ARTICLE
Journal of Cellular Biochemistry 110:985–994 (2010)

Journal of Cellular Biochemistry

Molecular Approaches to Examine the Phosphorylation State of the C Type Natriuretic Peptide Receptor

Abdel A. Alli[1,2,3] and William R. Gower Jr.[1,2,3*]

[1]*Research Service, James A. Haley Veterans Hospital, Tampa, Florida*

[2]*Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, Florida*

[3]*Cardiac Hormone Center, University of South Florida, Tampa, Florida*

ABSTRACT

The intracellular domain of the C type natriuretic peptide receptor (NPRC) contains one threonine and several serine residues where phosphorylation is thought to occur. Several phosphorylation consensus sequences for various kinases have been identified within the intracellular domain of NPRC, but the exact residues that are phosphorylated and the specific kinases responsible for their phosphorylation have not been thoroughly defined. Here we introduce a recombinant GST fusion protein and a rat gastric mucosa (RGM1) cell line as molecular tools to study the phosphorylation state of NPRC in vitro and in vivo, respectively. We utilize a previously characterized polyclonal antibody against NPRC to probe for total NPRC protein and various phosphospecific and substrate motif antibodies to probe for phosphorylation of NPRC. Phosphoprotein staining reagents were used with a phosphoprotein control set to detect phosphorylation of NPRC at serine and threonine residues. Recombinant GST-NPRC fusion protein was phosphorylated in vitro by RGM1 lysate in the presence of adenosine-5'-triphosphate (ATP). Western blot analysis using a monoclonal phospho-Thr antibody, which exclusively detects phosphorylated threonine residues, and does not cross-react with phosphorylated serine residues revealed NPRC immunoprecipitated from RGM1 lysate is phosphorylated on a threonine residue. Global analysis of the entire rat NPRC sequence using a protein kinase A (PKA) prediction algorithm, identified five putative PKA phosphorylation sites containing a serine residue and one containing a threonine residue, Thr 505. Taken together, the data presented here suggest that rat NPRC is a substrate for PKA and Thr 505 located within the intracellular domain of NPRC is a likely candidate site for the phosphorylation. J. Cell. Biochem. 110: 985–994, 2010.

KEY WORDS: NPRC; PHOSPHORYLATION; NATRIURETIC PEPTIDES

Phosphorylation is the most common form of protein posttranslational modification [Wan et al., 2008]. Protein phosphorylation plays a key role in many cellular processes, and the disruption of normal phosphorylation can result in disease [Cohen, 2002]. Receptor phosphorylation is known to regulate transmembrane signaling and may also regulate the ligand binding affinity as well as subcellular distribution of the receptor [Sibley et al., 1987].

The C type natriuretic peptide receptor (NPRC) is a single transmembrane spanning receptor that is responsible for systemic clearance of circulating natriuretic peptides (NPs) including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) and controlling local effects of the NP system [Matsukawa et al., 1999]. The topology of NPRC consists of a large amino terminal extracellular domain (ECD) for ligand binding, a hydrophobic transmembrane domain, and a short carboxy terminal intracellular domain [He et al., 2005]. Recent evidence has implicated NPRC in signal transduction [Murthy et al., 2000; Pagano and Anand-Srivastava, 2001; Anand-Srivastava, 2005]. Whether phosphorylation plays a role in NPRC signal transduction has yet to be determined. Moreover, the specific amino acid residues that are phosphorylated and the kinases responsible for their phosphorylation have not yet been fully characterized. One report has provided evidence that the intracellular domain of NPRC is phosphorylated in rat aortic smooth muscle cells and identified putative consensus sequences for protein kinase A (PKA), protein kinase C (PKC), and PKG phosphorylation [Pedro et al., 1998].

Abbreviations used: NPs, natriuretic peptides; NPRC, C type natriuretic peptide receptor; GST, glutathione-*S*-transferase (GST); RGM1, rat gastric mucosa; PEG, polyethylene glycol; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; IPTG, isopropyl-D-thiogalactoside; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; ATP, adenosine-5'-triphosphate; GPCR, G-protein-coupled receptor.

*Correspondence to: Dr. William R. Gower, Jr., James A. Haley Veterans Hospital, Research Service (151), 13000 Bruce B. Downs Blvd, Tampa, FL 33612. E-mail: wgower@health.usf.edu

Received 7 January 2010; Accepted 12 March 2010 • DOI 10.1002/jcb.22612 • © 2010 Wiley-Liss, Inc.
Published online 20 April 2010 in Wiley InterScience (www.interscience.wiley.com).

However, identification of a consensus sequence does not guarantee in situ phosphorylation of the protein by a particular kinase [Kennelly and Krebs, 1991].

Characterization of the phosphorylation state of a protein is becoming more practical with the availability of phosphospecific and substrate motif antibodies. A renewable source of the protein of interest may facilitate the study of phosphorylation of that protein. Our laboratory has previously identified the rat gastric mucosa (RGM1) cell line as a useful tool to investigate the expression, regulation, and physiological significance of NPRC [Gower et al., 2006; Alli and Gower, 2009a]. We have shown that RGM1 cells overexpress endogenous NPRC, but do not express any appreciable levels of NPRA or NPRB proteins. Alternatively, the expression of recombinant proteins in *Escherichia coli* offers the ability to obtain large quantities of the desired protein for use in various in vitro assays. Recent advancements leading to a better understanding of transcription, translation, and protein folding in *E. coli* have made it the system of choice for recombinant protein production [Baneyx, 1999]. However, membrane proteins often pose difficulties in their expression and purification in bacterial systems because of their hydrophobicity [Eshaghi et al., 2005]. Also, overexpression of foreign fusion proteins in bacterial cells frequently results in the production of insoluble and inactive precipitated aggregates termed inclusion bodies [Shi et al., 1997]. To circumvent these issues, various expression conditions of the protein can be manipulated to facilitate solubility and minimize the chance of protein misfolding.

In this report, we exercise a strategy to efficiently recover overexpressed foreign proteins from bacterial inclusion bodies. We investigate the phosphorylation state of rat NPRC in vitro using phosphospecific antibodies, a recombinant GST fusion protein, and lysate from the RGM1 cell line. We utilize a phosphorylation site prediction algorithm and several different substrate motif antibodies to identify the phospho-acceptor residue and specific kinases responsible for the phosphorylation of rat NPRC.

MATERIALS AND METHODS

MATERIALS

Plasmid pGEX-4T3, *E. coli* strain BL21, glutathione sepharose 4B medium, and Hyperfilm-ECL were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Mammalian protein extraction reagent (M-PER), phosphatase inhibitors, protease inhibitors, BCA protein assay reagents, SuperSignal West Pico chemiluminescent substrate, and the GelCode Phosphoprotein Stain Reagent Set were purchased from Pierce (Rockford, IL). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

CELL CULTURE AND CELL LYSATE PREPARATION

Rat gastric mucosal (RGM1) epithelial cells were grown in DMEM/F12 medium. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg amphotericin B (Gibco, Grand Island, NY). Cells were maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37°C. Only low passaged cells of less than 30 passages were used for experiments. For preparation of cell lysate, cells grown as a monolayer were scraped from 100 mm dishes in 500 μl ice cold M-PER containing 1× phosphatase inhibitor and protease inhibitors. The cell lysate was sonicated and protein concentration was determined using the BCA Protein Assay Reagent kit according to the manufacturer's instructions.

PLASMID CONSTRUCTION

The cDNA fragment (NM 012868) encoding rat C type natriuretic peptide receptor (rNPRC) (kindly provided by Dr. David Lowe, Genentech, San Francisco, CA) was subcloned into a pGEX4T3 expression vector between *Eco*R1 sites and confirmed by restriction analysis and DNA sequencing (Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL).

PREPARATION OF COMPETENT *E. COLI* CELLS

Competent BL21 cells were prepared using the DMSO method according to Chung et al. (1989) with modifications. Briefly, single colonies were used to inoculate 50 ml LB in a 250 ml Erlenmeyer flask and then grown at 37°C with shaking at 225 rpm. Cells were grown to an OD_{600} of 0.4, harvested by centrifugation at 2,500*g* for 15 min at 4°C, and resuspended in 5 ml of ice-cold TSS buffer (Luria–Bertani (LB) broth with 10% (w/v) polyethylene glycol (PEG), 5% (v/v) dimethyl sulfoxide (DMSO), 50 mM $MgCl_2$, pH 6.5). Single use aliquots of the competent cells were frozen at −80°C for use within 3 months.

EXPRESSION OF FUSION PROTEIN

The constructed expression vector was transformed into the host cell *E. coli* BL21 (DE3) strain for expression. Transformants were selected by growth on LB agar plates containing 100 μg/ml ampicillin. Single isolated colonies were used to inoculate 5 ml of 2XYTA medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0, containing 100 μg/ml ampicillin) and grown at 37°C with shaking at 225 rpm for 12 h. The starter culture was diluted 1:100 into fresh prewarmed 2XYTA. At an OD_{600} of 0.3–0.6, isopropyl-D-thiogalactoside (IPTG) was added to a final concentration of 0.1–1 mM for induction of the lac promoter.

SOLUBILIZATION OF FUSION PROTEIN FROM INCLUSION BODIES

Recombinant fusion protein was solubilized from inclusion bodies according to Frangioni and Neel [1993], with modifications. Briefly, bacterial cells were harvested at 5,000*g* for 10 min at 4°C. The supernatant was removed and the pellet was washed once with a volume of ice-cold PBS (11.9 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) (1/15 the original culture volume) (Table I). The pellet was resuspended in a volume of ice-cold STE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl) (1/50th the original culture volume) (Table I). A volume of lysozyme from chicken egg white was added (1/5,000th the original culture volume) (Table I) followed by a 15-min incubation on ice. A volume of freshly prepared dithiothreitol (DTT) (1 M) and Sarkosyl (10%, w/v) was added (1/5,000th and 1/350th the original culture volume, respectively) (Table I) and then mixed by inversion three times. The lysate was sonicated twice for 5-s intervals and then centrifuged at 13,000 rpm for 15 min to pellet cellular debris. The supernatant was transferred to a new tube and a volume of 10% Triton X-100 and

TABLE I. Solubilization and Purification Scheme for GST-NPRC

Culture volume	60 ml culture	500 ml culture
Centrifugation (5,000*g* for 10 min at 4°C)		
PBS wash buffer	4 ml	33 ml
Resuspension buffer (STE)	1.2 ml	10 ml
Lysozyme	12 μl	100 μl
1 M DTT	12 μl	100 μl
10% Sarkosyl	170 μl	1.43 ml
Sonication (2× for 5 s intervals)		
Centrifugation (13000 rpm for 15 min at room temp.)		
10% Triton X-100	480 μl	4 ml
STE	2.4 ml	20 ml
Incubation (30 min at room temp.)		
Elution		
Glutathione sepharose bed volume	30 μl	240 μl
Glutathione elution buffer	60 μl	500 μl
PBS wash buffer (2×)	300 μl	2.4 ml
Approx. GST-NPRC yield	30 μg	200 μg

Approximate volumes for a 60-ml culture and 500 ml culture are provided. Purification scheme for recombinant GST-NPRC are provided. A set of optimized conditions and the procedure for the expression and purification of GST-NPRC is given for a small- or large-scale culture.

STE was added (1/125th and 1/25th the original culture volume, respectively) (Table I) and allowed to incubate at room temperature for 30 min.

BATCH PURIFICATION OF FUSION PROTEIN

A volume of prewashed 50% glutathione sepharose 4B slurry (1/100 the lysate volume) (Table I) was added to the lysate and incubated at 4°C for 45 min with end-over-end rocking. Bound proteins were collected by centrifugation at 500*g* for 2 min. The complex was washed twice with a volume of ice-cold PBS (10× the initial slurry volume) (Table I). Protein was eluted by resuspending the complex in a volume of elution buffer (1/1,000 the culture volume) (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0) (Table I), incubating for 30 min at 4°C with end-over-end rocking, and collecting the supernatant after centrifugation for 2 min at 500*g*. The elution step was repeated twice and the resulting fractions were pooled.

IDENTIFICATION OF FUSION PROTEIN

Purity of the fusion protein was analyzed by SDS–PAGE and Coomassie blue staining using Colloidal Coomassie blue stain according to manufacturer's instructions (Genomic Solutions, Ann Arbor, MI). The NPRC portion of the fusion protein was identified by indirect Western blot analysis for NPRC using a polyclonal antibody (JAH84) directed against the carboxy terminal tail of NPRC [Alli and Gower, 2009a]. The GST portion of the fusion protein was identified by direct Western blot analysis for GST using peroxidase conjugated anti-GST polyclonal antibody (Sigma).

SDS–PAGE, NATIVE PAGE, AND IMMUNOBLOTTING

Twenty-five to 50 μg of protein was separated by electrophoresis on 5%, 7.5%, or 10% gradient polyacrylamide gels in electrode buffer (25 mM Tris–HCl, 192 mM glycine, pH 8.3, with or without 1% SDS). Proteins were analyzed by Coomassie blue staining or by Western blotting. For Western blotting, proteins were electrically transferred onto nitrocellulose membranes (Hybond-C extra; Amersham, UK) and stained with Ponceau solution (0.1% (w/v) Ponceau-S in 5% (v/v) acetic acid) to confirm the transfer of the bands. Membranes were destained by washing with Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5), blocked in 5% (w/v) nonfat powdered milk, incubated with anti-NPRC polyclonal antibody (JAH84) at a dilution of 1:1,000 in 5% BSA in TBS [Alli and Gower, 2009b]. Membranes were washed with TBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (BioRad, Hercules, CA) at a dilution of 1:3,000 in blocking solution. Immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions and developed using Hyperfilm-ECL.

ESTIMATION OF MOLECULAR WEIGHT OF GST-NPRC

In order to further confirm NPRC was intact we indirectly estimated the molecular weight of GST-NPRC by using a protein molecular weight marker kit and Ferguson plots [Ferguson, 1964]. The electrophoretic mobilities of GST-NPRC and different molecular weight markers were determined after electrophoresis on a set of gels of increasing polyacrylamide concentrations. The retardation coefficients of each protein were determined from the slope of the plot of the log of the electrophoretic mobilities against the percent gel concentrations. The logarithm of the negative slope was plotted against the logarithm of the molecular weight of each protein, and the resulting linear plot was used to estimate the molecular weight of GST-NPRC.

IN VITRO PHOSPHORYLATION OF GST-NPRC

GST-NPRC immobilized on glutathione sepharose 4B beads was incubated with 200 μg of RGM1 cell lysate in kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 5 mM DTT, 2 mM ATP) for a total volume of 300 μl, and incubated at 30°C for 1 h. The beads were rinsed three times with PBS, before the addition of 150 μl of elution buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0).

DETECTION OF PHOSPHORYLATED PROTEINS

Recombinant GST-NPRC fusion protein or RGM1 protein lysate was subjected to SDS–PAGE, then gels were stained using the GelCode Phosphoprotein Stain reagent set according to the manufacturer's instructions. Alternatively, proteins were transferred to nitrocellulose membranes and then membranes were probed with phosphospecific and substrate motif antibodies to identify phosphorylation of proteins.

OVERLAY ASSAY

Purified GST-NPRC was resolved on 7.5% SDS–PAGE gels and then electrically transferred onto nitrocellulose membranes. Membranes were stained with Ponceau S (Sigma) to confirm transfer. Membranes were blocked in far-Western Buffer (20 mM HEPES, pH 7.5, 1 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.02% sodium azide, and 5% (w/v) nonfat dry milk). Membranes were washed with TBS and then incubated with RGM1 cell lysate diluted in 5% BSA in TBS. Membranes were washed with TBS and then incubated with anti-phosphothreonine antibody (Cell Signaling Technology, Danvers, MA) in blocking solution. Membranes were washed with TBS and the immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate System.

MASS SPECTROMETRY

Mass spectrometry was performed to confirm the identity of the recombinant fusion protein (Proteomics Department at the Moffitt Cancer Center, Tampa, FL). Briefly, the gel band was subjected to in-gel tryptic digestion followed by liquid extraction of the gel fragments. LC–MS/MS was used to analyze collected peptides and was performed on an LTQ mass spectrometer (Thermo Electron Corporation, Waltham, MA) with an LC packings ultimate dual gradient nano-LC system (Dionex, Sunnyvale, CA). The Mascot algorithm (Matrix Science, London, UK) was used to search the collected data against the nonredundant rodentia database at the National Center for Biotechnology Information (NCBInr) with the following parameters: peptide mass tolerance, 2.5 Da; MS/MS ion mass tolerance, 0.8 Da; allowing up to two missed cleavages. Significant hits, defined by Mascot probability analysis and hits that exceeded the arbitrarily set acceptance threshold were regarded as positive identifications.

IMMUNOPRECIPITATION

Two hundred micrograms of total protein cell lysate was incubated with a 1:250 dilution of anti-NPRC polyclonal antibody at 4°C for 2–4 h with end-over-end mixing. The complexes were incubated with a 1:10 dilution of prewashed 50% protein G agarose slurry (Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C for 4–6 h with end-over-end mixing. Beads were washed 4× with ice-cold M-PER. Immune complexes were eluted in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) and analyzed by SDS–PAGE and Coomassie staining or Western blotting.

RESULTS

CONSTRUCTION, EXPRESSION, AND PURIFICATION OF GST-NPRC

In order to create a fusion protein that would maintain the signaling function mediated by the carboxy terminal domain of NPRC, a GST tag was strategically placed on the amino terminal end of the full-length sequence of rat NPRC (Fig. 1A). Several expression conditions of GST-NPRC, including temperature of bacterial cultivation, optical density of bacteria upon IPTG induction, IPTG concentration, and duration of IPTG induction were considered. The expression of GST-NPRC was favored at a higher temperature with IPTG induction at a lower optical density (Fig. 1B). Attempts to prevent misfolding of GST-NPRC by reducing the rate of expression of GST-NPRC by culturing the bacteria harboring the fusion protein at 30°C instead of 37°C resulted in less product, as indicated by the bands at 75 kDa in Coomassie stained gels (Fig. 1B). The expression of GST-NPRC appeared to be fivefold less at 30°C than at 37°C, as indicated by densitometric analysis (data not shown).

Despite the inclusion of a GST tag to facilitate solubilization, the overexpression of the GST-NPRC foreign protein in the bacteria resulted in aggregation and formation of insoluble inclusion bodies. The fusion protein was found exclusively within intracellular inclusion bodies and represented approximately 10% of total bacterial proteins as indicated by densitometric analysis (data not shown). Therefore, we took a similar approach as originally described by Frangioni and Neel [1993] to recover the fusion protein from bacterial inclusion bodies. The solubilization and purification scheme used is presented in Table I. GST-NPRC was purified to homogeneity, as indicated by a single band of an apparent molecular weight of 75 kDa (Fig. 1C). Furthermore, a desirable yield of purified fusion protein could not be achieved by conventional methods. As shown by the Coomassie stained band at 75 kDa in Figure 1C, GST-NPRC was efficiently recovered from inclusion bodies when compared to the amount of GST-NPRC recovered after purification of the soluble fraction by conventional methods.

The expected size of GST-NPRC was between 86 and 92 kDa, since NPRC is known to have a molecular mass between 60 and 66 kDa and GST is known to have a molecular mass of 26 kDa. In order to confirm the NPRC portion of GST-NPRC was intact after purification, indirect Western blot analysis was performed using a polyclonal antibody against the carboxy terminal domain of NPRC. NPRC was also shown to be intact by estimating the molecular weight of the purified recombinant protein by Ferguson plots (data not shown). Immunoreactive bands at 75 and 66 kDa were observed after probing for NPRC (Fig. 1D). The presence of at least a partial GST moiety associated with GST-NPRC was demonstrated by direct Western blot analysis using a peroxidase-conjugated antibody, as an immunoreactive band at 75 kDa was observed (Fig. 1E). Next, the purity of GST-NPRC was confirmed and the NPRC portion of the fusion was shown to be intact by mass spectrometry analysis. MASCOT results revealed several signature peptides corresponding only to rat NPRC and significant sequence coverage from the amino terminal domain to the carboxy terminal domain of NPRC (Fig. 1F). However, MASCOT results did not reveal sequence coverage for the GST moiety. The ability to remove the GST moiety of GST-NPRC was demonstrated. Isolation of the NPRC portion of the fusion protein was demonstrated after subjecting the GST-NPRC fusion protein to thrombin cleavage for 18 h and subsequent indirect Western blot analysis, as an immunoreactive band at 66 kDa was observed (Fig. 1G).

DIMERIZATION OF NPRC FROM THROMBIN CLEAVAGE OF GST-NPRC IS INDEPENDENT OF LIGAND

Since the GST tag itself can dimerize [Armstrong, 1997], the GST tag was removed from GST-NPRC by thrombin cleavage prior to investigating the dimerization of NPRC in vitro. We examined if the addition of the NPRC agonist cANF could induce dimerization of NPRC liberated from thrombin cleavage of GST-NPRC. Interestingly, the primary sequence of NPRC alone was sufficient to result in its spontaneous dimerization, as shown by native-PAGE analysis (Fig. 1H). The addition of ligand at increasing concentrations did not augment the dimerization of NPRC (Fig. 1H).

PHOSPHORYLATION OF GST-NPRC IN VITRO

The intracellular domain of rat NPRC contains several putative phosphorylation consensus sequences, as shown in Figure 2. Two PKA, one PKB, and one PKG phosphorylation sites were identified within the 37 amino acid intracellular domain of rat NPRC.

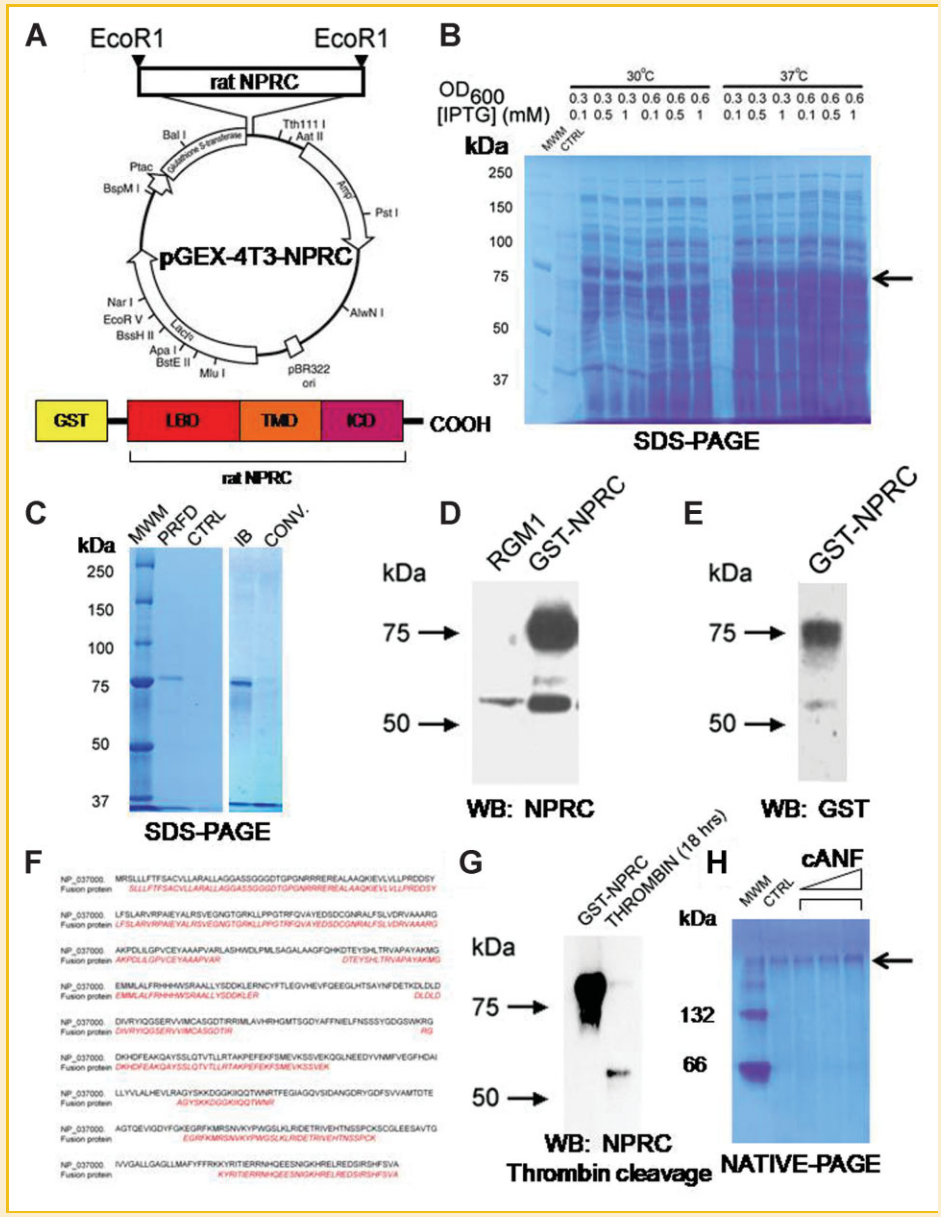

Fig. 1. Construction, optimization of expression conditions, and purification of GST-NPRC. A: Schematic representation of the pGEX4T3-NPRC construct and domains of GST-NPRC. The ligand-binding domain (LBD), transmembrane domain (TMD), and intracellular domain (ICD) of NPRC are depicted. B: Coomassie stained gel illustrating the effects of temperature, cell density, and IPTG concentration on the expression of GST-NPRC. The arrow indicates expression of the GST-NPRC fusion protein. Lysates were obtained from *E. coli* BL21 cells transformed with the pGEX4T3-NPRC construct. Lane 1: Unstained molecular weight markers (MWM). C: Coomassie stained gel illustrating the purification of GST-NPRC to homogeneity and comparing the yield of GST-NPRC solubilized from inclusion bodies compared to the conventional method. Left blot: Lane 1: Molecular weight markers (MWM). Lane 2: Purified fraction (PRFD) of GST-NPRC. Lane 3: Control in which lysate from empty vector was purified in parallel with lysate from bacteria harboring the GST-NPRC construct. Right gel: Lane 1: GST-NPRC purified after solubilization from inclusion bodies. Lane 2: GST-NPRC purified from the soluble fraction. D: Indirect Western blot showing an immunoreactive band of an apparent molecular mass of 75 kDa using anti-NPRC antibody. The band at 66 kDa from RGM1 lysate and purified GST-NPRC corresponds to the GST-NPRC fusion protein in which GST is truncated and NPRC is intact. E: Direct Western blot showing an immunoreactive band of an apparent molecular mass of 75 kDa using a peroxidase-conjugated anti-GST antibody. F: Sequence alignment/Mascot results from LC–MS/MS. Matched peptides are italicized in red. G: Indirect Western blot showing an immunoreactive band of an apparent molecular mass of approximately 60 kDa after thrombin cleavage of GST-NPRC. H: Native PAGE (nondenaturing conditions) of GST-NPRC using a 10% SDS–PAGE gel and after Coomassie blue staining. Lane 1: Albumin dimer at 132 kDa and monomer at 66 kDa. All results are representative of at least three experiments.

The phosphorylation of GST-NPRC was evaluated in vitro using a crude lysate from RGM1 cells. While immobilized to glutathione sepharose 4B beads, GST-NPRC was incubated with RGM1 whole cell lysate at increasing concentrations and increasing periods of time. Compared to untreated GST-NPRC, the addition of increasing concentrations of RGM1 lysate for increasing periods of time did not affect the ability to purify the fusion protein to homogeneity (Fig. 3A). However, compared to untreated

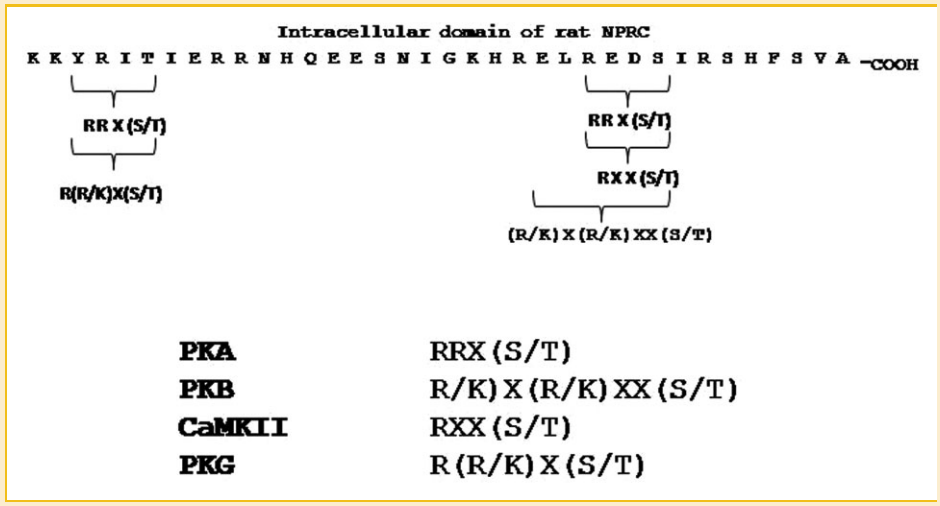

Fig. 2. Phosphorylation consensus sequences within the intracellular domain of rat NPRC. Putative PKA, PKB, CaMKII, and PKG phosphorylation sites flanking the 37-amino-acid carboxy terminal tail of rat NPRC, where X represents any amino acid residue.

GST-NPRC, incubation of GST-NPRC with 600 μg of phosphatase inhibitor treated RGM1 lysate in the presence of ATP for 1 h resulted in a phosphostained band corresponding to 75 kDa (Fig. 3B).

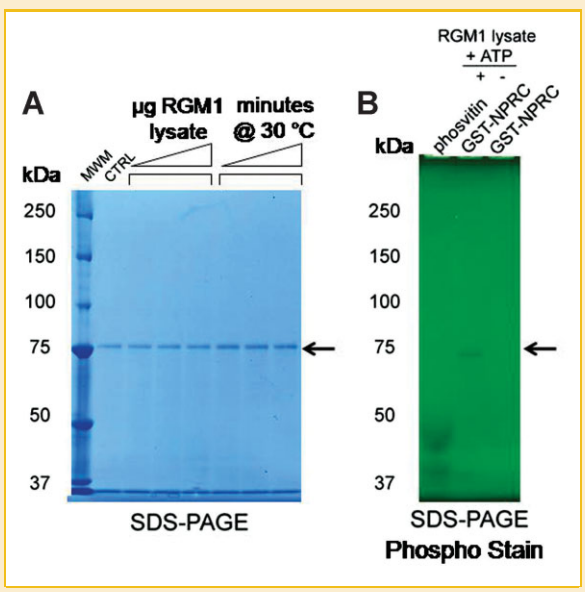

Fig. 3. In vitro phosphorylation of GST-NPRC. A: Representative Coomassie blue stained gel of three similar experiments. Lane 1: Molecular weight markers. Lane 2: Untreated control. Lanes 3–5: Purification of GST-NPR fusion protein after incubating the fusion protein, immobilized on glutathione sepharose 4B beads with increasing amounts of RGM1 cell lysate (200, 400, or 600 μg, respectively) for 30 min in the presence of ATP. Lanes 6–8: Purification of GST-NPRC fusion protein after incubating the fusion protein, immobilized on glutathione sepharose 4B beads for increasing time intervals (15 min, 30 min, 1 h, respectively) with 400 μg of RGM1 lysate in the presence of ATP. B: Representative phosphostained gel of three similar experiments. Immobilized GST-NPRC was treated with or without 400 μg RGM1 lysate and ATP for 1 h before being batch purified, subjected to SDS–PAGE, and analyzed after gel phosphostaining. Lane 1: Phosvitin-positive control.

THR 505 OF RAT NPR-C IS A LIKELY CANDIDATE SITE FOR PHOSPHORYLATION BY PKA

Several different monoclonal and polyclonal antibodies were utilized to investigate the phosphorylation state of NPRC, as indicated in Table II. In order to determine if rat NPRC exists as a constitutively phosphorylated protein, NPRC was immunoprecipitated from RGM1 cell lysate treated with or without phosphatase inhibitors and then probed for phosphorylated serine and threonine residues. NPRC phosphorylation at serine and threonine residues was markedly reduced in the absence of phosphatase inhibitors after probing with a phosphospecific polyclonal antibody that recognizes phosphorylated serine and threonine residues (Fig. 4A). Similarly, NPRC phosphorylation at threonine residues was essentially nondetectable in the absence of phosphatase inhibitors after probing with a phosphospecific antibody that recognizes only phosphorylated threonine residues (Fig. 4B). As a control, blots were probed for total NPRC protein using a polyclonal antibody, which resulted in immunoreactive bands for lysates treated with or without phosphatase inhibitors (Fig. 4C).

In order to determine if NPRC is a substrate for a specific kinase, NPRC was immunoprecipitated from RGM1 cell lysate and probed for phosphorylation using PKA, PKB, and PKC substrate motif antibodies (Table II). An immunoreactive band corresponding to NPRC was not observed when probing with PKB or PKC substrate antibodies (data not shown). However, an immunoreactive band at 66 kDa corresponding to NPRC was observed when probing with a PKA substrate motif antibody that was markedly reduced in the absence of phosphatase inhibitors (Fig. 4D). As a control, blots were probed for total NPRC protein using JAH84 antibody. In contrast, immunoreactive NPRC bands of equal intensity were detected by JAH84 antibody in both treated and untreated RGM1 cell lysates (Fig. 4E).

Next, global phosphorylation of NPRC by PKA was evaluated using a PKA consensus sequence prediction algorithm, pkaPS [Neuberger et al., 2007]. Six distinct phosphorylation sites were identified over the entire sequence of rat NPRC (Table III). However,

TABLE II. Antibodies Used in This Investigation

Antibody	Specificity/sensitivity	Identified motif	Source
JAH84	Total NPRC	None	Our laboratory
Phospho-Thr-polyclonal	Phospho-Tyr, Ser, Thr	None	Cell Signaling
Phospho-Thr-monoclonal	Phospho-Thr	None	Cell Signaling
Phospho-PKA substrate	Phospho-Ser, Thr	RRXS/T	Cell Signaling
Phospho-PKB (Akt) substrate	Phospho-Ser, Thr	(R/K)X(R/K)XX(T/S)	Cell Signaling
Phospho-PKC substrate	Phospho-Ser	(R/K)X(S)(Hyd)(R/K)	Cell Signaling

Characteristics of the various antibodies used in this study are given. Rabbit polyclonal NPRC (JAH84) antibody detects total NPRC protein with broad species cross-reactivity. Rabbit polyclonal phospho-Thr antibody detects proteins phosphorylated at Thr residues but may cross-react with some phospho-Tyr or phospho-Ser containing sequences. Mouse monoclonal phospho-Thr antibody detects proteins phosphorylated at Thr residues and does not cross-react with phospho-Ser containing sequences. Rabbit monoclonal phospho-PKA substrate antibody detects proteins containing a phospho-Ser/Thr residue with arginine at the −3 and −2 positions. Rabbit polyclonal phospho-PKB (Akt) substrate antibody detects proteins containing phospho-Ser/Thr preceded by Lys/Arg at positions −5 and −3. Rabbit polyclonal phospho-PKC substrate antibody detects proteins containing phospho-serine residues surrounded by Arg or Lys at the −2 and +2 positions and a hydrophobic residue at the +1 position.

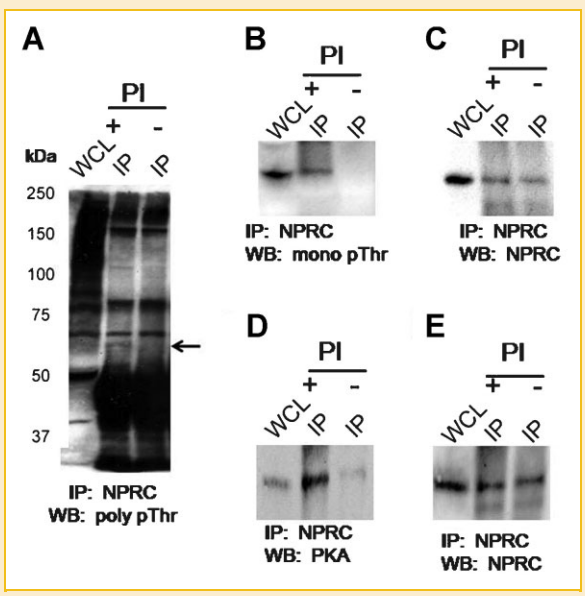

Fig. 4. Identification of putative residues and kinases involved in NPRC phosphorylation. A: IP Western blot analysis after immunoprecipitating NPRC from RGM1 whole cell lysate (WCL) treated with phosphatase inhibitors (+PI) and probing with polyclonal phospho-Thr (poly-pThr) specific antibody revealed an immunoreactive band at 66 kDa. B: IP Western blot analysis after immunoprecipitating NPRC from RGM1 WCL treated with PI and probing with monoclonal phospho-Thr (mono-pThr) specific antibody revealed an immunoreactive band at 66 kDa. The immunoreactive band at 66 kDa was absent after immunoprecipitating NPRC from untreated (−PI) RGM1 lysate and probing with mono-pThr-specific antibody. C: Similarly, IP Western blot analysis after immunoprecipitating with NPRC-specific antibody and probing with NPRC-specific antibody revealed an immunoreactive band at 66 kDa. D: IP Western blot analysis after immunoprecipitating NPRC from RGM1 lysate treated with PI and probing with a PKA substrate motif antibody revealed an immunoreactive band at 66 kDa. The immunoreactive band at 66 kDa was attenuated after immunoprecipitating NPRC from untreated RGM1 lysate and probing with PKA substrate motif antibody. E: Similarly, IP Western blot analysis after immunoprecipitating with NPRC-specific antibody and probing with NPRC-specific antibody revealed an immunoreactive band at 66 kDa. The data presented here are representative of three independent experiments.

only one of these sites included a threonine residue, Thr 505 (Table III).

DISCUSSION

Here we present a set of optimized procedures to efficiently recover intact GST fusion proteins from bacterial inclusion bodies. We introduce a recombinant GST-NPRC fusion protein as a tool to investigate the phosphorylation state of rat NPRC. We show that rat NPRC contains several serine and threonine residues, as well as phosphorylation consensus sequences for various kinases. A cAMP-dependent protein kinase (PKA) predictor algorithm, pkaPS, revealed five serine and one threonine containing putative PKA phosphorylation sites spanning the entire sequence of rat NPRC. Immunoprecipitation and Western blot analysis using RGM1 cell lysate and a phospho-Thr specific antibody suggested phosphorylation occurs at Thr residue(s) of NPRC. The phosphorylation of NPRC on Thr residues may contribute to the phosphorylation state of NPRC, as NPRC has already been reported to be phosphorylated on various Ser residues [Pedro et al., 1998]. Immunoprecipitation and Western blot analysis using RGM1 cell lysate and a PKA substrate motif antibody suggested that Thr-505 of rat NPRC is a potential site for the phosphorylation by PKA.

Overexpression of foreign proteins in bacterial cells frequently results in the production of insoluble and inactive precipitated

TABLE III. Prediction of NPRC Phosphorylation by PKA Using the pkaPS Algorithm

Position	Residue	Score	Profile
193	S	0.04	0.82
337	S	0.31	1.08
441	S	0.65	1.09
505	T	0.41	0.74
527	S	0.14	0.81
533	S	0.18	0.40

Prediction of NPRC phosphorylation by PKA using the pkaPS algorithm is given. Six putative phosphorylation sites containing either a Ser or Thr residue are shown for NPRC. The position of the Ser or Thr residue is given as well as the score and profile of the prediction.

aggregates termed inclusion bodies [Shi et al., 1997]. For this reason it was not surprising to find GST-NPRC in the pelleted fraction after performing SDS–PAGE analysis in initial experiments. The formation of inclusion bodies is not correlated with the size but is correlated to certain sequence-dependent features of the polypeptide being synthesized [Idicula-Thomas and Balaji, 2005]. Causes that may contribute to eukaryotic proteins being insoluble in *E. coli* include rapid overexpression of the recombinant protein under the control of bacterial promoters, improper folding of the recombinant protein in the considerable reducing intracellular environment, or absence of posttranslational modifications that could affect polarity [Mercado-Pimentel et al., 2002]. Our attempts to manipulate various parameters including expression temperature, concentration of IPTG, duration of IPTG induction, or initiation of IPTG induction at a specific optical density did not prevent the formation of inclusion bodies. Therefore, we used the formation of inclusion bodies as a means of purification of GST-NPRC, which may have otherwise been unstable or truncated in the soluble fraction. The yield of GST-NPRC fusion protein recovered from inclusion bodies was significantly greater when compared to that recovered by conventional methods (Fig. 1C). We exercised a denaturation and renaturation step as described by Frangioni and Neel [1993], using the ionic detergent Sarkosyl and the nonionic detergent Triton X-100, respectively, with the ratio of the Sarkosyl to Triton X-100 being determined empirically. The intensity and duration of sonication during cell lysis was carefully executed, as over sonication resulted in co-purification of *E. coli* host proteins and degradation of the fusion protein in initial experiments. The ratio of lysate to glutathione sepharose media was determined empirically and various elution conditions, including buffer molar concentration and pH, were optimized.

Since NPRC has a molecular mass between 60 and 66 kDa and GST has molecular mass of 26 kDa, we expected GST-NPRC would have a molecular mass between 86 and 92 kDa. However, we consistently observed a molecular mass of 75 kDa for GST-NPRC (Fig. 1B–E). Mass spectrometry sequence analysis and Western blot analysis confirmed NPRC was completely intact from the amino terminal to the carboxy terminal domain (Fig. 1F). Since there was no sequence coverage from mass spectrometry for the GST moiety and because the peroxidase conjugated antibody specific for GST used for Western blot analysis recognizes multiple epitopes of GST, the site of cleavage of the GST moiety of GST-NPRC could not be determined by these methods. Our results indicate that the GST moiety of the GST-NPRC fusion protein is cleaved after purification over time and/or is susceptible to degradation as lower molecular weight bands are present after analyzing and comparing GST-NPRC thawed from storage (Fig. 1D) and freshly prepared GST-NPRC immediately after batch purification (Fig. 1G). However, truncation of the GST moiety was not a concern since it was removed by thrombin cleavage in some experiments.

Instead of using a partial sequence of NPRC, the entire coding sequence of rat NPRC was used to construct the recombinant GST-NPRC fusion protein because we desired the ability to investigate posttranslational modifications of complete NPRC. Endogenous NPRC exists as a monomer and a disulfide-linked homodimer [Itakura et al., 1997; Anand-Srivastava, 2005], but the dimeric structure of NPRC is not necessary for ligand binding activity [Itakura et al., 1997]. The ECD of NPRC, which consists of multiple N-linked glycosylation sites, becomes activated and mediates NPRC homodimerization upon ligand binding [He et al., 2005]. Furthermore, the cytoplasmic tail of NPRC is phosphorylated upon ligand binding [Pedro et al., 1998]. However, under nondenaturing native-PAGE conditions we observed dimerization of GST-NPRC (Fig. 1H) or NPRC alone (data not shown) in both the absence and presence of increasing amounts of the NPRC ligand, cANF. It may be possible that the primary sequence of NPRC is sufficient to allow for the observed spontaneous dimerization of the fusion protein. Since the importance of NPRC glycosylation remains controversial, the NPRC fusion protein presented in this report, which lacks glycosylation, provides a simplified means to investigate the requirement of glycosylation for NPRC activation and function.

Since mammalian posttranslational modifications including phosphorylation are absent in bacteria, our GST-NPRC fusion protein was useful for the investigation of phosphorylation events of NPRC. Mass spectrometry and site-directed mutagenesis are commonly used to investigate the phosphorylation state of a protein. Alternatively, the availability of phosphospecific antibodies can be used to probe for protein phosphorylation. Phosphorylation motif antibodies are useful resources for the identification and characterization of the phosphorylation state of a protein. The carboxy terminal domain of NPRC is rich in serine and threonine residues and consensus sequence motifs for PKA, PKB, PKG, and CaMKII (Fig. 2). In one report, NPRC was shown to be phosphorylated exclusively on serine residues in rat aortic smooth muscle cells [Pedro et al., 1998]. Although it was not addressed, it is possible that multiple serine residues of NPRC are phosphorylated simultaneously or sequentially by one or more kinases. In agreement with a recent report [Zhou and Murthy, 2004], our data suggest that NPRC is phosphorylated on a Thr residue and that Thr 505, located within the intracellular domain of NPRC, is a likely site for the phosphorylation to occur. The discrepancy between the phosphorylated residues of NPRC may be attributed to the phosphorylation state of NPRC being species and cell type dependent, and the possibility of the various kinases sharing the same phosphorylation site, as there are similarities between the substrate-recognition sequences between the kinases. Phosphorylation of Thr-505 of NPRC may have significant implications because it is within the intracellular domain of NPRC. The intracellular domain of NPRC has been reported to contain G protein activator sequences and participate in signal transduction [Murthy et al., 2000; Pagano and Anand-Srivastava, 2001]. The Thr-505 residue of NPRC was reported to be part of a consensus sequence for phosphorylation by cGMP-dependent protein kinase (PKG) [Zhou and Murthy, 2004]. After expression of various NPRC mutants into COS-1 cells, desensitization by PKG was found to be mediated by phosphorylation of Thr-505 of NPRC [Zhou and Murthy, 2004].

In order to identify the kinase responsible for phosphorylation of Thr-505, we utilized substrate motif antibodies for various kinases. Only a PKA substrate motif antibody (Fig. 4D,E) and not PKB or PKC substrate motif antibodies (data not shown) resulted in immunoreactive bands after immunoprecipitating NPRC from RGM1 lysate with NPRC-specific antibody. PKA phosphorylates proteins on

Serine (Ser) and Threonine (Thr) residues within the motif Arg-X-X-Ser/Thr, where X may represent any amino acid residue. The motif is not absolute and some variations in basic residues and spacing are allowed [Slice and Taylor, 1989]. It has been reported that the second or third position prior to the phosphorylated serine or threonine should be occupied by an arginine [Kemp et al., 1977]. Several algorithms exist for predicting putative PKA phosphorylation sites. We utilized the pkaPS algorithm because it offers both high sensitivity and specificity for predicting PKA phosphorylation sites of a given protein sequence based on various parameters [Neuberger et al., 2007]. We used pkaPS to identify all putative PKA phosphorylation sites within the entire sequence of rat NPRC. Of the six predicted phosphorylation sites (Table III), only amino acid 505 contained a Thr residue. Since the phospho-Thr specific antibody we used in IP-Western blot analysis only recognizes phosphorylated Thr residues and does not cross-react with phosphorylated Ser residues, it is likely that Thr-505 of rat NPRC is phosphorylated by PKA. NPRC may be phosphorylated on multiple residues but does not exist as a constitutively phosphorylated protein in RGM1 cells, since treatment of RGM1 lysate with phosphatase inhibitors was necessary to detect phosphorylation (Fig. 4B,D). Future studies will be necessary to determine if this phosphorylation is conserved in other species and to determine sequential phosphorylation and dephosphorylation events.

The procedure used here to efficiently recover mammalian proteins from bacterial inclusion bodies may in itself have biological significance. It is often difficult to solubilize proteins with large hydrophobic domains and it is also difficult to recover mammalian proteins overexpressed in bacteria. Here, we demonstrate that it is possible to efficiently recover a mammalian protein with a hydrophobic transmembrane domain from bacterial inclusion bodies that could not otherwise be recovered using conventional methods. Also, there is biological significance associated with the GST-NPRC fusion protein itself since it represents a novel molecular tool to investigate the role of posttranslational modification in mediating NPRC function. Here we provide evidence for the phosphorylation of NPRC by PKA on Thr residue(s) which may eventually prove to be important for receptor homodimerization, activation, desensitization, internalization, and/or signaling.

Additional studies will also be necessary to determine if Thr phosphorylation of NPRC by PKA is necessary for the clearance and signaling functions of NPRC. Future studies will involve creating NPRC mutants in which Ser 527, Ser 533, and Thr 505 of rat NPRC are changed to Ala and subjecting them to phosphorylation by recombinant PKA, PKB, PKG, or CaMKII in vitro. The role of the phosphorylation at each putative site by each specific kinase in mediating NPRC function will be evaluated by performing ligand-binding experiments.

ACKNOWLEDGMENTS

We thank Gay Carter and Eric Thomas for technical assistance. We thank Dr. Andreas G. Seyfang for helpful discussions. This research was supported by an American Heart Association Predoctoral Fellowship (A.A.A.) and a Veterans Affairs Merit Award (W.R.G.).

REFERENCES

Alli AA, Gower WR, Jr. 2009a. The C type natriuretic peptide receptor tethers AHNAK1 at the plasma membrane to potentiate arachidonic acid induced calcium mobilization. Am J Physiol Cell Physiol 297:C1157–C1167.

Alli AA, Gower WR, Jr. 2009b. Characterization of a polyclonal antibody against the cytoplasmic domain of the C-type natriuretic peptide receptor. J Clin Ligand Assay 31:3–7.

Anand-Srivastava MB. 2005. Natriuretic peptide receptor-C signaling and regulation. Peptides 26:1044–1059.

Armstrong RN. 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem Res Toxicol 10:2–18.

Baneyx F. 1999. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 10:411–421.

Chung CT, Niemela SL, Miller RH. 1989. One-step preparation of competent Escherichias coli:transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA 86:2172–2175.

Cohen P. 2002. Protein kinases—The major drug targets of the twenty-first century? Nat Rev Drug Discov 1:309–315.

Eshaghi S, Hedren M, Nasser MI, Hammarberg T, Thornell A, Nordlund P. 2005. An efficient strategy for high-throughput expression screening of recombinant integral membrane proteins. Protein Sci 14:676–683.

Ferguson KA. 1964. Starch-gel electrophoresis—Application to the classification of pituitary proteins and polypeptides. Metabolism 13(Suppl):985–1002.

Frangioni JV, Neel BG. 1993. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal Biochem 210:179–187.

Gower WR, Jr., Carter GM, McAfee Q, Solivan SM. 2006. Identification, regulation and anti-proliferative role of the NPR-C receptor in gastric epithelial cells. Mol Cell Biochem 293:103–118.

He XL, Dukkipati A, Wang X, Garcia KC. 2005. A new paradigm for hormone recognition and allosteric receptor activation revealed from structural studies of NPR-C. Peptides 26:1035–1043.

Idicula-Thomas S, Balaji PV. 2005. Understanding the relationship between the primary structure of proteins and its propensity to be soluble on overexpression in *Escherichia coli*. Protein Sci 14:582–592.

Itakura M, Suzuki H, Hirose S. 1997. Structural analysis of natriuretic peptide receptor-C by truncation and site-directed mutagenesis. Biochem J 322(Pt 2): 585–590.

Kemp BE, Graves DJ, Benjamini E, Krebs EG. 1977. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J Biol Chem 252:4888–4894.

Kennelly PJ, Krebs EG. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J Biol Chem 266:15555–15558.

Matsukawa N, Grzesik WJ, Takahashi N, Pandey KN, Pang S, Yamauchi M, Smithies O. 1999. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. Proc Natl Acad Sci USA 96:7403–7408.

Mercado-Pimentel ME, Jordan NC, Aisemberg GO. 2002. Affinity purification of GST fusion proteins for immunohistochemical studies of gene expression. Protein Expr Purif 26:260–265.

Murthy KS, Teng BQ, Zhou H, Jin JG, Grider JR, Makhlouf GM. 2000. G(i-1)/G(i-2)-dependent signaling by single-transmembrane natriuretic peptide clearance receptor. Am J Physiol Gastrointest Liver Physiol 278:G974–G980.

Neuberger G, Schneider G, Eisenhaber F. 2007. pkaPS: Prediction of protein kinase A phosphorylation sites with the simplified kinase-substrate binding model. Biol Direct 2:1.

Pagano M, Anand-Srivastava MB. 2001. Cytoplasmic domain of natriuretic peptide receptor C constitutes Gi activator sequences that inhibit adenylyl cyclase activity. J Biol Chem 276:22064–22070.

Pedro L, Fenrick R, Marquis M, McNicoll N, De Lean A. 1998. Characterization of the phosphorylation state of natriuretic peptide receptor-C. Mol Cell Biochem 178:95–101.

Shi PY, Maizels N, Weiner AM. 1997. Recovery of soluble, active recombinant protein from inclusion bodies. Biotechniques 23:1036–1038.

Sibley DR, Benovic JL, Caron MG, Lefkowitz RJ. 1987. Regulation of transmembrane signaling by receptor phosphorylation. Cell 48:913–922.

Slice LW, Taylor SS. 1989. Expression of the catalytic subunit of cAMP-dependent protein kinase in *Escherichia coli*. J Biol Chem 264:20940–20946.

Wan J, Kang S, Tang C, Yan J, Ren Y, Liu J, Gao X, Banerjee A, Ellis LB, Li T. 2008. Meta-prediction of phosphorylation sites with weighted voting and restricted grid search parameter selection. Nucleic Acids Res 36:e22.

Zhou H, Murthy KS. 2004. Distinctive G protein-dependent signaling in smooth muscle by sphingosine 1-phosphate receptors S1P1 and S1P2. Am J Physiol Cell Physiol 286:C1130–C1138.