Phosphorylation of GTP Dissociation Inhibitor (GDI) by PKA Negatively Regulates RhoA

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Running Head: PKA-phosphorylated GDI negatively regulates RhoA

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The cAMP-PKA cascade is a recognized signaling pathway important in inhibition of inflammatory injury events such as endothelial permeability and leucocyte trafficking, and a critical target of regulation is believed to be inhibition of Rho proteins. Here, we hypothesize that PKA directly phosphorylates GDI (GTP Dissociation Inhibitor) to negatively regulate Rho activity. Amino acid analysis of GDI\(\alpha\) showed two potential PKA phosphorylation motifs, Ser\(^{174}\) and Thr\(^{182}\). Using \textit{in vitro} kinase assay and mass spectrometry, we found that the purified PKA catalytic subunit phosphorylated GDI\(\alpha\)-GST fusion protein and PKA motif-containing GDI\(\alpha\) peptide at Ser\(^{174}\), but not Thr\(^{182}\). Transfection of COS 7 cells with mutated full-length GDI\(\alpha\) at Ser\(^{174}\) to Ala\(^{174}\) (GDI\(\alpha\)-Ser\(^{174}\)A) abrogated the ability of cAMP to phosphorylate GDI\(\alpha\). However, mutation of Thr\(^{182}\) to Ala\(^{182}\) (GDI\(\alpha\)-Thr\(^{182}\)A) did not, and cAMP increased phosphorylation of GDI\(\alpha\) to a similar extent as wtGDI\(\alpha\) transfectants. The mutant GDI\(\alpha\)-Ser\(^{174}\)A, but not GDI\(\alpha\)-Thr\(^{182}\)A, was unable to prevent cAMP-mediated inhibition of Rho-dependent SRE reporter activity. Further, the mutant GDI\(\alpha\)-Ser\(^{174}\)A was unable to prevent the thrombin-induced RhoA activation. Co-precipitation studies indicated that neither mutation of the PKA consensus sites nor phosphorylation alter GDI\(\alpha\) binding with RhoA, suggesting that phosphorylation of Ser\(^{174}\) regulated pre-formed GDI\(\alpha\)-RhoA complexes. The findings provide strong support that the selective phosphorylation at Ser\(^{174}\) by PKA is a signaling pathway in the negative regulation of RhoA activity, and therefore could be a potential protective mechanism for inflammatory injury.

Keywords: cAMP-dependent protein kinase, PKA consensus phosphorylation sites, single-site mutated GDI\(\alpha\)
INTRODUCTION

Rho is a member of the superfamily of Rho GTPases (Rho, Rac, and Cdc42), and is a critical signaling intermediate in regulation of vascular inflammatory activities, such as increases in endothelial permeability \((7; 11; 21; 38; 39; 41)\) and leukocyte extravasation \((1; 2)\). Therefore, there is much current interest in understanding mechanisms that could inhibit its activity. In the vascular endothelium, the cAMP signaling cascade is a recognized protective pathway against inflammatory activities through its direct targets PKA \((26; 36; 40)\) \((8)\) and EPAC1 \((8; 13; 23; 37)\). We \((36)\) and others \((13; 18)\) have found that Rho proteins are potential direct targets of the cAMP-mediated protective pathway which could be responsible in prevention of inflammatory activities in endothelial cells.

Although the precise mechanisms by which cAMP inhibits Rho remain yet to be fully delineated, there is clear evidence that the cAMP-activated PKA can inhibit Rho by multiple pathways, including direct phosphorylation of RhoA \((17; 24)\) or phosphorylation of upstream determinants controlling Rho activity such as regulator of G protein signaling \((3)\) and \(G\alpha_{13}\) \((28)\). In endothelial cells, Essler and coworkers \((18)\) observed that RhoA is not phosphorylated by an elevation of intracellular cAMP, suggesting that the preferred target(s) of regulation are factors upstream of RhoA.

Therefore, we postulate that one such possible upstream regulator is GDI (\textit{GDP Dissociation Inhibitor}), which functions to prevent GDP dissociation from Rho proteins. It is well-established that the activation of Rho proteins is determined by binding of GTP, and inactivation by GDP, with this cycling of GTP/GDP binding controlled by GDI, GEF (\textit{GDP Exchange Factor}) which facilitates exchange of GDP for GTP, and GAP (GTPase-Activating Protein) which stimulates hydrolysis of GTP to GDP. Of these, GDI is a pivotal regulator that
controls access of Rho proteins to GEF and GAP, as well as effector targets (20). Recent evidence has linked specific sites phosphorylated by PKCα (22), PAK1 (16), and Src (15) to selective functions of GDI.

We have already reported that PKA increased GDI phosphorylation in endothelial cells (36), but a direct phosphorylation by PKA and specific GDI residues phosphorylated were not determined. Here, we hypothesize that PKA directly phosphorylates GDI to negatively regulate Rho activity. The specific goals of the study were to identify the residues on GDI phosphorylated directly by PKA, and the effects of their phosphorylation on Rho function. The findings indicate that of the two predicted PKA consensus phosphorylation sites (Ser^{174} and Thr^{182}), only Ser^{174} was phosphorylated as determined by in vitro kinase activity assay and transfection studies of single-point mutations of human GDIα. The phosphorylation of GDIα-Ser^{174} resulted in inhibition of RhoA activity as evaluated by SRE-reporter activity and affinity binding for rhotekin. The results indicate that phosphorylation of GDIα at Ser^{174} by PKA suppresses RhoA activity, providing a potential protective signaling mechanism for inflammatory injury.

**MATERIALS and METHODS**

**Materials**

The following reagents were purchased from commercial sources. Amersham Pharmacia Biotech (Piscataway, NJ): ECL Kit, protein A sepharose CL-4B, horseradish peroxidase-conjugated anti-rabbit IgG antibodies; Cytoskeleton, Inc. (Denver, CO): human GDIα-GST Protein; Gibco, BRL (Gaithersburg, MD): Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, phosphate-buffered saline (PBS), LipofectAMINE; HyClone
GDI peptide synthesis

PKA is a cAMP-dependent serine/threonine protein kinase and phosphorylates its substrates through recognition of consensus motifs (Arg-X-Ser/Thr or Arg-Arg/Lys-X-Ser/Thr). Our amino acid sequence analysis of GDIα showed that there are possibly two potential PKA consensus phosphorylation sites (Arg-Gly-Ser$_{174}$ and Arg-Phe-Thr$_{182}$) located near the C-terminus (Fig. 1). We designed GDIα peptides which contain the PKA phosphorylation consensus sites as follows. Peptide$^{S174/T182}$ contained both consensus PKA phosphorylation sites: Met-Leu-Ala-Arg-Gly-Ser$_{174}$-Tyr-Ser-Ile-Lys-Ser-Arg-Phe-Thr$_{182}$-Asp-Asp. A negative control peptide was made containing the same sequence except Ser$_{174}$ and Thr$_{182}$ were changed to Ala$_{174}$ and Ala$_{182}$, respectively. Peptides containing a single PKA phosphorylation site were synthesized as: Met-Leu-Ala-Arg-Gly-Ser$_{174}$-Tyr-Ser (Peptide$_{S174}$); Ile-Lys-Ser-Arg-Phe-Thr$_{182}$-Asp-Asp (Peptide$_{T182}$).

The peptides were synthesized by solid phase peptide synthesis with Fmoc (9-fluorenylmethyl-oxy carbonyl) chemistry (Research Resources Core Facility, University of
Illinois, Chicago, IL). Synthesis took place from C-terminus to N-terminus; the C-terminal Fmoc-amino acid (Anaspec, Inc., CA) was attached to an insoluble support resin via an acid labile linker. Fmoc group of this amino acid was deprotected by 20% piperidine. The second Fmoc-amino acid was coupled to the C-terminal residue using the activator, 0.1 M HBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] in DMF (dimethylformamide) containing 0.4 M 4-methyl morpholine for 60 min and later washed to remove all unbound amino acid. Fmoc groups were again removed and the cycle was continued to generate the peptide. The resin bound peptide was deprotected of its side chain protection groups as well as cleaved from the resin using trifluoroacetic acid (TFA). Ethyl ether was added to precipitate the peptide from the TFA solution. The precipitated peptide was lyophilized and was then characterized by MALDI (matrix assisted laser desorption/ionization) mass spectrometry (Voyager, DE PRO, Applied Biosystems, Foster City, CA). The peptide was verified by HPLC chromatogram and N-terminus sequencing.

**In vitro phosphorylation**

*In vitro* phosphorylation was made by reacting purified PKA catalytic subunit with purified recombinant GDIα-GST fusion protein or synthetic GDIα peptides. The reaction mixture contained either 5 μg GDIα-GST or 10 μg GDIα peptides, 25 U (units) of PKA from bovine heart (30-65 units/μg protein), and 20 μM ATP in a reaction buffer [50 mM Tris (pH 7.4), 10 mM MgCl]. For control, 7.7 μg Kemptide and 25 U PKA were added into the reaction buffer. The reactions were incubated at 30 °C for 10 min, and immediately analyzed by mass spectrometry. For determination of phosphorylation, a mass spectrometric value of an increase of 80 daltons (Da) would be indicative of incorporation of one phosphate into a
protein. In brief, samples were spotted onto a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) target and analyzed by a Voyager-DE PRO Mass Spectrometer (Applied Biosystems, Foster city, CA) equipped with a 337 nm pulsed nitrogen laser. Peptide mass was measured using a positive-ion linear mode over the 1000-6500 m/z range. External mass calibration was performed using peaks of a mixture of bradykinin fragments 1-7 at 757 Da (daltons), angiotensin II (human) at 1046 Da, P14R (synthetic peptide) at 1533 Da, and adrenocorticotropic hormone fragment 18-39 (human) at 2465 Da.

**Mutagenesis of GDI\(\alpha\)**

Single mutations of full-length human GDI\(\alpha\) were made with the use of the QuikChange® Site-directed Mutagenesis Kit. The wild-type GDI\(\alpha\) cDNA (wtGDI\(\alpha\)) (GenBank # AF498926) was obtained from Guthrie Research Institute (Sayre, PA) which has been cloned into pcDNA3.1+ (Invitrogen, Carlsbad, CA). One pair of mutagenic primers was designed to replace Ser\(^{174}\) with alanine (Sense: 5'-CTG GCC CGG GGC gcC TAC AGC ATC AAG-3'; Antisense: 5'-CTT GAT GCT GTA Ggc GCC CCG GGC CAG-3'). Another pair of mutagenic primers was designed to replace Thr\(^{182}\) with alanine (Sense: 5'-C AAG TCC CGC TTC gCA GAC GAC GAC AAG ACC-3'; Antisense: 5'- GGT CTT GTC GTC GTC TGc GAA GCG GGA CTT G-3'). The mismatched nucleotides are indicated by lower case letters. The PCR reaction was set up using the primers and plasmid pcDNA 3.1 containing wtGDI\(\alpha\) according to the KIT instruction. Extension of the oligonucleotide primers generated mutated plasmids containing either GDI\(\alpha\)-Ser\(^{174}\)A (Ser\(^{174}\) changed to Ala\(^{174}\)) or GDI\(\alpha\)-Thr\(^{182}\)A (Thr\(^{182}\) changed to Ala\(^{182}\)). Following temperature cycling, the products were treated with Dpn I to digest the parental DNA template and to select for mutation-containing synthesized DNA.
The plasmids, wtGDIα, GDIα-Ser^{174A} and GDIα-Thr^{182A} were transformed into XL1-Blue supercompetent cells and grown on agar plates containing the antibiotic ampicillin. Positive clones were selected and verified by sequencing.

**Transfection studies**

*GDIα constructs:* COS 7 cells were cultured in DMEM containing 4.5 g/l glucose, 5% FBS, 1% penicillin-streptomycin, and grown in culture dishes until 60-70% confluent. The medium was replaced with serum-free DMEM, and the cells were transfected with 1 ~ 2 μg of wtGDIα, GDIα-Ser^{174A} or GDIα-Thr^{182A} using LipofectAMINE according to standard transfection protocol. After incubation for 4 hr at 37°C, the medium was replaced with DMEM containing 10% FBS, and cells incubated overnight and used for studies as described.

*Rho-dependent SRE-driven reporter:* The luciferase reporter plasmid containing SRE (serum response element) was kindly provided by Dr. Dolly Mehta (University of Illinois, Chicago, IL) for evaluation of the Rho-dependent transcription activity. The SRE-driven transcriptional activation requires SRF, which is an ubiquitous transcription factor shown to be regulated by mediators and other factors (i.e., lysophosphatidic acid, thrombin, serum, and stress) in a Rho-dependent manner (19; 29). For these assays, COS 7 cells were plated on 12-well dishes, grown to 70-80% confluence, and cotransfected with 1 μg of the SRE-luciferase plasmid plus either 1 μg of wtGDIα, GDIα-Ser^{174A} or GDIα-Thr^{182A} as described. Following transfection, the cells were treated according to experimental protocol, and collected for assay of luciferase activity using Promega Luciferase Kit according the manufacture’s protocol. The luciferase activity was measured with a Femtomaster FB12
luminometer (Zylux Corporation; Maryville, TN). Assays were made in duplicates per group and luciferase activity was normalized to μg protein from each of the duplicates.

*Recombinant adenovirus:* For overexpression of the PKA inhibitor gene using the recombinant adenovirus, an E1-, E3- replication-deficient adenovirus containing full length PKI cDNA (AdPKI) was constructed and characterized as described previously (26). The gene product of PKI binds with high affinity and selectivity to PKA (9; 25; 26; 30). COS 7 cells were infected with AdPKI at 100 MOI (Multiplicities Of Infection = plaque forming units/target cell) for overnight, and were transfected with 1 μg of wtGDIα, GDIα-Ser<sup>174</sup>A or GDIα-Thr<sup>182</sup>A using LipofectAMINE according to transfection protocol. The Adnull, which has no inserted gene, served as control virus.

**In situ cellular phosphorylation**

The effects of cAMP on GDIα phosphorylation in cells were determined using the PhosphoProtein Purification Kit. COS 7 cells transfected with wtGDIα, GDIα-Ser<sup>174</sup>A or GDIα-Thr<sup>182</sup>A were treated according to experimental protocol, and lysed in PhosphoProtein Lysis Buffer which contained the zwitterionic detergent CHAPS and a mixture of protein inhibitors. The supernatant from the cell lysate (2 mg for each group) was poured into the PhosphoProtein Purification Column pre-equilibrated with PhosphoProtein Lysis Buffer. The columns were washed with Lysis Buffer to remove unphosphorylated proteins, and the phosphorylated proteins eluted with the PhosphoProtein Elution Buffer containing CHAPS. The phosphorylated proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western blot analysis made with anti-GDI antibody. Bands corresponding to
phosphorylated GDI (~25 Kd) were quantified by scanning densitometry (Scion Image, Beta, 4.0.2; Frederick, MD).

**Affinity-binding assay for RhoA-GTP**

The GTP-bound form of RhoA was determined by affinity-binding assay to evaluate RhoA activation as previously described (21; 36). In brief, glutathione-S-transferase-C21 fusion protein (rhotekin, a Rho target molecule) was prepared from induction of cultures of transformed *E. coli* with 0.1 mM isopropylthiogalactoside. COS 7 cells were grown to 60-70% confluence, and transfected with either pcDNA3.1 empty vector or containing wtGDIα, GDIα-Ser<sup>174</sup>A, GDIα-Thr<sup>182</sup>A, respectively using Lipofectamine. At 24 hr post-transfection, the cells were treated according to experimental protocol, and collected in GST-FISH buffer [50 mM Tris (pH 7.4), 10% glycerol, 100 mM NaCl, 1% NP-40, 2 mM MgCl₂, 25 mM NaF and 1 mM EDTA] plus protease inhibitor cocktail (10 μg/ml of pepstatin A, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF). Cell lysates were pelleted by centrifugation at 10,000 g at 4°C for 5 min, and equal volumes of supernatant were incubated with purified GST-rhotekin coupled to glutathione sepharose™ 4B beads at 4°C for 1 hr. The GTP-form of RhoA bound specifically to the rhotekin-sepharose beads was eluted by boiling in 2.5 x Laemmli sample buffer, electrophoresed on 12.5% SDS-PAGE, and Western blot made with affinity-purified antibody directed against RhoA.

**Immunoprecipitation**

COS 7 cells were plated on 60 mm dishes, grown to 70-80% confluence, and transfected with 2 μg of wtGDIα, GDIα-Ser<sup>174</sup>A or GDIα-Thr<sup>182</sup>A as described. Following
transfection, the cells were treated according to experimental protocol. The cells were then quickly washed with ice-cold PBS and lysed in radioimmune precipitation buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 1 mM EGTA, 1 mM EDTA, 1 mM orthovanadate, 50 mM NaF] plus protease inhibitor cocktail (10 μg/ml of pepstatin A, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF). The cell lysate was passed through a 21-gauge needle eight times, centrifuged at 4°C at 10,000 x g for 10 min. The supernatant was collected and protein determination made. Seven hundred micrograms of protein from each experimental group was incubated with 2.0 μg rabbit anti-GDI antibody for 1 hr at 4°C, 20 μl Protein A Sepharose CL-4B added and incubated overnight at 4°C on a rocker platform. The immunoprecipitated protein complex was collected by centrifugation at 2,500 rpm at 4°C for 5 min, washed four times with PBS, boiled in 1x electrophoresis sample buffer and separated by SDS-PAGE. Western blot analysis was made using anti-RhoA or anti-GDI antibodies to determine co-immunoprecipitation of RhoA with GDIα. As negative control, a separate group of cells was used for immunoprecipitation without the precipitating antibody.

Western blot

Cells were collected and lysates prepared in the appropriate extraction buffer. Protein concentration was determined using BCA Protein Assay kit with bovine serum albumin as standard. The cell lysates were loaded at constant protein concentrations, separated by SDS-polyacrylamide gel electrophoresis in 12.5% acrylamide as needed, and electrotransferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris buffered saline with 0.05% Tween-20 (TBST), and incubated with the
appropriate primary antibodies diluted in TBST with 1% nonfat dry milk for overnight at 4 °C in a rocker. The blot was washed 5x with TBST and incubated with the appropriate anti-IgG secondary antibody conjugated with horseradish peroxidase. The bands were detected using the ECL kit.

Statistics

Single sample data were analyzed by the two-tail t test; a multiple range test (Scheffe’s test was used for comparison of experimental groups with a single control group.

RESULTS

PKA directly phosphorylates GDIα

Purified GDIα protein: We initially determined the effects of the catalytic PKA subunit on phosphorylation of purified GDIα-GST fusion protein by mass spectrometry analysis. Results indicated that PKA (25 U) increased the MW (molecular weight) of GDIα-GST from 49,655 Da in control to 49,789 Da, a shift of 146±6 Da (Fig 2a; Table), suggesting incorporation of two phosphates since an increase in 80 Da corresponds to incorporation of one phosphate. Using Kemptide as a positive control, a specific substrate of PKA, we observed that incubation with the purified PKA catalytic subunit increased its MW from 773 Da (control) to 853 Da (with PKA), a shift of 80 Da (Fig. 2b; Table). Comparing mass spectra (Figs. 2a and 2b), those from the GDIα-GST reaction mixture did not resolve into the sharp peaks as with Kemptide. We suspect that this likely was attributed to varying amounts of unphosphorylated, mono- and di-phosphorylated GDIα-GST proteins in the reaction mix.
To address the possibility that GST was also phosphorylated, we used in vitro phosphorylation of GDIα-GST after cleavage of GST from the protein. Analysis of the amino acid sequence of GST in pGEX-2T (Accession number AAA57089; cloning vector used for producing GDIα-GST) for PKA phosphorylation consensus sites indicated that GST contains one putative PKA phosphorylation site which overlapped with the thrombin cleavage site. This suggests that thrombin cleavage will likely prevent GST phosphorylation by PKA. Following incubation of GDIα-GST with human α-thrombin (10^{-6} \mu M, 10 \text{ min}), which effectively cleaved GST off from the fusion protein, subsequent detection for phosphorylation yielded phosphorylated GDIα, auto-phosphorylated PKA, and non-phosphorylated GST (Fig. 2c), which were subsequently confirmed by mass spectrometry. The results indicated that GST contained very likely only one PKA phosphorylation site which is at the thrombin cleavage site.

**Motif-containing GDIα peptides:** Subsequently, we synthesized peptides that contained either Ser^{174} (Peptide^{S174}), Thr^{182} (Peptide^{T182}), or both residues (Peptide^{S174/T182}) (Fig. 1) to determine the specific sites phosphorylated by PKA. Preliminary experiments indicated that the in vitro PKA-catalyzed phosphorylation of the synthetic peptides was optimal at 100 U of PKA (data not shown), and we subsequently used that PKA concentration for phosphorylation studies of the synthetic GDIα peptides. Incubation of Peptide^{S174}, but not Peptide^{T182}, with the PKA catalytic subunit increased the MW by 80 Da (Fig. 3a and b, respectively; Table), indicating that Peptide^{S174} incorporated one phosphate, whereas Peptide^{T182} did not. To confirm the phosphorylation site, we fragmented the precursor ions of Peptide^{S174} using high energy collision (20 KeV) with an Axima TOF2 mass spectrometer (Shimadzu Corp., Kyoto, Japan). Analysis of the resulting fragment
ions using ProteinProspector software program (MS-Product, http://prospector.ucsf.edu)

confirmed phosphorylation at serine 174 (see Supplemental results, Figure A).

Mass spectrometry analysis of Peptide$^{S174/T182}$, which contained both phosphorylation sites showed that PKA catalyzed the incorporation of only one phosphate (Fig. 3c; Table). The negative control Peptide$^{A174/A182}$ was not phosphorylated by PKA (Fig 3d; Table). The overall in vitro kinase results support that PKA directly phosphorylated GDI$\alpha$, and the preferred residue was Ser$^{174}$.

**Ser$^{174}$ of GDI$\alpha$ is phosphorylated by intracellular cAMP**

To test whether PKA also phosphorylates Ser$^{174}$ in the in situ condition, we constructed single mutations of full-length GDI$\alpha$ at the PKA phosphorylation consensus sites, producing GDI$\alpha$ mutants with non-phosphorylatable residues at Ser$^{174}$ (GDI$\alpha$-Ser$^{174A}$) or Thr$^{182}$ (GDI$\alpha$-Thr$^{182A}$) (see Materials and Methods). COS 7 cells transfected with wtGDI$\alpha$, GDI$\alpha$-Ser$^{174A}$ or GDI$\alpha$-Thr$^{182A}$ were treated with 20 μM forskolin plus 2 μM IBMX (FI) for 30 min to increase intracellular cAMP levels, and the cells were analyzed for GDI$\alpha$ phosphorylation. The results showed that basal phosphorylation of GDI$\alpha$ was low and similar in the three transfectant groups. Treatment of wtGDI$\alpha$ transfectants with FI resulted in ~4-fold increased GDI$\alpha$ phosphorylation over non-stimulated control (Fig. 4). However, cells transfected with the mutant GDI$\alpha$-Ser$^{174A}$ were not responsive to FI treatment, showing GDI phosphorylation at similar levels as non-treated control. The non-significant slight increase was likely attributed to endogenous GDI$\alpha$ phosphorylated. With the mutant GDI$\alpha$-Thr$^{182A}$ transfectants, FI caused a robust increase in phosphorylation similar in extent to the wtGDI$\alpha$ transfectants (Fig. 4). The results, together with the in vitro phosphorylation
studies, provide strong evidence that Ser$^{174}$ on GDIα is the more important residue targeted by PKA.

**Phosphorylation of GDIα-Ser$^{174}$ inhibits RhoA**

*Effects of cAMP on SRE-luciferase reporter activity:* The functional significance of the PKA-mediated phosphorylation of GDIα-Ser$^{174}$ was tested using a Rho-dependent SRE-driven reporter plasmid (19). PKA specificity was determined by overexpression of COS 7 cells with a recombinant adenovirus containing PKI inhibitor gene (AdPKI) as described by us (26) (see Methods). Following infection with AdPKI or Adnull overnight, the cells were co-transfected with the SRE-luciferase reporter along with either wtGDIα or the GDI mutant constructs (GDIα-Ser$^{174A}$ or GDIα-Thr$^{182A}$). In control Adnull cells, results show that FI treatment of wtGDIα transfectants significantly decreased reporter activity compared to control cells, but not in the GDIα-Ser$^{174A}$ transfectants (Fig. 5). However, FI treatment of cells transfected with GDIα-Thr$^{182A}$ mutant resulted in significant inhibition of SRE reporter activity (Fig. 5). Following PKI overexpression, the FI-mediated inhibition in both wt-GDIα and GDIα-Thr$^{182A}$ transfectants was abrogated, indicating that the inhibited reporter activity was PKA-specific (Fig. 5). As expected, PKI overexpression had no effects on reporter activity in the GDIα-Ser$^{174A}$ transfectants (Fig. 5). The results indicate that PKA phosphorylation of Ser$^{174}$ enhanced GDIα’s ability to negatively regulate RhoA.

*Effects of cAMP on thrombin-stimulated RhoA:* We next tested whether phosphorylation of GDIα-Ser$^{174}$ can prevent agonist-stimulated activation of RhoA. COS 7 cells were transfected with wtGDIα or GDIα-Ser$^{174A}$ overnight, pretreated with FI, and then challenged with thrombin (100 nM) for 10 min. The cells were collected for affinity-binding
with rhotekin to determine the GTP-bound RhoA. Results showed that FI treatment significantly inhibited the thrombin-stimulated increase in RhoA-GTP (~40%) in wt-GDI\(\alpha\) transfectants; whereas in GDI\(\alpha\)-Ser\(^{174}\)A transfectants, FI treatment was unable to prevent the thrombin-induced RhoA activation (Fig. 6).

**Effects of phosphorylated GDI\(\alpha\)-Ser\(^{174}\) on interaction with RhoA:** We investigated whether phosphorylation of GDI\(\alpha\)^\(^{S174}\) alters GDI\(\alpha\) interactions with RhoA. COS 7 cell transfectants were treated with FI as previously described for phosphorylation determination, and cell lysates were immunoprecipitated with anti-GDI Ab, followed by Western blot detection for RhoA. Results showed that FI treatment of wtGDI\(\alpha\), GDI\(\alpha\)-Ser\(^{174}\)A, and GDI\(\alpha\)-Thr\(^{182}\)A transfectants did not alter GDI-RhoA complex formation (Fig. 7). Further, the mutation of these residues *per se* did not alter interaction between the two proteins either since cells in the absence of FI showed similar levels of co-precipitated RhoA as those in the presence of FI (Fig. 7). Negative controls were immunoprecipitated in the absence of anti-GDI Ab or with the isotype-matched IgG and showed absence of precipitated bands (data not shown).

**DISCUSSION**

The current study provides strong evidence that PKA directly phosphorylated GDI\(\alpha\) at Ser\(^{174}\), which was sufficient to inhibit, at least in part, RhoA function. Mutation of this residue to Ala\(^{174}\) effectively abrogated the cAMP-stimulated phosphorylation response in COS 7 cell transfectants. This nearly 100% inhibition of phosphorylation in the GDI\(\alpha\)-Ser\(^{174}\)A mutant indicated that cAMP phosphorylated mostly Ser\(^{174}\), and not other residues. The finding is further underscored by the observation that mutation of Thr\(^{182}\), the other PKA
consensus phosphorylation site did not prevent cAMP-mediated phosphorylation of GDI. The Thr\(^{182A}\) mutant construct was phosphorylated to the same extent as the wtGDI\(\alpha\).

Results from \textit{in vitro} phosphorylation of GDI peptides provide further evidence of the selectivity of Ser\(^{174}\) phosphorylation by PKA. The synthetic GDI\(\alpha\) peptide containing both PKA phosphorylation consensus sites (Ser\(^{174}\) and Thr\(^{182}\)) was phosphorylated at only one of these residues by PKA. This finding was substantiated with GDI\(\alpha\) peptides containing single PKA phosphorylation sites, which showed that Ser\(^{174}\), but not Thr\(^{182}\), was phosphorylated.

Surprisingly, when whole GDI\(\alpha\)-GST protein was used as substrate, PKA increased the MW by 146 D, indicating potential phosphorylation of two residues. This finding appears to be in contrast to the phosphorylation studies made with GDI\(\alpha\) peptides and the single site mutation studies. Analysis of the amino acid sequence of the GST component of the fusion protein revealed that it contains one putative PKA phosphorylation consensus site, located at the carboxy terminus and overlapping with the thrombin cleavage site, and suggests that this could account for the other phosphorylation site. The subsequent \textit{in vitro} phosphorylation assay following thrombin cleavage detected phosphorylation of GDI\(\alpha\), but not GST, confirming that GST contained only that one phosphorylation site which contributed to the overall phosphorylation of GDI-GST by PKA.

A key finding from the current work is that phosphorylation of GDI\(\alpha\)-Ser\(^{174}\) by PKA enhances GDI\(\alpha\)’s negative regulation of RhoA. We found that the mutation of Ser\(^{174}\) to Ser\(^{174A}\), rendering GDI\(\alpha\) not phosphorylatable by PKA, abrogated the ability of cAMP to inhibit basal RhoA and thrombin-stimulated RhoA activities. This observation is consistent with our previous report that PKA inhibited thrombin-induced RhoA activation, and
prevented the increase in endothelial permeability (36). Ser\textsuperscript{174} appears to be a critical residue for regulation of GDI\textsubscript{α} function, serving as a convergence point for multiple kinases (and phosphatases?). DerMardirossian and coworkers (16) found that PAK1 also phosphorylates GDI at Ser\textsuperscript{174}, and the combined phosphorylation with Ser\textsuperscript{101} causes dissociation of Rac1 from GDI, leading to a selective activation of Rac1. Although no data were shown in the publication nor in the supplementary results, the authors (16) additionally noted that PKA phosphorylated Ser\textsuperscript{174}, which corroborates this current important finding. In contrast to the consequences of PAK1-mediated phosphorylation (on both Ser\textsuperscript{101} and Ser\textsuperscript{174}), PKA phosphorylated only Ser\textsuperscript{174}. Together, these findings clearly suggest high selectivity of GDI function under control of specific residues as phosphorylated by different kinases.

GDI is believed to negatively regulate Rho proteins through its association with them, affecting both the cellular location and GDP/GTP cycling (14; 20; 31). However, in our model, the phosphorylation of GDI\textsubscript{α}-Ser\textsuperscript{174} by PKA did not increase GDI\textsubscript{α} association with RhoA, a finding consistent with our previous report in endothelial cells in which elevation of intracellular cAMP does not increase co-precipitated endogenous GDI\textsubscript{α} and RhoA (36). The results suggest that phosphorylated GDI inhibited RhoA not by formation of new complexes, but rather by exerting effects on pre-existing GDI-RhoA complexes. The hydrophobic geranylgeranyl-binding domain of GDI comprises the carboxy-terminal two-thirds of the molecule (residues 74-204) (Fig. 1), folding into an immunoglobulin-like β sandwich for binding Rho proteins. Ser\textsuperscript{174} lies adjacent to Tyr\textsuperscript{175}, a residue lining the hydrophobic binding pocket and forming hydrophobic contacts with Rho proteins (20). We speculate that phosphorylation of Ser\textsuperscript{174} may exert local steric changes on Tyr\textsuperscript{175}, which
could potentiate retention of Rho within GDI’s hydrophobic binding pocket and thereby preventing Rho activation.

The cAMP-PKA signaling pathway is important in preventing a wide range of inflammatory activities (4; 5; 10; 26; 32-36; 40). The current results show that a direct target of the cAMP-PKA pathway is Ser$^{174}$ of GDI, and this phosphorylation of GDI resulted in the inhibition of RhoA function. The findings are significant in that activation of RhoA is implicated in a variety of inflammation-linked diseases, including pulmonary hypertension, acute lung injury, atherosclerosis (27; 42), tumor progression and metastasis (12).
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FIGURE LEGENDS

Fig. 1. Putative PKA phosphorylation sites on GDIα

Two potential PKA consensus phosphorylation sites (underscored) are located in the hydrophobic binding domain of GDIα: Arg-Gly-Ser (amino acids 172-174) and Arg-Phe-Thr (amino acids 180-182). Additionally, two GDI regions bind Rho proteins: N-terminal regulatory domain binds the switch region of Rho; C-terminal hydrophobic binding domain binds the isoprenylated membrane-anchoring region of Rho.

Fig. 2. Purified PKA phosphorylates GDIα-GST

Representative mass spectrometric graphs of MW of a) purified GDIα-GST and b) Kemptide (positive PKA substrate control), n=3; c) Phosphorylation detected by Pro-Q Diamond Phosphoprotein Gel staining Kit (Lane 1=MW standards; Lane 2=GDIα-GST uncleaved; Lane 3=GDIα-GST cleaved with human α-thrombin, 10^-6 μM); purified PKA catalytic subunit=25 U; Control, absence of PKA.

Fig. 3. Purified PKA phosphorylates motif-containing GDIα peptides

Representative mass spectrometric graphs of MW of purified GDIα peptides containing PKA phosphorylation consensus sites: a) GDIα Peptide_S174 (n=3), b) GDIα Peptide_T182 (n=3), c) GDIα Peptide_S174/T182 (n=5), and d) control Peptide_A174/A182 (n=5); PKA = 100 U PKA catalytic subunit; Control = absence of PKA.
Fig. 4. **Mutation of GD\(\alpha\)-Ser\(^{174}\) abrogates cAMP-mediated phosphorylation**

COS 7 cells were transfected with wild type GD\(\alpha\) (wt) or the mutant constructs, GD\(\alpha\)-Ser\(^{174A}\) (S174A) or GD\(\alpha\)-Thr\(^{182A}\) (T182A) overnight, and treated with 20 \(\mu\)M forskolin and 2 \(\mu\)M IBMX (FI) to increase intracellular level of cAMP. GD\(\alpha\) phosphorylation was determined using the PhosphoProtein Purification Kit from Qiagen (Materials and Methods); a) representative Western blot showing detection of GD\(\alpha\) phosphorylation from the transfectants (top panel); bottom panel indicates total GD\(\alpha\) in cell lysates; b) bar graph summarizes results from 6 separate determinations; * \(p<0.01\) compared with Control.

Fig. 5. **Mutation of GD\(\alpha\)-Ser\(^{174}\) prevents cAMP-induced inhibition of Rho-dependent activity**

COS 7 cells were co-transfected overnight with the Rho-dependent SRE-luciferase reporter plasmid and with either wtGD\(\alpha\) (wt), GD\(\alpha\)-Ser\(^{174A}\) (S174A) or GD\(\alpha\)-Thr\(^{182A}\) (T182A). For PKA specificity, cells were overexpressed with the recombinant adenovirus containing PKI inhibitor gene; AdNull served as control (see Methods). One group was treated with 20 \(\mu\)M forskolin plus 2 \(\mu\)M IBMX (FI) for 8 hr to increase intracellular cAMP levels, whereas another remained as untreated control. Luciferase activity was measured in all cell groups, and results are reported as % FI/control; \(n=10\) -14 separate determinations; * \(p<0.01\) compare with non-treated control in wtGD\(\alpha\) and GD\(\alpha\)-Thr\(^{182A}\) transfected groups.
Fig. 6. Mutation of GDiα-Ser\textsuperscript{174} prevented cAMP's ability to inhibit mediator-induced RhoA activation

COS 7 cells were transfected with wtGDiα (wt) or GDiα\textsuperscript{S174A} (S174A) overnight. Controls and those treated with 20 μM forskolin plus 2 μM IBMX (FI) for 30 min were stimulated with human α-thrombin (Thr; 100 nM for 10 min). RhoA activation was determined by affinity-binding assay (see Materials and Methods), and densitometric scans of bands are summarized in the bar graph. A representative Western blot of the pull-downed RhoA-GTP. Values are reported as mean ± SEM of normalized RhoA-GTP; n = 5; * indicates p< 0.01.

Fig. 7. Phosphorylation of GDiα-Ser\textsuperscript{174} did not alter GDiα-RhoA complex formation

Co-precipitation analysis were determined from COS 7 cells transfected with wtαGDi (wt), GDiα-Ser\textsuperscript{174A} (S174A), or GDiα-Thr\textsuperscript{182A} (T182A) overnight, followed by treatment with 20 μM forskolin plus 2 μM IBMX (FI) to increase intracellular levels of cAMP. Affinity-purified anti-GDi Ab was used for immunoprecipitation, and separated proteins were detected by Western blot analysis with anti-RhoA or anti-GDi antibody; a representative Western blot from 5 separate determinations; densitometric scans of bands summarized in bar graph below.
REFERENCES


Regulatory domain

Hydrophobic binding domain

Binding of switch region of Rho protein

Binding of isoprenylated membrane-anchoring C-terminus of Rho protein

Fig. 1

Met Leu Ala Arg Gly Ser Tyr Ser Ile Lys Ser Arg Phe Thr Asp Asp

Ser^{174}

Thr^{182}
Fig. 2a

GST-GDI

Control: 49,655 D

PKA: 49,789 D
a)

**Kemptide**

**Control:** 773 D

**PKA:** 853 D
Fig. 2c
Fig. 3a

Peptide$^{S174}$

Control: 886 D

PKA: 966 D
Fig. 3b

Peptide^{T182}

Control: 983 D

PKA: 983 D
Peptide$^{S174/T182}$

Control: 1851 D

PKA: 1931 D
Fig. 3d

Control Peptide

Control: 1818 D

PKA: 1818 D
Table: Summary of *in vitro* kinase phosphorylation of GST-GDI

<table>
<thead>
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<th>Difference</th>
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<td>GDI-GST</td>
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<tr>
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<tr>
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<tr>
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<td>1818 ± 0</td>
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Values are daltons ± SE as determined by mass spectrometry; n = number of separate determinations.
Fig. 4

(a) Phosphorylated GDI and Lysate GDI

(b) Bar graph showing phosphorylated GDI/Lysate GDI for WT, S174A, and T182A with and without FL.
Fig. 5

WT □ S174A □ T182A

% SRE Luciferase activity (FI/Control)

0% 50% 100% 150%
Fig. 6

![Graph showing percentage of RhoA-GTP levels in WT and S174A with and without Thr and FI+Thr treatments. The graph includes error bars and a star indicating statistical significance.]

WT S174A

Control Thr FI + Thr

RhoA-GTP

Control Thr FI + Thr

WT S174A
Fig. 7

**IP: anti-GDI Ab**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
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<th>T182A</th>
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<tr>
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<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Blot:</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

**FI: - + - + - + - +**

**FI / Control**

- WT: 1.20 ± 0.05
- S174A: 1.10 ± 0.03
- T182A: 1.05 ± 0.04
N-terminal ions

b ions  ---  245.1310  316.1609  472.2700  529.2915  696.2099  859.3532  ---

- 1  2  3  4  5  6  7  8 -

- M L A R G S -

8 7 6 5 4 3 2 1

C-terminal ions

y ions  ---  833.3553  720.2712  649.2341  493.1330  436.1116  269.1132  106.0499

Supplemental Figure A