Regulation of the Atrial Natriuretic Peptide Receptor Guanylyl Cyclase (44042)

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The discovery of atrial natriuretic peptide (ANP) and the demonstration that its receptors were coupled to the activation of particulate guanylyl cyclase (GC) and that ANP receptors copurified with GC activity were important findings in the 1980s. With the molecular cloning of the ANP receptor, the knowledge gained of its structure has allowed new questions to be posed about how signaling through this receptor is regulated—and about signaling through a growing family of related receptors having intrinsic GC activity (1). Here, I review experiments addressing the role of the protein kinase–like domain of the ANP receptor, the regulation of receptor function by adenine nucleotides, the role of dimerization in receptor activation, and the identification of a novel protein-serine phosphatase that may mediate desensitization of the ANP receptor.

Topology of the ANP Receptor

The topology of the ANP receptor, also known as GC-A, is representative of that of a growing number of receptors found in mammals, Drosophila, and *Caenorhabditis elegans*. While additional C-terminal extensions of unknown function are found in some members of this receptor family, all contain the following: (i) an extracellular peptide hormone-binding domain; (ii) a single transmembrane domain; and, intracellularly, (iii) a 20–30–amino acid juxtamembrane region; (iv) a protein kinase-like domain having most of the hallmarks of an authentic protein kinase but lacking certain key residues and lacking protein kinase activity; (v) a C-terminal GC catalytic domain whose activity is stimulated by binding of peptide to the extracellular domain; and (vi) a dimerization domain, located between the kinase-like domain and the GC domain, that is predicted to form an amphipathic α helix (1).

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Protein Kinase–Like Domain

The presence of this structure was noted in the first membrane GC to be sequenced, from the sea urchin *Arbacia punctulata* (2). There was initially some confusion about its function, since the cDNA sequence contained an error resulting in a premature termination codon. The sequence terminated approximately 5 amino acids into what we now know to be the GC catalytic domain, so the intracellular domain of the sea urchin GC appeared to consist almost entirely of a protein kinase–related domain. It was assumed, therefore, that this kinase-like domain (KLD) contained GC activity and that a protein kinase had somehow evolved to perform a different enzymatic function. The molecular cloning of a second sea urchin membrane GC having additional carboxyl-terminal sequences related to the only subunit that had yet been cloned from the heterodimeric soluble GC (3), and our finding of closely related sequences in GC-A (4), set the stage for mutagenesis experiments testing which of these domains—the KLD or the carboxyl-terminal domain—actually contained GC activity.

The KLD itself is remarkably similar in sequence to authentic protein kinases, containing most of the residues conserved among these enzymes, and not being particularly more similar to protein-serine kinases than to protein-tyrosine kinases. Notably absent from the KLD of GC-A, and from the KLD of every known membrane GC, is a conserved aspartic acid residue in subdomain VI that has been shown to be essential for catalytic activity of authentic protein kinases. In general, the ATP-binding amino-terminal lobe of protein kinases is poorly conserved among KLDs, although in GC-A the ATP-binding lysine is conserved and a GXGXXXG sequence similar to the consensus GXGXXG sequence is present. To date, protein kinase activity has not been detected in GC-A or other receptor GCs (1).

It was first noted by Wilks *et al.*, upon their cloning of the first JAK tyrosine kinases, that these proteins contained, in addition to an authentic protein kinase domain, a KLD highly related to the KLD of the ANP receptor and other receptor GCs, often termed a "pseudokinase" domain (5). The function of this do-

main in the JAK kinases, proteins critical in cytokine signaling, is unknown (6).

One possible clue to the function of the KLD of GC-A came from the work of Kurose *et al.*, who had shown that in membrane preparations derived from various tissues ATP, ADP, or nonhydrolyzable ATP analogs were required for optimal stimulation of GC activity by ANP (7). We confirmed these observations using recombinant GC-A expressed in mammalian or insect cells, and hypothesized that since protein kinases bind ATP, perhaps the KLD, although it appeared to lack kinase activity, retained the ability to function as an allosteric ATP-binding site that mediated stimulation of GC-A (8, 9). This has remained a popular hypothesis despite the lack of any direct evidence for the binding of ATP by the KLD. The failure to obtain such evidence may be due to low affinity of ATP for this domain: millimolar levels of ATP are required for maximal activation of GC-A. If this reflects the dissociation constant for ATP, direct binding or affinity labeling studies might not be possible. Sharma and co-workers have published data they interpreted as showing direct binding of ATP by GC-A, but other interpretations are possible, In one set of experiments, higher levels of ATP binding were measured in crude particulate preparations from COS cells transfected with wild-type GC-A than in preparations from cells transfected with GC-A mutated in the KLD (10). It seems unlikely, however, that GC-A is the major ATP-binding protein in COS cell membranes or that these results reflect binding to the receptor. In a dot blot assay, the same group reported binding of ATP to a purified 180-kDa protein whose identity as a variant of the 130-kDa GC-A is controversial (11). Wong *et al.* have recently presented evidence consistent with the hypothesis that the KLD mediates the stimulatory effect of ATP on GC-A activity (12). These workers identified conditions under which GC-A could be solubilized and purified with retention of its ability to be stimulated by ANP and ATP; this had not previously been achieved. The purified receptor's ability to be stimulated by adenine nucleotides indicated that the ATP-binding site is present on the receptor itself, although it remains to be determined whether that site is within the KLD. The observation that mutagenesis of conserved glycine residues in the putative ATP-binding loop of the KLD blocks ANP receptor activation would, however, be consistent with this hypothesis (13).

An additional function of the KLD was identified by deletion mutagenesis experiments. As noted above, due to a sequencing error in the first membrane GC, it seemed possible that the KLD contained GC activity (2). We performed experiments deleting from GC-A either the carboxyl-terminal region related to a subunit of the soluble GC—not yet known to be a catalytic domain—or the KLD. We found that deletion of the carboxyl region resulted in loss of GC activity. Deletion of the KLD, on the other hand, led to a constitutively active GC that was no longer stimulated by ATP or ANP, although the extracellular domain retained the ability to bind ANP (8). These results indicated that the KLD serves an autoinhibitory function and were consistent with the idea that this domain mediates adenine nucleotide effects on GC-A activity. This led to a model of receptor activation in which, in the basal state, GC-A was inactive due to interactions between its KLD and its GC domain. Upon binding of ANP to the extracellular domain of GC-A, a conformational change would ensue allowing binding of ATP to the KLD. That, in turn, would lead to a further conformational change freeing the GC domain from the inhibitory constraint of the KLD and allowing production of the second messenger, cyclic GMP. Activation of GC-A, and of related receptors by deletion of the KLD has been confirmed by other laboratories (14, 15). This model for receptor activation has become somewhat more complex over the past several years, however, with the discovery of roles for receptor dimerization and phosphorylation in regulating GC-A activity.

Dimerization of GC-A Is Required for Its Activation

We and others have tested whether GC-A, like other single-transmembrane receptors, underwent ligand-induced dimerization in order to become active as a guanylyl cyclase. We found that when cells were co-transfected with FLAG-tagged and *myc*-tagged GC-A, the *myc*-tagged receptor co-immunoprecipitated with FLAG-tagged receptor in an ANP-independent manner. Using deletion mutagenesis in conjunction with this co-immunoprecipitation approach, we found that extracellular sequences alone were sufficient for the self-association of GC-A (16). This approach did not address the stoichiometry of the receptor complexes; a cross-linking approach used by Lowe suggested GC-A is present as a tetramer in the absence or presence of ANP (17).

In a separate series of experiments, we mapped sequences involved in dimerization of the intracellular portion of GC-A. Thorpe *et al.* had demonstrated that bacterial expression of a fragment of GC-A, containing the catalytic domain and sequences extending amino-terminal just into the kinase-like domain, migrated as a dimer during gel filtration chromatography and had GC activity (18). We expressed the soluble intracellular domain of GC-A in the baculovirus system and, similarly, found it to be an enzymatically active dimer. We then mapped the sequences responsible for dimerization by deletion mutagenesis. Various truncations of the intracellular domain of GC-A, deleted from the

amino- or carboxy-terminal ends of the protein, were expressed in the baculovirus system. Each construct was assayed for GC activity and, using gel filtration chromatography, for its dimerization state (19). Mutants possessing GC activity all had three things in common: (i) all contained the GC catalytic domain, defined based on its homology with soluble GCs and adenylyl cyclases; (ii) all were dimeric; (iii) all contained sequences between the KLD and GC domains predicted to form an amphipathic α helix. Amphipathic α helices often mediate protein-protein interactions, and all KLD constructs that migrated as dimers in these experiments contained the same interdomain sequences. These sequences were both necessary and sufficient for dimerization and activity of the GC domain of GC-A. They were also able to mediate the dimerization of heterologous proteins (19). These results defined an intracellular dimerization domain and suggested that intracellular dimerization would be critical for receptor activation. We tested this hypothesis using a dominant negative GC-A mutant.

For receptors requiring dimerization for activation, cytoplasmic truncations often act as dominant negative mutants by forming inactive heterodimers with wild-type receptor. We tested whether a cytoplasmically truncated GC-A could inhibit activation of wild-type receptor when co-transfected into COS cells (16). When we examined ANP-stimulated cyclic GMP production in cells transfected with wild-type receptor alone or co-transfected with wild-type and truncated receptors, we found that the mutant receptor inhibited signaling by shifting the ANP concentration-response curve to the right. This is typical of what is seen for receptors undergoing ligand-induced dimerization but was unexpected for the ANP receptor, which is a constitutive oligomer. Others have interpreted these data as suggesting the mutant acts by binding and sequestering ANP, reducing its effective concentration in the medium, rather than as a true dominant negative that forms an inactive heteromer (1). We disagree with that interpretation, based on a control co-culture experiment (16). In that experiment, cells expressing only wild-type receptor were co-cultured with cells expressing only the truncated receptor. Co-culture had no effect on signaling, indicating that the truncated receptor had to be in the same cell as the wild-type receptor to shift the concentration-response curve. This is not consistent with the truncated receptor blocking signaling by binding ANP and reducing its concentration in the medium. We believe that the truncated protein acts by forming inactive heteromers but that binding of ANP stimulates intracellular dimerization. Cooperativity between extracellular and intracellular dimerization would then push the equilibrium to favor wild-type dimers over heterodimers at high ANP concentrations. This would lead to the observed identical maximal response in the absence or presence of the truncated receptor. In our model, in the basal state intracellular dimerization would be blocked or inhibited by the KLD, and a conformation change after ANP binding would lead to a higher affinity intracellular dimerization, and activation of GC.

Thompson and Garbers have shown that an intracellular receptor fragment containing the catalytic domain rendered inactive by a point mutation can also function as a dominant negative mutant, presumably through the formation of inactive heterodimers (20). Thus, using two different types of dominant negative mutant, different laboratories have concluded that dimerization of the intracellular domain is critical to GC-A activation.

GC-A Dephosphorylation and Desensitization

Prolonged exposure of cultured cells either to ANP or to agents that activate protein kinase C results in desensitization of GC-A. Both homologous and heterologous desensitization has been shown to be due to dephosphorylation of GC-A at phosphoserine sites (1). While this phenomenon has been characterized only in tissue culture, it has potential physiological significance. First, GC-A has been reported to fail to undergo significant ligand-induced internalization and degradation, so another mechanism is required for turning off the ANP signal. Second, many agents that activate protein kinase C antagonize ANP effects on vascular smooth muscle tone, so heterologous desensitization by protein kinase C may play a role in cross-talk between different hormonal systems *in vivo*. Finally, in pathological states in which circulating ANP levels are high, physiological responses to ANP become blunted. It is not yet known whether this is due to the dephosphorylation/desensitization pathway seen in cultured cells, but this loss of responsiveness is of clinical interest.

By using the yeast two-hybrid system to identify proteins interacting with the KLD of GC-A, we cloned a novel protein-serine phosphatase designated PP5. PP5 binds to the KLD through an amino-terminal tetratricopeptide repeat (TPR) domain (21). TPR domains are thought to function in protein targeting, and we have hypothesized that PP5 is targeted to GC-A for the purpose of mediating its desensitization and dephosphorylation (21). PP5 has been cloned by others, using PCR and low stringency searches for novel protein-serine phosphatases (22, 23). The TPR domain of PP5 binds to both monomeric and dimeric KLD constructs both in the two-hybrid system and *in vitro* (21). Northern analysis suggests that while PP5 expression is ubiquitous, its expression is highest in tissues that express high levels of GC-A (21). We have purified PP5 expressed in a baculovirus system and characterized its enzymatic activity. Like others (23), we find that

PP5 is stimulated by Mn^{2+} and inhibited by low concentrations of okadaic acid. The binding of PP5 to GC-A and the co-expression of these proteins in the same tissues were consistent with the hypothesis that PP5 mediates GC-A dephosphorylation and desensitization. As further evidence for this hypothesis, we have found that overexpression of PP5 enhances either homologous or heterologous desensitization of GC-A.

Additional evidence is consistent with PP5's playing a role in the GC-A signaling pathway (our unpublished observations). PP5 has low basal activity that can be increased by removal of the TPR domain. This suggests that the TPR domain plays an autoinhibitory as well as a targeting role. Evidence has been published suggesting the existence of an okadaic acid-sensitive protein-serine phosphatase that is activated by the cyclic GMP–dependent protein kinase (PKG) (24, 25). Such a phosphatase mediates ANP effects on the large conductance voltage and Ca^{2+}-activated K^+ channels in GH4 cells (24). We have tested whether PP5 might be this PKG-activated phosphatase. *In vitro,* purified PKG can phosphorylate PP5, and thiophosphorylation of PP5 by PKG can lead to several-fold increases in PP5 activity measured against a phosphopeptide substrate. These experiments are technically difficult due to robust autodephosphorylation and instability of purified PKG. However, they suggest a possible feedback loop in which ANP-stimulated cyclic GMP production would lead to phosphorylation and activation of PP5. The activated PP5, bound to the ANP receptor, would then dephosphorylate and desensitize the receptor, leading to decreased cyclic GMP production. Further studies are necessary to determine whether this pathway is active *in vivo.* We speculate that, if PP5 is phosphorylated by PKG *in vivo,* it could also be involved in downstream signaling by dephosphorylating ion channels (24, 25) or as yet unidentified substrates.

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