Functional Inactivation of the Human Guanylyl Cyclase C Receptor: Modeling and Mutation of the Protein Kinase-like Domain†

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ABSTRACT: Receptor guanylyl cyclases possess an extracellular ligand-binding domain, a single transmembrane region, a region with sequence similar to that of protein kinases, and a C-terminal guanylyl cyclase domain. ATP regulates the activity of guanylyl cyclase C (GC-C), the receptor for the guanylin and stable toxin family of peptides, presumably as a result of binding to the kinase homology domain (KHD). Modeling of the KHD of GC-C indicated that it could adopt a structure similar to that of tyrosine kinases, and sequence comparison with other protein kinases suggested that lysine$_{516}$ was positioned in the KHD to interact with ATP. A monoclonal antibody GCC:4D7, raised to the KHD of GC-C, did not recognize ATP-bound GC-C, and its epitope mapped to a region in the KHD of residues 491−568 of GC-C. Mutation of lysine$_{516}$ to an alanine in full-length GC-C (GC-C$_{K516A}$) dramatically reduced the ligand-stimulated activity of mutant GC-C, altered the ATP-mediated effects observed with wild-type GC-C, and failed to react with the GCC:4D7 monoclonal antibody. ATP interaction with wild-type GC-C converted a high-molecular weight oligomer of GC-C to a smaller sized oligomer. In contrast, GC-C$_{K516A}$ did not exhibit an alteration in its oligomeric status on incubation with ATP. We therefore suggest that the KHD in receptor guanylyl cyclases provides a critical structural link between the extracellular domain and the catalytic domain in regulation of activity in this family of receptors, and the presence of K$_{516}$ is critical for the possible proper orientation of ATP in this domain.

Guanylyl cyclase C (GC-C)[1] is a member of the family of membrane-associated guanylyl cyclases, and serves as the receptor for the guanylin peptides and heat-stable enterotoxins (ST) (*1*). GC-C is predominantly expressed in intestinal epithelial cells (*2*). Binding of ligands to the extracellular domain of GC-C induces a conformational change in the receptor, leading to activation of the intracellular catalytic domain that harbors guanylyl cyclase activity. Increased intracellular levels of cGMP elicit a cascade of signaling events leading to an increased level of chloride secretion from the intestinal cell, and the characteristic diarrhea associated with the bacterial enterotoxin peptides (*3*).

Mammalian receptor guanylyl cyclases characterized so far include GC-A and GC-B, the receptors for the atrial natriuretic peptides, and retinal guanylyl cyclase, which controls cGMP levels in the retina cell during visual signal transduction (*4*). Precise molecular details about the mechanism of activation of these receptors are currently not available. Biochemical evidence suggests that receptor guanylyl cyclases are present as dimers or higher oligomers even in the absence of their cognate ligand (*5*), and the crystal structure of the extracellular domain of GC-A has demonstrated the presence of an ANF-bound dimer (*6*).

All receptor guanylyl cyclases possess a single transmembrane-spanning domain and a large intracellular domain (*7*). The extreme C-terminus of the intracellular domain harbors the catalytic guanylyl cyclase domain, and juxtaposed between the transmembrane and cyclase domain is a region with some degree of homology to protein kinases. This kinase homology domain (KHD) appears to play an important role in modulating the catalytic activity of these receptors. ANF-mediated activation of GC-A and GC-B requires the presence of ATP (*8*, *9*). GC-C is activated by its ligands in vitro even in the absence of ATP (*10*, *11*), but ATP, and nonhydrolyzable analogues of ATP, potentiate ligand-mediated activation of GC-C, indicating that this is mediated by ATP binding per se, and does not require the hydrolysis of ATP (*12*−*17*). ATP has also been shown to bind to and potentiate the activity of retinal guanylyl cyclase purified from bovine rod outer segment membranes (*18*). Therefore, the KHD in receptor guanylyl cyclases, in the absence of ATP, negatively regulates the guanylyl cyclase activity of this family of receptors.

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[1] Abbreviations: GC-A, guanylyl cyclase A; GC-B, guanylyl cyclase B; ST, heat-stable enterotoxin; GC-C, guanylyl cyclase C; KHD, kinase homology domain; ANP, atrial natriuretic peptide; cAPK, cAMP-dependent protein kinase; STh, heat-stable enterotoxin from human isolate; ST$_{Y72F}$, STh with replacement of tyrosine at amino acid position 19 in the mature peptide with phenylalanine; FSBA, 5′-(*p*-fluorosulfonylbenzoyl)adenosine; GST, glutathione *S*-transferase; PVDF, polyvinylidene fluoride; PCR, polymerase chain reaction; IBMX, isobutyl methyl xanthine; CFTR, cystic fibrosis transmembrane conductance regulator.

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The role of the kinase-like domain in modulating guanylyl cyclase function has been further investigated by the construction of KHD deletion mutants and chimeric guanylyl cyclases. In the case of the natriuretic peptide receptor, GC-A, deletion of the kinase homology domain resulted in a ligand-independent, constitutively active guanylyl cyclase, indicating that the KHD is a negative regulator of cyclase activity (*8*). A chimeric receptor in which the kinase homology domain of GC-A was replaced with that of GC-C, or with the catalytic domain of the EGF receptor tyrosine kinase, resulted in a protein that was unresponsive to natriuretic peptides (*19*). This suggests that despite a similarity in sequence, there are significant differences in the regulation of guanylyl cyclases by their cognate kinase-like domains. Deletion of the KHD of human GC-C also resulted in a constitutively active cyclase, as observed in the case of GC-A, indicating a similar negative modulation of GC-C by its kinase-like domain (*20*). A contrasting report, however, demonstrated that deletion of the kinase domain in rat GC-C resulted in a completely inactive guanylyl cyclase that is unresponsive to ST (*21*).

ATP binding sites in proteins may be classified into two broad types based on function (*22*). These are catalytic sites, which include protein kinases, ATP synthases, and adenyltransferases, and regulatory sites, such as those found in aspartate carbamoyl-transferase and phosphofructokinase. The prototype for a discussion of the three-dimensional structure of the catalytic core of protein kinases is cyclic AMP-dependent protein kinase (cAPK) (*23*). Kinases consist of two lobes, with the smaller lobe, derived primarily from the amino terminus, and a large C-terminal lobe. A conserved hydrophobic pocket is formed at the domain interface, which positions the adenine ring of MgATP in such a way as to allow transfer of the γ-phosphate of ATP to various substrates (*24*). Catalysis occurs at the cleft between the lobes, and binding of a substrate causes cleft closure. Many of the secondary structural elements found in cAPK are conserved in the structures of other protein kinases, including the Src and Hck tyrosine kinases, and structural changes that are observed are reflections of differences in the function or biological role of individual kinases (*25*).

Most of the residues that are conserved in the family of kinases have structural and functional relevance. The glycine-rich loop between β-strands 1 and 2 provides conformational flexibility for avoiding a steric clash with ATP (*26*). Furthermore, the γ-phosphate of ATP is anchored via hydrogen bonding to backbone amides in the glycine-rich loop (*24*). A lysine residue, at position 72 in cAPK, is fixed by its ionic interaction with Glu_{91}, and ion pairs with the α- and β-phosphates of ATP (*24*). Mutation of this lysine residue in many protein kinases completely abolishes protein kinase activity (*27*, *28*), indicating its critical role in the positioning of ATP for phospho transfer. Two residues that are involved in chelating the Mg ions, namely, Asn_{171} and Asp_{184} in cAPK, are also conserved in all protein kinases (*29*). Therefore, homology modeling of the KHD of GC-C would allow the identification of structural features common to this domain and protein kinases, and identify this region as being involved in binding ATP and thereby regulating guanylyl cyclase activity.

In this paper, we have examined the regulation of human GC-C by ATP and the importance of the KHD of GC-C in mediating the effects of ATP on guanylyl cyclase activity. We have modeled the structure of the kinase-like domain of GC-C on the basis of its similarity to the catalytic domains of protein kinases. We have mutated a lysine residue in the KHD of GC-C, which is conserved in all known protein kinases and is known to stabilize the position of the ATP, and this mutation leads to dramatic alterations in the signaling properties of the receptor.

EXPERIMENTAL PROCEDURES

Materials. All fine chemicals were from Sigma Chemical Co. (St. Louis, MO). Tissue culture media, protein G agarose, and Lipofectamine were from Life Technologies. Fugene transfection reagent was obtained from Roche Molecular Biochemicals. Restriction enzymes were obtained from New England Biolabs. T84 (CCL 247) and COS7 cell lines were obtained from ATCC (Rockville, MD). Stable toxin of the human variety (STh) and a mutant form of the STh peptide (ST_{Y72F}) were purified from strains hyperexpressing the peptide, as described previously (*30*, *31*).

Comparative Modeling of the Kinase Homology Domain of GC-C. All the known structures of kinases were aligned, using *COMPARER* (*32*, *33*), which performs the alignment on the basis of the structural features (such as solvent accessibility and secondary structure) and relationships such as hydrogen bonding patterns. The sequence of the KHD was then aligned with the kinase structures by matching the template representing the known kinase structures and by considering the probability of a residue in the KHD adopting the structural environment of equivalent residues in the known kinase structures.

The suite of programs encoded in *COMPOSER* (*34*−*37*) and incorporated in *SYBYL* (Tripos Inc., St. Louis, MO) was used to generate the three-dimensional model of the KHD. The structures of the conserved regions of Src kinases, which are most similar to the KHD, were used to define a framework of Src kinase structures as the weighted average of equivalent Cα atoms in the conserved regions (*36*). A given conserved region of the KHD was modeled by superimposing the equivalent region of a known Src kinase structure, whose sequence is the most locally similar to that of the KHD, onto the equivalent framework region. The variable regions were modeled by identifying a suitable segment from a known kinase structure or by searching for a suitable fragment in the data bank of known protein structures, and using a template matching approach to rank the candidate loops (*38*). Loops with no short contacts with the rest of the protein were fitted using a ring-closure procedure.[2] Side chains were modeled either by extrapolating from the equivalent positions in the basis structure or by using rules derived from the analysis of known protein structures (*35*).

Since the Src family of kinases was crystallized along with analogues of ATP, the binding of ATP to the KHD was modeled by using the structure of cAPK with the bound ATP (*24*). Positions of side chains at the ATP-binding site in the KHD were manually adjusted using the interactive graphics in SYBYL and analyzed using SETOR (*39*) to optimize the interactions between ATP and the KHD.

[2] F. Eisenmenger, unpublished results.

The *COMPOSER*-generated model was energy minimized in *SYBYL* using the *AMBER* force field (*40*). During the initial cycles of energy minimization, the backbone was kept rigid and side chains alone were moved. Subsequently, all atoms in the structure were allowed to move during minimization. Energy minimization was performed until all short contacts, and inconsistencies in geometry were rectified. During the initial stages of minimization, the electrostatic term was not included, since the main objective was to relieve steric clashes and rectify bad geometry. The stereochemical quality of the final model was ensured by the program *PROCHECK* (*41*).

Analysis of Monoclonal Antibody GCC:4D7 Binding to GC-C Expressed in HEK 293 Cells. A stable cell line of HEK293 cells expressing wild-type GC-C was generated as described previously (*42*) and called HEK293:hGCC-C5. This cell line was responsive to the stable toxin in terms of accumulation of cGMP on application of the toxin to monolayer cultures of the cells. Membranes were prepared from this cell line as described previously (*42*), and the amount of protein was estimated using a modification of the Bradford protein assay (*43*). GC-C content in the membrane was monitored by performing receptor binding analysis with a radiolabeled ST peptide as described previously (*31*). Binding data were analyzed with GraphPad *PRISM* (San Diego, CA) to determine the K_d and B_{max}.

GC-C from these cells was solubilized from the prepared membrane fraction (300 μg) in the presence of 1% Triton X-100, 2 mM $MgCl_2$, and varying concentrations of ATP or 1 mM GTP, for 1 h at 4 °C. Solubilized protein (150 μg) was incubated with GCC:4D7 (2 μg/mL), and incubation was continued for 6 h, after which the immunocomplex was recovered by immunoprecipitation with protein A agarose. The protein A beads were washed and subjected to SDS gel electrophoresis and Western blot analysis using the monoclonal antibody to GC-C, GCC:C8, as described previously (*42*). In parallel experiments, a solubilized membrane preparation was incubated directly without or with 4 mM Mg and 1 mM ATP for 16 h, and then subjected to Western blotting.

Mutation of Lysine$_{516}$ to Alanine. Lysine$_{516}$ in the protein kinase-like domain of GC-C was mutated to alanine by PCR-based mutagenesis using the megaprimer method (*44*). Human GC-C cDNA (*45*) was used as the template, and the following oligonucleotides were used as primers to generate a 198 bp fragment of the intracellular domain of GC-C containing the point mutation: forward primer, 5′AGAAAATAGGTACCAGATTATGAACTTCGT3′ starting at position 1480; and reverse primer, 5′GTGCTTGAGATCAGCGAGAATCACTCG3′ starting at position 1608. This fragment was used as the megaprimer along with another reverse primer (5′CTCATCTCGAGTTAAAAATAGGTGC3′) (starting at position 3350) to generate a PCR fragment which was then digested with the restriction enzymes, *Cla*I and *Bam*HI, flanking the mutation site and subcloned into pBluescript KS+ (Stratagene). The mutation was confirmed by DNA sequencing using an automated DNA sequencer (Applied Biosystems). GC-C cDNA was digested with *Kpn*I and *Sma*I and inserted into *Kpn*I- and *Eco*RV-cut pCDNA3. This was digested with the *Cla*I−*Bam*HI fragment and replaced with the *Cla*I−*Bam*HI fragment carrying the mutation. An N-terminal *Kpn*I−*Kpn*I fragment from GC-C was subsequently inserted into the *Kpn*I site of this clone to obtain full-length GC-C with the point mutation K516A in pCDNA3 to generate pCDNA3:GC-C$_{K516A}$. Wild-type GC-C was also subcloned into pCDNA3 as described previously (*42*).

Epitope Delineation of the GCC:4D7 Monoclonal Antibody. A fusion protein of glutathione *S*-transferase (GST) with the kinase homology domain of GC-C has been described previously (*55*). The protein was localized to inclusion bodies, and therefore purified in a denatured state by electroelution from SDS gels. A GST fusion protein with amino acids from position 491 to 568 of GC-C was generated by digesting wild-type pBSK containing the GC-C cDNA with a *Cla*I−*Bam*HI fragment and cloning the 227 bp fragment into pBluescript II KS+ (Stratagene), digested with the same enzymes. From this clone (pBKS GC-C$_{491-568}$), a fragment released on digestion with *Sal*I and *Not*I (252 bp) was cloned into pGEX-5X3 (Amersham Pharmacia) digested with *Sal*I and *Not*I, to generate the plasmid pGEX GC-C$_{491-568}$. Expression of the protein was achieved on addition of isopropyl thiogalactoside to DH5α cells harboring plasmid pGEX GC-C$_{491-568}$. Fusion protein, with a molecular mass of 35 kDa, was purified from the inclusion body fraction and used for Western blot analysis with the GCC:4D7 antibody, as described above.

Generation of a Stable HEK293 Cell Line Expressing GC-C$_{K516A}$ and Measurement of the Levels of ST-Stimulated cGMP Production and ST Binding. HEK293 cells were cultured in DMEM:F12 containing 10% fetal calf serum. Mutant GC-C (GC-C$_{K516A}$) cDNA cloned into pCDNA3 was transfected using Lipofectamine or Fugene, per the manufacturer's protocol (*42*), and cells were treated with 400 μg/mL G418 72 h after transfection. Clones were isolated, and a cell line (HEK293:hGCC$_{K516A}$) was generated following screening of clones by checking the presence of GC-C by Western blot analysis with the GCC:C8 monoclonal antibody. HEK293:hGCC-C5 cells or HEK293:hGCC$_{K516A}$ cells were washed with serum-free DMEM:F12 and incubated in medium containing 1 mM IBMX for 30 min at 37 °C in a 5% CO_2 humidified incubator. STh (10^{-7} M) was then added to the cells and incubation continued for 1 h, after which the cells were lysed and the cGMP content was measured by radioimmunoassay (*46*).

GC-C$_{K516A}$ content in membranes prepared from cells was monitored as described previously (*31*).

Preparation of Membranes from Cells and in Vitro Guanylyl Cyclase Assays. Guanylyl cyclase assays were carried out with membranes prepared from HEK293 cells expressing GC-C or GC-C$_{K516A}$ as described previously (*13*, *42*). Briefly, the crude membrane fraction was isolated from cells (*42*) and protein content estimated (*43*). For assays involving ligand-mediated activation of GC-C, membrane protein (5−30 μg) was incubated in assay buffer [60 mM Tris-HCl (pH 7.6) containing 500 μM IBMX and an ATP/GTP regenerating system consisting of 10 μg of creatine phosphokinase and 7.5 mM creatine phosphate] in the presence or absence of 10^{-7} M STh and 1 mM ATP. Substrate (1 mM GTP and 10 mM $MgCl_2$) was then added, and the tubes were incubated at 37 °C for 10 min. For assays which monitored ATP effects on Lubrol PX-stimulated activity, membranes were incubated with 0.3% Lubrol-PX in the absence or presence of varying concentrations of ATP for 10 min at 4 °C, after which substrate (1 mM GTP and

20 mM $MgCl_2$) was added and incubation continued for a further 10 min at 37 °C. In some experiments, 20 mM $MnCl_2$ was used along with 1 mM GTP as the substrate instead of $MgCl_2$, following incubation of the membranes in the presence or absence of varying concentrations of ATP, as described above. The reaction was terminated by the addition of 50 mM sodium acetate (pH 4.0); samples were heated in a boiling water bath for 5 min, and the supernatant was assayed for cGMP by radioimmunoassay (*46*).

Monitoring the Oligomeric Status of GC-C. The oligomeric status of GC-C expressed in HEK293 cells was studied using a modification of the method described by Vaandrager et al. (*15*). Membranes were prepared as described above, but DTT was excluded from the cell lysis buffer. Membrane protein (600 μg from HEK293:hGCC-C5 and 300 μg from HEK293:$hGCC_{K516A}$) was solubilized by incubation at 4 °C for 1 h in immunoprecipitation buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X100, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 100 μM sodium orthovanadate], in the presence or absence of ATP (1 mM). The solubilized protein was then incubated with an antibody to the C-terminal domain of GC-C (GCC-CTD; 20 μg/mL) for 16 h, followed by collection of the immunocomplexes with protein A agarose beads. The beads were washed in immunoprecipitation buffer with or without ATP (1 mM), as required, followed by incubation in Laemmli buffer without β-mercaptoethanol at 60 °C for 10 min, and resolution on a 6% SDS−polyacrylamide gel. The proteins were transferred to PVDF membrane, and Western blot analysis was carried out using the GC-C monoclonal antibody GCC:C8.

RESULTS

Modeling of the KHD of GC-C. We worked with the assumption that the KHD could adopt a conformation similar to that of tyrosine kinases, and therefore could bind ATP. The sequence of the KHD is more similar to that of protein tyrosine kinases, such as the insulin and fibroblast growth factor receptor kinases (*47*, *48*) as well as variants of the Src family tyrosine kinases (*49*−*51*). Structures of full-length c-Src and Hck tyrosine kinases are available in the autoinhibited form (*50*, *51*), and used during the modeling.

An alignment of the sequence of the KHD with the kinase domains of the tyrosine kinases of the Src family, and the serine-threonine cAMP-dependent kinase (cAPK), is shown in Figure 1. The alignment begins at residue 490 in GC-C, corresponding to the start of the kinase-like domain (the first amino acid of the signal peptide is taken to be residue 1). The level of sequence identity between the KHD and Src tyrosine kinases varies between 26 and 28%, but the level of sequence similarity (computed by scoring amino acid substitutions) is as high as 64%. The sequence of the KHD could be comfortably accommodated in the kinase fold (Figure 2a), and almost all the conserved helical and β-strand regions of tyrosine kinases were conserved in the KHD. However, the first β-strand seen in tyrosine kinases (*50*, *51*) was not modeled in the KHD, because of poor sequence similarity.

Most of the core-forming residues are well conserved between Src kinases and the KHD, but a few significant amino acid changes are observed. The replacement of buried, apolar residues of Src kinases with polar residues in the KHD appears to be accompanied by compensatory mutations in the KHD. For example, Phe_{307} of Src kinase is buried, and this feature is conserved among tyrosine kinases of known structure (Figure 1). This residue is replaced with Gln_{529} in the KHD, but the inclusion of Asp_{509} in an insertion of six residues (504−509) in the KHD allows side chains of Asp_{509} and Gln_{529} to form a hydrogen bond. In addition, Thr_{550} of the KHD replaces a buried and conserved valine residue (position 328 in Src), and is also in a position to interact with Gln_{529} (Figure 1). Thr_{545} in the KHD replaces Val_{323} of Src kinases, which is conserved and usually buried in tyrosine kinases, and the compensatory mutation is Thr_{631} of the KHD, which replaces a largely conserved Ala (corresponding to position 403 of Src) in tyrosine kinases.

The critical catalytic base-containing sequence motif of protein kinases, HRD, is replaced by HGR in the KHD. A multiple-sequence alignment of all the known related guanylyl cyclase receptor sequences (*13*) shows that His and Gly are absolutely conserved among the homologues. The Arg is replaced with Ser or Asn in some of the receptor cyclases. The absence of the HRD motif in the KHD suggests that a phospho transfer catalytic mechanism, if present, is likely to be completely different from that of protein kinases. Indeed, there is only a single report so far of a kinase activity in a receptor cyclase (*18*).

The KHD lacks the Gly-rich motif in the smaller lobe that is associated with ATP binding (*52*). The aminoglycoside kinase, APH(3′)-IIIa, which catalyzes the phosphorylation of a broad spectrum of aminoglycoside antibiotics, lacks the Gly-rich loop, but has the same fold as protein kinases despite poor sequence similarity (*53*). Thus, the absence of the Gly-rich motif does not necessarily indicate the inability of a protein to bind ATP. We modeled the ATP found in the cAPK crystal structure (*24*) in the kinase fold of the KHD, and indeed, ATP could be accommodated without any steric hindrance or unfavorable interactions (Figure 2a).

A number of conserved residues in protein kinase structures are conserved or conservatively substituted in the KHD (Table 1). Some of these residues are directly involved in hydrogen bonding to ATP. One such critical residue is Lys_{516} (corresponding to Lys_{72} in cAPK), which is involved in interaction with the α- and β-phosphates of ATP. This lysine residue is conserved in all kinases (*52*), including the bacterial aminoglycoside kinase that has a low level of sequence similarity with other protein kinases, but has the same structural fold (*53*). Figure 2b highlights the interaction of this Lys (K_{516}) in the KHD with ATP. Glu_{532} in the KHD corresponds to Glu_{91} in cAPK, which has been shown to form an ion pair with Lys_{72} in active forms of protein kinases (*25*). This interaction is, however, disrupted in the down-regulated forms of the Src kinases, for which crystal structures are available, due to a change in the orientation of α-helix C (*54*). There are other residues in the KHD that are not conserved in protein kinases, such as Arg_{496} and Arg_{614}, that could interact with ATP in the KHD (Figure 2b). In addition, Tyr_{563} of the KHD is found in the vicinity of the adenine base in ATP, and could interact with the ring of adenine. On the basis of this modeled structure of the KHD, we suggest that this domain of GC-C can adopt a structure that is the binding site for ATP, and thereby regulate the activity of the receptor.

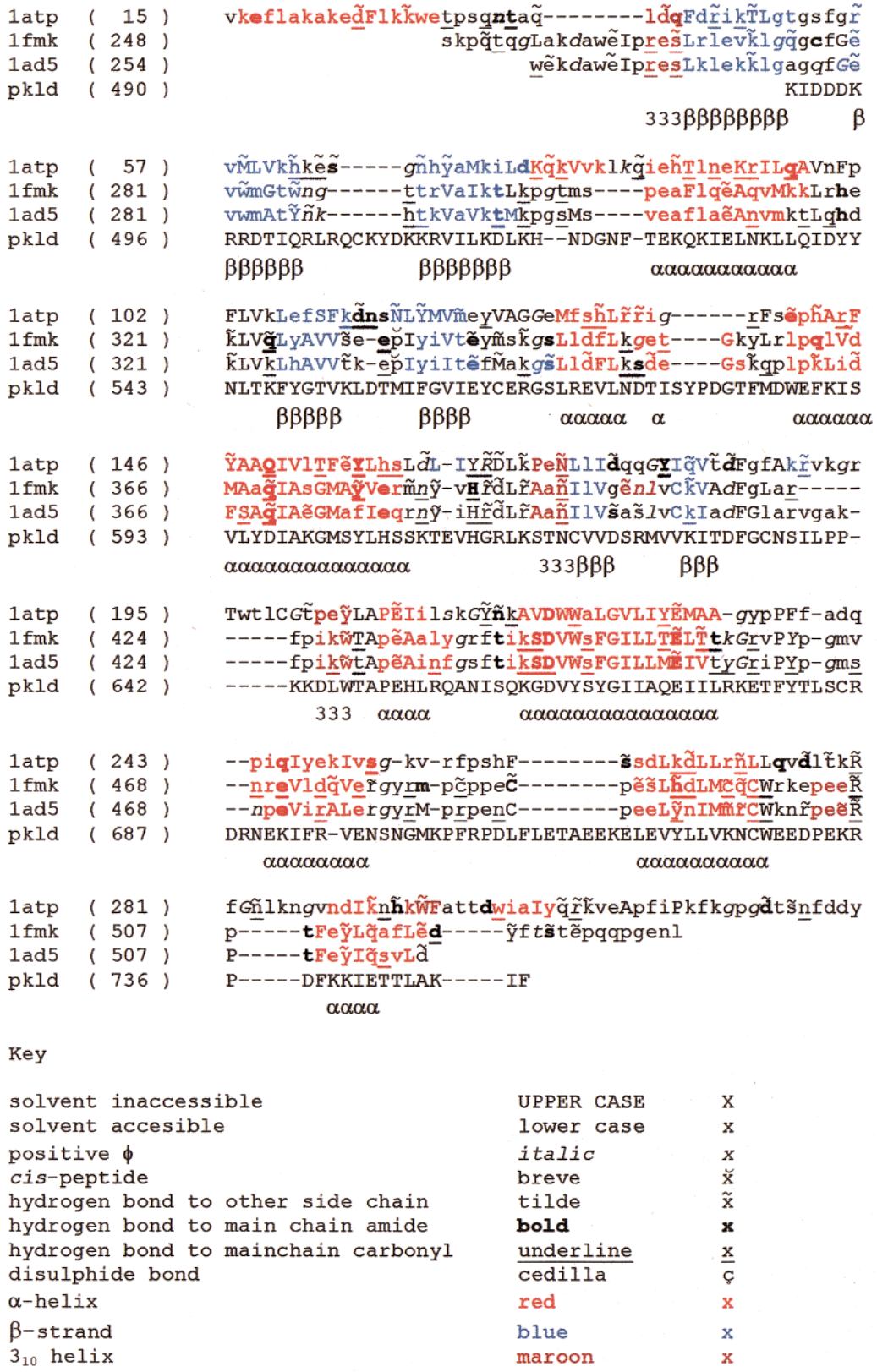

FIGURE 1: Alignment of the amino acid sequence of the KHD of GC-C with some of the known three-dimensional structures of protein kinases. 1atp, 1fmk, and 1ad5 correspond to the crystal structures of cAPK, src, and hck kinases, respectively. PKLD corresponds to the protein kinase-like domain of GC-C. The known structures are aligned on the basis of the three-dimensional structures. The structural environment at every residue position is shown for the known structures. The number of the first residue in a line within an alignment block is indicated within parentheses following the protein code in the same line. Conserved α-helices and *β*-strands are indicated. This figure was produced using the program JOY (*63*).

A Monoclonal Antibody to GC-C Does Not Recognize the ATP-Bound Form of GC-C. We have earlier described the generation of a monoclonal antibody, GCC:4D7, raised to the protein kinase-like domain of GC-C (*55*). This antibody

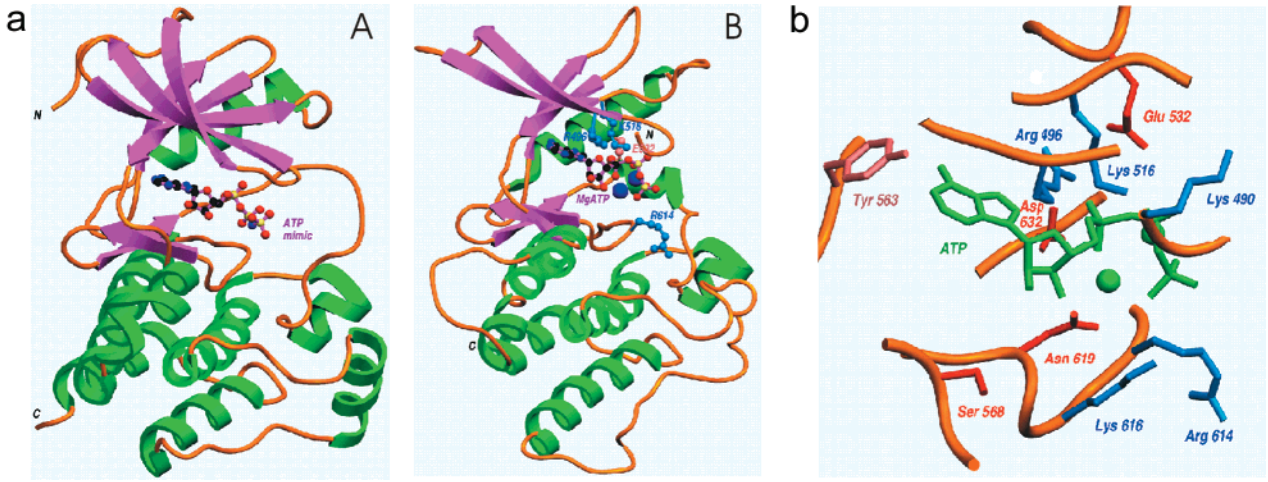

FIGURE 2: (a) Three-dimensional model of the KHD of GC-C. (A) Ribbon representation of the Hck tyrosine kinase structure with bound AMP-PNP (*51*). (B) Ribbon representation of the KHD of GC-C with bound MgATP. Helices and strands are shown in green and pink, respectively, and loops are in orange. Various atoms in the ATP/ATP mimic are black (carbon), red (oxygen), blue (nitrogen), and yellow (phosphate). The metal ion is represented by a large blue dot. Side chains of residues mentioned in the text are shown. The figure was produced using SETOR (*39*). (b) Closeup view of the ATP-bound region in the structural model of the KHD. ATP is shown in green, and the backbone is shown in orange. The basic residues that are conserved among protein kinases and are in the vicinity of ATP are shown. Lys_{490}, Arg_{496}, and Arg_{614} are not conserved compared to classical protein kinases. However, they are in the vicinity of ATP in the three-dimensional model, and these are capable of interacting with the phosphate group in ATP. This figure was produced using SETOR (*39*).

Table 1: Comparison of Conserved Residues in Src Kinases and the KHD of GC-C[a]

residue in the KHD	residue in Src kinases
Lys_{490}	Gln_{275}
Lys_{516}	Lys_{295}
Glu_{532}	Glu_{310}
Ser_{568}	Ser_{345}
Lys_{616}	Arg_{388}
Asn_{619}	Asn_{391}
Asp_{632}	Asp_{404}

[a] Column 1 indicates the residues in the KHD of GC-C that are conserved or conservatively substituted with respect to Src kinases. Residue numbers are as indicated in Figure 1. Column 2 indicates the analogous residues and their positions in the Src family kinases.

was able to react with GC-C expressed in HEK293 cells and identified the characteristic doublet of bands with M_rs of 145 and 130 kDa, representing the alternatively glycosylated forms of GC-C expressed in this and other cell lines (*42*). We hypothesized that interaction of GC-C with ATP may alter the conformation of the KHD and thereby regulate the interaction of GCC:4D7 with GC-C. We therefore solubilized GC-C from membranes prepared from HEK293:hGCC-C5 cells in the absence or presence of various concentrations of ATP or 1 mM GTP and 10 mM $MgCl_2$, prior to immunoprecipitation with GCC:4D7. The ability of GC-C bound to ATP to interact with GCC:4D7 was determined by the level of GC-C detected following Western blot analysis of immunocomplexes that formed. The data in Figure 3 show that interaction of GC-C with MgATP dramatically reduced the extent of reactivity of GCC:4D7 with the receptor, since smaller amounts of GC-C were recovered in immunocomplexes. The extent of interaction with the GCC:4D7 antibody was reduced when solubilization of GC-C was performed in the presence of 250 μM ATP, in agreement with the extent of inhibition observed in the detergent-stimulated activity of GC-C in the presence of ATP (see below and Figure 6). Incubation of GC-C with 1 mM GTP did not appreciably reduce the reactivity with GCC:4D7, since GTP is likely to

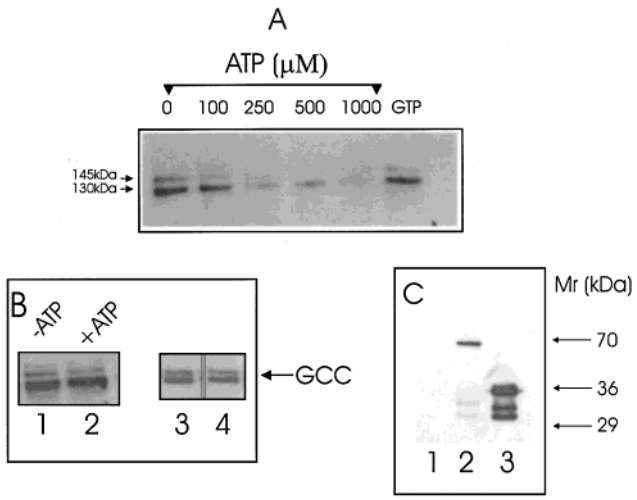

FIGURE 3: ATP inhibits the binding of GCC:4D7 to GC-C. (A) Membranes prepared from HEK293:hGCC-C5 cells were solubilized in the presence of varying concentrations of ATP or GTP and 2 mM $MgCl_2$, followed by immunoprecipitation with the GCC:4D7 antibody. Immunocomplexes were collected on protein A agarose and resolved by SDS−PAGE, followed by Western blot analysis with the GC-C specific monoclonal antibody, GCC:C8: lane 1, no ATP; lane 2, 100 μM ATP; lane 3, 250 μM ATP; lane 4, 500 μM ATP; lane 5, 1 mM ATP; and lane 6, 1 mM GTP. Data are representative of three experiments. (B) Membranes from HEK293:hGCC-C5 cells were solubilized and incubated in the absence (lane 1) or presence (lane 2) of 10 mM $MgCl_2$ and 1 mM ATP for 16 h. The samples were resolved by SDS−PAGE and Western blot analysis carried out with the GCC:4D7 monoclonal antibody. In lanes 3 and 4, Western blot analysis was performed with GCC:4D7 alone (lane 3), or with 1 mM ATP during the interaction with the GCC:4D7 antibody (lane 4). (C) Western blot analysis with purified GST fusion proteins corresponding to the entire KHD of GC-C (GST-GC-$C_{460-784}$, lane 2), or residues 491−568 (GST-GC-$C_{491-568}$, lane 3), and GST alone (lane 1).

interact only with the catalytic domain of GC-C, and therefore not alter the structure of the KHD.

The reduction in the reactivity of GCC:4D7 with ATP-bound GC-C was not due to a covalent modification of GC-C

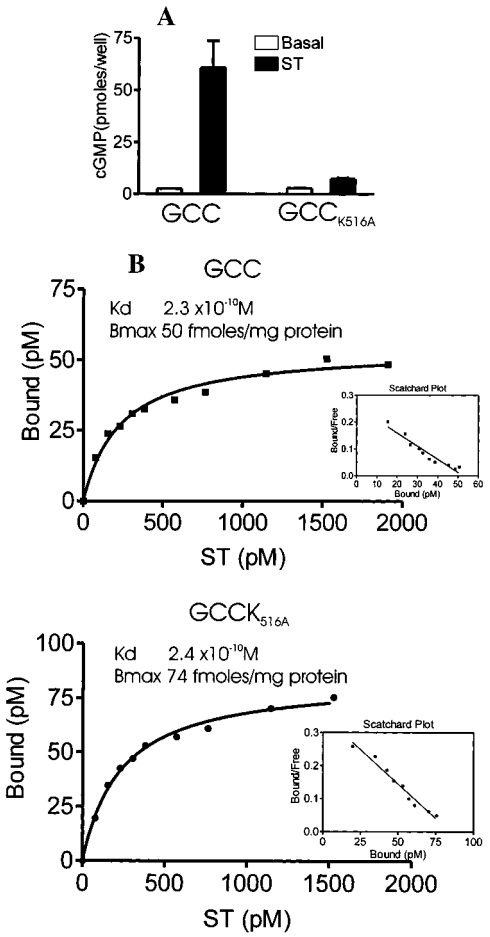

FIGURE 4: Characterization of the HEK293:hGCC$_{K516A}$ cell line. (A) HEK293 cells expressing either wild-type GC-C or GC-C$_{K516A}$ were treated with IBMX (1 mM) for 30 min, followed by incubation in the absence (white bars) or presence (black bars) of ST (10^{-7} M) for 1 h. Cells were lysed in 0.1 N HCl and cGMP levels monitored. Assays were carried out twice, and values correspond to means ± standard error of the mean of triplicate determinations carried out in a single representative experiment. (B) Membranes prepared from HEK293 cells expressing wild-type GC-C (upper panel) or mutant GC-C$_{K516A}$ (lower panel) were incubated with varying concentrations of ^{125}I-labeled ST$_{Y72F}$ for 1 h. Following the incubation, samples were filtered and the amount of bound radioligand was monitored. Saturation binding and Scatchard analyses were carried out using Graph Pad PRISM.

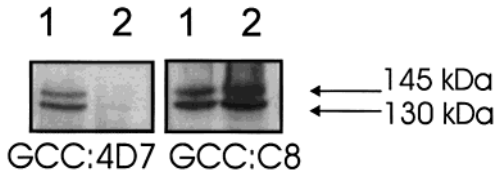

FIGURE 5: GC-C$_{K516A}$ does not react with the GCC:4D7 monoclonal antibody. Membranes prepared from HEK293 cells expressing either wild-type GC-C (100 μg of protein, lane 1) or mutant GC-C$_{K516A}$ (60 μg of protein, lane 2) were resolved on SDS−polyacrylamide gels in duplicate lanes and subjected to Western blot analysis with the GCC:4D7 monoclonal antibody, or with the GC-C monoclonal antibody GCC:C8.

following prolonged incubation of the receptor with MgATP along with other proteins in the crude solubilized preparation. GC-C incubated with and without MgATP for 16 h at 4 °C and then subjected to a direct Western blot (without a step of immunoprecipitation with GCC:4D7) was able to react equally efficiently with GCC:4D7 (Figure 3). In addition, GCC:4D7 did not react with ATP alone, since inclusion of 1 mM ATP in the reaction with primary antibody did not inhibit reactivity with GC-C, as monitored by Western blot analysis (Figure 3).

To identify the epitope in GC-C to which GCC:4D7 binds, we generated a recombinant protein that expressed residues 491−568 of GC-C as a fusion protein with GST. This region includes K_{516}, which from the modeling studies is poised to interact with ATP. Western blot analysis with GCC:4D7 indicated that the antibody recognized the GST−KHD fusion protein as well as the GST fusion protein encompassing residues 491−568 (Figure 3C). Since ATP interaction with native GC-C reduced GCC:4D7 reactivity, it appears that the epitope for GCC:4D7 is masked on ATP binding to native GC-C, perhaps precluding access of GCC:4D7 to key residues required in the epitope. These results therefore suggest that ATP does indeed interact with the KHD of GC-C, in a region that harbors the epitope for GCC:4D7.

Mutation of Lysine$_{516}$ to Alanine Leads to Reduced ST-Stimulated Activity in Intact Cells. Our modeled structure of the KHD indicates that Lys$_{516}$ plays an important role in interacting with ATP. With the assumption that ATP binding to GC-C occurred in the KHD, and with the available information that implicates a critical role for an equivalent lysine residue in protein kinases (*25*), we mutated this lysine residue in the KHD to alanine, and analyzed the consequences of this mutation on the modulation of GC-C activity by ATP.

To characterize and compare the effects of ATP on the catalytic activity of wild-type GC-C with GC-C$_{K516A}$, we generated an HEK293 cell line that stably expressed the mutant receptor (HEK293:hGCC$_{K516A}$). Remarkably, this point mutation of lysine$_{516}$ to alanine led to HEK293:hGCC$_{K516A}$ cells responding poorly to ST, in terms of elevation of intracellular cGMP levels (Figure 4A). Membranes were prepared from HEK293:hGCC-C5 and HEK293:hGCC$_{K516A}$ cells, and binding of radiolabeled ST to these membranes was monitored. The data were subjected to a Scatchard analysis. Receptor content was higher (1.5−2.5-fold) in HEK293:hGCC$_{K516A}$ cells than in the cell line expressing the wild-type receptor, but there was no change in the affinity of the mutant receptor for the ST peptide compared to that of the wild-type receptor (Figure 4B). This showed that the mutation of the lysine residue dramatically impaired in vivo ligand-stimulated activity, especially in view of the fact that higher levels of receptor were present in HEK293:hGCC$_{K516A}$ cells. In biochemical experiments performed subsequently, a correction was made to account for different levels of receptor expression in the two cell lines, and the protein used for assays corresponded to equal ST-binding activity in membranes prepared from the two cell lines.

Since monoclonal antibody GCC:4D7 was a sensitive monitor of ATP interaction with GC-C, we tested the reactivity of this antibody with HEK293:hGCC$_{K156A}$ cells by Western blot analysis. Most interestingly, this antibody reacted very poorly with the mutant receptor (Figure 5), while another monoclonal antibody also raised to the KHD of GC-C, GCC:C8, demonstrated equivalent reactivity for both the wild-type and mutant receptor (Figure 5). These results suggested that the epitope of GCC:4D7 requires the lysine residue at position 516 for recognition.

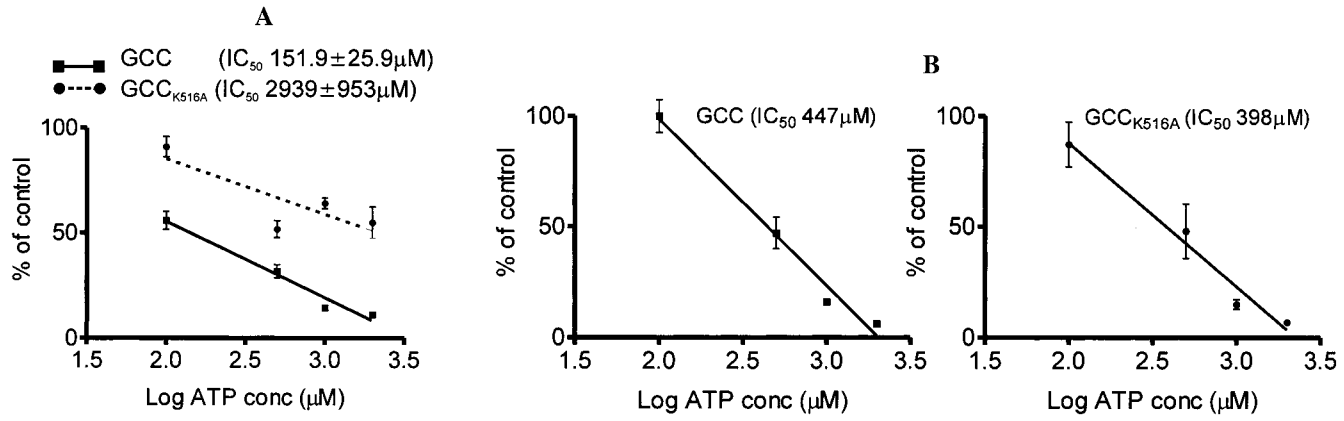

FIGURE 6: Effect of the K516A mutation on ATP modulation of detergent- and MnGTP-stimulated GC-C activity. (A) Membranes were prepared from HEK293 cells expressing GC-C or GC-C_{K516A}, and in vitro guanylyl cyclase assays were performed. Preincubation was carried out in the absence or presence of varying concentrations of ATP at 4 °C for 10 min, in the presence of Lubrol-PX (0.3%). $MgCl_2$ (20 mM) and GTP (1 mM) were then added, and incubation was continued for a further 10 min at 37 °C. The amount of cGMP produced was monitored by radioimmunoassay. The experiments were performed thrice, and values represent the mean ± standard error of the mean of duplicate determinations in a single experiment. Data are represented as a percentage inhibition of the activity seen in the absence of ATP. (B) Membrane proteins prepared from HEK293:hGCC-C5 and HEK293:hGCC_{K516A} cell lines were incubated without or with various concentrations of ATP for 10 min at 4 °C, followed by the addition of 20 mM $MnCl_2$ and 1 mM GTP. Samples were then incubated for 10 min at 37 °C, and the amount of cGMP produced was monitored by radioimmunoassay. Experiments were repeated twice with duplicate determinations, and data are means ± standard error of the mean.

Table 2: Effects of ST and ATP on the Activity of Wild-Type and Mutant GC-C cGMP (picomoles per tube)[a]

preincubation	addition	GC-C	GC-C_{K516A}
−	MgGTP	4.1 ± 0.6	20.5 ± 0.7
−	ST and MgGTP	17.5 ± 1.0	25.4 ± 0.4
ST	MgGTP	8.8 ± 0.8	23.3 ± 1.7
ATP and ST	MgGTP	16.3 ± 0.1	17.1 ± 0.9

[a] Membranes prepared from HEK293 cells expressing GC-C or GC-C_{K516A} were used for in vitro guanylyl cyclase assays, and the amount taken corresponded to the equivalent receptor concentration. Membranes were preincubated at 30 °C for 10 min in the absence or presence of ST (10^{-7} M) or ST and ATP (1 mM), as indicated. Following this, substrate (10 mM $MgCl_2$ and 1 mM GTP) was added along with ST (10^{-7} M), where indicated, and incubation was continued at 37 °C for 10 min. cGMP production was monitored by a radioimmunoassay. Assays were performed in duplicate, and values correspond to means ± standard error of the mean of triplicate determinations carried out in a single representative experiment.

Effect of the K516A Mutation: In Vitro Guanylyl Cyclase Activity. We then carried out in vitro guanylyl cyclase assays to examine the effects of the K516A mutation on modulation of GC-C activity by ATP, using membranes prepared from the two cell lines. Wild-type GC-C demonstrated characteristic ST-mediated activation of guanylyl cyclase activity (Table 2), showing a 4-fold stimulation of guanylyl cyclase activity in the presence of ST. Preincubation of GC-C with ST in the absence of MgGTP, prior to addition of the substrate, led to a reduction in the level of cGMP production by GC-C, when compared to that in membranes where ST and substrate are added simultaneously (2- vs 4-fold activation over basal activity). This phenomenon is known as "ligand-induced inactivation in vitro" (*5*, *42*). The presence of ATP along with ST during the preincubation alleviated this ST-mediated inactivation, and led to equivalent cGMP production by GC-C, as seen in membranes without preincubation (Table 2).

Interestingly, membranes prepared from cells transfected with GC-C_{K516A} cDNA exhibited high levels of basal cGMP production (Table 2), when assays were performed with the same amount of mutant receptor and wild-type receptor, as measured by ST-binding activity. In addition, only a slight increase in guanylyl cyclase activity was observed on ST addition to membranes prepared from cells expressing GC-C_{K516A} . This is in agreement with that observed on application of ST to monolayer cultures of cells expressing GC-C_{K516A} (Figure 4A). Preincubation of GC-C_{K516A} with ST prior to addition of MgGTP did not lead to any ligand-induced inactivation. The presence of ATP along with ST during the preincubation step did not alter cGMP production by GC-C_{K516A} significantly. These results clearly demonstrate the requirement of a kinase-like domain in GC-C that is competent in interacting with ATP in a manner that elicits ligand-mediated activation of the receptor.

In addition to stimulation by ligand, GC-C is activated in the presence of nonionic detergents such as Lubrol-PX (*31*), as well as in a ligand-independent manner, when Mn^{2+} is used as the cofactor in the substrate instead of Mg^{2+} (*42*, *56*). ATP has been shown to inhibit activation of GC-C by nonionic detergents as well as MnGTP (*5*, *57*). We monitored the inhibition of Lubrol-stimulated guanylyl cyclase activity in membranes prepared from HEK293:hGCC-C5 and HEK293:hGCC_{K516A} cells, in the presence of varying concentrations of ATP. For an equivalent amount of receptor taken for assays, the detergent and MnGTP-stimulated activity was higher in membranes prepared from HEK293:hGCC_{K516A} cells (2-fold). Therefore, the data shown in Figure 6 are expressed as a percentage of catalytic activity seen in the presence of ATP to that in its absence (control). The catalytic activity of wild-type GC-C was effectively inhibited by ATP, with an IC_{50} of approximately 150 μM (Figure 6A). This is similar to the concentration required for inhibition of GCC:4D7 binding to GC-C (Figure 3). In contrast, the concentration of ATP that would have been required for 50% inhibition of catalytic activity of GC-C_{K516A} was more than 2 mM, a concentration of ATP at which almost 90% inhibition of catalytic activity of wild-type GC-C was seen.

Using MnGTP as a substrate, guanylyl cyclase activity in both wild-type and mutant GC-C was equally efficiently

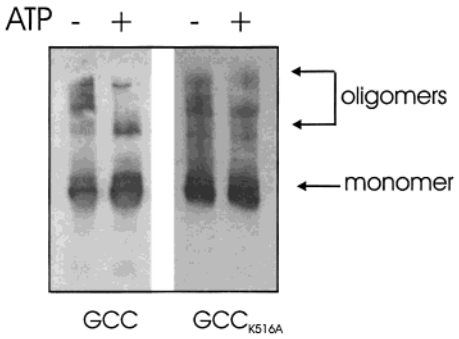

FIGURE 7: The oligomeric status of the mutant GC-C$_{K516A}$ is not affected in the presence of ATP. Membranes prepared from HEK293 cells expressing wild-type GC-C or mutant GC-C$_{K516A}$ were solubilized in 1% Triton X-100 in the absence or presence of ATP (1 mM) for 1 h at 4 °C. The proteins were then precipitated with an antiserum raised against the C-terminal region of GC-C, and immunocomplexes were collected on protein A agarose and washed in the absence or presence of ATP (1 mM). The samples were subsequently resolved on a 6% polyacrylamide gel under nonreducing conditions followed by Western blot analysis using the GCC:C8 monoclonal antibody.

inhibited at similar concentrations of ATP (Figure 6B). The differential effects of the mutation of Lys$_{516}$ on ATP modulation of detergent- and MnGTP-stimulated activity indicate that these modes of activation and inhibition by ATP of GC-C activity have different mechanisms. It is clear from these biochemical results, as well as results with the GCC:4D7 monoclonal antibody, that the regulation of GC-C activity by ATP is mediated by direct binding of ATP to the KHD of GC-C, and consequent conformational changes that could occur. The mutation of the lysine residue in the KHD alters the affinity of ATP for the KHD of GC-C, and/or changes the conformation of the domain and its consequent interaction with the catalytic domain of GC-C.

ATP Modulates the Oligomeric Status of GC-C. GC-C exists as an oligomer even in the absence of ligand, and it is suggested that ligand binding could alter the oligomeric status of GC-C, leading to its activation (*15*). We have also provided evidence recently that sequences mediating the oligomerization of GC-C reside in the intracellular domain, possibly in the linker sequence between the KHD and the catalytic domain (*55*). Since the results described above clearly indicate the role of the KHD in regulating both the ligand-stimulated activity of GC-C and its activation by detergents, we tested the ability of ATP to modulate the oligomeric status of both wild-type GC-C and GC-C$_{K516A}$. The methodology was similar to that used in earlier studies (*15*) with some modifications, and involved solubilization and immunoprecipitation of equal amounts of GC-C from HEK293:hGCC-C5 and HEK293:hGCC$_{K516A}$ cells either in the absence or in the presence of MgATP, subjecting the receptor preparation to SDS gel electrophoresis in the absence of a reducing agent, followed by Western blotting with the GCC:C8 monoclonal antibody. The results are shown in Figure 7. As can be seen, GC-C solubilized from membranes prepared from both cell lines exists as high-molecular weight complexes. The addition of ATP to wild-type GC-C resulted in the conversion of the high-molecular weight oligomer to that of a smaller size. This is in agreement with results that have been described previously by other groups (*15*, *57*). In contrast, no change in the oligomeric status of GC-C$_{K516A}$ was observed in the presence of ATP, even at concentrations as high as 5 mM (data not shown). Higher-molecular weight oligomers could be the active form of GC-C (since detergent is present in the preparation as a result of solubilization), and this is converted to a smaller oligomer by ATP, thereby reducing the catalytic activity of the receptor.

It is therefore clear that a single mutation in the KHD of GC-C can have profound effects in regulating ATP-mediated effects on activation of GC-C by both ligand and detergents, thus emphasizing the role of the KHD in GC-C, and perhaps in other guanylyl cyclases as well, in coordinating the interaction of the extracellular domain of these receptors with the catalytic domain.

DISCUSSION

Using a monoclonal antibody to GC-C and site-directed mutation of GC-C, the results described above have suggested that the KHD of GC-C is the site for ATP interaction and regulation of guanylyl cyclase activity. Since three-dimensional modeling suggested that this domain of GC-C could adopt a structure similar to that of protein kinases, we 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 in turn may result in altered interactions of the KHD with the guanylyl cyclase domain within a single receptor molecule, or between different chains of GC-C, thereby regulating guanylyl cyclase activity.

We have attempted to demonstrate direct binding of an analogue of ATP, FSBA, to both wild-type GC-C and the mutant GC-C generated in this study. However, it appears that at the concentrations of FSBA required for this type of assay, the analogue binds nonspecifically to the receptor, and ATP-mediated effects on GC-C catalytic activity are not mimicked by FSBA (unpublished observation). The reduced immunological cross-reactivity of GCC:4D7 with GC-C$_{K516A}$ could suggest that this lysine residue present in the epitope of GCC:4D7 is necessary for recognition of the antibody, since an alanine residue at position 516 dramatically reduces GCC:4D7 reactivity.

The low level of sequence similarity between the extreme N-terminus of the KHD and the catalytic domains of protein kinases indicates that this region of the KHD may have a structure completely different from that of protein kinases. Furthermore, there is also a very low level of sequence similarity in this region between the different receptor guanylyl cyclases (*13*). The glycine-rich loop is present in GC-A and partly conserved in GC-B, but is absent in retGC and GC-C. The natriuretic peptide receptors have an absolute requirement for ATP in activating the guanylyl cyclase domain following ligand binding (*8*, *9*). GC-C and retinal guanylyl cyclase, on the other hand, are activated by ligands in the absence of ATP in vitro, but show an increased activity in the presence of ATP (*12*, *18*). It is possible therefore that the presence of the Gly-rich motif in the natriuretic peptide receptors results in a unique structure of the kinase-like domain such that there is an absolute requirement for ATP binding to relieve the inhibition of the cyclase domain by the kinase-like domain. This may be one of the reasons why the kinase-like domains of GC-A and GC-C are not interchangeable (*19*).

Deletion of the entire KHD of human GC-C resulted in a protein that was constitutively active and refractory to further stimulation by ligand (*20*), as seen in the lysine to alanine mutation seen here. In contrast to assays performed with membranes prepared from cells, there was no change in intracellular basal levels of cGMP in cells expressing mutant GC-C, but there was a dramatic reduction in ligand-stimulated activity in whole cells. It is likely that despite the high level of basal guanylyl cyclase activity that is observed, the intracellular levels of cGMP are regulated by degradation mediated by phosphodiesterases (*58*), but measurement of cGMP phosphodiesterase activity in HEK293:hGCC-C5 and HEK293:hGCC$_{K516A}$ cells showed similar activity in both cell lines (data not shown). We as of now do not have an explanation for normal levels of intracellular cGMP present in the two cell lines, but this could be related to other intracellular factors which could regulate the catalytic activity of GC-C.

The loss of ligand-stimulated activity in GC-C$_{K516A}$ suggests that interaction of ATP with the KHD may be essential to bringing about normal levels of GC-C activation. Therefore, ATP levels in the cell may play a role in modulating GC-C activity. A downstream effect of the activation of GC-C is an increased level of chloride secretion from the cystic fibrosis transmembrane conductance regulator, CFTR (*3*). CFTR activation is also known to require the presence of ATP (*59*), suggesting that ATP levels within the cell can concomitantly regulate both cGMP production and chloride secretion. Such a system could provide a mechanism for coordinated regulation of ionic balance with the energy levels within the cell.

GC-C$_{K516A}$ does interact with ATP, albeit with a lower affinity, since inhibition of detergent-stimulated activity was seen at higher concentrations of ATP. It is possible that ATP still binds to the KHD of mutant GC-C, but in an altered conformation that results in inefficient interaction with the catalytic domain. Using stereochemical considerations, we were able to model ATP into the binding site in GC-C$_{K516A}$, but significant differences in the positions of the phosphates and those of many of the side chains of the protein near ATP were observed (data not shown). Replacement of Lys$_{516}$ with Ala means that the positive charge of the side chain is lost, and the side chain in the mutant is much smaller than in the wild type. Therefore, residues around the site of mutation are likely to repack in a way to avoid the void volume. The conformation of GC-C now achieved would be different from that of wild-type GC-C bound to ATP, and may be unfavorable for efficient ATP modulation of ligand- and detergent-stimulated GC-C activity. ATP-mediated modulation of manganese-stimulated GC-C activity may, however, occur via a different mechanism, which is not clear at present, and it is therefore possible that ATP binding, even in a different orientation, is able to bring about this effect efficiently. Goldsmith and co-workers (*60*), who replaced the Lys of MAP kinase-ERK2 (equivalent to Lys$_{516}$ of the KHD) with Ala or Arg, observed no difference in the apparent K_m of the mutants for ATP. However, crystallographic analysis of the lysine to arginine mutant revealed that there was a significant alteration in the position of the phosphate group of ATP so that the mutant showed a much reduced level of the phospho transfer reaction (*60*).

Binding of ATP appears to alter the oligomeric status of GC-C (Figure 7), and this could be the underlying cause for the modulation of activity of GC-C by ATP. We, and others, have shown that GC-C exists as a trimer in the absence of the ST peptide, and regions mediating association of GC-C, and indeed other receptor guanylyl cyclases, could lie in a linker domain between the KHD and the catalytic domain. A change in the oligomeric status of GC-C on ATP binding appears to lead to the inactivation of the enzyme, as shown by biochemical assays (Figure 7). The presence of ATP almost completely inhibits the Lubrol-stimulated guanylyl cyclase activity of the full-length receptor, and the intracellular domain expressed in insect cells (*55*), raising the possibility that the lower oligomeric form of GC-C could have a specific activity far reduced compared to that of higher oligomers.

The modeled structure of the guanylyl cyclase domain of the retinal guanylyl cyclase predicts that this domain functions as a homodimer (*61*). Since there is significant sequence similarity between the cyclase domains of the various receptor guanylyl cyclases, it is probable that the cyclase domains of all membrane guanylyl cyclases adopt a similar structure. The kinase domains of most receptor tyrosine kinases have been shown to function as dimers (*62*). It is therefore probable that functionally, the kinase-like domains of the receptor guanylyl cyclases are able to adopt a dimeric structure. It would now be interesting to determine how the cyclase and the kinase-like domains are organized with respect to each other, and how this may affect their functional interactions.

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