A Cysteine-rich LIM-only Protein Mediates Regulation of Smooth Muscle-specific Gene Expression by cGMP-dependent Protein Kinase*

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Vascular smooth muscle cells (VSMCs) undergo phenotypic modulation, changing from a differentiated, contractile to a de-differentiated, synthetic phenotype; the change is associated with decreased expression of smooth muscle (SM)-specific genes and loss of cGMP-dependent protein kinase (PKG), but transfection of PKG into de-differentiated VSMCs restores SM-specific gene expression. We show that small interference RNA-mediated down-regulation or pharmacologic inhibition of PKG reduced SM-specific gene expression in differentiated VSMCs and provide a mechanism for cGMP/PKG regulation of SM-specific genes involving the cysteine-rich LIM-only protein CRP4. PKG associated with CRP4 and phosphorylated the protein in intact cells. CRP4 had no intrinsic transcriptional activity, but exhibited adaptor function, because it acted synergistically with serum response factor (SRF) and GATA6 to activate the SM-α-actin promoter. cGMP stimulation of the promoter required PKG and CRP4 co-expression with SRF and GATA6. A phosphorylation-deficient mutant CRP4 and a CRP4 deletin mutant deficient in PKG binding did not support cGMP/PKG stimulation of the SM-α-actin promoter. In the presence of wild-type but not mutant CRP4, cGMP/PKG enhanced SRF binding to a probe encoding the distal SM-α-actin promoter CarG (CC(AT)₆GG) element. CRP4 and SRF associated with CarG elements of endogenous SM-specific genes in intact chromatin. Small interference RNA-mediated down-regulation of CRP4 prevented the positive effects of cGMP/PKG on SM-specific gene expression. In the presence of CRP4, cGMP/PKG increased SRF- and GATA6-dependent expression of endogenous SM-specific genes in pluripotent 10T1/2 cells. Thus, CRP4 mediates cGMP/PKG stimulation of SM-specific gene expression, and PKG plays an important role in regulating the phenotype of VSMCs.

Vascular smooth muscle cells (VSMCs)⁴ can reversibly change their phenotype from a differentiated, “contractile” phenotype with high levels of smooth muscle (SM)-specific gene expression to a de-differentiated, “synthetic” phenotype with reduced levels of SM-specific gene expression (1, 2). De-differentiated cells also show increased expression of growth factor receptors, extracellular matrix, and inflammatory adhesion proteins and have increased proliferative and migratory potential (1, 2). This phenotypic switching plays an important role in the development of vascular diseases: in acutely injured blood vessels, e.g. after balloon angioplasty, VSMCs proliferate and migrate from the medial layer of the vessel wall contributing to a “neo-intimal” layer, and the majority of SM-like cells found in atherosclerotic plaques appear to represent de-differentiated VSMCs originating from the medial layer (1, 3, 4). The regulation of VSMC phenotypic switching is complex and mediated by multiple factors, but it is clear that de-differentiated VSMCs are a major cell type responsible for the generation of vascular lesions (2).

Compared with normal resting VSMCs in the medial layer, neo-intimal smooth muscle-like cells in vascular lesions show decreased transcription of SM-specific genes such as SM-myosin heavy chain (SM-MHC), SM-α-actin (SMA), and calponin, as well as decreased levels of cGMP-dependent protein kinase (PKG) and cGMP-generating soluble guanylate cyclase (2, 3, 5–7). Primary VSMCs cultured in vitro undergo changes similar to those observed in neo-intimal smooth muscle-like cells, including phenotypic de-differentiation, decreased expression of SM-specific genes, and loss of PKG I (3, 8, 9). When these de-differentiated, PKG-deficient VSMCs are transfected with expression vectors encoding PKG I to restore physiologic levels of PKG activity, the cells develop a more contractile phenotype, increased expression of SM-specific genes, and reduced production of extracellular matrix proteins and growth-related genes (3, 9–11). These results suggest that PKG may contribute...
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to phenotypic switching of VSMCs, but the mechanism(s) whereby PKG regulates SM-specific gene expression remain unknown.

In VSMCs, cGMP is generated by nitric oxide (NO) stimulation of soluble guanylate cyclase, and by natriuretic peptide activation of receptor guanylate cyclases (12). The main effect of increased intracellular cGMP is smooth muscle relaxation, mediated by PKG I phosphorylation of multiple target proteins (12). Mice with homozygous null mutations for PKG I die at an early age from severe intestinal dysfunction due to loss of NO/cGMP-dependent smooth muscle relaxation (13). Surprisingly, postnatal ablation of PKG I in VSMCs does not lead to hypertension but attenuates development of atherosclerotic lesions in apoe-deficient mice. Analyses of plaque composition in PKG I-deficient and control mice suggest PKG I regulates factors secreted by VSMCs that affect matrix remodeling and recruitment of other plaque cells such as macrophages (14). Although this study suggests a pro-atherogenic role for PKG I, the preponderance of data suggests that the NO/cGMP/PKG pathway inhibits proliferation and de-differentiation of VSMCs in vitro and limits neo-intimal thickness in various models of arterial injury in vivo (3, 5, 15−20).

Most SM-specific promoters, including the SM-MHC, SMA, and calponin promoter, contain multiple CArG (CC(AT)6GG) elements recognized by the ubiquitously expressed serum response factor (SRF) (21−23). Expression of these genes depends on the interaction of SRF with multiple cofactors, including myocardin family members, homeodomain transcription factors, GATA4 and -6, and the cysteine-rich LIM-only proteins CRP1 and CRP2/smLIM (smooth muscle LIM protein) (22, 24−26). Cysteine-rich LIM-only proteins contain two LIM domains separated by a spacer of ~60 amino acids, with each LIM domain containing two zinc fingers that function as protein interaction modules (27, 28). CRP1 and CRP2/smLIM act as adaptor proteins that associate with SRF and GATA4 or -6 and enhance SRF- and GATA6-induced transcription of SM-specific genes; a dominant negative version of CRP2/smLIM (32), we will refer to it as CRP4 (CRP3 is a family member of the CRP family was identified through a yeast two-hybrid screen that used PKG I as bait (29). This protein was phosphorylated by PKG I and the unique N terminus of PKG I (29). TRP4 can be phosphorylated by PKG I in vitro and in vivo, but its function is unknown (29).

We found that PKG was required for maintaining SM-specific gene expression in several differentiated smooth muscle cell lines, and enhanced SRF- and GATA6-induced differentiation of pluripotent embryonal cells into smooth muscle cells. CRP4 was associated with SM-specific promoters and mediated positive transcriptional effects of PKG I on SM-specific gene expression.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs, Antibodies, and Reagents**—Expression vectors encoding PKG Iα and Iβ were described previously (34). Vectors for SRF and GATA4/6 were provided by L. Sealy and M. Nem, respectively (35, 36); the coding sequences were amplified by PCR with introduction of an appropriate restriction site at the 5′-end to allow in-frame insertion of either a Myc-, HA-, or FLAG-epitope tag in pXJ40, provided by Z. S. Zhao (37). CRP4 was cloned by reverse transcription-PCR using total RNA isolated from C2C12 myoblasts with the following primer pair: 5′-GGATCCATGGCCCTCAAAGTGGTCCCCAAGTGGT3′ (sense); 5′-GACGAGCATCTCGAGATCT-AGG-3′ (antisense). All PCR products were sequenced and were identical to published sequences (the GenBankTM accession numbers for murine and rat CRP4 are AK002484 and D17512, respectively; the murine and rat proteins differ by only one amino acid residue). Truncated CRP4 mutants were generated by PCR, and the products were inserted into pXJ40-Myc (37). The plasmid (−2.8 to +3.0)PromInt-LacZ carrying the SMA promoter and intron I sequence was provided by G. K. Owens (38); the SMA promoter sequence from −125 to +44 (containing two tandem CArG sequences) was amplified by PCR and inserted into the pGL2-basic luciferase reporter vector.

The anti-C-terminal PKG I antibody was from Calbiochem, and an antibody specific for CRP4 was from BD Transduction Laboratories (BD #612079). Antibodies against SRF, RhoA, α-tubulin, glutathione S-transferase (GST), and the HA and Myc epitopes were from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-FLAG antibody was from Sigma, and the anti-phospho-Ser239 VASP antibody was from NanoTools. 8-(4-Chlorophenylthio)guanosine-3′,5′-cyclic monophosphate (CPT-cGMP) and 8-(4-chloro-phenylthio)-β-phenyl-1,2′,3′,4′-ethenoguanosine-3′,5′-cyclic monophosphate, R isoform (R-cpt-PET-cGMPs) were from Biolog.

**Cell Culture, DNA Transfections, and Reporter Gene Assays**—PAC1 rat pulmonary artery smooth muscle cells were from A. Rothman (39); A10 and A7r5 rat aortic smooth muscle cells (40), CV1 African green monkey kidney fibroblasts, and C3H/10T1/2 mouse embryonic fibroblasts were from ATCC. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. CV-1 cells were transformed with 0.8 μg of DNA and 3 μl of Polyfect (Qiagen) per 6-well dish, according to the manufacturer’s protocol. Full serum-containing media was added 5 h later, and cells were harvested at 48 h.

**siRNA Transfection**—The sequences targeted by siRNA in the C terminus of PKG I and the unique N terminus of PKG Iα were 5′-CCGGACAUUUAAGACACACAA-3′ and 5′-AAGAGGAAAUCUCCCAUAUGC-3′, respectively. The target sequence for the CRP4-specific siRNA was 5′-CAGCUAGUCUAGAAUUUCA-3′. siRNA oligoribonucleotides, including a control siRNA targeting green fluorescent protein (GFP), were produced by Qiagen. PAC1 cells plated at 8 × 105 cells per 6-well dish were transfected 18 h later (at ~40% confluency) with 100 pmol of siRNA and 3 μl of Lipofectamine™ 2000 (Invitrogen) in 1 ml of serum-free media per well according to the manufacturer’s protocol. Full serum-containing media was added 5 h later, and cells were harvested at 48 h.
**Lentivirus Transduction**—The PKG Iα cDNA was subcloned into the feline immunodeficiency virus-based vector pVE-FcKrES-GFPpuro under control of the EF1α promoter. Using calcium phosphate co-precipitation, 293T cells were transfected with either empty vector or the PKG Iα viral transfer vector, together with the feline immunodeficiency virus packaging construct pC34N, pCMV-Rev, and a plasmid encoding a VSV-G-pseudotyped envelope (41). After 48 h, culture supernatants were filtered, and virus was concentrated by centrifugation for 2 h at 50,000 × g. The concentrated viral suspension was used to infect PAC1 cells overnight (15 μl/ml of culture medium containing 8 μg/ml polybrene). Transduction efficiency was 20–25% as determined by counting GFP-positive cells by fluorescence-activated cell sorter.

**Quantitative RT-PCR**—Total cytoplasmic RNA was extracted using TRI-Reagent from Molecular Research Center Inc., and 1 μg of RNA was subjected to reverse transcription with random hexamer primers as described (42). Quantitative RT-PCR was performed using an Mx3000 real-time PCR detector (Stratagene); reactions contained appropriate dilutions of cDNA, IQ SYBR Green Supermix (Bio-Rad), and 0.2 μM of primers (supplemental Table S1) (42). DNA was denatured at 95 °C for 30 s, with annealing and extension occurring at 60 °C for 45 s; each primer pair generated a single product as determined by analyzing melting curves after a 40-cycle control reaction. Standard curves were generated by plotting Ct values versus the amount of input RNA for each primer pair and demonstrated similar amplification efficiencies. Relative changes in mRNA expression were analyzed using the 2-ΔΔCt method, with GAPDH serving as an internal reference to correct for differences in RNA extraction or reverse transcription efficiencies (42).

**Protein Interaction Studies**—Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 10 mM β-mercaptoethanol, 0.5% Triton X-100, and a protease inhibitor mixture (Calbiochem), and cell lysates were cleared by centrifugation at 47,000 × g for 20 min. For PKG-CRP4 interaction studies, cell extracts were subjected to immunoprecipitation using the indicated antibodies on protein G-agarose beads, or were incubated with glutathione Sepharose beads for GST pulldown experiments. For SRF-CRP interaction studies, GST-tagged versions of CRP4 or CRP2/smLIM or GST alone were expressed in bacteria, immobilized on glutathione-Sepharose beads, and incubated with cell lysates of CV1 cells transfected with Myc-tagged SRF. Washed precipitates were analyzed by SDS-PAGE and Western blotting with the indicated antibodies (34).

**ChIP**—Approximately 10⁷ PAC1 cells were incubated in situ with 1% formaldehyde for 10 min at room temperature to cross-link DNA and proteins; cells were washed and scraped into 1 ml of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 85 mM KCI, 0.5% Nonidet P-40, and protease inhibitor mixture. Nuclei were spun through a cushion of 12.5% glycerol in lysis buffer, resuspended, and sonicated in a chromat precipitation buffer as described previously (43). A 16,000 × g supernatant was preabsorbed with 25 μl of protein A/G-agarose, which had been blocked with 1 mg/ml bovine serum albumin and 1 mg/ml sheared salmon sperm DNA. The supernatant was shaken gently for 16 h at 4 °C with 5 μg of either control immunoglobulin G or antibodies specific for SRF or CRP4; after centrifugation, 25 μl of blocked protein A/G-agarose was added for an additional 2 h. Immunoprecipitates were washed, eluted, and heated, and eluates were digested with proteinase K and purified with phenol/chloroform as described previously (43). Semi-quantitative PCR was performed with varying amounts of input template using published primers flanking the two conserved CArG elements located at −62 and −112 of the SMA promoter, and located at −1.3 kb of the SM-MHC promoter, or a region of the β-globin promoter that is devoid of CArG sequences (21). Primer sequences and amplicon sizes are described in supplemental Table S2.

**Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared, incubated with 5'-end-labeled double-stranded oligodeoxynucleotide (oligodNT) probes, and analyzed by non-denaturing PAGE and autoradiography as described previously (42). OligodNTs corresponding to −113 to −97 of the SMA promoter (5'-TGAGGTCTCCCTATATGTTGCG-3') for upper strand, containing one CArG site) were synthesized, annealed, and used as the probe designated SMA CArG “B-site.” The CArG consensus oligodNT (5'-GGATGCCTCAATAGCACT-3') and the CRE consensus oligodNT (5'-AGAAGGGGCTGAGGAGAGCTAG-3') were from Santa Cruz Biotechnology and Promega Life Sciences, respectively. For supershift assays, nuclear extracts were preincubated with the indicated antibodies (42).

**Data Presentation**—All results presented in bar graphs represent the means ± S.D. of at least three independent experiments performed in duplicate. Autoradiographs and Western blots demonstrate a representative experiment, performed at least three times with similar results. Statistical analyses were performed using Prism 5 software (GraphPad, Carlsbad, CA). The Student t test was employed for pair-wise comparisons, and a one-way analysis of variance with Dunnett’s post-test analysis for multiple comparisons to the control group; a p value of <0.05 was considered to indicate statistical significance.

**RESULTS**

**siRNA-mediated Suppression of PKG Expression Reduces SM-specific Gene Expression without Affecting RhoA**—The mechanism of PKG I-mediated induction of contractile protein expression in de-differentiated VSMCs has not been determined, and it is unknown whether PKG I expression in differentiated VSMCs is necessary to maintain high levels of SM-MHC expression. We used the pulmonary artery smooth muscle cell line PAC1, which maintains a differentiated phenotype with stable expression of PKG I and SM-MHC during passage in vitro (39). These cells express both PKG Iα and Iβ, with PKG Iα being the predominant isoform (42); PKG Iα and Iβ are derived from a single gene by differential splicing of the first exon (44). Transfecting PAC1 cells with siRNA oligoribonucleotides targeted against a common C-terminal sequence of PKG I, or against the unique N terminus of PKG Iα, reduced PKG mRNA and protein levels by ~70% or ~50%, respectively (Fig. 1A; PKG I expression was compared with that in cells transfected with a control siRNA targeted against GFP). SM-MHC
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FIGURE 1. siRNA-mediated suppression of PKG expression reduces SM-MHC mRNA levels without affecting RhoA. PAC1 cells were transfected with siRNA oligoribonucleotides targeting GFP (control), the C terminus of PKG I (common to PKG Iα and β), or the unique N terminus of PKG Iα. After 48 h, total RNA was extracted and endogenous mRNA expression of PKG I (panel A, top), SM-MHC (panel B), and RhoA (panel C, top) was determined by real-time RT-PCR. Expression of each gene was normalized to GAPDH, and a value of 100 was assigned to the relative mRNA level found in cells transfected with GFP siRNA. We obtained p < 0.05 for the comparison of SM-MHC mRNA between cells transfected with GFP versus PKG siRNAs. In parallel experiments, cell extracts were analyzed by Western blotting using antibodies directed against the C terminus of PKG (panel A) or RhoA (panel C). D, A7r5 cells were transfected with siRNA duplexes specific for GFP (open bars) or PKG Iα + β (filled bars), and mRNA levels of SM-MHC, SMA, calponin, and RhoA were quantified by real-time RT-PCR as described in A. For SM-MHC, SMA, and calponin, the comparison between cells transfected with GFP versus PKG siRNA yielded p < 0.05. E, PAC1 cells were transfected with siRNA duplexes targeting GFP (lanes 1 and 2) or PKG Iα + β (lanes 3 and 4), and 8 h later, cells were infected with either control lentivirus (lanes 1 and 3) or a lentivirus expressing human PKG Iα (lanes 2 and 4). PKG expression was analyzed by Western blotting using an antibody directed against the C terminus of PKG I (upper panel), and loading was determined by reprobing the blot with an anti-α-tubulin antibody (lower panel). Control experiments showed that the PKG virus infected ~25% of the cells. F, cells were transfected with the indicated siRNA, and infected with either control lentivirus (gray bars) or lentivirus expressing human PKG Iα (black bars) as described in panel E. After 48 h, total RNA was extracted and endogenous SM-MHC expression was measured by real-time RT-PCR. *, p < 0.05 for the comparison between control virus- and PKGα virus-infected cells.
and slightly increased SM-MHC mRNA (Fig. 1, E and F). In cells transfected with the PKG Ia+β siRNA, expression of human PKG Ia increased PKG I protein levels (Fig. 1E) and SM-MHC mRNA expression more dramatically (Fig. 1F, \( p < 0.05 \) for the comparison between PKG virus- and control virus-transduced cells). The level of SM-MHC mRNA did not return fully to the level found in cells transfected with GFP siRNA; this is likely due to only 25% of the cells being successfully infected with the PKG Ia virus. However, “rescue” of SM-MHC mRNA expression by re-introduction of PKG I suggests that down-regulation of the SM-specific gene was a specific effect of the siRNA-mediated down-regulation of PKG I.

**Pharmacologic Inhibition of PKG Activity Reduces SM-specific Gene Expression**—PAC1 cells produce NO in culture and contain soluble guanylate cyclase activity, leading to significant basal PKG activity in unstimulated cells (42, 47). To determine whether basal PKG activity is important for SM-specific gene expression in PAC1 cells, we treated cells with the membrane-permeable PKG inhibitor \( R_p \)-CPT-PET-cGMPS (48). Culturing cells for 48 h in the presence of 100 \( \mu M \) \( R_p \)-CPT-PET-cGMPS reduced SM-MHC, SMA, and calponin mRNA levels by 65%, 40, and 47%, respectively (Fig. 2A, \( p < 0.05 \) for the comparison between cells cultured in the absence and presence of inhibitor, represented by open and filled bars). RhoA mRNA levels were not affected by the drug. To determine if the drug inhibited PKG activity effectively, we examined phosphorylation of vasodilator-stimulated phosphoprotein (VASP) on serine 239, a preferred PKG phosphorylation site (48). VASP phosphorylation was detectable in untreated cells, and increased markedly when cells were treated with CPT-cGMP for 1 h to maximally stimulate PKG activity (Fig. 2B, lanes 1 and 2). In contrast, in cells cultured with \( R_p \)-CPT-PET-cGMPS for 48 h, basal VASP phosphorylation was undetectable, and CPT-cGMP-stimulated VASP phosphorylation was almost completely suppressed, indicating effective inhibition of PKG (Fig. 2B, lanes 3 and 4). Thus, long term inhibition of PKG activity resulted in reduced SM-specific gene expression.

**PKG Ia and Iβ Associate with CRP4 in Intact Cells in a Phosphorylation-independent Manner**—CRP4 was originally isolated from a rat brain and a human intestinal cDNA library on the basis of its homology to the cysteine-rich protein family (30, 31). CRP1 and CRP2/smLIM regulate SM-specific gene transcription, whereas CRP3/MLP is expressed exclusively in cardiac and striated muscle and regulates transcription during myogenic differentiation (49). Because CRP4 is widely expressed and efficiently phosphorylated by PKG, we hypothesized that CRP4 might participate in PKG regulation of SM-specific gene expression (29, 30). To determine whether CRP4 interacted with PKG I in intact VSMCs, we performed co-immunoprecipitation experiments using a CRP4 antibody, which we showed did not cross-react with CRP2 (Fig. 3A is a Western blot from transfected cells expressing epitope-tagged CRP2 and CRP4). PKG I co-immunoprecipitated with endogenous CRP4 from PAC1 cells but was not present in control IgG immunoprecipitates (Fig. 3B, compare lanes 1 and 2, immunoprecipitation with control IgG versus anti-CRP4 antibody). To determine whether CRP4 selectively associated with one PKG I isoform, we transfected PKG I-deficient, late passage CV1 cells with empty vector or expression vectors encoding PKG Ia or Iβ. Some cells were co-transfected with Myc epitope-tagged CRP4, and CRP4 was isolated by anti-Myc immunoprecipitation (Fig. 3C). We found that both PKG I isoforms associated with CRP4 to a similar extent (compare lanes 5 and 6, top panel).

To determine the effect of CRP4 phosphorylation on PKG I association with CRP4, CV1 cells were co-transfected with epitope-tagged CRP4 and either empty vector or PKG Ia; to activate the kinase, some cultures were treated for 1 h with CPT-cGMPS. We found that CGMPS had no effect on PKG I association with CRP4 (Fig. 3D, top panel, compare lanes 2 and 4, cells co-transfected with CRP4 and PKG Ia cultured in the absence and presence of CPT-cGMPS, respectively). To examine CRP4 phosphorylation under the same conditions, we incubated the CV1 cells with \( ^{32}P \)-PO4 and added CPT-cGMPS during the last hour of labeling. We observed significant \( ^{32}P \)-PO4 incorporation into CRP4 only in cGMPS-treated cells expressing PKG Ia, but not in PKG-deficient CV1 cells or in PKG-transfected
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A. PAC1 cells were transfected with empty vector (lanes 1 and 4), or expression vectors encoding FLAG epitope-tagged CRP2/smlIM (lanes 2 and 5) or CRP4 (lanes 3 and 6). Cell lysates were analyzed by Western blotting using either anti-FLAG antibody (lanes 1–3) or anti-CRP antibody (lanes 4–6). The epitope-tagged CRP4 migrates with a slightly higher apparent molecular weight than endogenous CRP4. B, whole cell lysates of PAC1 cells were subjected to immunoprecipitation using control IgG (lane 1), or the anti-CRP4 antibody described in A (lane 2). Immunoprecipitates were analyzed by Western blotting with antibodies for PKG I (top panel) or CRP4 (bottom panel), and 1% of total input lysates were analyzed in parallel (lanes 3 and 4). C, late passage, PKG-deficient CV1 cells were co-transfected with empty vector (lanes 1 and 4), or expression vectors encoding PKG Iα (lanes 2 and 5), or PKG Iβ (lanes 3 and 6). Cells received additional empty vector (lanes 1–3), or Myc-epitope-tagged CRP4 (lanes 4–6). Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and immunoprecipitates were analyzed by Western blotting with antibodies for PKG I (top panel) or Myc (middle panel). The lower panel shows 1% of total input lysates analyzed by Western blotting for expression of PKG Iα (lanes 1) and Iβ (lanes 4), late passage CV1 cells were co-transfected with FLAG-epitope-tagged CRP4 and either empty vector (lanes 1 and 3) or PKG Iα expression vector (lanes 2 and 4); some of the cultures were treated with 10 μM CPT-cGMP for 1 hr to activate PKG (lanes 3 and 4). Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and PKG association with the immunoprecipitates was tested as in C. E, late passage CV1 cells were co-transfected with FLAG-epitope-tagged CRP4 and either empty vector (lanes 1 and 2) or PKG Iα (lanes 3 and 4). After 36 h, cells were labeled with [32P]PO₄ for 4 h, and cells were treated with 100 μM CPT-cGMP for the last hour (lanes 2 and 4) or left untreated (lanes 1 and 3). CRP4 was immunoprecipitated using anti-FLAG antibody and analyzed by SDS-PAGE/electrotransfer/autoradiography (upper panel). The amount of CRP4 present in the immunoprecipitates was determined by Western blotting with anti-FLAG antibody (lower panel). F, PAC1 cells were transfected with FLAG-epitope-tagged wild-type CRP4 (lanes 1 and 2) or CRP4 with an alanine substitution for serine 104 (CRP4ΔA104, lanes 3 and 4). Cells were labeled with [32P]PO₄ in the absence or presence of CPT-cGMP. Wild-type CRP4 showed significant basal [32P]PO₄ incorporation in the absence of cGMP suggesting high basal PKG activity in PAC1 cells, but phosphorylation was enhanced in the presence of cGMP (Fig. 3F, compare lanes 1 and 2). In contrast, the mutant CRP4ΔA104 showed no phosphorylation in the absence or presence of cGMP (lanes 3 and 4), confirming serine 104 as the major PKG phosphorylation site (29). The association of PKG I with CRP4 was not affected by the mutation of serine 104 to alanine (data not shown). We conclude that CRP4 and PKG I associate in VSMCs; the association is independent of the PKG I isoform, and is not affected by cGMP-induced CRP4 phosphorylation.

PKG Association with CRP4 Requires the Third Zinc Finger of CRP4 and SRF Associates with CRP4—Cysteine-rich proteins are composed of two tandem copies of an unusual double zinc finger motif (CXXCXX/CXXCXX) and are thought to act as adaptor proteins (27). We mapped the CRP4 domain(s) required for PKG I association using a series of deletion mutants lacking one or several zinc fingers (ZF) domains; these constructs were co-transfected with GST-tagged PKG Iα into CV1 cells (Fig. 4, A and B). We found that full-length CRP4, as well as CRP4 containing a deletion of ZF1, ZF1 and -2, ZF4, or ZF1 and -4 associated with PKG, whereas a CRP4 construct containing a deletion of ZF3 and -4 did not interact with PKG I (Fig. 4B, lane 7). The protein expressed from the CRP4ΔZF3/4 was soluble and present at levels comparable to the other constructs (Fig. 4B, lower panel). In reciprocal experiments, the Myc epitope-tagged CRP4 constructs were isolated by anti-Myc immunoprecipitation, and Western blotting with anti-PKG I antibody demonstrated PKG I association with all constructs, except CRP4ΔZF3/4 (data not shown). We conclude that ZF3 is required for PKG I interaction with CRP4.

Alignment of CRP4 with other members of the cysteine-rich protein family shows a high degree of homology of the four zinc fingers (27, 29). Previous work demonstrated association of SRF and GATA4 with CRP2/smlIM ZF1 and ZF4, respectively (26).
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Using GST-tagged versions of CRP4 and CRP2/smLIM immobilized on glutathione-Sepharose beads and incubated with Myc epitope-tagged SRF expressed in CV1 cells, we found that SRF bound to CRP4 as efficiently as to CRP2/smLIM, but it did not bind to GST-loaded control beads (Fig. 4C). Because PKG I appeared to bind to ZF3 of CRP4, it is possible that CRP4 may act as an adaptor protein connecting SRF and GATA6 with PKG I signaling.

CRP4 Cooperates with SRF and GATA6 in Transcriptional Activation of the SMA Promoter and Associates with the SMA Promoter CArG Box—Chang et al. (26) showed that CRP1 and CRP2/smLIM enhance transcriptional effects of SRF and GATA4 (or -6) on SM-specific promoters. To determine whether CRP4 has similar transcriptional activity, we used a luciferase reporter gene under control of the SMA promoter and epitope-tagged SRF, GATA6, and/or CRP4; these studies were done in CV1 cells, which express low levels of SRF and do not express GATA6. SRF transactivated the SMA promoter 12-fold, whereas GATA6 or CRP4 alone, or in combination, found SM-MHC and SMA promoter sequences in both anti-SRF and anti-CRP4 immunoprecipitates, but no signal was detected in immunoprecipitates obtained with control IgG (Fig. 5C, compare lanes 5 and 6, anti-SRF and anti-CRP4 immunoprecipitates to lane 4, control IgG). The promoter of the β-globin gene was used in control reactions, because it is transcriptionally silent in VSMCs and does not contain CArG elements. β-Globin sequences were undetectable in the anti-SRF and anti-CRP4 immunoprecipitates, demonstrating specificity of the ChIP assay (Fig. 5C, lower panel). Thus, both SRF and CRP4 associated with CArG element-containing chromatin regions of the SM-MHC and SMA promoters in PAC1 cells.

CRP4 Mediates cGMP/PKG I Stimulation of the SMA Promoter—in intact cells, PKG I phosphorylates CRP4 on serine 104 located in the linker region between the two LIM domains, and the in vitro kinetics of this phosphorylation compare favorably with established PKG I substrates (29). To determine whether PKG I phosphorylation influenced the transcriptional effects of CRP4, we transfected early passage CV1 cells had minimal effects (Fig. 5A). SRF in combination with GATA6 acted synergistically to activate the promoter 90-fold, as previously reported (36). Adding CRP4 further enhanced the effect of SRF and GATA6, resulting in ~200-fold activation of the SMA promoter (Fig. 5A, last column). Co-expression of CRP4 did not alter expression levels of SRF or GATA6 (Fig. 5B). Thus, CRP4 cooperated with SRF and GATA6 to activate the SMA promoter, although by itself, had little effect.

To examine the subcellular localization of CRP4, we performed immunofluorescence staining of PAC1 cells transfected with small amounts of epitope-tagged CRP4. CRP4 was found both in the cytosol, where it appeared to be associated with actin filaments, and in the nucleus (supplemental Fig. S1). This distribution is very similar to that described for CRP2/smLIM and other cysteine-rich proteins (26, 27).

To assess whether CRP4 was associated with endogenous SM-specific promoters in VSMCs, we performed chromatin immunoprecipitation (ChIP) assays using primers corresponding to the SM-MHC and SMA promoter regions containing the critical CArG sequence elements. Cross-linked chromatin from PAC1 cells was immunoprecipitated with control IgG, or anti-SRF, or anti-CRP4 antibody. We
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FIGURE 5. CRP4 cooperates with SRF and GATA6 in transcriptional activation of the SMA promoter and associates with SM-specific promoter CArG elements. A, CV1 cells were co-transfected with a luciferase reporter gene under control of the SMA promoter, a β-galactosidase-expressing control vector, and either empty vector, Myc-tagged SRF (10 ng), HA-tagged GATA6 (200 ng), and/or FLAG-tagged CRP4 (300 ng). Luciferase and β-galactosidase activities were determined, and the luciferase/β-galactosidase ratio obtained in cells transfected with empty vector (first column) was assigned a value of one. B, cell extracts from the experiment described in A were analyzed by Western blotting using the indicated epitope-specific antibodies to determine expression levels of the transfected SRF, GATA6, and CRP4. C, association of SRF and CRP4 with the CArG-containing region of the endogenous MHC or SMA promoter in PAC1 cells was determined by ChIP assay. Cell extracts containing equal amounts of cross-linked, sheared chromatin were amplified by PCR using primers spanning the CArG-containing regions of the endogenous MHC or SMA promoter (first and second panels, respectively). Primers specific for the β-globin promoter, which lacks CArG boxes, served as a negative control (lower panel). Input DNA diluted 1:30, 1:100, and 1:300 was amplified in parallel to demonstrate that the PCR conditions were semi-quantitative (lanes 1–3). PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. Similar results were obtained in four independent experiments.

with the SMA-luciferase reporter, SRF, GATA6, and wild-type CRP4 or the phosphorylation-deficient mutant CRP4A104 described above. Early passage CV1 cells express endogenous PKG I (Fig. 6B, lower panel), and we treated some cultures with 100 μM CPT-cGMP for 3 h to activate the kinase. SRF- plus GATA6-stimulated SMA promoter activity was only minimally enhanced by cGMP (Fig. 6A, compare open and filled bars, cells cultured in the absence and presence of cGMP). However, when wild-type CRP4 was co-transfected with SRF and GATA6, SMA promoter activity was stimulated ∼200-fold in the absence of cGMP and >400-fold in the presence of cGMP. In the absence of cGMP, the phosphorylation-deficient mutant CRP4A104 enhanced SRF- and GATA6-stimulated SMA promoter activity to a similar extent as wild-type CRP4, but no further increase occurred in the presence of cGMP. Control Western blots demonstrated that cGMP did not affect expression of SRF, GATA6, or CRP4, and that wild-type and mutant CRP4 were present in similar amounts (Fig. 6B). These data suggest that cGMP enhancement of SMA promoter activity required CRP4 phosphorylation on serine 104.

To determine whether PKG phosphorylation affected the subcellular localization of CRP4, we performed immunofluorescence staining on PAC1 cells transfected with epitope-tagged wild-type or phosphorylation-deficient mutant CRP4A104. The subcellular localization of wild-type and mutant CRP4 were indistinguishable, and there was no detectable change when cells were treated with CPT-cGMP to activate PKG (supplemental Fig. S1).

In VSMCs, some of the effects of cGMP may be mediated by cross-activation of cAMP-dependent protein kinase (50). To determine whether the cGMP effects on the SMA promoter were mediated by PKG, we used late passage, PKG-deficient CV1 cells. Like other cell types, CV1 cells lose PKG expression with prolonged passage in tissue culture (8, 43). In PKG-deficient CV1 cells, SRF- and GATA6- plus CRP4-mediated stimulation of the SMA promoter was only slightly enhanced by cGMP, but co-transfection of PKG I restored cGMP responsiveness of the promoter (Fig. 6C, the lower panel shows expression of the transfected kinase). Similar results were obtained with PKG Iβ (data not shown). Thus, the cGMP effect on the SMA promoter required CRP4 and PKG I.

To determine whether cGMP stimulation of SMA promoter activity required PKG I association with CRP4, we used the CRP4ΔZF3/4 mutant, which is missing the second LIM domain and is unable to bind PKG I (Fig. 4). In the absence of cGMP, this truncated CRP4 construct enhanced SRF- and GATA6-induced SMA promoter activity less than wild-type CRP4 (75-fold versus 194-fold); the difference was more pronounced in cGMP-treated cells, because no cGMP response occurred in cells transfected with the truncated CRP4ΔZF3/4 (Fig. 6D; expression of wild-type CRP4 and mutant CRP4ΔZF3/4 are shown in the lower panel). Control experiments indicated that the subcellular distribution of CRP4 was not altered by the deletion of zinc fingers 3 and 4 (supplemental Fig. S1). We conclude that CRP4 mediates cGMP/PKG I stimulation of SRF- and GATA6-dependent transcription of the SMA promoter.

cGMP/PKG I Enhance SRF-DNA Complex Formation in the Presence of CRP4—SRF binds to CArG sequences in SM-specific promoters with relatively low affinity and requires stabilization by other cofactors (22, 23). CRP4 by itself did not transactivate the SMA promoter in the absence of GATA and SRF (Fig. 5A), but CRP4 could enhance SRF- and GATA6-stimu-
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for a summary of three independent experiments). Additional transfection of either GATA6 or wild-type CRP4 had no significant effect (Fig. 7A, lanes 3 and 4), and GATA6 and CRP4 did not appear to bind the probe by themselves (lane 5). Adding both wild-type CRP4 and GATA6 to SRF significantly increased the protein-DNA complex, and co-transfection of PKG Iα with cGMP increased DNA binding further: ~3-fold above the level seen in cells transfected with CRP4, GATA6, and SRF (lanes 7 and 8 for cells co-transfected with PKG Iα cultured in the absence or presence of cGMP, respectively). In contrast, transfection of mutant CRP4ΔZF3/4 with GATA6 and SRF did not enhance protein-DNA complex formation above that seen in cells transfected with SRF alone (compare lanes 9 and 2), and co-transfection of PKG Iα in the presence or absence of cGMP had only a minimal effect (lanes 10 and 11). Similar results were obtained with the SMA promoter CARG element. Control experiments showed equal expression of CRP4, GATA6, and SRF in the presence and absence of PKG I/cGMP (data not shown).

To show specificity of the protein-DNA complex formed in the presence of SRF, GATA6, and CRP4, we performed competition and supershift experiments (Fig. 7B). When nuclear extracts were preincubated with excess unlabeled CARG probe prior to adding radioactively labeled SMA CARG sequence, protein binding to the radioactive probe was prevented (Fig. 7B, compare lane 1, no competition, with lanes 2 and 3, competition with SMA CARG “B-site” or CARG consensus sequence, respectively). Preincubation with the same amount of unlabeled, unrelated oligodNT sequence did not affect the protein-DNA complex (Fig. 7B, lane 4). Preincubation with an anti-Myc antibody resulted in a supershifted complex (Fig. 7B, lane 5) indicating the presence of transacted, Myc epitope-tagged SRF in the protein-DNA complex.

These results indicate that CRP4 and GATA6 cooperatively increase SRF DNA binding activity. PKG I activation by cGMP further increased the abundance of SRF-containing protein-DNA complexes in the presence of wild-type CRP4, but not mutant CRP4ΔZF3/4, which cannot bind the kinase.

FIGURE 6. cGMP/PKG I stimulation of the SMA promoter is mediated by CRP4. A, early passage CV1 cells expressing endogenous PKG I were co-transfected as described in the legend of Fig. 5A with the SMA-luciferase reporter, β-galactosidase control vector, and empty vector, or expression vectors encoding SRF, GATA6, wild-type CRP4, or a phosphorylation-deficient CRP4 mutant (CRP4ΔA104). Cultures were treated for 3 h with 100 μM CPT-cGMP (filled bars) or were left untreated (open bars). Luciferase activity was normalized to β-galactosidase activity, and the luciferase/β-galactosidase ratio obtained in untreated cells transfected with empty vector was assigned a value of one. B, cell lysates from selected conditions of the experiment described in A were analyzed by Western blotting using the indicated epitope-specific antibodies to determine expression levels of transfected SRF (lanes 2–8, top panel), GATA6 (lanes 3–8, second panel), and CRP4 (lanes 5 and 6 for wild-type, and lanes 7 and 8 for mutant CRP4ΔA104, third panel). Endogenous PKG I expression is shown in the lowest panel. C, late passage CV1 cells, lacking PKG I, were transfected and treated as described in A, but some of the cultures were co-transfected with an expression vector encoding PKG Iα. The lower panel shows a Western blot demonstrating PKG I expression in PKG-transfected cells (lanes 3 and 4). D, early passage CV-1 cells were co-transfected with SMA-luciferase, RSV-βgal, SRF, GATA6, and either wild-type CRP4 or a mutant CRP4 lacking the PKG I-interaction domain (CRP4ΔZF3/4); cGMP treatment was as in panel A (filled bars). Expression of wild-type CRP4 (lanes 1 and 2) and mutant CRP4ΔZF3/4 (lanes 3 and 4) was analyzed by Western blotting using anti-FLAG antibody as shown in the lower panel.
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siRNA-mediated Knockdown of CRP4 Reduces SM-specific Gene Expression and Prevents cGMP/PKG I Stimulation of SM-MHC Expression—PAC1 cells express CRP4 as well as CRP1 and CRP2/smLIM mRNA, but it is not known whether the three CRP proteins share similar functions in differentiated VSMCs. Differences in tissue distribution and regulation by cellular stresses and the phenotype of CRP2/smLIM-deficient mice suggest that the three proteins may not have redundant functions (27, 51). The PKG phosphorylation site in CRP4 is not conserved in the other two CRP proteins (29).

To determine the contribution of CRP4 to SM-specific gene expression, we designed a siRNA oligonucleotide that effectively reduced CRP4 mRNA and protein levels in PAC1 cells (Fig. 8, A and C). In cells transfected with the CRP4-specific siRNA, SM-MHC mRNA expression was reduced by ~50% compared with control siRNA-transfected cells; SMA and calponin mRNA levels were reduced even more effectively, but CRP2 and PKG I mRNA expression and PKG I protein levels were not affected, demonstrating specificity of the CRP4 siRNA (Fig. 8, A and C). These results suggest that CRP4 expression is required for maintaining basal SM-specific gene expression in differentiated PAC1 cells and that CRP1 and -2 cannot replace CRP4 in this function.

If CRP4 is required for PKG functions at SM-specific promoters, siRNA knockdown of CRP4 should prevent the effect of PKG on SM-MHC expression. As shown in Fig. 1F, PKG I overexpression in differentiated VSMCs, which express already high levels of the kinase, had little effect on SM-MHC mRNA expression. Therefore, we treated PAC1 cells with either CRP4- or PKG I-specific siRNAs, or with a combination of the two siRNAs to reduce CRP4 and/or PKG I expression, prior to infecting cells with a virus encoding siRNA-resistant, human PKG I α subunit. As shown previously in Fig. 1F, PKG I partly rescued the expression of SM-MHC mRNA in cells transfected with PKG I siRNA, but it failed to do so in cells transfected with CRP4 siRNA or a combination of PKG I and CRP4 siRNAs (Fig. 8B). Thus, CRP4 is required for PKG I regulation of SM-MCH mRNA expression in PAC1 cells.

cGMP/PKG I Increase SRF/GATA6/CRP4-dependent Expression of Endogenous SM-specific Genes in Pluripotent 10T1/2 Cells—10T1/2 embryonal mesenchymal cells have the potential for myogenic differentiation and can be induced to express SM-specific genes when co-transfected with SRF and GATA6 in combination with CRP1 or CRP2, but not CRP3 (26). To determine whether cGMP/PKG I can modulate expression...
of endogenous SM-specific genes in this model, we transfected undifferentiated 10T1/2 cells with SRF, GATA6, and CRP4 in the absence and presence of PKG I/cGMP. Although cells transfected with empty vector showed no detectable SM-MHC mRNA, cells transfected with SRF and GATA6 showed a clear signal, and adding CRP4 slightly enhanced SM-MHC mRNA levels (Fig. 9A, top panel; the second panel shows equal loading of RNA by amplification of GAPDH; Fig. 9C shows a summary of three independent experiments). When cells were additionally co-transfected with PKG I and treated with cGMP, the level of SM-MHC mRNA doubled compared with the level found in the absence of PKG I/cGMP (Fig. 9A, lane 5 and C). CPT-cGMP treatment had no effect on 10T1/2 cells transfected with empty vector, but it significantly increased SM-MHC mRNA levels in cells expressing SRF, GATA6, CRP4, and PKG I (Fig. 9B). PKG I/cGMP had a similar, albeit less pronounced, effect on calponin mRNA expression. The presence of PKG I/cGMP did not affect SRF, GATA6, or CRP4 protein levels (Fig. 9D). Thus, in the presence of CRP4, PKG I/cGMP enhanced SM cell lineage-specific gene expression induced by SRF and GATA6 in pluripotent 10T1/2 cells.

DISCUSSION

Changes in the differentiation state of VSMCs play a critical role in the pathogenesis of vascular diseases, including atherosclerosis and post-injury repair. Phenotypic modulation of VSMCs following vascular injury requires multiple environmental cues, and injury-induced down-regulation of SM-specific genes is mediated at the transcriptional level (2, 52). Boerth et al. (9) showed that the gradual decline of SM-specific gene expression during in vitro culture of primary aortic VSMCs correlates with a gradual loss of PKG I expression, and that restitution of physiologic PKG levels by transfection restores...
SM-specific gene expression. PKG I re-expression also restores VSMC contractile function, and inhibits migration and extracellular matrix synthesis, suggesting that cultured primary VSMCs may represent a model system for reversible phenotypic modulation (9–11). These studies implied that decreased PKG I expression leads to a de-differentiated VSMC phenotype, but this was not formally tested, and the one or more mechanisms whereby PKG I regulates SM-specific gene expression were not addressed.

We found that endogenous PKG I activity regulates SM-specific gene expression in cloned populations of PAC1 pulmonary artery and A7r5/A10 aortic VSMCs, which maintain a differentiated phenotype during in vitro culture (39, 40, 53). PKG activity was required to maintain high levels of SM-specific gene expression, because both siRNA-mediated suppression of PKG I mRNA and protein, and pharmacologic inhibition of PKG activity, significantly decreased SM-specific gene expression. We previously demonstrated basal PKG I activity in PAC1 cells due to constitutive production of NO and cGMP generation (47), and we now found basal phosphorylation of the PKG substrates VASP and CRP4 in unstimulated PAC1 cells. PKG overexpression and membrane-permeable cGMP analogs only slightly increased SM-MHC mRNA levels above those observed in control cells, suggesting that basal PKG I activity in unstimulated PAC1 cells was sufficient for near-maximal SM-MHC expression. Since the original submission of our manuscript, Zhou et al. (54) published a report describing that possibly other proteins (Fig. 10). CRP4 is homologous to CRP1 and CRP2/smLIM, and their high degree of sequence similarity suggests similar functions. Solution structures of CRP1 and -2 demonstrate that the proteins’ two LIM domains are independent folding units bridged by a flexible linker region, suggesting an adaptor or linker role for the proteins (55, 56). Similar to CRP4, CRP1, and CRP2/smLIM co-operate with SRF and GATA factors to transactivate SM-specific genes; CRP2 exists in a trimeric complex, with SRF and GATA4 (or -6) docking to the N-terminal and C-terminal LIM domains, respectively (26). However, CRP1 and -2 do not contain the PKG binding-deficient CRP4ΔZF3/4 mutant indicated that PKG I phosphorylation of CRP4 Ser104 was required for cGMP regulation of the SMA promoter. In vitro kinetics of CRP4 phosphorylation by PKG I indicate CRP4 is an excellent substrate for the kinase (29). siRNA-mediated down-regulation of CRP4 expression in primary ovine pulmonary venous smooth muscle cells correlated with repressed SM-specific gene expression, while overexpression of PKG I reversed the effect. In agreement with our results, they showed that inhibition of PKG activity with the membrane-permeable inhibitor peptide DT-3 attenuated SM-MHC, SMA, and calponin protein expression in these cells. These results confirm a requirement of basal PKG activity for maintenance of SM-specific gene expression.

We found that cGMP stimulation of the SMA promoter required PKG I and CRP4 co-expression with SRF and GATA6. CRP4 acted synergistically with SRF and GATA6 to transactivate the SMA promoter, although CRP4 by itself had no significant transcriptional activity. Because the PKG binding-deficient CRP4ΔZF3/4 mutant did not support cGMP stimulation of the SMA promoter, we propose a model wherein CRP4 acts as a scaffolding protein that aids complex formation between SRF, GATA6, PKG I, and hypoxia-induced reduction in PKG expression in primary ovine pulmonary venous smooth muscle cells.
the effect described for CRP2/smLIM (26). However, the mechanism whereby CRP and GATA proteins stabilize SRF DNA binding *in vitro* is unclear, and we could not detect formation of ternary or quaternary complexes, similar to the results of Chang *et al.* (26).

Association of protein kinases with anchoring proteins places the enzymes close to their substrates and ensures signaling specificity; this has been well demonstrated for cAMP-dependent protein kinase, but there are also examples of PKG anchoring (34, 57–59). Binding of PKG I to CRP4 may position the kinase to phosphorylate other proteins associated with CRP4 that affect transcription of SM-specific genes. For example, both SRF and CRP2/smLIM interact with the protein inhibitor of activated STAT1 (PIAS1), which is a ligase for small ubiquitin-like modifier-1 and activates SM-specific genes (60, 61). More work is required to determine the different functions of CRP1, -2, and -4 in VSMCs. After vascular injury, CRP2/smLIM expression is down-regulated in neo-intimal VSMCs, and CRP2/smLIM-deficient mice show increased neo-intima formation; these findings suggest that CRP proteins play an important role in the phenotypic modulation of VSMCs *in vivo* (32, 51).

SRF activation of SM-specific genes involves multiple cofactors, some of which compete with each other (22). SRF by itself produces only subtle changes in gene expression, and over 50 different cofactors have been described that restrict SRF-mediated transcription to specific genes and environmental conditions (23). It is presently unknown how SRF-GATA-CRP complexes relate to SRF complexes with other cofactors, but our data suggest that CRP4, together with SRF, is bound to CArG sequences of the SMA and SM-MHC promoter in intact cells.

In pluripotent 10 T1/2 cells, cGMP/PKG enhanced SRF- and GATA6-dependent expression of endogenous SM-specific genes in the presence of CRP4. These results suggest that PKG may modulate SM-specific differentiation of mesenchymal “stem cells.” PKG is also involved in skeletal muscle differentiation, because PKG interaction with the transcription factor FoxO1a regulates myoblast cell fusion in C2C12 cells (62). Overexpression of CRP2/smLIM (or CRP3) promotes myogenic differentiation in the same cell type (33).

The small GTPase RhoA regulates SRF activity through changes in actin dynamics and activation of myocardin-related cofactors (23), but we found that the effect of PKG I inhibition on SM-specific gene expression cannot be explained by changes in RhoA expression or activity. siRNA-mediated down-regulation or pharmacologic inhibition of PKG I had no effect on RhoA expression, and we previously showed in PAC1 cells that PKG I inhibits Rho activation in response to agonists with little effect on basal RhoA activity (63).

De-differentiated VSMCs in neo-intimal lesions express decreased levels of soluble guanylate cyclase and PKG I in ath erosclerotic and vascular injury models (5–7, 15). In addition, atherosclerotic vessels produce low amounts of NO and show defective cGMP signaling with reduced VASP phosphorylation (7, 64). Our results suggest that decreased PKG activation in atherosclerotic or injured vessels could facilitate de-differentiation of VSMCs. Restoration of soluble guanylate cyclase or PKG I expression in the injured vessel wall reduces neo-intima formation after vascular injury in intact animals (5, 15); pharmacologic or genetic manipulations that enhance cGMP production in injured blood vessels yield similar results (16–18, 20). Delivery of C-type natriuretic peptide to rabbit femoral arteries at the time of balloon injury reduces neo-intima formation and enhances SM-MHC expression in the residual neo-intimal cells (16). These results suggest that increased cGMP production in de-differentiated neo-intimal VSMCs can inhibit growth and induce differentiation. Although the majority of data suggest an atheroprotective role of the NO/cGMP/PKG signaling pathway, atheromatous plaques were attenuated when apoe-deficient mice were mated with mice containing a conditional, SM-specific deletion of PKG I; unfortunately, SM-specific gene expression was not examined in these animals (14). The effects of cGMP/PKG on SM-specific gene expression and SM phenotype could differ depending on the experimental conditions and the origin of the VSMCs.

In conclusion, PKG I plays a central role in cardiovascular (patho)physiology, because PKG I regulates the contractility, proliferation, survival, and phenotype of VSMCs (3, 12). Previous studies investigated the effects of PKG I in de-differentiated VSMCs but did not address the mechanism(s) whereby PKG I regulates SM-specific genes. We show that basal PKG activity is required for maintaining high SM-specific gene expression in differentiated VSMCs and provide evidence that CRP4 mediates at least some of the effects of PKG I on SM-specific gene expression.

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