Phosphorylation of Serine 188 Protects RhoA from Ubiquitin/Proteasome-Mediated Degradation in Vascular Smooth Muscle Cells

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Abstract—cAMP and cyclic GMP-dependent kinases (PKA and PKG) phosphorylate the small G protein RhoA on Ser188. We have previously demonstrated that phosphorylation of Ser188 inhibits RhoA-dependent functions and positively regulates RhoA expression, and that the nitric oxide (NO)/cGMP-dependent protein kinase pathway plays an essential role, both in vitro and in vivo, in the regulation of RhoA protein expression and functions in vascular smooth muscle cells. Here we analyze the consequences of Ser188 phosphorylation on RhoA protein degradation. By expressing Ser188 phosphomimetic wild-type (WT-RhoA-S188E) and active RhoA proteins (Q63L-RhoA-S188E), we show that phosphorylation of Ser188 of RhoA protects RhoA, particularly its active form, from ubiquitin-mediated proteasomal degradation. Immunoprecipitation experiments indicate that the resistance of the phosphorylated active form of RhoA to proteasome-mediated degradation is because of its cytoplasmic sequestration through enhanced RhoGDI interaction. In rat aortic smooth muscle cells, stimulation of PKG and inhibition of proteasome by lactacystin, induce nonadditive increases in RhoA protein expression. In addition, stimulation of PKG leads to the accumulation of GTP-bound RhoA in the cytoplasm. In vivo stimulation of the NO/PKG signaling by treating rats with sildenafil increased RhoA level and RhoA phosphorylation, and enhanced its association to RhoGDI in the pulmonary artery, whereas opposite effects are induced by chronic inhibition of NO synthesis in Nω-nitro-L-arginine-treated rats. Our results thus suggest that Ser188 phosphorylation-mediated protection against degradation is a physiological process regulating the level of endogenous RhoA and define a novel function for RhoGDI, as an inhibitor of Rho protein degradation. (Circ Res. 2005;96:1152-1160.)

Key Words: Rho-GTP-binding proteins ■ signal transduction ■ phosphorylation ■ cGMP ■ ubiquitin

The small G proteins of the Rho family are identified as tightly regulated molecular switches that cycle between an active GTP-bound form, and an inactive GDP-bound form. Rho proteins are recognized as major regulators of the actin cytoskeleton.1,2 A large body of evidence has now been obtained regarding the important functions of Rho proteins in the vasculature.3-5 The activity of Rho is under the direct control of a large set of other regulatory proteins.2,6 In the inactive GDP-bound form, Rho is locked in the cytosol by guanine dissociation inhibitors (RhoGDI).7 The guanine nucleotide exchange factors (RhoGEFs) catalyze the exchange of GDP for GTP to activate RhoA.8 In the active GTP-bound form, Rho translocates to plasma membrane where it interacts with effectors to transduce the signal downstream. GTPase-activating proteins that hydrolyze GTP to GDP then turn off activation. In addition to this regulation, recent reports have proposed that the phosphorylation/dephosphorylation cycle also controls small G protein activity. cAMP-dependent protein kinase (PKA) has been shown to phosphorylate Ser180 of Rap1, Ser179 of Rap1B, and Ser188 of RhoA causing their relocalization in the cytosol.9-11 In vitro experiments have indicated that RhoA phosphorylation on Ser188 increases the ability of RhoGDI to extract RhoA from membrane.11,12 Studies from this laboratory have shown that cGMP-dependent protein kinase (PKG) also phosphorylates RhoA on Ser188 and that the NO/PKG pathway plays an essential role, both in vitro and in vivo, in the regulation of RhoA protein expression and functions in vascular smooth muscle.13-15 PKG-mediated inhibition of RhoA-dependent functions such as actin cytoskeleton organization, vascular smooth muscle contraction and serum-response factor-dependent transcription is, at least partially, due to Ser188 phosphorylation of RhoA.13,16,17 In addition, we showed that activation of PKG increases the stability of RhoA.15 There is only very limited published data on the regulation of stability...
or production/degradation of Rho proteins. The inducible RhoB protein has been reported to have rapid turnover because of ubiquitin-mediated destruction by the 26S proteasome and carboxyl methylation of RhoA and Cdc42 has been reported to increase their half-life. Recently, it has been described that Rac, constitutively activated by the cytoxic necrotizing factor 1, is subjected to enhanced ubiquitin/proteasome-mediated degradation. In addition, Cdc42-mediated recruitment of the E3 ubiquitin ligase SmurF1 to cellular protrusion has been shown to induce degradation of RhoA in lamellipodia and filopodia to prevent RhoA signaling during dynamic membrane movements. These data suggest that regulation of Rho protein degradation could participate in the regulation of Rho protein functions.

Recent studies reported that the C-terminal polybasic region of Rho proteins could be involved in the regulation of Rho protein degradation. Since Ser188 immediately follows the basic amino acids of the C-terminal region of RhoA, we hypothesized that phosphorylation of RhoA at Ser188 affects its susceptibility to degradation. We report that phosphorylation of Ser188 of RhoA protects RhoA, particularly its active form, from ubiquitin-mediated proteasomal degradation. The resistance of the active, phosphorylated form of RhoA to proteasome-mediated degradation results from its cytoplasmic sequestration through enhanced RhoGDI interaction. Consistently, stimulation of PKG activity in vascular smooth muscle cell leads to the accumulation of GTP-bound RhoA in the cytoplasm.

Materials and Methods
For expanded Materials and Methods, see the online data supplement available at http://circres.ahajournals.org.

Cell Culture and Transfection
Rat aortic smooth muscle cells were isolated as previously described and used at passage 2 as we previously showed that they expressed PKG. Human umbilical vein endothelial cells, obtained from PromoCell (Heidelberg, Germany) were cultured and transfected. Swiss 3T3 fibroblasts were plated 24 hours before transfection to reach 60% to 70% confluence the day of transfection.

Western Blot Analysis
After treatment, cells were harvested and homogenized in lysis buffer. Proteins were resolved on SDS-PAGE and transferred to nitrocellulose membranes, which were incubated with specific antibodies. Signals from immunoreactive bands were detected by ECL (Amersham) and quantified using QuantityOne (BioRad). When needed, cell fractionation was performed according to Dignam et al.

Measurement of RhoA Activity
RhoA activity was assessed in homogenized cell samples by pull-down assays using the Rho-binding domain of the Rho effector Rhotekin.

Plasmids and Site-Directed Mutagenesis of RhoA
DNA vectors corresponding to HA-pcDNA3-RhoA-WT (WT-RhoA), or HA-pcDNA3-RhoA-Q63L (Q63L-RhoA) have been used. In vitro site-directed mutagenesis was performed according to the QuikChange site-directed mutagenesis kit instruction manual (Stratagene).

Coimmunoprecipitation
Protein extracts were precleared with 40 μL of protein A- or G-Sepharose beads, and immunoprecipitations were performed with monoclonal anti-HA antibody, anti-RhoGDI antibody, or antiphosphoserine antibody preadsorbed on protein A- or G-Sepharose beads.

RESULTS

The protein A/G-Sepharose-bound immune complexes were then washed. Pellets from the immunoprecipitations were heated at 95°C for 5 minutes in 70 μL of Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot for RhoA, RhoGDI, or HA.

RhoA Ubiquitylation Measurements
RhoA ubiquitylation measurements were performed as previously described.

Animal Model
Three groups of male Wistar rats (250 g) were used. The control group (8 rats) did not receive any treatment and the sildenafil-treated group (5 rats) received sildenafil (25 mg/kg/d orally administered) throughout 3 days. The third group (5 rats) was given 0.5 g/L Nω-nitro-L-arginine (L-NNA) in their drinking water for 10 days (L-NNA treated rats). At completion of this time, the main pulmonary was taken out, dissected, and prepared as indicated for immunoprecipitations and Western blot analysis.

Chemicals and Drugs
Mouse monoclonal anti-RhoA antibody (26C4) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif). Mouse monoclonal anti-HA (HA.11) was from Berkely Antibody Co. (BabCO, Richmond, Calif) and rabbit polyclonal anti-RhoGDI antibody from Upstate Biotechnology (Lake Placid, NY). Rp-8-Br-PET-cGMPS, Rp-8-CPT-cAMPS, and 8-pCPT-cGMP were purchased from Biolog Life Science Institute (Bremen, Germany). Lactacystin and H-89 (N-[2-((bromocinnamyl)amino)ethyl]-5-isouquinolinesulfonamide were purchased from Calbiochem (Darmstadt, Germany). All other reagents were purchased from Sigma (Saint-Quentin, Fallavier, France).

Statistics
All results are expressed as the mean±SD of sample size n. Significance was tested by ANOVA or Student’s t test.

RESULTS

PKG Activation Increases RhoA Stability
The stability of RhoA in rat aortic smooth muscle cells has been analyzed by RhoA immunoblotting in the presence of cycloheximide (CHX, 10 μg/mL). Figure 1A shows that stimulation of smooth muscle cells with sodium nitroprusside (SNP, 10 μmol/L) for 6 hours prevented the degradation of endogenous RhoA. A similar effect was observed in PKG-expressing Swiss 3T3 fibroblasts transfected with the WT-RhoA, stimulated with the cGMP analog 8-pCPT-cGMP (100 μmol/L) (Figure 1B). In contrast, the effect of 8-pCPT-cGMP on RhoA stability was not observed in cells expressing the phosphorylation resistant RhoA mutant (WT-RhoA-S188A) (Figure 1B). This result shows the essential role of the phosphorylation of RhoA at Ser188 in PKG stimulation-mediated RhoA stabilization. Moreover, these results suggest that the mechanisms regulating RhoA stability/degradation in vascular smooth muscle cells are also found in Swiss 3T3 fibroblasts. To confirm the stabilizing effect of phosphorylation of Ser188 of RhoA in vascular smooth muscle cells, we next used DETA NONOate (2,2’(hydroxynitrosohydrazino)bis-ethanamine), which allows a more prolonged NO release and PKG activation. Figure 1C shows that DETA NONOate (100 μmol/L, 6 hours) in the presence of CHX induced a stabilization of RhoA much stronger than that obtained with SNP, that was inhibited in the presence of the PKG inhibitor Rp-8-Br-PET-cGMPS (100 nmol/L). The stabilizing effect of
phosphorylation of Ser188 of RhoA was further confirmed by using the PKA activator forskolin (FSK, 25 μmol/L), the effect of which was prevented by the PKA inhibitor H-89 (3.5 μmol/L) (Figure 1C).

**RhoA Phosphorylation Slows-Down RhoA Degradation**

To directly analyze how phosphorylation of Ser188 affects RhoA protein stability, we next transfected fibroblasts with HA-tagged Ser 188 phosphomimetic RhoA (S188E). Since Rho protein activity has been shown to affect its susceptibility to degradation,21,22 both wild-type (WT-RhoA-S188E) and constitutively active GTPase-deficient (Q63L-RhoA-S188E) RhoA mutants have been used. To address the stability of RhoA mutants, we examined the fate of transfected RhoA proteins by Western blotting with anti-HA tag antibody, in the presence of CHX (10 μg/mL). Under this condition, the half-life of WT-RhoA was 5 hours (Figure 2A and 2B). A similar time course was obtained with the WT-RhoA-S188A (not shown). The active Q63L-RhoA showed a shorter half-life (T1/2≈3 hours) compared with the WT-RhoA. In contrast, the nonprenylated C190A-RhoA,

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**Figure 1.** Activation of the PKA or PKG signaling pathway controls RhoA protein levels. Western Blot and corresponding densitometric analyses for RhoA expression in rat aortic smooth muscle cells (endogenous RhoA) (A and C), or Swiss 3T3 fibroblasts transfected with wild-type (WT-RhoA), or the phosphorylation-resistant mutant (WT-RhoA-S188A) (B), preincubated with 10 μg/mL cycloheximide (CHX, 30 minutes) then stimulated for 6 hours with 10 μmol/L SNP (A), 50 μmol/L 8-pCPT-cGMP (B), 100μmol/L DETA NONOate and 25 μmol/L FSK in the absence, or in the presence of Rp-8-Br-PET-cGMPS (100 nmol/L), or H-89 (3.5 μmol/L), respectively (C). Results are expressed relative to the control in the absence of CHX taken as 1. The data presented are representative of at least 3 independent experiments. **P<0.005.

**Figure 2.** Ser188 phosphorylation of RhoA increases its stability. A, Swiss 3T3 fibroblasts transfected with WT-RhoA, Q63L-RhoA, C190A-RhoA, or their phosphomimetic mutants WT-RhoA-S188E, Q63L-RhoA-S188E, C190A-RhoA-S188E, were incubated with 15 μg/mL cycloheximide for 0, 4, or 8 hours. Expression of these RhoA mutants was assessed by Western blot analysis using anti-HA antibody. B, RhoA protein level, normalized to β-actin expression, is expressed relative to the control (0 hour) taken as 100%. The data presented are representative of at least 3 independent experiments. *P<0.05 and **P<0.005, phosphomimetic vs nonphosphomimetic form at the same time.
shown to have a low level of GTP loading, displayed an increased stability with T1/2 longer than 8 hours (Figure 2A and 2B). For the 3 forms of RhoA, phosphoserine mimetic RhoA mutants were significantly more stable (Figure 2A and 2B). The strongest effect was obtained with the active Q63L-RhoA-S188E, with a T1/2 that became longer than 8 hours. These data thus suggest that phosphorylation of Ser188 increases RhoA protein stability independently of GTP/GDP loading and prevents the increased susceptibility to degradation of GTP-loaded RhoA.

**Cytoplasmic Localization of Phosphoserine Mimetic RhoA Mutants**

To examine whether a relationship exists between the stability of RhoA protein and its subcellular distribution, the presence of RhoA mutants has been assessed in membrane and cytosolic fractions from Swiss-3T3-transfected cells. All the RhoA mutants have similar expression levels (Figure 3A). As expected, WT-RhoA and C190A-RhoA were mainly found in the cytosol (Figure 3A and 3B). Phosphomimetic mutation of Ser188 further enhanced this cytosolic distribution of both WT- and C190A-RhoA (Figure 3A and 3B). In contrast, 90% of the constitutively active Q63L-RhoA mutant was associated to the membrane (Figure 3A and 3B). However, this subcellular distribution was completely compromised in cells expressing the phosphoserine mimetic Q63L-RhoA mutant as 67.8 ± 7.5% (n = 3) of Q63L-RhoA-S188E was localized in the cytosolic fraction (Figure 3A and 3B). These results provide evidence that phosphorylation of Ser188 in intact cells is sufficient to extract constitutively active GTP-loaded RhoA from the membrane to the cytosol.

**Phosphoserine Mimetic RhoA Mutants Bind to RhoGDI**

Subcellular RhoA distribution can be related to its binding to RhoGDI and in vitro experiments using recombinant proteins have suggested that phosphorylation on Ser188 favors the interaction with RhoGDI. These previous observations thus prompted us to examine binding of phosphoserine mimetic RhoA mutants to endogenous RhoGDI in intact cells. Figure 4 shows that a similar quantity of RhoGDI coimmunoprecipitated with WT-RhoA and C190A-RhoA, while the amount of RhoGDI coimmunoprecipitated with Q63L-RhoA was reduced by 50% compared with WT-RhoA. The 3 different phosphomimetic RhoA mutants displayed an increased association with RhoGDI, while the expression level of RhoGDI is unchanged (Figure 4A and 4B). The amount of RhoGDI associated with WT-RhoA-S188E and C190E-RhoA-S188E was doubled compared with the corresponding control mutant. The association of the nonprenylated C190A-RhoA with RhoGDI confirms a previous report showing that prenylation is not required for RhoGDI binding, and suggests that protein-protein interactions, which are increased with the phosphomimetic mutants, are sufficient to form a complex between the 2 proteins. A dramatic 4-fold increase in RhoGDI association was observed with Q63L-RhoA-S188E compared with the Q63L-RhoA (Figure 4A and 4B). This observation provides evidence that phosphorylation of Ser188 in intact cells is sufficient to promote the binding of constitutively active GTP-loaded RhoA to endogenous RhoGDI. These results indicate that the greater stability of phosphoserine mimetic RhoA mutants is associated with redistribution of the protein in the cytosolic fraction and its binding to RhoGDI.

**Decreased Ubiquitylation of Phosphoserine Mimetic RhoA Mutants**

To investigate whether the increased stability of phosphoserine mimetic RhoA mutants depended on a decreased RhoA ubiquitylation, we compared the ubiquitylation sensitivity of phosphomimetic RhoA mutants to their corresponding controls. Cells were cotransfected with histidine-tagged ubiquitin and WT-RhoA or RhoA mutants. We observed that ubiquitylation of the phosphorylation resistant WT-RhoA-S188A mutant is similar to that of the WT-RhoA (Figure 5A and 5B). In contrast, both WT- and Q63L-RhoA phosphomimetic
mutants were less ubiquitylated than WT-RhoA and Q63L-RhoA, respectively. The WT-RhoA-S188D and Q63L-RhoA-S188D showed a 40% reduction of ubiquitylation level, and the ubiquitylation of the WT-RhoA-S188E and Q63L-RhoA-S188E mutants was almost abolished (Figure 5A and 5B). These observations provide evidence that phosphorylation of Ser188 desensitizes RhoA to ubiquitylation and subsequently might decrease its proteasomal degradation, in particular in its activated GTP-bound form. This prompted us to investigate the effect of proteasome inhibitor on the level of RhoA protein in vascular smooth muscle cells, in the absence or presence of PKG stimulation.

**Proteasome Inhibition Induces RhoA Protein Accumulation**

The proteasome inhibitor lactacystin has been used to assess the role of proteasome in the regulation of RhoA level in vascular smooth muscle cells and in the stabilizing effect of PKG stimulation. Under control conditions, lactacystin significantly induced time-dependent accumulation of RhoA, indicating that proteasomal degradation of RhoA participates in the regulation of RhoA expression (Figure 6A and 6B). Quantitatively, the increase in RhoA protein level induced by lactacystin was similar to that produced by 8-pCPT-cGMP treatment for the same time (Figure 6A and 6B). However, the effect of lactacystin and 8-pCPT-cGMP were not additive, indicating that an increase in RhoA protein level induced by both lactacystin and 8-pCPT-cGMP-mediated PKG stimulation were underlain by common mechanism (Figure 6A and 6B). A similar increase in RhoA protein level, that was also not additive with lactacystin effect, was obtained by stimulation of PKA with forskolin (20 μmol/L; not shown). On the other hand, inhibition of PKA or PKG activity decreased the

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Ser188 phosphorylation of RhoA promotes Rho-RhoGDI interaction. A, RhoA mutants (WT-RhoA, Q63L-RhoA, C190A-RhoA, or their phosphomimetic mutants WT-RhoA-S188E, Q63L-RhoA-S188E, C190A-RhoA-S188E) were immunoprecipitated with anti-HA antibody. RhoGDI binding was tested using anti-RhoGDI antibody. Specificity of the detected signal was analyzed by blotting extract from cells transfected with an empty vector (mock). Mutants or RhoGDI expression was analyzed by Western blotting of cell lysates using anti-HA or anti-RhoGDI antibodies respectively. B, Each phosphomimetic mutant bound more RhoGDI than its nonphosphomimetic form. RhoGDI binding is normalized to HA immunoprecipitations and expressed relative to the binding of the WT considered as 100%. The data presented are representative of 3 independent experiments. *P<0.05 phosphomimetic vs nonphosphomimetic form.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Decreased ubiquitylation of Ser188 phosphomimetic mutants of RhoA. Ubiquitylated proteins are purified by metal-affinity precipitation using histidine-tagged ubiquitin and ubiquitylated RhoA is revealed using anti-HA antibody. A, S188E phosphomimetic mutants (WT-RhoA-S188D, WT-RhoA-S188E, Q63L-RhoA-S188D, Q63L-RhoA-S188E) are less ubiquitylated than their respective control (WT-RhoA and Q63L-RhoA) whereas the phosphorylation-resistant mutant (WT-RhoA-S188A) is as ubiquitylated as the WT-RhoA. Specificity of the detected signal is analyzed by blotting extracts from cells transfected with an empty vector instead of the pRBG4–6HisUb. B, Ubiquitylation is expressed relative to the ubiquitylation of the nonphosphomimetic form considered as 100%. The data corresponded to 3 independent experiments.
basal RhoA protein level (not shown). Together, these data suggest that activity of kinases that phosphorylate RhoA on Ser188 in intact cells protects RhoA from proteasome-mediated degradation and this mechanism participates to the control of RhoA protein level in vascular smooth muscle cells.

**PKG Stimulation Induces Accumulation of Cytosolic GTP-Bound RhoA Protein**

Our results with phosphomimetic RhoA mutants suggest a major protective role of the phosphorylation of Ser188 against degradation of the active GTP-bound form of RhoA through enhanced RhoGDI interaction (Figure 2 and 3). Accordingly, it could be expected that phosphorylation of endogenous RhoA might lead to increased association with GDI and accumulation of GTP-bound RhoA. To assess this hypothesis, we first analyzed RhoA/RhoGDI interaction in vascular smooth muscle cells submitted to PKG stimulation. Figure 7A shows that stimulation of aortic smooth muscle cells by 8-pCPT-cGMP increased the amount of endogenous RhoA that coimmunoprecipitated with RhoGDI, attesting the enhancement of RhoA to RhoGDI interaction. This increased RhoA/RhoGDI interaction was mediated by PKG activity, as it was lost in the presence of Rp-8-Br-PET-cGMPS. We next examined the activity and the subcellular distribution of RhoA in vascular smooth muscle cells submitted to PKG stimulation. Stimulation with serum has been used as a positive control. Pull-down assays show that, as expected, stimulation of cells with serum led to an increase in the amount of GTP-bound RhoA (Figure 7B). Furthermore, isolation of cytosolic and membrane fractions indicated that the increased amount of GTP-RhoA in serum-stimulated cells is located in the membrane fraction (Figure 7B). In contrast to that observed in serum-stimulated cells, stimulation by 8-pCPT-cGMP induced an increase in GTP-RhoA that was localized in the cytosolic fraction (Figure 7B). This effect was mediated by PKG activity as it was abolished in the presence of Rp-8-Br-PET-cGMPS (Figure 7B). This result demonstrates that PKG activity, by phosphorylating RhoA, protected GTP-RhoA from degradation and led to the accumulation of cytosolic GTP-RhoA.

**NO/PKG Signaling Mediates RhoA Phosphorylation and Regulates RhoA Association to RhoGDI In Vivo**

We next assessed the existence of NO/PKG mediated-RhoA phosphorylation and regulation of its association to GDI in rat arteries in vivo. For this purpose, we analyzed RhoA expres-
sion, RhoA phosphorylation, and RhoA binding to RhoGDI in pulmonary artery of sildenafil- and L-NNA treated rats. The potent and selective inhibitor of the type 5 phosphodiesterase sildenafil has been used to stimulate cGMP-PKG signaling, and in contrast, L-NNA has been used to inhibit NO production and depress cGMP-PKG signaling in vascular smooth muscle cells. Analysis of RhoA expression in total lysates of pulmonary artery confirmed the rise in the amount of RhoA in sildenafil treated rats and the decrease in RhoA level in L-NNA treated rats (Figure 8A and 8B). To analyze serine phosphorylation of RhoA, RhoA-serine-phosphorylated proteins were immunoprecipitated from pulmonary artery of control, sildenafil- and L-NNA-treated rats, and RhoA was detected with specific anti-RhoA antibodies. Figure 8 shows that serine-phosphorylated RhoA is detected under control condition. The amount of serine-phosphorylated RhoA immunoprecipitated from pulmonary artery of sildenafil-treated rats was strongly increased compared with control (Figure 8A and 8C). This increase in serine-phosphorylated RhoA correlated with an increase in the amount of RhoA that coimmunoprecipitated with RhoGDI (Figure 8A and 8C). On the contrary, in pulmonary artery of L-NNA treated rats, the amount of serine-phosphorylated RhoA and the amount of RhoA bound to RhoGDI were both decreased (Figure 8B and 8D). Similar results were obtained in aorta (not shown). Although the effect of sildenafil was less pronounced in aorta than in pulmonary artery. This could be related to the different expression level of PDE5 or different relative contribution of PDE5 to cGMP hydrolysis in aorta and in pulmonary artery. These results thus provide evidence that NO-PKG signaling phosphorylates RhoA in vivo, and that PKG-mediating phosphorylation of RhoA regulates its interaction to RhoGDI.

### Discussion

Both RhoA-dependent signaling and NO/PKG-signaling pathway are identified as essential determinants of vascular functions. The small G protein RhoA is a convergence point for multiple signals that regulate smooth muscle cell functions, and NO/PKG signaling plays a major role in the structure and function of normal adult vessel wall. We have previously demonstrated that PKG phosphorylated RhoA on Ser188 and inhibited RhoA-dependent functions but also positively regulated RhoA expression, in part through stimulation of RhoA gene transcription. Here we show that phosphorylation of Ser188 protects RhoA from ubiquitin-mediated proteasomal degradation by forcing cytosolic localization through enhanced RhoGDI binding. Our results indicate that this mechanism is a physiological process regulating the level of RhoA and that stimulation of PKG activity in vascular smooth muscle cells leads to the accumulation of GTP-bound RhoA in the cytoplasm. In vivo experiments demonstrate that this process is active under basal conditions, and that stimulation or inhibition of NO/PKG signaling regulates RhoA phosphorylation and localization in arteries.

The precise temporal and spatial coordination of Rho proteins is an important determinant for a large range of functions.
cellular activity. The isoprenylation of the CAAX-Box at the C-terminus of Rho proteins is a crucial factor of membrane localization. Activation of downstream RhoA effectors requires translocation of RhoA to the membrane fraction. We found that active form of RhoA is mainly associated to the membrane fraction (Figure 3), poorly associated to RhoGDI (Figure 4), and subjected to enhanced degradation (Figure 2). However, completely opposite properties have been found for the phosphoserine mimic active RhoA mutant, which was found essentially in the cytosolic fraction, mainly associated to RhoGDI, and slowly degraded. These data indicate that in intact cells, Ser188 phosphorylation of RhoA forces the cytosolic sequestration of RhoA through enhanced RhoGDI interaction, regardless of the activation state (GTP or GDP loading) of the protein. Therefore, the phosphorylation state of RhoA overrides the activation state to define the Rho protein localization. Our observations thus suggest that RhoA undergoes proteasomal degradation at the plasma membrane and that the cytosolic accumulation of phosphorylated RhoA protects it from degradative pathways. This is in agreement with the limited degradation of the nonphosphorylated, inactive RhoA (C190A-RhoA) and indicates that, as described for Rac23 and for RhoB,19 the C-terminal region of RhoA and the isoprenol moiety are important for its degradation. Addition of a charged group to the C-terminal region on phosphorylation of Ser188 could thus alter recognition of RhoA by the ubiquitin/proteasome machinery or its binding of a regulatory protein. The correlation between the increased stability of phosphomimetic RhoA mutants and enhanced RhoGDI interaction indeed suggests that binding of phosphorylated RhoA to RhoGDI is the key determinant of the phosphorylation-mediated proteasomal degradation. Our results are thus consistent with the hypothesis recently drawn from in vitro experiments suggesting that RhoGDI binding could inhibit RhoA interaction with the E3 ubiquitin ligase Smurfl and subsequent ubiquitin-mediated proteasomal degradation.23

A small fraction of cellular proteins, essentially proteins involved in regulatory pathway undergo rapid turnover. Their limited life span, in concert with other control mechanisms, provides regulations of their biological functions. In addition, controlling the turnover rate of signaling proteins, thereby influencing their steady-state levels, could strongly affect signal transduction pathways in which these proteins participate. Our observation that endogenous RhoA is subjected to proteasomal degradation (Figure 6) indicates that RhoA degradation is a physiological process that may serve to regulate the level of RhoA and consequently RhoA-dependent signaling in living cells. In addition, the changes in RhoA protein levels induced by stimulation of PKG and PKA in vascular smooth muscle cells (Figure 6 and 7) suggests that phosphorylation-mediated protection from proteasomal degradation of RhoA is an additional physiological mechanism involved in the regulation of RhoA level and function. In agreement with the results obtained with the phosphomimetic RhoA mutants, PTK-mediated phosphorylation leads to accumulation of GTP-bound RhoA in the cytosolic fraction of smooth muscle cells (Figure 7). The presence of GTP-bound Rho protein in the cytosol is consistent with other recent reports showing that in suspended cells, growth factors induce GTP loading of Rac but GTP-Rac is found in the cytosol.33 Binding of RhoGDI prevents GTP-Rac from coupling to effectors, and therefore blocks the stimulation of downstream cytosolic events.34 The release of RhoGDI and membrane translocation allows the coupling of activated Rac to effectors. In the present study, we suggest that the presence of RhoGDI-GTP-bound RhoA in the cytosol is related to PKG-mediated Ser188 phosphorylation of RhoA. This PKG-induced increase in the amount of cytosolic, GTP-loaded RhoA is associated with a low level of RhoA signaling pathway activity (transcription and actin cytoskeleton organization),13,17 in agreement with the association of phosphorylated GTP-RhoA to RhoGDI and the inability of RhoGDI-bound Rho protein to interact with downstream effectors.34 According to the results obtained with Rac,33,34 it could be envisaged that activation of mechanism leading to dephosphorylation of Ser188 would allow release of RhoGDI, membrane translocation and activation of RhoA effectors. Further studies are now required to assess this hypothesis and more generally, to define the functional relevance of the RhoA regulation mechanism described in this study.

In summary, we have determined a new physiological mechanism for the regulation of endogenous RhoA expression. Phosphorylation of Ser188 of RhoA prevents its ubiquitin-mediated proteasomal degradation through enhanced RhoGDI association and cytosolic sequestration. Accordingly, we have defined a novel function for RhoGDI, as an inhibitor of Rho protein degradation. By this mechanism, the NO/PKG pathway controls the steady-state level of RhoA in vascular smooth muscle cells and therefore, could strongly affect signal transduction pathways in which RhoA participate.

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Cell Culture and transfection

Rat aortic smooth muscle cells were isolated by enzymatic dissociation as previously described. Only smooth muscle cells at passage 2 were used in this study as we previously showed that they expressed PKG. Stimulation or inhibition of PKA (forskolin, 25 µmol/L) or PKG (8-pCPT-cGMP, 100 µmol/L) pathways were performed in the absence of serum in the culture medium. Human umbilical vein endothelial cells (HUVECs), obtained from PromoCell (Heidelberg, Germany) were cultured and transfected as previously described. Swiss 3T3 fibroblasts were plated 24 h before transfection in 100 mm plates to reach 60-70% confluence the day of transfection. The different constructions of RhoA were transfected in 3T3 fibroblasts with jetPEI (Qbiogen, Illkirch, France) according to the manufacturer's instructions, leading to transfection efficiency of 60-80%. Twenty four hours after transfection, the culture medium is replaced by fresh culture medium and cells are treated for different time, harvested and analyzes by Western Blotting. To avoid contamination by endogenous, basal PKA activity, all experiments (except Figure 1) were performed in the presence of PKA inhibitor (Rp-8-CPT-cAMPS, 100 nmol/L).

Western Blot Analysis

After treatment, cells were harvested and homogenized in lysis buffer containing 20 mmol/L Hepes-NaOH, 10 mmol/L KCl, 10 mmol/L NaCl, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, and protease inhibitors. Nuclei and unlysed cells were removed by low speed centrifugation at 10,000g for 15 min at 4 °C. Proteins were resolved on SDS-PAGE and transferred to nitrocellulose membranes, which were incubated with specific antibodies. Signals from
immunoreactive bands were detected by ECL (Amersham) and quantified using QuantityOne (BioRad). Equal loading was checked by reprobing the membrane with monoclonal anti β-actin antibody. When needed cell fractionation were performed according to Dignam et al.⁴

**Measurement of RhoA activity**

RhoA activity was assessed in homogenized cell samples by pull-down assays using the Rho-binding domain of the Rho effector Rhotekin as described previously.⁵ Cells were washed with ice-cold PBS three times and lysed in a lysis buffer (50 mmol/L Tris, pH 7.2, 500 mmol/L NaCl, 10 mmol/L MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Sigma) 1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 12000 g at 4°C for 10 minutes, the extracts were incubated at 4°C for 45 minutes with glutathione-Sepharose 4B beads coupled with glutathione-S-transferase (GST)-rhotekin fusion protein to determine RhoA activity. Precipitated GTP-bound RhoA and total RhoA were then analyzed by Western Blotting. Signals from immunoreactive bands were detected by ECL (Amersham) and quantified using QuantityOne (BioRad).

**Plasmids and Site-directed Mutagenesis of RhoA**

DNA vectors corresponding to HA-pcDNA3-RhoA-WT or HA-pcDNA3-RhoA-Q63L have been used.⁶ The Q63L mutation renders the RhoA protein constitutively active. The HA tag allows recognition of transfected proteins by a monoclonal anti-HA antibody. To analyze the effect of phosphorylation of RhoA on Ser188, we created RhoA proteins containing phosphomimetic residues (glutamic acid [E] and Aspartic Acid [D]) in the place of Ser188, and non-phosphorylatable RhoA protein in which ser188 was replaced by alanine (A). *In vitro* site-directed mutagenesis was carried out according to the QuikChange site-directed mutagenesis kit instruction manual (Stratagene, La Jolla, CA) using the following PAGE-
purified primers: S188A up 5’GGGAAGAAAAAAGCTGGTTGCTTGTC3’; S188A down 5’GACAAGGCAACCACTTCTTTTCTTCCC3’; S188D up 5’GGGAAGAAAAAAGATGGTTGCTTGTC3’; S188D down 5’GACAAGGCAACCATCTTTTTTTCTTCCC3’; S188E up 5’GGGAAGAAAGAAGGTGCCCTTGTC3’. C190A up 5’GGGAAGAAAAATCTGGTGCCTTGTC3’; C190A down 5’CAAGACAAAGGGCACCAGATTCTCTTGCCC3’. The absence of other mutations was verified by sequencing the entire coding region.

**Coimmunoprecipitation**

Cells or tissues were harvested in NETF buffer (100 mmol/L NaCl, 2 mmol/L EGTA, 50 mmol/L Tris-Cl pH 7.4 and 50 mmol/L NaF) containing 1% NP-40, 2 mmol/L orthovanadate, protease inhibitors and phosphatase inhibitor cocktail (Sigma). Samples were precleared with 40 µl of protein A- or G-sepharose beads (which have been washed in NETF buffer to give a 50% slurry), and immunoprecipitations were carried out with monoclonal anti-HA antibody, polyclonal anti-RhoGDI antibody or monoclonal anti-phosphoserine antibody preadsorbed on protein A- or G-sepharose beads. The protein A/G-sepharose-bound immune complexes were washed twice in NETF buffer containing NP-40 (1% w/v) and once in NETF without detergent. Pellets from the immunoprecipitations were heated at 95 °C for 5 min in 70 µl of Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot for RhoA, RhoGDI and/or HA. Signals from immunoreactive bands were detected by ECL (Amersham) and quantified using QuantityOne (BioRad).

**RhoA ubiquitylation measurements**
RhoA ubiquitylation measurements were performed as previously described. Briefly, cells were transfected by electroporation with pRBG4-6HisUb (15 µg/5 × 10⁶ cells) and HA-pcDNA3-RhoA wild-type and mutants (15 µg/5 × 10⁶ cells). Transfection efficiency was around 35-50%. Transfected cells were lysed in ULB (8 M Urea, 20 mmol/L Tris-HCl [pH 7.5], 200 mmol/L NaCl, 10 mmol/L imidazole, and 0.1% Triton X-100). The quantities of RhoA mutants present in the clarified lysates were first determined on immunoblots in order to use equal quantities of RhoA mutants in the histidine-tag purifications. Normalized HA-RhoA lysates were purified for histidine-tagged ubiquitin on 30 µl cobalt chelated resin (Clontech), previously incubated in 5% bovine serum albumin (RIA grade, Sigma). After lysate incubation, the beads were washed four times in ULB and once in PBS and resuspended in one volume of Laemmli buffer. Proteins were resolved on a 12% SDS-PAGE, and polyubiquitylated RhoA was visualized by immunoblotting anti-HA. The efficiency of RhoA mutant ubiquitylation was assessed after 16 h expression. Graphs correspond to the ratio of the RhoA-Ubn signal to the total HA-RhoA signal.

Animal model
Three groups of male Wistar rats (250 g) were used. The control group (8 rats) did not receive any treatment and the sildenafil-treated group (5 rats) received sildenafil (25 mg/kg/d orally administrated) throughout 3 d. The third group (5 rats) was given 0.5 g/L N-ω-nitro-L-arginine (L-NNA) in their drinking water for 10 d (L-NNA treated rats). At completion of this time, the main pulmonary was taken out and dissected under binocular. Tissues were then prepared as indicated for immunoprecipitations and Western blot analysis.

Chemicals and Drugs
Mouse monoclonal anti-RhoA antibody (26C4) was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Mouse monoclonal anti-HA (HA.11) was purchased from Berkely Antibody Co. (BabCO, Richmond, CA, USA) and rabbit polyclonal anti-RhoGDI antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rp-8-Br-PET-cGMPS, Rp-8-CPT-cAMPS and 8-pCPT-cGMP were purchased from Biolog Life Science Institute (Bremen, Germany). Lactacystin and H-89 (N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide were purchased from Calbiochem (Darmstadt, Germany). All other reagents were purchased from Sigma (Saint-Quentin Fallavier, France).

Statistics

All results are expressed as the means±SD of sample size $n$. Significance was tested by ANOVA or Student's $t$ test.

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Online Data Supplement

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**References**


