Biochemical Characterization of the Intracellular Domain of the Human Guanylyl Cyclase C Receptor Provides Evidence for a Catalytically Active Homotrimer †

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Biochemical Characterization of the Intracellular Domain of the Human Guanylyl Cyclase C Receptor Provides Evidence for a Catalytically Active Homotrimer†

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ABSTRACT: Guanylyl cyclase C (GCC) is the receptor for the family of guanylin peptides and bacterial heat-stable enterotoxins (ST). The receptor is composed of an extracellular, ligand-binding domain and an intracellular domain with a region of homology to protein kinases and a guanylyl cyclase catalytic domain. We have expressed the entire intracellular domain of GCC in insect cells and purified the recombinant protein, GCC-IDbac, to study its catalytic activity and regulation. Kinetic properties of the purified protein were similar to that of full-length GCC, and high activity was observed when MnGTP was used as the substrate. Nonionic detergents, which stimulate the guanylyl cyclase activity of membrane-associated GCC, did not appreciably increase the activity of GCC-IDbac, indicating that activation of the receptor by Lubrol involved conformational changes that required the transmembrane and/or the extracellular domain. The guanylyl cyclase activity of GCC-IDbac was inhibited by Zn^{2+}, at concentrations shown to inhibit adenylyl cyclase, suggesting a structural homology between the two enzymes. Covalent cross-linking of GCC-IDbac indicated that the protein could associate as a dimer, but a large fraction was present as a trimer. Gel filtration analysis also showed that the major fraction of the protein eluted at a molecular size of a trimer, suggesting that the dimer detected by cross-linking represented subtle differences in the juxtaposition of the individual polypeptide chains. We therefore provide evidence that the trimeric state of GCC is catalytically active, and sequences required to generate the trimer are present in the intracellular domain of GCC.

Membrane-associated guanylyl cyclases comprise a family of enzymes that serve as receptors for a number of diverse polypeptide ligands (*1*). These receptors possess a large extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic domain that is composed of two regions. Sequences adjacent to the transmembrane domain show significant similarity to protein kinases (*2*), and therefore this region has been named the protein kinase-like domain. Toward the C-terminal region of these receptors is the catalytic guanylyl cyclase domain, which has sequence similarity to the adenylyl cyclases (*3*). Ligand binding to these receptors leads to activation of the catalytic domain and a consequent increase in intracellular cGMP, which serves as the second messenger in initiating a variety of signaling events (*4*).

Heat-stable enterotoxins are a family of low molecular weight peptides that are produced by toxigenic strains of *Escherichia coli* and other pathogenic bacteria (*5*). These peptides are one of the major causes of watery diarrhea the world over and bind to a member of the receptor guanylyl cyclase family, guanylyl cyclase C (GCC)[1] (*6*), expressed at high levels in intestinal epithelial cells (*7*). Endogenous ligands for GCC have been identified and are called guanylin and uroguanylin (*8*). Binding of these peptides to GCC increases intracellular cGMP, activation of cAMP- and cGMP-dependent protein kinases, and phosphorylation of the cystic fibrosis transmembrane conductance regulator, leading to increased chloride and fluid efflux from the intestinal cell (*9*).

An understanding of the structural features of both the ST and the guanylin family of peptides and GCC would aid in the design of inhibitors of this pathway and therefore serve as therapeutics for diarrhea mediated by the toxins. GCC has been cloned from the rat and human intestinal tissue, as well as human colonic cell lines (*10*−*12*), and analysis of the sequence suggests a topology similar to that predicted

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[1] Abbreviations: DSS, disuccinimidyl suberate; GCA, guanylyl cyclase A, receptor for the atrial natriuretic peptides; GCC, guanylyl cyclase C; GCC-IDbac, intracellular domain of GCC expressed in Sf21 cells; GST, glutathione *S*-transferase; IBMX, isobutyl methyl xanthine; PBST, sodium phosphate buffer, pH 7.2, containing 0.9% NaCl and 0.1% Tween 20; ST, stable toxin peptide.

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for other members of the guanylyl cyclase receptor family. The extracellular domain has been expressed in both bacteria and insect cells and the recombinant protein bound the ST peptides with an affinity comparable to the native receptor (*13*, *14*). The extracellular domain of GCC is heavily glycosylated (*15*), but since the expression of this domain in bacteria yielded a protein that bound ST with high affinity, it appears that glycosylation of the receptor is not critical for ligand binding (*13*). However, results with the baculovirus expression system suggest that glycosylation could impart stability to the protein (*16*). More recent results have identified critical residues near the transmembrane domain of GCC that are involved in ligand binding (*17*).

There are no reports so far describing the expression of the intracellular domain of GCC in a catalytically active form. We have expressed the intracellular domain in bacteria, but the protein was localized in inclusion bodies and did not show guanylyl cyclase activity (*7*). Indeed, the catalytic domain of the receptor for the atrial natriuretic peptides, guanylyl cyclase A, when expressed in bacteria, also was found in an insoluble form that had to be refolded to generate active protein (*18*).

Information on the catalytic properties of the intracellular domain of receptor guanylyl cyclases, obtained with purified recombinant protein, would not only be useful in understanding the regulation of catalytic activity, but also provide information on the oligomeric status of the protein. It is believed that both adenylyl and guanylyl cyclases function as dimers (*19*). The crystal structure of adenylyl cyclase provides evidence for the existence of a dimer (*20*). The cytosolic guanylyl cyclases are heterodimeric proteins and the computational modeling of a membrane associated guanylyl cyclase, retinal GC, also allows for the formation of the dimer (*19*). Regions thought to be involved in the dimerization of the atrial natriuretic factor receptor, guanylyl cyclase A (GCA), have been identified and are present in a stretch of amino acids that are found between the protein kinase-like domain and the cyclase domain (*21*). Full-length GCC has been shown to exist as a trimer under native conditions, and radiolabeled ST binding identified both dimeric and trimeric structures (*22*). Expression of the extracellular domain of porcine GCC in COS7 cells suggested that a dimeric form of the protein was found in these cells (*23*). Expression of the ligand binding domain in insect cells and purification of the protein indicated that addition of ST peptide led to the formation of a trimer (*16*). It was therefore concluded that sequences mediating oligomerization were also found in the extracellular region of GCC.

In this study, we chose to express the intracellular domain of GCC in insect cells, with the hope that high level expression could be achieved, allowing us to purify the protein to monitor and characterize its catalytic activity. Our results provide evidence that the intracellular domain of GCC can exist as a trimer, which is catalytically active. Analysis of the sequence of the intracellular domain could putatively identify a region that could exist as a coiled-coil structure, allowing for the possible formation of a catalytically active dimer in the trimeric structure.

MATERIALS AND METHODS

Expression of GCC-IDbac in Sf21 Cells. Expression of GCCID-bac was achieved using the Bac-to-Bac baculovirus expression system (Gibco BRL, USA). The intracellular domain clone GGC:ID6, representing a region beginning with amino acid 460 to 1073 in pRSETC has been described earlier (*7*) and was generated from the cDNA of human GCC (GenBank Accession S57551). A *Pst*I fragment from this clone was excised and cloned into pBSII-KS+ (Stratagene) to generate plasmid pBS-ID7, and orientation of the fragment was checked by suitable restriction enzyme digestion, such that sequences corresponding to the N-terminal region of the protein were present next to the *Pst*I site in pBSII KS+. The clone pBS-ID7 was digested with *Sal*I and *Xba*I, and the released fragment was cloned into pFastBac HTc, to generate a clone that would express the entire intracellular domain of GCC, GCC-IDbac, fused to a hexahistidine tag at the N-terminus, present in the vector. Further manipulations to generate the recombinant bacmid DNA in DH10Bac were performed as described by the manufacturers. Bacmid DNA was transfected into Sf21 cells using Cellfectin reagent (Gibco BRL, USA), and virus was harvested from the culture supernatant 72 h following transfection. Varying concentrations of the virus were used for infection of monolayer cultures of Sf21 cells, and protein expression was monitored by guanylyl cyclase assays and Western Blot analysis as described below. Intracellular cGMP was monitored in lysates of cells prepared in 0.1 N HCl, as described earlier (*24*). Protein was monitored by a modification of the method of Bradford (*25*).

Cloning of the Protein Kinase-Like Domain of GCC and Generation of Monoclonal Antibody GCC:4D7. A fragment comprising the protein kinase-like domain of GCC was cloned as a fusion protein with glutathione *S*-transferase (GST). A DNA fragment encoding a region encompassing the protein kinase-like domain of GCC was generated by excising a *Pvu*II fragment from pRSET-ID6 and then cloned into the *Sma*I site of pGEX-5 × 2 (Amersham Pharmacia Biotech,UK). This construct, pGEX-PKLD, expresses the N-terminal 325 amino acids of the intracellular domain of GCC, from residues 460 to 784 and encompasses the protein kinase-like domain of GCC, fused to GST. Protein was expressed in BL21 cells following induction with 1 mM IPTG. Inclusion body was obtained by centrifugation of lysed cells at 12000*g*, and the pellet was subjected to SDS−gel electrophoresis. The band corresponding to GST-PKLD was electroeluted, and purified protein was used for immunizing Balb/c mice. A monoclonal antibody, GCC:4D7, was generated by established procedures (*7*). This antibody reacts in Western blots with native GCC expressed in T84 cells (data not shown).

Purification of GCC-IDbac. Infected Sf21 cells (multiplicity of infection 5) were harvested 72 h following infection, and extracts prepared by lysing cells were grown in a 10-cm culture dish in 1 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 5 μg/mL aprotinin, 5 μg/mL leupeptin) containing 1% NP-40. Cells were subjected briefly to sonication and centrifuged at 12000*g* for 45 min at 4 °C. The supernatant was applied to Ni-NTA resin (Quiagen, Germany) and mixed for 3 h at 4 °C. The beads were washed with 10-bed volumes of 20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, and 10% glycerol. Bound protein was eluted with 20 mM Tris-HCl, pH 8.5, 100 mM NaCl, 100 mM imidazole, and 10% glycerol. Protein was dialyzed

against buffer without imidazole and stored at −70 °C until use. Aliquots were monitored for purity by silver staining (*26*).

Western Blot Analysis of GCC-IDbac using GCC:4D7 Monoclonal Antibody. Purified protein or crude cell lysate was subjected to SDS−gel electrophoresis in 7.5% polyacrylamide gels, and proteins were transferred to PVDF membrane (NEN, USA). The membrane was blocked with 2% blocking reagent (Amersham Pharmacia Biotech, UK) prepared in 10 mM sodium phosphate buffer, pH 7.5, containing 0.1% Tween 20 and 0.9% NaCl (PBST), for 1 h at 25 °C. The blot was incubated with purified GCC:4D7 IgG (200 ng/mL) in PBST containing 0.2% bovine serum albumin. Incubation was continued for 2 h at 25 °C, and the membrane was washed in PBST and incubated with anti-mouse IgG peroxidase (Amersham Pharmacia Biotech, UK) diluted to 1:2500 in PBST/0.2% bovine serum albumin. Following incubation for 1 h, the blot was washed extensively and bound antibody was detected by the enhanced chemiluminescence reagent as per the manufacturer's instructions (Amersham Pharmacia, UK).

In Vitro Guanylyl Cyclase Assays. Crude lysate, prepared from infected cells lysed in lysis buffer, or purified protein was taken for a guanylyl cyclase assay in a buffer of 60 mM Tris-HCl, pH 7.6, containing 1 mM isobutyl methyl xanthine (IBMX), 7.5 mM creatine phosphate, and 20 μg creatine phosphokinase. The assay was initiated by the addition of MgGTP solution to a final concentration of 1 mM GTP and 10 mM $MgCl_2$. For kinetic analysis, varying concentrations of MgGTP were used, maintaining the ratio of Mg^{2+}/GTP as 10:1. Tubes were incubated at 37 °C for 10 min, the reaction was terminated by the addition of 400 μL of 50 mM sodium acetate buffer, pH 4.5, and the sample was boiled for 5 min and centrifuged, and supernatant was taken for cGMP radioimmunoassay as described earlier (*27*). Some assays were performed by replacing $MgCl_2$ with 4 mM $MnCl_2$. In some experiments, following incubation of samples for 10 min with varying concentrations of Lubrol-PX or Triton-X100, MgGTP or MnGTP was added as substrate. To monitor inhibition of guanylyl cyclase activity by Zn^{2+}, samples were incubated with varying concentrations of $ZnCl_2$ in assay buffer, for 10 min, prior to addition of the substrate.

Cross-Linking of GCC-IDbac with Disuccinimidyl Suberate (DSS). For cross-linking GCC-IDbac in crude lysates of infected Sf21 cells, lysis of Sf21 cells was performed in lysis buffer prepared with 50 mM HEPES buffer, pH 7.5, instead of 50 mM Tris-HCl. Varying amounts of protein were incubated with DSS (2 mM) on ice for 5 min, following which 1× SDS sample buffer was added. Samples were then subjected to SDS−gel electrophoresis, transferred to PVDF membrane, and processed for Western blot analysis with GCC:4D7 monoclonal antibody, as described earlier.

Gel Filtration Analysis of GCC-IDbac. A Superdex-200 column (16 mm × 62 cm; Amersham Pharmacia Biotech, UK) was equilibrated with 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, and 10% glycerol (vlv). Crude lysate (approximately 3 mg) from infected Sf21 cells was applied to the column, and fractionation was performed at a flow rate of 0.5 mL/min using an Akta FPLC system (Amersham Pharmacia Biotech, UK). Fractions (1 mL) were collected, guanylyl cyclase assays were performed on fractions using MnGTP as a substrate, and aliquots of each fraction were also subjected to Western blot analysis with GCC:4D7 monoclonal antibody as described earlier. The molecular weights of protein eluting in different fractions were estimated following calibration of the column using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome C (12.3 kDa) as standards.

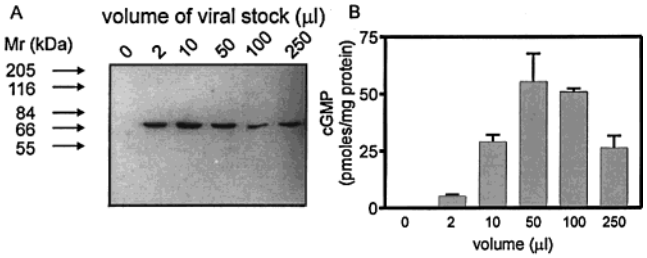

FIGURE 1: Expression of GCC-IDbac in Sf21 cells leads to intracellular accumulation of cGMP. (A) Varying volumes of a viral stock generated from infection of Sf21 cells with GCC-IDbac were used for infection and 72 h following infection, cell lysates were prepared. 10 μg of protein was fractionated on a 7.5% SDS−polyacrylamide gel, and proteins were transferred to a PVDF membrane and analyzed by Western blotting using GCC:4D7 monoclonal antibody raised to the protein kinase-like domain of GCC. Data shown is representative of experiments performed thrice. (B) Sf21 cells were infected with varying volumes of a viral stock and 72 h following infection, cells were lysed in 0.1 N HCl and aliquots were assayed for cGMP by radioimmunoassay. Data shown is the mean ± SEM of individual wells infected in duplicate, with the experiment performed twice.

RESULTS

There have been no reports so far describing the recombinant expression of a catalytically active, intracellular domain of GCC in any expression system, but the full-length receptor has been expressed in mammalian and insect cells in a form that is biologically active (*14*, *16*). We cloned the entire intracellular domain of GCC into the vector pFAST-Bac-His, where protein is expressed along with a hexahistidine N-terminal tag. We prepared the recombinant bacmid and used Sf21 cells for the expression of the recombinant protein.

To monitor protein expression, we generated a monoclonal antibody to the protein kinase-like domain domain of GCC, called GCC:4D7. As shown in Figure 1, intracellular levels of cGMP increase following viral infection in Sf21 cells, indicating that the expressed protein, GCC-IDbac, is constitutively active in Sf21 cells. Western blot analysis, using GCC:4D7 monoclonal antibody in lysates prepared from uninfected and infected cells, indicated that an immunoreactive band of molecular mass 76 kDa, a size predicted from the sequence of the cloned fragment, was detected only in infected cells, and densitometric analysis if the immunoreactive bands levels of expression correlated with the amount of intracellular cGMP accumulation (data not shown).

Sf21 cells were infected with the optimum amount of virus, and expression was monitored by in vitro guanylyl cyclase performed at various times following infection. Maximum expression of protein was observed 3−4 days following infection (Figure 2), as measured by a marked increase in guanylyl cyclase activity in cell lysates. Cells were henceforth harvested at this time. GCC-IDbac was purified from cell

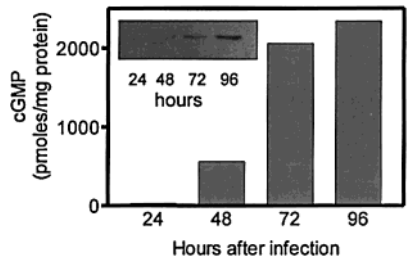

FIGURE 2: Optimization of production of catalytically active GCC-IDbac. Sf21 cells were infected with recombinant virus and cells were lysed in lysis buffer at various times following infection. In vitro guanylyl cyclase assays were performed using MgGTP as the substrate. Data shown is mean of duplicate determinations with the assay performed thrice with different batches of protein. Inset: aliquots of the lysate (10 μg) were subjected to SDS−gel electrophoresis and Western blot analysis using GCC:4D7 monoclonal antibody as described earlier. Data shown are representative of three experiments.

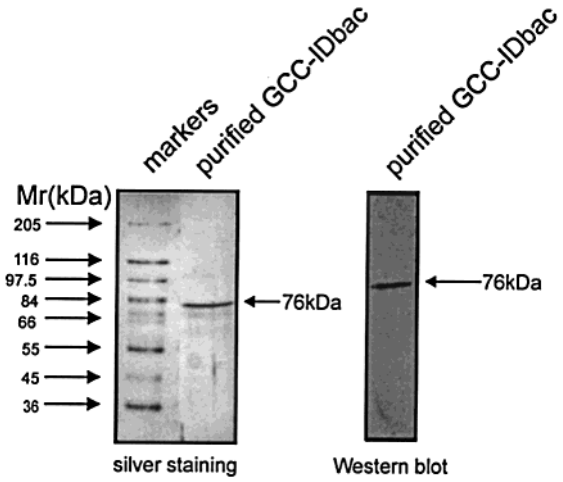

FIGURE 3: Purification of GCC-IDbac from infected SF21 cells. Lysate from cells infected with GCC-IDbac bacmid was prepared and GCC-IDbac was purified by Ni-chelate affinity chromatography. Purified protein (50 ng) was subjected to SDS−gel electrophoresis, silver staining, and Western blot analysis with GCC:4D7 monoclonal antibody.

lysates by nickel-chelate affinity chromatography. Figure 3 shows a silver-stained gel indicating the purity of the protein obtained from infected cell lysates. Yields were routinely around 100 μg of purified GCC-IDbac/10^8 cells with a fold purification of 400. The specific activity of the purified protein was of the order of 9600 pmol of cGMP produced min^{-1} (mg of protein)$^{-1}$. As expected, the purified protein was recognized in Western blot analysis by GCC:4D7, and the molecular mass of the purified protein was 76 ± 3 kDa, which is in close agreement to that predicted from the amino acid sequence of the cloned protein (77 344 Da).

Catalytic activity of GCC-IDbac was monitored using MgGTP and MnGTP as substrates. As shown in Figure 4, panel A, the catalytic activity was enhanced when MnGTP was used as the substrate, as has been reported for all cyclases so far (*28*, *29*). Nonionic detergents have been shown to enhance the activity of membrane-associated guanylyl cyclases, leading to marked stimulation of the catalytic activity, even in the absence of ligands (*27*). Interestingly, the catalytic activity of GCC-IDbac was increased only 2-fold in the presence of either 1% Lubrol-PX or 1% Triton X-100, using either MgGTP or MnGTP as

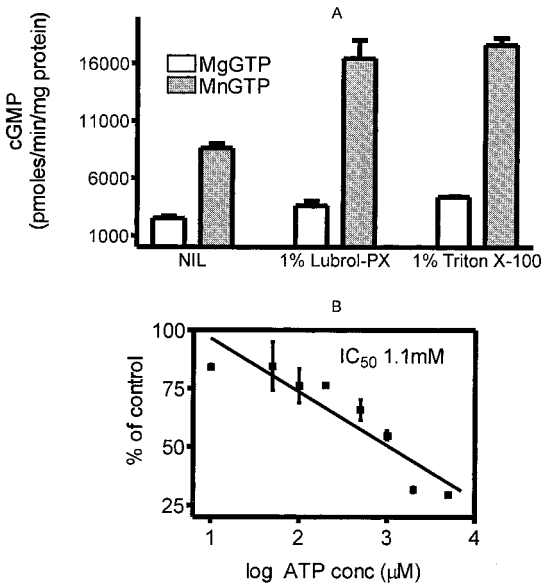

FIGURE 4: Catalytic activity of purified GCC-IDbac. (A) GCC-IDbac was assayed for guanylyl cyclase activity in vitro, using MgGTP (10 mM $MgCl_2$, 1 mM GTP) or MnGTP (4 mM MnGTP, 1 mM GTP) as substrate with or without preincubation of purified protein with indicated concentrations of Lubrol-PX or Triton-X100. Data shown are mean ± SEM of duplicate determinations with the assay performed thrice with different batches of protein. (B) In vitro guanylyl cyclase assays were performed with MgGTP as substrate in the presence of varying concentrations of ATP. Activity detected is depicted as a percent of the activity observed in the absence of ATP. Data shown are mean ± SEM of duplicate determinations with the assay performed twice. Data were analyzed by linear regression analysis using GraphPad software (San Diego, USA).

substrate. A similar fold activation was observed with lower concentrations of either detergent (0.1%; data not shown). The modest 2-fold activation seen with GCC-IDbac in the presence of detergents is in contrast to the nearly 100-fold increase in activity seen with full-length GCC (*27*). This indicates that marked activation of GCC by detergents could require a conformational change induced by regions of the receptor in the extracellular/transmembrane domains of GCC, which are absent in the intracellular domain of GCC-IDbac.

Working with the T84 cell line as a model system to study GCC activity in our earlier studies, and assuming a concentration of GCC of 200 fmol/mg of total membrane protein, we routinely obtain a catalytic activity of 300 pmol of cGMP/200fmol of GCC, in guanylyl cyclase assays using MnGTP as substrate (*27*). The catalytic activity of purified GCC-IDbac that we have measured under similar conditions is of the order of 900 pmol cGMP/200 fmol of protein. This activity that we have measured in purified GCC-IDbac suggests that its guanylyl cyclase activity is at least as high as the native receptor expressed in intestinal cell lines.

Earlier reports have indicated that the presence of ATP during the guanylyl cyclase assay potentiated ST-stimulated activity of GCC (*30*, *31*). We have also earlier provided evidence that ATP was able to inhibit Lubrol-PX stimulated activity (*31*), suggesting that ATP could inhibit a constitutively activated form of GCC. GCC-IDbac presumably represents the constitutively active form of GCC, since we could detect increased intracellular accumulation of cGMP in infected Sf21 cells (Figure 1). We therefore performed guanylyl cyclase assays of the purified recombinant protein

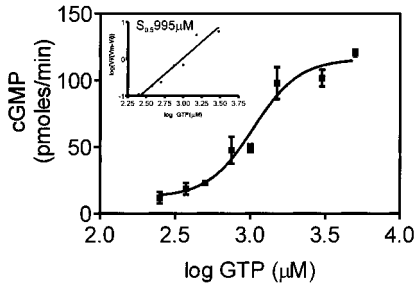

FIGURE 5: Kinetic analysis of the guanylyl cyclase activity of purified GCC-IDbac. In vitro guanylyl cyclase assays were performed with varying concentrations of MgGTP in a ratio of $MgCl_2$/GTP of 10:1. Assays were performed with 10 ng of purified protein, and values represent the mean ± SEM of duplicate determinations with the assay performed thrice. Inset: Double-reciprocal plot of representative data obtained.

in the presence of ATP. The data shown in Figure 4, panel B, demonstrate that the catalytic activity of GCC-IDbac is inhibited in the presence of ATP, at concentrations that have been shown to inhibit the full-length receptor (*30*, *32*). Therefore, the conformation of GCC-IDbac must represent a state that is attained during constitutive activation of GCC, as, for example, in the presence of detergents, but is distinct from that of the ligand-stimulated conformation, where ATP potentiates activity (*31*).

To study the catalytic properties of the recombinant protein, we performed kinetic analysis using varying concentrations of MgGTP as the substrate. The data obtained is shown in Figure 5. The enzyme shows positive cooperativity, as has been reported earlier for the full-length receptor (*33*), and the $S_{0.5}$ for MgGTP is approximately 1 mM, again similar to that reported for full-length human GCC (*33*). A lower ratio of Mg/GTP (1:1), at 1 mM GTP concentrations, showed around 25% of the activity seen with ratios of Mg/GTP of 2:1 and 5:1 (data not shown).

A comparison of the sequences of the catalytic domain of GCC and adenylyl cyclase indicates significant similarity, which is presumably reflected in their three-dimensional structures. It can therefore be anticipated that metal ions known to inhibit the catalytic activity of adenylyl cyclases could regulate the activity of the catalytic domain of GCC. Zn has been shown to inhibit the catalytic activity of adenylyl cyclase, and crystal structures of adenylyl cyclase with the metal ion-ATP complex bound have been recently reported (*34*). Residues involved in binding the metal ion are conserved in both GCC and adenylyl cyclase, as shown in Figure 6, panel A. We therefore tested the ability of Zn to inhibit GCC activity, and the data shown in Figure 6, panel B, indicate that Zn does in fact inhibit GCC-IDbac, with the IC_{50} for $ZnCl_2$ being approximately 80 μM. This is of the order of concentrations required to inhibit adenylyl cyclase (*34*). Interestingly, this is the first report of the inhibition of a receptor guanylyl cyclase by Zn and strongly predicts

A

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          *                                                  *
GCC  YEEVTIYFSDIVGFTTICKYSTPMEVVDMLNDIYKSFDHIVDHHDVYKVETIGDAYM
     ++ V+I F+DI GFT++    T  E+V  LN+++  FD +   +   +++ +GD Y
 AC  HDNVSILFADIEGFTSLASQCTAQELVMTLNELFARFDKLAAENHCLRIKILGDCYY

GCC  VASGLPKRNGNRHAIDIAKMALEILSFMGTFELEHLPGLPIWIRIGVHSGPCAAGVV
       SGLP+   + HA    +M ++++  +    +  + G+ + +R+G+HSG    GV+
 AC  CVSGLPEARAD-HAHCCVEMGMDMIEAISL--VREVTGVNVNMRVGIHSGRVHCGVL

GCC  GIKMPRYCLFGDTVNTASRMESTGLPLRIHVSGSTIAILKRTECQFLYEVR----GE
     G++  ++ ++ + V  A+ ME+ G   RIH++ +T+  L        YEV     GE
 AC  GLRKWQFDVWSNDVTLANHMEAGGKAGRIHITKATLNYLNGD-----YEVEPGCGGE

GCC  --TYLKGRGNET
        YLK    ET
 AC  RNAYLKEHSIET
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B

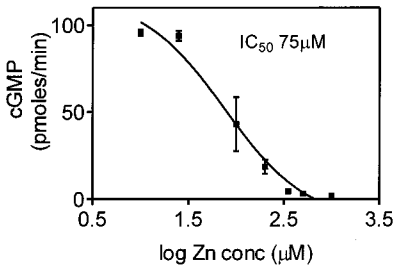

FIGURE 6: Inhibition of guanylyl cyclase activity by Zn. (A) Alignment of the protein sequence of adenylyl cyclase (AC, rabbit Type V, GenBank accession Z29371) and the catalytic domain of GCC. The asterisks identify the aspartate residues shown to be involved in interacting with Zn in adenylyl cyclase (*43*). (B) Inhibition of guanylyl cyclase activity of purified GCC-IDbac by $ZnCl_2$. In vitro guanylyl cyclase assays were performed with purified GCC-IDbac (10 ng) using MgGTP as substrate, in the presence of varying concentrations of $ZnCl_2$. Values represent the mean ± SEM of duplicate determinations with the assay performed thrice. The IC_{50} was determined by a double reciprocal plot of the data obtained from three experiments.

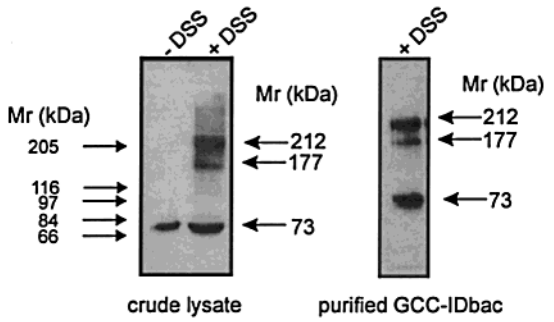

FIGURE 7: The catalytic domain of GCC exists as a homotrimer. Crude lysates from infected Sf21 cells (100 μg) or purified GCC-IDbac (20 ng) were treated with DSS (2 mM) for 5 min on ice. Samples were subjected to Western blot analysis with GCC:4D7 monoclonal antibody. Data shown are representative of experiments performed thrice.

structural similarities between adenylyl cyclase and GCC in the catalytic domain.

The oligomeric status of GCC has been investigated in some detail utilizing co-immunoprecipitation techniques and radiation inactivation methodologies (*22*, *35*). It appears from these studies that the receptor exists as an oligomer even in the absence of the stable toxin peptide, and ligand binding does not appear to change the oligomeric status of GC (*35*). Earlier experiments have suggested that the extracellular domain of GCC contributes to oligomerization (*23*), while other investigators have shown that the oligomerization of GCC is brought about by sequences in the catalytic domain of GCC (*36*). We therefore monitored the oligomeric status of GCC-IDbac, since this would represent a form of GCC where oligomerization could be studied in the absence of sequences from the extracellular domain of GCC.

We added disuccinimidyl suberate to the crude Sf21 lysate containing GCC-IDbac, as well as the purified protein, and monitored the formation of oligomers by subjecting the cross-linked samples to electrophoresis and Western blot analysis with GCC:4D7 monoclonal antibody. As shown in Figure 7, recombinant protein corresponding in size to predicted trimeric and dimeric forms were seen. A significant fraction of the protein continued to migrate as a monomer, indicating that this could represent either monomeric protein, or a fraction of GCC-IDbac, which failed to cross-link under the conditions that we employed here. Cross-linking performed in the presence of SDS completely inhibited the formation of covalently cross-linked dimer or trimer (data not shown) indicating that the formation of the dimer/trimer requires the native conformation of GCC-IDbac to be intact. In agreement with this is the fact that the catalytic activity of GCC-IDbac did not change following cross-linking, and protein treated with SDS failed to demonstrate catalytic activity as would be expected (data not shown). However, to prove conclusively that catalytic activity of GCC-IDbac was present in the trimeric form of the protein, we subjected the crude Sf21 lysate containing GCC-IDbac to gel filtration analysis.

Gel filtration was performed under conditions expected to retain the native oligomeric status of the protein, and fractions were tested for guanylyl cyclase activity and GCC-IDbac content by Western blot analysis. As shown in Figure 8, maximum guanylyl cyclase activity was associated with fractions eluting at the position of proteins expected to have a size of 200 kDa, indicating that the major form of GCC-

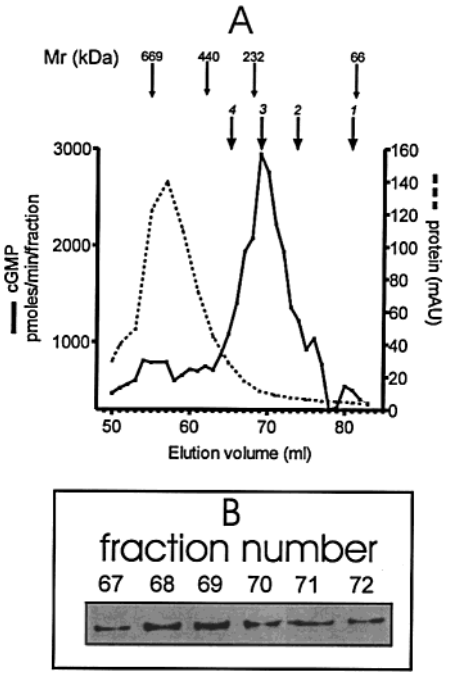

FIGURE 8: Gel filtration profile and Western Blot analysis of GCC−IDbac. Cytosol from infected Sf21 cells (2−3 mg of protein) was applied to a calibrated Superdex 200 column equilibrated with 50 mM HEPES, pH 7.5, 5 mM β-mercaptoethanol, and 100 mM NaCl. Elution was performed at a flow rate of 500 μL/min, and 1 mL fractions were collected. 5 μL of each fraction was subjected to an in vitro guanylyl cyclase assay using MnGTP as substrate (A) and Western blot analysis with GCC:4D7 monoclonal antibody (B). Results shown are representative of four experiments. Arrows on top in panel A indicate the elution positions of thyroglobulin, ferritin, catalase, and bovine serum albumin. The lower arrows indicate the predicted elution positions of the tetramer (*4*), trimer (*3*), dimer (*2*), and monomer (*1*).

IDbac was a trimer, in agreement with the cross-linking data. By Western Blot analysis of fractions with catalytic activity, it appeared that the maximum protein was also associated with fractions representing the trimeric form of the protein. Guanylyl cyclase activity was also associated with fractions eluting at the position of a dimer, but the activity in this fraction and GCC-IDbac content was lower than that of the fraction containing the trimeric protein. Cross-linking data had indicated that a small amount of dimeric GCC-IDbac was however present in the crude protein lysate (Figure 7). This raises the possibility that the regulation of the oligomeric status of GCC could modulate the guanylyl cyclase activity of the receptor, and such changes in oligomerization could be brought about by ligand binding to the ECD or ATP binding to the PKLD. Interestingly, no GCC-IDbac protein or guanylyl cyclase activity eluted in the position corresponding to a monomer, indicating that the monomer detected in cross-linking studies represented protein that was not cross-linked by DSS treatment.

DISCUSSION

We have shown in this study that the cytoplasmic domain of GCC has the potential to form a trimer, in a highly active form of the enzyme produced using baculovirus expression methodologies. Insect cells have been used earlier for the expression of active forms of guanylyl cyclases, including the heterodimeric soluble guanylyl cyclase (*37*), as well as

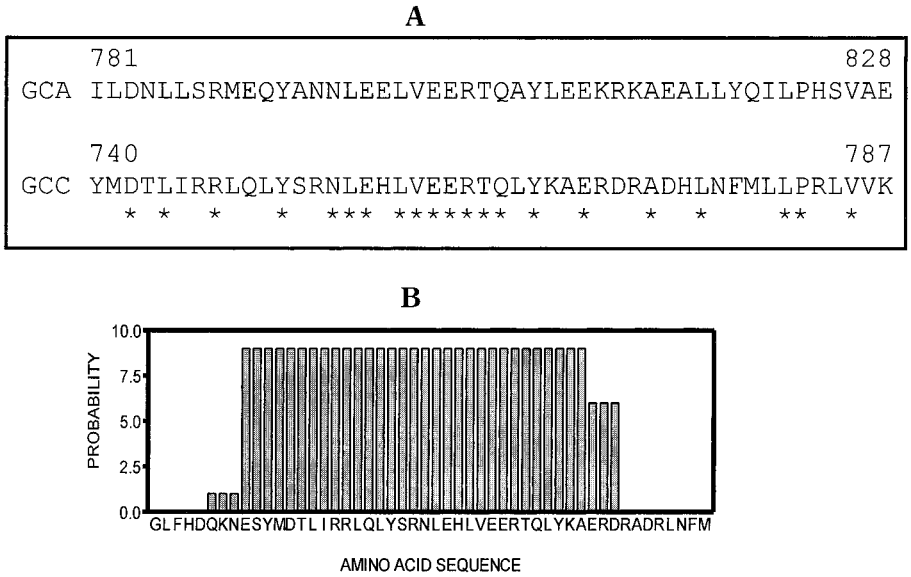

FIGURE 9: Prediction of region mediating oligomerization of GCC. (A) Alignment of regions of GCA and GCC predicted to lie in the linker between the protein kinase-like domain and the catalytic domain of each receptor. Asterisks indicate identical residues. (B) A prediction of a coiled-coil region in GCC based on the program of Lupas (*46*).

the full-length GCA (*38*). In the case of the soluble enzyme, high expression levels were achieved and the enzyme was catalytically active (*37*). The specific activity of the intracellular domain of GCC that we report in this study is higher than that of the soluble heterodimeric guanylyl cyclase, indicating that the catalytic domains of receptor cyclases are effectively inhibited when fused to the extracellular domain. In agreement with this observation, significant activity of full-length GCC expressed in Sf21 cell membranes was observed only on ST stimulation, though guanylyl cyclase activity was higher in cell membranes prepared from cells expressing GCC than in Sf21 cells alone (*16*). Our earlier experiments had indicated that the activation of full-length GCC by detergents could be inhibited by ATP (*31*), suggesting that ATP could regulate the constitutively activated form of GCC in a manner opposite to that of ST-stimulated activity. The fact that ATP could also inhibit the activity of GCC-IDbac argues for the fact that the conformation of the enzyme represents the constitutively active form.

The oligomeric status of receptor guanylyl cyclases, including GCC, has been studied earlier (*22*, *39*). It was realized that the catalytic mechanisms of adenylyl cyclases and guanylyl cyclases were likely to be similar, and following the demonstration that adenylyl cyclases are dimeric (*40*), it was anticipated that guanylyl cyclases exist as dimers as well. Indeed, GCA was shown to be oligomeric in the absence of its ligand, and sequences mediating the formation of oligomers were present in the extracellular domain, since proteins consisting of only the kinase-like or catalytic domain were unable to associate with the wild-type receptor (*39*). Dimers, trimers, and tetramers were identified in cross-linking experiments. Additional experiments performed with the cytoplasmic domain of GCA identified a region between the kinase-like and the catalytic domain of GCA that mediated the formation of dimers (*21*). This sequence corresponds to a stretch of amino acids that we predict to aid in the formation of the coiled-coil structure in GCC (see below). In agreement with this result is the report that the catalytic domain of GCA along with this hinge region could form dimers, and these dimers exhibited catalytic activity (*41*). Deletion of the hinge region led to a complete loss of catalytic activity as well as the formation of dimeric proteins (*41*). Thus, evidence is strong that catalysis of guanylyl cyclases requires the formation of a dimer and consequently, induction of dimer formation can lead to increased catalytic activity.

The oligomeric status of full-length GCC as well as fragments comprising the extracellular domain has also been investigated. Earlier experiments performed with rat GCC expressed in HEK 293 cells had shown that the full-length receptor existed as a trimer, both by radiolabeled ST-cross-linking studies and gel filtration experiments (*22*). Interestingly, the addition of ST seemed to stabilize a dimeric form of the receptor, without dissociation of the individual polypeptide chains, suggesting that a rearrangement of subunits within the inactive trimer to form a stable dimer accounted for the activation of the enzyme by ST (*22*). The report on the cloning and expression of the extracellular domain of porcine GCC, both in mammalian cells as well as in insect cells, suggested that sequences mediating oligomerization of GCC were present in the extracellular domain of GCC (*23*), but our results presented here clearly indicate that a region in the intracellular domain of GCC could also contribute to its trimeric status (*22*). Moreover, it is also clear from our studies that no dissociation of the trimer to a dimer is required for catalytic activity, in agreement with the model proposed earlier, since catalytic activity was associated with a fraction that eluted in gel filtration studies at the position of a trimer (Figure 8).

Our observation that the intracellular domain of GCC can form a trimer has interesting structural implications. On the basis of the crystal structure of adenylyl cyclase, a model of the catalytic domain of retinal GC was built, and point mutations were generated in the guanylyl cyclase core, that allowed the conversion of retinal guanylyl cyclase to an adenylyl cyclase (*42*). These results therefore emphasize that guanylyl cyclases could indeed adopt a similar structure to

adenylyl cyclases. There is significant sequence homology between retinal guanylyl cyclase and GCC, suggesting that the retinal GC model could also apply to GCC. However, the model does not account for the formation of a trimeric structure, since sequences taken for homology modeling did not include the hinge or linker region in retinal guanylyl cyclase (*19*).

We have analyzed the sequence of amino acids present in the intracellular domain of GCC using programs that predict the presence of a coiled-coil structure in a sequence of amino acids (*43*). The results shown in Figure 9, panel A, show that a stretch of amino acids present from residues 740 to 787 in GCC has a probability of forming a coiled-coil structure. Interestingly, there is a significant degree of sequence identity in this region with GCA, accounting perhaps for the higher order oligomers that have been observed with full-length GCA even in the absence of ligand (*6*). Cross-linking of the trimeric GCC-IDbac in the presence of the substrate for GCC, MgGTP, did not change the ratio of dimeric to trimeric GCC (data not shown), thus suggesting that the catalytic activity of the enzyme is perhaps a result of two polypeptide chains within the trimer structure that associate and form the catalytically active, dimeric core.

Of physiological interest in the current study is the demonstration that Zn can inhibit guanylyl cyclase activity. Adenylyl cyclases are inhibited by Zn ions and the crystal structure of the adenylyl cyclase dimer in the presence of Zn has identified residues in the enzyme that coordinate with Zn (*44*). A sequence alignment of GCC and adenylyl cyclase (Figure 6, panel A) identifies aspartate residues that interact with Zn in adenylyl cyclase in equivalent positions in GCC, again emphasizing the structural similarity that is likely to exist in the two enzymes. Interestingly, Zn supplementation is an effective therapy for the treatment of diarrhea (*45*). Cholera toxin mediates its action through activation of adenylyl cyclase and elevations in cAMP levels in intestinal cells, leading to increased chloride secretion via the cystic fibrosis transmembrane conductance regulator, CFTR, along with copious loss of fluid, characteristic of cholera. Thus, inhibition of adenylyl cyclase by Zn should prove an effective way of lowering cAMP accumulation in the cell. Similarly, inhibition of GCC by Zn should also lower cGMP accumulation, thereby again inhibiting chloride secretion and fluid loss. Perhaps the therapeutic effects of Zn supplementation are partly accounted for by the inhibition of enzymes that synthesize these cyclic nucleotides.

In conclusion, our results provide evidence for the first time that the intracellular domain of GCC can form a stable trimer, which presumably allows a catalytically active dimeric structure to form within the trimeric complex. We have identified putative sequences in the intracellular domain that could allow the formation of a coiled-coil structure, and it remains to be investigated if disruption of these sequences alters the activity of GCC. The role of the trimeric (or higher order oligomeric) structure of GCC in terms of regulation of GCC activity remains to be elucidated, both in terms of ligand binding affinity and induction of conformational changes in GCC by other signaling cascades.

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