Identification of Sequences Mediating Guanylyl Cyclase Dimerization†

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ABSTRACT: Deletion mutagenesis was used to identify sequences required for dimerization and enzymatic activity of the intracellular domain of the membrane guanylyl cyclase, GC-A. The intracellular domain of GC-A contains a protein kinase-like domain near its amino terminus, a guanylyl cyclase catalytic domain near its carboxyl terminus, and, between these domains, a region of unknown function predicted to form an amphipathic α-helix. Gel filtration analysis of deletion mutants of the GC-A intracellular domain suggested that a 43 amino acid sequence within the interdomain region was both necessary and sufficient for dimerization and was required for guanylyl cyclase catalytic activity. The ability of this sequence to mediate protein dimerization was confirmed in the yeast two-hybrid system, in which its fusion to the lexA DNA-binding domain and to the VP16 transcriptional activation domain led to their dimerization and consequent activation of a lexA-HIS3 gene. Thus, we have identified sequences responsible for dimerization of the intracellular domain of a guanylyl cyclase and shown that they are required for enzyme activity. Modulation of their interaction may be important in guanylyl cyclase activation.

Membrane forms of guanylyl cyclase are single-transmembrane-domain receptors; binding of peptide ligands to the extracellular domains of these receptors stimulates their intracellular guanylyl cyclase activity (Garbers, 1992). In theory, single-transmembrane receptors must form dimers, either between themselves or with other transmembrane proteins, in order to transduce a signal across the membrane. Both intracellular and extracellular interactions between receptor subunits are necessary for this process. GC-A, a membrane guanylyl cyclase which is a receptor for atrial natriuretic peptide (Chinkers et al., 1989; Lowe et al., 1989), is oligomeric in the basal state (Iwata et al., 1991; Chinkers & Wilson, 1992; Lowe, 1992). The stoichiometry of the receptor oligomer has not been rigorously determined; receptor dimers, trimers, and tetramers have been detected in cross-linking experiments (Iwata et al., 1991; Lowe, 1992). We have shown that the ligand-independent dimerization of GC-A is required for receptor activation and that the extracellular domain is able to dimerize (Chinkers & Wilson, 1992). Thorpe et al. (1991) have shown that an intracellular fragment of GC-A containing the catalytic domain and some additional intracellular sequences is dimeric and enzymatically active when expressed in bacteria.

In addition to the theoretical requirement for dimerization of single-transmembrane receptors for signal transduction to occur, considerable evidence suggests that guanylyl and adenylyl cyclase catalytic subunits must be dimeric to be active. Soluble forms of guanylyl cyclase, which are heterodimeric proteins that function as NO receptors, contain one putative catalytic domain per subunit on the basis of sequence similarities to the catalytic domain of GC-A (Koesling et al., 1991). Since both subunits must be coexpressed to obtain NO-stimulated guanylyl cyclase activity, their dimerization, and specifically the dimerization of their catalytic domains, has been suggested to be essential for their activity (Koesling et al., 1991). It is not clear whether catalytic activity resides in one or both subunits of the soluble guanylyl cyclases. Mammalian adenylyl cyclases also contain two putative catalytic domains on the basis of sequence similarities to the catalytic domain of GC-A, though it is not known whether one or both of these domains are enzymatically active (Krupinski, 1991). Experiments in which the amino half (containing one putative catalytic domain), the carboxyl half (containing the other putative catalytic domain), or both halves of a recombinant adenylyl cyclase were expressed in mammalian cells demonstrated that neither half alone showed adenylyl cyclase activity but that the two half-molecules could combine to form a functional adenylyl cyclase (Tang et al., 1991). All of these data suggest that dimerization of catalytic domains may be important for the activity of guanylyl and adenylyl cyclases, although direct evidence for interactions between catalytic domains has been lacking.

The intracellular portion of each membrane guanylyl cyclase contains a protein kinase-like regulatory domain and a guanylyl cyclase catalytic domain (Garbers, 1992). A region between those two domains that is predicted to form an amphipathic α-helix has been speculated to function as a dimerization domain (Chinker & Wilson 1992; Garbers, 1992). Such a domain could be critical to receptor activation. In order to test this hypothesis, we have systematically mutagenized this domain to determine which sequences are required for dimerization and whether dimerization is required for enzyme activity. In this report, we demonstrate that a short sequence within the putative amphipathic helical region is required for guanylyl cyclase activity of GC-A and is both necessary and sufficient for dimer formation.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses Encoding Deletion Mutants of the GC-A Intracellular Domain. We have previously described (Chinkers & Wilson, 1992) the

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construction of several plasmids encoding portions of the GC-A intracellular domain, each tagged at their amino terminus with the sequence MDYKDDDDKMQ, the underlined residues representing the FLAG peptide epitope (Prickett et al., 1989). These constructs, denoted IN, KIN, and CYC in Chinkers and Wilson (1992), are referred to herein as IN, KIN4, and CYC1. The IN and KIN constructs described here all initiate at residue 464 of GC-A (the linker/adaptor encoding residues 464−466, KMQ); KIN4 terminates at residue 770. All of the CYC constructs described here terminate at the carboxyl end of wild-type GC-A (Gly^{1029}). Numbering of amino acids is based on the sequence of mature rat GC-A after signal peptide cleavage (Chinkers et al., 1989). These constructs were cloned into the baculovirus expression vectors pVL941 (IN, CYC1) or pVL1393 (KIN4).

Systematic deletions from the amino end of CYC1 were prepared, using the polymerase chain reaction to amplify sequences encoding proteins extending from residues 763 (CYC2), 781 (CYC3), 797 (CYC4), or 814 (CYC5) to the carboxyl terminus of GC-A. The synthetic linker/adaptor described above, containing a *Bam*HI site and encoding the sequence MDYKDDDDKMQ, was then ligated to the reaction products. The 3′ primer used in the polymerase chain reactions introduced an *Nhe*I site just 3′ to the wild-type termination codon. After digestion with *Nhe*I, the reaction products were cloned into the *Bam*HI and *Xba*I sites of Bluescript KS+ and sequenced to confirm the absence of undesired mutations. Inserts were excised with *Bam*HI and *Not*I and cloned into the baculovirus expression vector pVL1393.

Deletions from the carboxyl terminus of the GC-A intracellular domain were performed as follows. To prepare KIN1, the *Xba*I/*Eco*RI fragment of pVL-KIN4 (above) was replaced with the 0.9-kb *Xba*I/*Eco*RI fragment of pSVL-cyc$^-$ (Chinkers & Garbers, 1989), a previously described carboxy-truncated GC-A terminating at residue 868. KIN2 (terminating at residue 823) and KIN3 (terminating at residue 805) were prepared by digesting KIN1 with *Dra*III or *Hin*dIII, respectively, blunt-ending with T4 DNA polymerase or Klenow fragment, respectively, and ligating an *Nhe*I linker (GGCTAGCC) to the 3′ end of each construct. The constructs were sequenced to confirm that the termination codon from the *Nhe*I linker had been inserted in the proper reading frame and then were cloned into pVL1393.

Recombinant baculoviruses expressing the constructs described above were prepared by homologous recombination of the various plasmids with linearized BaculoGold DNA (Pharmingen) according to the manufacturer's instructions. In all experiments, second-passage virus stocks grown from purified plaques were used to infect Sf9 cells for 3 days at a multiplicity of infection of 5.

Preparation of Sf9 Cell Extracts. Cells were washed twice with cold 20 mM Hepes, pH 7.4, and 150 mM NaCl and centrifuged at 1400*g* for 10 min at 4 °C. Cells were then resuspended in 350 μL of 20 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 μg/mL leupeptin, and 20 mM benzamidine and lysed by passage 10 times through a 22-gauge needle. Lysates were clarified by centrifugation at 18500*g* for 20 min at 4 °C.

Western Blotting. One hundred microliters of clarified cell extracts or of column fractions was electrophoresed through 12% SDS−polyacrylamide gels using Rainbow molecular weight markers (Amersham) as standards and transferred to nitrocellulose. Incubations of filters with monoclonal antibody M2, directed against the FLAG epitope, and with peroxidase-conjugated second antibody were performed as described (Chinkers & Wilson, 1992). Bound antibodies were detected by chemiluminescence using Renaissance reagents (Dupont NEN).

Guanylyl Cyclase Assays. Forty microliters of clarified cell extracts or of column fractions was brought to a volume of 50 μL containing final concentrations of 1 mM GTP, 0.2 mM isobutylmethylxanthine, and 5 mM $MgCl_2$. After incubation for 20 min at 30 °C, reactions were terminated by addition of 450 μL of boiling 30 mM EDTA, pH 7.0, and cyclic GMP was determined by radioimmunoassay (Domino et al., 1991). Results are presented as the average of duplicate determinations.

Gel Filtration Chromatography. Clarified extracts from 10-cm culture dishes seeded with 5×10^6 cells were passed through a 0.2-μm syringe filter, and 200 μL of each extract was loaded onto a 25-mL Superose-12 column (Pharmacia) equilibrated at 4 °C with 20 mM Hepes, pH 7.4, 150 mM NaCl, and 20 mM benzamidine and run at 0.4 mL/min. Fractions of 0.5 mL were collected. The column was calibrated with molecular weight markers as indicated in the figure legends.

Yeast Two-Hybrid System. Methods and reagents for two-hybrid experiments were as described (Vojtek et al., 1993). A *Pvu*II fragment excised from CYC3, encoding GC-A residues 781−828, was cloned into the *Bam*HI sites of the lexA-fusion vector pBTM116 and the VP16-fusion vector pVP16, after blunt-ending the linearized plasmids with Klenow fragment. In each case, the presence of a single insert in the correct orientation was verified by restriction analysis. L40 yeast cells were transformed with the indicated plasmids and grown on plates containing 5 mM 3-aminotriazole and lacking the indicated amino acids.

RESULTS

Construction and Guanylyl Cyclase Activity of Epitope-Tagged Deletion Mutants. Portions of the intracellular domain of GC-A truncated at the amino or carboxyl ends, and tagged with the FLAG epitope at their amino terminus, were expressed in Sf9 cells using recombinant baculoviruses (Table 1). Cell extracts were analyzed by Western blotting with a monoclonal antibody against the FLAG epitope (Figure 1). Immunoreactive proteins of the expected molecular weights (Figure 1 legend) were expressed at similar levels in all cases (Figure 1). The same extracts used for Western blotting in Figure 1 were used for the guanylyl cyclase assays shown in Table 1. The data in Table 1 indicate that the soluble intracellular domain of GC-A has guanylyl cyclase activity, as expected (6-fold over background levels in this experiment), but this activity is enhanced by an order of magnitude when the protein kinase-like domain (residues 467−745) is deleted (Table 1, CYC1). This is consistent with our previous observation that the protein kinase-like domain is autoinhibitory in intact transmembrane GC-A in the absence, but not in the presence, of bound ligand (Chinkers & Garbers, 1989). Further deletions from the amino terminus extending to residues 762 (CYC2) or 780 (CYC3) reproducibly resulted in further enhancement of guanylyl cyclase activity (Table 1). When the deletions were

Table 1: Guanylyl Cyclase Activity of Sf9 Cell Extracts Expressing Various Epitope-Tagged GC-A Deletion Mutants[a]

Cells infected with	Guanylyl cyclase activity (nmol cGMP formed per 20 min)
Control wild-type baculovirus	1.0
IN	6.0
CYC1	75
CYC2	147
CYC3	151
CYC4	3.3
CYC5	0.2
KIN1	ND
KIN2	ND
KIN3	ND
KIN4	ND

[a] Enzyme activity was determined as described in Experimental Procedures, and the same cell extracts assayed here were used for the Western blots in Figure 1. Samples here and in Figure 1 were normalized for volume rather than for total protein, which amounted to 4 μg (CYC2, CYC3, CYC4), 8 μg (control wild-type baculovirus, IN, CYC5), or 9 μg (CYC1) in this assay. Hatched boxes represent the kinase-like domain, and shaded boxes represent the guanylyl cyclase catalytic domain. ND, not determined.

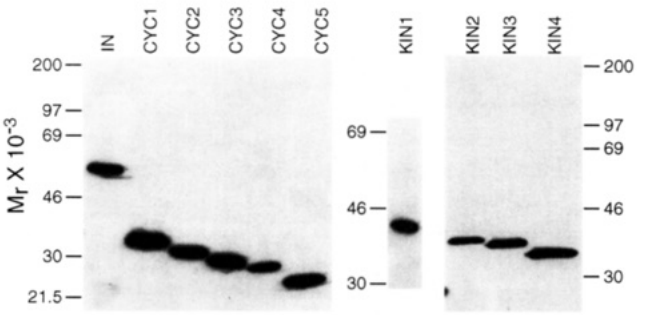

FIGURE 1: Western blots of Sf9 cell extracts containing various deletion mutants of the GC-A intracellular domain. Sf9 cells seeded at 10^6 cells per 35-mm well were infected with the indicated recombinant baculoviruses, and Western blotting of clarified cell extracts was performed as described in Experimental Procedures. Positions of molecular weight markers are indicated. Predicted molecular weights for the expressed proteins, based on their deduced amino acid sequences, were as follows: IN, 65 246; CYC1, 33 456; CYC2, 31 345; CYC3, 29 334; CYC4, 27 431; CYC5, 25 359; KIN1, 47 280; KIN2, 42 466; KIN3, 40 272; KIN4, 36 153.

extended to residue 796, however (CYC4), activity was almost completely destroyed, though it remained slightly above background levels (Table 1). A deletion extending to residue 813 (CYC5) completely destroyed activity; for unknown reasons, extracts of cells expressing this deletion mutant consistently showed lower guanylyl cyclase activity than control cells infected with a wild-type baculovirus (Table 1). We concluded from these data that residues 781−1029 of GC-A are sufficient for guanylyl cyclase activity and that residues 781−796 may be required for activity. Since these residues are predicted to lie outside the catalytic domain, we tested the hypothesis that they were required because they are important for protein dimerization.

Gel Filtration Analysis of Deletion Mutants. We tested the ability of the full intracellular domain of GC-A, and of mutants of the intracellular domain containing amino-terminal deletions, to dimerize. Extracts of Sf9 cells expressing the various proteins were fractionated by gel filtration chromatography, and fractions were analyzed for guanylyl cyclase activity (Figure 2A) and, by Western blotting with an antibody to the FLAG epitope, for the presence of the various epitope-tagged proteins (Figure 2B). Guanylyl cyclase activity was found in fractions corresponding to dimeric proteins for the full intracellular domain and each of the enzymatically active truncated proteins (Figure 2A). The immunoreactive protein peaks were found in the same fractions (Figure 2B). Although in crude extracts CYC4 reproducibly showed trace guanylyl cyclase activity, we were unable to detect this activity after gel filtration (Figure 2A). It is possible that the slightly active CYC4 protein in crude extracts is unstable when diluted during chromatography. This could involve a very weak tendency of CYC4 to self-associate that is only observable in concentrated solution. The amino-truncated intracellular proteins lacking guanylyl cyclase activity after gel filtration chromatography migrated as monomers (Figure 2B). Thus, only those proteins capable of dimerization were active as guanylyl cyclases, and sequences between residues 781 and 796 were necessary for dimerization as well as for enzyme activity.

In order to further delimit the sequences required for dimerization, we analyzed FLAG-tagged carboxyl truncations of the intracellular domain by gel filtration chromatography (Figure 2B). A carboxyl truncation of the intracellular domain at residue 868 (KIN1) resulted in a protein which migrated as an apparent dimer (Figure 2B). Truncation at residue 823 (KIN2) resulted in a protein which migrated as a broad peak at a position consistent with a slowly dissociating dimer (Figure 2B). Carboxyl truncations at residue 805 (KIN3) or 770 (KIN4) resulted in a protein which migrated as a monomer (Figure 2B). This experiment, in conjunction with the gel filtration data analysis of amino-terminal trucations described above, suggests that sequences between

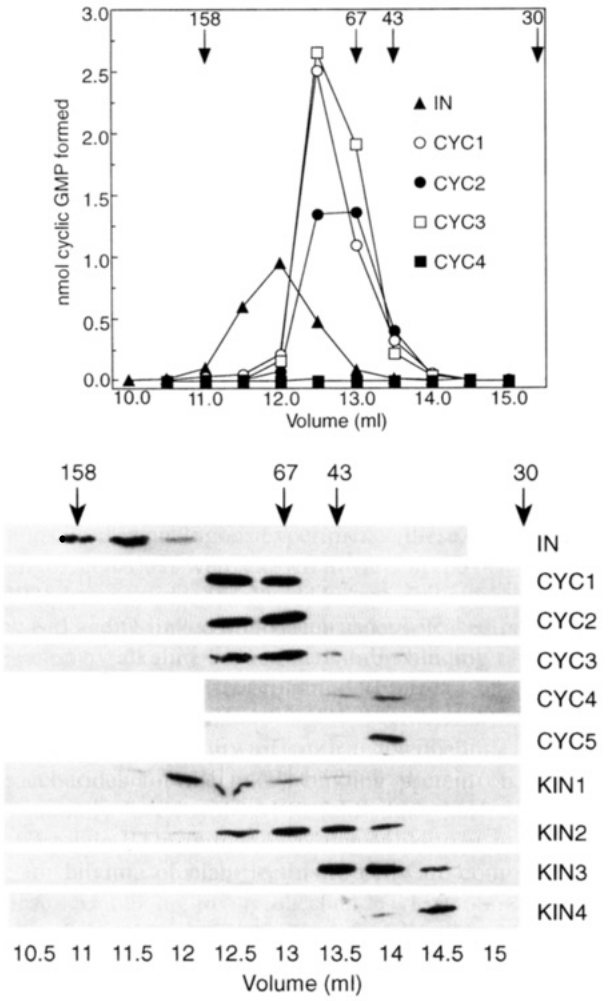

FIGURE 2: Gel filtration chromatography of various deletion mutants of the GC-A intracellular domain. Extracts of Sf9 cells infected with the indicated recombinant baculoviruses were fractionated by fast-performance liquid chromatography over a Superose-12 column, and fractions were analyzed for guanylyl cyclase activity (A, top) or by Western blotting (B, bottom) as described in Experimental Procedures. Elution volumes of molecular weight markers are indicated: aldolase (158 000); bovine serum albumin (67 000); ovalbumin (43 000); carbonic anhydrase (30 000).

residues 781 and 823 are necessary and sufficient for dimerization of the intracellular domain of GC-A. We used another method, the yeast two-hybrid system, to confirm the ability of these residues to mediate dimerization.

Residues 781−828 of GC-A Mediate Dimerization of Heterologous Proteins in the Yeast Two-Hybrid System. The sequence between residues 781 and 821 of GC-A, suggested by the above experiments to form the major part of a dimerization domain (Figure 3A), is potentially capable of forming an amphipathic α-helix (Figure 3B). Amphipathic helices often mediate protein−protein interactions (Erickson-Viitanen et al., 1987; Landschulz et al., 1988; Carr et al., 1991). We tested whether this region could mediate dimerization of heterologous proteins, using a modification (Vojtek et al., 1993) of the yeast two-hybrid system (Fields & Song, 1989) (Figure 3C).

We used a yeast strain containing the HIS3 gene under the control of lexA upstream activating sequences. Proteins postulated to interact with each other are fused to the lexA DNA-binding domain and to the VP16 transcriptional activation domain. The DNA-binding domain and transcriptional activation domain are unable to interact with each other

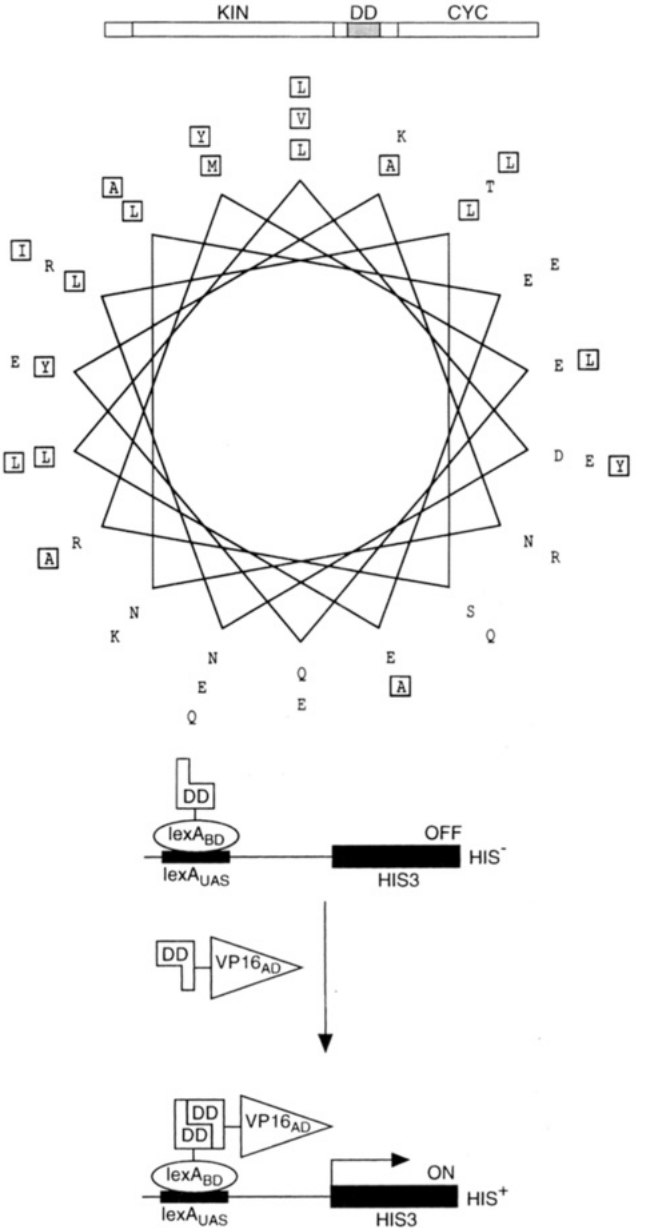

FIGURE 3: Schematic diagrams showing the putative GC-A dimerization domain and the use of the yeast two-hybrid system to test its ability to self-associate. (A, top) The protein kinase-like, guanylyl cyclase, and interdomain regions of the intracellular portion of GC-A are drawn to scale. The putative dimerization domain (DD) is shaded. (B, middle) A helical wheel diagram of the putative dimerization domain, showing its potential amphipathic nature. Hydrophobic residues are boxed. (C, bottom) Use of the yeast two-hybrid system to detect self-association of the putative dimerization domain (DD). Forty-eight amino acids, including the 41 residues shown in (B), were fused to both the lexA DNA-binding domain and the VP16 transcriptional activation domain. The L40 yeast strain contains a HIS3 gene under the control of lexA upstream activating sequences (lexA$_{UAS}$). At the top, in the presence of the lexA−DD fusion, the HIS3 gene is not transcribed due to the lack of transactivating sequences. At the bottom, after cotransformation with a plasmid encoding a VP16−DD fusion, HIS3 transcription is activated if the DD sequences self-associate, and cells may be grown on medium lacking histidine.

except when fused to interacting proteins. If the fusion proteins bind to each other, DNA-binding and transcriptional activation domains are brought together, resulting in the activation of HIS3 transcription (Figure 3C). Dimerization of the fusion proteins is measured as yeast cell growth in the absence of histidine. To test the ability of the putative

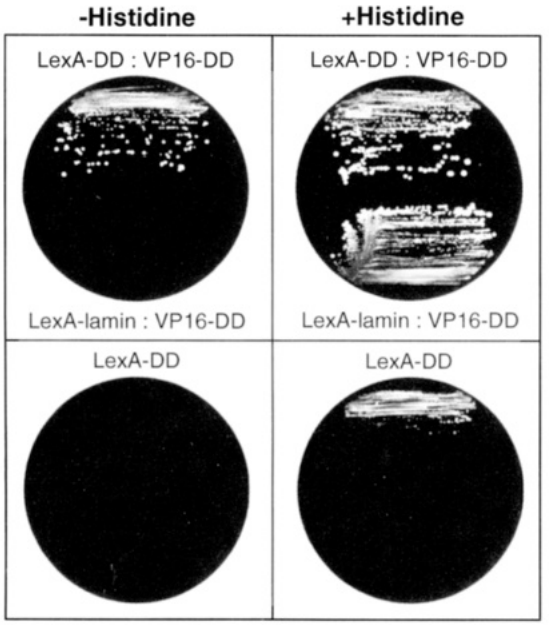

FIGURE 4: Residues 781−828 of GC-A mediate dimerization of heterologous proteins in the yeast two-hybrid system. Yeast were transformed with plasmids encoding fusion of the lexA DNA-binding domain to the putative dimerization domain (DD, residues 781−828) of GC-A, the VP16 transcriptional activation domain to the putative dimerization domain of GC-A, and the lexA DNA-binding domain to lamin, as indicated. Growth in the absence of tryptophan selected for cells containing lexA fusion plasmids (lower panels), while growth in the absence of tryptophan and leucine selected for cells containing both lexA fusion plasmids and VP16 fusion plasmids (upper panels). Identical cultures were plated in the absence (left) or presence (right) of histidine to test for activation of the HIS3 gene.

dimerization domain of GC-A to self-associate, we fused GC-A residues 781−828 to both the lexA DNA-binding domain and to the VP16 transcriptional activation domain. The plasmids encoding these fusion proteins were transformed into yeast, which were then cultured in the absence or presence of histidine (Figure 4).

As shown in Figure 4, cells cotransformed with a plasmid encoding a fusion of the putative GC-A dimerization domain (DD, residues 781−828) to the lexA DNA-binding domain and a plasmid encoding a VP16 activation domain−DD fusion were able to grow in the absence of histidine. This indicated formation of heterodimers between the two fusion proteins. Both fusion proteins were required for activation of the HIS3 gene: cells containing only the lexA−DD fusion or cells containing a control lexA−lamin fusion and the VP16-DD fusion were unable to grow in the absence of histidine (Figure 4). The inability of lexA−lamin to substitute for lexA−DD indicates specific binding of lexA−DD to VP16−DD (Bartel et al., 1993). These results confirm that residues 781−828 of GC-A self-associate in a specific manner and are sufficient to mediate protein dimerization.

DISCUSSION

Within the intracellular region of GC-A and other membrane guanylyl cyclases, a region of approximately 84 amino acids lies between the carboxyl terminus of the protein kinase-like domain and the amino terminus of the putative catalytic domain (Figure 3A). This definition of domain boundaries is based on the observations that Phe^{762} of GC-A is the most carboxyl residue highly conserved among protein tyrosine kinases (Hanks et al., 1988) and that when the sequence of GC-A is aligned with an enzymatically active adenylyl cyclase from *Rhizobium meliloti* (Beuve et al., 1990), Phe^{847} of GC-A corresponds to the amino terminus of the bacterial cyclase. We and others have previously suggested that a potential amphipathic α-helix in this interdomain region (Figure 3B) could mediate dimerization of the intracellular domain of GC-A (Chinkers & Wilson, 1992; Garbers, 1992). Such dimerization may be critical in regulating the enzymatic activity of GC-A, since dimerization is required for the activity of soluble guanylyl cyclases (Koesling et al., 1991) and adenylyl cyclases (Krupinski, 1991; Tang et al., 1991).

We have shown here, by deletion mutagenesis and gel filtration analysis, that a 43 amino acid sequence within this interdomain region is necessary for guanylyl cyclase activity, as well as for dimerization of either the kinase-like domain or the guanylyl cyclase catalytic domain. We then used a separate technique, the yeast two-hybrid system, to confirm that this sequence was sufficient to mediate protein dimerization (Figure 4). In conjunction with the conclusion, from comparison of GC-A with an adenylyl cyclase sequence, that this dimerization domain lies outside the cyclase catalytic domain (above), these data lead us to suggest that dimerization of GC-A catalytic domains, mediated by a sequence outside those domains, is required for enzyme activity. We cannot exclude the possibility that other weak interactions occur between subunits but were not detected due to their being disrupted by cell lysis and gel filtration chromatography.

GC-A is oligomeric in the basal state (Chinkers & Wilson, 1992; Lowe, 1992), and both extracellular and intracellular contacts between subunits in the basal state seem necessary to allow transduction of a conformational change from the extracellular domain to the intracellular domain after ligand binding. It is probable that the major site of intracellular contacts is the dimerization domain described here. This would be consistent with previous models that take into consideration the ability of the kinase-like domain to inhibit cyclase activity in the absence, but not in the presence, of bound ligand (Chinkers & Garbers, 1989). In one such model, conformational changes after ligand binding would free guanylyl cyclase catalytic domains from inhibitory interactions with kinase-like domains within the same receptor subunit or within another subunit in the same receptor oligomer. The data presented here would also be consistent with an alternative model: Intracellular contacts between the kinase-like domain of one subunit and the guanylyl cyclase or dimerization domain of another subunit, in the inactive receptor oligomer, could prevent interactions between the dimerization domains described here. Conformational changes following ligand binding would then allow functional dimerization of catalytic domains through the sequences described here. This could be an attractive model for activation of guanylyl cyclases in general; sequences related to the dimerization domain described here are present upstream of the catalytic domains of soluble guanylyl cyclases. Activation of soluble guanylyl cyclases by NO could involve dimerization of these sequences. If so, the use of peptide inhibitors of dimerization (Rosenmund et al., 1994; Lemmon et al., 1992) could be useful in studies of the physiological effects of cyclic GMP.

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