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Conservation of the Kinaselike Regulatory Domain Is Essential for Activation of the Natriuretic Peptide Receptor Guanylyl Cyclases

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The natriuretic peptide receptors, NPR-A and NPR-B, are two members of the newly described class of receptor guanylyl cyclases. The kinaselike domain of these proteins is an important regulator of the guanylyl cyclase activity. To begin to understand the molecular nature of this type of regulation, we made complete and partial deletions of the kinase domain in NPR-A and NPR-B. We also made chimeric proteins in which the kinase domains of NPR-A and NPR-B were exchanged or replaced with kinase domains from structurally similar proteins. Complete deletion of the kinase homology domain in NPR-A and NPR-B resulted in constitutive activation of the guanylyl cyclase. Various partial deletions of this region produced proteins that had no ability to activate the enzyme with or without hormone stimulation. The kinase homology domain can be exchanged between the two subtypes with no effect on regulation. However, structurally similar kinaselike domains, such as from the epidermal growth factor receptor or from the heat-stable enterotoxin receptor, another member of the receptor guanylyl cyclase family, were not able to regulate the guanylyl cyclase activity correctly. These findings suggest that the kinaselike domain of NPR-A and NPR-B requires strict sequence conservation to maintain proper regulation of their guanylyl cyclase activity.

The natriuretic peptides (NPs) are a family of hormones involved in the control of body fluid homeostasis (28). Atrial NP (ANP) is secreted from atrial myocytes in response to increased intravascular volume and arterial blood pressure and induces natriuresis, diuresis, and vasorelaxation (5). Brain NP (BNP), first isolated from porcine brain, is found predominantly in the heart (15, 33). Its biological activities in vivo and in vitro parallel those of ANP (24, 31, 33), suggesting both of these peptides may interact with a common receptor. The third member of this hormone family, C-type NP (CNP), appears to be expressed exclusively in the central nervous system (18). CNP is significantly less potent at inducing natriuretic, diuretic, and hypotensive effects in rat and chick bioassay systems than is either ANP or BNP (34); therefore, CNP, interacting with specific receptors in the brain, may coordinate central aspects of the regulation of salt and water balance and blood pressure.

Three different NP receptors (NPRs) have been described. NPR-C is a 496-amino-acid single-transmembrane-domain protein with a short 37-amino-acid cytoplasmic tail (12, 21). It can bind the three known NPs and most NP analogs (4), and it has been proposed to function as a clearance receptor (23). The other two NPRs, NPR-A and NPR-B (also called GC-A and GC-B), are members of the newly described family of receptor guanylyl cyclases (GCs) (7, 9, 22, 30). Activation of these two receptors causes a dose-dependent increase in intracellular accumulation of cyclic GMP (cGMP), which presumably serves as a second messenger for downstream signalling events within the cell (19). The GC activity of NPR-A is most efficiently stimulated by ANP; BNP is as efficacious but is approximately 10-fold less potent (30). CNP does not bind or activate human NPR-A at physiological concentrations (19). Conversely, the GC activity of NPR-B is selectively stimulated by CNP; neither ANP nor BNP can activate NPR-B except at very high concentrations (19, 30).

NPR-A and NPR-B are single-transmembrane-domain proteins whose intracellular regions can be divided into two domains (7, 9, 22, 30). The carboxyl-terminal portion of NPR-A and NPR-B comprises the GC enzymatic activity of these proteins and has the highest level of sequence conservation (88% identity) between the two receptor subtypes (8, 35). The approximately 280 amino acids between the transmembrane and GC domains, the kinase homology domain, possess characteristic homology to the catalytic domain of protein kinases. These domains from NPR-A and NPR-B are 63% identical to each other and are approximately 30% identical to a wide range of protein kinases. Each contain 28 or 27, respectively, of the 33 highly conserved individual amino acids of these kinases, as described by Hanks et al. (14). Whether NPR-A or NPR-B has intrinsic kinase activity, however, is unknown.

Studies on NPR-A have suggested that the kinase homology domain serves an important role in transducing the hormone binding signal to activate the GC. Mutant NPR-A proteins that completely lack the kinase homology domain constitutively produce cGMP, independent of the presence of activating hormone (8). This observation suggests that the kinase homology domain normally represses the enzymatic activity of the receptor and that binding of hormone to the extracellular domain releases this inhibition. A similar type of regulation has been postulated for the enzymatic activity of the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor with a single membrane-spanning region (2, 3). The protein kinase activity of this receptor is suppressed by interaction of its catalytic domain with its carboxy terminus. Binding of epidermal growth factor releases this inhibitory interaction, perhaps as a result of receptor dimer-

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ization (37), and stimulates the receptor's tyrosine kinase activity.

To clarify the role of the kinase homology domain in regulating the GC activity of the NPRs, we studied whether the kinaselike domains of various proteins were interchangeable. We made chimeric proteins in which the kinase domains of the two human NPR subtypes were exchanged. Similar chimeras were also made by using the kinase domains from the human EGFR and a new member of the GC receptor family, the human intestinal *Escherichia coli* enterotoxin (heat-stable toxin type a) receptor (STaR) (11, 29). To determine which regions of the kinaselike domain may be important for modulation of the activity of the GC domain, mutant receptors with partial or complete deletions of the kinase homology domain were studied. The data obtained support the hypothesis that the entire kinaselike domain of the NPRs is essential for proper regulation of the production of their second messenger, cGMP.

MATERIALS AND METHODS

Materials. Human NPs (hANP, hBNP, and hCNP [=pCNP-22]) were obtained from Peninsula Laboratories (Belmont, Calif.). ^{125}I-hANP was purchased from Amersham Corp. (Arlington Heights, Ill.). The cGMP radioimmunoassay kit was from Biomedical Technologies, Inc. (Stoughton, Mass.). Protein A-Sepharose CL-4B was purchased from Pharmacia (Piscataway, N.J.). The monoclonal antibody specific for the hNPR-A extracellular domain was prepared as described elsewhere (22a). The rabbit anti-mouse antibody was obtained from Cappel (West Chester, Pa.). Other laboratory supplies were obtained from standard suppliers.

Cell culture and transfections. COS-7 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum under 5% CO_2–95% air at 37°C in a humidified atmosphere. Cells were plated at 2.5×10^5 cells per well in six-well dishes (for cGMP assays), at 8×10^5 cells per 10-cm dish (for binding studies), or at 5×10^5 cells per 60-mm dish (for immunoprecipitation or membrane GC experiments) 18 h before transfection by lipofection with the appropriate plasmid DNA (1.5 μg of DNA/15 μg of Lipofectin reagent per 35-mm well; 5 μg of DNA/50 μg of Lipofectin reagent per 10-cm dish; 3 μg of DNA/30 μg of Lipofectin reagent per 60-mm dish) as instructed by the manufacturer (GIBCO-BRL, Bethesda, Md.). Forty-eight hours after addition of DNA, cells were either stimulated and assayed for intracellular cGMP production or harvested for binding or membrane GC assays.

Receptor constructs. All receptor constructs were made in the pRK expression vector. In this vector, the cDNA of interest is under the control of the cytomegalovirus immediate-early promoter, and the plasmid contains the simian virus 40 origin of replication to increase transient expression of the encoded protein in COS-7 cells (7).

Silent mutations in both pRKANP-AR (22) and pRKANP-BR (7) were made to generate new unique restriction sites to facilitate subsequent receptor constructs. For pNPR-A, an *Nhe*I site at codons 472 to 473 and an *Xho*I site at codons 795 to 796 were inserted. For pNPR-B, a *Bsu*36I site at codons 696 to 698 and an *Xho*I site at codons 790 to 791 were made. A region of these cDNAs encompassing these codons was subcloned into a phagemid vector (pBluescript or pTZ18U; Promega and Bio-Rad, respectively), single-stranded DNA was isolated, and mutagenesis was performed by using phosphorylated mutagenic primers with the Bio-Rad Muta-gene phagemid in vitro mutagenesis kit according to the manufacturer's instructions. Mutant primers were as follows:

A-NheI	5′-(bp 1527) CCGCCACAGCTC**gcta**GCCAGTTCCTTCTCC (bp 1497)-3′
A-XhoI	5′-(bp 2491) CCAGTTCCTC**g**AGATTGTTCGC (bp 2469)-3′
B-Bsu 36I	5′-(bp 2162) GAAAGGACCACT**cC**t**A**AG**g**CTATCTCCTGC (bp 2142)-3′
B-XhoI	5′-(bp 2446) CCAGCTTCTC**g**A**g**GTTATTGGC (bp 2434)-3′

The lowercase nucleotides represent the mutated residues. The resultant mutants were screened by restriction digestion and sequencing analysis (Sequenase kit; U.S. Biochemical Corp., Cleveland, Ohio).

The pAΔKHD and pBΔKHD constructs (see Fig. 4A) were made by digesting pNPR-A and pNPR-B with *Nhe*I and *Xho*I, isolating the large vector band, and religating the fragments in the presence of a synthetic 15-codon linker (amino acids 780 to 794 in NPR-A and 775 to 789 in NPR-B) to reconstitute the coding region from the beginning of the GC domain to the *Xho*I site. This linker contains a 5′ *Nhe*I site and a 3′ *Xho*I site to allow for directional cloning. The final constructs were verified by sequence analysis and resulted in the deletion of amino acids 474 to 779 for pAΔKHD or 468 to 774 for pBΔKHD.

The kinase domain exchange constructs, pA[B]A and pB[A]B (Fig. 1A), were made by digesting pNPR-A and pNPR-B with *Nhe*I and *Xho*I. The vector and cDNA fragment containing the extracellular and GC domains was ligated with the *Nhe*I-*Xho*I fragment encoding the kinaselike domain from the alternate receptor. The pA[B]A construct encodes a protein with amino acids 1 to 473 of NPR-A, 468 to 789 of NPR-B, and 795 to 1029 of NPR-A. The pB[A]B-encoded protein contains amino acids 1 to 467 of NPR-B, 474 to 794 of NPR-A, and 790 to 1025 of NPR-B.

For the pA[EGF]A and pB[EGF]B constructs (see Fig. 3A), the region of cDNA encoding the kinase domain from the human EGFR (36) (amino acids 684 to 950) was isolated from EGFR cDNA by using the polymerase chain reaction (PCR). The 5′ PCR primer encoded amino acids 684 to 693, including an *Eco*RI site at codons 686 to 687. The 3′ PCR primer encompassed amino acids 936 to 950 and included a silent mutation at codon 942 to eliminate an *Eco*RI site which was approximately 20 bp upstream from the 3′ end of the kinase domain. In addition, an *Nhe*I site was added to the downstream portion of this primer. The PCR product was generated in an automated thermal cycler (Perkin Elmer-Cetus) (30 cycles of 1 min at 94°C, 30 sec at 55°C, and 1 min at 72°C) and was digested with *Eco*RI and *Nhe*I. The subsequent 800-bp fragment was isolated. Two 69-bp synthetic linkers were made that encoded the region from the *Nhe*I site to the amino terminus of the kinaselike domains of the two NPRs (amino acids 472 to 493 of NPR-A and amino acids 476 to 487 of NPR-B) and the Thr-685 and *Eco*RI sites (codons 686 and 687) of the EGFR. These linkers were digested with *Nhe*I and *Eco*RI, ligated to the 800-bp EGFR PCR product, and redigested with *Nhe*I. The appropriate bands of 869 bp were isolated and ligated to either pAΔKHD or pBΔKHD that had been digested with *Nhe*I. The expression constructs were analyzed for correct orientation by restriction digestion and verified by complete sequence analysis across the 869 bp of EGFR and linker DNA. The final constructs (pA[EGF]A and pB[EGF]B) encoded proteins that contained amino acids 1 to 493 of NPR-A and 1 to 477 of NPR-B, respectively, followed by amino acids 685 to 950 of the EGFR kinase domain, a two-amino-acid insertion of Ser-Ala, and finally amino acids 780 to 1029 of NPR-A and 775 to 1025 of NPR-B, respectively.

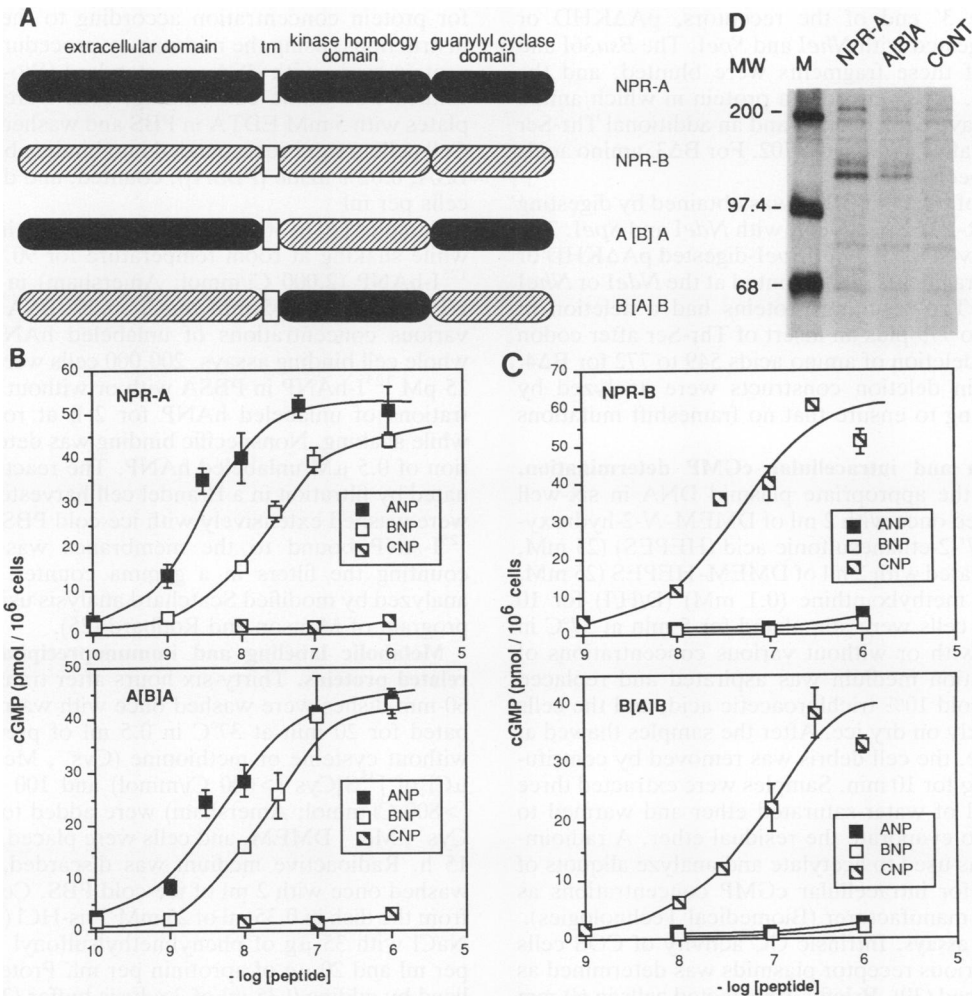

FIG. 1. GC activities of the A[B]A and B[A]B chimeric receptors. (A) Schematic representation and nomenclature of the wild-type and chimeric receptors. The kinase homology domains of the wild-type receptors were exchanged, and the expression plasmids were transfected into COS-7 cells. Cells expressing NPR-A or A[B]A (B) and NPR-B or B[A]B (C) were stimulated with increasing amounts of human NPs, and intracellular cGMP was measured. Each experimental value was determined in triplicate and expressed as picomoles of cGMP per 10^6 cells ± standard error of the mean. (D) Immunoprecipitation of NPR-A and A[B]A from COS cell lysates. [^{35}S]Cys- and [^{35}S]Met-labeled cell lysates (50 μg) were immunoprecipitated with a monoclonal antibody specific for the extracellular domain of NPR-A and analyzed on a 10% polyacrylamide gel as described in Materials and Methods. Molecular weight (MW) is shown in thousands; size markers are represented by lane M.

For the A[STa]A chimeric receptor (see Fig. 3A), the cDNA encoding the kinaselike domain of the human STaR (11) was prepared by PCR as described above. The 5′ PCR primer encoded amino acids 458 to 465 and included a Thr-to-Phe mutation at codon 461 to generate a 5′ *Eco*RI site. The 3′ PCR primer encompassed amino acids 730 to 739 and generated a 3′ *Nhe*I site by mutating Glu-735 to Ala. After digestion of the PCR product with *Eco*RI, the approximately 830-bp fragment was purified and ligated to the *Nhe*I-*Eco*RI-digested 69-bp synthetic linker described above that encoded the region of NPR-A (amino acids 472 to 493) from the *Nhe*I site to the amino-terminal portion of the kinaselike domain. This ligation was digested with *Nhe*I, and the 900-bp fragment was isolated and ligated to *Nhe*I-digested pAΔKHD. The resulting construct contained amino acids 1 to 493 of NPR-A, followed by amino acids 461 to 736 of the StaR kinaselike domain (including the T461F and E735A point mutations) and the carboxyl 780 to 1,029 amino acids of NPR-A.

The partial kinase homology domain deletions were made by using convenient restriction sites (see Fig. 4A). pAΔ1 and pBΔ1 were made by digesting pNPR-A or pNPR-B with *Nhe*I and *Nde*I, treating the digested DNA with DNA polymerase to form blunt ends, and religating the vector after purification of the large fragment away from the small *Nhe*I-*Nde*I fragment. These constructs generate proteins in which amino acids 474 to 554 for AΔ1 and 468 to 548 for BΔ1 have been deleted.

For pAΔ2 and pBΔ2, the appropriate wild-type receptor plasmid was digested with *Nde*I and *Bsu*36I. After formation of blunt ends as described above, the large cDNA and vector fragment was isolated and religated. pAΔ2 encodes a protein with amino acids 556 to 702 deleted. pBΔ2 will produce a mutant receptor in which amino acids 549 to 696 have been deleted and Leu-697 has been mutated to Ile.

For pAΔ3 and pBΔ3, the 5′ portion of the receptor cDNA was obtained by digesting either pNPR-A or pNPR-B with *Bsu*36I and *Spe*I. The *Spe*I site is in the vector portion of the

plasmid. For the 3′ end of the receptors, pAΔKHD or pBΔKHD was digested with *Nhe*I and *Spe*I. The *Bsu*36I and the *Nhe*I ends of these fragments were blunted, and the DNA was ligated. pAΔ3 encodes a protein in which amino acids 703 to 780 have been deleted and an additional Thr-Ser has been inserted after amino acid 702. For BΔ3, amino acids 698 to 772 have been deleted.

The 5′ portion of pAΔ4 or pBΔ4 was obtained by digesting pNPR-A or pNPR-B, respectively, with *Nde*I and *Spe*I. The 3′ region was derived from *Nhe*I-*Spe*I-digested pAΔKHD or pBΔKHD. Both fragments were blunted at the *Nde*I or *Nhe*I site and ligated. The resulting proteins had a deletion of amino acids 555 to 779 plus an insert of Thr-Ser after codon 554 for AΔ4 or a deletion of amino acids 549 to 772 for BΔ4. All partial domain deletion constructs were analyzed by dideoxy sequencing to ensure that no frameshift mutations had occurred.

GC stimulation and intracellular cGMP determination. Cells expressing the appropriate plasmid DNA in six-well plates were washed once with 2 ml of DMEM–*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (25 mM, pH 7.2) and incubated with 2 ml of DMEM–HEPES (25 mM, pH 7.2)–isobutyl methylxanthine (0.1 mM) (D/H/I) for 10 min at 37°C. The cells were stimulated for 5 min at 37°C in 0.5 ml of D/H/I with or without various concentrations of NPs. The incubation medium was aspirated and replaced with 1 ml of ice-cold 10% trichloroacetic acid, and the cells were frozen quickly on dry ice. After the samples thawed at room temperature, the cell debris was removed by centrifugation at 2,500 × *g* for 10 min. Samples were extracted three times with 0.5 ml of water-saturated ether and warmed to 55°C for 20 min to evaporate the residual ether. A radioimmunoassay kit was used to acetylate and analyze aliquots of the cell extracts for intracellular cGMP concentrations as instructed by the manufacturer (Biomedical Technologies).

Membrane GC assays. Intrinsic GC activity of COS cells expressing the various receptor plasmids was determined as previously described (30). Briefly, transfected cells in 60-mm dishes were washed once with 2 ml of HEPES-buffered saline. The cells were scraped into 1 ml of 20 mM HEPES (pH 7.2)–50 mM NaCl–1 mM EDTA–1 mM dithiothreitol and disrupted by sonication (30 s at 50% setting). The homogenate was spun at 15,000 × *g* for 10 min, and the pellet washed once in the same buffer. The membrane proteins were solubilized by resuspending the pellet into 150 μl of 20 mM HEPES (pH 7.2)–100 mM NaCl–1% Triton X-100–10% glycerol–1 mM dithiothreitol and incubating the samples for 30 min on ice. The samples were spun at 5,000 × *g* for 5 min. Ten micrograms of supernatant protein was assayed for GC activity in a total volume of 100 μl containing 20 mM HEPES (pH 7.2), 0.1 mM GTP, 0.5 mM $MnCl_2$, and 0.2 mM isobutyl methylxanthine at 30°C for 15 min. Each reaction was stopped by adding 400 μl of 50 mM sodium acetate (pH 6.2) and heating to 95°C for 3 min. An aliquot of each reaction was assayed for cGMP content by radioimmunoassay as described above.

^{125}I-ANP binding to whole cells or to cell membranes. Cells expressing the appropriate plasmid DNA in 10-cm dishes were washed twice in ice-cold phosphate-buffered saline (PBS). For cell membrane preparations, cells were scraped into 50 mM Tris-HCl (pH 7.5) and disrupted with a Polytron (setting of 5 for 30 s). Membranes were pelleted by spinning in a Sorvall ultraspeed centrifuge at 18,000 rpm in an SS-34 rotor for 30 min. Each pellet was resuspended into binding buffer (50 mM HEPES [pH 7.2], 5 mM $MgCl_2$) without bovine serum albumin (BSA), and an aliquot was analyzed for protein concentration according to the manufacturer's instructions, using the microassay procedure of the Bio-Rad protein assay with BSA as a standard (Bio-Rad, Richmond, Calif.). For whole cell binding, cells were harvested from plates with 5 mM EDTA in PBS and washed once with PBS. Cell pellets were resuspended into binding buffer (PBS, 0.1% BSA, 0.02% azide [PBSA]), counted, and diluted to 400,000 cells per ml.

Samples (10 to 40 μg) of membrane protein were incubated while shaking at room temperature for 90 min with 15 pM ^{125}I-hANP (2,000 Ci/mmol; Amersham) in 1 ml of 50 mM HEPES (pH 7.2)–5 mM $MgCl_2$–0.2% BSA with or without various concentrations of unlabeled hANP or hBNP. In whole cell binding assays, 200,000 cells were incubated with 15 pM ^{125}I-hANP in PBSA with or without various concentrations of unlabeled hANP for 2 h at room temperature while shaking. Nonspecific binding was determined by addition of 0.5 μM unlabeled hANP. The reactions were terminated by filtration in a Brandel cell harvester, and the filters were washed extensively with ice-cold PBS. The amount of ^{125}I-ANP bound to the membranes was determined by counting the filters in a gamma counter. The data were analyzed by modified Scatchard analysis using the LIGAND program of Munson and Rodbard (25).

Metabolic labeling and immunoprecipitation of NPR-A-related proteins. Thirty-six hours after transfection, cells in 60-mm dishes were washed once with warm PBS and incubated for 20 min at 37°C in 0.5 ml of prewarmed DMEM without cysteine or methionine (Cys^-, Met^- DMEM); 100 μCi of [^{35}S]Cys (>600 Ci/mmol) and 100 μCi of [^{35}S]Met (>800 Ci/mmol; Amersham) were added to cells in 2 ml of Cys^-, Met^- DMEM, and cells were placed at 37°C for 12 to 15 h. Radioactive medium was discarded, and cells were washed once with 2 ml of ice-cold PBS. Cells were scraped from the dish in 0.35 ml of 25 mM Tris-HCl (pH 7.5)–250 mM NaCl with 35 μg of phenylmethylsulfonyl fluoride (PMSF) per ml and 20 μg of aprotinin per ml. Proteins were solubilized by adding 0.35 ml of 2× lysis buffer (2% Triton X-100, 1% deoxycholate, 10 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl [pH 7.5], 35 μg of PMSF per ml, 20 μg of aprotinin per ml), vortexing, and placing on ice for 10 min. Cell nuclei were pelleted at 10,000 × *g* for 10 min, and sodium dodecyl sulfate (SDS) was added to the supernatant to a final concentration of 0.1%. The protein concentration of the cell lysates was determined by using the microassay procedure of the Bio-Rad protein assay with BSA as a standard as instructed by the manufacturer.

Lysate protein (50 to 150 μg) was precleared by incubating with 10 μl of Pansorbin (equilibrated with 1× lysis-SDS buffer (1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl [pH 7.5], 0.1% SDS, 35 μg of PMSF per ml, 20 μg of aprotinin per ml) for 30 min at 4°C while rocking. The Pansorbin was removed by centrifugation, and the precleared supernatant was incubated with 10 μl of anti-NPR-A specific monoclonal antibody (approximately 0.5 μg of murine immunoglobulin G) for 30 min at 4°C while rocking; 2.5 μg of rabbit anti-mouse antibody was added to the solution, and incubation was continued an additional 30 min at 4°C. Thirty microliters of a 50% slurry of protein A-Sepharose CL-4B was added to the solution, and incubation continued for 1 h at 4°C. The immunoprecipitation reaction mixture was spun at 10,000 × *g* for 1 min, and the supernatant was aspirated. The pellet was washed three times with 1 ml of 1× lysis-SDS buffer, and the final pellet was resuspended into SDS sample buffer with β-mercaptoethanol. Proteins were removed from the protein A-Sepha-

rose by heating to 85°C for 10 min and analyzed by electrophoresis on a 10% polyacrylamide Tris-glycine gel. Gels were fixed in 10% acetic acid–10% methanol and enhanced by soaking in Amplify (Amersham) as instructed by the manufacturer. After drying at 60°C under vacuum, the gel was exposed to film at −70°C with intensifying screens. Size standards used were high-molecular-weight ^{14}C-protein markers (GIBCO-BRL) and high-molecular-weight prestained protein markers (Bio-Rad).

RESULTS

GC activity of chimeric NPR-A and NPR-B proteins. To study the effect of the kinase homology domains on the regulation of ligand-stimulated GC activity, expression vectors were constructed that encode chimeric receptors in which the kinase homology domains of NPR-A and NPR-B had been exchanged. The structures of the chimeric receptors (A[B]A and B[A]B) are shown schematically in Fig. 1A. Intracellular cGMP was measured in COS-7 cells transiently expressing either the wild-type NPRs (NPR-A and NPR-B) or the chimeric receptors (Fig. 1B and C). The presence of the alternate kinase homology domain did not change the ligand specificity for either NPR-A or NPR-B. The GC activity of both NPR-A and A[B]A was stimulated by ANP and BNP in a dose-dependent manner, while CNP did not increase cGMP levels over background at concentrations as high as 1 μM (Fig. 1B). The half-maximal effective concentration (EC_{50}) value of stimulation for both proteins with BNP was approximately 12 nM. ANP demonstrated EC_{50} values of approximately 2 nM for NPR-A and 6 nM for A[B]A. The significance, if any, of this threefold difference in potency for activation is unclear. NPR-B and B[A]B are each activated by CNP with EC_{50} values of 50 nM. Neither ANP nor BNP significantly increased cGMP levels of NPR-B- or B[A]B-expressing cells except at very high levels (Fig. 1C).

While ligand preference and potency are not altered with domain exchanges between the two receptor subtypes, expression levels appear to be lower in COS cells expressing the chimeric proteins compared with the wild-type receptors. The maximum amount of cGMP produced by the wild-type receptors after stimulation with the most efficacious hormone is 55 and 60 pmol/10^6 cells for NPR-A and NPR-B, respectively, while A[B]A and B[A]B produce only 45 and 40 pmol/10^6 cells, respectively. This decrease in enzymatic activity could be caused by an altered specific activity of the chimeric enzymes. However, immunoprecipitation of metabolically labeled NPR-A and A[B]A expressed in COS cells shows that the chimeric protein is expressed at a lower level than is the wild type (Fig. 1D). Two proteins are specifically immunoprecipitated from cells expressing either NPR-A or A[B]A with molecular sizes of approximately 135 and 120 kDa.

Effect of kinase homology domain exchange on ligand binding to NPR-A. Since GC stimulation by ANP appeared to have a threefold higher EC_{50} in the A[B]A receptor than in NPR-A, we studied the ability of the two proteins to bind ANP. As shown in Fig. 2, ^{125}I-ANP bound to both proteins was displaced by unlabeled ANP with the same potency (K_i = 112 versus 128 pM for NPR-A and A[B]A, respectively). BNP also interacted identically with both receptors (data not shown). While the affinities of these two receptors for ANP are the same, membranes from NPR-A-expressing cells bound twice as much ^{125}I-ANP as did membranes from A[B]A-expressing cells (B_{max} = 2.62 versus 1.31 pmol/mg of protein, respectively); therefore, the wild-type receptor appears to express better than the A[B]A chimera.

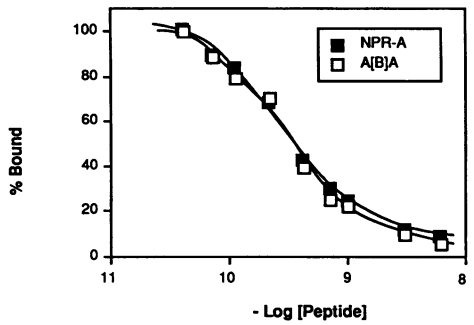

FIG. 2. ^{125}I-ANP binding to membranes of COS cells expressing either NPR-A or A[B]A. COS cells expressing the appropriate receptor were harvested, and membranes were prepared for binding; 15 pM ^{125}I-ANP was incubated in the presence or absence of various concentrations of unlabeled ANP for 90 min at room temperature. The reaction was terminated by filtration, and the amount bound was determined by counting filters in a gamma counter. The results are from a representative experiment that was reproduced two times in duplicate and are expressed as the percentage of counts per minute specifically bound at each peptide concentration.

Regulation of GC activity by heterologous kinase domains. To determine whether homologous portions of structurally related receptors can regulate the catalytic activity of NPR-A or NPR-B, chimeric constructs were made by replacing the kinase homology domain of the NPRs with either the catalytic domain of the EGFR or the kinaselike domain of the STaR (Fig. 3A). COS cells transiently expressing these constructs were stimulated with high concentrations of ANP or CNP, and intracellular cGMP concentrations were measured (Fig. 3B). The EGFR kinase domain was unable to regulate the GC activity of either NPR-A or NPR-B (A[EGF]A and B[EGF]B, respectively). These chimeric proteins had no ability to generate cGMP after stimulation with either hormone tested. The NPR-A/STaR kinase homology domain exchange chimeric protein (A[STa]A) was also unable to produce cGMP. Therefore, similar domains from either the EGFR or the STaR are unable to transduce the hormone binding signal and fail to activate second-messenger production of the NPRs.

To determine whether these proteins were expressed, cell lysates from metabolically labeled cells expressing the NPR-A chimeras were immunoprecipitated with a monoclonal antibody specific to the extracellular domain of NPR-A (Fig. 3C). While a band of the expected size is specifically precipitated from cells expressing either A[EGF]A and A[STa]A, the higher-molecular-weight band of the doublet precipitated from cells expressing NPR-A is not present in the cells expressing the inactive chimeras.

To assess protein expression in another manner, the intrinsic levels of GC activity independent of hormone stimulation in COS cells expressing the chimeric receptors were assayed by using solubilized membrane proteins in the presence of manganese (Table 1). For the NPR-A constructs, A[EGF]A and A[STa]A produce cGMP at levels two- and sixfold over background (1.33 and 4.37 versus 0.67 pmol/mg of protein per min, respectively); however, both proteins have much lower activity than does the wild-type receptor (257.8 pmol/mg of protein per min). While immunoprecipitation results suggest that A[EGF]A and A[STa]A

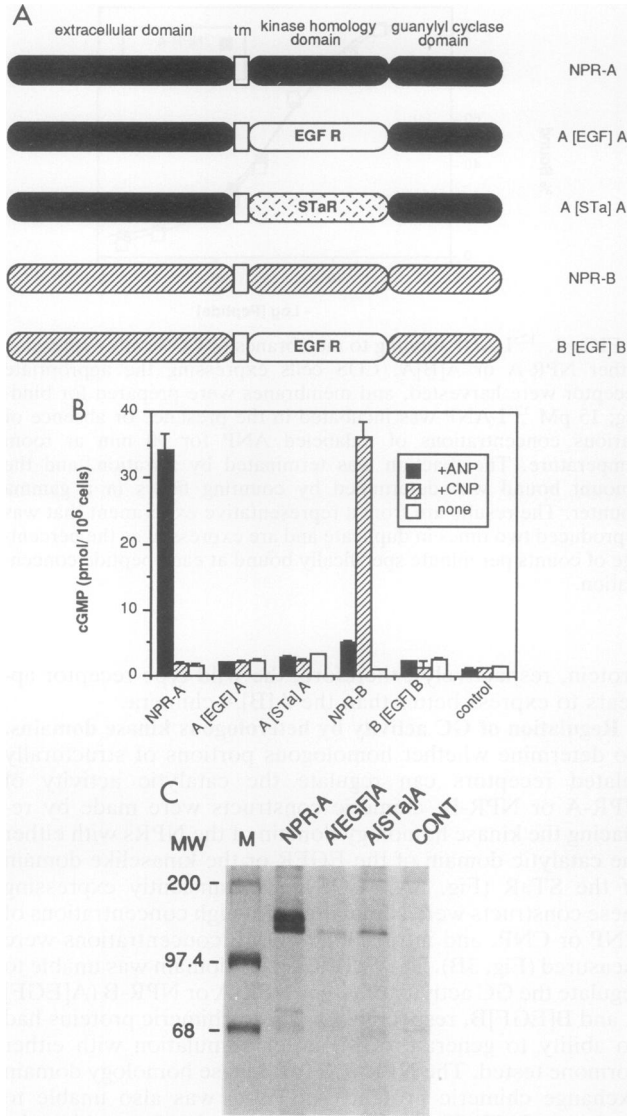

FIG. 3. GC activities of the A[EGF]A, B[EGF]B, and A[STa]A chimeric proteins. (A) Schematic representation of chimeric constructs made by replacing the kinase homology domain of NPR-A or NPR-B with either the kinase domain of the EGFR or the kinaselike domain of STaR. (B) COS cells expressing the appropriate receptor were stimulated with 1 μM ANP or CNP or with medium alone, and intracellular cGMP was determined. Experiments were performed in triplicate, and values are expressed as picomoles per 10^6 cells ± standard error of the mean. (C) Immunoprecipitation of NPR-A, A[EGF]A, or A[STa]A from COS cell lysates. [^{35}S]Cys- and [^{35}S]Met-labeled cell lysates (50 μg) were immunoprecipitated and analyzed on a 10% polyacrylamide gel. Molecular weight (MW) is shown in thousands; size markers are represented by lane M.

are expressed more poorly than NPR-A, the difference in the level of expression does not appear to be as great as is indicated by intrinsic GC activity. Perhaps the activity measured in the presence of nonionic detergent and manganese reflects not only expression level of the protein but also the disrupted regulation of the GC activity of these mutants. For NPR-B and B[EGF]B, both proteins are expressed, and cells expressing the chimeric protein also have much lower intrinsic activity than do those expressing the wild-type receptor (18.48 versus 1,161.5 pmol/mg of protein per min).

TABLE 1. Hormone-independent membrane GC activity and cell surface receptor number of mutant proteins

Receptor	cGMP produced (pmol/mg of protein/min)	Binding sites/cell	Receptor	cGMP produced (pmol/mg of protein/min)
NPR-A	257.80	15,185	NPR-B	1,161.50
A[EGF]A	1.33	ND[a]	B[EGF]B	18.48
A[STa]A	4.37	4,868	Control	0.67
AΔKHD	956.70	10,243	BΔKHD	337.55
AΔ1	15.38	6,186	BΔ1	29.85
AΔ2	9.85	8,842	BΔ2	25.92
AΔ3	0.36	ND	BΔ3	14.92
AΔ4	10.59	8,111	BΔ4	9.06

[a] ND, not determined.

Effect of partial deletions of the kinase homology domains on the regulation of GC activity. In an attempt to determine whether a particular region of the kinase homology domain is sufficient for regulation of the GC activity, we produced a series of partial deletions for both NPR-A and NPR-B. A schematic representation of these proteins for NPR-A, their terminology (AΔ1 to AΔ4), and the predicted sizes of the nonglycosylated protein products are shown in Fig. 4A. The analogous constructs were made for NPR-B (BΔ1 to BΔ4). Cells expressing the pNPR-A and pNPR-B constructs were stimulated with ANP and CNP, respectively, and the intracellular cGMP concentrations were determined (Fig. 4B and C). Deletion of the complete kinase homology domain (ΔKHD) activated the GC of NPR-A, as shown previously (8), as well as NPR-B independently of hormone stimulation. However, none of the partially deleted proteins could be activated in the presence or absence of hormone. Immunoprecipitation of the pNPR-A-derived proteins (Fig. 4D) demonstrated that cells transfected with plasmids pNPR-A, pAΔKHD, pAΔ1, pAΔ2, and pAΔ4 express proteins of the expected sizes. No specific band is seen from cells transfected with plasmid pAΔ3, suggesting that this construct is expressed poorly. The two proteins with GC activity, NPR-A and AΔKHD, are immunoprecipitated as a doublet, while the inactive proteins (AΔ1, AΔ2, and AΔ4) are synthesized as a single-molecular-weight species.

Detailed studies on the expression levels of the NPR-B proteins await the production of an NPR-B-specific antibody; however, GC activities of solubilized membrane proteins from cells expressing these constructs are shown in Table 1. For NPR-B, all receptor deletion constructs appear to be expressed, since the intrinsic activity is 15- to 500-fold over background. The GC activities of the NPR-A proteins correlate with the immunoprecipitation results; cells expressing all of the constructs except pAΔ3 have GC activity over background levels. These results indicate that not only are the majority of the NPR-A and NPR-B constructs expressed, but the disruptions in their tertiary structure caused by deletion of large portions of the receptor molecule do not completely prohibit the GC domain from folding properly.

Cell surface expression of the mutant receptors. To assess whether the inability of the inactive chimeric or mutant receptors to respond to hormone stimulation was due to a lack of expression of the proteins on the cell surface, we studied binding of ^{125}I-hANP to whole cells. The number of cell surface binding sites estimated from Scatchard analysis for the NPR-A proteins is shown in Table 1. All of the expressed receptors are present on the cell surface, and the

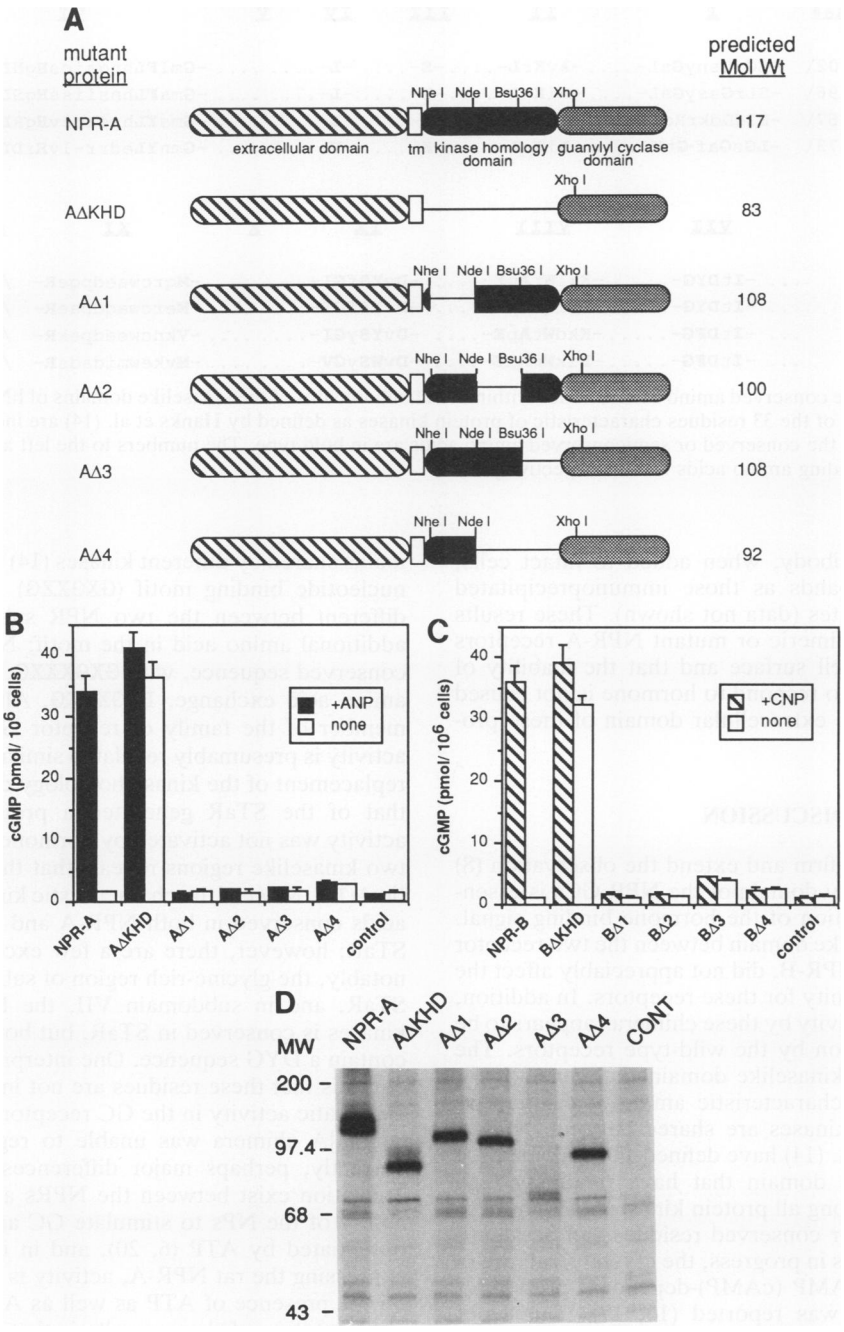

FIG. 4. Effects of complete or partial deletions of the kinase homology domain on the GC activities of NPR-A and NPR-B. (A) Schematic representation of the NPR-A deletion constructs. Restriction sites that were used to make the constructs are indicated. Thin lines correspond to area that has been deleted. Names of the constructs are on the left, and the predicted molecular weights (Mol Wt; in thousands) of the unmodified proteins are on the right. Identical constructs were made for NPR-B. COS cells expressing the appropriate receptors (NPR-A [B] and NPR-B [C]) were stimulated with medium alone or with 1 μM ANP (for NPR-A constructs) or CNP (for NPR-B constructs), and intracellular cGMP levels were determined. Results are expressed as the mean of three separate determinations ± standard error of the mean. (D) Immunoprecipitation of NPR-A receptor deletion variants from COS cell lysates. [^{35}S]Cys- and [^{35}S]Met-labeled cell lysates (50 μg) were immunoprecipitated and analyzed on a 10% polyacrylamide gel. Molecular weight (MW) is shown in thousands.

level of expression as measured by binding correlates well with the immunoprecipitation results. In addition, we studied the ability of the NPR-A-specific antibody to recognize cell surface receptor either by fluorescence-activated cell sorting (FACS) analysis or by whole cell immunoprecipitation. In FACS experiments, incubation of COS cells transiently expressing either NPR-A or A[STa]A with the specific antibody caused a significant shift in fluorescence intensity of a subpopulation of cells compared with incubation with the secondary fluorescent antibody alone (data not shown). In cells expressing the wild-type (NPR-A) or the three chimeric receptors, (A[B]A, A[EGF]A, or A[STa]A),

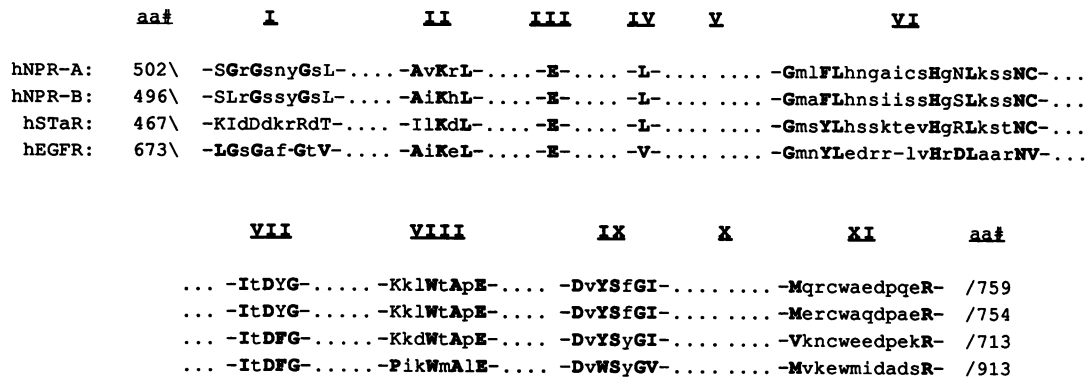

FIG. 5. Alignment of the conserved amino acid residues within the 11 subdomains of the kinaselike domains of hNPR-A, hNPR-B, hSTaR, and hEGFR. The positions of the 33 residues characteristic of protein kinases as defined by Hanks et al. (14) are indicated by capital letters. Those residues identical to the conserved or semiconserved amino acids are in bold type. The numbers to the left and right of the sequences refer to the starting and ending amino acids listed, respectively.

the NPR-A-specific antibody, when added to intact cells, recognized the same bands as those immunoprecipitated from detergent cell lysates (data not shown). These results demonstrate that the chimeric or mutant NPR-A receptors are expressed on the cell surface and that the inability of some of these proteins to respond to hormone is not caused by inaccessibility of the extracellular domain of these proteins to ligand.

DISCUSSION

In this report, we confirm and extend the observation (8) that the kinase homology domain of the NPR GCs is essential for proper transduction of the hormone binding signal. Exchange of the kinaselike domain between the two receptor subtypes, NPR-A and NPR-B, did not appreciably affect the ligand preference or affinity for these receptors. In addition, the regulation of GC activity by these chimeras appears to be identical to the regulation by the wild-type receptors. The similarity of these two kinaselike domains is over 60% (7), and a majority of the characteristic amino acids that are shared by the protein kinases are shared between NPR-A and NPR-B. Hanks et al. (14) have defined 11 subdomains (I to XI) of the catalytic domain that have relatively high sequence homology among all protein kinases. Within these domains, 33 invariant or conserved residues can be identified. While our work was in progress, the crystal structure of the mammalian cyclic AMP (cAMP)-dependent protein kinase catalytic domain was reported (16, 17). This work demonstrated that the topology of this protein is composed of interwoven β strands and α helices which form two distinct protein domains. A smaller amino-terminal region includes the first three α helices and the first five β strands and contains most of the residues important for ATP binding. A larger carboxy-terminal domain contains the remaining seven α helices and four β strands and plays an important role in protein substrate recognition (13, 16, 17). Most of the conserved residues found in all kinases (14) either contribute to MgATP binding and catalysis or form internal salt bridges within the tertiary structure (16, 17). We can infer that the kinase domain of the receptor GCs will have an overall architecture similar to that of the catalytic domain of bona fide protein kinases.

A comparison of the conserved amino acids present in NPR-A, NPR-B, STaR, and EGFR within the 11 subdomains shared by different kinases (14) is shown in Fig. 5. The nucleotide binding motif (GXGXXG) in region I is slightly different between the two NPR subtypes. Both have an additional amino acid in the motif; NPR-A is closer to the conserved sequence, with GXGXXXG, while NPR-B has one amino acid exchange, LXGXXXG. Although the STaR is a member of the family of receptor GCs and its enzymatic activity is presumably regulated similarly to that of the NPR, replacement of the kinase homology domain of NPR-A with that of the STaR generated a protein whose enzymatic activity was not activated by hormone. Comparison of these two kinaselike regions reveals that they are only 30% identical (11). Most of the characteristic kinaselike domain amino acids conserved in both NPR-A and NPR-B are present in STaR; however, there are a few exceptions (Fig. 5). Most notably, the glycine-rich region of subdomain I is missing in STaR, and in subdomain VII, the DFG motif of protein kinases is conserved in STaR, but both NPR-A and NPR-B contain a DYG sequence. One interpretation of these differences is that these residues are not important for control of enzymatic activity in the GC receptors. However, since the A[STa]A chimera was unable to regulate its GC activity correctly, perhaps major differences in the mode of this regulation exist between the NPRs and STaR. In fact, the ability of the NPs to stimulate GC activity is known to be potentiated by ATP (6, 20), and in insect cell membranes expressing the rat NPR-A, activity is completely dependent on the presence of ATP as well as ANP (10). The simplest interpretation of these results is that ATP binds directly to the kinase domain of NPR-A. While it is unclear whether activation of STaR is modulated by ATP, this receptor completely lacks the glycine-rich nucleotide binding consensus sequence and therefore may not interact with ATP. More generally, the GC domains of these receptors may need to interact with several important structural features within the appropriate kinase homology domains. Notably, while the GC regions of NPR-A and NPR-B are 88% identical, both of the NPR GC domains are only approximately 55% identical to the GC domain of STaR (11). Our findings imply that GC regulation occurs by direct interaction of the kinaselike domains with specific GC domains and that several structural or functional regions within these two domains must be conserved in order for the receptors to regulate their enzymatic activity properly.

The EGFR is a single-transmembrane-domain receptor tyrosine kinase whose kinase activity is believed to be inhibited by interaction with the carboxy terminus of the protein (2, 3). Since this type of receptor self-modulation of enzymatic activity is similar to the negative regulation of GC activity of the NPRs by their kinaselike domains, chimeric proteins were made in which the kinase homology domain of either NPR-A and NPR-B was exchanged with the catalytic domain of the EGFR (A[EGF]A and B[EGF]B, respectively). Like the A[STa]A chimera, the similarity between the EGFR kinase domain and the kinaselike domains of the NPRs is fairly low (approximately 30%). Apparently, the structural components necessary for proper regulation of the GCs are not present in the EGFR kinase region, as these chimeras had no ligand-stimulated GC activity. Figure 5 compares the conserved kinase domain residues in the EGFR with those in NPR-A and NPR-B. Unlike NPR-A and NPR-B, the EGFR contains all 33 of these consensus residues (14). This comparison reveals that within subdomain VI, the highly conserved acidic residue (aspartate) present in the EGFR is replaced with neutral nonpolar residues in NPR-A and NPR-B (asparagine and serine, respectively). This aspartate residue has been postulated to be involved in transferring the phosphate group from ATP to the appropriate substrates (14). Since neither of the NPRs contain this residue, these proteins may have no intrinsic ability to phosphorylate proteins. In fact, no kinase activity has as yet been associated with the NPRs.

While complete deletions of the kinaselike domain in both NPR-A and NPR-B constitutively activated the GC activity of these proteins, partial deletions within these regions created inactive proteins. These experiments may indicate that the complete kinaselike domain is essential to regulate the GC activity of these proteins correctly. When comparing the deletion constructs presented here with the three-dimensional structure of the catalytic domain of the mammalian cAMP-dependent protein kinase (16, 17), we find that the mutant receptors contain deletions of portions of either the small amino-terminal or the large carboxy-terminal regions of the kinaselike domains, as described above (AΔ1 and BΔ1 or AΔ3 and BΔ3, respectively), of portions of both the small and large regions (AΔ2 and BΔ2), or of a portion of the small region and the complete large region (AΔ4 and BΔ4). For the NPR-A proteins, all of the expressed receptors (AΔ1, AΔ2, and AΔ4) have at least a portion of the small ATP binding region deleted. Since normal GC activity for NPR-A is dependent of the presence of both ATP and ANP (10), perhaps these mutant receptors cannot be activated by hormone because of their inability to interact correctly with ATP. Alternatively, these deletions may disrupt the tertiary structure of the protein such that the GC domain is not folded properly and therefore has no ability to synthesize cGMP. This possibility appears unlikely, since intrinsic cyclase activity can be measured in cells expressing these proteins (Table 1). However, the amount of protein expressed does not correlate directly with this GC activity, indicating some compromise of GC function in these proteins. The results presented here suggest that the entire kinaselike domain, as a whole, is responsible for correct regulation of enzymatic activity.

Expression of these chimeric and mutant NPRs has been studied by using a specific monoclonal antibody against the extracellular domain of NPR-A to immunoprecipitate receptor proteins from COS cell lysates. From ^{35}S-labeled cells expressing the wild-type NPR-A, two bands are specifically immunoprecipitated. In the active chimeric protein, A[B]A, this doublet is also seen; however, only a single band is precipitated from cells expressing either the inactive chimeric proteins or the inactive kinase domain deletion receptors. We do not yet know the chemical nature of this difference in molecular weight, but it may correspond to complete versus partial use of the seven available glycosylation sites present in NPR-A. This type of heterogeneous glycosylation has been observed for other proteins such as tissue plasminogen activator (26, 32). Alternatively, the presence of two bands may be due to other posttranslational events such as partial proteolysis or phosphorylation. For example, band mobility changes as a function of phosphorylation have been observed with the GC receptor from sea urchin spermatozoa (1, 27). The active form of this protein is highly phosphorylated (17 mol of phosphate per mol of enzyme), and stimulation of the receptor by its ligand causes a large increase in intracellular cGMP followed by a rapid dephosphorylation of the receptor. Concomitant with this dephosphorylation is a molecular size shift of the purified proteins from 160 kDa to 135 kDa.

The studies presented here suggest that the kinase homology domain of the NPRs is essential for proper regulation of the GC activity of these receptors. Experiments with chimeric proteins indicate that specific sequences within the overall structure of the kinase domains of either NPR-A or NPR-B are necessary for correct regulation. Studies on site-specific mutants made within the kinase domains of these proteins may help to define important residues for the interaction between the kinase homology and the GC domains of these receptors.

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