Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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31. The authors thank V. Graziano for analyzing the binding affinities of the knob variants; M. Rossmann, D. Engelman, C. Anderson, J. Dunn, and J. Kuryian for critically reading the manuscript and suggesting improvements; and L. Berman, R. Sweet, and J. Berendsen for access to beamlines X25, X12C, and X8C, respectively. This research was supported by NIH grant AI36251 to P.F. and by the Office of Biological and Environmental Research of the U.S. Department of Energy under Prime Contract DE-AC02-98CH10886 with Brookhaven National Laboratory. The macromolecular crystallography beamlines X25, X12C, and X8C, at the National Synchrotron Light Source, are also supported by NSF and by NIH grant 1P41 RR12408-01A1. Coordinates have been deposited in the Protein Data Bank with accession codes 1NOB and 1KAC for Ad12 knob and Ad12 knob in complex with CAR D1, respectively.

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Regulation of Myosin Phosphatase by a Specific Interaction with cGMP-Dependent Protein Kinase Iα

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Contraction and relaxation of smooth muscle are regulated by myosin light-chain kinase and myosin phosphatase through phosphorylation and dephosphorylation of myosin light chains. Cyclic guanosine monophosphate (cGMP)–dependent protein kinase Iα (cGKIα) mediates physiologic relaxation of vascular smooth muscle in response to nitric oxide and cGMP. It is shown here that cGKIα is targeted to the smooth muscle cell contractile apparatus by a leucine zipper interaction with the myosin-binding subunit (MBS) of myosin phosphatase. Uncoupling of the cGKIα-MBS interaction prevents cGMP-dependent dephosphorylation of myosin light chain, demonstrating that this interaction is essential to the regulation of vascular smooth muscle cell tone.

Smooth muscle cells are critical to the normal physiology of many of the organs of the body. Smooth muscle cells are the principal component of blood vessels, where they regulate vascular tone and play a central role in the pathogenesis of atherosclerosis and vascular diseases. Smooth muscle contraction and relaxation are regulated by the rise and fall of intracellular calcium levels (*1, 2*). An increase in intracellular calcium causes smooth muscle cell contraction by activation of the calcium/calmodulin-dependent myosin light-chain kinase, which phosphorylates myosin light chain and activates the contractile myosin adenosine triphosphatase (ATPase). A decrease in intracellular calcium causes inactivation of myosin light-chain kinase, accompanied by dephosphorylation of myosin light chain by the myosin light-chain phosphatase, PP1M (*2*). PP1M is a trimer comprising a 130-kD regulatory myosin-binding subunit (MBS), a 37-kD catalytic subunit (PP1c), and a 20-kD protein of uncertain function (M20) (*3*).

In smooth muscle, the sensitivity of the contractile apparatus to calcium is modulated by intracellular messengers that alter PP1M activity. Contractile agonists acting through signaling molecules such as protein kinase C, arachidonic acid, and rho kinase increase the sensitivity of vascular smooth muscle cells to contractile stimuli by inhibiting PP1M (*4*). Conversely, endogenous nitric oxide and related nitrovasodilators regulate blood pressure by activation of soluble guanylate cyclase, elevation of cGMP, and activation of cGMP-dependent protein kinase Iα (cGKIα), which is required for nitric oxide–mediated vasodilatation and leads to vasorelaxation by an unknown mechanism (*5*). Cyclic GMP–mediated vascular smooth muscle cell relaxation is characterized by both a reduction of intracellular calcium concentration and by activation of PP1M, which reduces the sensitivity of the contractile apparatus to intracellular calcium (*5, 6*). The mechanism by which cGMP increases PP1M activity and myosin light-chain dephosphorylation is unknown.

Kinases and phosphatases are targeted to subcellular locations by binding to specific targeting proteins that restrict the subcellular locale of these signaling enzymes (*7*). Anchoring proteins, such as the A-kinase anchoring pro-

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tein AKAP79 in mammals and STE5 in yeast, colocalize signaling enzymes to a specific subcellular region and thereby integrate multiple components of a signaling pathway (*8*). Anchoring proteins have been identified and cloned recently for cAMP-dependent protein kinase, for protein kinase C, and for the serine-threonine phosphatase PP1 (*7, 8*).

To identify potential cGKIα binding proteins, the full-length coding sequence of cGKIα was used in the yeast two-hybrid system to screen 2.5×10^6 clones from a human activated T cell library (*9–12*). Clone AL9, which was found to transactivate both histidine and β-galactosidase reporters with cGKIα, proved upon sequencing to encode the COOH-terminal 181 amino acids of the MBS of myosin phosphatase (PP1M) (*13*). MBS is the 130-kD regulatory subunit of PP1M that confers the specificity of PP1 for myosin light chain and is the site of PP1M regulation by rho kinase (*3, 4*). Cotransformation of AL9 and cGKIα into *Saccharomyces cerevisiae* (*9*) confirmed the interaction of full-length cGKIα with the COOH-terminal 181 amino acids of MBS (Fig. 1A), which includes a leucine zipper domain (amino acids 1007 through 1028 of human MBS) (*13*).

Yeast two-hybrid protein interaction assays with truncation mutants of cGKIα were used next to define the cGKIα domain that interacts with MBS (*14*). Amino-terminal cGKIα fragments of 446 ($cGKI\alpha_{1-446}$), 256 ($cGKI\alpha_{1-256}$), and 59 ($cGKI\alpha_{1-59}$) amino acids all interacted with AL9 in these studies (Fig. 1B). In contrast, an internal fragment of cGKIα, including amino acids 68 through 446, ($cGKI\alpha_{68-446}$) and a cGKIα clone in which the first 67 amino acids were deleted ($cGKI\alpha_{68-667}$) both failed to associate with AL9 (Fig. 1B). The peptide $cGKI\alpha_{1-59}$ includes a leucine/isoleucine zipper domain (*15*). These experiments show that the COOH-terminus of MBS interacts with the NH_2-terminal regulatory region of cGKIα (Fig. 1E).

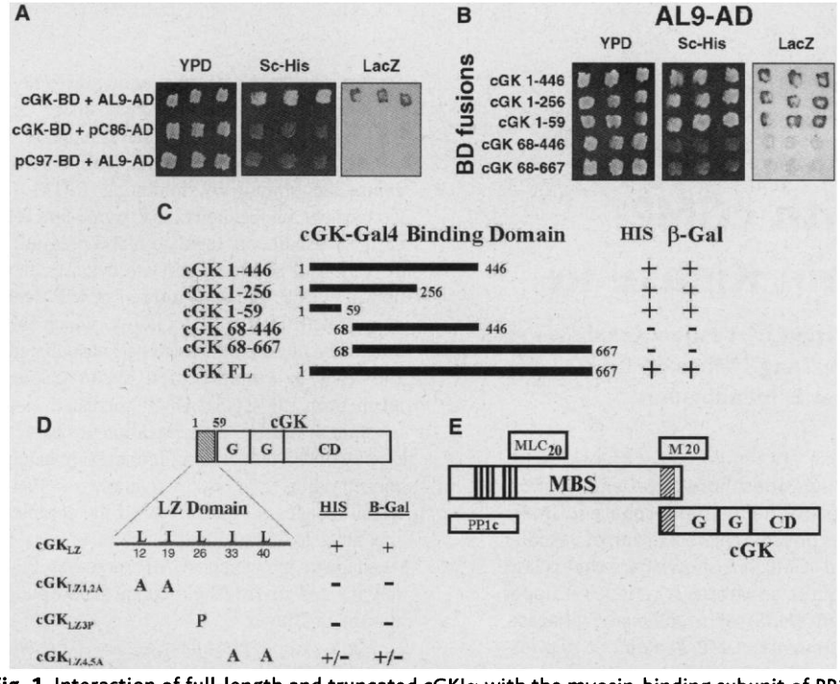

Fig. 1. Interaction of full-length and truncated cGKIα with the myosin-binding subunit of PP1M in yeast. (**A**) Yeast transformed with cGKIα-GalDB or GalDB alone in combination with AL9-GalAD or GalAD alone and plated on complete media (YPD) and media lacking histidine (Sc-HIS). Also shown is the β-galactosidase assay (LacZ) of yeast from the YPD plate. (**B**) Yeast transformed with AL9-GalAD in combination with one of five cGKIα-GalDB truncations and plated on complete media (YPD) and media lacking histidine (Sc-His). The β-galactosidase assay (LacZ) for the yeast colonies growing on the YPD plate is shown. (**C**) Summary of HIS and β-Gal reporter activation in yeast cotransformed with cGKIα-GalDB truncations and AL9-GalAD in (B). (**D**) Summary of site-directed mutagenesis experiments in which selected leucine and isoleucine residues in $cGKI\alpha_{1-59}$ were mutated to either alanine (A) or proline (P) (*20*). The binding of the wild-type cGKIα (cGK_{LZ}) and the site-directed mutants $cGK_{LZ1,2A}$, cGK_{LZ3P} and $cGK_{LZ4,5A}$ to AL9, assayed by His and β-Gal reporter activation in yeast is shown on the right (*20*). +, strong binding; +/−, weak binding, and −, no binding; G, cGMP binding site; CD, catalytic domain. (**E**) Schematic diagram showing binding of cGKIα to the MBS of PP1M. The NH_2-terminal leucine-isoleucine zipper in cGKIα interacts with the COOH-terminal MBS domain, which also contains a leucine zipper. PP1M binds myosin light chain (MLC_{20}) through the NH_2-terminal ankyrin repeat region of MBS (*3*). PP1M also contains the catalytic subunit of PP1 (PP1c) and a third subunit of uncertain significance at present (M20). Other symbols are as indicated in (D).

The interaction of cGKIα and MBS also was examined by GST-fusion protein binding studies (*16*). GST-AL9, but not GST alone (negative control), bound cGK from human saphenous vein smooth muscle cell lysates (Fig. 2A). The 690–amino acid NH_2-terminal half of MBS (MBS_{1-690}) showed no interaction with cGK in similar experiments (*17*). Conversely, GST-$cGKI\alpha_{1-59}$ also specifically bound MBS from human vascular smooth muscle cell lysates (Fig. 2B) (*16*). Mammalian tissues contain two additional cGK isoforms, cGKIβ and cGKII, which share considerable sequence homology to cGKIα (*5*), and contain leucine zippers in their NH_2-termini that differ substantially in primary sequence. The cGK isoforms cGKIβ and cGKII were tested for binding to MBS (*5, 16*). Neither GST-cGKIβ nor GST-cGKII bound MBS from vascular smooth muscle cell lysates (Fig. 2B), or interacted with MBS in the yeast two-hybrid assay (*17*), revealing that the interaction with MBS is specific to the Iα isoform of cGK. The stoichiometry of the binding of cGKIα and MBS was explored using fluorescence spectroscopy (*18*). Binding of labeled cGKIα to GST-MBS was specific and saturable, with a K_d of 62 nM. Linear transformation of the data (*19*) demonstrates that cGKIα, which exists as a dimer (*5, 15*), binds MBS in a 1:1 molar ratio, indicating that each dimer of cGKIα binds a dimer of MBS.

Leucine and leucine-isoleucine zipper motifs are α helical heptad repeats known to mediate protein-protein interactions (*15*). The

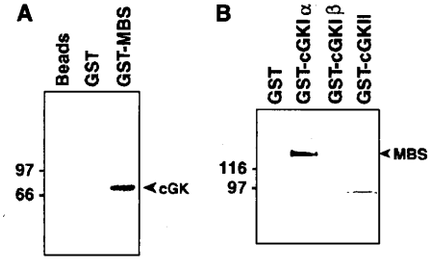

Fig. 2. Specific interaction between MBS and cGKIα in human vascular smooth muscle cells. (**A**) Protein-protein interaction studies with human saphenous vein smooth muscle cell lysates and the MBS fragment AL9. Lysates were incubated with glutathione agarose beads (lane 1), GST beads (lane 2), or GST-MBS beads (lane 3) followed by SDS-PAGE and immunoblotting with anti-cGK antibody (*16*). (**B**) Protein-protein interaction studies with human saphenous vein smooth muscle cell lysates and peptides derived from cGK isoforms Iα, Iβ, and II. Lysates were incubated with GST beads (lane 1), GST-$cGKI\alpha_{1-59}$ beads (lane 2), GST-$cGKI\beta_{1-92}$ beads (lane 3), and GST-$cGKII_{1-272}$ beads (lane 4) (*16*) and immunoblotted for MBS (*16*). One of two similar experiments is shown.

NH_2-terminus of cGKIα contains a leucine-isoleucine zipper between amino acids 12 and 40 (*5, 10, 15*), and the COOH-terminus of MBS contains a leucine zipper from residues 1007 through 1028 (*3*). Replacement of leucine residues with alanine, valine or proline has been shown in some proteins to abrogate leucine zipper-mediated binding (*15*). To determine whether the leucine-isoleucine zipper in the NH_2-terminus of cGKIα is essential for binding to MBS, we used site-directed mutagenesis to replace leucine or isoleucine residues of cGKIα (*20*). Three mutants of the leucine/isoleucine zipper of cGKIα were prepared: Leu^{12} and Ile^{19} to Ala ($cGK_{LZ1,2A}$); Leu^{26} to Pro (cGK_{LZ3P}); and Ile^{33} and Leu^{40} to Ala ($cGK_{LZ4,5A}$) (Fig. 1D). Binding to MBS was tested using both GST-fusion protein (Fig. 2) and yeast two-hybrid interaction assays. None of the leucine zipper mutants interacted with MBS from vascular smooth muscle cell lysates in GST-fusion protein studies (*17*). In yeast two-hybrid interaction assays, $cGK_{LZ4,5A}$ showed some association with MBS, whereas $cGK_{LZ1,2A}$ and cGK_{LZ3P} both failed to interact with MBS, confirming the data of the GST-fusion protein studies (Fig. 1D). These experiments indicate that the leucine-isoleucine zipper motif of cGKIα specifically mediates the interaction with the leucine zipper–containing COOH-terminal domain of MBS.

Immunoprecipitation methods also were employed to detect whether cGKIα and MBS interact in vascular smooth muscle cells. Immunoprecipitates of cGKIα from human vascular smooth muscle cell lysates contained MBS (Fig. 3A) (*21*). Similarly, when MBS was immunoprecipitated, cGKIα was detected in the immunopellet (*21*) (Fig. 3B). PP1M phosphatase activity in the cGKIα immunoprecipitates also was measured against two known PP1M substrates, myosin light chain and phosphorylase a (*22*). Phosphatase activity was present in the cGKIα immunopellets and was only minimally inhibited by 2 nM okadaic acid (12 ± 10%, P = NS, $n = 3$), but was significantly inhibited by 1 μM okadaic acid (79 ± 2%, $P < 0.001$, $n = 3$) (Fig. 3C), characteristic of the effects of this inhibitor on PP1 phosphatases (*23*). These experiments demonstrate that cGKIα is complexed with fully functional PP1M phosphatase activity.

To examine potential cGKIα substrates in the cGKIα-PP1M complex, phosphorylation studies also were performed. Addition of cGMP and cGKIα (final concentration, 350 nM) to the anti-MBS immunopellets in the presence of [γ-^{32}P]ATP led to markedly increased phosphorylation of the MBS itself (Fig. 3D) (*24, 25*). Four other proteins of 72, 57, 42, and approximately 20 to 26 kD in size also were phosphorylated to lesser degrees in these studies (Fig. 3D). The significance of these smaller phosphoproteins is currently under investigation. Since the NH_2-terminal domain of cGKIα mediates binding to MBS (Figs. 1 and 2), and MBS is a substrate of cGKIα, we also examined whether the NH_2-terminal domain of cGKIα is important to target the kinase to its substrate, MBS. Proteolysis of cGKIα with trypsin removes the first 77 amino acids of the enzyme, including both the leucine-isoleucine zipper and autoinhibitory domains, and results in a constitutively active cGKIα (cGK-CA) (*26*). In phosphorylation assays (*24*), cGK-CA or full-length cGKIα were incubated with either the MBS immunopellet or histone F2b, a substrate for several protein kinases. Phosphorylation of MBS by cGK-CA was substantially reduced in comparison to phosphorylation of MBS by cGKIα (76 ± 3%, $P < 0.003$, $n = 3$). However, cGK-CA and cGKIα both phosphorylated histone F2b to a similar extent (Fig. 3E). These data further indicate that MBS is a substrate for cGKIα, and that the NH_2-terminal leucine-isoleucine zipper domain of cGKIα is important in targeting cGKIα for phosphorylation of MBS.

Double-labeling immunofluorescence and confocal microscopy were used to explore the subcellular localization of cGKIα and MBS in human vascular smooth muscle cells (Fig. 4) (*27*). cGKIα and MBS colocalized consistently to two regions: a circumferential ring adjacent to the plasma membrane (Fig. 4, A through C) and to actin-myosin stress fibers in the vascular smooth muscle cells (Fig. 4, D

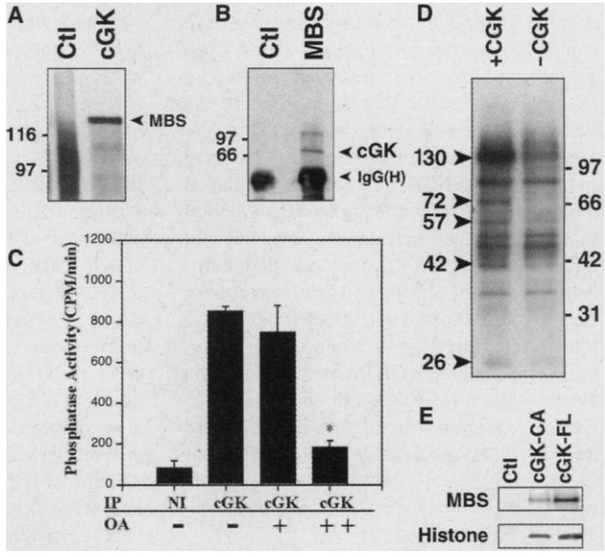

Fig. 3. Coimmunoprecipitation of MBS, PP1 phosphatase activity, and cGK. (**A**) Lysates from cultured saphenous vein smooth muscle cells were immunoprecipitated with either nonimmune IgG, or anti-cGKIα antibodies, then resolved on SDS-PAGE and immunoblotted for MBS (arrow) (*21*). (**B**) Lysates from saphenous vein smooth muscle cells were immunoprecipitated with either nonimmune IgG or anti-MBS antibodies, resolved by SDS-PAGE, and immunoblotted for cGK (arrow) (*21*). (**C**) Association of PP1 phosphatase activity with cGKIα. cGKIα was immunoprecipitated and phosphatase activity was assayed in the immunopellet (*22*). NI, nonimmune IgG; cGK, anti-cGKIα immunopellets. OA = okadaic acid, 2 nM (+) or 1 μM (++; 79 ± 2% decrease, * = $P < 0.001$ versus untreated). One of two similar experiments is shown. (**D**) Phosphorylation of proteins in the MBS immunopellet by cGKIα. Kinase assays (*24*) demonstrate marked phosphorylation by cGKIα of MBS, as well as four other proteins to lesser degrees (72, 57, 42, and 20 to 26 kD) (arrowheads). (**E**) In vitro MBS phosphorylation assays without cGK (Ctl), or with constitutively active cGKIα (cGK-CA) (*26*) or full-length cGKIα (cGK-FL) (*24*). Control phosphorylations with the general cGKIα substrate histone F2b are shown in the lower panel (*24*).

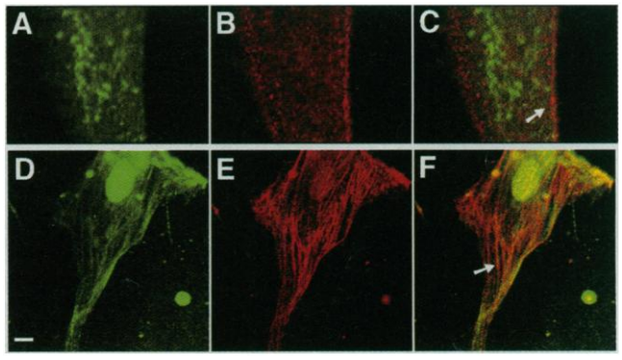

Fig. 4. Intracellular distribution and colocalization of cGK and MBS in vascular smooth muscle cells. [(A) through (C)] Vascular smooth muscle cells immunostained (*27*) with either (**A**) anti-cGKIα or (**B**) anti-MBS, (**C**) and the two images superimposed to reveal colocalization of MBS and cGKIα near the plasma membrane. [(D) through (F)] Vascular smooth muscle cells permeabilized prior to fixation to reveal actin-myosin stress fibers (*27*) and immunostained with (**D**) anti-cGKIα, (**E**) anti-MBS, and (**F**) the two images superimposed demonstrating colocalization of MBS and cGKIα on cellular stress fibers. Bar, 20 μm.

through F), where myosin light-chain kinase and PP1M already are known to colocalize and regulate contraction (*2*). Colocalization of MBS and cGKIα near the plasma membrane demonstrates that MBS is found in a site in addition to stress fibers in vascular smooth muscle cells and suggests that this may position cGKIα nearby membrane protein substrates, such as G-protein coupled receptors, which have been shown recently to be regulated by cGKIα phosphorylation (*28*). The localization of cGKIα to stress fibers (Fig. 4, D and F) has not been appreciated previously, and shows further that cGKIα is targeted within the cell to a site where it may catalyze phosphorylation of MBS and other proteins important to the regulation of vascular smooth muscle cell relaxation.

In vascular smooth muscle cells, phosphorylation of the regulatory myosin light chain is the key determinant of actomyosin ATPase activity and smooth muscle cell contraction (*2*). Because MBS targets cGKIα to the smooth muscle cell contractile apparatus, and activation of cGKIα increases PP1M activity (*6*), the cGKIα-MBS interaction may play an important role in the regulation of smooth muscle cell contractile state by NO and cGMP. The extent of agonist-stimulated myosin light-chain phosphorylation was quantified in intact vascular smooth muscle cells transfected with either vector alone or a plasmid expressing the cGKIα leucine/isoleucine zipper domain (cGK_{1-59}), to examine the effects of disrupting the cGKIα-MBS interaction on cGMP-mediated inhibition of myosin light chain phosphorylation (*29*). The thromboxane analog U46619 caused an increase in myosin light-chain phoshorylation from 10 ± 2% to 68 ± 2% ($P < 0.001$, $n = 3$) in both vector alone and cGK_{1-59} transfected vascular smooth muscle cells (Fig. 5). In vector-alone transfected vascular smooth muscle cells, 8-Br-cGMP inhibited U46619 mediated myosin light-chain phosphorylation by 79 ± 17% ($P < 0.001$, $n = 3$) (Fig. 5). However, expression of cGK_{1-59} significantly diminished the ability of 8-Br-cGMP to inhibit myosin light-chain phosphorylation following U46619 stimulation (from 79% to 35% inhibition, $P = 0.001$, $n = 3$). Thus, disruption of the cGKIα-MBS interaction prevents cGMP-mediated dephosphorylation of myosin light chain, the central determinant of contractile state in intact vascular smooth muscle cells.

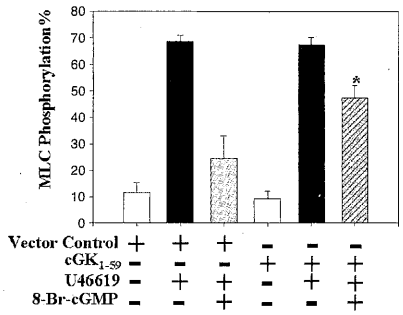

Fig. 5. Effect of cGMP on myosin light-chain phosphorylation in native vascular smooth muscle cells and following disruption of the cGK-MBS interaction: Rat aortic smooth muscle cells were transfected with either vector alone or cDNA for the leucine/isoleucine zipper peptide from cGK (cGK_{1-59}). Cells were stimulated with the thromboxane analog U46619 in the absence or presence of 8-Br-cGMP pretreatment and myosin light-chain phosphorylation state was quantified (*29*). Data represent the means ± standard error of three separate experiments in duplicate. The thromboxane analog U46619 increases myosin light-chain phosphorylation from 10 to 68% in both vector control and CGK_{1-59}–transfected cells. Overexpression of cGK_{1-59} significantly impairs cGMP inhibition of myosin light-chain phosphorylation (from 79 to 35% inhibition, $^*P = 0.001$).

These studies show for the first time that cGKIα binds specifically to the MBS of the phosphatase PP1M via a leucine zipper interaction, which targets cGKIα to stress fibers to mediate smooth muscle cell relaxation and vasodilation in response to rises in intracellular cGMP. In addition, these studies demonstrate MBS and several other proteins are substrates of cGKIα, and disruption of the cGKIα-MBS interaction impairs cGMP-mediated dephosphorylation of myosin light chain, the critical determinant of smooth muscle cell contractile state. MBS, which contains NH_2-terminal ankyrin repeats in addition to a COOH-terminal leucine zipper, is also complexed with the 37-kD catalytic subunit of PP1M, the 20-kD subunit of the phosphatase (M20), the regulatory MLC, and RhoA/Rho kinase (*3, 4*). Thus, MBS assembles a multienzyme complex, tethering a phosphatase and at least two distinct kinases with counter-regulatory effects on PP1M activity to the contractile apparatus to regulate smooth muscle contraction and relaxation.

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16. The 586–base pair (bp) cDNA insert from AL9, the 177-bp $cGKI\alpha_{1-59}$ (*14*), the NH_2-terminal 274 bp of cGKIβ, and the NH_2-terminal 816 bp of cGKII were cloned into pGEX (Pharmacia Biotech). GST-fusion proteins were expressed in bacterial strain BL21 (Stratagene) in standard fashion. Expression and concentrations of the GST fusion proteins were confirmed by SDS-PAGE and Coomassie blue staining. Lysates for GST-AL9 experiments were derived from frozen human saphenous vein homogenized in RIPA buffer with protease inhibitors and 1% Triton-X at 4°C, followed by centrifugation at 2500*g* for 6 min at 4°C. Lysates used for all other GST-fusion protein

experiments were from cultured human saphenous vein smooth muscle cells. Human saphenous vein smooth muscle cells (passage 3) were grown in Dulbecco's modified Eagle's medium (DMEM) to near-confluency. Cells were scraped into phosphate-buffered saline (PBS), centrifuged at 500*g* for 6 min at 4°C, resuspended in 0.5 ml of lysis buffer A [50 mM Tris-Cl (pH 7.5), 7 mM $MgCl_2$, 2 mM EDTA, 2 mg/ml *N*-dodecyl-B-maltoside, 0.4 mg/ml cholesteryl hemisuccinate, 0.6 M NaCl, 10 mM Na Molybdate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml chymostatin, 200 μg/ml aprotinin, 50 μg/ml leupeptin] and incubated for 1 hour at room temperature. Lysates were microfuged for 15 min at 4°C, and the supernatant was incubated with 100 μl of GST-fusion protein beads overnight, followed by washing in RIPA buffer containing 1% NP40, and boiling for 5 min in SDS sample buffer. Associated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either rabbit anti-human cGPK-CT (Upstate Biotechnology, Lake Placid, NY) or rabbit anti-MBS antibody (Berkelely Antibody Company). The membranes were developed with ECL (Amersham Life Science).

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18. Fluorescence spectroscopy was performed as previously described for the binding of cGK and vimentin [L. A. MacMillan-Crow and T. M. Lincoln, *Biochemistry* **33**, 8035 (1994)].
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20. $cGKI\alpha_{1-59}$ was cloned into vector pcDNA3.1 (Invitrogen) for site-directed mutagenesis using the method of Kunkel *et al.* (*30*). Three mutants were constructed: Leu^{12} and Ile^{19} to Ala ($LZ_{1,2A}$); Leu^{26} to Pro (LZ_{3P}); Ile^{33} and Leu^{40} to Ala ($LZ_{4,5A}$). All mutants were confirmed by DNA sequencing, then subcloned into vectors pGEX-4T-2 (Pharmacia Biotech) and PC97 for expression in bacteria and yeast. Equal amounts of GST, $GSTcGK_{1-59}$, $GSTcGKLZ_{1,2A}$, $GSTcGKLZ_{3P}$, and $GSTcGKLZ_{4,5A}$ were incubated with saphenous vein smooth muscle cell lysate, and GST pulldown experiments were performed as in (*16*). $cGKLZ_{1,2A}$, $cGKLZ_{3P}$, and $cGKLZ_{4,5A}$ were each cotransformed with AL9 in yeast strain Y190, and reporter gene activation assayed as in (*9*).
21. For cGK co-immunoprecipitation experiments, lysates from human saphenous vein smooth muscle cells in culture were prepared as in (*16*) using buffer A. Lysates were precleared with protein A Sepharose beads then incubated overnight with 5 μg of nonimmune goat serum or goat polyclonal anti-cGK, followed by harvest with protein A beads. SDS-PAGE and immunoblots were performed as above. For MBS coimmunoprecipitation experiments, a cell pellet prepared as in (*16*) was resuspended in lysis buffer B (25 mM Tris pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 1% Triton X and protease inhibitors as in buffer A). The lysate was incubated for 1 hour at room temperature, microcentrifuged 5 s, and the supernatant precleared with 12.5 μg rabbit IgG followed by protein A beads. The precleared supernatant was incubated with either rabbit nonimmune IgG, or rabbit polyclonal anti-MBS (Berkeley Antibody Company) overnight, followed by harvest with protein A beads. Equal amounts of rabbit nonimmune and anti-MBS antibodies were added, and verified by Ponceau staining. SDS-PAGE and cGK immunoblots were performed as above.
22. Immunoprecipitation of cGK was performed by addition of antibody to human saphenous vein smooth muscle cell culture lysates prepared in lysis buffer A, as above. After washing, the immunopellets were resuspended in phosphatase assay buffer (20 mM MOPS, 20 mM glucose, 1 mM dithiothreitol, 1 mM theophylline, 1mg/ml BSA, and 5mM azide, pH 7.5). We added [^{32}P]-labeled phosphorylase a (final concentration = 10 μM; gift of D. Brautigan) in buffer A to the immunopellet, and in some samples, the immunopellet was supplemented with 2 nM okadaic acid or 1 μM okadaic acid. Reactions were incubated at 30°C for 30 min, then terminated by the addition of trichloroacetic acid. In separate experiments, ^{32}P-myosin light chains were used as substrate in place of phosphorylase a, as previously described (*4*), and similar results were obtained. Data are presented as mean ± standard error.
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