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Human Natriuretic Peptide Receptor-A Guanylyl Cyclase Is Self-Associated prior to Hormone Binding

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ABSTRACT: The human natriuretic peptide receptor-A (NPR-A) guanylyl cyclase is specifically activated to synthesize cGMP by binding of atrial natriuretic peptide (ANP) to the receptor's extracellular domain. In this report, NPR-A monoclonal and polyclonal antibodies were used to assess the aggregation status of wild-type NPR-A and a truncation mutant lacking most of the NPR-A cytoplasmic domain. On intact human embryonic kidney 293 cells, in the absence of ANP, recombinant human NPR-A is self-aggregated through disulfide bonds in an M_r >500 000, possibly tetrameric, complex. Under nonreducing conditions, truncated NPR-A was a monomer, indicating that the cytoplasmic domain is necessary for NPR-A self-association. In the presence of the homobifunctional cross-linker dithiobis(succinimidyl propionate), or disuccimidyl suberate, truncated NPR-A could be cross-linked as a dimer and trimer only in the presence of ANP. Wild-type NPR-A was cross-linked with disuccinimidyl suberate to an M_r >500 000 species in the absence of ANP, and with ANP, a smaller, M_r ≈400 000 receptor trimer cross-linking product was observed, together with the larger, possibly tetrameric complex. When whole cell stimulation of cGMP production by ANP was tested on the low level of endogenous 293 cell NPR-A, maximal stimulation was observed regardless of truncated NPR-A overexpression. The absence of a dominant negative effect by the truncated NPR-A, together with the cross-linking data, demonstrates that preassociated NPR-A is the functionally relevant form of this receptor.

Membrane guanylyl cyclases are a family of three single transmembrane domain receptors. Two of these guanylyl cyclases are natriuretic peptide receptors (NPR's),[1] termed NPR-A or GC-A (Chinkers et al., 1989; Lowe et al., 1989) and NPR-B or GC-B (Chang et al., 1989; Schulz et al., 1989). The third member of this family is the intestinal receptor for *Escherichia coli* heat-stable enterotoxin STa (Schulz et al., 1990). Although the STa receptor is not homologous to the NPR's in their extracellular domains, these three receptors share related cytoplasmic domains that divide into two subdomains. The membrane-proximal kinase homology domain is related to protein kinases, and the carboxyl GC catalytic domain of approximately 250 amino acids is homologous to soluble GC and adenylyl cyclase (Chinkers & Garbers, 1991; Krupinksi et al., 1991).

ANP binding to the extracellular domain of NPR-A activates the receptors' guanylyl cyclase domain. The mechanism of signal transduction is not known, but the structural similarities between membrane GC's and receptor tyrosine kinases suggest that the state of receptor aggregation could control cGMP production (Koesling et al., 1991). For class I, III, and IV receptor tyrosine kinases (Ullrich & Schlessinger, 1990), hormone-induced receptor dimerization is the mechanism for signal transduction (Kashles et al., 1991; Ueno et al., 1991, 1992). Along these lines, both soluble GC and adenylyl cyclase have two catalytic domains. Soluble GC is a heterodimer of M_r = 70 000 and 73 000 subunits that share a homologous C-terminal GC domain (Koesling et al., 1988,

[1] Abbreviations: ANP, human atrial natriuretic peptide (28 amino acids); hBNP, human brain natriuretic peptide (32 amino acids); CNP, C-type natriuretic peptide (22 amino acids); NPR, natriuretic peptide receptor; GC, guanylyl cyclase; ΔKC, deletion of *k*inase homology domain and guanylyl *c*yclase domain; DSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; cGMP, guanosine cyclic 3′,5′-monophosphate; GTP, guanosine triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ED_{50}, median effective dose.

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1990). Coexpression of both subunits is required for catalytic activity (Harteneck et al., 1990). Adenylyl cyclase is predicted to be an intrinsic membrane protein with 12 transmembrane domains and two large cytoplasmic loops homologous to the catalytic domain of membrane and soluble GC's (Krupinski et al., 1991). Coexpression of both domains is necessary for adenylyl cyclase activity (Tang et al., 1991). The presence of at least two catalytic domains in soluble GC and adenylyl cyclase suggests that membrane GC's may also be found in a multimeric complex (Koesling et al., 1991). Consistent with this idea, bovine adrenal NPR-A exists as a tetramer when chemically cross-linked to ANP (Iwata et al., 1991), and the rat NPR-A GC domain spontaneously forms a catalytically active dimer when expressed as a single domain (Thorpe et al., 1991).

In this report, I have examined the aggregation state of human NPR-A before and after ANP binding. The results demonstrate that NPR-A is preassociated before ANP binding. Consequently, ANP-dependent interreceptor association is not the mechanism of human NPR-A signal transduction.

EXPERIMENTAL PROCEDURES

Receptor Antibodies and Cell Lines. For immunoblotting of NPR-A, the synthetic peptide antibody A-4/1 (Bennett et al., 1991) was employed. The A-4/1 polyclonal antiserum was raised by immunizing rabbits with a 16 amino acid sequence beginning at residue 31 of the human NPR-A extracellular domain. Immunoprecipitations were performed with mouse monoclonal antibody 1G5, raised against the native extracellular domain of human NPR-A (Lowe & Fendly, 1992).

The human embryonal kidney cell line 293 (American Type Culture Collection, CRL 1573) was grown in F12/Dulbecco's minimal essential medium (50/50) supplemented with 10% (v/v) dialyzed fetal bovine serum (culture medium). The NPR-A expression vector was pFANX (Koller et al., 1992). An expression vector for the cytoplasmic truncation mutant NPR-AΔKC (deletion of *k*inase homology domain and guanylyl *c*yclase domain) that has only a 23 amino acid cytoplasmic domain was constructed in the vector pCIS (Gorman et al., 1990). Using $CaHPO_4$ precipitation, 293 cells were cotransfected with pNeoDHFR (Lowe & Goeddel, 1987) and the receptor expression vector, or just pNeoDHFR for control cell lines. Clones were selected in 400 μg/mL of G418 (Geneticin, Gibco/BRL) in culture medium.

Receptor Cross-Linking and Detection. The cross-linkers DSS and DSP (Pierce Chemical Co.) were initially dissolved to a concentration of 200 mM in dimethyl sulfoxide, then diluted to 1 mM, and used immediately. For cross-linking of [^{35}S]Cys/[^{35}S]Met-labeled receptors, 35-cm-diameter monolayer cultures of 293 Neo, 293 NPR-AΔKC, or 293 NPR-A cells were metabolically labeled as described (Lowe & Goeddel, 1987). Cultures were chilled on ice for 5 min and incubated with 0.5 μM ANP and/or 1 mM DSP in PBS at 4 °C for 15 min. Nonionic detergent extracts were prepared in 1% Triton X-100, 0.05% (w/v) deoxycholic acid, 250 mM NaCl, 24 mM Tris-HCl, and 5 mM EDTA, pH 7.5 (lysis buffer), as described (Bennett et al., 1991), with the inclusion of 60 mM ammonium acetate to stop the cross-linking reaction. Immunoprecipitations were performed in lysis buffer with 0.1% SDS using NPR-A monoclonal antibody 1G5 as described (Koller et al., 1992). For analysis, samples were dissolved in sample mix [4% SDS, 120 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue] either with or without 0.5 M β-mercaptoethanol and fractionated by SDS–PAGE on a 7.5% gel. The gel was then fixed in 50% (v/v) methanol and 10% (v/v) acetic acid, dried under vacuum, and exposed to a storage phosphor imaging plate, and the plate was developed using a Fuji BAS 2000 Bio-Image Analyzer (Fuji Medical Systems, Stamford, CT).

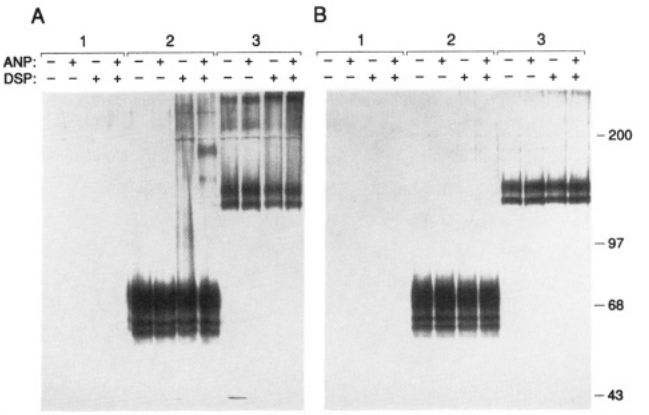

FIGURE 1: DSP cross-linking and immunoprecipitation of full-length and truncated NPR-A. Cells labeled with [^{35}S]Cys and [^{35}S]Met were treated either with (+) or without (–) 0.1 μM ANP or 1 mM DSP as indicated across the top. Immunoprecipitates from 293 Neo (1), 293 NPR-AΔKC (2), and 293 NPR-A (3) cells were fractionated by SDS–PAGE on a 7.5% gel under nonreducing (A) or reducing (B) conditions. Size standards are shown on the right ($M_r \times 10^{-3}$).

For immunoblot analysis, cells in 35-mm wells were treated with 0.5 μM ANP and/or 1 mM DSS (see above), and reactions were stopped by adding to the cell monolayer 500 μL of 2 × sample mix without β-mercaptoethanol. Samples were fractionated by SDS–PAGE on a 7.5% gel, transferred to nitrocellulose, and probed with antibody A-4/1 according to Bennett et al. (1991).

Whole Cell Stimulation of cGMP Production. For 293 cells, cultures in 35-mm-diameter wells were treated with varying concentrations of ANP, hBNP, or CNP at 37 °C for 10 min. 293 Neo or 293 NPR-AΔKC cell lines growing in 35-mm-diameter wells were treated for 10 min at 37 °C with 0.5 μM ANP. Cultures were treated exactly as described (Chang et al., 1989), and intracellular cGMP was measured by radioimmunoassay (Biomedical Technologies, Inc., Stoughton, MA).

RESULTS AND DISCUSSION

To examine the intermolecular association of wild-type NPR-A, or the truncated receptor NPR-AΔKC, in response to ANP binding, immunoprecipitation of ^{35}S-labeled protein was performed after treatment of intact cells with ANP and/or the reducible homobifunctional cross-linker DSP. Cells were treated with nothing, 0.5 μM ANP, 1 mM DSP, or ANP plus DSP. Monoclonal antibody immunoprecipitates were treated either without (Figure 1A) or with β-mercaptoethanol (Figure 1B) to cleave the cross-linker prior to gel electrophoresis. The control cell line 293 Neo does not show detectable endogenous NPR-A by immunoprecipitation. For NPR-AΔKC, without ANP treatment, there is a triplet of proteins with M_r = 64 000, 66 000, and 75 000, either without or with β-mercaptoethanol reduction (Figure 1). The size heterogeneity of NPR-AΔKC is due to differences in N-linked glycosylation (Lowe & Fendly, 1992). In the presence of ANP, NPR-AΔKC migrated as a monomer, but with DSP alone, some higher M_r material is evident as a smear. In the presence of both ANP and DSP, there is cross-linking of NPR-AΔKC to a dimer of $M_r \approx 140\,000$ and a more abundant trimer of $M_r \approx 200\,000$ (Figure 1A). Reduction of the cross-

linker results in only NPR-AΔKC receptor monomers (Figure 1B). Other discrete protein species present in DSP-treated nonreduced NPR-AΔKC samples (Figure 1A) are not evident in immunoblotting experiments (see below), suggesting that they do not represent receptor protein.

Without reduction, NPR-A migrates as a prominent M_r = 135 000 species with two closely spaced smaller species of M_r = 124 000 and 126 000. This size heterogeneity is due to glycosylation differences, as in the case of NPR-AΔKC (Lowe & Fendly, 1992). In addition to receptor monomer, nonreduced NPR-A also exhibited a distinct smear starting at M_r ≈250 000, the size of receptor dimer, and a more sharply resolved species of M_r >500 000, possibly receptor tetramer (Figure 1A). This result is in contrast to the nonaggregated state of NPR-AΔKC and suggests that NPR-A is self-associated without ANP. Cross-linking with DSP appeared to retard the mobility of the M_r >500 000 complex, either without or with ANP binding, and ANP may have increased the amount of M_r >250 000 material (Figure 1A). When electrophoresed after sample reduction, almost all of the high M_r receptor species migrated as monomer (Figure 1B).

The experiment in Figure 1 demonstrates that ANP-dependent cross-linking products for NPR-AΔKC are due to covalent interreceptor linking and not cross-linking to other proteins. Sample reduction shows only NPR-AΔKC monomers and no other associated proteins (Figure 1B). Although NPR-AΔKC clearly showed ANP dependence for intermolecular DSP cross-linking, the reducible aggregated state of NPR-A prior to ANP binding may have obviated detection of cross-linked receptors with DSP. Note that there is an apparent reduction in the amount of NPR-A monomers when the cross-linker DSP is present (Figure 1A, panel 3), suggesting that there may be recruitment of receptor monomers into high M_r complexes. Sample reduction reveals that NPR-A does not cross-link to other ^{35}S-labeled cellular proteins.

To examine receptor aggregation states independent of immunoprecipitation, extracts were analyzed by immunoblotting after treatment with ANP and/or cross-linking with DSS, the nonreducible analog of DSP. For NPR-AΔKC, in the absence of DSS, almost all of the receptor migrates as monomer either with or without reduction (Figure 2A). Untreated NPR-AΔKC shows a very minor disulfide-bonded trimer on this western blot (Figure 2A), but this species is not evident by immunoprecipitation (Figure 1A). The reason for this difference is most likely due to the two different detection methods used. Antibody binding in the immunoprecipitation reaction may have caused reduction of this minor population of NPR-AΔKC trimers. When ANP is added to NPR-AΔKC, the minor trimer population is no longer present. ANP binding may therefore be causing a change in reactivity of sulfhydryl groups to disulfide formation. In the presence of DSS alone, there is significant dimer and an increase in the trimer species, together with very high M_r aggregates of NPR-AΔKC. However, after reduction, most of these species are lost to the monomer size with very little of the dimer visible on the gel. This result suggests that DSS cross-linking is promoting disulfide exchange among receptors without actual bifunctional cross-linking. In the presence of ANP, DSS treatment causes trapping of NPR-AΔKC dimers and trimers (Figure 2A). These cross-linking products are nonreducible, but the very high M_r receptor aggregate of NPR-AΔKC is still lost on reduction (Figure 2A). Similar results are therefore obtained for DSP or DSS cross-linking of NPR-AΔKC in the presence of ANP. However, the immunoblotting experiment suggests that a minor amount of NPR-AΔKC may be self-associated

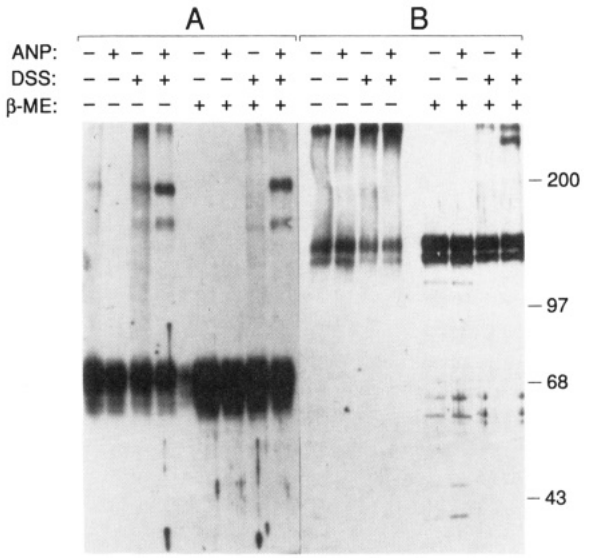

FIGURE 2: Receptor immunoblot detection after DSS cross-linking. 293 NPR-AΔKC (A) or 293 NPR-A (B) cells were treated either with (+) or without (−) 0.1 μM ANP or 1 mM DSS, and lysates were fractionated by SDS–PAGE on a 7.5% gel under either nonreducing (β-ME −) or reducing (β-ME +) conditions. Proteins were electrophoretically transferred to nitrocellulose and probed with anti-NPR-A synthetic peptide antibody A-4/1. Size standards are shown on the right ($M_r \times 10^{-3}$).

prior to ANP binding.

For NPR-A, a nearly identical situation is observed on immunoblotting (Figure 2B) compared to immunoprecipitation (Figure 1), when samples are not reduced prior to electrophoresis. A significant amount of the immunoreactive material migrated as an M_r >500 000 aggregate; however, ANP treatment alone does cause an increase in the amount of aggregate receptor (Figure 2B). This was not observed by immunoprecipitation (Figure 1). There is also an apparent reduction in the amount of NPR-A monomer with DSS treatment (Figure 2B, β-ME −), similar to what is observed by immunoprecipitation (Figure 1A). When samples are reduced, the effects of cross-linking are apparent; DSS traps M_r >500 000 NPR-A, and ANP binding causes cross-linking of an additional M_r ≈400 000 species that may represent receptor trimers (Figure 2B). In Figure 2B, there are immunoreactive bands below the NPR-A monomer. These proteins correspond to antibody-cross-reactive endogenous 293 cell proteins that do not cross-link to NPR-A (Figure 1B). These proteins are absent in Figure 2A due to the shorter exposure time for chemiluminescent detection of NPR-AΔKC.

The data presented in Figures 1 and 2 demonstrate that a portion of NPR-A is self-associated in a reducible manner prior to ANP binding. The absence of abundant NPR-AΔKC aggregation before ANP binding demonstrates that the cytoplasmic domain of NPR-A is at least necessary for disulfide bond formation between NPR-A monomers. The minor amount of NPR-AΔKC trimer seen by western blotting could be indicative of association for the entire population of receptors; however, unlike NPR-A, there is very little NPR-AΔKC oligomer cross-linked in the absence of ANP (Figures 1A and 2A).

Not all of the available NPR-A is covalently aggregated, suggesting that disulfide bond formation between NPR-A monomers is inefficient and may be indicative of association but not a necessary consequence thereof. When analyzed under nonreducing conditions, there is a decrease in the amount of NPR-A monomer with DSP or DSS (Figures 1A and 2B). This observation is consistent with the idea that disulfide

formation is inefficient and that most of the receptor is in a noncovalent associated state. Cross-linking would then decrease the amount of monomer observed on a nonreducing gel. Presumably this would be cross-linking to other NPR-A molecules, since there is no cross-linking to other prominent cellular proteins (Figure 1B).

For receptor tyrosine kinases such as the epidermal growth factor receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor (Kashles et al., 1991; Ueno, 1991, 1992) and for the non-kinase, tumor necrosis factor receptor (Tartaglia & Goeddel, 1992), deletion of the cytoplasmic domain results in a dominant negative mutation for inhibition of wild-type receptor signaling. In this activation paradigm, hormone-driven dimerization of monomeric receptors is necessary and sufficient for signal transduction. For NPR-A, if receptor association prior to ANP binding is functionally significant, then ANP-driven aggregation of receptor monomers may not be the mechanism of activation. In this model, the deletion mutant NPR-AΔKC should not be a dominant negative mutation for signal transduction. To determine if NPR-A and NPR-AΔKC could be cross-linked together in an ANP-dependent manner, cell lines coexpressing both receptor forms were developed. With DSP as a cross-linker, ^{35}S-labeled cells were treated with ANP, and full-length NPR-A was immunoprecipitated with an NPR-A C-terminal synthetic peptide antibody (Potter & Garbers, 1992) that does not recognize NPR-AΔKC. No evidence was obtained for full-length and truncated receptor association (unpublished data).

To determine if there is a functional effect of NPR-AΔKC coexpression on NPR-A activation, ANP stimulation of the low level of endogenous NPR-A in human embryonic kidney 293 cells was tested. Whole cell stimulation of cGMP production in 293 cells showed a natriuretic peptide rank order of potency, ANP > hBNP >>> CNP, with ED_{50} = 1.1 nM for ANP and 64 nM for hBNP (Figure 3a). This level of receptor expression is not detectable by immunoprecipitation (Figure 1) but displays the same sensitivity to natriuretic peptide stimulation as rat and human NPR-A (Schulz et al., 1989; Koller et al., 1992). By polymerase chain reaction amplification of 293 cell complementary DNA, NPR-A is the only membrane GC or NPR expressed in these cells (unpublished experiments), confirming the pharmacological identification of NPR-A in 293 cells (Figure 3A).

According to the dimerization model for receptor activation, if x is the ratio of truncated to wild-type receptors, then at saturating concentrations of ligand, the probability of forming normal homodimers is $1/(1 + x)^2$ (Ueno et al., 1991). If ANP-induced receptor association is the mechanism of activation, then it may be expected that in 293 NPR-AΔKC cells there should be complete inhibition of the low level of endogenous NPR-A ($x \gg 1$). Whole cell stimulation of cGMP production with 0.5 μM ANP was measured on three 293 Neo clones as a positive control and four 293 NPR-AΔKC clones (Figure 3B). Testing of several different cell lines provides an indication of the clonal variation for ANP stimulation of cGMP production. Expression of NPR-AΔKC did not suppress the activation of endogenous NPR-A, demonstrating that the NPR-A cytoplasmic truncation mutant is not a dominant negative mutation for ANP-stimulated signal transduction by NPR-A.

ANP causes both NPR-A and NPR-AΔKC to become sensitive to interreceptor DSS cross-linking. With ANP bound, there is either a change in reactivity or a juxtaposition of the extracellular domain reactive groups to within the 12-Å

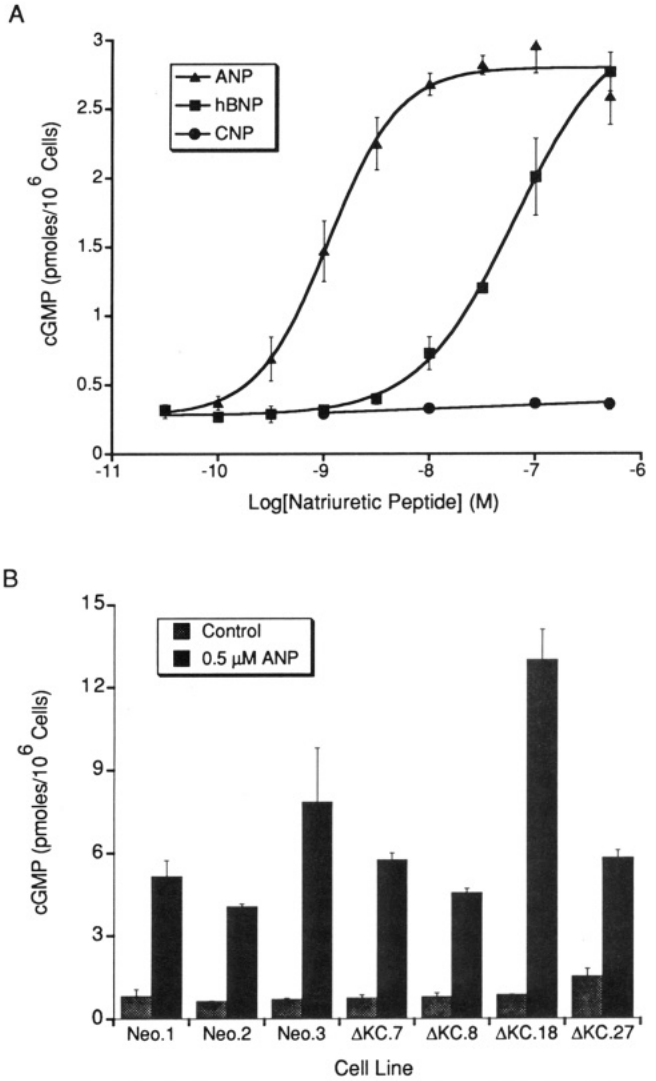

FIGURE 3: Stimulation of endogenous NPR-A on 293 cells. Dose response for whole cell stimulation of cGMP production in 293 cells (A). Basal and 0.5 μM ANP stimulated cGMP levels in 293 Neo clones (Neo 1, Neo 2, and Neo 3) and 293 NPR-AΔKC clones (ΔKC.7, ΔKC.8, ΔKC.18, ΔKC.27) (B). Values are the mean ± standard error (n = 3).

cross-linking radius of DSS (Brenner et al., 1985). The wild-type NPR-A is preassociated and does exhibit cross-linking in the absence of ANP. With ANP, a trimeric complex is cross-linked with DSS, possibly as a result of trapping a signal transduction intermediate form of the receptor. ^{125}I-ANP cross-linking to NPR-A with DSS only shows receptor monomer (Chinkers et al., 1989), possibly as a result of the low concentration of ANP (0.5 nM) used, relative to the experiments described here (0.1 μM). Interreceptor cross-linking is not observed at 0.5 nM ANP (unpublished observations), suggesting that, at the lower ANP concentrations, there is not enough of the reactive intermediate present for detection with DSS cross-linking.

The human NPR-A is more like the class II receptor tyrosine kinases, such as the insulin receptor (Ullrich & Schlessinger, 1990). In this case, the receptor is preassociated before ligand binding, and activation requires two identical kinase domains (Treadway et al., 1991; Chin et al., 1991). Implicit in our model for activation is the idea that the activated GC domain of NPR-A is at least a dimer, consistent with the cooperativity of membrane GC for GTP as substrate (Waldman & Murad, 1987) and the GTP cooperativity of the dimeric catalytic core of NPR-A expressed in *E. coli* (Thorpe et al., 1991). Since

the cytoplasmic kinase homology domain of NPR-A acts to repress the catalytic activity of the GC domain in the absence of ANP (Chinkers & Garbers, 1989; Koller et al., 1992), this kinase-like domain may serve to inhibit GC domains from spontaneous association in the preformed receptor complex.

The common theme for soluble GC, and membrane adenylyl cyclase of multiple catalytic units in the quiescent enzyme, is now reflected in the membrane GC NPR-A. Given that NPR-A does exist in a preassociated state, it will be important to determine if ANP binding to only one extracellular domain can induce activation with unbound receptors in the same complex. Such a reaction could have profound implications for the mechanism of signal transduction.

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