MOLECULAR AND CELLULAR BIOLOGY, Apr. 1998, p. 2164–2172 Vol. 18, No. 4
0270-7306/98/$04.00+0

Phosphorylation of the Kinase Homology Domain Is Essential for Activation of the A-Type Natriuretic Peptide Receptor

LINCOLN R. POTTER* AND TONY HUNTER

Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037

Received 30 September 1997/Returned for modification 3 November 1997/Accepted 8 January 1998

Natriuretic peptide receptor A (NPR-A) is the biological receptor for atrial natriuretic peptide (ANP). Activation of the NPR-A guanylyl cyclase requires ANP binding to the extracellular domain and ATP binding to a putative site within its cytoplasmic region. The allosteric interaction of ATP with the intracellular kinase homology domain (KHD) is hypothesized to derepress the carboxyl-terminal guanylyl cyclase catalytic domain, resulting in the synthesis of the second messenger, cyclic GMP. Here, we show that phosphorylation of the KHD is essential for receptor activation. Using a combination of phosphopeptide mapping techniques, we have identified six residues within the ATP-binding domain (S497, T500, S502, S506, S510, and T513) which are phosphorylated when NPR-A is expressed in HEK 293 cells. Mutation of any one of these Ser or Thr residues to Ala caused reductions in the receptor phosphorylation state, the number and pattern of phosphopeptides observed in tryptic maps, and ANP-dependent guanylyl cyclase activity. The reductions were not explained by decreases in NPR-A protein levels, as indicated by immunoblot analysis and determinations of cyclase activity in the presence of detergent. Conversion of Ser-497 to Ala resulted in the most dramatic decrease in cyclase activity (~20% of wild-type activity), but conversion to an acidic residue (Glu), which mimics the charge of the phosphoserine moiety, had no effect. Simultaneous mutation of five of the phosphorylation sites to Ala resulted in a dephosphorylated receptor which was unresponsive to hormone and had potent dominant negative inhibitory activity. We conclude that phosphorylation of the KHD is absolutely required for hormone-dependent activation of NPR-A.

Atrial natriuretic peptide (ANP) is a cardiac hormone which regulates several physiologically important processes, including fluid balance, vascular smooth muscle tone, and cell growth (3, 5). These effects are mediated by the binding of ANP to one of two known cell surface receptors. The natriuretic peptide clearance receptor consists of an extracellular ligand-binding domain, a single membrane-spanning region, and a 37-amino-acid intracellular tail (17). Its primary function is to clear natriuretic peptides from the circulation (39), although a role for this receptor in signalling has been reported (1). Natriuretic peptide receptor A (NPR-A), also known as guanylyl cyclase A, is a member of the expanding guanylyl cyclase family (18, 20). It is thought to be the primary ANP signalling molecule because most physiological effects of the hormone can be mimicked by cell-permeable cyclic GMP (cGMP) analogs (5). In addition, recent gene disruption experiments indicate that the major cardiovascular functions of ANP are absent in NPR-A knockout mice (27). For these reasons, it is sometimes referred to as the biological ANP receptor.

NPR-A is composed of four discrete structural domains: an amino-terminal ligand-binding domain, a single hydrophobic membrane-spanning region, a juxtamembrane kinase homology domain (KHD), and a carboxyl-terminal guanylyl cyclase catalytic domain (18). Of these four domains, the ~280-amino-acid KHD is the least well understood. Originally named the KHD because it has homology to the eukaryotic protein kinase superfamily, it is most similar to the protein-tyrosine kinase domain of the platelet-derived growth factor receptor, with 31% of the amino acids being identical between the comparable regions (9, 37). Although NPR-A contains 30 of the 33 highly conserved or invariant residues originally identified in known protein kinases, there are some notable exceptions. In particular, the invariant aspartate found in subdomain VI of known protein kinases is replaced with an asparagine in NPR-A. Based on the crystal structure of protein kinase A, this residue is thought to function as the catalytic base (28). Interestingly, mutation of the corresponding aspartate to asparagine in c-Kit (murine white spotting locus) (50) or v-Fps (41) results in a loss of kinase activity. Thus, in the absence of any data suggesting otherwise, it is currently thought that the KHD does not have intrinsic protein kinase activity (18).

The activation mechanism of NPR-A is poorly understood. It exists as a higher-ordered homomeric structure in the absence of ligand, and ANP binding does not lead to further aggregation (11, 25, 36). Thus, the role of hormone binding is not to simply stimulate receptor oligomerization as has been shown for some growth factor receptors (45). Early studies demonstrated that in addition to ANP, ATP is required for maximal enzyme activity (6, 33). These initial observations were extended when it was found that ATP is absolutely required for ANP-dependent activation of NPR-A overexpressed in insect cells (10). The nucleotide does not appear to be a substrate in a phosphotransferase reaction, since nonhydrolyzable adenine nucleotide analogs effectively substitute for ATP (6, 10, 33). It is currently hypothesized that ATP serves as an intracellular allosteric regulator of NPR-A (18, 21). The KHD appears to be required for the ATP effect because receptor constructs lacking this domain have an elevated basal activity and are not further stimulated by ANP and ATP (8,

* Corresponding author. Mailing address: The Salk Institute for Biological Studies, Molecular Biology and Virology Laboratory, 10010 North Torrey Pines Road, La Jolla, CA 92037. Phone: (619) 453-4100, ext. 1613. Fax: (619) 457-4765. E-mail: lpotter@aim.salk.edu.

30). Because the KHD contains the sequence GRGSNYG, which is closely related to the known ATP-binding motif GX GXXG found in most protein kinases (22), several groups have speculated that this glycine-rich region is part of an ATP-binding site (18, 21). However, mutations within this subdomain have produced mixed results. In one study, mutation of all three conserved glycines to alanine had little or no effect on the ability of the receptor to be activated by ANP and ATP (31). In contrast, a separate study found that mutation of the same sequence to GRVNNYG dramatically reduced the ability of the receptor to respond to hormone (21).

ATP is also known to decrease ANP binding to NPR-A by reducing the number of high-affinity binding sites (13, 26, 34, 40). This process is mediated, in part, by increasing the off rate of ANP from receptor-ligand complexes (34), a process that is speculated to play a role in the deactivation of the receptor (29). In contrast, the diuretic drug amiloride increases ANP binding and antagonizes the effect of ATP on binding (13, 26, 29). The competitive nature of these two molecules is tantalizing because amiloride has been shown to be a competitive inhibitor of ATP binding to known protein kinases (12, 24). Like the enzymatic effects, the modulatory properties of both ATP and amiloride on hormone binding require the KHD (26). Whether ATP binds directly to KHD or to another protein that associates with the KHD is not absolutely clear because experiments describing the binding or cross-linking of labeled ATP analogs to NPR-A have not been reported. However, if the 50% effective concentration for ATP activation (~0.5 mM) is indicative of its affinity for NPR-A, direct association may be difficult to demonstrate. Nonetheless, the observation that the ATP effects on guanylyl cyclase activation (54) and ANP binding (34) are maintained in highly purified receptor preparations is consistent with a direct binding model.

Phosphorylation-dependent regulation of membrane guanylyl cyclases was first demonstrated in sea urchin spermatozoa (19). In this system, the receptor is highly phosphorylated in the basal state, and binding to its cognate egg peptide hormone causes a dramatic stimulation of guanylyl cyclase activity followed by a rapid desensitization response. The deactivation is temporally correlated with massive receptor dephosphorylation (from 15 to 17 mol of phosphate to 2 mol of phosphate/receptor) and can be mimicked by phosphatase treatment. Thus, it appears that dephosphorylation mediates the desensitization. NPR-A has also been shown to be regulated by phosphorylation. However, the functional effects of phosphorylation on enzymatic activity are unclear. Early in vitro studies suggested that phosphorylation of NPR-A by protein kinase C (PKC) inhibited ANP-dependent guanylyl cyclase activity in a manner similar to the desensitization of many serpentine receptors (16, 35, 47). Subsequent studies using a cell culture model showed that NPR-A, like the sea urchin receptor, was phosphorylated in the basal state, and hormone binding caused both dephosphorylation and desensitization (31, 43, 44). Although the exact stoichiometry of phosphorylation has not been determined, the molar ratio of phosphate to receptor is likely to be at least 1:1, since both desensitization and dephosphorylation result in a slight increase in the electrophoretic mobility of NPR-A (43). In addition, phosphatase treatment of NPR-A in crude membranes has been shown to dephosphorylate and desensitize the receptor. As in whole cells, the dephosphorylation is associated with an increase in the electrophoretic mobility of NPR-A (43). Surprisingly, activation of PKC in the cell culture system has also been shown to correlate with dephosphorylation and desensitization of NPR-A (44). Hence, the current understanding of phosphorylation-dependent regulation of NPR-A is controversial.

In an attempt to clarify the effect of phosphorylation on NPR-A activity, we have identified four serine and two threonine residues located in or around the putative ATP-binding site of NPR-A that are phosphorylated when expressed in unstimulated human epithelial kidney (HEK) 293 cells. Mutation of these individual sites to alanine resulted in a decreased phosphorylation state of the receptor, changes in tryptic phosphopeptide maps, and reduced hormone-dependent guanylyl cyclase activity. Simultaneous mutation of four or five of the sites to alanine resulted in a completely dephosphorylated receptor which was unresponsive to ANP. These data indicate that receptor phosphorylation is absolutely required for hormone-dependent activation of NPR-A and are consistent with a desensitization by dephosphorylation model. Finally, to our knowledge these are the first phosphorylation sites identified for any guanylyl cyclase molecule, and this information may therefore be useful in assessing the role of phosphorylation on additional members of the guanylyl cyclase receptor family.

MATERIALS AND METHODS

Site-directed mutagenesis and transient transfections. Mutations within the KHD were generated on the ~700-bp *Bam*HI-*Xba*I fragment of NPR-A, which was subcloned into pBluescript II (Stratagene, San Diego, Calif.). The mutations were generated by using either the Muta-Gene kit from Bio-Rad (Hercules, Calif.) or a Quikchange kit from Stratagene according to the manufacturer's protocols. The mutant *Bam*HI-*Xba*I fragments were then subcloned back into the corresponding region of the expression plasmid pCMV3-GC-A (43). All indicated mutations and the absence of unwanted mutations were confirmed by manual or automated nucleic acid sequencing. HEK 293 cells were grown to ~40% confluence in 6- or 10-cm-diameter dishes and then transfected with 2.5 or 5 μg of the various pCMV3-NPR-A constructs, using the BES-buffered calcium phosphate coprecipitation method (42); 24 to 48 h later, the cells were either metabolically labeled or harvested for membrane preparation.

Preparation of crude membranes. Ten-centimeter-diameter plates of transfected HEK 293 cells were washed once with 10 ml of phosphate-buffered saline and then scraped off the plate in 0.5 ml of phosphatase inhibitor buffer (50 mM HEPES [pH 7.4], 20% glycerol, 50 mM NaCl, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 μg of pepstatin per ml, 10 mM $NaPO_4$ [pH 7.0], 0.1 M NaF, 1 mM Na_3VO_4, 80 μM β-glycerol phosphate, 0.1 μM okadaic acid), sonicated with a Branson Sonifier cell disrupter at 4°C, and centrifuged at 15,800 × *g* for 20 min at 2°C. The resulting membrane pellet was resuspended in HGPB (50 mM HEPES [pH 7.4], 20% glycerol, 50 mM NaCl, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 μg of pepstatin per ml at a protein concentration of approximately 1.5 to 2.5 mg/ml as estimated by the bicinchoninic acid protein assay (Pierce Chemical Company, Rockford, Ill.).

Metabolic labeling, phosphoamino acid analysis, and phosphopeptide mapping. Transfected HEK 293 cells were washed twice with phosphate-deficient Dulbecco's modified Eagle's medium (D-DMEM), then placed in a mixture of 95% D-DMEM, 5% dialyzed fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and $^{32}P_i$ (1 to 2 mCi/ml; NEN), and incubated at an atmosphere of 5% CO_2 and 95% air at 37°C overnight. NPR-A was isolated from metabolically labeled cells by immunoprecipitation with rabbit polyclonal antiserum R1215 and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (44). NPR-B was immunoprecipitated with rabbit polyclonal antiserum Z658 as described elsewhere (42a). For phosphoamino acid analysis, ^{32}P-labeled NPR-A was immunoblotted to Immobilon-P, cut out of the membrane, and hydrolyzed in 5.7 N HCl at 110°C for 1.5 or 2 h. The resulting phosphoamino acids were separated together with exogenously added phosphoserine, phosphothreonine, and phosphotyrosine standards by two-dimensional high-voltage electrophoresis as described by Boyle et al. (4). The phosphoamino acids were visualized by ninhydrin staining followed by autoradiography using Kodak XAR film. Phosphopeptide mapping was performed essentially as described by Boyle et al. (4). Briefly, labeled NPR-A was isolated as described above and electroblotted to nitrocellulose. The membrane was then exposed to film to localize NPR-A. In all subsequent procedures, it was very important to use 1.5-ml snap-cap tubes (Sarstedt no. 72.690) that had been previously coated with the siliconizing agent Sigmacote (Sigma, St. Louis, Mo.) to prevent the phosphopeptides from sticking to the tubes. The band corresponding to NPR-A was then cut out and incubated with 0.5% polyvinylpyrrolidone (average molecular weight of 360,000) dissolved in 0.1 M acetic acid for 30 min at 37°C. The membrane fragments were then washed five times with water and two times with 50 mM $(NH_4)_2CO_3$ (pH 8.0); 5 μg of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was added to each sample, which was then incubated for 4 h at 37°C; 5 more μg of trypsin was added, and the tube was incubated overnight at 37°C. The remaining $(NH_4)_2CO_3$ was removed by repeated lyophilization in a SpeedVac. The phosphopeptides were dissolved in a small volume of distilled water and spotted on 100-μm-thick cellulose plates (Merck). The peptides were then separated in the

horizontal dimension by high-voltage electrophoresis (1,000 V) for 25 min in 1% ammonium carbonate (pH 8.9). The plate was dried for 1 h, and the phosphopeptides were separated in the vertical dimension by ascending chromatography in phosphochromatography buffer (Fig. 2 and 5) or isobutyric acid buffer (Fig. 1) (4). The phosphopeptides were visualized by exposing the plates to Kodak XAR film for approximately 1 week at −80°C with one intensifying screen.

Immunoblot analysis. NPR-A was isolated as described above and electroblotted to a polyvinylidene difluoride (Immobilon-P) membrane. The membrane was then blocked for 1 h in TBST (20 mM Tris, 500 mM NaCl, 0.05% polyoxyethylene sorbitan monolaurate [pH 7.5]) containing 3% bovine serum albumin (BSA), washed three times for 5 min with TBST, then incubated with rabbit antiserum 5936 diluted 1/500 in TBST containing 1% BSA for 2 h at 25°C. Rabbit 5936 was immunized with a glutathione *S*-transferase fusion protein encoding the cyclase domain (amino acids 781 to 1029) of NPR-A. The membrane was washed three times for 10 min each with TBST and incubated for 45 min at 25°C with an affinity-purified goat anti-rabbit immunoglobulin G-directed antibody conjugated to horseradish peroxidase diluted 1 to 10,000 in TBST. The membrane was then washed once for 15 min and twice for 5 min in TBST. The NPR-A antibody complex was detected by chemiluminescence using the ECL Western blot detection system from Amersham Life Sciences (Arlington, Ill.).

In vitro phosphorylation of synthetic peptide. The peptide SAGSRLTLSGR was synthesized on a Applied Biosystems 432A peptide synthesizer and purified according to the manufacturers' protocol. The purified peptide was then incubated in the presence of 140 ng of purified bovine protein kinase A catalytic subunit, 20 mM Tris, 10 mM $MgCl_2$, and 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reaction was terminated by the addition of EDTA (2 mM, final concentration). The peptide was separated from the protein kinase and free ATP by thin-layer electrophoresis on a cellulose plate for 25 min at 1 kV in solvent buffered to pH 3.5 as described by Boyle et al. (4). The resulting labeled phosphopeptide was purified from the plate, cleaved with trypsin, spotted on a new cellulose plate, and separated by electrophoresis and ascending chromatography as described above. The phosphopeptide migrated as a distinct spot on this plate. It was then repurified from the cellulose and used in the comigration assay shown in Fig. 6. Although we have not determined whether this peptide is phosphorylated on the first or fourth serine, it is likely that this peptide is SAGS(P)R, since protein kinase A phosphorylates an amino-terminal serine very poorly. Moreover, since migration is primarily determined by mass and charge (4), both isoforms are predicted to migrate the same.

Guanylyl cyclase assays. All guanylyl cyclase assays were at 37°C in the presence of 25 mM HEPES (pH 7.4), 50 mM NaCl, 0.25 mM 1-methyl-3-isobutylxanthine, 0.1% BSA, 5 mM creatine phosphate, 5 to 10 U of creatine phosphokinase per assay, 1 mM GTP, and 0.1 to 0.2 μCi of [α-^{32}P]GTP; 5 mM $MgCl_2$, 1 mM ATP, and 1 μM ANP or 1% Triton X-100 and 3 mM $MnCl_2$ were also included in the reaction mixtures. Basal levels were determined in the presence of only 5 mM $MgCl_2$. Assays were initiated by the addition of a solution of the above-specified reagents to approximately 50 μg of crude membrane protein in a total volume of 0.1 ml. cGMP accumulation was analyzed as described by Domino et al. (14). For the basal and some stimulated determinations, no [α-^{32}P]GTP was included in the reaction mixtures, and the amount of cGMP accumulated was estimated with a radioimmunoassay kit from DuPont NEN Life Science Products (Boston, Mass.).

RESULTS

The phosphorylation sites of NPR-A and NPR-B are located within the first 132 intracellular residues. Our initial attempts to determine the phosphorylated region of NPR-A relied on deletion constructs which were missing large segments of the intracellular domain. We found that a carboxyl-terminal deletion at amino acid 675 which removes the cyclase domain resulted in a receptor which was phosphorylated and had wild-type tryptic phosphopeptide maps (data not shown). These data indicated that the phosphorylated residues are not contained in the deleted portion of the receptor. However, subsequent deletion constructs which removed additional carboxyl-terminal residues resulted in a dephosphorylated receptor. One interpretation of these results is that the phosphorylation sites are located within the deleted portion of the receptor. However, since we could not rule out the possibility that the deletion resulted in a conformational change of the receptor which made it either unstable or unable to be phosphorylated due to steric considerations, we could not draw any firm conclusions from these subsequent deletion experiments.

Initial observations indicated that tryptic phosphopeptide maps of NPR-A and NPR-B (7, 46), a closely related homolog of NPR-A, were totally different. Therefore, we reasoned that chimeric constructs of the two receptors might be useful reagents for identifying their phosphorylated domains. We speculated that this approach would be less likely to result in improperly folder receptor mutants because Koller and colleagues had previously shown that swapping the KHD between NPR-A and NPR-B resulted in chimeras that maintained wild-type functional properties (30). The chimeras were constructed by taking advantage of an *Xba*I restriction site that is conserved in the cDNAs of both NPR-A and NPR-B (Fig. 1A). The chimera named NPR-A/B was engineered by fusing the extracellular domain, transmembrane region, and first 132 intracellular amino acids of NPR-A (residues 1 to 593) to the intracellular domain of NPR-B (residues 610 to 1047) which was missing the corresponding 132 juxtamembrane residues (Fig. 1A). The converse construct, composed of the extracellular domain of NPR-B (residues 1 to 609) and intracellular domain of NPR-A (residues 594 to 1029), was also made and named NPR-B/A (Fig. 1A). When expressed in HEK 293 cells, both the wild-type receptors and chimeric receptors were phosphorylated (Fig. 1B). Furthermore, the tryptic phosphopeptide maps of NPR-A and NPR-A/B were very similar, if not identical (Fig. 1C). Likewise, the maps of NPR-B and NPR-B/A were strikingly similar (Fig. 1C). These data indicate that the phosphorylation sites of both NPR-A and NPR-B are located within the first 132 intracellular amino acids of each receptor, because these are the only identical regions contained in the receptors with similar maps.

The putative ATP-binding domain contains several possible phosphorylation sites. NPR-A has been shown to contain primarily phosphoserine and lower amounts of phosphothreonine (31, 43, 44), but whether both phosphoamino acids were contained in the same or different tryptic peptides was not known. Therefore, we performed phosphoamino acid analysis on the major tryptic phosphopeptides of NPR-A (Fig. 2). Note that the pattern of NPR-A tryptic phosphopeptides shown in Fig. 2A is different from the one shown in Fig. 1C. This is because a different buffer was used for the ascending chromatography step in each experiment. Of the 10 phosphopeptides analyzed, 8 contained both phosphoserine and phosphothreonine, and 2 (1 and 10) contained only detectable amounts of phosphoserine. These data are summarized in Fig. 2C and suggest that the phosphorylation sites are in a region that contain both serine and threonine residues flanked by arginine or lysine residues. Examination of the first 132 intracellular amino acids of NPR-A revealed a region that satisfied these criteria (Fig. 3). This region contains a high degree of identity with NPR-B, but not the heat-stable enterotoxin receptor (STa-R), a guanylyl cyclase receptor whose KHD does not appear to be functionally equivalent to those found in natriuretic peptide receptors (30). Interestingly, this region also contains the putative ATP-binding domain, which is speculated to play a role in the ATP-dependent activation of NPR-A (Fig. 3).

Serine- and/or threonine-to-alanine mutations within the KHD reduce the ^{32}P content of NPR-A. To determine if any of the serine or threonine residues within this region are phosphorylated, we generated individual mutant constructs consisting of either single serine- or threonine-to-alanine changes, e.g., S497A or multiple changes, e.g., 500/502/506/510/A. Alanine was chosen as the replacement residue because it cannot be phosphorylated and is unlikely to impose constraints on protein conformation due to its small side chain. Expression constructs were transiently transfected into HEK 293 cells, which were subsequently metabolically labeled overnight with $^{32}P_i$. Wild-type and mutant receptors were purified from cell extracts by immunoprecipitation, fractionated by SDS-PAGE, and electroblotted to Immobilon membrane. The amount of

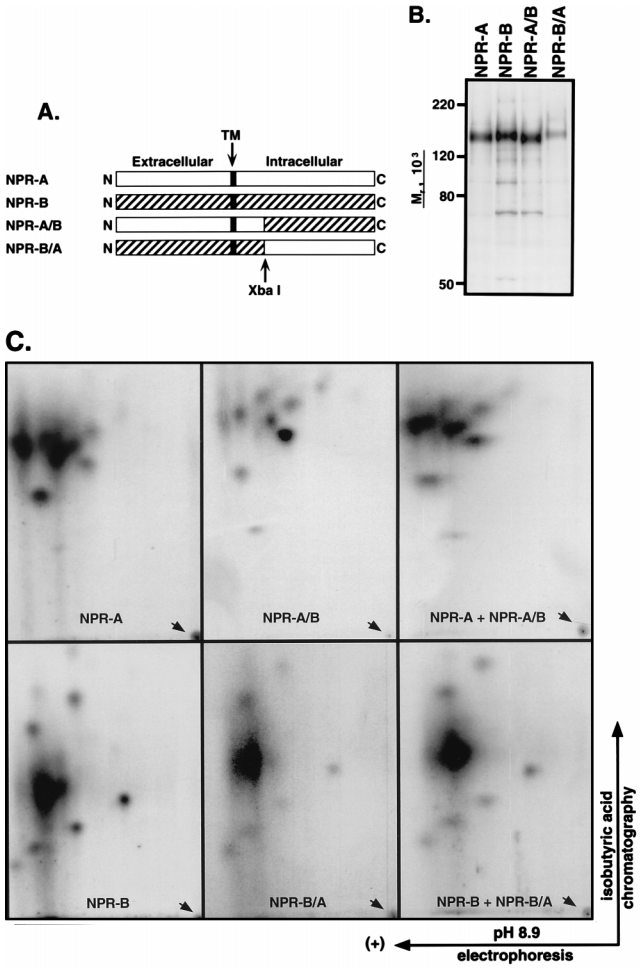


FIG. 1. Chimeric natriuretic peptide receptors yield tryptic phosphopeptide maps which are similar to only one parent receptor. (A) Schematic representation of wild-type and chimeric natriuretic peptide receptors. Chimeras were generated by cloning into a conserved *Xba*I restriction site contained in both NPR-A and NPR-B. NPR-A/B yields a protein that derives its extracellular domain, transmembrane (TM) domain, and first 132 intracellular amino acids from NPR-A (residues 1 to 593) and its remaining carboxyl-terminal residues from NPR-B (residues 610 to 1047). Conversely, NPR-B/A derives its extracellular domain, transmembrane domain, and first 132 intracellular amino acids from NPR-B (residues 1 to 609), with the remaining residues coming from NPR-A (residues 594 to 1029). (B) Wild-type and chimeric receptors are phosphorylated when isolated from transiently transfected HEK 293 cells. Transfected HEK 293 cells were labeled with $^{32}PO_4$ (1 mCi/ml) overnight. The following day the cells were lysed and the receptors were immunoprecipitated, fractionated by SDS-PAGE, electroblotted to nitrocellulose, and visualized by exposure to Kodak XAR film overnight. (C) Tryptic phosphopeptide maps of NPR-A/B resemble those of NPR-A, and maps of NPR-B/A are similar to those of NPR-B. The purified receptors were digested with 10 μg of TPCK-treated trypsin overnight. Approximately 500 cpm was spotted on each origin. For mixes, approximately 250 cpm from each of the two preparations was added to a single origin. The resulting phosphopeptides were separated electrophoretically on a thin-layer cellulose plate for 25 min at 1 kV (pH 8.9), followed by ascending chromatography in an isobutyric acid-based solvent. The arrows indicate the origins of application. The phosphopeptides were visualized by exposing the plates to Kodak XAR film for 1 week at −70°C with one intensifying screen.

^{32}P associated with the receptors (NPRA-^{32}P) was visualized by autoradiography. Alanine substitutions of serine or threonine residues at positions 497, 500, 502, 506, 510, and 513 resulted in receptors with a decreased phosphorylation state (Fig. 4). In contrast, mutation of residues just amino (position 494) or carboxyl (position 514) terminal to this region had no significant effect (data not shown). Mutation of four or five of

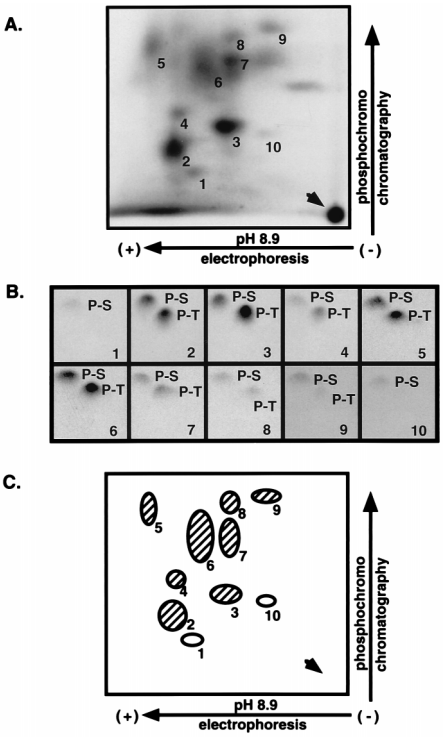


FIG. 2. The major tryptic phosphopeptides of NPR-A contain phosphoserine and phosphothreonine. (A) Tryptic phosphopeptide map of NPR-A. NPR-A was isolated from ^{32}P-labeled cells and digested with 10 μg of TPKC-treated trypsin at 37°C overnight. The resulting phosphopeptides were separated electrophoretically on a thin-layer cellulose plate for 25 min at 1 kV (pH 8.9), followed by ascending chromatography in phosphochromatography solvent. The arrow head denotes the origin of application. (B) Phosphoamino acid analysis of individual NPR-A tryptic phosphopeptides. The numbered peptides from panel A were scraped from the cellulose plate, eluted in water, dried, and then hydrolyzed in 6 N HCl for 90 min at 110°C. The resulting phosphoamino acids were redissolved in 5 μl of pH 1.9 buffer, separated electrophoretically in the first dimension at pH 1.9, and then separated in the second dimension at pH 3.5. P-S and P-T denote phosphoserine and phosphothreonine, respectively. (C) Graphic representation of the results of the phosphoamino acid analysis. Hatched ellipses contain both phosphoserine and phosphothreonine. Ellipses with no hatches contain only detectable amounts of phosphoserine.

the potential phosphorylation sites to A resulted in receptors that contained very low levels of ^{32}P. The reductions in ^{32}P were not explained by decreased receptor protein levels since immunoblot analysis on the same membrane used for the ^{32}P determinations indicated that the wild-type and mutant receptors were present in approximately equal amounts (Fig. 4B). These data suggest that S497, T500, S502, S506, S510, and T513 are in vivo phosphorylation sites of NPR-A.

Serine- and/or threonine-to-alanine mutations within the KHD dramatically alter tryptic phosphopeptide maps of NPR-A. To further characterize the effects of the phosphorylation site

FIG. 3. The putative ATP-binding domain of NPR-A contains multiple serine and threonine residues which are conserved in NPR-B but not Sta-R. Shown is alignment of the putative ATP-binding domain of NPR-A with the analogous amino acid sequences of NPR-B and Sta-R. Black boxes indicate residues contained in two or more receptors. Underlines represent residues contained in the putative ATP-binding motif GXGXXXG. All sequences are derived from translations of rat cDNAs. The numbers above the sequences correspond to the primary amino acid sequence of rat NPR-A (9).

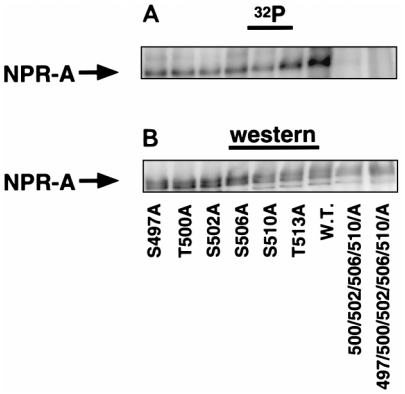


FIG. 4. Serine- and/or threonine-to-alanine mutations within the putative ATP-binding domain reduce the phosphorylation state of NPR-A. HEK 293 cells were transiently transfected with mutant or wild-type NPR-A constructs and then labeled with $^{32}PO_4$ overnight. NPR-A was then immunoprecipitated, separated by SDS-PAGE, blotted to an Immobilon membrane, and visualized by exposure to Kodak XAR film (A). The same membrane was subsequently probed with an antibody specific for NPR-A to indicate the amount of receptor protein present (B).

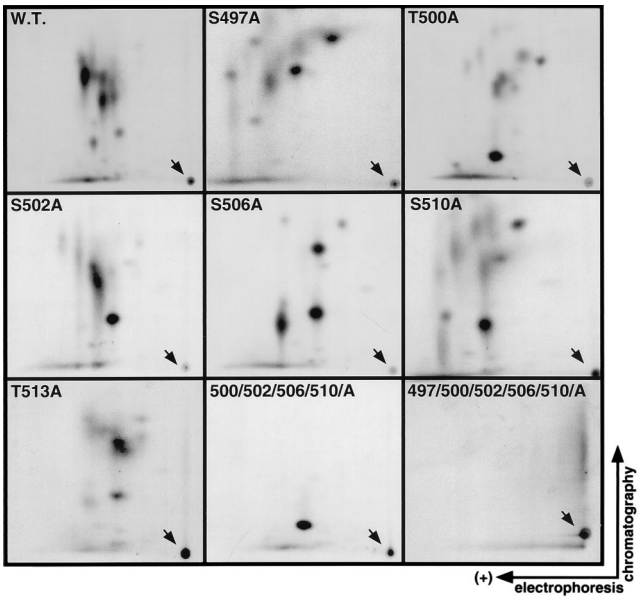


FIG. 5. Serine- and/or threonine-to-alanine mutations within the putative ATP-binding domain dramatically change NPR-A tryptic phosphopeptide maps. HEK 293 cells were transiently transfected with mutant or wild-type (W.T.) NPR-A constructs. The following day, the cells were labeled with $^{32}PO_4$ overnight. NPR-A was then immunoprecipitated, separated by SDS-PAGE, blotted to a nitrocellulose membrane, and visualized by exposure to Kodak XAR film. The fragments containing the labeled NPR-A protein were then cut out of the membrane and digested with 10 μg of trypsin overnight at 37°C. The resulting phosphopeptides were then applied to thin-layer cellulose plates and separated electrophoretically at pH 8.9 and chromatographically in phosphochromatography buffer. The phosphopeptides were visualized by placing the plates against Kodak XAR film with one enhancing screen at −70°C for 1 week.

mutations, we performed tryptic phosphopeptide mapping experiments. We reasoned that the loss of a phosphorylation site should either eliminate a phosphopeptide or change the migration pattern of a remaining phosphopeptide(s), since it would result in the loss of negative charge. The maps were generated by digesting the purified receptors off nitrocellulose membranes with trypsin and separating the resulting phosphopeptides on thin-layer cellulose plates in the first dimension by electrophoresis at pH 8.9 and in the second dimension by ascending chromatography. A phosphopeptide map of wild-type NPR-A is shown in Fig. 5 (panel W.T.). Wild-type maps are characterized by two lower spots that are diagonally separated, sloping up to the right, and two upper spots that are diagonally separated, sloping up to the left. As shown in Fig. 5, every mutant that reduced the phosphorylation state of NPR-A also yielded an altered phosphopeptide map. However, mutants that failed to reduce the phosphate content of NPR-A did not significantly change the maps (data not shown). The simultaneous substitution of A for the residues at positions 500, 502, 506, and 510 resulted in a map that consisted of only one major phosphopeptide (Fig. 5, panel 500/502/506/510/A). A receptor that contained an A substitution at S497 in addition to the other four mutations resulted in a map that was completely devoid of any visible phosphopeptides (Fig. 5, panel 497/500/502/506/510/A). These data suggest that S497, T500, S502, S506, S510, and T513 are in vivo phosphorylation sites of the NPR-A and that the major phosphopeptide observed in the 500/502/506/510/A map contains S497.

Comigration of a synthetic and in vivo phosphorylated peptide. Since the tryptic phosphopeptide mapping experiments suggested that S497 was the major phosphorylated residue in the 500/502/506/510/A mutant receptor, we initiated studies to identify the sequence of this phosphopeptide. Assuming complete digestion by trypsin, S497 should be the second serine in the peptide SAGSR. In vitro phosphorylation of the synthetic peptide SAGSRLTLSGR (amino acids 494 to 504 of NPR-A) with the catalytic subunit of protein kinase A followed by trypsin digestion resulted in a peptide that migrated as predicted for the monophosphorylated form of the peptide SAGS(P)R (data not shown). When this peptide was scraped from the plate and refractionated, it migrated as a single species (Fig. 6B). Phosphoamino acid analysis of this phosphopeptide indicated that it contained only detectable amounts of phosphoserine (Fig. 6B, inset). To test whether the peptide observed in the in vivo maps from the 500/502/506/510/A mutant receptor was SAGS(P)R, we performed a comigration exper-

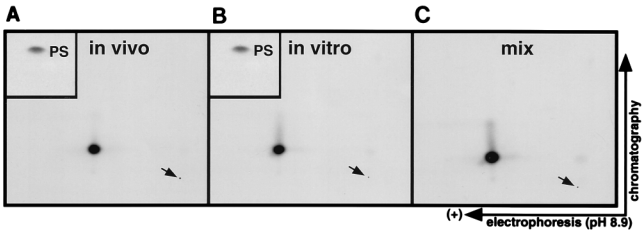
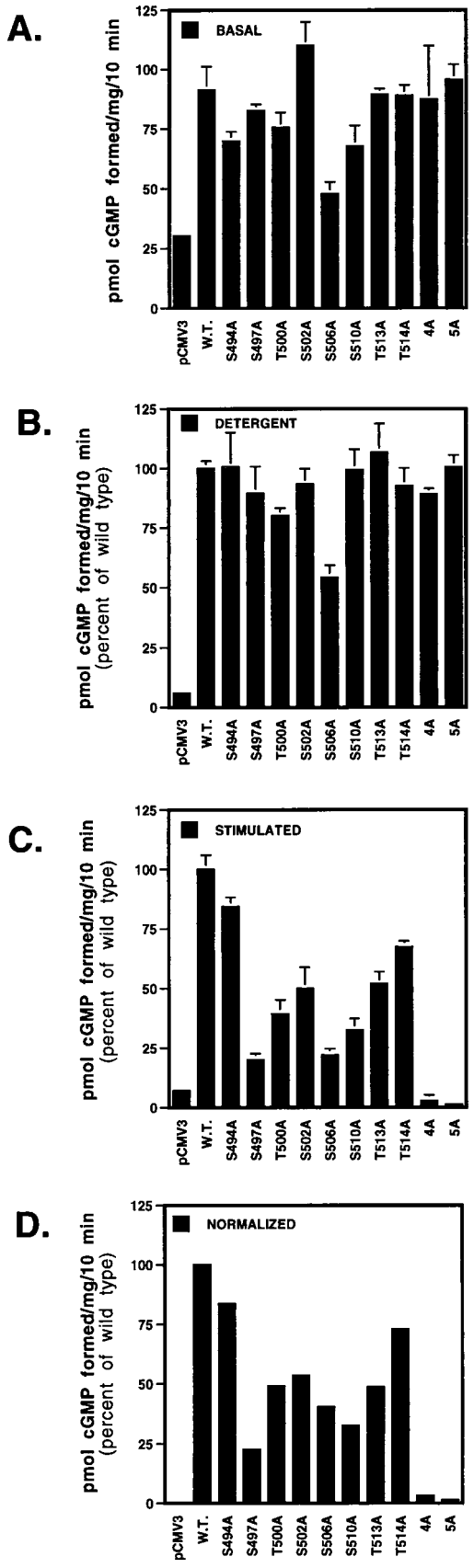


FIG. 6. The synthetic peptide SAGS(P)R comigrates with the major phosphopeptide isolated from tryptic digestions of the 500/502/506/510/A mutant receptor. The peptide SAGSRLTLSGR was phosphorylated with the catalytic subunit of protein kinase A and then cleaved with trypsin to produce the peptide SAGS(P)R. This phosphopeptide was purified from the other digestion products by thin-layer electrophoresis and ascending chromatography as described in Materials and Methods; 100 cpm of the purified SAGS(P)R peptide was then spotted on a thin-layer cellulose plate and separated electrophoretically for 25 min at 1 kV (pH 8.9) followed by ascending chromatography in phosphochromatography solvent (B). Similarly, 100 cpm of the major phosphopeptide isolated from the tryptic phosphopeptide map of the 500/502/506/510/A mutant receptor was isolated, spotted on a cellulose plate, and refractionated as described for the in vitro-phosphorylated peptide (A). Phosphoamino acid analysis was performed on 50 cpm of each sample as described in Materials and Methods (A and B, insets). PS denotes phosphoserine. For the mixing experiment (C), 100 cpm of each peptide was added to the same origin (200 cpm, total) and separated electrophoretically and chromatographically as described for the individual phosphopeptides. The phosphopeptides were visualized by exposing the plates to Kodak XAR film for 12 days with one intensifying screen.



iment (Fig. 6). When the major peptide from the mutant receptor maps was purified from the original cellulose plate and refractionated, it ran as a single species (Fig. 6A). Phosphoamino acid analysis indicated that it, like the in vitro-phosphorylated peptide, contained only phosphoserine (Fig. 6A, inset). When equal numbers of counts/minute of the in vivo- and in vitro-phosphorylated peptides were fractionated together on the same plate, the peptides migrated as a single entity. These data, together with mutagenesis data, indicate that the sequence of the major phosphopeptide observed in the maps of the 500/502/506/510/A mutant receptor is SAGS(P)R.

Analysis of the phosphopeptide mapping experiments. One of the major conclusions from these studies is that several of the phosphopeptides contain the same phosphorylation site(s). This is most likely due to the presence of a phosphorylated serine or threonine residue located two amino acids carboxyl terminal to an arginine residue. This configuration is known to inhibit trypsin cleavage (4) and greatly complicates the maps. For example, if T500 is phosphorylated, then trypsin may inefficiently cleave after R498. This would result in peptides with the sequence SAGSR and SAGSRLTLSGR. If both T500 and S506 are phosphorylated, then the peptide SAGSRLTLSGRG SNYGSLLTTEGQFQVFAK may also be observed. Furthermore, peptides with the same peptide backbone but different numbers of phosphates will also migrate as separate species due to charge differences (4). Thus, it is theoretically possible for S497 to be contained in more than 10 separate peptides! Finally, it is important to note that of the 17 S and T residues contained in the first 132 intracellular amino acids of NPR-A, we have mutated 9 to A. Six of them (497, 500, 502, 506, 510, and 513) were found to be phosphorylated, and three (494, 514, and 587) were not (data not shown). The remaining eight residues are not contained in a predicted tryptic peptide that contains both S and T. Hence, they are unlikely candidates for the phosphorylation sites. Therefore, we have mapped these sites not only by identifying serine or threonines that change the phosphorylation state of the receptor; we have also eliminated the other possibilities from consideration.

Serine- and/or threonine-to-alanine mutations within the KHD reduce or abolish hormone-dependent but not basal or detergent-dependent guanylyl cyclase activity of NPR-A. To determine what effects, if any, the phosphorylation site mutations have on the enzymatic activity of NPR-A, we performed guanylyl cyclase assays. Crude membranes isolated from HEK 293 cells that had been transiently transfected with the various constructs were assayed in the presence of Mg^{2+}-GTP alone (basal), ANP, ATP, and Mg^{2+}-GTP (stimulated), or Triton X-100 and Mn^{2+}-GTP (detergent). The latter conditions are traditionally known to artificially stimulate guanylyl cyclases to

FIG. 7. Specific serine- and/or threonine-to-alanine mutations within the putative ATP-binding domain reduce or abolish hormone-dependent but not basal or detergent-dependent guanylyl cyclase activities of NPR-A. HEK 293 cells were transfected with the indicated NPR-A expression constructs; 48 h later, crude membranes were prepared and assayed for guanylyl cyclase activity for 10 min at 37°C in the presence of Mg^{2+}-GTP only (basal; A), Mn^{2+}-GTP and Triton X-100 (detergent; B) or ANP, ATP, and Mg^{2+}-GTP (stimulated; C). The mean values for wild-type basal, detergent-dependent, and hormone-dependent activities were 1,000, 27,200, and 12,420 pmol/mg, respectively. The vertical bars centered above the columns represent the range of values obtained from two separate transfections, which were assayed in duplicate for panel A, and the standard error of the mean (n = 4 to 8) for panels B and C. In an effort to control for the different expression levels of the various constructs, the values shown in panel D were normalized to a constant amount of detergent-dependent wild-type activity. This was accomplished by dividing the detergent-dependent activity of the wild-type receptor by the detergent-dependent activity of each mutant receptor and multiplying this number by the stimulated values shown in panel C.

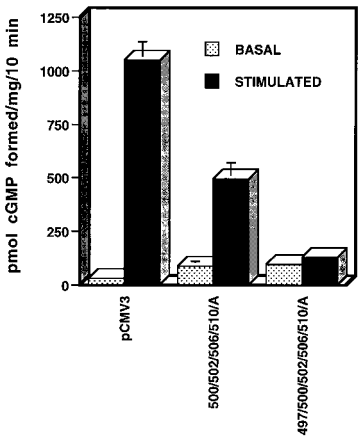


FIG. 8. Multiple serine- and threonine-to-alanine mutations within the KHD of NPR-A result in a receptor with dominant negative inhibitory activity. HEK 293 cells were transfected with either vector alone (pCMV3) or vector with the full-length cDNA for NPR-A containing alanine substitutions at the indicated positions. Two days later, crude membranes were prepared from these transfected cells, which were assayed for guanylyl cyclase activity in the presence of Mg^{2+}-GTP only (basal) or Mg^{2+}-GTP, ANP, and ATP (stimulated). The vertical bars centered above the columns represent the range of values obtained from two separate transfections which were assayed in duplicate.

their maximum levels (23). As shown in Fig. 7A, HEK 293 cells transfected with vector alone (pCMV3) contained low but detectable levels of basal guanylyl cyclase activity. Transfection of these cells with the same vector containing the full-length cDNA for wild-type or mutant forms of NPR-A yielded basal guanylyl cyclase activities that were two- to three-fold higher than those for vector alone. Guanylyl cyclase assays conducted in the presence of detergent yielded activities for wild-type and mutant NPR-A constructs that were more than 10-fold higher than those obtained with the vector alone (Fig. 7B). Both detergent-dependent and basal activities were roughly similar between those for wild-type and the various mutant constructs with the exception of S506A, which yielded activities that were about half of wild-type levels. Addition of hormone and ATP dramatically stimulated the activity of the wild-type receptor, and the mutants that did not affect the phosphorylation state of the NPR-A (S494A and T514A) had only marginally reduced hormone-stimulated activities (Fig. 7C). However, the hormone-dependent activities of the mutants which did reduce the phosphorylation state of NPR-A (Fig. 4) were markedly diminished in comparison to the wild-type receptor (Fig. 7C). Mutation of S497 to A had the greatest effect, resulting in only about 20% of wild-type activity. Strikingly, the mutation of four (500/502/506/510/A) or five (497/500/502/506/510/A) of the phosphorylation sites to A resulted in receptors that showed no detectable increases in hormone-dependent guanylyl cyclase activity over mock-transfected levels. Since it could be legitimately argued that the effects of some of the mutations were due to decreased expression levels of catalytically active cyclase, we normalized the data shown in Fig. 7C by multiplying each value by the ratio of the detergent-dependent activity of the wild-type receptor divided by the detergent-dependent activity of the mutant receptor (Fig. 7D). We believe that this value more closely approximates the effect that each mutation has on the physiological activity of NPR-A because it takes into account the relative expression level of each construct. As shown in Fig. 7D, even when the reduced expression level of the S506A mutation is taken into account, the hormone-dependent activity of this mutant is decreased to less than half of wild-type activity. Taken together, these data indicate that phosphorylation of residues within the putative ATP-binding domain has a dramatic effect on the activity of the NPR-A and suggest that phosphorylation is absolutely required for hormone-dependent enzyme activation.

Multiple phosphorylation site mutations within the KHD of NPR-A result in a receptor with dominant negative inhibitory activity. In the process of studying the effect of the phosphorylation site mutations on the mutant receptors, we found that they also inhibit the ability of the endogenous receptors to respond to hormone. As shown in Fig. 8, the line of HEK 293 cells used in this study possesses a relatively robust response to ANP and ATP, being activated approximately 35-fold from 30 pmol of cGMP/mg/10 min in the absence of ANP and ATP to 1,051 pmol of cGMP/mg/10 min in the presence of the activators. When these cells were transfected with constructs containing alanine mutations at four or five of the phosphorylation sites, basal cyclase activities were elevated to 87 and 96 pmol of cGMP/mg/10 min, respectively. However, when assayed in the presence of ANP and ATP, their activities were reduced from 1,051 to 495 and 128 pmol of cGMP/mg/10 min (Fig. 8). Thus, high expression of the five alanine mutant relative to the wild-type receptor can completely inhibit the ability of the wild-type receptor to respond to agonist. Since NPR-A is known to exist in an homo-oligomeric state and is thought to require oligomerization for activity (11, 25, 36), it is likely that the dominant negative effect of these mutant receptors is a function of their ability to physically associate with the wild-type receptor. Thus, the sequestration of the wild type by mutant receptors effectively reduces the number of oligomeric complexes capable of responding to hormone.

Glutamate functionally substitutes for serine at position 497. Since the mutation of S497 to A resulted in the most dramatic reduction in hormone-dependent activity, we asked whether the substitution of a glutamate residue at this position would preserve, at least partially, the capacity of this receptor to respond to ANP. The ability of a carboxylic acid moiety to mimic the negative charge of phosphate group has been previously demonstrated (51). To our surprise, the substitution of glutamate for serine 497 completely restored the ability of NPR-A to respond to ANP and ATP (Fig. 9). It is not a constitutively activating mutation, since the S497E mutant, like the wild-type receptor, is tightly repressed in the absence of the hormone (Fig. 9, Mg^{2+}). These data suggest that the purpose

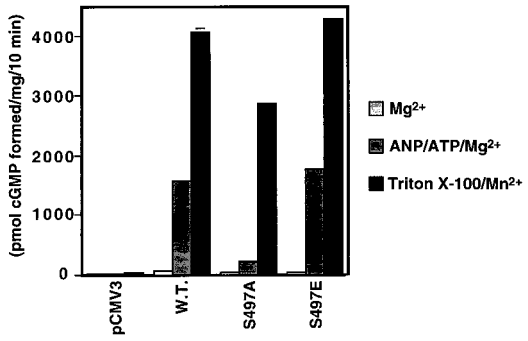


FIG. 9. Substitution of serine 497 with glutamate, but not alanine, retains wild-type ANP-dependent guanylyl cyclase activity. HEK 293 cells were transfected with the wild-type (W.T.), S497A, or S497E construct. Two days later, crude membranes were prepared from these cells, and guanylyl cyclase assays were conducted for 10 min at 37°C in the presence of the activators indicated. The vertical bars centered above the columns represent the ranges of values obtained from two separate transfections which were assayed in duplicate.

of the phosphoserine residue at position 497 is to provide a localized negative charge around the putative ATP-binding domain and provide additional evidence to support a role for the phosphorylation of this residue in the regulation of NPR-A enzymatic activity.

DISCUSSION

In this report, we have identified four serine and two threonine residues located within the KHD of NPR-A that are phosphorylated when the receptor is expressed in HEK 293 cells. Mutational analysis of these sites indicated that they are all required for maximal ANP-dependent enzyme activity and that the phosphorylation of multiple sites is obligatory for the signal transduction process. That phosphorylation is required for maximal hormonal activation of NPR-A had been previously suggested based on whole and broken cell desensitization and dephosphorylation assays (31, 43, 44). Unfortunately, these experiments could not rule out the possibility that another regulatory protein was also being dephosphorylated with kinetics similar to that of NPR-A. The data presented in this report suggest that the dephosphorylation of only NPR-A is sufficient to mediate the desensitization response. These data do not, however, rule out the possibility that NPR-A can be desensitized by other mechanisms as well.

Our results now indicate that receptor activation requires at least three separate processes: (i) phosphorylation of the KHD, (ii) ANP binding to the extracellular domain, and (iii) ATP binding to the intracellular domain. If any one of these three processes does not occur, the receptor is inactive. The fact that mutation of any individual site to a nonphosphorylatable residue greatly decreases hormone-stimulated guanylyl cyclase activity indicates that most of these sites have to be phosphorylated to obtain a hormonally responsive receptor. This suggests that a cluster of negative charges is required in this region. The substitution of serine 497 with glutamate apparently provides the negative change at this site, but this mutant receptor presumably still requires phosphorylation at the other sites for activity since the receptor mutant 500/502/506/510/A is hormone insensitive. We do not know that identity of the protein kinase(s) that phosphorylates these sites, but the high stoichiometry of phosphorylation suggests that this might be a cooperative or processive event such as that observed for ribosomal protein S6.

Interestingly, our data may explain why the initial purifications of NPR-A resulted in a hormonally unresponsive enzyme (32, 40, 49). Since NPR-A was not known to be phosphorylated at the time, no precautions were taken to keep the receptor in its fully phosphorylated state during the purification process. In contrast, NPR-A was recently purified to apparent homogeneity in the presence of phosphatase inhibitors, and this preparation, unlike previous attempts, retained the ability to be activated by ANP and ATP (54). These data may also shed some light on recent experiments by Sharma and colleagues, who have identified a region within both NPR-A and NPR-B which they call the ATP regulatory module; they mutated G505 and S506 of NPR-A to V and N, respectively, and found that the mutant receptor was no longer stimulated by ANP and ATP (15, 21). They concluded that the mutation of the second G (G505) in the GXGXXXG motif disrupted the ability of NPR-A to bind ATP, and that was the reason why NPR-A was inactive (15, 21). However, it is now apparent that they also mutated a critical phosphorylation site (S506) in the process of modifying the putative ATP-binding module. Whether additional phosphorylation sites were affected is unknown, but the replacement of two amino acids that contain relatively small side chains with residues with much larger side chains is likely to cause major changes in the steric properties of this critical regulatory domain.

Are other cell surface guanylyl cyclases regulated by phosphorylation? NPR-B, the other known guanylyl cyclase linked natriuretic peptide receptor, has been shown to be desensitized by dephosphorylation in a manner similar to NPR-A (42a). This is not surprising, since these receptors are 78% identical at the intracellular amino acid level (46) and the KHDs of the receptors have been shown to be functionally equivalent (30). In fact, we have found that many of the sites that are phosphorylated in NPR-A are also phosphorylated in NPR-B. Thus, it is likely that the two guanylyl cyclase-linked natriuretic peptide receptors are regulated similarly. It has also been recently reported that a guanylyl cyclase purified from bovine retina is an autophosphorylating kinase (2). This is an extremely interesting finding and, if confirmed, almost certainly means that this receptor is also regulated by phosphorylation. Since two distinct but similar guanylyl cyclases have been cloned from retinal tissue libraries (38, 48, 55), the primary amino acid sequence for the autophosphorylating cyclase is not known. However, it is interesting that a serine corresponding to S497 of NPR-A is conserved in both of the known retinal guanylyl cyclases. Sta-R, also known as guanylyl cyclase C, has been reported to be regulated by PKC-dependent phosphorylation, but unlike the case for NPR-A, PKC-dependent phosphorylation appears to increase ligand-dependent activity of this receptor (53). Thus, it is unlikely that Sta-R and NPR-A are regulated similarly.

In conclusion, it is remarkable that it has taken 15 years since guanylyl cyclases were first shown to be phosphorylated (52) to identify a guanylyl cyclase phosphorylation site. The lack of information regarding the responsible protein kinase, the inherent difficulties of purifying these enzymes, the incomplete tryptic digestions, and the large number of sites are all likely contributors to this delay. Nonetheless, the identification of the phosphorylation sites of NPR-A is a major step toward the understanding of how this enzyme is regulated. In the future, it will be particularly informative to test the effects of glutamate substitutions at phosphorylation sites on the homologous and heterologous desensitization processes. The heterologous desensitization pathway may be more amenable to this analysis, since, in contrast to homologous desensitization, only a subset of sites are dephosphorylated. In addition, the identities of the protein kinase(s) and phosphatase(s) involved in modulating the phosphorylation state of NPR-A are of great interest. The phosphorylation sites information may be useful for designing affinity supports or peptide substrates for the isolation of these enzymes.

ACKNOWLEDGMENTS

This work was initiated in David L. Garbers' laboratory in the Department of Pharmacology at the University of Texas Southwestern Medical Center. We thank Dr. Garbers for helpful comments during the early stages of this work and for the generous donation of numerous reagents that were necessary for these studies. We also thank Jill Meisenhelder for peptide synthesis and purification.

L.R.P. was supported by NRSA fellowship CA-67452 from the National Cancer Institute. T.H. is an American Cancer Society Research Professor. This work was supported by USPHS grants CA14195 and CA39780 to T.H.

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