Serine Phosphorylation Negatively Regulates RhoA in Vivo*

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Previous work indicates that RhoA phosphorylation on Ser188 by cAMP or cGMP-dependent kinases inhibits its activity. However, these studies lacked the possibility to directly study phosphorylated RhoA activity in vivo. Therefore, we created RhoA proteins containing phosphomimetic residues in place of the cAMP/cGMP-dependent kinase phosphorylation site. RhoA phosphorylation or phosphomimetic substitution did not affect Rho guanine nucleotide exchange factor, GTPase activating protein, or geranylgeranyl transferase activity in vitro but promoted binding to the Rho guanine-dissociation inhibitor as measured by exchange factor competition assays. The in vitro similarities between RhoA phosphomimetic proteins and phosphorylated RhoA allowed us to study function of phosphorylated RhoA in vivo. RhoA phosphomimetic proteins display depressed GTP loading when transiently expressed in NIH 3T3 cells. Stable-expressing RhoA and RhoA(S188A) clones spread significantly slower than mock-transfected or RhoA(S188E) clones. RhoA(S188A) clones were protected from the morphological effects of a cAMP agonist, whereas phosphomimetic clones exhibit stress fiber disassembly similar to control cells. Together, these data provide in vivo evidence that addition of a charged group to Ser188 upon phosphorylation negatively regulates RhoA activity and indicates that this occurs through enhanced Rho guanine-dissociation inhibitor interaction rather than direct perturbation of guanine nucleotide exchange factor, GTPase activating protein, or geranylgeranyl transferase activity.

The intracellular protein RhoA, a member of the Ras superfamily of low molecular weight G proteins, regulates cell cycle progression, gene expression, focal adhesion assembly/disassembly, and the acto-myosin generated contraction and tension events of cell motility, matrix remodeling, and cytokinesis (1). RhoA cycles between an active GTP-bound and an inactive GDP-bound state through nucleotide exchange and intrinsic GTPase activity. During cellular events of RhoA activation, guanine nucleotide exchange factors (GEFs)³ can bind and activate RhoA by promoting uptake of free nucleotide (2). GTPase activating proteins (GAPs) negatively regulate RhoA by binding and stimulating GTP hydrolysis leading to an inactive GDP-bound state (3). RhoA signals within the cell by binding to a variety of effector molecules, such as mDia and the Rho kinases ROKα/ROCK2 and ROKβ/ROCK1 (4–6). The vast majority of RhoA (>95%) resides in the cytosolic fraction of the cell (7, 8), with the remainder associated with lipid membranes via a geranylgeranylated moiety attached to a carboxyl Cys¹⁹⁰ residue (9). Prenylation of RhoA is required for promotion of cell growth, transformation, and cytoskeletal organization, indicating that membrane cycling is a critical component of Rho regulation (10). A primary mechanism of cytosolic sequestration of RhoA is through binding of Rho guanine-dissociation inhibitors (RhoGDIs) (11). RhoGDI contacts RhoA through two distinct domains, a flexible amino-terminal domain that impedes guanine nucleotide dissociation/hydrolysis and a highly folded carboxyl domain that stabilizes the RhoA geranylgeranylated moiety (12). RhoGDI is postulated to extract RhoA from membranes through concerted actions of both domains (11). Administration of forskolin, an activator of adenylate cyclase, or dibutyryl cAMP to cells stimulates morphological changes that are strikingly similar to those observed upon introduction of the Rho-specific inhibitor C3-transferase (13). From this initial observation, cAMP- and cGMP-dependent kinase (PKA and PPK) were demonstrated to phosphorylate RhoA on Ser¹⁸⁸ (13, 14), thereby joining RhoA with Rap1a and Rap1b as small GTPases regulated by carboxyl PKA phosphorylation (15, 16). Although PKA phosphorylation is linked to Rap activation (17, 18), evidence indicates that it negatively regulates RhoA function. RhoA phosphorylation promotes formation of RhoA/RhoGDI complexes (13, 19) and enhances the ability of RhoGDI to extract RhoA from membranes (13, 20). In support of enhanced RhoGDI binding, the ability of RhoA to cycle from membranes has been linked to cAMP and cGMP signaling within cells (21, 22). Functionally, constitutively active RhoA containing an S188A mutation is more effective in blocking actin dissolution promoted by dibutyryl cAMP (23) or 8-Bromo-cGMP (24), and constitutively active RhoA require a S188A mutation to promote stress fibers in cells co-transfected with constitutively active PKG (14).

On the other hand, the significance of RhoA phosphorylation to PKA/PKG regulation of its signaling is unclear considering recent reports. Thromboxane receptor stimulation promotes RhoA activation through a Gq₁₃ and a PKA-sensitive pathway(s) (25). Manganello et al. (26) demonstrated recently that PKA phosphorylates Gq₁₃ subunit to promote βγ subunit uncoupling, thereby effectively shutting down receptor activation.

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§ The abbreviations used are: GEF, guanine nucleotide exchange factor; cPKA, catalytic domain of protein kinase A; GAP, GTPase activating protein; DH, Dbl homology; PH, pleckstrin homology; CRD, cysteine-rich domain; GST, glutathione S-transferase; mDia, N-methylanthraniloyl; RBD, Rho-binding domain of Rhotekin; RhoGD1, Rho guanine-dissociation inhibitor; ggRhoA, geranylgeranylated RhoA; PKA, cAMP-dependent kinase; PKG, cGMP-dependent kinase.

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of RhoA. Moreover, PKG inhibited G$_{T52}$ activation of serum response factor transcription by hyperphosphorylation of RhoA (27). Notably, PKG also inhibited serum response factor transcriptional activity promoted by constitutively active forms of Rho kinase, protein kinase N, or protein kinase C-related kinase 2, indicating that PKG antagonizes RhoA signaling downstream of effector regulation. Thus, an emerging picture is that PKA and PKG differentially regulate RhoA at multiple levels.

In this work, we expressed Ser$^{188}$ phosphomimetic RhoA proteins, in the absence of constitutive RhoA activity and aberrant PKA/PKG signaling, to assess the contribution of PKA phosphorylation to RhoA function. We report here that addition of a charged group to Ser$^{188}$ upon phosphorylation non-specifically regulates RhoA activity in vivo and indicates that this occurs through enhanced RhoGDI interaction rather than direct perturbation of GEF, GAP, or geranylgeranyl transference activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Puromycin, bovine serum albumin, and buffer reagents were acquired from Sigma. Polyvinylidene fluoride membranes were purchased from Millipore. RhoA monoclonal antibodies (clone 55) were purchased from BD Biosciences. NIH 3T3 cells were maintained in growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum [Sigma]).

**Constructs**—RhoA mutations (S188A, S188D, S188E, C190A) and RhoG2 mutation (S187A) were created through PCR mutagenesis using the QuikChange mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing, and cDNAs were subcloned into either pGEX-4T-1 (Amersham Biosciences) or pCMV-Mye (Clontech) using EcoRI and XhoI restriction sites. pGEX-4T-1-RhoGDI (human) was created by subcloning RhoGDI cDNA into pGEX-4T-1 using BamHI and EcoRI restriction sites. pPro-HT-Dbl (DH/PH) was a gift of Dr. K. Rossman, University of North Carolina, Chapel Hill, NC.

**Antibody Production**—Antibody production and purification was performed by Covance Research Products Inc. Rabbis were immunized with a phosphoserine peptide containing the proximal nine residues of RhoA (RRGGKKEGSO) thiol bonded to keyhole limpet hemocyanin. Antibodies were isolated by negative affinity purification with immobilized unphosphorylated RhoA peptide followed by positive affinity purification with immobilized phosphorylated RhoA peptide and low-temperature pH elutions.

**Fusion Proteins**—GST-Rho fusion proteins (GST, GST-RhoA, GST-RhoG, GST-Cdc42, GST-Rac1, GST-RhoGDI) were purified from BL21 *Escherichia coli* cells (Stratagene) using glutathione-Sepharose 4B (Amersham Biosciences). Proteins were eluted with free and reduced glutathione in Tris-buffered saline medium (50 mM Tris, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM dithiothreitol) and stored in 30% glycerol. His$_6$-Dbl DH/PH was purified from BL21 *E. coli* cells using nickel-nitrilotriacetic acid-Sepharose (Qiagen) and eluted with 20 units of tobacco etch virus phosphatase (Invitrogen) according to the manufacturer’s specifications. RhoGDI was produced by cleaving RhoGDI from GST-RhoGDI-Sepharose with 5 units of bovine thrombin (Sigma). RhoGDI was subsequently cleared with benzamidine-agarose (Sigma). RhoGDI was subsequently cleared with benzamidine-agarose (Sigma) to remove thrombin from incubation buffer. Recombinant geranylgeranyl transference was purchased from Sigma. Phosphate-binding protein carrying an A197C mutation was purified and fluorescently labeled with N-[2-[(2-maleimidyl)ethyl]-7-(diethylamino)-coumarin-3-carboxamide as described previously (28) from E. coli ANCC-77 bacteria (a kind gift of Dr. M. R. Webb, National Institute for Medical Research, London). His$_6$-Larg DH/PH and His$_6$-Vav2 DH/PH/CRD were kind gifts of Dr. M. Booden, University of North Carolina, Chapel Hill, NC. Full-length p190RhoGAP was a generous gift of Dr. J. Settleman, Harvard Medical School.

**Rho Protein Phosphorylation**—To measure CPKA phosphorylation of Rho fusion proteins (see Fig. 1), 2.0 pmols of fusion protein phosphorylation buffer for 90 min at 25 °C. Phosphorylation efficiency was evaluated by scaling down reaction to 1% of the amount of GST-RhoA and PKA. Specifically, 1.2 μg (25 pmols) of GST-RhoA-Sepharose, 1.5 units of cPKA, 2.12 nmols of ATP, and 3.3 pmols of [γ$^3$P]ATP (1.645 dilution) were incubated in 10 μl of phosphorylation buffer with rotation for 30 min at 25 °C. Reactions were washed extensively, and incorporation of [γ$^3$P] into the GST-RhoA-Sepharose was measured. Under these conditions, incorporation of [γ$^3$P]ATP was linear for at least 1 h. PKA incorporation was added to a concentration of 1%, and phosphatase was performed as described previously for Rho proteins (29). The definitive phosphorylation was measured with 20-fold volumes of phosphate-buffered saline to ensure removal of non-prenylated RhoA. Both aqueous and detergent phases were aliquoted and analyzed for RhoA phosphorylation by measuring release of radioactivity and visualizing by SDS-PAGE and autoradiography.

**In Vitro Guanine Nucleotide Exchange Factor Assays**—Fluorescence spectroscopic analysis of N-methylanthraniloyl (mant)-GTP (Biomol) incorporation into GDP-preloaded GST-Rho proteins was carried out using a FLUOSstar fluorescence microplate reader at 25 °C similar to that described previously (30). 2 μM GST-RhoA or 1 μM prenylated GST-RhoA was prepared and allowed to equilibrate in exchange buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1% glycerol). 500 nM mant-GTP and varying amounts of DH/PH (Larg, Dbl, or Dbs) or DH/PH/CRD (Vav2) protein were added at the indicated time, and the relative mant fluorescence (excitation = 360 nm, emission = 460 nm) was monitored. Experiments were performed in duplicate for every condition.

**GAP Assays**—GAP assays were performed in a Spectramax Gemini XS fluorescence microplate reader (Molecular Devices) (excitation = 425 nm, emission = 465 nm) at 25 °C by incubating 1 μM GTP-loaded GST-RhoA together with 2.5 μM fluorophore-label peptide-binding protein in the absence of prenyl 250 or 750 μl purified recombinant GAP in a buffer containing 20 mM Tris, 50 mM MgCl$_2$ at a total volume of 100 μl. Upon hydrolysis of GTP, the resulting free F$_{360}$ is bound rapidly (1.36 × 10$^{-5}$ M$^{-1}$ s$^{-1}$ at 22 °C) and with high affinity (about 100 nM) by the phosphate-binding protein, resulting in a 13-fold increase in the fluorescence at 465 nm (28). Because of this, the observed change in fluorescence corresponds to the rate and amount of GAP released from the GTPase.

**RhoGDI Competition Assays**—2 μM of the indicated GST-RhoA fusion protein was incubated for 10 min in exchange buffer containing 0.5, 1.0, or 2.0 μM RhoGDI to promote RhoA-RhoGDI complexes. Vav2 DH/PH/CRD and mant-GTP nucleotides were then added and allowed to equilibrate with mixing for 15 s, and the rate of mant-GTP incorporation was monitored. Linear velocity of exchange was determined as described previously (31). Briefly, baseline and GEF-induced nucleotide exchange rates were calculated by dividing the change in emission at 460 nm by change in time. Values were averaged and standard deviations were calculated for each reaction. Reaction velocity was considered linear as long as the regression value of the exchange slope was greater than 0.97. Data from these and all other experiments were considered significantly different if the p values, as determined by two-tailed t tests, were <0.02.

**Geranylgeranyl Transferase Assays**—GST or the indicated GST-RhoA-Sepharose (5 pmols), 100 pmols of [H$^2$]-geranylgeranyl pyrophosphate (PerkinElmer Life Sciences), and 0.36 units of geranylgeranyl transferase in Tris-buffered saline containing 0.25% deoxycholate. Through dilutions, reactions were washed extensively, and placed in scintillation buffer, and the extent of [H$^2$]-geranylgeranyl was quantified. Geranylgeranyl transferase geranylgeranylated GST-RhoA or phosphorylated GST-RhoA were incubated in solution with geranylgeranyl pyrophosphate (25 nmols) and in the absence or presence of 10 units of geranylgeranyl transference overnight at 30 °C. Proteins were either GDP-loaded (GEF assays) or GTP-loaded (GAP assays) and eluted with small volumes of free glutathione in Tris-buffered saline containing 0.25% deoxycholate, pH 7.6, for 20 or 60 min. Samples were then resolved by 15% SDS-PAGE, and gels were fixed, incubated in Amplify solution (Amersham Biosciences), and then exposed by autoradiography. Alternatively, reactions were collected at 20, 40, 60, and 80 min in duplicate, washed extensively, and placed in scintillation buffer, and the extent of [H$^2$]-geranylgeranyl was quantified.
Transfections and Production of Stable Cells—NIH 3T3 fibroblast cells were transfected with the expression vectors indicated in each experiment according to the manufacturer’s protocol using LipofectAMINE PLUS (Invitrogen). After introduction of the expression vectors for 3 h, the transfection medium was replaced with growth medium for 18 h. For creation of stable cell lines, NIH 3T3 cells were transfected with 1 μg of pPUR (Clontech) only or cotransfected with 0.05 μg of pPUR and 1 μg of pCMV-Myc RhoA construct and then selected in 10 μg/ml puromycin (Sigma). Clonal lines were established and screened for expression by Western blotting lysates with anti-c-Myc (clone 9E10; Sigma) monoclonal antibodies.

**RhoA GTP Profile Assays**—The amount of activated, GTP-bound RhoA protein was measured using a technique similar to the method described by Ben et al. (32). Briefly, transfected or untransfected stable cells were lysed in 300 μl of 50 mM Tris, pH 7.4, 10 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, and protease inhibitors. 500–750 μg of lysates were cleared at 16,000 × g for 5 min, and the supernatant was rotated for 30 min with 30 μg of GST-RBD (GST fusion protein containing the Rho-binding domain (RBD; amino acids 7–89) of Rhotekin) bound to glutathione-Sepharose beads. Samples were washed in 50 mM Tris, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. GST-RBD pulldowns and lysates were then Western blotted with anti-c-Myc. To quantify GST-RBD pull-downs, Western blots of lysates and corresponding GST-RBD pull-downs from three unique experiments done in duplicate were scanned, and densitometry was performed using Metamorph imaging software.

**Calculation of Cell Spreading**—For all experiments cells were replated in the absence of puromycin the day before experiments, trypsinized, washed twice in Dulbecco’s modified Eagle’s medium, and then suspended before plating for 30 min in Dulbecco’s modified Eagle’s medium and 0.5% fatty acid-free bovine serum albumin. Suspended cells were plated on fibronectin-coated coverslips for 20, 40, and 60 min. Coverslips were fixed and stained with Coomassie Blue (2% Brilliant Blue, 45% methanol, and 10% acetic acid) for 10 min and then washed with water and mounted. The relative areas of individual cells from Metamorph images were quantified with the use of NIH Image software. At least 50 cells taken from 10 arbitrary fields were counted from each coverslip. Two coverslips counted every condition were used in the assay, demonstrating that prenylation does not interfere with the catalytic activity of cPKA against Ser188.

**RESULTS**

**PKA Phosphorylation of Rho Proteins**—We confirmed that PKA phosphorylates RhoA on Ser188 (Fig. 1A) as described previously (13). Although Forget et al. (19) demonstrated that PKA also phosphorylates Cdc42, we found Cdc42 to be a poor substrate for PKA in vitro, in agreement with an initial report (33). Further, Rac1 was not phosphorylated, whereas PKA efficiently phosphorylates RhoG on Ser187 (Fig. 1A), indicating that both RhoA and RhoG carboxyl termini are targets of PKA phosphorylation. The proximity of the Rho prenylation site (residue 190) to the site of PKA phosphorylation prompted us to examine whether phosphorylation affects PKA phosphorylation. Although RhoA is efficiently phosphorylated in our reactions (60–80% by molar incorporation of [3H]geranylgeranyl pyrophosphate), phosphorylated RhoA or geranylgeranylated RhoA (ggRhoA) were subjected to TX-114 phase extraction. TX-114 phase extraction partitions prenylated RhoA into the detergent phase because of the hydrophobic modification (29), ensuring that ggRhoA will be specifically examined as a substrate. Phosphorylation efficiency of RhoA and ggRhoA was comparable (Fig. 1, B and C), demonstrating that prenyl modification of RhoA does not impede PKA phosphorylation. To measure cellular RhoA phosphorylation, we generated affinity-purified antibodies that selectively recognize phospho-Ser188 RhoA (Fig. 2A). Characterization of two unique antisera revealed that geranylgeranylation of phosphorylated GST-RhoA proteins abrogated antibody binding (Fig. 2A), indicating that the presence of a C20 isoprenoid moiety sterically blocks antibody recognition. Therefore, we elected to use NIH 3T3 cells that stably express Myc epitope-tagged-RhoA(C190A) to measure cellular PKA phosphorylation of Ser188. Forskolin stimulation of cAMP production strongly enhanced Myc-RhoA(C190A) phosphorylation as measured by Western blotting of whole cell lysates or immunoprecipitated proteins (Fig. 2B). We and others (32, 34) have established previously that RhoA activity is reduced during the initial 15–30 min of cell spreading. Interestingly, PKA activity is transiently stimulated during the same time course (35). To address whether RhoA is a PKA target during spreading, cells expressing Myc-RhoA(C190A) were allowed to spread on fibronectin and at various times collected for immunoprecipitation with phospho-Ser188 antibodies. We observed that cellular phosphorylation of RhoA was significantly increased within the initial 20 min of NIH 3T3 cell spreading on fibronectin (Fig. 2C). These data support the conclusion that cellular PKA activity is elevated early in spreading (35, 36) and indicates that RhoA is a target of cellular PKA regulation.

**GEF Exchange against Phosphorylated RhoA**—GEF-binds GDP-bound Rho proteins and induce a transition state that promotes uptake of free GTP nucleotide, thereby stimulating Rho activation. It has been reported that CAMP inhibits GEF activation of RhoA in leukocytes (37), thereby raising the possibility that RhoA phosphorylation impairs GEF exchange. To address this hypothesis, GST-RhoA was phosphorylated in vitro with cPKA and compared with control protein for the ability of DH/PH domain-containing GEFs to promote nucleo-
tide uptake. Using \(^{32}\)P-ATP, GST-RhoA was estimated to be at least 80–90% phosphorylated by cPKA. Basal incorporation of mant-GTP was identical for phosphorylated and control GST-RhoA proteins (Fig. 3A). Larg (Fig. 3A), Vav-2 (Table I), Dbl, and Dbs (not shown) all displayed similar exchange activity against control and phosphorylated GST-RhoA at multiple GEF concentrations. This finding also extended to phosphorylated and control geranylgeranylated RhoA proteins (Fig. 3B). Lastly, the phosphomimetics GST-RhoA(S188E) and GST-RhoA(S188D) were equivalent substrates as wild-type GST-RhoA or GST-RhoA(S188A) controls (Fig. 3C). These data demonstrate that phosphorylation of the RhoA tail does not directly interfere with GEF-induced exchange.

RhoA Ser\(^{188}\) Phosphomimetics Display Increased Binding to RhoGDI—cPKA-phosphorylation of RhoA has been reported to increase its affinity toward RhoGDI, therefore we examined the ability of RhoGDI to bind RhoA proteins using solution-phase binding competition of RhoGDI and GEF molecules. Briefly, GST-RhoA proteins were pre-incubated with no or varying amounts of full-length RhoGDI for 10 min to promote the formation of RhoA/RhoGDI complexes. Vav2 DH/PH/CRD and mant-GTP nucleotides were subsequently added and allowed to equilibrate with mixing for 15 s and then mant-GTP incorporation was measured. Vav2 exchange was linear over the initial 150–200 s for phosphorylated, phosphoserine-mimetics, and control GST-RhoA proteins (see Fig. 4A and Tables I and II). As RhoGDI concentrations were increased, the velocity of nucleotide incorporation was both reduced and lengthened for all RhoA molecules (Tables I and II). Importantly, both phosphorylated and phosphomimetic GST-RhoA proteins exhibited a significant reduction in GEF exchange compared with control proteins at equal concentrations of RhoGDI (Fig. 4B). The bulkier S188E mutation resulted in slightly greater inhibition than the S188D mutation, suggesting tighter binding to RhoGDI. As prior experiments demonstrated that GEF ex-
GTP-hydrolyzing activity of Rho proteins. As cPKA phosphorylation and GAP activity—GTP loading.

TABLE I

| Condition | RhoA   | RhoA-phosphorylated |
|-----------|--------|---------------------|
| Basal     | 0.98 ± 0.15 | 0.87 ± 0.13         |
| 150 nM Vav2 | 3.84 ± 0.21 (3.9-fold) | 3.52 ± 0.27 (4.1-fold) |
| + 0.5 μM GDI | 3.08 ± 0.30 (81%) | 3.26 ± 0.33 (93%) |
| + 1.0 μM GDI | 2.83 ± 0.22 (74%) | 2.37 ± 0.34 (67%) |
| + 2.0 μM GDI | 2.34 ± 0.28 (61%) | 1.51 ± 0.28 (43%) |

*p < 0.01.

Fig. 4. RhoA phosphorylation promotes RhoGDI interaction. A, GST-RhoA or GST-RhoA serine mutants, as indicated, were allowed to equilibrate for 15 min prior to the addition of the DH/PH/CRD Vav2 and mant-GTP. Vav2 promoted an equivalent linear exchange velocity for all GST-RhoA proteins. B, equal molar amounts of RhoGDI and GST-RhoA proteins (2 μM) were equilibrated with GST-RhoA proteins for 15 min prior to addition of DH/PH/CRD of Vav2. RhoGDI inhibition of GEF exchanges results in a slower and more sustained linear exchange velocity for all RhoA proteins compared with A. The linear exchange velocities of the two phosphomimetics, GST-RhoA(S188D) and GST-RhoA(S188E), were significantly depressed compared with GST-RhoA and GST-RhoA(S188A) protein controls.

change was equivalent for all RhoA proteins at multiple concentrations, these data provide additional evidence to previous reports that RhoA phosphorylation promotes or stabilizes RhoA-RhoGDI complex formation. Moreover, as these proteins are not post-translationally modified, these data indicate that adding a negative charge to the carboxyl terminus of RhoA enhances the protein-protein interactions of RhoA and RhoGDI.

RhoA Phosphorylation Does Not Affect GAP Activity—RhoGAPs negatively regulate Rho activity by promoting the intrinsic GTP-hydrolyzing activity of Rho proteins. As cPKA phosphorylation was also hypothesized to negatively regulate RhoA, we analyzed whether p190RhoGAP activity against phosphorylated RhoA is perturbed in vitro. Basal and p190RhoGAP-induced GTP hydrolysis by phosphorylated, phosphoserine-mimetic, and control GST-RhoA proteins was essentially identical (Fig. 5, A and B). Moreover, p190RhoGAP activity against geranylgeranylated RhoA was also insensitive to PKA phosphorylation, although prenylated RhoA had a slightly lower basal and GAP-induced hydrolysis rate (Fig. 5, C and D). Together, these data indicate that phosphorylation of RhoA does not influence the ability of p190RhoGAP to bind and stimulate GTPase activity.

RhoA Phosphorylation Does Not Affect Geranylgeranyl Transferase Activity—Incubation of purified cellular membranes with cPKA promotes RhoA extraction from membranes by RhoGDI (13, 20). As non-prenylated RhoA is a cellular PKA target, we analyzed whether cPKA phosphorylation of RhoA impairs addition of a geranylgeranyl moiety as an additional level of regulation. Geranylgeranyl transferase possesses equivalent activity against phosphorylated and control protein GST-RhoA proteins (Fig. 6, A and B). Further, Ser188 mutants were also comparable substrates (Fig. 6C). Together, these data indicate that phosphorylation of the RhoA tail does not directly alter geranylgeranyl transferase modification of RhoA.

Transient Expression of Ser188 RhoA Mutants—NIH 3T3 cells transiently transfected with Myc epitope-tagged RhoA, RhoA(S188A), RhoA(S188D), or RhoA(S188E) displayed enhanced stress fibers compared with mock-transfected cells (data not shown), indicating that all transient proteins were functional. The extent of Myc-RhoA GTP loading was evaluated using GST-RBD pulldowns (32). Both phosphoserine-mimetic Myc-RhoA proteins, with Myc-RhoA(S188E) being the more significant, displayed reduced GTP loading in comparison with Myc-RhoA and Myc-RhoA(S188A) proteins (Fig. 7). Myc-RhoA(C190A) proteins were not appreciably GTP-loaded under these conditions and are included as a negative control for GTP loading. These data provide evidence that addition of a negative charge by phosphorylation of Ser188 is sufficient to negatively regulate RhoA activation.

Stable Expression of Ser188 RhoA Mutants—To examine whether a functional differences exists between Ser188 mutant RhoA proteins, stable clones of NIH 3T3 cells expressing Myc-RhoA, Myc-RhoA(S188A), or Myc-RhoA(S188E) were isolated. Two independent clones were chosen for each based solely on expression level and characterized further. Western blots revealed that Myc-RhoA migrates at higher apparent molecular weight than the endogenous RhoA proteins; therefore it was evident that all clones express Myc-RhoA protein at a significantly lower level than endogenous RhoA (Fig. 8A). GST-RBD pulldowns established that, contrary to transiently expressed proteins; there is little or no difference in the GTP-loading profile of stably expressed mutant Myc-RhoA proteins, with the exception of the non-prenylated mutant, Myc-RhoA(C190A) (Fig. 8B).

Activity of Stably Expressed RhoA Protein—As RhoA is hypothesized to antagonize cell spreading (34), NIH 3T3 clones were allowed to spread on fibronectin and collected at the indicated time, and rates of cell spreading were quantified (Fig. 9A). Both Myc-RhoA(S188E) clones spread at a similar rate as a pool of mock-transfected clones, whereas all the Myc-RhoA and Myc-RhoA(S188A) clones spread significantly slower. Additionally, a pool of Myc-RhoA(S188E)-expressing cells (five clones) that express a higher amount of Myc-RhoA (Fig. 9B, inset) still spread significantly faster than a corresponding pool of Myc-RhoA(S188A)-expressing cells (Fig. 9B).

RhoA signaling has been found to antagonize the morpholog-
RhoA Phosphorylation and in Vivo Activity

Table II
RhoGDI inhibition of GEF-exchange activity (units s⁻¹ ± S.D.)

| Condition | wtRhoA | RhoA-S188A | RhoA-S188D | RhoA-S188E |
|-----------|--------|------------|------------|------------|
| Basal     | 1.09 ± 0.11 | 0.98 ± 0.12 | 0.89 ± 0.06 | 0.93 ± 0.08 |
| + 150 nM Vav2 | 6.84 ± 0.33 (6.3-fold) | 6.75 ± 0.31 (6.9-fold) | 6.67 ± 0.40 (7.4-fold) | 6.47 ± 0.17 (6.9-fold) |
| + 0.5 μM GDI | 5.74 ± 0.30 (81%) | 5.55 ± 0.36 (79%) | 5.89 ± 0.22 (86%) | 4.50 ± 0.13 (64%)a |
| + 1.0 μM GDI | 4.27 ± 0.18 (55%) | 4.16 ± 0.13 (55%) | 3.28 ± 0.19 (41%)a | 2.89 ± 0.23 (35%)a |
| + 2.0 μM GDI | 2.74 ± 0.19 (29%) | 2.53 ± 0.14 (27%) | 1.72 ± 0.12 (14%)a | 1.53 ± 0.10 (11%)a |

a p < 0.01.

2 μM GST-RhoA or GST-RhoA serine mutant proteins were incubated with the indicated amount of RhoGDI (GDI) prior to the addition of Vav2. Data are presented as described for Table I. RhoGDI inhibited Vav2-induced nucleotide exchange of phosphomimetic proteins to a greater extent than control proteins. RhoGDI inhibition was more pronounced for GST-RhoA(S188E) than GST-RhoA(S188D).

**FIG. 5. RhoA phosphorylation does not affect p190RhoGAP activity.** A, Rho GTPase activity was indirectly examined by incubating cPKA phosphorylated (white symbol) or control GST-RhoA (black symbol) preloaded with GTP in the presence of a coumarin-tagged phosphate-binding protein whose fluorescence increases (465 nm) following binding of phosphate released from GTP. Addition of 250 or 750 μM p190RhoGAP-stimulated GAP activity was not affected by RhoA serine phosphorylation. B, GST-RhoA (black symbol), GST-RhoA(S188A) (gray symbol), and GST-RhoA(S188E) (white symbol) were treated as in A. The presence of a phosphomimetic residue does not directly influence p190RhoGAP activity. GFP-loaded RhoA (C) or GFP-loaded RhoA (prenylated) (D) were stimulated with p190RhoGAP (500 μM) as indicated. Basal and GAP-induced activity of phosphorylated ggRhoA (gray curves) was consistent with control ggRhoA (black curves). Both basal and stimulated GAPase activities of unmodified RhoA proteins were slightly higher than their prenylated counterparts (basal initial linear velocity (% hydrolysis min⁻¹ ± S.D.) of RhoA, phosphorylated RhoA, ggRhoA, and phosphorylated ggRhoA was 1.95 ± 0.15, 1.66 ± 0.18, 1.66 ± 0.17, and 1.50 ± 0.26, and stimulated was 5.50 ± 0.40, 5.13 ± 0.32, 3.96 ± 0.32, and 3.96 ± 0.20).

Discussion

In agreement with previous work (13, 19, 23), we found that RhoA is an excellent substrate for PKA. Additionally, we discovered that RhoG, but not Cdc42 or Rac1, was also phosphorylated by PKA. Although RhoA, RhoG, and Cdc42 possess carboxyl serine residues, the preceding three amino acids that target PKA diverge. Although RhoA, Rap1a, and Rap1b, all contain three lysines (KKKS), Cdc42 (PKKS) contains a proline turn in place of the initial basic residue, a divergence that may explain why Cdc42 is a poorer substrate than RhoA in vitro (33). On the other hand, RhoG (KRGRS) contains a glycine flanked by three basic residues, a motif more consistent with KKKS. The significance of RhoG phosphorylation is unknown and currently under investigation.

Unlike Rap1a and Rap1b, which are easily isolated as phosphoproteins from cells (15, 16), a clear demonstration of intracellular RhoA phosphorylation is lacking. Lang et al. (13) were able to immunoprecipitate only small amounts of 32P-labeled RhoA from intact cells, whereas Essler et al. (38) reported recently that a significant charge shift of RhoA was not observed following cAMP stimulation of HUVEC cells. To address this problem, we created antibodies that specifically recognize the phosphorylated form of RhoA. When expressed in cells, the non-prenylated RhoA (C190A) was a cellular target of PKA following forskolin stimulation and during cell spreading, an event associated with low RhoA (32, 34) and high PKA activity (35, 36). Although limited by antibody constraints, these results provide the first conclusive evidence, at least in the case of non-prenylated RhoA, that Serⁱ⁸⁸ is phosphorylated in response to cellular PKA activation in vitro.

Treatment of cells with cAMP or cGMP agonists is associated

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)
with translocation of RhoA from membranes (13, 14). Non-prenylated RhoA is efficiently phosphorylated within the cell (Fig. 2), suggesting that it may be a target of PKA regulation. We therefore analyzed whether Ser188 phosphorylation controls RhoA cycling by impeding prenylation. In vitro assays demonstrated that geranylgeranyl transferase activity was not sensitive to the presence of a phosphate on Ser188. As events of Rho protein prenylation are poorly understood, it remains possible that phosphorylation of this residue affects post-translational modification of RhoA through mechanisms not reflected by in vitro assays. However, regulating RhoA cycling at the step of prenylation appears to be an inefficient and long term approach for a kinase that depends on localized and transient activity (reviewed in Ref. 39).

We found that transiently expressed phosphomimetic Myc-RhoA proteins were poorly loaded with GTP nucleotides compared with control proteins. We also demonstrated that Myc-RhoA(S188E) protein signaling is significantly attenuated compared with control proteins and that this difference was not reflected in the GTP-loading profiles of stably transfected cells. Both of these results are consistent with enhanced sequestration of Myc-RhoA(S188E) by RhoGDI. In the case of transiently expressed protein, sequestration by RhoGDI would reduce the accessibility of GEFs, as is observed in vitro (Tables I and II). In the case of stably expressed proteins, Myc-RhoA(S188E) that has become GTP-loaded over time could still be aberrantly sequestered by RhoGDI. This would be reflected by low RhoA activity in the cell (see Figs. 9 and 10) but not by RBD pulldowns because of a limitation of the assay. The lysis conditions of RBD pulldown assays requires disruption of both RhoA(GTP)$\gamma$H1 and RhoA(GTP)$\gamma$GDI complexes to form RhoA(GTP)$\gamma$GST-RBD complexes that are driven by the vast molar excess of GST fusion proteins.
protein. Not unexpectedly, we found that the lysis conditions completely disrupt RhoA/Rhodi complexes, and have been unable to find lysis conditions that liberate RhoA(GTP) for RBD pulldown assays and preserve RhoA/Rhodi binding. It is probable, therefore, that a portion of the stably expressed and GTP-loaded Myc-RhoA(S188E) protein is released from inactive RhoA(GTP)/Rhodi pools, in addition to active RhoA(GTP)/Rhodi effector complexes. Thus, GST-RBD pulldowns, while an effective tool for measuring RhoGAP-mediated inhibition (34) or GEF activation of RhoA (31), will not reflect Rhodi inhibition by sequestration.

In addition to enhanced binding to Rhodi, Myc-RhoA(S188E) proteins may have lower binding affinity to an effector, such as Rho kinase (23). However, we found that constitutively active GST-RhoA(63L, S188E) was as efficient as GST-RhoA(63L, S188A) proteins in pulling down Rho kinase from NIH cell lysates. Further, stably expressed phosphomimetic RhoA proteins bound the RBD domain of the Rho effector Rhotekin (Fig. 8B). Therefore, we propose that attenuation of phosphomimetic RhoA activation and activity is because of cytosolic sequestration of RhoA driven by enhanced Rhodi interactions that are observed in vitro.

The data presented here provide information regarding RhoA phosphorylation in context of regulatory protein interactions and demonstrate that the addition of a negative charge to Ser188 is sufficient to diminish both RhoA activation and activity within the context of a cell. As PKA also uncouples GEF activation from receptor stimulation (26, 37) and antagonizes microfilament integrity by directly phosphorylating and inhibiting myosin light chain kinase (40), it is becoming clear that PKA inhibits RhoA signaling pathways at multiple levels. It will be important in future work to establish where and when RhoA phosphorylation occurs within the cell and to address whether PKA phosphorylation works in parallel with RhoGAP-mediated inhibition of RhoA during events of cell protrusion and migration.

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