Identification and Characterization of the Major Phosphorylation Sites of the B-type Natriuretic Peptide Receptor*

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C-type natriuretic peptide (CNP) is a newly discovered factor that stimulates vasorelaxation and inhibits cell proliferation. Natriuretic peptide receptor-B (NPR-B) is the primary signaling molecule for CNP. Recently, the guanylyl cyclase activity of NPR-B was shown to correlate with its phosphorylation state, and it was suggested that receptor dephosphorylation is a mechanism of desensitization. We now report the identification and characterization of the major NPR-B phosphorylation sites. Mutagenesis and comigration studies using synthetic phosphopeptides were employed to identify five residues (Ser-513, Thr-516, Ser-518, Ser-523, and Ser-526) within the kinase homology domain that are phosphorylated when NPR-B is expressed in human 293 cells. Mutation of any of these residues to alanine reduced the receptor's phosphorylation state and CNP-dependent guanylyl cyclase activity. The reductions were not explained by decreases in receptor protein level as indicated by immunoblot analysis and determinations of cyclase activity in the absence of CNP or in the presence of detergent. Elimination of all of the phosphorylation sites resulted in a completely dephosphorylated receptor whose CNP-dependent cyclase activity was decreased by >90%. However, unlike NPR-A, the dephosphorylated receptor was not completely unresponsive to hormone. Finally, two additional residues (Gly-521 and Ser-522) were identified that when mutated to alanine reduced the overall phosphorylation state and hormone responsiveness of the receptor without abolishing the phosphorylation of a specific site. These data indicate that phosphorylation of the kinase homology domain is a critical event in the regulation of NPR-B.

The natriuretic peptide family consists of atrial natriuretic peptide (ANP),1 B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (1–3). ANP and BNP are stored primarily in the cardiac atria and ventricles, respectively, and are released into the circulation upon an increase in cardiac volume (1–3). CNP on the other hand, is found primarily in vascular endothelial cells (4), seminal plasma (5), and brain tissue (6). It does not seem to be stored in significant quantities. Instead, it appears to be regulated at the level of transcription by various cytokines, such as TGF-β (4). The physiological responses elicited by these natriuretic peptides are similar but not identical. For instance, all three have been shown to cause vascular relaxation, but although both ANP and BNP have been shown to cause diuresis and natriuresis, CNP's role in these processes is dubious. Likewise, both ANP and CNP have been shown to inhibit the proliferation of various cell types (7–9) but only CNP has thus far been shown to be a potent inhibitor of intimal thickening after vascular injury (10–12).

The cognate signaling receptor for ANP and BNP is the natriuretic peptide receptor A (NPR-A), which is also known as guanylyl cyclase A (13, 14). The primary signaling receptor for CNP is the natriuretic peptide receptor B (NPR-B), which is also known as guanylyl cyclase B (15, 16). NPR-A and NPR-B are approximately 40 and 78% identical within their extracellular and intracellular regions, respectively (16). Motifs present in the primary amino acid sequence of both NPR-A and NPR-B suggest that they contain five primary functional domains: an extracellular ligand-binding domain, a single membrane-spanning region, a juxtamembrane protein kinase homology domain (KHD), an α-helical hinge region involved in oligomerization (17), and a carboxyl-terminal guanylyl cyclase catalytic domain (18, 19). Maximal activation of these receptors is thought to require ligand binding extracellularly and ATP binding intracellularly (20–23). The effect of ATP is presumably mediated allosterically by the KHD, because nonhydrolyzable adenine nucleotide analogs effectively substitute for ATP (20–22), and deletion mutants lacking the KHD are unresponsive to hormone (24, 25). Within the KHD of NPR-A is the sequence GXGXXG, which is similar to the known ATP-binding motif GXGXXG found in many protein kinases (26). Sharma and colleagues (27, 28) have coined this corresponding region in natriuretic peptide receptors the ATP-regulatory module. They have reported that the mutation of some but not all of the residues within the ATP-regulatory module region dramatically reduces hormone-dependent activation of these receptors. The mechanism for this effect has not been demonstrated, but it has been speculated that it is a result of the disruption of the putative ATP-binding domain.

Both NPR-A and NPR-B are known to be phosphorylated on serine and threonine residues in unstimulated cells (29–32). Upon binding to their cognate hormone, the guanylyl cyclase activity of these receptors is markedly enhanced, resulting in dramatic elevations in intracellular cGMP concentrations. After the initial stimulation, the activity of these enzymes de-
creases with kinetics that are coincident with receptor dephosphorylation (29, 31). Thus, it has been suggested that dephosphorylation mediates their desensitization. We recently, identified six phosphorylation sites within the KHD of NPR-A (33). As was predicted from the desensitization studies, the mutation of these sites to alanine dramatically decreased the ANP-dependent activity of NPR-A. Furthermore, the conversion of four or more of the phosphorylation sites to alanine resulted in an hormonally unresponsive receptor. In this report, we describe the identification of five in vivo phosphorylation sites for NPR-B. The evidence for these sites is based primarily on deletion and site-specific mutagenesis studies. However, for two of the sites we were able to show comigration with synthetic phosphopeptides. Four of the five sites correspond to residues present in NPR-A, and the additional site is only one amino acid carboxyl-terminal to a known NPR-A site. All of the single phosphorylation site mutations decreased CNP-dependent activity, and a completely dephosphorylated form of NPR-B retained less than 10% of its wild type activity. Finally, we show that a previously described inactivating mutation within the so called ATP- regulatory module of NPR-B (G521A) also results in receptor dephosphorylation, suggesting that dephosphorylation may explain the functional consequence of this mutation as well.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Transient Transfections—Mutations within the KHD were generated on the 304-base pair EcoRV-XbaI fragment of NPR-B, which was subcloned into pBluescript II (Stratagene, San Diego, CA). The mutations were generated using the Quikchange™ kit from Stratagene according to the manufacturer’s protocols. The mutant EcoRV-XbaI fragments were then subcloned back into the corresponding region of the expression plasmid pRK5-NPR-B. This plasmid was engineered by subcloning the full-length NPR-B cDNA (5′ EcoRI to HindIII 3′ fragment) into the plasmid pRK5 (34). All indicated mutations and the absence of unwanted mutations were confirmed by manual or automated nucleic acid sequencing. HEK 293 cells were grown to ~50% confluence in 6- or 10-cm dishes, and then transfected with 2.5 or 5 μg of the various pRK5-NPR-B constructs using the BES-buffered calcium phosphate coprecipitation method (35).

Metabolic Labeling, Phosphoamino Acid Analysis, and Phosphopeptide Mapping—Transfected HEK 293 cells were washed twice with phosphate-deficient Dulbecco’s modified Eagle’s medium then changed to 95% phosphate-deficient Dulbecco’s modified Eagle’s medium, 5% dialyzed fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml iodoacetamide, and 0.5 mM 1-methyl-3-isobutylxanthine, 0.1% bovine serum albumin for 2 h at 5 °C. This antiserum (29) was raised against a fragment of NPR-B, which was subcloned into pBluescript II (Stratagene, San Diego, CA). The mutations were generated using the Quikchange™ kit from Stratagene according to the manufacturer’s protocols. The predicted trypsin cleavage site within the so called ATP-regulatory module of NPR-B (G521A) also results in receptor dephosphorylation, suggesting that dephosphorylation may explain the functional consequence of this mutation as well.

In Vitro Synthesis of Phosphopeptides—The peptides LTLS(P)GR and LVPS(L)PGR were synthesized on an Applied Biosystems 432A peptide synthesizer and purified according to the manufacturer’s protocol. The full-length species were then separated from breakdown and spurious products by HPLC on a Vydac C18 column using a 5–60% gradient of increasing buffer B, where buffer A = water with 0.1% trifluoroacetic acid and buffer B = acetonitrile with 0.1% trifluoroacetic acid. The mass composition of each phosphopeptide was verified by laser desorption mass spectroscopy.

Guanyl Cyclase Assays—Crude membranes were prepared as described previously (33). All guanyl 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% bovine serum albumin, 5 mM creatine phosphate, 5–10 units/assay creatine phosphokinase, 1 mM GTP, and 0.1–0.2 μCi of [α-32P]GTP. 5 mM MgCl2, 1 mM ATP, and 1 μM CNP or 1% Triton X-100 and 5 mM MgCl2 were also included in the reaction mixtures. Basal levels were determined in the presence of only 5 mM MgCl2, or 1 mM ATP and 5 mM MgCl2. Assays were initiated by the addition of a solution of the above reagents to approximately 50 μg of crude membrane protein in a total volume of 0.1 ml. Cyclic GMP accumulation was analyzed as described previously (37).

RESULTS

All of the Known NPR-A Phosphorylation Sites Are Conserved in NPR-B—Previous experiments involving chimeric constructs between NPR-A and NPR-B indicated that the major phosphorylation sites of both receptors are conserved within the first 132 amino acids past their respective transmembrane domains (33). Further experiments identified and characterized six phosphorylation sites within subdomain I of the KHD of NPR-A. Because all six of these sites are conserved in NPR-B, we asked whether these analogous serine and threonine residues were also phosphorylated in this receptor. An alignment of the NPR-A phosphorylation sites with the analogous NPR-B residues is shown in Fig. 1A. The predicted trypsin cleavage sites within this region of NPR-B are shown in Fig. 1B. The solid lines indicate that complete cleavage is expected, and the dashed lines indicate that a partial cleavage is likely because of the presence of a potentially phosphorylated serine or threonine residue located 2-amino acids carboxyl-terminal to an arginine residue. This scenario is known to inhibit trypsin cleavage in general (36) and was found to inhibit the cleavage of specific sites within NPR-A in a phosphorylation-dependent manner (33). Based on the characteristics of the individual amino acids comprising the predicted tryptic phosphopeptides, we generated a hypothetical tryptic phosphopeptide map for NPR-B (Fig. 1C). The bold letters within each peptide indicate the residues that are phosphorylated. The two upper spots represent the mono- and diphosphorylated forms of the peptide LTLSLR. The three phosphopeptides at the bottom represent the mono-, di-, and triphosphorylated forms of the peptide GSSYGMVMTAGK. The two spots in the middle of the map represent the mono- and diphosphorylated forms of the phosphopeptide GSGSRLTLSLR. This peptide would only be observed if Thr-516 is phosphorylated, because trypsin would otherwise be expected to cleave after arginine 514. The most highly phosphorylated phosphoisoform of each peptide is shown as the species that migrates furthest to the left (anode) because of its increased negative charge and the shortest distance in the vertical dimension because of its increased hydrophilicity.

The Phosphopeptide Map of NPR-B Isolated from in Vivo
Identification of the NPR-B Phosphorylation Sites

Labeled 293 Cells Resembles the Predicted Map—A typical tryptic phosphopeptide map of NPR-B that was isolated from metabolically 32P-labeled transiently transfected 293 cells is shown in Fig. 2A. The major phosphopeptides are labeled A-F. The similarity of this map to the predicted map is striking and is very similar to maps of NPR-B isolated from stably transfected NIH 3T3 cells (31). Phosphoamino acid analyses of the individual phosphopeptides that were scraped from this map are shown in Fig. 2B, and a cartoon summary is shown in Fig. 2C. All of the scraped phosphopeptides contained both phosphoserine and phosphothreonine except for B and F, which contained only phosphoserine, and D, which contained only phosphothreonine. Because of the low number of cpm associated with phosphopeptide B', we were unable to isolate it in the absence of B. Therefore, phosphoamino acid analysis of this sample represents the phosphoamino acid composition of both species (Fig. 2B, panel B + B'). However, because B isolated alone contains only phosphoserine, it is likely that B' contains only phosphothreonine. In Fig. 2C, we have indicated that it contains only phosphothreonine for reasons that will be described below.

Comigration with Synthetic Phosphopeptides—Because phosphopeptides A and B migrated with mobilities that were similar to those predicted for the di- and monophosphorylated forms of the peptide LTLSLR, we tested whether they would comigrate with chemically synthesized versions of these phosphopeptides (Fig. 3). When 5 μg of HPLC purified LTLSLR was mixed with 1000 cpm of NPR-B tryptic phosphopeptides isolated from 32P-labeled 293 cells, the major ninhydrin staining spot comigrated with phosphopeptide B. Ninhydrin staining is indicated by the dashed ellipses (Fig. 3, left panel). Likewise, when 5 μg of HPLC purified LT(P)LS(P)LR was fractionated with 1000 cpm of the in vivo NPR-B phosphopeptides, the major ninhydrin staining spot comigrated with phosphopeptide A (Fig. 3, right panel). No ninhydrin staining was observed in the absence of either synthetic peptide. These comigration data, together with the consistent phosphoamino acid data (phosphoserine and phosphothreonine for A and only phosphoserine for B), strongly suggest that phosphopeptides A and B are LT(P)LS(P)LR and LTLSLR, respectively.

Serine or Threonine to Alanine Mutations within the Putative ATP-binding Subdomain Decrease the Phosphorylation State of NPR-B—To test whether Thr-516 and Ser-518 as well as other residues in this region were phosphorylated (e.g., Ser-516) or not (e.g., Thr-516), we engineered individual constructs consisting of either single serine or threonine to alanine mutations, e.g., S513A or multiple changes, e.g., 7A (513/516/518/522/523/526/529 to A). These expression constructs were transiently transfected into 293 cells, which were then metabolically labeled overnight with [32P]orthophosphate. The wild type and mutant receptors were purified from the labeled cells by immunoprecipitation, SDS-PAGE, and electroblotting to Immobilon membrane. The amount of 32P associated with each receptor was visualized by...
Fig. 3. Comigration of tryptic NPR-B phosphopeptides with known synthetic phosphopeptides. Approximately 5 µg of the synthetic phosphopeptides LTLS(P)LR (left panel) or LT(P)LS(P)LR (right panel) were mixed with 1000 cpm of NPR-B tryptic phosphopeptides that were obtained from 32P-labeled 293 cells. The mixes were spotted on cellulose plates and separated electrophoretically for 35 min (left panel) or 25 min (right panel) at 1 kV (pH 8.9), followed by ascending chromatography in phosphochromatography solvent. The 32P-labeled phosphopeptides were visualized by autoradiography. The synthetic phosphopeptides were visualized by ninhydrin staining (0.25% in acetone) and are indicated by dashed ellipses.

Fig. 4. Serine and/or threonine to alanine mutations within the KHD reduce the phosphorylation state of NPR-B. HEK 293 cells were transiently transfected with the indicated mutant or the wild type NPR-B constructs and then labeled with 32P04 overnight. NPR-B was then immunoprecipitated, separated by SDS-PAGE, blotted to an Immobilon membrane, and visualized by exposure to Kodak XAR film (32P-content). The same membrane was subsequently probed with an antibody that recognizes NPR-B to indicate the amount of receptor protein present (western).

Fig. 5. Serine and/or threonine to alanine mutations within the KHD domain of NPR-B result in the loss of specific tryptic phosphopeptides. The fragments containing the labeled NPR-B proteins shown in Fig. 4 were cut out of the membrane and digested with 10 µg 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.

Phosphorylation of protein (Fig. 4, western).

Serine and/or Threonine to Alanine Mutations within the KHD Alter the Tryptic Phosphopeptide Maps of NPR-B—To characterize these putative phosphorylation sites further, we generated tryptic phosphopeptide maps for each of the mutants (Fig. 5). We reasoned that if our predictions were correct (Fig. 1C), these mutations would result in the loss of the phosphopeptide(s) that contained the substituted amino acid. The maps were produced by trypsinizing the purified receptors off the membranes and separating the resulting phosphopeptides on thin layer cellulose plates in the first dimension by high voltage electrophoresis at pH 8.9 and in the second dimension by ascending chromatography. Phosphopeptide maps of the wild type receptors are shown in the top left and bottom right panels of Fig. 5 (W.T.) and are very similar to the map shown in Fig. 2A. The conversion of serine 513 to alanine resulted in the loss of phosphopeptides C and D. This is consistent with our prediction that Ser-513 is contained in these phosphopeptides (Fig. 1C). Likewise, substitution of alanine for threonine at position 516 resulted in the loss of phosphopeptides A, B', C, and D. Again, these results are consistent with our predictions. Mutation of serine 518 to alanine resulted in the loss of phosphopeptides A and B, but not B', C, or D. This confirms our synthetic camigration data, and it suggests that the sequence of spots C and D are GAGS/PRLT/P/LSLR and GAGSRLT-(P)LSLR, respectively. In addition, because the mutation of Thr-516, but not Ser-518, results in the loss of B', it is likely that sequence of this peptide is LT(V)PLSLR. This conclusion is also in agreement with the phosphoamino acid analysis data (phosphothreonine) for this peptide. The mutation of serine 522 to alanine yielded maps that were not significantly different from wild type maps. This was surprising, because this mutation markedly reduced the phosphorylation state of the receptor (Fig. 4), and its analogous residue (Ser-506) is a known phosphorylation site in NPR-A (33). However, because the mutant maps are unchanged from wild type maps, it is unlikely that this residue is phosphorylated in NPR-B under the conditions tested. The reduced phosphorylation state of this mutant receptor is likely to be the result of its decreased ability to be phosphorylated in general and not the loss of a specific site. In contrast, the conversion of serine 523 to alanine resulted in the loss of the phosphopeptides E and F. We hypothesized that phosphopeptides E and F were the diphosphorylated and monophosphorylated forms of the peptide GSSYGS(L/T)AHGK, respectively (Fig. 1C). The absence of these phosphopeptides in the S523A mutant is consistent with this prediction. Likewise, the conversion of serine 526 to alanine resulted in the loss of...
Again, this is what would be expected for this mutation. Based on these data, it is likely that phosphopeptide F is the monophosphorylated form of GSSYGSLMTAHGK where Ser-523 is phosphorylated, and F' is the monophosphorylated form of GSSYGSLMTAHGK where Ser-526 is phosphorylated. The mutation of threonine 529 to alanine, on the other hand, had no obvious effect on the phosphopeptide map of NPR-B. Because this mutation had no effect on the phosphate content of NPR-B either, it is unlikely that it is a major phosphorylation site. Finally, we note that the mutation of all the possible phosphorylation sites in this region (Ser-513, Thr-516, Ser-518, Ser-522, Ser-523, Ser-526, and Thr-529) to alanine results in the complete loss of all phosphopeptides.

**Effect of the Phosphorylation Site Mutations on the Guanylyl Cyclase Activity of NPR-B—**

We next tested whether these phosphorylation site mutations affected the guanylyl cyclase activity of NPR-B. Crude membranes isolated from 293 cells that had been transiently transfected with the various constructs were assayed in the presence of: Mg\(^{2+}\)-GTP and ATP (panel A), Triton X-100 and Mn\(^{2+}\)-GTP (panel B), or CNP, ATP, and Mg\(^{2+}\)-GTP (panel C). Triton X-100 and Mn\(^{2+}\) have been shown to stimulate NPR-B to maximal levels in a manner independent of the phosphorylation state of the receptor (31). Thus, these conditions are an excellent measure of the total amount of guanylyl cyclase present in any given membrane preparation. As shown in Fig. 6A, 293 cells transfected with vector alone (pRK5) contained very low levels of basal guanylyl cyclase activity compared with the NPR-B transfected cells. Transfection of these cells with the same vector containing the full-length cDNA for wild type or mutant forms of NPR-B yielded basal guanylyl cyclase activities that were markedly higher than the vector alone but were generally similar. We have included ATP in the basal cyclase reaction mixture, because it is highly likely that ATP is always present at saturating concentrations with respect to NPR-B activation in whole cells. However, we have also performed cyclase assays in the presence of only Mg\(^{2+}\)-GTP and found no significant differences between the mutant and wild type receptors under these conditions as well (data not shown). Guanylyl cyclase assays conducted in the presence of detergent yielded activities for wild type and mutant NPR-B receptors that were dramatically higher than those obtained with the vector alone (Fig. 6B). As with the basal determinations, the activities of the wild type and various mutant receptors were generally similar, suggesting that their expression levels were not significantly different. However, in both the basal and detergent stimulated guanylyl cyclase experiments there were some mutants that had slightly reduced activities compared with the wild type receptor. These differences were not consistently observed and are most likely due to differing transfection efficiencies and not the ability of the cell to express the mature protein once it has taken up the plasmid.

**The Addition of CNP Markedly Stimulated the Activity of the**

**FIG. 6.** Phosphorylation site mutations markedly reduce hormone-dependent but not basal or detergent-dependent guanylyl cyclase activities of NPR-B. Human 293 cells were transfected with

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

the indicated NPR-B 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 and ATP (panel A), Mn\(^{2+}\)-GTP and Triton X-100 (panel B), or CNP, ATP, and Mg\(^{2+}\)-GTP (panel C). The vertical bars centered above the columns represent the range of values obtained from two separate transfections, which were assayed in duplicate for panels A and B and the standard error of the mean (n = 4) for panel C. To control for the differing 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.
wild type receptor, and the activities of the mutant receptors were diminished to varying extents in comparison (Fig. 6C). To help control for the fluctuating transfection and expression levels of the individual mutant receptors, we normalized the data shown in Fig. 6C 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. 6D). This value more closely approximates the effect that each mutation has on the biological activity of NPR-B, because it helps to control for both the transfection efficiency as well as the relative expression level of each receptor. Serine or threonine to alanine substitutions at positions 513, 516, 518, 522, and 529 all reduced hormone-dependent activities of NPR-B to between 55 and 80% of wild type levels. The single mutations that had the greatest inhibitory effect on the hormone-dependent activity of NPR-B were S523A and S526A (Fig. 6, C and D). These mutations also had the most profound effect on the phosphorylation state of NPR-B (Fig. 4) and resulted in the loss of a specific phosphopeptide (Fig. 5). The double mutant containing substitutions at both Thr-516 and Ser-518 resulted in CNP-dependent activities that were similar to the S526A levels. The hormone-dependent activity of the receptor lacking all the known phosphorylation sites (7A) was the most dramatically affected, possessing only 6% of wild type activity. However, in contrast to NPR-A, the completely dephosphorylated version of NPR-B was still slightly responsive to hormone. The stimulation is very slight (2.6-fold) compared with the wild type receptor (49-fold), but it was consistently observed.

**Mutation of Glycine 521 to Alanine Decreases the Phosphate Content of NPR-B**—The substitution of the glycine residue at position 521 with alanine has been reported to dramatically reduce the CNP-dependent guanylyl cyclase activity of NPR-B by disrupting its putative ATP regulatory motif (28). To determine whether this mutation also results in receptor dephosphorylation, we expressed this mutant in 32P-labeled 293 cells and measured the amount of 32P associated with it (Fig. 7A, 32P content). Compared with the wild type receptor, the mutant receptor’s 32P content was reduced by 50%. As with the previous mutations, this decrease was not explained by diminished protein expression as indicated by immunoblot analysis (Fig. 7B, western). The decreased 32P content does not appear to be explained by the ablation of a specific site, because the phosphopeptide map of this mutant contained all the phosphopeptides normally observed in wild type maps (Fig. 7C, phosphopeptide map). Hence, it is appears that G521A and S522A are similar in that they both decrease the phosphorylation state of NPR-B without inhibiting the phosphorylation of a specific site. Finally, we note that in our hand this mutation only reduced the CNP-dependent guanylyl cyclase activity of NPR-B to 50% of wild type levels (Fig. 7D). This is a much less severe effect than that reported by Sharma and colleagues (28) who observed a greater than 85% reduction in hormone-dependent activity in receptors expressing the same mutation.

**DISCUSSION**

In this report, we describe the identification and characterization of five phosphorylated residues located in the putative ATP-binding portion of the KHD of NPR-B. The data for these conclusions are based on a number of experiments including: deletion mutagenesis (33), single site mutagenesis, and comparison studies involving synthetic phosphopeptides. The experiments involving the conjugation of synthetic phosphopeptides with tryptic phosphopeptides of NPR-B isolated from 32P-labeled cells provide strong evidence for the phosphorylation of Thr-516 and Ser-518. Furthermore, the high correlation of the mutagenesis data with the predicted phosphopeptide maps together with the corroborating phosphoamino acid analysis, make a very strong case for the correct identification all five sites.

Although most of the NPR-A phosphorylation sites were conserved in NPR-B, we were unable to find conclusive evidence for the phosphorylation of Thr-529. Mutation of the corresponding residue in NPR-A, Thr-513, was shown to decrease the phosphorylation state and change the tryptic phosphopeptide maps of this receptor, whereas mutation of Thr-529 to alanine in NPR-B had little or no effect. However, because
phosphoamino acid analysis of peptides that are predicted to contain this residue revealed the presence of low levels of phosphothreonine, we cannot rule out the possibility that Thr-529 may be a minor phosphorylation site. Furthermore, the replacement of this residue with alanine resulted in CNP-dependent guanylyl cyclase activities that were similar to those observed for the S513A, T516A, S518A, and S522A mutations. Whether the reduction associated with the T529A mutant is because of the loss of a specific phosphorylation site or is simply because of structural differences between the threonine and alanine residues is not known. It is also interesting that Ser-522 does not appear to be phosphorylated in NPR-B, because the corresponding residue in NPR-A (Ser-506) has been shown to be a major site. It is possible that the function of this residue is subserved by the adjacent residue, Ser-523, which lacks a consensus phosphorylation site. Whether the reduction associated with the T529A mutant is because of structural differences between the threonine and alanine residues is not known. It is also interesting that Ser-521 to alanine mutation in NPR-B, the effect of this replacement of this residue with alanine resulted in CNP-dephosphorylation (31). We have now extended our molecular understanding of this process by determining the exact location of the phosphorylated residues. Because the selective removal of these sites via site-directed mutagenesis mimics the effect of phosphatase treatment on NPR-B in crude membranes, it appears likely that dephosphorylation of only NPR-B is sufficient to inhibit its ability to respond to hormone. Now that these sites are known, the role of phosphorylation/dephosphorylation in the regulation of this receptor can be further addressed. It will be interesting to determine whether or not the removal of specific sites can abrogate the protein kinase C-mediated heterologous desensitization response. Likewise, the effect of phosphorylation/dephosphorylation on hormone binding and receptor trafficking and can now be effectively investigated using receptor mutants. Acknowledgments—We thank Jill Meisenhelder for peptide synthesis, Nigel Carter for HPLC purification of the synthetic phosphopeptides, and Anthony Craig for mass spectroscopy. We are also grateful to Dr. David L. Garbers for the generous donation of antisera Z658 and R1215.

REFERENCES

1. Brenner, B. M., Ballermann, B. J., Gunning, M. E., and Zeidel, M. L. (1990) Physiol. Rev. 70, 665–699
2. Baskoohi, H. (1992) Pharmacol. Rev. 44, 479–602
3. McDowell, G., Shaw, C., Buettner, K. D., and Nicholle, D. P. (1995) Eur. J. Clin. Invest. 25, 291–298
4. Sugai, S., Nakao, K., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., and Imura, H. (1992) J. Clin. Invest. 90, 1145–1149
5. Christian, T. D., Schulz, S., Potter, L. L., and Garbers, D. L. (1993) J. Biol. Chem. 268, 3698–3703
6. Sudoh, T., Minamino, N., Kangawa, K., and Matsuo, H. (1990) Biochem. Biophys. Res. Commun. 183, 863–870
7. Appel, R. G. (1992) Am. J. Physiol. 262, F911–F918
8. Furuya, M., Yoshida, M., Hayashi, O., Ohnuma, N., Minamino, N., Kangawa, K., and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. 177, 927–931
9. Hagiwara, H., Sakaguchi, H., Ikagaki, M., Yoshihito, T., Furuya, M., Tanaka, S., and Hirose, S. (1994) J Biol Chem 269(14), 10729–33
10. Furuya, M., Miyazaki, T., Honobu, N., Kawashima, K., Ohno, T., Tanaka, S., Kangawa, K., and Matsuo, H. (1996) Am. J. Physiol. 270(2 Pt 1), H2919–31
11. Ueno, H., Haruno, A., Morisaki, N., Furuya, M., Kangawa, K., Takeshita, A., and Saito, Y. (1997) Circulation 96, 2272–2279
12. Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H. M., Goeddel, D. V., and Schulz, S. (1989) Nature 338, 78–83
13. Lowe, D. G., Chang, M. S., Hellmies, R., Chen, S., Singh, S., Garbers, D. L., and Goeddel, D. V. (1989) Nature 341, 68–72
14. Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., and Garbers, D. L. (1989) Proc. Natl. Acad. Sci. USA 86, 1155–1162
15. Wilson, E. M., and Chinkers, M. (1995) Biochemistry 34, 4969–4971
16. Garbers, D. L., and Lowe, D. G. (1994) J. Biol. Chem. 269, 30741–30744
17. Garbers, D. L., Koseoglu, D., and Schultz, G. (1994) Mol. Biol. Cell. 5, 1–5
18. Kurose, H., Inagami, T., and Hirose, S. (1994) J. Clin. Invest. 94, 1522–1529
19. Chang, C. H., Kohse, K. P., Chang, B., Hirata, M., Jiang, B., Douglas, J. E., and Murad, F. (1980) Biochem. Biophys. Acta 645, 159–165
20. Chinkers, M., Singh, S., and Garbers, D. L. (1991) J. Biol. Chem. 266, 4088–4093
21. Duda, T., and Sharma, R. K. (1995) Mol. Cell. Chem. 152, 179–183
22. Chinkers, M., and Garbers, D. L. (1989) Science 245, 1392–1394
23. Koller, K. J., de Sauvage, F. J., Lowe, D. G., and Goeddel, D. V. (1992) Mol. Cell. Biol. 12(6), 2581–2590
24. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
25. Goraciakni, R. M., Duda, T., and Sharma, R. K. (1992) Biochem. J. 282, 523–537
26. Duda, T., Goraciakni, R. M., and Sharma, R. K. (1993) FEBS Lett. 315, 143–148
27. Potter, L. R., and Garbers, D. L. (1992) J. Biol. Chem. 267, 14531–14534
28. Potter, L. R., and Garbers, D. L. (1994) J. Biol. Chem. 269, 14636–14642
29. Potter, L. R. (1998) Biochemistry 37, 2422–2429
30. Koller, K. J., Lipari, M. T., and Goeddel, D. V. (1995) J. Biol. Chem. 269, 5997–6005
31. Potter, L. R., and Hunter, T. (1998) Mol. Cell. Biol. 18, 2164–2172
32. Lin, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schleif, S. (1993) Nature 363, 55–58
33. O’Malone, J. V., and Adams, T. E. (1994) DNA Cell Biol. 13, 1227–1232
34. Boyle, W. J., van der, Geer, P, and Hunter, T. (1991) Methods Enzymol. 209(110), 110–149
35. Domino, S., Tubb, D. J., and Garbers, D. L. (1991) Methods Enzymol 195(25), 345–355
36. Fenrich, R., Babinski, K., McNicoll, N., Therrien, M., Drouin, J., and De Lean, A. (1994) Mol. Cell. Biochem. 137, 173–192
37. Katafuchi, T., Takashima, A., Kashiwagi, M., Hayakawa, H., Takey, I., and Hirose, S. (1994) Eur. J. Biochem. 222, 835–842
38. Duda, T., and Sharma, R. K. (1995) Biochem. Biophys. Res. Commun. 209, 286–292
