Csk Mediates G-Protein-Coupled Lysophosphatidic Acid Receptor-Induced Inhibition of Membrane-Bound Guanylyl Cyclase Activity†

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ABSTRACT: Natriuretic peptides (NPs) are involved in many physiological processes, including the regulation of vascular tone, sodium excretion, pressure−volume homeostasis, inflammatory responses, and cellular growth. The two main receptors of NP, membrane-bound guanylyl cyclases A and B (GC-A and GC-B), mediate the effects of NPs via the generation of cGMP. NP-stimulated generation of cGMP can be modulated by intracellular processes, whose exact nature remains to be elucidated. Thus, serum and lysophosphatidic acid (LPA), by unknown pathways, have been shown to inhibit the NP-induced generation of cGMP. Here we report that the nonreceptor-tyrosine-kinase Csk is an essential component of the intracellular modulation of atrial natriuretic peptide (ANP)-stimulated activation of GC-A. The genetic deletion of Csk ($Csk^{-/-}$) in mouse embryonic fibroblasts blocked the inhibitory effect of both serum and LPA on the ANP-stimulated generation of cGMP. Moreover, using a chemical rescue approach, we also demonstrate that the catalytic activity of Csk is required for its modulatory function. Our data demonstrate that Csk is involved in the control of cGMP levels and that membrane-bound guanylyl cyclases can be critically modulated by other receptor-initiated intracellular signaling pathways.

Many cellular processes, such as cell migration, smooth muscle contraction, cellular growth, and proliferation, are under the control of the second messenger cGMP (*1*). In eukaryotes, cGMP is synthesized by two distinctive classes of guanylyl cyclases (GC)[1]: membrane-bound guanylyl cyclases and soluble guanylyl cyclases (*2*). Soluble guanylyl cyclases are regulated by intracellular nitric oxide. Among membrane-bound forms, the receptor guanylyl cyclases, GC-A and GC-B, represent the most widely expressed enzymes (*3*, *4*). Their activity is primarily regulated through a set of natriuretic peptide hormones, namely, atrial natriuretic peptide (ANP), brain-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (*5*, *6*). GC-A binds both ANP and BNP, whereas the specific ligand for GC-B is CNP. Binding of natriuretic peptides to the extracellular domains of GC-A and GC-B results in the activation of receptors to produce cGMP (*7*). The most well-studied physiological role of natriuretic peptides is the maintenance of the cardiovascular pressure−volume homeostasis (*8*, *9*). Natriuretic peptides lower the blood pressure, increase renal salt excretion, glomerular filtration rates, and vascular smooth-muscle relaxation, and antagonize all known actions of the renin−angiotensin−aldosterone system (*8*). In addition, natriuretic peptide receptor guanylyl cyclases have attracted a great deal of attention in recent years for their ability to modulate cell proliferation and cardiomyocyte hypertrophy (*1*, *10*−*13*). Gene knockout experiments have shown that the disruption of GC-A in mice results in a hypertensive and/or cardiac hypertrophic phenotype (*12*, *14*, *15*). Moreover, ANP has been shown to inhibit the cardiomyocyte hypertrophy induced by growth factors and other stimuli through a cGMP-dependent mechanism (*16*).

Both GC-A and GC-B consist of an extracellular ligand-binding domain, a short membrane-spanning domain, a kinase homology domain, a hinge region, and a catalytic cyclase domain (*2*). Phosphorylation of the kinase homology domain is critical for the ligand-induced activation of GC-A and GC-B (*17*, *18*). Extensive studies in the past have shown that desensitization of GC-A and GC-B involves the dephosphorylation of the receptor without significant changes in basal activity (*17*, *19*). Protein kinase C (PKC) and the PP2C family of phosphatases have been implicated in the desensitization process (*20*). In fibroblast cells, growth regulatory and mitogenic signals, such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), and serum, have been shown to inhibit the ANP-induced activation of GC-A through a mechanism that at least in part involves dephosphorylation (*21*). This study also suggests that tyrosine kinase receptor-mediated pathways play a key role in the desensitization of GC-A. More recently, it was reported that in NIH3T3 fibroblasts serum, lysophosphatidic acid (LPA), and PDGF desensitized GC-B through an unknown mechanism (*22*).

Nonreceptor tyrosine kinase Csk (*C*-terminal Src kinase) was originally purified as a kinase capable of phosphorylating Src and other Src family kinases at their *C*-terminal tyrosine

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[1] Abbreviations: NP, natriuretic peptide; GC, guanylyl cyclase; LPA, lysophosphatidic acid; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; IBMX, 3-isobutylmethylxanthine; MEF, mouse embryonic fibroblast; PMA, phorbol myristate acetate.

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residues (*23*). Csk is ubiquitously expressed in mammalian cells and is evolutionarily conserved from early-diverging metazoan Hydra to humans (*24*). Mice that are deficient in Csk exhibited developmental defects (*25*, *26*). The Csk-deficient mouse embryos died around day 10, post gestation. Csk has Src-dependent and -independent physiological functions (*27*). Indeed, Csk is positively required for the normal development of lymphoid cells. Csk deficiency blocks T- and B-cell differentiation as is the case with an Src-family kinase deficiency (*28*).

We have previously demonstrated that the activation of nonreceptor tyrosine kinases in response to G-protein-coupled receptors such as receptors for LPA is a key step in the regulation of cellular growth, proliferation, and cytoskeletal reorganization (*29*−*34*). Given that several studies have indicated that the activation of nonreceptor tyrosine kinases is a critical event leading to the development of cardiomyocyte hypertrophy (*35*−*37*), we investigated the possibility of the existence of a nonreceptor-tyrosine-kinase-mediated pathway in the regulation of GC-A activity. Herein we show that the nonreceptor-tyrosine-kinase Csk negatively modulates the ANP-induced increases in GC-A activity and critically controls the serum- and LPA-induced inhibition of ANP-stimulated GC-A activity in mouse embryonic fibroblasts. Furthermore, we demonstrate that the catalytic activity of Csk is required for this regulation.

MATERIALS AND METHODS

Materials. Imidazole, 3-isobutyl-methylxanthine (IBMX), Triton X-100, leupeptin, pepstatin, aprotinin, PMSF, EDTA, creatine phosphate, creatine phosphokinase, ATP, and GTP were purchased from Sigma Aldrich (St.Louis, MO). Calphostin C was from Calbiochem. Rat ANP1-28 and CNP were purchased from Peninsula Laboratories or Bachem Laboratories. The direct cGMP EIA system was purchased from Assay Designs, Inc. All reagents used for cell cultures were of tissue-culture grade.

Cell Culture and Transient Transfection. All cell lines used for the experiments were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin−streptomycin, and L-glutamine at 37 °C and 5% CO_2. Cells, 80% confluent in six well dishes, were transfected with 2 μg of plasmid DNA using a Lipofectamine-2000 reagent following the manufacturer's instructions (*38*). The transfection efficiency was 30−60% based on transfection with a control GFP plasmid. Briefly, 1 h before transfection, the cells were washed once with PBS and incubated in DMEM without the serum and antibiotics. In two separate tubes, 2 μg of plasmid DNA and 6 μL of transfection reagent were dissolved in 100 μL each of DMEM. The solutions were allowed to stand at room temperature for 5 min. The two solutions were then gently mixed, and the DNA−reagent mixture was allowed to stand at room temperature for 20 min. This mixture was added to the medium over the cells, and the plate was rocked five to six times to ensure the uniform distribution of the reagent−DNA complex. The cells were incubated at 37 °C and 5% CO_2 for 5−6 h. The medium was aspirated and replaced with fresh growth medium. The cells were allowed to grow at 37 °C and 5% CO_2 for 18−24 h before switching to the starvation medium (DMEM supplemented with penicillin−streptomycin and L-Glutamine).

$Csk^{-/-}$ cells and the stable $Csk^{-/-}$/CskR318A cell line were described previously (*30*). The R318A mutant of human Csk cDNA in pcDNA3.1/hyg was transfected into $Csk^{-/-}$ cells. Stable cell lines (a pool of many clones) were selected with hygromycin.

Measurement of cGMP Levels in Whole-Cell Extracts. Eighteen to twenty-four hours after transfection, the cells were washed once with PBS and switched to the starvation medium. Sixteen to twenty-four hours after starvation, the cells were treated for 30 min with a DMEM medium containing 0.5 mM IBMX in the presence or absence of 10% serum or 10 μM LPA. This was followed by treatment with 200 nM ANP or vehicle for 3 min at 37 °C (*39*, *40*). The cells were then washed once with PBS at room temperature and lysed with 0.5% Triton X-100 containing 0.5 mM IBMX. The cGMP content present in the samples was determined by using the Direct cGMP EIA system following the acetylation method (Assay Designs). For comparison, cGMP levels obtained from separate experiments were normalized with respect to the amount of proteins present in the lysates. For assays in the presence of imidazole, 50 mM imidazole (pH 7.5) and 0.5 mM IBMX in growth medium was used (*30*). In some experiments, 200 nM PMA was used.

In Vitro Kinase Assays. Purified Csk (final concentration 200 nM) or Src (500 nM) was incubated with 2.5 μg of purified GST-GC-A-intra (amino acid residues 495−1061) in 30 mM Hepes at pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 100 nM sodium orthovanadate along with 5 μCi of [γ-^{32}P] ATP (3000 Ci/mmol) (Perkin-Elmer) or 1 mM cold ATP for 15 min at 30 °C (*30*, *41*). The reaction was stopped by adding an SDS−PAGE sample buffer and incubated at 90 °C for 5 min. Samples were then separated on a 10% SDS−PAGE gel. The gels were dried and autoradiographed or transferred to a nitrocellulose membrane and western blotted with anti-phospho-tyrosine antibodies (Cell Signaling). GST-CDB3 (2.5 μg) was used as a positive control (*29*, *34*).

Western Blots with Anti-Phospho-Ser/Thr Antibody. $Csk^{-/-}$ cells grown in 10-cm plates were cotransfected with pAX-Neo-GC-A and pcDNA3.1/Hygromycin-Csk or pAX-Neo-GC-A and pcDNA3.1/Hygromycin empty vector. After 24 h of starvation, the cells were treated with 10 μM LPA or 1 μM PMA for 30 min. The cells from one 10 cm plate were lysed in 1 mL of RIPA buffer containing 50 mM Tris at pH 8, 150 mM NaCl, 1 mM EDTA, 5 mM *n*-dodecyl-D-maltoside, 50 mM NaF, 200 μM Na_3VO_4, and protease inhibitors (*29*, *42*). The lysate was briefly sonicated and centrifuged at 13 000 rpm for 10 min. The supernatant was transferred to a fresh tube and pre-cleared by incubating with 30 μL of 50% protein A-agarose slurry. After centrifugation, the supernatant was collected and incubated with 1.5 μg of antiserum against GC-A (FabGennix, Inc., International, Shreveport, LA) overnight at 4 °C. Protein A-agarose (30 μL) was added, and the mixture was incubated for an additional 3 h at 4 °C. The agarose beads were collected by centrifugation and washed three times with a RIPA buffer. Proteins were eluted with an SDS sample buffer and subjected to western-blot analyses. Anti-phospho-serine/threonine antibodies were from BD Clontech.

RESULTS

LPA and Serum Do Not Exert an Inhibitory Effect on ANP-Stimulated cGMP Synthesis in Csk$^{-/-}$ Cells. To investigate the possibility that GC-A may be regulated by tyrosine kinases, we used cultured mouse embryonic fibroblasts (MEFs). MEF cells were grown to 80% confluence and serum-starved overnight. Treatment of the cells with 200 nM ANP for 3 min at 37 °C resulted in about a 30−60-fold increase in cGMP production, whereas similar concentrations of CNP resulted in only a modest increase in the intracellular cGMP content (Figure 1A). The relatively lower elevation in intracellular cGMP levels when MEF cells were treated with CNP compared to that obtained when stimulated with similar concentrations of ANP shows that the majority of endogenously expressed NP receptors in MEF cells are GC-A. Previous reports showed that serum, LPA, and PDGF were able to desensitize GC-B in fibroblast cells (*4*, *22*). Therefore, we tested if endogenous GC-A in MEF cells is also inhibited by LPA and serum. Treatment of serum-starved MEF cells with 10% FBS or 10 μM LPA for 30 min resulted in about 60% inhibition of ANP-induced elevation in the intracellular cGMP levels (Figure 1B). Serum and LPA also inhibited CNP-stimulated elevations in the intracellular cGMP levels in MEF cells to a similar extent (data not shown).

Serum and LPA initiate downstream signaling including tyrosine-kinase- and G-protein-mediated pathways (*43*−*45*). Previous studies from our laboratory have established that nonreceptor-tyrosine-kinase Csk is essential for the cytoskeletal rearrangements induced by serum and LPA in MEF cells (*30*). Nonreceptor tyrosine kinases are also implicated in the regulation of sarcomeric organization as well as hypertrophic gene expression (*37*). Therefore, we examined whether Csk plays a role in the regulation of ligand-dependent activity of GC-A in MEF cells. For this purpose, we used embryonic fibroblasts derived from knockout mice that lack the nonreceptor-tyrosine-kinase Csk (Csk$^{-/-}$) (*30*). Treatment of Csk$^{-/-}$ cells with 200 nM ANP resulted in about an 80−100-fold increase in cellular cGMP levels (Figure 1C), whereas treatment with 200 nM CNP resulted in only a modest increase in the cGMP content (data not shown). The relatively lower elevation in the intracellular cGMP levels upon stimulation with CNP again shows that the majority of the receptors expressed in Csk$^{-/-}$ cells are GC-A. In contrast to the observation in MEF cells, treatment of Csk$^{-/-}$ cells with 10% FBS or 10 μM LPA for 30 min did not result in any statistically significant changes in ANP-stimulated cGMP production (Figure 1C). These data strongly indicate that Csk is a negative modulator of the ANP-induced increase in cGMP and is an essential mediator of serum- and LPA-induced attenuation of ANP-stimulated cGMP synthesis in MEF cells.

To further confirm this conclusion, we transiently transfected Csk$^{-/-}$ cells with a DNA plasmid encoding human Csk and measured the changes in ANP-induced cGMP production. Re-expression of Csk in Csk$^{-/-}$ cells resulted in ANP-stimulated cGMP levels about 3-fold lower than that observed in vector-transfected cells (Figure 2). Moreover, re-expression of Csk rescued the inhibitory effect of LPA on ANP-induced cGMP production (Figure 2).

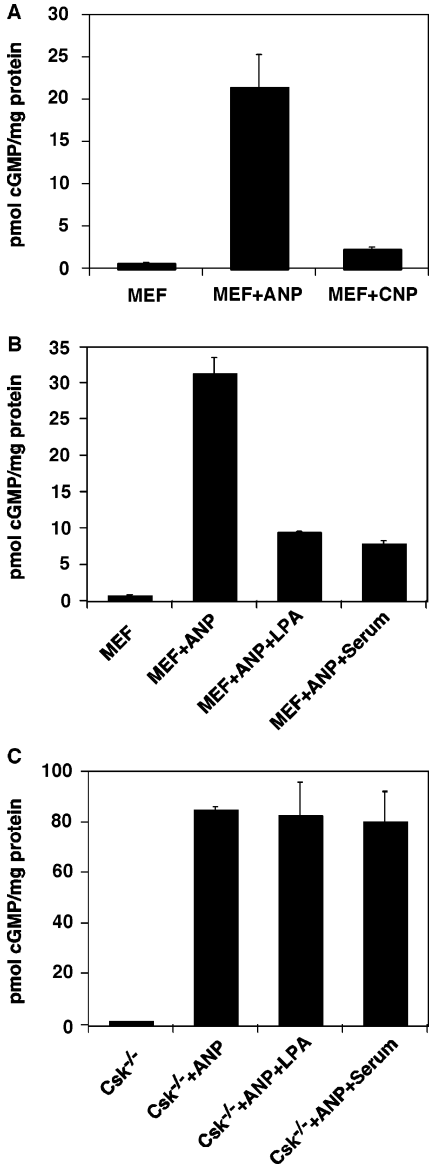

FIGURE 1: LPA and serum do not exert an inhibitory effect on ANP-stimulated cGMP synthesis in Csk$^{-/-}$ cells. (A) Elevation in the intracellular cGMP levels in MEF cells in response to ANP and CNP treatment. MEF cells were cultured in six-well dishes to 80% confluence. After serum starvation for 18−24 h, the cells were treated with a starvation medium containing 0.5 mM IBMX for 30 min at 37 °C, followed by treatment with 200 nM ANP or 200 nM CNP for 3 min at 37 °C. The cells were washed once with PBS and lysed with 0.5% Triton X-100 containing 0.5 mM IBMX, and the cGMP content was determined. (B) LPA (10 μM) and serum (10% FBS) attenuate ANP-induced cGMP synthesis in MEF cells. (C) ANP response is enhanced, and LPA and serum do not exert an inhibitory effect on ANP-stimulated cGMP synthesis in Csk$^{-/-}$ cells. Data represent the means ± SD of three experiments.

Catalytic Activity of Csk Is Essential for Its Regulation of Receptor-Guanylyl-Cyclase Activity. To determine if the catalytic activity of Csk is essential for its inhibitory effect on GC-A, we used Csk$^{-/-}$ cells stably expressing the Csk R318A mutant, whose catalytic activity is impaired but can be chemically rescued and activated by small organic compounds such as imidazole (*30*, *46*). Using this mutant cell line, we previously demonstrated that the catalytic activity of Csk is required for LPA- and serum-induced cytoskeletal reorganization in MEF cells (*30*). As shown in Figure 3A, ANP-stimulated cGMP production in CskR318A

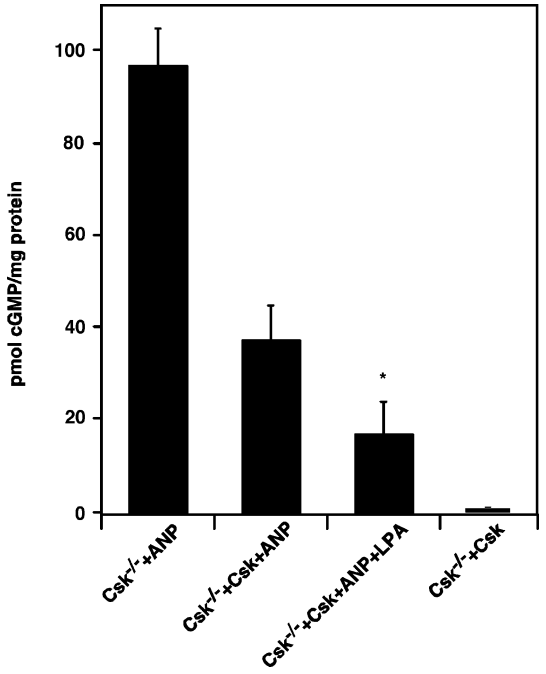

FIGURE 2: Transient re-expression of Csk in $Csk^{-/-}$ cells results in attenuation of ANP-stimulated cGMP production and reconstitution of LPA inhibition. $Csk^{-/-}$ cells grown to 70−80% confluence in six-well dishes were transiently transfected with a vector plasmid (Column 1) or plasmids carrying human Csk (Columns 2 and 3) as described in experimental protocols. Sixteen to eighteen hours after transfection, the cells were serum-starved overnight and treated for 30 min at 37 °C and 5% CO_2 with a medium containing LPA and 0.5 mM IBMX. This was followed by treatment with 200 nM ANP at 37 °C for 3 min. The medium was aspirated, and the cells were washed once with PBS, lysed with 0.5% Triton X-100 containing 0.5 mM IBMX, and the cGMP present in the samples was determined. The symbol * indicates that the cGMP level from $Csk^{-/-}$ + Csk + ANP is significantly different from that from $Csk^{-/-}$ + Csk + ANP + LPA (*t*-test: $p < 0.05$). The values shown represent the means ± SD of three experiments.

cells was upto 10-fold lower in the presence than in the absence of imidazole. Imidazole had no effect on the EC_{50} of ANP for GC-A (EC_{50} ~28 nM). As a control, the treatment of $Csk^{-/-}$ cells (without the CskR318A mutant) with imidazole did not result in a statistically significant change in ANP-stimulated cGMP production (Figure 3B). The transient expression of CskR318A in $Csk^{-/-}$ cells (without imidazole addition) did not lead to any significant changes in ANP-stimulated cGMP synthesis (Figure 3B). These results demonstrate that the catalytic activity of Csk is essential for its observed inhibitory effect on ANP-stimulated cGMP synthesis in MEF cells.

Attenuation of ANP-Stimulated cGMP Synthesis by Csk Does Not Require PKC Activity. Because GC-A is known to be heterologously desensitized by PKC, possibly working through a phosphatase to dephosphorylate GC-A, we used a general PKC inhibitor, Calphostin C, to determine if the inhibition of GC-A by Csk is occurring through a PKC-dependent pathway. As shown in Figure 4A, Calphostin C blocked the phorbol-myristate-acetate (PMA, a pharmacological activator of PKC)-induced inhibition of GC-A but not the LPA-initiated inhibition (Figure 4A). The treatment of $Csk^{-/-}$ cells with Calphostin C had no effect on ANP-induced cGMP production (Figure 4B). The expression of the CskR318A mutant in $Csk^{-/-}$ cells (without the addition

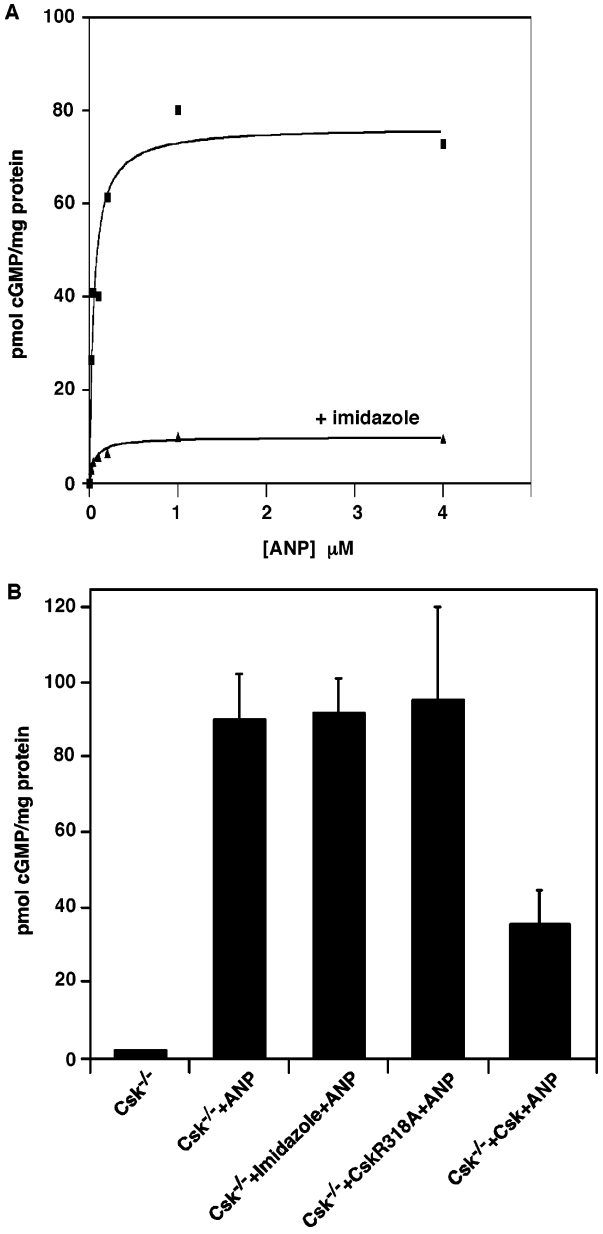

FIGURE 3: Catalytic activity of Csk is essential for its regulation of receptor-guanylyl-cyclase activity. (A) $Csk^{-/-}$ cells stably expressing the CskR318A mutant were grown to 80% confluence in six-well dishes. Following serum starvation overnight, the cells were treated with a medium containing LPA and 0.5 mM IBMX for 30 min at 37 °C in the presence (▲) or absence (■) of 50 mM imidazole. This was followed by treatment with indicated concentrations of ANP for 3 min at 37 °C. The cells were then washed once with PBS and lysed with 0.5% Triton X-100 containing 0.5 mM IBMX, and the cGMP content present in the samples was determined. (B) Treatment of $Csk^{-/-}$ cells with imidazole (Column 3) or the transient expression of CskR318A in $Csk^{-/-}$ cells (without imidazole addition) (Column 4) did not lead to significant changes in ANP-stimulated cGMP production. Data are representative of three similar experiments.

of imidazole) did not suppress ANP stimulation of GC-A, confirming the impaired catalytic activity of the CskR318A mutant. The addition of imidazole rescued the catalytic activity of CskR318A, leading to the inhibition of ANP-induced cGMP production. Moreover, the treatment of CskR318A cells with imidazole in the presence Calphostin C resulted in no significant change in the magnitude of Csk inhibition on ANP-induced cGMP production (Figure 4B).

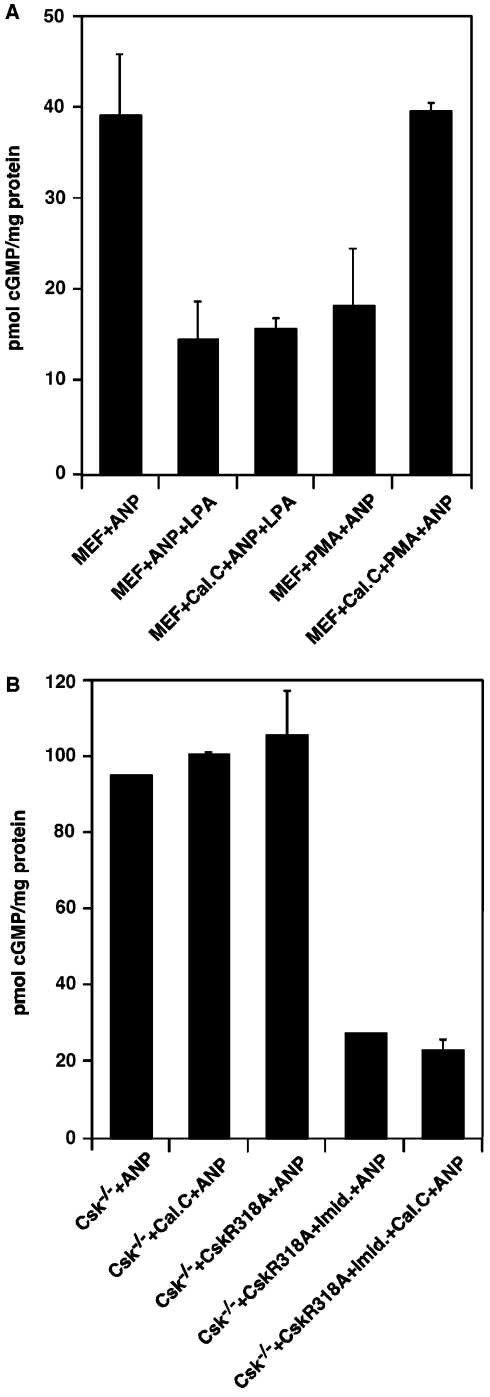

FIGURE 4: Attenuation of ANP-stimulated cGMP synthesis by Csk does not require PKC activity. (A) MEF cells were serum-starved overnight. After pretreatment with Calphostin C or the vehicle, the cells were treated with ANP, LPA, and then ANP, or PMA and then ANP for 3 min. The cells were then washed once with PBS and lysed with 0.5% Triton X-100 containing 0.5 mM IBMX, and the cGMP content present in the samples was determined. (B) $Csk^{-/-}$ cells grown to 70−80% confluence in six-well dishes were transiently transfected with CskR318A or an empty vector (pcDNA3.1). Sixteen to twenty-four hours after transfection, the cells were serum-starved overnight. After pretreatment with Calphostin C or the vehicle, the cells were treated with a growth medium containing 50 mM imidazole and 0.5 mM IBMX for 30 min at 37 °C. This was followed by treatment with 200 nM ANP for 3 min at 37 °C and 5% CO_2. The cells were then washed once with PBS and lysed with 0.5% Triton X-100 containing 0.5 mM IBMX, and the cGMP content present in the samples was determined. Data represent the means ± SD of three experiments.

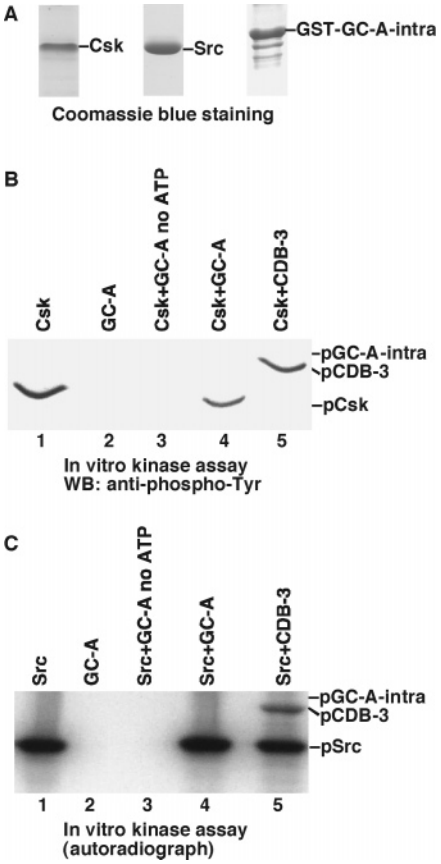

FIGURE 5: Csk does not directly phosphorylate GC-A. (A) Coomassie blue staining of purified recombinant Csk, Src, and GST-GC-A intra (the intracellular domain of GC-A). (B) In vitro kinase assay of Csk using GST-CDB3 and GST-GC-A as exogenous substrates. The in vitro kinase assay was performed in the presence of ATP, and the results were analyzed with SDS−PAGE and western blotted with anti-phospho-Tyr antibody. (C) In vitro kinase assay of Src using GST-CDB3 and GST-GC-A-intra as substrates. The kinase assay was performed in the presence of γ-^{32}P-ATP, and the results were analyzed by SDS−PAGE and autoradiography.

This implies that the observed inhibitory effect of Csk on ANP-induced cGMP synthesis in MEF cells is not mediated by PKC.

Csk Does Not Phosphorylate GC-A. To learn the biochemical mechanism by which Csk mediates the LPA inhibitory effect on GC-A, we first tested the possibility that Csk might directly phosphorylate GC-A, leading to the inhibition of GC-A activity. We purified the recombinant Csk and the GST-fusion of the intracellular domain of GC-A (residue 495 to the *C*-terminal end) from *E. coli* (*30*) (Figure 5A). In the presence of ATP, Csk phosphorylated an exogenous purified substrate GST-CDB3 or autophosphorylated itself (*29*) (Figure 5B). We noticed that in the presence of a phosphorylatable exogenous substrate Csk would phosphorylate the exogenous substrate with little autophosphorylation. In the absence of a phosphorylatable exogenous substrate, Csk autophosphorylated. These protein tyrosine phosphorylation events were monitored with an anti-phospho-tyrosine antibody. However, Csk did not phosphorylate GST-GC-A-intra (Figure 5B). Therefore, Csk could not directly phosphorylate GC-A.

Csk was originally purified as a tyrosine kinase capable of phosphorylating the *C*-terminal tyrosine residue of Src-family tyrosine kinases (*23*). We next sought to examine

whether Src could directly phosphorylate GC-A. We purified the recombinant Src from Sf9 insect cells (*34*) (Figure 5A). In the presence of [γ-^{32}P]ATP, Src autophosphorylated itself and the exogenous substrate GST-CDB3 but not GST-GC-A-intra (Figure 5C). Furthermore, immunoprecipitated GC-A from HEK-293 cells and MEF cells was not tyrosine phosphorylated when the immunoprecipitates were probed with anti-phospho-tyrosine antibodies (data not shown). Hence, these data demonstrate that direct tyrosine phosphorylation of GC-A is not the mechanism by which Csk mediates LPA's inhibitory effect.

LPA Decreased the Ser/Thr Phosphorylation of GC-A in the Presence of Csk. Previously, it has been shown that the activation of protein kinase C (by PMA) led to the activation of protein Ser/Thr phosphatase to decrease the Ser/Thr phosphorylation of GC-A and the inhibition of GC-A activity (*17*). Furthermore, it is known that Ser/Thr phosphorylation of GC-A is required for ANP activation of GC-A (*17*). Therefore, there is a correlation of a decrease in Ser/Thr phosphorylation of GC-A and a decrease of GC-A activity. Although we had shown that Csk-mediated inhibition does not involve PKC (Figure 4), we investigated whether Csk-mediated inhibition involves a decrease in Ser/Thr phosphorylation of GC-A (Figure 6). We treated starved Csk$^{-/-}$/Csk cells with LPA or PMA. The GC-A protein was immunoprecipitated with an anti-GC-A antibody and western blotted with anti-phospho-Ser/Thr antibody (Figure 6 A and B). Both LPA and PMA induced a decrease of Ser/Thr phosphorylation of GC-A, indicating that LPA-induced inhibition of GC-A involves a decrease in Ser/Thr phosphorylation of GC-A, similar to protein kinase C activation. Similar results were obtained with MEF cells (data not shown). More importantly, this LPA-induced reduction of Ser/Thr phosphorylation of GC-A was dependent on Csk. In Csk$^{-/-}$ cells, although PMA induced a decrease in GC-A Ser/Thr phosphorylation, LPA treatment did not (Figure 6 C and D). These results demonstrate that Csk is required for LPA-induced reduction of Ser/Thr phosphorylation of GC-A. Furthermore, comparing the percentage of Ser/Thr phosphorylated GC-A in total GC-A, there was a 50% increase in GC-A Ser/Thr phosphorylation in Csk$^{-/-}$ cells (Figure 6 B and D). This result is consistent with the higher ANP-induced activity of GC-A in Csk$^{-/-}$ cells than in MEF cells (Figure 1). Therefore, the molecular mechanism by which Csk mediates LPA-induced GC-A inhibition likely involves a reduction of the Ser/Thr phosphorylation of GC-A.

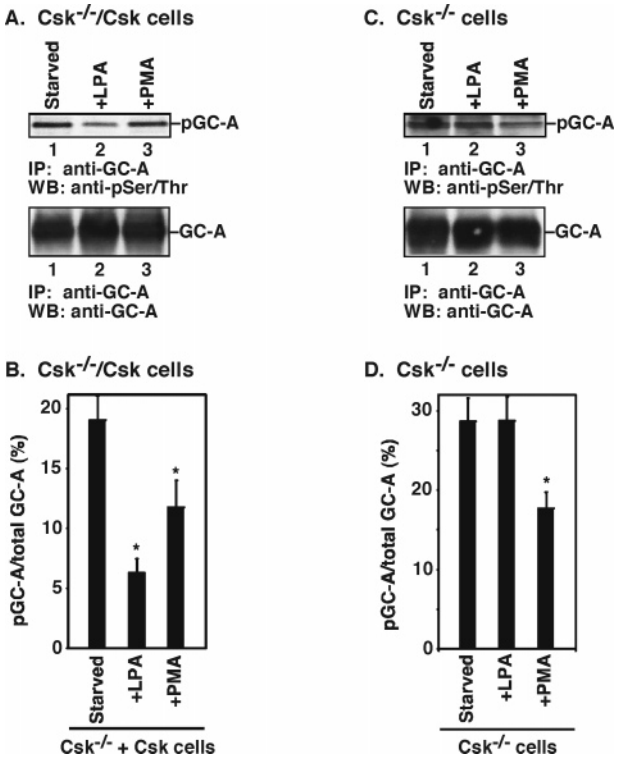

FIGURE 6: LPA-induced decrease of Ser/Thr phosphorylation of GC-A depends on Csk. (A) Csk$^{-/-}$/Csk cells were serum-starved. The cells were then treated with LPA or PMA. The cell lysates were immunoprecipitated with anti-GC-A antibody. After SDS−PAGE, the filters were probed with anti-phospho-Ser/Thr antibody (top panel) or anti-GC-A antibody (bottom panel). (B) Intensity of each band in A was quantified by an image analyzer. The percentage of phosphorylated GC-A is corrected by the amount of the total GC-A. (C) Csk$^{-/-}$ cells were serum-starved. The cells were then treated with LPA or PMA. The cell lysates were immunoprecipitated with anti-GC-A antibody. After SDS−PAGE, the filters were probed with anti-phospho-Ser/Thr antibody (top panel) or anti-GC-A antibody (bottom panel). (D) The intensity of each band in C was quantified by an image analyzer. The percentage of phosphorylated GC-A is corrected by the amount of the total GC-A. The symbol * indicates a significant difference from the untreated cells (*t*-test: $p < 0.05$). Data represent the means ± SD of three experiments.

DISCUSSION

In this study, we show that a nonreceptor tyrosine kinase is involved in the regulation of natriuretic peptide-induced activity of membrane-bound guanylyl cyclase. We showed that the marked inhibition of the ANP-induced generation of cGMP by serum and LPA was completely blocked by the deletion of Csk in MEF cells. The inhibitory effect of Csk was fully restored by the reintroduction of Csk in Csk$^{-/-}$ cells. These observations demonstrate that Csk is a negative modulator of GC-A activity and that Csk activation is essential for serum and LPA attenuation of ANP-stimulated GC-A activity in MEF cells.

Using a newly developed chemical rescue approach, we also demonstrated that the catalytic activity of Csk is necessary for its negative modulatory effect on GC-A. It has previously been shown that the catalytic activity of an inactive Csk mutant, CskR318A, can be rescued by chemical compounds such as imidazole in vitro and in vivo (*30*, *46*). Csk$^{-/-}$ MEF cells transfected with the inactive CskR318A failed to show the characteristic inhibitory action of serum and LPA on ANP-stimulated GC-A activity. This inhibitory action was rescued upon treatment of the cells with imidazole, demonstrating that the rescued catalytic activity of CskR318A is critical to the inhibitory effect of Csk.

It has been reported that PKC, possibly working through a phosphatase to dephosphorylate GC-A, inhibits GC-A activity (*20*). However, we observed no effect of a general PKC inhibitor, Calphostin C, on Csk-mediated inhibition of GC-A activity. Nevertheless, our data suggests that the mechanism of inhibition of GC-A by Csk involves changes on GC-A modification. Indeed, we have shown that in the absence of Csk GC-A is highly Ser/Thr phosphorylated. It is known that GC-A under basal conditions is phosphorylated on six known Ser/Thr sites within its kinase homology domain (*17*, *47*). A single mutation of any one of these phosphorylation sites to alanine decreases receptor activity.

The mutation of all of these phosphorylation sites to alanine yields a receptor that is unresponsive to the ligand ANP (*17*). Desensitization of GC-A is correlated with a decrease in Ser/Thr phosphorylation (*48*). Our finding reveals that Csk-mediated LPA inhibition of GC-A might also involve a decrease in Ser/Thr phosphorylation of GC-A. It would be interesting to investigate whether Csk is involved in GC-A homologous desensitization after ANP treatment. Given that the re-expression of Csk in $Csk^{-/-}$ cells resulted in ANP-stimulated cGMP levels about 3-fold lower than that observed in vector-transfected cells (Figure 2), Csk might be involved in GC-A homologous desensitization. However, Csk could lead to the de-phosphorylation of GC-A and thus reduce the ANP-induced cGMP accumulation without being directly involved in the GC-A homologous desensitization after ANP treatment. Because both PKC and Csk could lead to GC-A desensitization, though via different pathways, PKC and Csk could employ the same or different phosphatases to inhibit GC-A. The underlying biochemical mechanism is the same for both PKC and Csk-mediated desensitization, that is, the dephosphorylation of GC-A.

Currently, it is not clear which kinase(s) or phosphatase(s) is responsible for the phosphorylation and dephosphorylation of GC-A. Interestingly, in a yeast two-hybrid screen, protein phosphatase 5 (PP5) was shown to interact with the kinase homology domain of GC-A (*49*). Although this interaction has not been reported in mammalian cells, PP5 has also been reported to interact with $G\alpha_{12}$ and $G\alpha_{13}$, which mediate the signaling from G-protein-coupled LPA receptors (*50*). Further investigation is needed to examine whether PP5 indeed mediates the LPA-initiated inhibition of GC-A.

ANP, via the activation of GC-A receptors, inhibits cardiomyocyte growth, promotes the relaxation of preconstricted vasculature, and is a potent antihypertensive and antihypertrophic agent. However, overactivation of several nonreceptor tyrosine kinases has been implicated in abnormal cellular growth, vasoconstriction, and development of cardiovascular diseases such as hypertension and cardiac hypertrophy (*35*−*37*). Taking these findings in conjunction with the present data, we postulate that the effects of Csk and possibly other tyrosine kinases on contraction, vascular tone, activation of the MAPK pathway, growth, and hypertrophy may be mediated in part by their inhibition of the powerful vasodilatory, antiproliferative, and antihypertrophic effects of natriuretic peptides.

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