cGMP-dependent protein kinase and the regulation of vascular smooth muscle cell gene expression: possible involvement of Elk-1 sumoylation

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Although the regulation of smooth muscle cell (SMC) gene expression by cGMP-dependent protein kinase (PKG) is now recognized, the mechanisms underlying these effects are not fully understood. In this study, we report that PKG-I stimulates myocardin/serum response factor (SRF)-dependent gene expression in vascular SMCs. The expression of PKG in PKG-deficient cells enhanced myocardin-induced SM22 promoter activity in a concentration-dependent fashion. However, neither SRF nor myocardin expression was affected. To investigate alternative mechanisms, we examined whether PKG affects the phosphorylation of E26-like protein-1 (Elk-1), a SRF/myocardin transcription antagonist. The activation of PKG caused an increase in a higher molecular mass form of phospho-Elk-1 that was determined to be small ubiquitin-like modifier (sumoylated) Elk-1. PKG increased Elk-1 sumoylation twofold compared with the PKG-deficient cells, and Elk-1 sumoylation was reduced using dominant-negative sumo-conjugating enzyme, DN-Ubc9, confirming PKG-dependent sumoylation of phospho-Elk-1 in vascular SMCs. In addition, PKG stimulated Elk-1 sumoylation in COS-7 cells overexpressing Elk-1, sumo-1, and PKG-I. The increased expression of PKG in vascular SMCs inhibited Elk-1 binding to SMC-specific promoters, SM22 and smooth muscle myosin heavy chain, as measured by EMSA and chromatin immunoprecipitation assay, and PKG suppressed the Elk-1 inhibition of SM22 reporter gene expression. Taken together, these data suggest that PKG-I decreases Elk-1 activity by sumo modification of Elk-1, thereby increasing myocardin-SRF activity on SMC-specific gene expression.

guanosine 3’,5’-cyclic monophosphate-dependent protein kinase; small ubiquitin-like modifier; myocardin; E26-like protein-1

VASCULAR SMOOTH MUSCLE CELLS (SMCs) undergo dramatic phenotypic changes in both culture and in vivo (8, 37). It has been shown that in response to arterial injury in vivo or exposure to growth conditions in vitro, SMCs become more proliferative and express lower levels of contractile proteins and other signaling molecules associated with the more contractile “differentiated” phenotype (7, 9, 38, 43). These changes in gene expression profiles are believed to underlie SMC phenotypic modulation, a phenomenon that is associated with several vascular disorders such as atherosclerosis and vascular fibrosis. Understanding the mechanisms that control SMC-specific gene expression is therefore an important aspect for understanding vascular disease.

Over the last several years, a significant amount of progress has been made toward understanding the mechanisms regulat-
In this study, we explore one possible mechanism of action of cGMP/PKG in the regulation of SMC-specific gene expression. The results reported here suggest that cGMP/PKG suppresses the activity of Elk-1 on myocardin-SRF transcription through the sumoylation of Elk-1.

EXPERIMENTAL PROCEDURES

**DNA constructs.** The PKG-Iα construct was cloned into pcDNA3.1 (Invitrogen). The pCMV5-Elk-1 construct tagged with FLAG and His was provided by Dr. Andrew Sharrocks (Univ. of Manchester, UK) and recloned into pcDNA3.1. The expression plasmid for mouse myocardin isoform B was provided by Dr. Eric Olson (Univ. of Texas, Dallas, TX), whereas hemagglutinin (HA) -tagged sumo-1 was provided by Dr. Edward Yeh (Univ. of Texas, MDACC, TX). The dominant negative (DN)-Ubc9 cDNA was provided by Dr. Ronald Hay (Univ. of St. Andrews, St. Andrews, UK). Mouse SM22α-luciferase (∼447 ± 62 bp) construct was provided by Dr. Joseph Miano (Univ. of Rochester Medical School, Rochester, NY).

**Cell culture, transfection, and reporter gene assays.** Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama. SMCs were isolated from the thoracic and abdominal aortas of Sprague-Dawley rats (200 to 250 g) as described previously (1, 14). A7r5 and COS-7 cells were cultured in DMEM (Cellgro)-10% FBS (Invitrogen) and passaged routinely. Transient and stable plasmid transfection of cells was performed by using Lipofectamine 2000 (Invitrogen) or FuGene6 (Roche Diagnostics, Indianapolis, IN). To acquire stably PKG-transfected rat aortic SMCs, the cells were plated at a density of 800,000 cells per 100-mm plate and grown overnight. On the next day, adenovirus carrying the coding sequence of PKG-Iα or green fluorescent protein was produced and used for the infection of rat aortic SMCs according to the manufacturer’s protocol (Vector BioLabs). To express PKG-Iα in rat aortic SMCs, the cells were plated at a density of 800,000 cells per 100-mm plate and grown overnight. On the next day, either control adenovirus or recombinant PKG-Iα adenovirus was added directly to the culture medium at a multiplicity of infection of 20 after changing to the fresh complete medium. After 1 day of incubation, the culture medium was replaced with serum-deprived DMEM including 1 mg/ml of BSA and cultured for 2 to 3 days. The level of PKG-Iα expression was constantly monitored by Western blot analysis.

**Preparation of nuclear and cytoplasmic extracts.** Nuclear and cytoplasmic fractionation was performed using nuclear and cytoplasmic extraction reagent (Pierce) supplemented with 1× protease inhibitors and 1× phosphatase inhibitors (Pierce) according to the manufacturer’s protocol. In brief, treated cells were placed on ice, washed three times with cold PBS, and harvested directly or snap frozen until use. The cells were harvested by scraping off the dish in an appropriate volume of cytoplasmic extraction reagent I containing protease inhibitors, phosphatase inhibitors, and 20 μM of N-ethylmaleimide (NEM; Sigma) and incubated on ice for 10 min. After cytoplasmic extraction reagent II was added with an additional incubation on ice for 1 min, the nuclei were collected by centrifugation at 16,000 g for 5 min and the supernatants were collected for cytoplasmic extracts. The nuclei were resuspended by adding nuclear extraction reagent and incubated on ice for a total of 50 min with vortexing for 15 s every 10 min. The nuclear extracts were collected as the supernatant by centrifugation at 16,000 g for 10 min. All extracts were used directly or snap frozen and kept at −80°C until use.

**SDS-PAGE and immunoblot analysis.** Cells (SMCs or COS-7) were lysed in a denaturing lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% SDS, containing 1× protease inhibitor cocktail (Pierce), 1× phosphatase inhibitor cocktail (Pierce), and 20 μM of NEM (Sigma) or in SDS sample buffer and were briefly sonicated (Virti Ultrasonic Cell Disruptor 100) to reduce sample viscosity. After being heated to a boil, the samples were applied to 8% or −8−17.5% gradient SDS-PAGE and transferred to nitrocellulose membranes for the molecules of interest using the following primary antibodies: anti-PKG-I (Stressgen), anti-SRF, anti-ERK-1, anti-myocardin (Santa Cruz), anti-Elk-1,-sumo-1,-anti-phospho-ERK-1, anti-phospho-vasodilator-stimulated phosphoprotein (Ser239), anti-phospho-ERK, anti-FLAG (cell signaling), anti-β actin (Sigma). Protein bands were visualized using the enhanced chemiluminescence system (Pierce).

**Immunoprecipitation.** For immunoprecipitation, the total volume of nuclear extracts (∼50−100 μg) were made to 0.5 ml with radioimmunoprecipitation assay buffer consisting of 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.2% SDS, 2% Nonidet P-40, and 2% sodium deoxycholate, supplemented with protease inhibitor cocktail and phosphatase inhibitors. To this lysate, 2 μg of normal rabbit IgG (Santa Cruz) were added to preclar the lysate. After incubation at 4°C for 30 min, 40 μl of protein A/G agarose (Santa Cruz) were added and incubated for an additional 30 min and centrifuged at 1,000 g for 5 min. The resulting supernatant was incubated with primary antibody (10 μl of anti-sumo-1 antibody, Cell Signaling) at 4°C for 1 h following overnight incubation after adding 20 μl of protein A/G agarose. The agarose beads were collected by centrifugation at 1,000 g for 5 min, and the pellet was resuspended in cold radioimmunoprecipitation assay buffer and washed four times with the same buffer by centrifuging at 1,000 g for 5 min before boiling in the SDS sample buffer. Immunoblotting following 8% SDS-PAGE was conducted to detect the sumoylated Elk-1.

**Denaturing purification and analysis of in vivo sumo-Elk conjugates.** Control or stably PKG-I expressing COS-7 cells were transfected with plasmids expressing HA-sumo-1 and His-Elk-1. Two days later, the cells were harvested in 1 ml of lysis buffer consisting of 6 M guanidine-HCl, 100 mM...
Na$_2$HPO$_4$, 0.3 M NaCl, 10 mM Tris·Cl (pH 8.0), 20 mM β-mercaptoethanol, and 20 mM imidazole, containing 1× protease inhibitor cocktail, calyculin A, and 20 μM of NEM. One-twentieth amounts of cell lysates were precipitated with 10% trichloroacetic acid for total protein. His-tagged Elk-1 was purified by overnight incubation with 30 μl of nickel-nitrilotriacetic acid agarose (Qiagen) prewashed with lysis buffer. Collected agarose-bound proteins were washed with serial wash buffer containing 8 M urea, 100 mM Na$_2$HPO$_4$, 0.3 M NaCl, 10 mM Tris·Cl, and 0.2% Triton X-100 at pH 8.0, pH 6.3, and pH 5.9 and then wash buffer at pH 4.5. All wash buffers were made to be 20 mM β-mercaptoethanol and 20 mM imidazole before use, except for the final wash buffer. The final wash buffer at pH 4.5 was made to be 720 mM β-mercaptoethanol and 200 mM imidazole to elute the proteins. After heat denaturation with SDS sample buffer, the proteins were separated by SDS-PAGE, followed by immunoblotting against HA antibody to detect sumo-modified Elk-1.

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared, incubated with double-stranded oligonucleotide probe corresponding to the SM22α promoter for 20 min at room temperature, and analyzed by 4.5% nondenaturing PAGE. The sequences of the SM22-promoter probe contain the CC (A/T) GG (CarG) box (underlined) and the wild- or mutant-type of ternary complex factor (TCF) site (bold) and are shown below: wild-type, AGCTGTTCAGGCTTCGC-CCATAAAAGGTITTTCCGCGCCGC; and the TCF mutant, AAGCTGTTCAGGCTTCGCGCCATAAAAGGTITTTAAGGCGGC-GC (51). In the antibody supershift assays or competitive EMSA, nuclear extracts were preincubated with 0.6 μg of the indicated antibodies or 50-fold excess of the unlabeled probe for 20 min on ice, respectively, before the addition of the labeled probe. After electrophoresis, the gels were dried and imaged by autoradiography.

**Chromatin immunoprecipitation assay.** The chromatin immunoprecipitation (ChIP) assay kit and protocol were from Upstate Biotechnology and used with minor modification. In brief, protein and chromatin from SMCs were cross-linked by adding formaldehyde directly to culture medium at a final concentration of 1% and incubated for 10 min at room temperature. To terminate the cross-linking process, glycine was added to a final concentration of 125 mM. The cells were washed three times with ice-cold PBS containing 1× protease inhibitor cocktail, calyculin A, and NEM. The cells were harvested in cell lysis buffer containing 5 mM PIPES-KOH (pH 8.0), 85 mM KCl, and 0.5% Nonidet P-40, including the same supplements. After incubation on ice for 20 min, the lysates were centrifuged at 5,000 rpm for 5 min. Nuclear pellets were resuspended in 200 μl of SDS lysis buffer containing 1% SDS, 10 mM EDTA, and 50 mM Tris·HCl (pH 8.1), including the same supplements per two million cells. After incubation for 10 min on ice, four cycles of sonication for 15 s at a setting of 4 using VirSonic Ultrasonic Cell Disrupter were conducted to obtain about 500 bp of chromatin fragments. The sonicated DNA protein complexes were then centrifuged at 4°C for 10 min at 16,000 g to remove insoluble material. The supernatant was diluted 1:10 with dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris·Cl (pH 8.1), and 167 mM NaCl, including the same supplements. The diluted supernatant was precleared by incubation with 80 μl of protein A agarose/salmon sperm DNA (PAA/SSD) at 4°C for 1 h. After centrifugation at 4,000 g for 1 min, 2.5% of the supernatant was saved to serve as an input control and the remaining supernatant was subsequently incubated with 8 μg of Elk-1 antibody (Santa Cruz) at 4°C overnight. As a negative control, 60 μl of precleared PAA/SSD was added to diluted supernatant without antibody and incubated at 4°C for 1 h and subsequently washed with washing buffer as described below. Unbound supernant resulting from control incubation was used for the immunoprecipitation with SRF antibody (4 μg, Santa Cruz) at 4°C overnight. On the next day, the antibody-agarose complex was obtained by incubation with precleared PAA/SSD at 4°C for 1 h. After being washed with four different washing buffers [low-salt wash buffer contained 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·Cl (pH 8.1), and 150 mM NaCl; high-salt wash buffer contained 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·Cl (pH 8.1), and 500 mM NaCl; LiCl wash buffer contained 0.25 M LiCl, 1% IGEPEL-Ca630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris·Cl (pH 8.1); TE buffer twice], the DNA protein complex was eluted with 100 μl of fresh elution buffer, containing 1% SDS and 0.1 M NaHCO$_3$, from agarose by shaking at room temperature for 15 min. After the supernant was collected by centrifugation at 4,000 g for 1 min, another elution was performed, and the eluates were combined. The combined eluates along with input control were reverse cross-linked to release the DNA from the protein by incubating tubes in a 65°C water bath for 4 h to overnight. After RNase A and Proteinase K treatment, the remaining DNA was purified using PCR purification kit (Qiagen). The amounts of purified DNA fragments bound to immunoprecipitated proteins of interest were determined by PCR using primers specific for the promoter sequence of interest. Primer sequences for ChIP assays were as follows: SM22α promoter region, GTGC-CTGCCCTAAAGGTTTTT and TGGCCATGGAAGTCTGC-TTGG; the exon 5 region, AAGCAGGAGCATAAGGAGG-GACT and GAAAGAGCTGGGCTGCCCCATCAG (51); SM-MHC promoter region, CTGAGGACTTCTATTAGTACT-GGGTCC and ACTCACGCCCCATTAAAAAGGACTGAG-GCAGATTTGGG; SM-MHC promoter region lacking CarG, ATGTCAGATGTCTCTCTACTGTTATTCG and AGCA-CAACAGACTTTAATAACGTATTTGCTTC (32); and c-fos CarG region, CATGTGACGGGCTGCGAAGCTGACGAGGAGAAGTCC and GCGTGGAAACCTGCTGAGCGA (57). The PCR products were separated on a 3% agarose gel.

**Statistics.** Data are presented as means ± SD. Statistical analyses were done using a Student’s t-test or with a one-way analysis of variance with post hoc (Newman multiple comparison) test when multiple groups were compared. A P value of <0.05 was taken as statistically significant.

**RESULTS**

**PKG-I enhanced myocardin-induced SM22 promoter activity.** Previous work by our laboratory (3) and that of Zhang et al. (56) demonstrated that the expression of PKG-I in passaged SMCs, deficient in endogenous PKG-I, induced SMC-specific gene expression. Because myocardin is a key transcriptional regulator of the expression of SMC-specific gene expression, we tested whether PKG-I expression affected myocardin-induced SMC gene promoter activity in COS-7 cells. COS-7 cells are useful to examine the specific effects of PKG-I because in
addition to not expressing myocardin, the cells express no PKG-I mRNA or protein. As shown in Fig. 1A, myocardin significantly increased SM22 promoter activity some 100- to 300-fold. PKG-I expression alone had no effect on the SM22 promoter activity in the cells. When PKG-I was cotransfected with myocardin, SM22 promoter activity was increased two- to threefold over myocardin alone (Fig. 1A). We also examined whether PKG-I increased myocardin-induced SM22 promoter activity in rat aortic SMCs stably transfected with PKG-I vector. Unlike COS-7 cells, rat aortic SMCs express myocardin, although more highly passaged cells express lower levels than lower passaged cells (10). As shown in Fig. 1B, SMCs stably transfected with PKG-I had no detectable effect on the level of endogenous myocardin expression but had higher levels of myocardin-induced SM22 promoter activity (~3-fold) compared with control-transfected SMCs.

PKG-I increases the unusual size of phospho-Elk-1 species in rat aortic SMCs. Olson’s laboratory proposed that the ets-binding protein, Elk-1, when phosphorylated by MAP kinases competes with myocardin binding to SRF (51). According to this model, Elk-1 phosphorylation suppresses SMC-specific gene expression by dislodging myocardin from SRF, thus reducing transcription. To increase myocardin-SRF specific gene expression, our initial hypothesis was that PKG may decrease Elk-1 phosphorylation, possibly by inhibiting MAP kinases or stimulating protein phosphatases (46, 52, 60). Thus we examined the level of phospho-Elk-1 (pElk-1) in rat aortic SMCs in response to PDGF by immunoblotting using a specific pElk-1 antibody. In primary cultures of rat aortic SMCs, PDGF increased the phosphorylation of Elk-1 as expected (arrowhead in Fig. 2A). However, in nontreated SMCs (Cont), there was an unexpected higher molecular mass species (molecular mass, ~90 kDa) that reacted with the pElk-1 antibody (arrow, Fig. 2A), which was decreased by PDGF treatment. Immunoblot using Elk-1 antibody was performed to show that this higher molecular mass species was Elk-1. As shown in the Elk-1 blot of Fig. 2A, anti-Elk-1 (as opposed to anti-pElk-1) also reacted with the higher molecular mass protein that showed a similar decrease in response to PDGF (asterisks). That this higher molecular mass species was authentic, Elk-1 was supported by other observations: 1) preincubation of anti-pElk-1 antibody with blocking peptide (pElk-1 peptide, Santa Cruz) completely abolished the pElk-1 signals, and 2) PDGF specifically decreased the presence of the higher molecular mass band while increasing the level of the 65 kDa protein, suggesting that this protein was not an unrelated, nonspecific protein.

When primary cultured rat aortic SMCs were treated with the cGMP analog 8-(p-chlorophenylthio)-cGMP together with treatment with PDGF for comparison, there was an increase in the higher molecular mass species in response to 8-(p-chlorophenylthio)-cGMP (arrow in pElk-1 blot of Fig. 2B). The Elk-1 blot was again performed to show the similar effects of PDGF or the cGMP analog and to confirm that it reacted with Elk-1 antibody (asterisk in Elk-1 blot of Fig. 2B).

To confirm that PKG increased the higher molecular mass form of pElk-1 in vascular SMCs, we examined the effects of overexpression of PKG-I on the abundance of the higher molecular mass pElk-1 species in more highly passaged rat aortic SMCs. In cells stably overexpressing PKG-I, there was a two- to threefold increase in the higher molecular mass pElk-1 that also reacted with anti-Elk-1 antibody (asterisk in Elk-1 blot), compared with control transfected SMCs (Fig. 3A, top, and B), confirming the effect of cGMP/PKG to increase the levels of this species. Also shown is the effect of PKG-I to increase the expression of the SMC-specific phenotypic marker, SM-MHC, without changing the levels of SRF protein in whole cell extracts (Fig. 3A, bottom). These results demonstrate two species of pElk-1 and show that PDGF reduced the higher molecular mass form. The mechanism of this effect of PDGF is not clear. The results also suggest that cGMP/PKG
antagonized the effect of PDGF not by inhibiting Elk-1 phosphorylation but by shifting pElk-1 to the unusual higher molecular mass form. These observations led us to define the characteristics of these species.

The higher molecular mass species is sumoylated Elk-1. Previous work has shown that Elk-1 undergoes posttranslational modification by sumoylation (54), raising the possibility that the higher molecular mass form of pElk-1 may have been a posttranslationally sumo-modified form of pElk-1. Our results regarding the unusual size of pELK-1 species thus led us to explore the possibility that PKG expression stimulates sumo-modification of pElk-1 in SMCs. As shown in Fig. 4A,
left, immunoprecipitation of nuclear extracts using anti-sumo-1 antibody followed by immunoblotting with pElk-1 antibody demonstrated the higher molecular mass species of Elk-1 was indeed sumoylated pElk-1. Furthermore, adenoviral-induced PKG-Iα expression in rat aortic SMCs increased sumoylated pElk-1 by approximately twofold compared with control rat aortic SMCs (Fig. 4A, right). Of note, sumo proteins have molecular masses of ~15 kDa. However, sumo-modified proteins generally migrate anomalously on SDS-PAGE as proteins of 20 kDa larger than the unmodified protein. Hence, the migration of pElk-1 in the 90-kDa range suggested a mono-sumoylated species, as has been reported previously (17, 28, 44).

To further study the effect of PKG expression on Elk-1 sumoylation, we transfected SMCs with a dominant-negative form of the E2 sumo ligase, DN-Ubc9, which is unable to transfer sumo proteins to the target proteins (48). As shown in Fig. 4B, a similar decrease in higher molecular mass pElk-1 (arrow) and an increase in lower molecular mass pElk-1 (arrowhead) were observed in accordance with an increased expression of DN-Ubc9, confirming that the higher molecular form of pElk-1 was PKG-induced sumoylated Elk-1.

**Sumoylation of Elk-1 by PKG-I in COS-7 cells.** To further explore the effects of PKG-I on Elk-1 sumoylation in intact cells, we examined the effect of PKG-I expression on Elk-1 sumoylation in COS-7 cells, where the direct effect of the kinase could be more easily studied. COS-7 cells were transfected with plasmids encoding His-tagged Elk-1 plus HA-tagged sumo-1 in control (COS/C) or PKG-Iα (COS/Iα) stably transfected cells. Following nickel affinity chromatography and immunoblotting using anti-HA antibody, we observed that there was a large increase in the level of sumoylated Elk-1 in PKG-I expressing COS-7 cells when both Elk-1 (E) and sumo-1 (S) were expressed compared with control cells (Fig. 5, top, lanes 2 and 4).

**PKG-I modulates Elk-1 inhibition on the myocardin-induced SM22 promoter activity.** It is well established that Elk-1 regulates cell proliferation, increases growth-related gene expression, and suppresses SMC-specific gene expression (5, 51, 57). To test whether PKG-I inhibits the effects of Elk-1 on myocardin-induced SM22 promoter activity, COS-7 cells were cotransfected with a combination of expression plasmids for myocardin, Elk-1, and PKG-I together with the SM22 promoter-reporter construct. Consistent with previous studies and our

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**Fig. 4. Sumoylation of a higher molecular mass pElk-1 in the PKG-I overexpressing rat aortic SMCs.** Immunoprecipitation was performed with nuclear extracts from adenoviral-mediated green fluorescent protein (Cont) or PKG-Iα (PKG)-expressing rat aortic SMCs using anti-small ubiquitin-related modifier-1 (sumo-1) antibody or normal IgG (IgG) followed by immunoblotting with anti-pElk-1 antibody. A, left: increased levels of sumoylated Elk-1 in the PKG-overexpressing cells was shown and indicated with arrow. Arrowhead indicates IgG heavy chain. A, right: results are expressed as means ± SD and typical of 3 independent experiments. *P < 0.05, PKG-Iα-expressing cells vs. Cont. Inset: lysates from control- and PKG-transfected cells were immunoblotted for PKG levels. B, top: rat aortic SMCs were transfected with dominant negative (DN)-Ubc9 expressing plasmids (0, 0.5, 1, or 2 μg). After 2 days, whole cell extracts were prepared with 1× denaturing lysis buffer and subjected to 8 – 17.5% of gradient SDS-PAGE and immunoblotting. Arrow indicates high molecular mass pElk-1, and arrowhead indicates DN-Ubc9-mediated pElk-1. B, bottom: pooled data from 3 separate experiments quantifying higher molecular mass species of pElk-1 (arrow). *P < 0.01, DN-Ubc9 vs. control.
plexes, not the SRF-alone complexes, were completely erased.

In the binding reaction with mutant oligos containing a TCF-site mutation, thus confirming the PKG-I effect on the reduced Elk-1 binding (Fig. 7, lanes 4 and 5). Nuclear extracts from control rat aortic SMCs (C) were used in the EMSA immuno-shift assay to confirm that PKG-mediated reduction of the complex was SRF-Elk-1 complex. As shown in Fig. 7A, lane 9, and previously (51), Elk-1 antibody reduced only the higher band intensity, whereas SRF antibody erased both bands completely and induced a supershift (Fig. 7, lane 10), suggesting that PKG-I expression was responsible for the reduced binding of Elk-1 to the smooth muscle-specific SM22 promoter.

To further test a possible role of PKG-I in Elk-1 binding to the SMC-specific gene promoters in vivo, we performed ChIP assays. In stably transfected PKG-I-expressing rat aortic SMCs, less Elk-1 binding to its specific binding sites on both the SM22 and SM-MHC promoters was observed compared with control stably transfected cells (Fig. 7B, lanes 5 and 6). Furthermore, there was also decreased binding of Elk-1 to the c-fos promoter in PKG-expressing SMCs, suggesting an overall effect of PKG-I to reduce Elk-1 availability for gene expression. It was also noted that SRF binding was increased, particularly to the SM-MHC promoter, in PKG-I-expressing SMCs compared with control cells. Taken together, these results suggest that the suppressive effect of PKG-1α on Elk-1 binding to CArG regions in SMC gene promoters mediates the increased myocardin-SRF activity on SMC-specific gene expression.

Results (Fig. 1), myocardin increased SM22 promoter activity (Fig. 6, lane 3) and this effect was reduced by Elk-1 cotransfection (Fig. 6, lane 4). Elk-1 repression of myocardin-induced SM22 promoter activity was reversed in the presence of PKG-I expression (Fig. 6, lane 5). These results show that PKG-I reduced the Elk-1 suppression of myocardin-induced SM22 promoter activity, suggesting a more direct role of PKG in SMC-specific gene regulation through the inhibition of Elk-1 activity.

Elk-1 binding to SMC-specific gene promoters is decreased by PKG-I in rat aortic SMCs. The inhibitory effect of Elk-1 on SMC-specific gene promoter activity and its reversal by PKG led us to examine the role of PKG in modulating SRF and Elk-1 binding to SMC-specific gene promoters using EMSA (in vitro) and ChIP assay (in vivo). In EMSA analysis, the incubation of nuclear extracts from PKG-Iα-expressing rat aortic SMCs (Iα) with a radiolabeled probe corresponding to the SM22 promoter region that contains a consensus CArG box and the TCP site revealed a decreased SRF-Elk-1 complex (SRF/Elkα) compared with the incubation with control extracts (C) (compare lanes 2 and 3 in Fig. 7A). These complexes, not the SRF-alone complexes, were completely erased...
DISCUSSION

The unique characteristic of vascular SMCs known as phenotypic plasticity plays a critical role in development as well as in phenotype modulation between differentiated SMCs and proliferative, synthetic SMCs (38). The differentiated phenotype is typically distinguished from the synthetic phenotype by the expression of high levels of various contractile proteins including SM-MHC, calponin, and smooth muscle α-actin. Knowledge of the cellular pathways that control SMC-specific gene expression and differentiation is important for understanding this phenotypic switch. In this study, we investigated mechanisms relating to the cGMP/PKG pathway in SMC-specific gene expression because studies in our laboratory (3) and others (56, 58) have shown that this pathway stimulates vascular SMC differentiation.

Our studies were prompted by progress in defining the molecular mechanisms of SMC-specific gene expression at the nuclear transcription level. SRF is the key transcription factor that is required for transcription of SMC-specific genes (34). In turn, SRF is regulated by numerous cotranscriptional and regulatory proteins that include myocardin and related proteins (19, 29), GATA proteins (35), and Elk-1 (5). Because SRF is a ubiquitous transcription factor that regulates gene expression in a wide variety of cell types, the specificity for SRF to regulate SMC-specific gene expression depends on myocardin (19, 29). However, SRF is also under the control of transcription factors such as Elk-1 that inhibit SMC-specific gene expression (51, 57). The activation of SMCs by growth factors that downregulate SMC-specific gene expression and promote vascular SMC phenotypic modulation to the fibroproliferative “synthetic” phenotype is associated with the activation of the MAP kinase pathway and the phosphorylation of Elk-1. Phosphorylated Elk-1 inhibits myocardin association with SRF on SMC promoters (51). Thus there has been much interest in defining the cytosolic signaling pathways that control both SRF and Elk-1 in SMCs.

The NO/cGMP/PKG signaling pathway has been shown to contribute to numerous SMC functions including relaxation, cell proliferation, and gene expression (26, 30, 31, 41). Previous studies from our laboratory (3) and that of Zhang et al. (56) and Zhou et al. (58) have shown that the cGMP/PKG pathway stimulates SRF-dependent SMC-specific gene expression and promotes a more “contractile” (differentiated) phenotype for SMCs. In this study, we examined the hypothesis that PKG decreases Elk-1 suppressive activity on SMC-specific gene expression. Specifically, we studied the posttranslational modification of Elk-1 by the cGMP/PKG pathway.

One posttranslational signaling pathway that has been shown to regulate the activity of a number of transcription factors including Elk-1 is sumoylation (21, 50). Like ubiquitination, protein sumoylation involves the covalent binding of sumo proteins to ε-lysine residues of target proteins. Unlike ubiquitination, however, sumoylation rarely targets proteins for proteasome degradation. Rather, sumoylation promotes structural changes in target proteins that allow the proteins to be directed to specific cellular organelles and/or to be changed in their activities (23, 33). It has been reported that a sumo-modification of Elk-1 suppresses its binding to nuclear targets (45). In this report, we show evidence that PKG increases sumo-modification of Elk-1 using immunoprecipitation, intact cell
sumoylation, and dominant-negative approaches. These data suggest that increased SMC-specific gene expression is due in part to PKG-activated sumo-modification of Elk-1. Our data demonstrate that an overexpression in COS-7 cells of sumo-1, Elk-1, and PKG-I were all required to increase the sumoylation of Elk-1 and Elk-1 inhibition of myocardin-dependent SM22 promoter activity (Figs. 5 and 6). Reporter gene, EMSA, and ChIP assays demonstrate that PKG suppresses the inhibitory effect of Elk-1 on SRF/myocardin-induced SMC-specific gene expression (Figs. 6 and 7). These results provide a possible molecular mechanism for PKG in the upregulation of smooth muscle gene expression and modulation to the contractile phenotype.

The precise mechanism by which PKG stimulates Elk-1 sumoylation is not yet known. In vitro sumoylation carried out with purified PKG and Elk-1 using the sumoylation kit (Bio-mol) did not stimulate Elk-1 sumoylation (data not shown), suggesting that perhaps PKG increases the sumoylation of Elk-1 through a more specific sumo E3 ligase pathway in the cell. For example, the nucleoporin RAN binding protein 2 (RanBP2) has been shown to strongly enhance the sumoylation of SP100 with the ability to confer substrate specificity, (40) and, of interest, human RanBP2 contains a PKG consensus sequence (RRIT) at the COOH-terminal region (55), suggesting RanBP2 as a possible target of PKG action in Elk-1 sumoylation. More work needs to be directed toward identifying the specific target of PKG action.

It was initially surprising that two posttranslational regulation pathways, sumoylation (repressive) and phosphorylation (active), operated simultaneously with Elk-1. The phosphorylation of Elk-1 by the MAP kinase pathway is transient (5) and leads to a decreased sumo conjugation, resulting in Elk-1 regaining transcriptional activity (54). Perhaps this is the mechanism underlying the effect of PDGF to decrease the amount of higher molecular mass Elk-1 (Fig. 2). However, the coordinated regulation of transcription factors by two different modifications (phosphorylation and sumoylation) has been reported in a number of cases including heat shock factors, myocyte enhancer factor 2D (MEF2D), and estrogen-related receptor-α and -γ (22, 24, 25, 49). In the case of MEF2D, the serine residue adjacent to the sumoylation motif was required for the sumoylation of MEF2D, and the transcriptional activity of MEF2D was inhibited by sumoylation that was increased by the phosphorylation. Likewise, it has been demonstrated that sumoylation suppresses a Ser383 phosphorylation-independent transcriptional capacity of Elk-1 (54), suggesting a possible coexistence of two modifications on Elk-1.

In the numerous reports describing the effects of posttranslational modification of proteins by sumoylation, an interesting regulatory mechanism was recently described in which oxidative stress induced or decreased reversible sumoylation of cellular substrates depending on the concentration of reactive oxygen species (ROS) (2, 59). ROS have also been recognized to mediate vascular SMC differentiation, and recently, the NAD(P)H oxidase, Nox4, was found to be required for differentiation marker gene expression in vascular SMCs (12, 53). Of interest is that the NO/cGMP/PKG pathway is known to be regulated by oxidative stress, and H₂O₂ oxidizes PKG-Iα to directly activate the kinase itself (6). Based on these reports, it is possible that PKG-mediated Elk-1 sumoylation might be regulated by ROS, resulting in the SMC differentiation.


