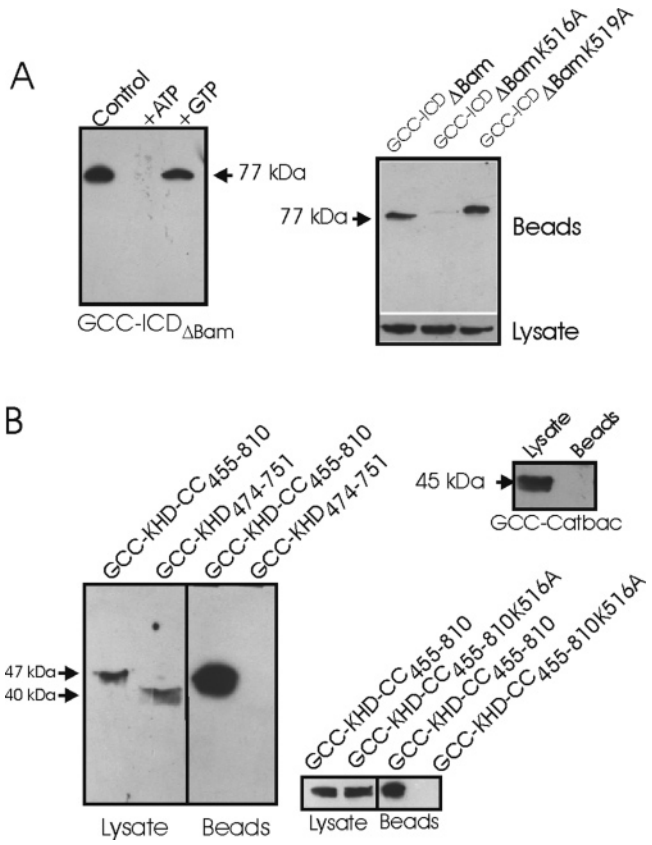

FIGURE 7: Binding of the intracellular domain of GC-C to ATP. (A) Lysates prepared from Sf21 cells expressing the indicated proteins were interacted with ATP-agarose beads, in either the absence or presence of 2 mM ATP or 2 mM GTP. Following interaction, the beads were washed and bound proteins subjected to Western Blot analysis with GCC:C8 antibody. The left panel shows that the intracellular domain of GC-C binds to ATP-agarose, and this binding is inhibited in the presence of excess ATP, but not GTP. In the right panel, wild-type and mutant proteins were used for interaction. Aliquots of the crude lysates were taken for direct Western Blot analysis to normalize for the levels of expressed proteins taken for interaction. Data shown are representative of experiments performed at least three times. (B) Individual domains representing the KHD, the KHD-CC (wild-type and mutant K516A proteins), and the catalytic domain of GC-C were expressed in insect cells and interacted with ATP-agarose beads. Western Blot analysis was performed with aliquots of the lysates taken for interaction and proteins bound to the beads using either GCC:C8 antibody (for KHD proteins) or GCC-CTD antibody for GCC-Catbac (representing the catalytic domain of GC-C). Data shown are representative of experiments performed at least three times.

are indeed brought about by binding of ATP to the KHD of GC-C.

To show the specificity of the interaction of the KHD with ATP, we mutated Lys516 to an Ala in the KHD-CC construct and interacted the expressed protein with ATP-agarose. As shown in Figure 7B, the mutant protein was unable to bind to the affinity matrix, clearly showing the critical role of this Lys residue in the KHD of GC-C in binding ATP. It is interesting to note at this time that mutations of the corresponding lysine residue in active protein kinases, such as protein kinase A and Erk, did not appear to abolish ATP binding (*40*, *43*). It perhaps can be suggested that the requirement for this Lys residue in pseudokinase domains may be more stringent than that seen in active protein kinases, where additional residues could allow ATP binding in the cleft between two kinase lobes, albeit in a position not favorable for phosphotransfer.

In summary, with these experiments, we have shown that the KHD of GC-C is the site for ATP binding and identified a region of the KHD that undergoes a conformational change on ATP interaction, thereby bringing about a reduction in the catalytic activity of GC-C.

DISCUSSION

The studies described here are the first to show direct ATP binding to the KHD of GC-C and indeed ATP binding to any pseudokinase domain, indicating that despite the low degree of sequence identity (~20%) between protein kinases and pseudokinases, structural features required for ATP binding appear to be conserved. The successful expression of the KHD of GC-C in insect cells in a functional form, and its enrichment through ATP-agarose that we describe here, may facilitate the structural elucidation of KHDs and, indeed, the intracellular domains of proteins that possess kinase-like domains fused to additional catalytic domains. We have also shown the critical role that Lys516 plays in binding ATP.

There is earlier evidence to suggest that monoclonal antibodies can be used to infer structural alterations in proteins as a consequence of ATP binding. In one study, decreased reactivity of mAb UIC2 specific to the ATP-dependent multidrug transporter P-glycoprotein (PgP) is seen in the presence of ATP, ADP, and nonhydrolyzable ATP analogues. In this protein, conformational changes may occur in the presence of ATP, which regulates the process of pumping drugs out of the cell (*44*). In an independent study, the epitope of a mAb raised against the nucleotide binding subunit MalK of the maltose ATP-binding cassette transporter of *Salmonella typhimurium* became less accessible in the presence of ATP, suggesting that there is an ATP-induced structural alteration (*45*). To our knowledge, our study is the first to show that a monoclonal antibody is sensitive to changes on ATP binding in a pseudokinase domain. An antibody to phosphorylase kinase shows increased reactivity on activation of the kinase in the presence of magnesium, but similar studies were not performed in the presence of ATP, and neither was the epitope for this antibody mapped (*46*).

The JH2 domain is the site for interaction of Jaks with STATs (*47*). It has also been demonstrated that an intramolecular interaction between the JH2 domain and the catalytic kinase domain occurs through co-immunoprecipitation and independent co-expression of pseudokinase and kinase domains (*3*), and this could account for the autoinhibitory effects of the pseudokinase domain. Similarly, in GC receptors, the KHD could have a regulatory role in signaling and may serve as a docking region for interacting proteins, or perhaps the guanylyl cyclase domain itself, in an intramolecular interaction, rather than being involved in a phosphorylation reaction. Indeed, there is evidence to suggest that HSP90 (*48*) and a specific phosphatase (*49*) are associated with GC-A in its KHD region.

The results described in this report have identified a region in the KHD of GC-C that alters its conformation with ATP interaction. On the basis of the homology modeling that we have performed earlier, as well as our results with ATP-

agarose beads suggesting the KHD can adopt a structure similar to that of protein kinases, it is instructive to consider conformational changes that have been shown to occur in kinases with ATP binding and activation and extrapolate these to the KHD of GC-C.

Increasing numbers of crystal structures of several protein kinases provide an insight into the regulation and specificity of kinases. The conformational flexibility generated in the absence or presence of ATP allows switching of the kinase from an "off" to "on" state, which is a central feature of the regulatory mechanism (*40*). Crystal structures of protein kinases have revealed a common two-domain core structure with enzyme-specific variations (*8*). The small amino-terminal domain consists of five antiparallel β strands and a C-helix. The large carboxyl-terminal domain consists of the substrate-binding site and is predominantly helical. ATP binds in the deep cleft between these two domains (*50*). There is no single conformation for the ATP-bound complexes of various protein kinases, but it is clear from extensive crystallographic evidence that the mechanism of regulation utilizes the upper domain of the conserved three-dimensional template. In several kinases, there is a considerable difference in the conformation of the protein in the presence or absence of ATP (*51*, *52*).

In some kinases, two critical residues, Lys and Glu, interact in the presence of ATP, inducing a slight closure of the cleft (*50*). For example, in the case of active, ATP-bound protein kinase A, Lys72 that makes crucial contacts with the α- and β-phosphate groups of ATP is buried deep within the interlobe cleft, where it is stabilized and oriented properly by an ionic interaction with Glu91 (*39*). In the absence of ATP, the two lobes are swung apart. On the basis of our previous analysis by computational modeling, it can be hypothesized that, in the presence of ATP, the phosphate groups of ATP could interact with the conserved Lys516, which could form a salt bridge with Glu532, as predicted by the homology model of the KHD of GC-C (*32*). Hence there could be closure of the lobes and the region around Lys516, whereby the epitope for GCC:4D7 is no longer accessible for interaction with the antibody.

The crystal structures of active CDK (*53*) and inactive CDK (*54*) suggest that, in the presence of ATP, Lys33 and Glu51 come together, and this induces a slight closure of the cleft. In the absence of ATP, these invariant residues are positioned apart. In inactive CDK2 and inactive down-regulated Src and Hck, the C-helix is swung outward and rotated by about 90° relative to its position in the active kinase structures. Thus the ion pair between the Glu from the C-helix and the Lys that chelates the α- and β-phosphates of ATP is not made (*52*). The openness of lobes was also observed in inactive FGF receptor kinase (*55*), insulin receptor tyrosine kinase (*56*), MAP kinase ERK2 (*57*), and p38 mitogen-activated protein kinase (*58*). On the basis of these findings, it can be suggested that Lys516 and the GC-C:4D7 epitope in GC-C may be available for the antibody interaction in the absence of ATP due to an "open" structure of the two lobes, and "closure" of the lobes occurs on ATP binding, thereby precluding antibody access.

These conformational changes produced in the receptor in the presence of ATP could facilitate a change in the oligomeric state of the receptor, which in turn regulates the activity. This is in agreement with the fact that, in the presence of ATP, there is a change in the oligomeric state of the receptor (*30*, *32*). Both full-length GC-C and the intracellular domain of GC-C (*35*) are expressed in monomeric and higher oligomeric forms, and interaction with ATP converts the larger oligomer into a smaller-sized protein (*30*, *32*). In the case of ERK2 kinase, the activation loop has the capacity to undergo large conformational changes that result in rearrangement of molecular interactions and modulates its oligomeric state, resulting in the formation of dimers (*57*). It is conceivable that the presentation of hydrophobic surfaces on GC-C is also altered in the presence of ATP, and this accounts for changes in its oligomeric state, perhaps masking the epitope of GCC:4D7 and resulting in loss of antibody interaction.

We can therefore propose a model where membrane-associated GC-C is present in a conformation that is unable to bind ATP, in a manner similar to that seen in the inactive conformations of many protein kinases. Ligand binding to GC-C may then induce a conformational change that *activates* the guanylyl cyclase domain, as well as alters the conformation of the KHD in such a way as to allow ATP to bind since ATP concentrations are high intracellularly (1−3 mM). This ATP binding may then stabilize the active conformation, thereby leading to *enhanced* cGMP production. In contrast, the detergent-stimulated state of GC-C, as well as the entire intracellular domain of GC-C, may adopt entirely different and perhaps nonphysiological conformations, which allow ATP binding to the KHD, resulting in an *inhibition* of the catalytic activity. These remain speculations as of now, and we are attempting to demonstrate ATP binding to the membrane-associated form of GC-C, as has been demonstrated recently for GC-A (*59*).

In conclusion, these studies represent the first evidence that shows ATP binding with the KHD of GC-C and identify a critical residue that is required for ATP interaction. Further mutational analyses will reveal the role of other residues in the KHD vis-à-vis their role in the protein kinases. The conservation of both the sequence and structural elements across diverse groups in the protein kinase family provides evidence that these sites involved in modulation of activity represent evolved physiological control mechanisms. Using a monoclonal antibody to GC-C, the results described in this study predict that the dramatic alteration in conformation that is observed upon the binding of ATP in protein kinases is also mimicked in the KHD of GC-C. This could bring about both local and global conformational changes in the receptor, which could result in alteration of the interaction of KHD with other domains of GC-C or its associated proteins, thereby regulating GC-C activity. In addition, these conformational changes which appear to occur upon binding of ATP to the KHD of GC-C could be mimicked in the large number of pseudokinase domains that have been identified in diverse proteins.

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