A Sensitive Method for Determining the Phosphorylation Status of Natriuretic Peptide Receptors: cGK-Iα Does Not Regulate NPR-A†

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Received June 29, 2005; Revised Manuscript Received November 21, 2005

ABSTRACT: Natriuretic peptide receptor A (NPR-A) and natriuretic peptide receptor B (NPR-B) are transmembrane guanylyl cyclases that catalyze the synthesis of cGMP in response to natriuretic peptides. Phosphorylation and dephosphorylation regulate these receptors and have been traditionally studied by $^{32}PO_4$ labeling of transfected cells. However, this approach cannot be used to determine the phosphorylation state of receptors isolated from unlabeled sources. Here, we use Pro-Q Diamond and SYPRO Ruby dyes to quantify the phosphorylation status and protein levels, respectively, of natriuretic peptide receptors from tissues and cells. Strong Pro-Q Diamond signals for NPR-A and NPR-B were obtained when receptors were isolated from lung tissue, liver tissue and overexpressing cells. The level of NPR-A Pro-Q staining was also high in kidney but was much lower in heart tissue. In contrast, the SYPRO Ruby protein signal was weaker and more variable. In a direct comparison, Pro-Q Diamond staining was as sensitive as but more specific than the $^{32}PO_4$ labeling method. The two approaches were highly correlated ($R^2 = 0.98$). We exploited these techniques to measure the effect of cGMP-dependent protein kinase Iα on the phosphate content and guanylyl cyclase activity of NPR-A. Neither value was significantly affected in cells overexpressing cGK-Iα or in tissues from mice lacking cGK-I. We conclude that cGK-I does not regulate the cyclase activity or phosphorylation state of NPR-A. Furthermore, we find that Pro-Q Diamond staining is a sensitive method for measuring the phosphate levels of natriuretic peptide receptors, but protein levels are best detected by Western blot analysis, not SYPRO Ruby staining.

The mammalian natriuretic peptide family consists of three members: atrial natriuretic peptide (ANP),[1] B-type natriuretic peptide, and C-type natriuretic peptide (*1*). ANP and B-type natriuretic peptide are stored in the atria and ventricles of the heart, respectively, and are released into the bloodstream in response to increased cardiac wall stretch (*2*). Together, they decrease blood pressure by increasing the rates of renal sodium and water excretion, stimulating vascular vasorelaxation, and inhibiting aldosterone secretion. C-type natriuretic peptide stimulates vasorelaxation and inhibits cell proliferation (*3*), but it does not stimulate natriuresis (*4*). In chondrocytes, C-type natriuretic peptide stimulates endochondral ossification (*5*). This process is essential for normal long bone growth in humans because homozygous loss-of-function mutations in NPR-B result in a form of short-limbed dwarfism called acromesomelia dysplasia, type Maroteaux (*6*).

The signaling effects of natriuretic peptides are mediated through the activation of the particulate guanylyl cyclases, natriuretic peptide receptor-A (NPR-A), also known as guanylyl cyclase A, and natriuretic peptide receptor-B (NPR-B), also known as guanylyl cyclase B (*1*, *7*−*10*). NPR-A is activated by both ANP and brain natriuretic peptide, whereas NPR-B is activated by C-type natriuretic peptide. Both receptors consist of an extracellular ligand binding domain, a single transmembrane-spanning region, and intracellular kinase homology-regulatory, hinge-dimerization, and guanylyl cyclase-catalytic domains. The kinase homology-regulatory domains of NPR-A and NPR-B contain six and five known sites of serine and threonine phosphorylation, respectively (*11*, *12*). Previous studies have demonstrated that in the absence of natriuretic peptides, NPR-A and NPR-B are highly phosphorylated and that phosphorylation of these receptors is absolutely required for their activation (*11*, *12*). Cellular exposure to natriuretic peptides or antagonizing hormones results in a time-dependent decrease in phosphate

† National Institutes of Health Grant RO1HL66397 to L.R.P. provided financial support for these studies.

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[1] Abbreviations: NPR-A, natriuretic peptide receptor A; NPR-B, natriuretic peptide receptor B; ANP, atrial natriuretic peptide; cGK-I, cGMP-dependent protein kinase I.

10.1021/bi051253d CCC: $33.50
Published on Web 01/07/2006

content that is tightly correlated to the receptor's guanylyl cyclase activity (*13*).

Natriuretic peptide receptors activate three classes of cGMP binding proteins: cGMP-dependent protein kinases (cGKs), cGMP binding phosphodiesterases, and cyclic nucleotide-regulated ion channels (*1*, *7*). Gene deletion studies in mice suggest that cGK-I mediates the vasorelaxant effects of cGMP (*14*), whereas cGK-II mediates the bone growth effect of CNP (*15*, *16*). Recently, it has been suggested that cGK-I phosphorylates NPR-A in a gain-of-function feedback loop, which results in further activation of NPR-A (*17*). However, no evidence was presented to show that expression of cGK-I modulates the phosphorylation state of NPR-A. The role of phosphorylation in the regulation of natriuretic peptide receptors has been established using cell lines that overexpress NPR-A or NPR-B. Indeed, the observation that dephosphorylation leads to desensitization and the identification of sites of phosphorylation on NPR-A and NPR-B was achieved by metabolically labeling overexpressing 293HEK cells with [^{32}P]orthophosphate (*18*, *19*). Unfortunately, metabolic labeling is not a feasible method for determining the phosphate content of natriuretic peptide receptors from endogenous sources since tissues cannot be labeled. With the advent of the Pro-Q Diamond phosphoprotein dye technology, the initial observations made in overexpressing cell lines can be validated and extended in tissue extracted from animals or humans undergoing various physiological challenges. In this report, we compare the ability of Pro-Q Diamond stain to measure the phosphorylation state of NPR-A and NPR-B against the "gold standard", metabolic labeling. We find that the extent of Pro-Q Diamond staining is highly correlated with the ^{32}P content of these receptors. Furthermore, we find that Pro-Q Diamond staining is as sensitive as ^{32}P detection but more specific because of a lower level of background staining. Finally, we use Pro-Q staining of NPR-A to dispute the recently proposed "feed-forward" mechanism for cGK-I-mediated phosphorylation and regulation of NPR-A.

EXPERIMENTAL PROCEDURES

Materials. SYPRO Ruby and Pro-Q Diamond stains were from Molecular Probes. Anti-VASP antibody was from Calbiochem.

Statistical Analysis. GraphPad Prism was used for statistical analysis of the data in this report. Significance was determined by performing unpaired *t*-tests ($p \leq 0.05$) on mean values.

Cell Culture and Transfections. 293T cells were transiently transfected, or stably transfected 293T NPR-A cells were used as previously described (*20*).

Crude Membrane Incubation. Crude membranes were prepared by washing 10 cm plates two times with PBS and scraping cells in HEPES/glycerol/protease inhibitor buffer (HGPB). Cells were sonicated and centrifuged at 20000*g* for 20 min at 4 °C. The supernatant was removed, and the cells were resuspended in HGPB. The crude membranes were then incubated on ice or at 37 °C with or without $MgCl_2$ for 30 min. To immunoprecipitate NPR-A, 1 mL of modified RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM EDTA, 1× protease inhibitor cocktail (Roche), and 0.5 μM microcystin] was added to the tubes after the incubation. Fifty microliters of a 50% slurry of protein A−agarose beads was added to the lysate and the mixture rotated for 20 min at 4 °C. The cleared lysate was centrifuged at 20000*g* for 20 min at 2 °C. Eight hundred microliters of the extract was incubated with 1 μL of polyclonal antiserum from rabbit 6326 (*21*) with constant end-over-end tumbling overnight at 4 °C. Fifty microliters of a 50% slurry of protein A−agarose beads was added to the extract and incubated for an additional 1 h as described above. The protein A immunocomplex was pelleted by low-speed centrifugation and washed two times with RIPA. The immunocomplex was released from the agarose beads by boiling for 3 min in the presence of 2× reducing sample buffer and fractionated by SDS−PAGE on an 8% resolving gel.

Preparation of Crude Membranes from Animal Tissues. Rat and mouse tissues were homogenized using a Polytron homogenizer in phosphatase inhibitor buffer (PIB) [25 mM HEPES (pH 7.4), 50 mM NaCl, 20% glycerol, 50 mM NaF, 2 mM EDTA, 1 μM microcystin, and 1× Complete EDTA free protease inhibitor cocktail from Roche Diagnostics]. The suspensions were pelleted by centrifugation at 20000*g* for 20 min at 4 °C. Pellets were resuspended in PIB and centrifuged two more times. Membranes were used immediately or frozen at −80 °C until they were used.

Immunoprecipitation of NPR-A and NPR-B from Rat Tissues. NPR-A or NPR-B was immunoprecipitated from 100 mg of rat or mouse tissue. Pellets were solubilized in 0.8 mL of modified RIPA buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaH_2PO_4, 50 mM NaF, 1 mM EDTA, 0.1 μM microcystin, and 1× Complete EDTA free protease inhibitor (Roche)]. After the mixture had been rotated for 30 min with 30 μL of a 50% protein A slurry and 3 μL of rabbit preimmune serum at 4 °C, the extract was cleared by centrifugation at 20000*g* for 15 min at 2 °C. The cleared extract (0.7 mL) from each treatment was incubated with 3 μL of polyclonal rabbit antiserum against NPR-A (antiserum 6325) or NPR-B (antiserum 6328) overnight at 4 °C. For negative controls, we used antiserum preincubated with 0.01 mg of synthetic peptides corresponding to the last 17 or 10 carboxyl-terminal amino acids of NPR-A (antiserum 6325) or NPR-B (antiserum 6328), respectively. In some samples, the incubation volume was increased to 10 or 11 mL total. Fifty microliters of a 50% protein A slurry was added to the extract and the mixture incubated for an additional 1 h. The protein A immunocomplex was pelleted by low-speed centrifugation and washed two times with 1 mL of RIPA-modified buffer and one time with RIPA-modified buffer without NaCl. NPR-A or NPR−B was released from the immunocomplex by boiling for 3 min in the presence of 30 μL of 2× reducing SDS sample buffer and fractionated by SDS−PAGE.

VASP Western Blots. Cellular extracts from transfected 293T cells were fractionated by SDS−PAGE, transferred to PVDF, and blocked for 20 min with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 3% bovine serum albumin. The membrane was then probed with anti-VASP antibody at a dilution of 1:1500 for 1.5 h in TBST. After four 5 min washes with TBST, the membrane was incubated with anti-rabbit HRP secondary antibody for 45 min at a dilution of 1:10000 and then was washed four times for 5

min with TBST. The blot was treated with an ECL solution and exposed to autoradiography film.

Guanylyl Cyclase Assays. Cyclase assays were conducted at 37 °C in the presence of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 50 mM NaCl, 0.5 mM isobutylmethylxanthine, 0.1% bovine serum albumin, 5 mM creatine phosphate, 0.1 μg/μL creatine phosphokinase, 1 mM GTP, 1 mM ATP, 1 μM ANP, and ~10 μCi of [^{32}P]-α-GTP. The reactions were stopped with 0.5 mL of 110 mM ZnOAc, and the amount of cGMP formed was purified and quantified as previously described (*21*).

Immunoblot Analysis. NPR-B was detected by Western blot analysis using antiserum from rabbit 6328 as previously described (*21*).

Gel Staining. Eight percent resolving gels were fixed in a 30 mL solution of 50% methanol and 10% acetic acid for 30 min with gentle rocking. The solution was changed two times for a total of three washes in the fixing solution. The gels were then washed twice in 100 mL of water for 10 min. Ten milliliters of Pro-Q Diamond phosphoprotein gel stain was added, and the gels were incubated with gentle rocking for 1.5 h in the dark. The gels were then destained with 80 mL of a solution of 20% acetonitrile and 50 mM sodium acetate (pH 4.0) for 15 min. This wash was repeated two times for a total of three washes. The gels were then rinsed in water, scanned with a 532 nm laser, and imaged with FUJI FLA 5000 software. After imaging, 10 mL of SYPRO Ruby protein stain was added, and the gels were incubated overnight with gentle rocking protected from light. After incubation, the gel was washed once with 100 mL of water, then scanned with a 473 nm laser, and imaged again.

Metabolic Labeling. 293T cells were transfected with 20 μg of pCMV3, pCMV3-NPR-A, or pcDNA3-cGK-Iα. Twenty-four hours post-transfection, the cells were washed twice with phosphate-free medium; then 5 mL of 0.5 mCi/mL [^{32}P]orthophosphate was added to the plates, and they were incubated at 37 °C overnight. The following day the cells were incubated with 0.5 μM ANP for 0, 30, or 90 min. The plates were washed twice with cold PBS, then scraped in 1 mL of RIPA buffer, and transferred to microcentifuge tubes. Forty microliters of protein A beads and 3 μL of preimmune serum were added to the tubes, and the contents were rotated for 20 min at 4 °C. The lysates were centrifuged at 36000*g* for 15 min, and 800 μL of the supernatant was incubated with 6325 rabbit serum and 50 μL of protein A beads overnight at 4 °C with rotation. The immunocomplex was washed twice with 1 mL of RIPA buffer. Fifty microliters of 2× reducing sample buffer was added, and the samples were boiled for 5 min. Twenty-five microliters of the sample was loaded onto gels.

Production of cGK-I Deficient Mice. Homozygous cGK-I deficient mice were produced as described in ref *31*. Mice were sacrificed at 3 weeks of age, and the kidneys were excised and snap-frozen in liquid nitrogen. The samples were stored at −80 °C until they were assayed for guanylyl cyclase activity and NPR-A phosphorylation.

RESULTS

Pro-Q Diamond Stain Is an Accurate and Sensitive Method for Assessing the Phosphorylation State of Natriuretic Peptide Receptors in Transfected Cells. Previous studies have examined the effect of individual phosphorylation sites on the phosphorylation state and guanylyl cyclase activity of NPR-A and NPR-B (*11*, *12*). Alanine was used to mimic a dephosphorylated serine or threonine, and glutamate was used to mimic the negative charge of a phosphorylated residue. A form of NPR-A-containing alanines substituted for the first five phosphorylation sites (NPR-A-5A) is dephosphorylated and unresponsive to natriuretic peptide stimulation, whereas a mutant containing glutamates substituted for all six known phosphorylation sites (NPR-A-6E) is hormonally responsive but resistant to desensitization (*22*).

To determine if the Pro-Q Diamond stain can distinguish between these receptor variants, 293T cells were transfected with the empty vector, wild-type NPR-A, NPR-A-6E, or NPR-A-5A. Membrane fractions were prepared from these cells and incubated on ice or at 37 °C for 30 min with or without 5 or 10 mM $MgCl_2$, conditions known to decrease both the phosphorylation state and cyclase activity of NPR-A (*23*). Following the incubation, NPR-A was immunoprecipitated and fractionated by SDS−PAGE. The gel was then fixed, stained with Pro-Q Diamond dye, and imaged (Figure 1A, top panel). Immediately afterward, the same gel was rinsed and incubated with SYPRO Ruby stain to detect NPR-A protein levels (Figure 1A, bottom panel). A strong Pro-Q Diamond signal was observed when NPR-A was immunopurified out of ice-cold membranes from cells transfected with wild-type NPR-A. The intensity of this signal was moderately reduced when the membranes were incubated at 37 °C and dramatically reduced when the membranes were incubated at 37 °C in the presence of magnesium. The decline in the Pro-Q Diamond signal was not due to NPR-A degradation because the SYPRO Ruby signal was unaffected by the treatments (Figure 1A, bottom panel). Importantly, no Pro-Q Diamond or SYPRO Ruby signals were observed in cells transfected with vector alone. These results are strikingly similar to previous published data recorded for membranes obtained from ^{32}P-labeled cells (*23*). Importantly, no significant Pro-Q Diamond staining was observed for NPR-A-5A, which also is consistent with data from metabolic labeling experiments. In addition, no staining was observed for NPR-A-6E, which indicates that the dye is not simply recognizing negatively charged residues.

We also found that wild-type NPR-B stained nicely with Pro-Q Diamond, whereas a version lacking the known phosphorylation sites (NPR-B-7A) failed to be significantly stained with Pro-Q Diamond (Figure 1B, top panel). Again, the protein levels were similar in the two mutants as evidenced by SYPRO Ruby staining (Figure 1B, middle panel), which correlated nicely with expression levels detected by Western blot analysis (Figure 1B, bottom panel).

In a separate series of experiments, we compared the Pro-Q Diamond staining method versus the ^{32}P metabolic labeling method of measuring the phosphorylation state of NPR-B (Figure 1C). HEK293T cells stably expressing NPR-B were metabolically labeled with [^{32}P]orthophosphate overnight as previously described (*11*). Membranes were prepared from these cells and incubated at 37 °C for 0, 2, 5, 10, 15, or 30 min to stimulate time-dependent NPR-B dephosphorylation. The receptor was then purified by immunoprecipitation and SDS−PAGE. The resulting gel was stained with Pro-Q Diamond dye and imaged (Figure 1C, top panel). The same wet gel was rinsed and exposed to a phosphorimager to

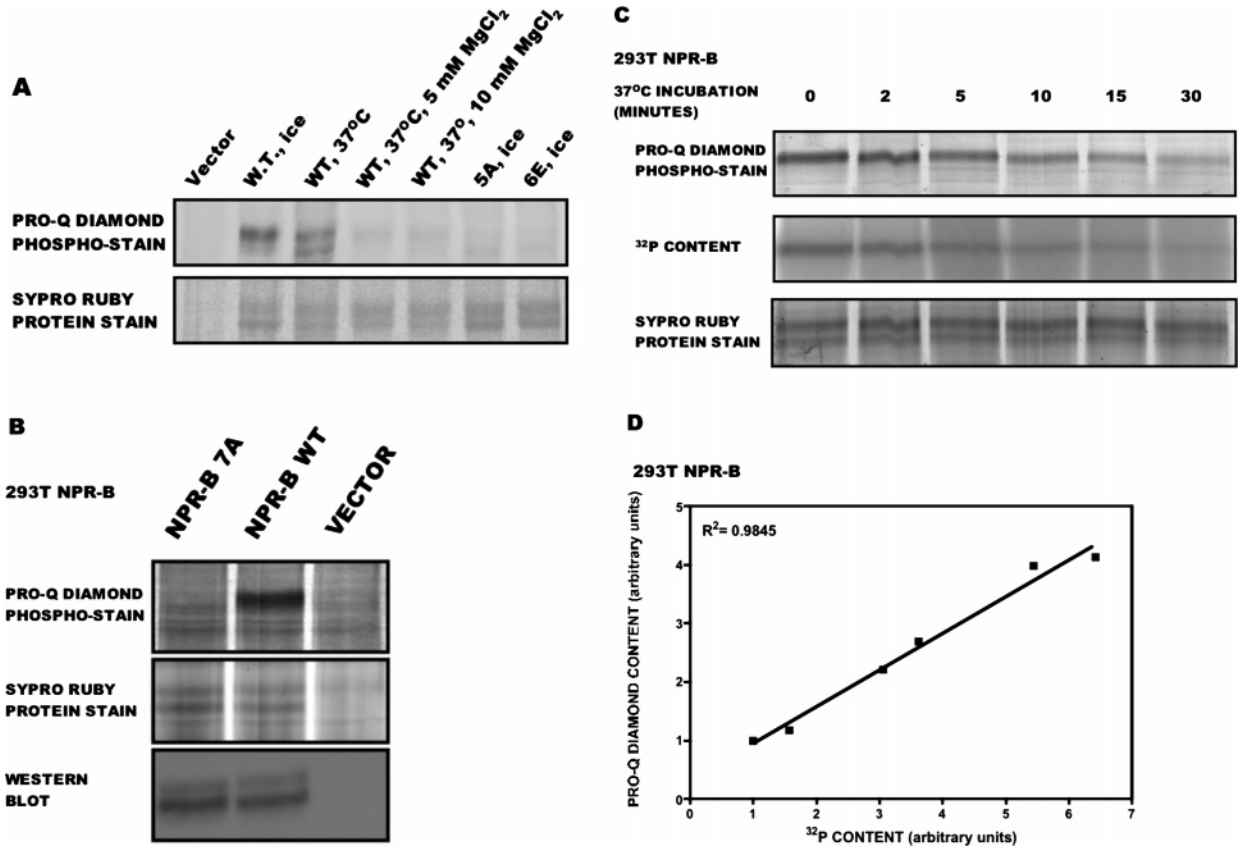

FIGURE 1: Pro-Q Diamond stain is an accurate and sensitive method for measuring the phosphorylation state of NPR-A and NPR-B. (A) 293T cells were transiently transfected with empty vector, wild-type NPR-A, NPR-A-5A, or NPR-A-6E. Cells were sonicated, and the membrane fraction was incubated on ice or at 37 °C for 30 min with or without $MgCl_2$. NPR-A was then immunoprecipitated, and the immunocomplex was fractionated by SDS−PAGE. The resulting gel was stained with Pro-Q Diamond dye. After being imaged, the gel was stained with SYPRO Ruby protein binding dye. (B) 293T cells were transfected with wild-type NPR-B, NPR-B-7A, or empty vector. NPR-B was immunoprecipitated and fractionated by SDS−PAGE. The phosphate content was determined using Pro-Q Diamond phosphoprotein gel stain, and the total protein was determined using SYPRO Ruby protein gel stain as well as by Western blot analysis. (C) Pro-Q Diamond staining was compared to ^{32}P labeling in HEK293T cells stably expressing NPR-B. Membranes were prepared from these cells and incubated at 37 °C for the indicated periods of time. NPR-B was purified by immunoprecipitation and SDS−PAGE. The gel was stained with Pro-Q Diamond phosphoprotein gel stain and imaged. The same gel was then exposed to film to determine the amount of ^{32}P incorporated into NPR-B. The gel was then incubated with SYPRO Ruby protein stain and imaged. (D) The relationship between the Pro-Q Diamond dye intensity and the ^{32}P content of NPR-B was estimated by determining the correlation coefficient ($R^2 = 0.98$). These results are representative of at least two experiments.

measure the ^{32}P content of NPR-B (Figure 1C, middle panel). Then the gel was rinsed and stained with SYPRO Ruby to visualize NPR-B protein levels (Figure 1C, bottom panel).

Both phosphate determination methods clearly indicated that the phosphorylation state of NPR-B was decreased as a function of time and that the decreases were not due to protein degradation. To quantify the relationship between the two methods, we plotted the relative signal intensity as a function of time as determined by the Pro-Q Diamond staining versus the relative signal intensity of the ^{32}P signal as a function of time. We found that the two methods were highly correlated, having a correlation coefficient (R^2) value of 0.98. Visual inspection of the top two panels of Figure 1C suggests that the sensitivity of the two techniques is comparable. On the other hand, we found that the "signal to noise" ratio was better with the Pro-Q Diamond staining. The higher background in the metabolic labeling experiment may be due to the fact that [^{32}P]orthophosphate is incorporated into many biological molecules in addition to proteins, such as nucleic acids and lipids, whereas Pro-Q Diamond binds only phosphoproteins (*24*).

Pro-Q Diamond Staining of NPR-A and NPR-B in Rat and Mouse Tissues. The ability of Pro-Q Diamond to detect the phosphorylation state of natriuretic peptide receptors from endogenous (untransfected) sources also was tested. Initially, we isolated NPR-A from rat lung, kidney, and heart tissues (Figure 2A) by immunoprecipitation and SDS−PAGE in the presence or absence of excess peptide antigen to specifically block receptor binding. Clear Pro-Q Diamond staining of NPR-A was apparent in samples lacking the blocking peptide and was not present when the blocking peptide was included in the binding reactions. In all tissues, diluting the extract from 0.7 to 10 mL reduced the extent of background staining (Figure 2A). In contrast, SYPRO Ruby staining of NPR-A was less distinct. In the lung, a nonspecific band migrated in a manner similar to that of NPR-A. In the kidney, many nonspecific bands were present, but only one band was blocked when the peptide antigen was included in the reaction, suggesting that the blocked band was NPR-A. In the heart, however, a specific SYPRO Ruby staining band was not apparent.

We also purified NPR-B from rat lung by immunoprecipitation and SDS−PAGE (Figure 2B). From this tissue, we saw very distinctive Pro-Q Diamond staining, and unlike the case with NPR-A, we observed clear SYPRO Ruby staining as well. The reason for the distinctive SYPRO Ruby

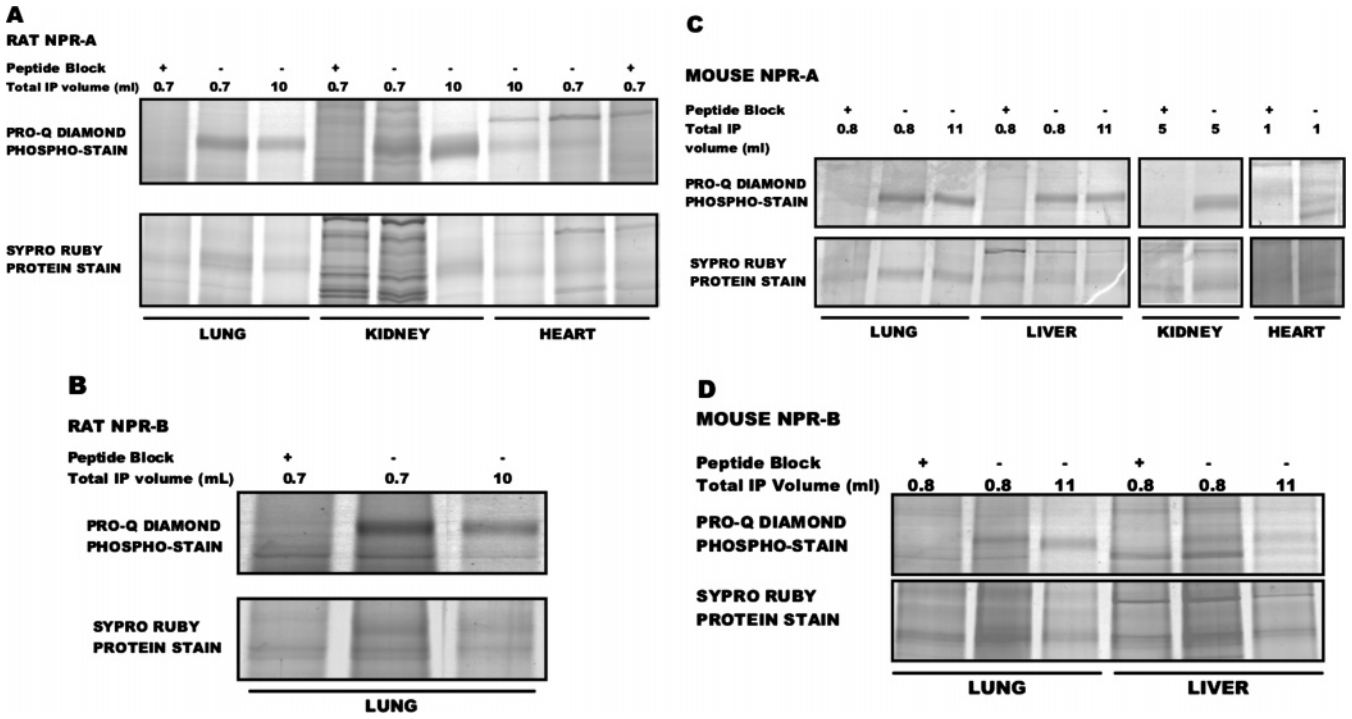

FIGURE 2: Pro-Q Diamond dye effectively measures the phosphorylation status of natriuretic peptide receptors in various tissues. (A) NPR-A was isolated from rat lung, kidney, and heart tissues by immunoprecipitation and SDS−PAGE. To decrease the level of background staining, some of the immunoprecipitations were performed in larger volumes. Addition of blocking peptide to reaction mixtures served as negative controls. Immunocomplexes were fractionated by SDS−PAGE, and the resulting gel was stained with Pro-Q Diamond dye and imaged. The same gel was then stained with SYPRO Ruby protein gel dye and imaged to estimate receptor levels. (B) The phosphorylation state and protein levels of NPR-B from rat lung were determined as described above. (C) The phosphorylation state and protein levels of NPR-A from mouse lung, kidney, liver, and heart were determined. (D) The phosphorylation state and protein levels of NPR-B from mouse lung and liver were determined as described above. These results are representative of at least two experiments.

staining for NPR-B, but not for NPR-A, is not known. In mouse lung, liver, and kidney tissues, we observed distinct Pro-Q Diamond staining for NPR-A, but again the SYPRO Ruby staining was difficult to interpret because of the presence of a nonspecific protein that migrated only slightly faster than NPR-A (Figure 2C). In mouse heart, a diffuse Pro-Q Diamond band that was blocked by the antigenic peptide was visible. However, no specific SYPRO staining was apparent.

Specific phosphostaining was observed when NPR-B was purified from mouse lung and liver as well (Figure 2D). Diluting the extract from 0.8 to 11 mL significantly reduced the extent of background staining. However, we were unable to detect specific SYPRO Ruby staining of NPR-B in these samples.

Expression of Active or Inactive cGK-Iα Does Not Affect the Guanylyl Cyclase Activity of NPR-A. Because cGK-Iα has been reported to phosphorylate and activate NPR-A in a feed-forward manner, we tested whether the coexpression of an active or kinase-dead version of cGK-Iα containing a lysine to alanine substitution at position 390 (K390A) would affect the catalytic activity of NPR-A. To confirm that cGK-I was activated by ANP in our 293T cell system, we used vasodilator-stimulated phosphoprotein (VASP) as a positive control for cGK-I activation (Figure 3). VASP is a well-characterized in vivo substrate for cGK-I and has been described as a useful indicator of cGK-dependent phosphorylation (*25*). 293T cells were transfected with plasmids encoding NPR-A and/or cGK-Iα and stimulated with 0.5 μM ANP for 1 h. The cells were then lysed in reducing sample

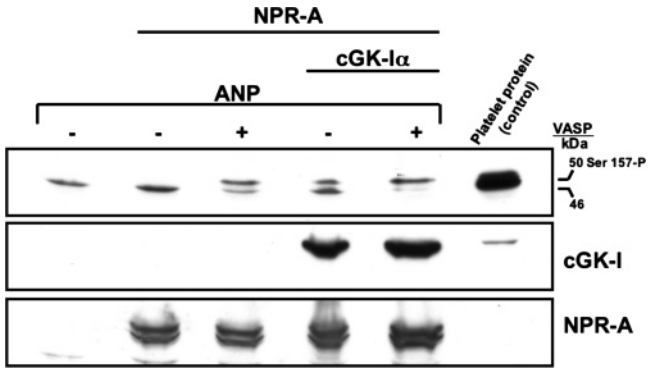

FIGURE 3: Phosphorylation of VASP confirms activation of cGK-I in 293T cells. 293T cells were transfected with GFP or NPR-A with or without cGK-Iα. Forty-eight hours post-transfection, cells were stimulated with or without 0.5 μM ANP for 1 h, and cells were harvested in reducing sample buffer and subjected to SDS−PAGE. The proteins were transferred to a PVDF membrane and sequentially probed with anti-VASP, cGK-I, and NPR-A antibodies. The data shown are representative of four experiments.

buffer, fractionated by SDS−PAGE, and transferred to a PVDF membrane. The membrane was probed with an antibody against total VASP. ANP stimulation of cells overexpressing NPR-A activated endogenous cGK-I because the amount of the upper phosphorylated form of VASP increased while that of the lower dephosphorylated version of VASP was decreased. Coexpression of cGK-Iα with NPR-A resulted in partial activation of VASP in the absence of ANP, perhaps due to increased basal levels of cGMP due to NPR-A overexpression. ANP stimulation of cells coexpressing NPR-A and cGK-Iα resulted in the greatest cGK-I

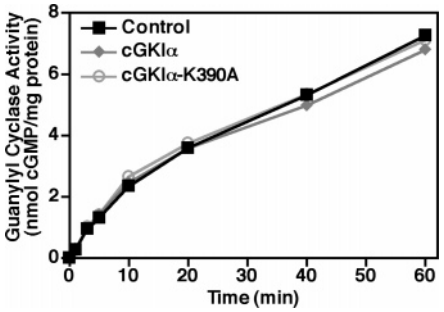

FIGURE 4: NPR-A activity is not regulated by cGK-I. 293T NPR-A cells were transiently transfected with empty vector (control), wild-type cGK-Iα, or an inactive version of cGK-Iα (cGK-Iα K390A). Crude membranes were then prepared from the transfected cells and assayed for ANP/ATP-dependent guanylyl cyclase activity for the indicated periods of time. The data shown are representative of at least four separate experiments.

activation because almost all of VASP was shifted to the higher-molecular weight form.

Having confirmed that cGK-I is activated in our cell line, we tested if overexpression of activated cGK-I affected the ANP-dependent guanylyl cyclase activity of NPR-A (Figure 4). We found that neither the overexpression of the wild type nor inactive versions of cGK-I had any effect on the ANP-dependent guanylyl cyclase activity of NPR-A at any time point measured. These data are not consistent with a previous report in a similar 293 model system where cGK-Iα was found to increase the guanylyl cyclase activity of NPR-A (*17*).

Expression of cGK-Iα Does Not Affect the Phosphorylation State of NPR-A. It has been suggested, but not shown, that cGK-Iα phosphorylates the kinase homology domain of NPR-A (*17*). To directly test this hypothesis, we employed both metabolic labeling and Pro-Q Diamond techniques simultaneously to assess the phosphorylation state of NPR-A in the presence of endogenous or overexpressed cGK-Iα. 293T cells were cotransfected with NPR-A and either empty vector or cGK-Iα. Twenty-four hours later, the cells were metabolically labeled with [^{32}P]orthophosphate overnight and then incubated with ANP or vehicle for 0, 30, or 90 min. The phosphorylation state of NPR-A was assessed by immunoprecipitating the receptor, fractionating the immunocomplex by SDS−PAGE, and staining the gel with Pro-Q Diamond (Figure 5, top panel). The wet gel was then rinsed and exposed to an imaging plate to capture the ^{32}P signal from NPR-A (Figure 5, middle panel). To make sure that any changes in phosphoprotein staining were not due to different amounts of purified receptor, the same gel was then stained with SYPRO Ruby protein stain (Figure 5, bottom panel). In previous reports, exposure to ANP was shown to cause a decrease in the phosphate content of NPR-A (*18*). In cells overexpressing NPR-A only (Figure 5, top panel, lanes 3−5), a decrease in the level of NPR-A phosphorylation is apparent after ANP stimulation for 90 min. It is important to note that although there appears to be more phosphate associated with the receptor after ANP stimulation for 30 min (see lane 4), the SYPRO Ruby signal indicates that this is due to a higher NPR-A protein level. If cGK-Iα is indeed the kinase responsible for phosphorylating NPR-A, the increase in cGMP concentrations brought about by hormonal stimulation of NPR-A should cause an increase in the amount of phosphate associated with the receptor. To test this hypothesis, NPR-A was coexpressed with cGK-Iα and stimulated for 0, 30, and 90 min with ANP (see lanes 6−8). We found that overexpression of cGK-Iα did not increase the phosphorylation state of NPR-A with ANP stimulation. In other words, there was no significant difference in the phosphorylation of NPR-A with or without overexpression of cGK-Iα.

Deletion of cGK-Iα Does Not Affect the Activity or Phosphorylation of NPR-A. Overexpression of cGK-Iα did not demonstrate any effect on NPR-A activity or phosphorylation, suggesting cGK-Iα does not regulate NPR-A. If this is true, then the absence of cGK-Iα should not affect NPR-A phosphorylation or activity either. To test this hypothesis, we utilized cGK-Iα knockout mice. Membranes were prepared from the kidneys of six wild-type and six cGK-Iα knockout mice, and a guanylyl cyclase assay was performed (Figure 6, top panel). With the exception of the 10 min time point, there was no significant difference between the activity

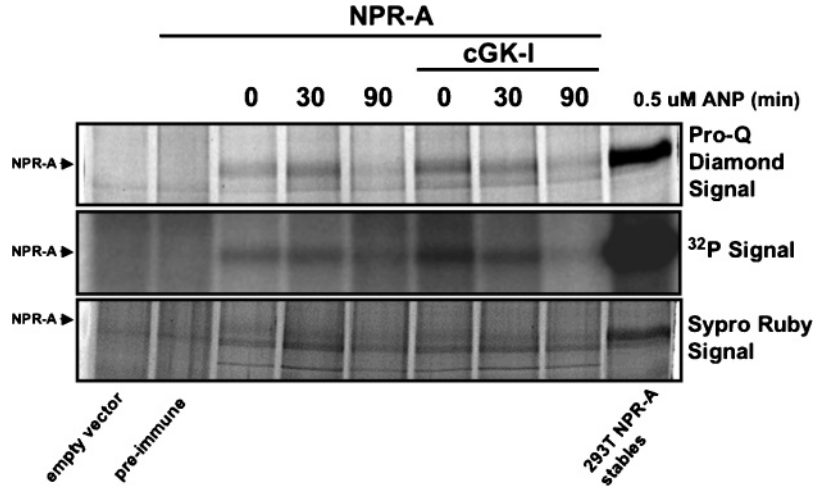

FIGURE 5: Expression of cGK-Iα does not affect NPR-A phosphorylation. 293T cells were transiently transfected with NPR-A and empty vector or cGK-Iα. Cells were metabolically labeled with [^{32}P]orthophosphate overnight and then incubated with or without 0.5 μM ANP for 0, 30, or 90 min. NPR-A was then purified by immunoprecipitation and SDS−PAGE. The gel was stained with Pro-Q Diamond dye and imaged (top panel). The same wet gel was rinsed and exposed to an imaging plate to capture the ^{32}P signal (middle panel). The same gel was then stained with SYPRO Ruby protein binding dye and imaged to measure receptor protein levels (bottom panel). These results are representative of three experiments.

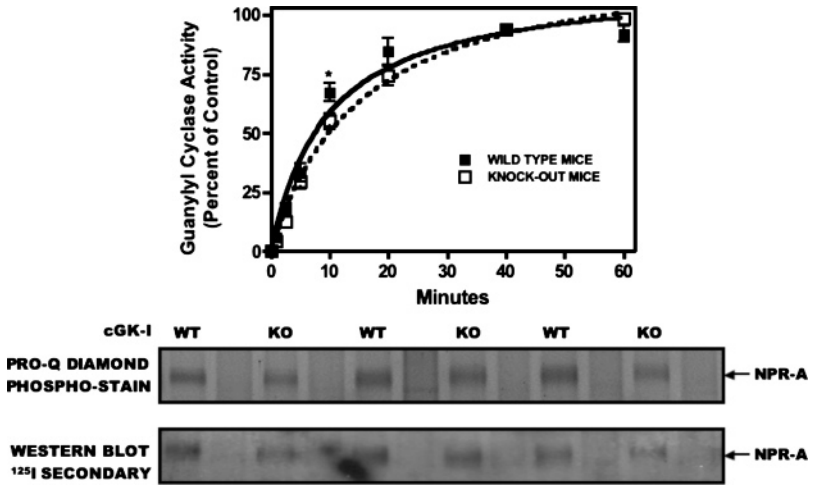

FIGURE 6: Deletion of cGK-Iα does not affect NPR-A activity or phosphorylation. Membranes were prepared from the kidneys of wild-type and cGK-Iα knockout mice, and guanylyl cyclase activity was measured in the presence of 1 μM ANP and 1 mM ATP for the indicated times (top panel). NPR-A was immunoprecipitated from the membranes of three wild-type and three knockout mouse kidneys and subjected to SDS−PAGE and Pro-Q Diamond staining (middle panel). The same samples were fractionated on another SDS−PAGE gel and blotted onto a PVDF membrane, and a western blot for NPR-A was performed using ^{125}I-labeled anti-rabbit secondary. The blot was exposed to film (bottom panel); the corresponding areas on the PVDF membrane were excised, and the amount of ^{125}I was measured in a gamma counter to determine protein levels. The guanylyl cyclase assay experiment is representative of two experiments with an *n* of 12. Student's *t*-tests were performed on both the cyclase and phosphorylation data where $p \leq 0.05$.

of NPR-A from the wild-type or cGK-I knockout kidneys ($p < 0.05$). To observe any possible changes in the phosphorylation state of NPR-A, the receptor was purified from the membrane preparations and subjected to SDS−PAGE, and the gel was stained for phosphoprotein content (Figure 6, middle panel). The same samples were also run on another SDS−PAGE gel and were transferred to a PVDF membrane. A Western blot for NPR-A was conducted using ^{125}I-labeled anti-rabbit secondary antibody to quantify protein levels (Figure 6, bottom panel). The amount of phosphate in each sample was quantified, and the ratio of phosphorylation to protein was calculated. An unpaired *t*-test ($p \leq 0.05$) was performed on these ratio values. The statistical analysis showed that there were no significant differences in the phosphorylation state of NPR-A isolated from tissues from wild-type or cGK-I knockout mice. On the basis of the data presented in Figures 3−6, we find no evidence to support a role for cGK-Iα in the direct regulation of NPR-A.

DISCUSSION

In this report, we have demonstrated that Pro-Q Diamond phosphoprotein stain is an effective tool for measuring the phosphorylation state of natriuretic peptide receptors. This technology can be used to measure the phosphorylation state of receptors isolated from cell lines or tissues. This is important because, until now, the ability to measure natriuretic peptide receptor phosphorylation from tissues was not possible since phosphorylation state specific antibodies are not available for NPR-A or NPR-B. Although we have previously used this technique to measure the phosphorylation status of NPR-B from NIH3T3 cells exposed to hyperosmotic medium (*26*), this is the first report to compare the metabolic labeling method versus the Pro-Q method of phosphate detection and to measure the phosphate content of NPR-A and NPR-B isolated from tissues. We anticipate that Pro-Q Diamond staining will facilitate research on the role of receptor dephosphorylation in the desensitization of NPR-A and NPR-B observed in patients and/or animal models with congestive heart failure, diabetes, and cirrhosis of the liver. Sequential SYPRO Ruby dye staining of the same gel facilitated quantification of the receptor protein levels. However, although this approach worked nicely in transfected cells, it was only marginally effective when the receptors were isolated from tissues. In the latter scenario, quantification of protein levels is probably best accomplished by western blot analysis.

One difference between the metabolic labeling and the Pro-Q Diamond technique is that the latter stains all of the phosphorylated natriuretic peptide receptors in the cell, whereas the former only assesses the phosphorylation of receptors that were synthesized during the labeling period. This may inhibit the ability of Pro-Q Diamond to detect small decreases in natriuretic peptide receptor phosphorylation if cells contain large amounts of intracellular and/or incompletely processed forms of the receptors, which occurs in highly overexpressing cells. Interestingly, in some cases (Figure 1A) we observed a doublet for NPR-A when stained with Pro-Q Diamond. This differs from the single band that is normally observed when NPR-A is isolated from ^{32}P-labeled cells (*18*, *27*, *28*). The reason for this discrepancy is unknown.

Surprisingly few proteins have been shown to associate with NPR-A. Protein phosphatase 5 was found to interact with the kinase homology domain of NPR-A in a yeast two-hybrid screen in 1994 (*29*), but subsequent studies demonstrating its ability to dephosphorylate or regulate NPR-A in whole cells have not been reported. Recently, heat shock protein 90 and its cochaperone p50^{cdc37} were found in an immunocomplex with NPR-A, consistent with a role for these proteins in facilitating the folding of the nascent receptor (*30*). Airhart and colleagues reported results from a yeast two-hybrid screen where cGK-Iα was used as bait to identify a single positive clone containing the cyclase and hinge portion of NPR-A (*17*). They found that NPR-A expression increases the amount of cGK-Iα in both crude membranes from broken cell preparations and plasma membranes of

intact cells using fluorescence-coupled antibody methods. Furthermore, they found that incubation of cells with ANP increased the amount of cGK-Iα in the membrane fraction. They also found that addition of recombinant cGK-Iα increased the guanylyl cyclase activity of NPR-A via a feed-forward activation mechanism. We saw absolutely no effect of coexpression of active or inactive versions of cGK-Iα on NPR-A activity, although we both used similar 293 cell expression systems. The reason for this discrepancy may be related to the poor ANP-dependent activation of NPR-A (1.5-fold) reported in their assay compared to the more normal >40-fold activation observed in our assay (Figure 4). Finally, it should be pointed out that in contrast to Airhart and colleagues, we actually measured the level of cGK-I activation as well as the phosphorylation state of NPR-A in the presence or absence of overexpressed cGK-Iα. As in the cyclase data, we found no evidence for cGK-Iα-dependent modulation of NPR-A phosphorylation. Finally, by measuring NPR-A activity from the kidneys of cGK-Iα knockout mice, we show that when cGK-Iα is absent, NPR-A activity and phosphorylation are not affected. Hence, our data do not support a model in which cGK-Iα phosphorylates or regulates NPR-A.

One important question that is difficult to resolve is whether NPR-A and cGK-Iα are physically associated in cells. Our initial studies employing a sequential immunoprecipitation−Western blot strategy suggested that this might be the case. However, many subsequent experiments employing extracts from overexpressing cell lines as well as various rat tissues failed to reproduce these results. Airhart and co-workers were unable to coprecipitate NPR-A and cGK-Iα as well. Of course, it is possible that the detergent required to solubilize NPR-A from the membrane fraction disrupts the interaction. Nonetheless, to the best of our knowledge, no published data support the idea of NPR-A and cGK-Iα being stably associated in whole cells. On the basis of the data presented in Figures 3−6, we find no evidence to support a role for cGK-Iα in the direct regulation of NPR-A. Instead, we suggest that if cGK-Iα associates with NPR-A, its main purpose would be to shape the sensitivity and specificity of the natriuretic peptide response, not to directly regulate the activity of the receptor.

ACKNOWLEDGMENT

We thank Alyssa Cody for excellent technical assistance.

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BI051253D