Reciprocal regulation controlling the expression of CPI-17, a specific inhibitor protein for the myosin light chain phosphatase in vascular smooth muscle cells

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Kim Ji, Urban M, Young GD, Eto M. Reciprocal regulation controlling the expression of CPI-17, a specific inhibitor protein for the myosin light chain phosphatase in vascular smooth muscle cells. Am J Physiol Cell Physiol 303: C58–C68, 2012. First published April 25, 2012; doi:10.1152/ajpcell.00118.2012.—Cellular activity of the myosin light chain phosphatase (MLCP) determines agonist-induced force development of smooth muscle (SM). CPI-17 is an endogenous inhibitor protein for MLCP, responsible for mediating G-protein signaling into SM contraction. Fluctuations in CPI-17 expression occur in response to pathological stresses, altering excitation-contraction coupling in SM. Here, we determined the signaling pathways regulating CPI-17 expression in rat aorta tissues and the cell culture using a pharmacological approach. CPI-17 transcription was suppressed in response to the proliferative stimulus with platelet-derived growth factor (PDGF) through the ERK1/2 pathway, whereas it was elevated in response to inflammatory, stress-induced and excitatory stimuli with transforming growth factor-β, IL-1β, TNFα, sorbitol, and serotonin. CPI-17 transcription was repressed by inhibition of JNK, p38, PKC, and Rho-kinase (ROCK). The mouse and human CPI-17 gene promoters were governed by the proximal GC-boxes at the 5′-flanking region, where Sp1/Sp3 transcription factors bound. Sp1 binding to the region was more prominent in intact aorta tissues, compared with the SM cell culture, where the CPI-17 gene is repressed. The 173-bp proximal promoter activity was negatively and positively regulated through PDGF-induced ERK1/2 and sorbitol-induced p38/JNK pathways, respectively. By contrast, PKC and ROCK inhibitors failed to repress the 173-bp promoter activity, suggesting distal enhancer elements. CPI-17 transcription was insensitive to knockdown of myocardin/Kruppel-like factor 4 small interfering RNA or histone deacetylase inhibition. The reciprocal regulation of Sp1/Sp3-driven CPI-17 expression through multiple kinases may be responsible for the adaptation of MLCP signal and SM tone to environmental changes.

smooth muscle contraction and differentiation; excitation-transcription coupling; protein phosphatase-1; inflammation

EXCITATION-CONTRACTION COUPLING in smooth muscle (SM) is governed through the signaling pathways determining reversible phosphorylation of the myosin regulatory light chains (MLC20). This coupling is altered through a subset of gene expression regulated in response to environmental cues, such as physiological stimuli and pathological stresses. The specific MLCP20 phosphatase (MLCP) is highly expressed in SM cells (18,19). G-protein activation suppresses MLCP activity, causing augmented MLC20 phosphorylation and force development, named Ca2⁺ sensitization (28). Conversely, MLCP activity is enhanced in response to cGMP elevation, causing Ca2⁺ desensitization (31,63). Cellular MLCP activity is regulated through the phosphorylation of myosin phosphatase targeting protein 1 regulatory subunit (MYPT1) (54) and a switch in specific splicing variants of MYPT1 confers the functional transition of MLCP during SM development (24). In addition, CPI-17, an endogenous inhibitor protein for MLCP (15), is predominantly expressed in SM, where it mediates agonist stimuli into MLCP inhibition. Several kinases, such as PKC, Rho-kinase (ROCK), integrin-linked kinase, zipper-interacting protein kinase, and p21-activated kinase, are identified to phosphorylate CPI-17 at Thr38, converting this protein into a potent inhibitor of MLCP (15). Conversely, CPI-17 is dephosphorylated and inactivated in response to nitric oxide production, leading to SM relaxation, playing a key role in determining cellular MLCP activity and modulating SM responsiveness (29).

CPI-17 expression varies among SM tissues. The expression is higher in arteries with the estimated concentration at 7 μM, which is above the level of MLCP (1–3 μM; Ref. 61). Less CPI-17 is expressed in phasic SMs, such as intestines and urinary detrusor SM, at < 1 μM (61). CPI-17 expression is regulated in response to environmental factors. During embryonic development, CPI-17 expression increases in arteries in parallel with a gain of the cardiac output and decreases in response to SM de-differentiation in the tissue cultures and neointimal cells (16, 27). Furthermore, lines of evidence suggest alterations in CPI-17 expression in response to pathological stresses. An increase in CPI-17 expression occurs in hypoxic pulmonary artery (9), hyperglycemic or obstructed urinary bladder (1,4), bronchus and airway under inflammation (38,47), and kidney cortex treated with testosterone (51). By contrast, the downregulation of CPI-17 expression occurs in inflamed ileum and colon SM, suggesting the change in CPI-17 expression depending on the SM tissue types (39,40). In addition to SM tissues, CPI-17 expression is low in embryonic heart, neurons, endothelium, and epithelium, but it is not detectable in mature skeletal and cardiac muscles (27,61). CPI-17 expression is elevated in epithelium-derived cancer cells, in which the proliferation is upregulated through the inhibition of MLCP (22,53). Thus it is evident that CPI-17 expression is tightly regulated through tissue-specific signaling pathways and determines cellular MLCP activity, whereas little is known about mechanisms controlling the expression.

Alterations in the transcription of SM-specific genes, including ion channels, cytoskeletal, and signaling proteins trigger a reversible transition between proliferative and contractile phenotypes of SM cells in response to environmental stimuli (42). The majority of SM-specific gene expression are driven through the binding of a ubiquitous transcription factor, serum-response factor (SRF), complexed with a SM-specific transcription cofactor myocardin or myocardin-related transcription factors, to the specific DNA motif CArG box, which is found at the 5′-flanking region of most SM-specific genes with...
The results suggest that myocardin-independent Sp1/Sp3 pathways and the regulatory circuit controlling SM tone are regulated through the expression of CPI-17 in SM (30, 64). Yet, non-myocardin pathways remain to be resolved. To understand the mechanisms by which MLCP activity and SM tone are regulated through the expression of CPI-17 in response to environmental changes, we determined the molecular basis of CPI-17 transcription in SM. The mouse and human CPI-17 gene promoters and the regulatory circuit controlling the activity in rat aorta tissue and cell culture were defined using quantitative qRT-PCR, chromatin immunoprecipitation (ChIP), EMSA, and luciferase-reporter gene assay. The results suggest that myocardin-independent Sp1/Sp3 pathways regulate the CPI-17 transcription through multiple kinases, such as ERK1/2, p38, JNK, PKC, and ROCK in response to environmental changes.

MATERIALS AND METHODS

Cell culture. Rat aorta smooth muscle cell (AoSMC) was prepared from inbred Sprague-Dawley rats (4- to 6-wk-old, male) using the explant method (13), following the protocol approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. AoSMC was cultured with the growth medium DMEM/Ham’s F-12 (DMEM/F12, Mediatech) supplemented with 10% FBS (Atlanta Biologicals) and penicillin-streptomycin (Mediatech). The endogenous CPI-17 was stably expressed in the cell culture until passage 15. AoSMC was also prepared using the enzymatic method with a mixture of collagenase and elastase (Worthington Biologicals; Ref. 62). The expression of CPI-17 was higher in the dispersed cells, and the expression declined during passage, as described previously (62). The response of the CPI-17 transcription was essentially indistinguishable between the two preparations (data not shown). The quiescent AoSMC was prepared by overnight conditioning with MCDB 131 medium (Mediatech) in the presence of 1% FBS. Drosophila S2 cells were gifted by Dr. James B. Jaynes in the institution and harvested in DMEM with 5% FBS.

Table 1. List of primers/probes for qRT-PCR and qChIP

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>qRT-PCR</td>
<td>CPI-17 CDS*</td>
</tr>
<tr>
<td>Tub-ß CDS</td>
<td>AGATGSGCATCAGCTTCGATGAA</td>
</tr>
<tr>
<td>Myocardin CDS</td>
<td>AGATGSGCATCAGCTTCGATGAA</td>
</tr>
<tr>
<td>KLFA4 CDS</td>
<td>CTTCATCTGGATGGAATAATG</td>
</tr>
<tr>
<td>sm22α CDS</td>
<td>AGGACGTGGAGATATGTGAA</td>
</tr>
<tr>
<td>sm-α-actin CDS</td>
<td>CTAGCAGAAAGGACCTCATTAGC</td>
</tr>
<tr>
<td>Rel-A CDS</td>
<td>CTATGCTGAATGTCGCCTT</td>
</tr>
<tr>
<td>ChIP</td>
<td>CPI-17 proximal</td>
</tr>
<tr>
<td></td>
<td>CPI-17 distal</td>
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Upper and lower limits of each data point were obtained using the method described by Yuan et al. (66).

Promoter assay. Cells were seeded in 24-well plate and transiently transfected using 2 μl of FuGeneHD (Roche) with 0.5 μg of the promoter DNA segment cloned in pGL4.20 reporter gene vector (Promega) plus 0.5 μg of CMV-SEAP, encoding secreted embryonic alkaline phosphatase (SEAP) cDNA driven by a CMV promoter (Addgene plasmid24595). After 48-h transfection with the growth medium, the cells were further incubated for 24 h under the desired conditions. The culture medium was transferred to a tube and subjected to SEAP assay. Remaining cells were rinsed once with PBS and then lysed for 5 min at room temperature with 75 μl of One-Glo lysis buffer (Promega). The cell lysate (50 μl) was transferred into 96-well black plate and mixed with 50 μl of One-Glo assay buffer (Promega). After a 5-min incubation, the luminescence was determined using the plate reader with luminometer module (Safire2, Tecan). In parallel, the culture medium was heated for 5 min at 65°C. An aliquot (2 μl) of the medium was incubated with 100 μl of SEAP reagent, including 50% PhosphaGLO, 0.1 M Na2CO3, 0.5 mM MgCl2, and 10 mM homogarginine, pH 10 in 96-well black plate and subjected to lumino-metry. Promoter activity was defined as change in relative fluorescence units (ΔRFU) (Luciferase assay)/ΔRFU (SEAP assay), where ΔRFU = RFU (sample) – RFU (background). Background value was obtained using the empty vector of pGL4.20 and pCMV.

EMSA. Nonradioactive EMSA was performed using LightShift chemiluminescence EMSA kit (Pierce) with biotinylated DNA duplexes as probes, following the manufacturer’s protocol. The 5’-biotinylated primers were used for PCR to amplify the desired portion. Biotinylated PCR products were purified using MinElute PCR purification kit (Qiagen). For the preparation of nuclear protein extracts, AoSMC or HEK293 cells were homogenized with the buffer including 0.32 M sucrose, 3 mM MgCl2, and 10 mM HEPES, pH 7.2, with 0.5 mM Tris-(2-carboxyethyl)phosphine (TCEP), 4 mM Pefabloc. 1 mM microcystin LR, and 0.3 mM sodium orthovanadate. Nuclei were collected by centrifugation for 10 min at 700 g and washed three times with the same buffer. The nuclear proteins were extracted for 30 min on ice with the buffer including 0.4 M NaCl, 3 mM MgCl2, 0.1% Tween20, 10% glycerol, 0.1 mM EGTA, and 10 mM HEPES buffer pH 7.2, supplemented with 0.5 mM TCEP, 4 mM Pefabloc, 1 mM Tween.
microcystin LR, and 0.3 mM sodium orthovanadate. The homogenates were spun for 15 min at 20,000 g, and the supernatant was used as the nuclear extract. The optimized conditions for EMSA were 10 μg of nuclear extracts mixed with 100 fmol of biotinylated DNA probe, 1 μg of poly(dextran-cinocin-dextran-cidin) acid, 40 μM ZnCl2, 0.2 mM EGTA, 100 mM NaCl, 0.1% Tween20, and 20 mM HEPES buffer, pH 7.2. After a 30-min incubation on ice, the reaction mixture was loaded to 0.5 × TBE-5% acrylamide gel (Bio-Rad), and the labeled DNA was visualized on nylon membrane using chemiluminescence method. For the competition and the supershift assay, nonlabeled DNA (2.5 pmol) and specific antibody (1 μg) were added to the mixture, respectively.

ChIP assay. ChIP assays were carried out using rat aorta tissues or AoSMC. Briefly, for rat aorta tissue, a cleaned fresh rat aorta (~15 mg) was immediately fixed for 1 h at room temperature with 10 ml of 1% formaldehyde in PBS, quenched for 5 min with 2% glycerol in PBS, and then pulsed under liquid nitrogen. The frozen tissue powder was homogenized with the lysis buffer [0.1 M NaCl, 50 mM MOPS-NaOH (pH 7.0), 1 mM EGTA, 0.1% Tween20, 5% glycerol, 2 mM MgCl2, 0.5 mM TCEP, and 4 mM Fetalbo mix. For AoSMC, the cells in a 10-cm dish were fixed with 1% formaldehyde, quenched with 2% glycerol, and homogenized with the lysis buffer using Dounce homogenizer. The crosslinked chromatin fraction was collected by centrifugation, washed twice using the lysis buffer, and treated for 20 min at room temperature with mung bean nuclease (New England Biolab). The homogenates were suspended using sonicator and the sheared chromatin was subjected to immunoprecipitation. An aliquot (100 μl) of the sheared chromatin was diluted with RIPA buffer, supplemented with 10 μg/ml salmon sperm DNA and 0.1 mg/ml BSA, and mixed with 4 μg of immunoprecipitation-grade antibodies as indicated in RESULTS. The mixture was rocked overnight at 4°C and mixed with protein A/G magnetic beads (Pierce) to capture the immunocomplex. After being extensively washed with the buffer with RIPA buffer supplemented with 0.25 M LiCl, the DNA fragments were released from the complex by incubation for 2 h at 65°C with proteinase K (New England Biolab). The DNA fragments were purified using QIA Quick PCR purification kit, and the eluant was subjected to PCR or qPCR analysis. For qPCR analysis, we used Taclman primer/probe (IDT) with the primers above. Preimmune IgG (Millipore) was used as blank, and the extent of DNA was normalized against the blank as fold enrichment. All PCR primers and Taclman probes used in the assay are listed in Table 1.

Others. Mouse and human genomic DNA fragments were prepared by PCR using the mouse genomic BAC clone (RP23-400K20) and human genomic DNA preparation (Clontech) as templates, respectively. Small interfering (si)RNA knockdown was performed using synthetic siRNA fragments for rat myocardin, and KLF4 and Rel-A were obtained from Siencer library of Ambion. AoSMC in 35-mm dish was incubated for 3 days with the mixture of 50 pmol of siRNA and 2 μl of Lipofectamine2000 (Invitrogen) and then subjected to qRT-PCR. Immunoblotting was done as described previously (25). Antibodies for Sp1 and Sp3 and Ac-histone H4 were obtained from Millipore. Anti-GAPDH, anti-ERK, anti-phospho-ERK, and recombinant Sp1 were from Novus, SantaCruz, Abcam, and Promega, respectively. Anti-CPI-17 was prepared previously (50). Statistical analyses of Student’s t-test and ANOVA were performed using Microsoft Excel, and P < 0.05 was considered as significant.

RESULTS

ERK1/2 mediates PDGF stimulus into the repression of the CPI-17 gene. Previous studies (27, 62) using immunoblotting and immunohistochemistry showed a decrease in CPI-17 protein in response to de-differentiation of SM cell culture and in neointimal cells. Figure 1A shows the simultaneous analysis of the levels of CPI-17 protein and mRNA in rat aorta SM tissue and the primary cell culture. The extent of the protein and mRNA was normalized against that of GAPDH as an internal control. The reduction in CPI-17 protein coincided with the level of mRNA, indicating the transcriptional regulation of CPI-17 expression (Fig. 1A). The CPI-17 mRNA level in rat aorta cell culture was reduced after a 24-h incubation under the growth condition (10% FBS), compared with the quiescent condition (1% FBS). PDGF-BB, a potent repressor of SM-specific genes, suppressed the CPI-17 transcription in parallel to a SM-marker gene, sm22α, but not a housekeeping gene, GAPDH (Fig. 1B). Because PDGF stimulation showed a tendency of GAPDH gene activation, we used β-tubulin as an internal control for the rest of assays. PDGF-BB is known to activate MAPK signaling, including ERK1/2, p38, and JNK. As shown in Fig. 1C, treatment of the quiescent AoSMC with U0126, a MEK inhibitor, did not affect the basal level of CPI-17 expression, whereas it cancelled PDGF-induced repression (Fig. 1C). Neither inhibitor of JNK [SP600125 (SP)] or SB203580 (SB)] eliminated the action of PDGF. Angiotsin-II (ANG II) is known to induce SM cell proliferation through MAPK pathways in parallel with the contraction.
CPI-17 transcription was not affected by ANG II stimulation but was enhanced in the presence of U0126 (Fig. 1C). These results suggest that PDGF-induced activation of ERK1/2 causes the repression of CPI-17 gene under growth condition, whereas ANG II stimulus induced both positive and negative signals in the gene regulation.

**JNK, p38, ROCK, and PKC are involved in CPI-17 transcription.** We tested whether inflammatory cytokines, stress, and excitatory stimulus activate CPI-17 transcription in AoSMC (Fig. 2). As shown in Fig. 2A, CPI-17 transcription in the quiescent AoSMC was elevated moderately by TGFβ stimulus and prominently in response to the inflammatory cytokines IL-1β and TNFα but not IL-6. Osmotic stress induced by the addition of sorbitol caused fourfold elevation of CPI-17 mRNA (Fig. 2B), reaching nearly 50% of that in aorta tissues. Sorbitol-induced increase in CPI-17 mRNA was eliminated in the presence of SP600125 and SB203580, whereas these inhibitors had minimal impacts on the basal transcription (Fig. 2B). These results suggest that sorbitol stress activates JNK/p38, inducing CPI-17 transcription. The inflammatory signals are also known to activate NF-κB, a transcription factor complex consisting of Rel-A and p50, in parallel to JNK and p38. However, the contribution of NF-κB in the CPI-17 expression was negligible, because the relative CPI-17 expression was unchanged in cells treated with siRNA of Rel-A, a subunit of NF-κB (1.3 ± 0.20 fold; P > 0.05 vs. control; n = 3).

Excitatory stimulation with serotonin (5-HT) also enhanced CPI-17 transcription by 1.7 fold (Fig. 2A). 5-HT receptors dominating in rat aorta SM cells (type-2A and 2B) are coupled with Gq/11 and trigger the activation of PKC and ROCK (55).

We further determined whether PKC and ROCK also activate CPI-17 transcription. The treatment with the pan PKC inhibitors calphostin C (Calph) and GF109203x (GFx) or with the ROCK inhibitors H1152 (H) and Y27632 (Y), significantly reduced CPI-17 transcription in AoSMC under the growth conditions (Fig. 2C), whereas the PKCα/β-specific inhibitor Go6976 (Go) failed to block the transcription. These results suggest that a subset of PKC isoforms and ROCK positively regulate CPI-17 expression.

**Proximal GC-rich motifs are necessary for the CPI-17 promoter.** The primary structures of 5'-untranslated region (5'-UTR) of mouse, rat, and human CPI-17 genes were analyzed using TRANSFAC, the database for transcription factor binding sites (Fig. 3 and see Fig. 6). There is no evidence of TATA-box or CArG box at the proximal region. CCAT sequence that obyes the initiation motif for TATA-less promoter was found within 50 bp from the initiation codon. A cluster of GC-rich motifs (GC box), which is often found in TATA-less promoter, exists within 100 bp at the proximal region, adjacent to a pair of GATA motifs.

Figure 3 shows luciferase-reporter gene activities driven by the 5'-flanking DNA segments of mouse (A and B) and human (C) CPI-17 genes. The sequential 5'-deletion from 3 kb to 510 bp did not alter the activity of mouse CPI-17 promoter (Fig. 3A). The further deletion from 510 to 235 bp, which eliminated a pair of GATA (open circle) and GC-box (closed circle), partially reduced the activity to 50%. The deletion of next pair of GATA motifs (GC box), which is often found in TATA-less promoter, exists within 100 bp at the proximal region, adjacent to a pair of GATA motifs.

Figure 3B shows effects of adenine substitutions at each GC box and GATA in the mouse 510-bp promoter activity. The mutation at one of three proximal GC boxes (GC-a, -b, and -c) or the proximal GATA adjacent to GC-d reduced the activity to almost basal levels, compared with the mutations at others, such as GC-d, -e, and -f, suggesting the dominant role of the

**Fig. 2.** Excitation-transcription coupling of CPI-17 mRNA in AoSMC. Extent of CPI-17 mRNA in AoSMC was determined by qRT-PCR using β-tubulin as reference. A: quiescent cells were stimulated overnight with TGFβ (5 ng/ml), IL-1β (20 ng/ml), IL-6 (10 ng/ml), TNFα (10 ng/ml), and sorbitol (0.2M) (n = 8–9) and subjected to qRT-PCR assay. B: unstimulated and sorbitol stimulated cells were treated overnight with SP600125 (SP; 10 μM) and SB203580 (SB; 10 μM) (n = 6). C: cells in the growth medium supplemented with 10% FBS were harvested with calphostin-C (Calp) 50 nM, GF109203x (GFx; 3 μM), Go6976 (Go; 1 μM), H1152 (H; 3 μM), and Y27632 (Y; 10 μM; n = 9). **P < 0.05 and #P < 0.05, compared with untreated and stimulated cell, respectively.
proximal GC boxes and a GATA motif in the CPI-17 promoter activity. Consistent with the mouse gene, a pair of the proximal GC boxes in human promoter region (Fig. 3C) was necessary for the maximum promoter activity, suggesting that the binding of transcription factors to the conserved GC-box cluster drives the CPI-17 transcription. In the human gene, the promoter activity was significantly enhanced when the 1-kb fragment was deleted from 5'-end, to 550, and 118 bp, suggesting repressor elements at 1 kb/550 bp and 364/118 bp.

There is no apparent GATA motif in the proximal human promoter, unlike the mouse gene (Fig. 3, B and C).

As shown in Fig. 4, the mouse 510-bp promoter was active in SM cells but not in non-SM cells, such as HEK293 and COS1 cells. Although the GC-rich motif ubiquitously functions in many gene expressions, neither the proximal GC-box cluster in mouse 173-bp nor human 118-bp CPI-17 gene was active in COS1 cells. These results suggest that the CPI-17 promoter is selectively activated in SM cells through the proximal GC-box cluster.

Sp1 transcription factor binds to the proximal GC boxes. The binding of specific transcription factors to the proximal GC boxes was examined by nonradioactive EMSA using biotinylated DNA probes illustrated in Fig. 5A, and the supershift assay with specific antibodies (Fig. 5). Addition of recombinant Sp1 protein or the nuclear extracts from AoSMC resulted in upward mobility shifts of the DNA fragment of the −169/−1 region (Fig. 5B), which was eliminated with unlabeled DNA competitor, indicating specific binding to the DNA

Fig. 3. Luciferase reporter gene assay for CPI-17 gene 5'-flanking region. pGL4.20 luciferase reporter gene vectors used in the assay are illustrated at left, pGL4.20 vector and CMV-secreted embryonic alkaline phosphatase (SEAP) were cotransfected for 72 h under the growth condition and subjected to luciferase and SEAP assay. Promoter activity was defined as luciferase activity/SEAP activity and normalized against mouse 510 bp (A and B) and human 1 kb (C). WT, wild type. *P < 0.05 against mouse 510 bp or human 1 kb (n = 6–12).

Fig. 4. Specific activity of the CPI-17 promoter in AoSMC. AoSMC, HEK293, and COS1 cells were transfected with a pair of vectors of pGL4.20 and CMV-SEAP. Mouse 510-bp construct (A and B) and 173-bp (B) and human 118-bp reporter constructs (B) were used for the assay.
Unlike 

probe. Neither shorter fragment, −97/−1 nor −169/−90, showed prominent binding to Sp1 (Fig. 5C). Recombinant Sp1 also failed to recognize the GC boxes between −257/−150 bp (Fig. 5C). Thus the intact DNA structure at −169/−1 region (Fig. 5A) is required for the strong binding of Sp1 to the proximal GC-box cluster. Addition of anti-Sp1 and anti-Sp3 antibodies to the AoSMC nuclear extracts mixed with the −169/−1 (Fig. 5D) or −97/−1 (Fig. 5E) fragment caused supershift of the dominant band at the top and the band below, respectively (Fig. 5, D and E), indicating the specific binding of Sp1 and Sp3 to the DNA probes containing the proximal GC boxes. Unlike 32P-labeled EMSA, the signals of the complex with antibodies (labeled with an asterisk in Fig. 5) indicated.}

analysis of the ChIP assay using real-time PCR showed 4.0-fold and 53-fold enrichment of the DNA segment with anti-Sp1 beads in the chromatin from AoSMC and aorta tissues, respectively (Fig. 6B). Ac-H4 binding to the CPI-17 promoter was also lower in AoSMC, compared with aorta tissue. These results suggest that Sp1 binding to the proximal GC-box cluster causes remodeling of the chromatin and activates CPI-17 transcription. We further determined the role of Sp1 in CPI-17 transcription using drosophila S2 cells, which lack endogenous Sp1 and Sp3 (Fig. 6C) (8). The mouse CPI-17 173-bp fragment was activated in S2 cells, as the extent of ectopic Sp1 cDNA increased (Fig. 6C). These results strongly suggest the positive regulation of the CPI-17 promoter through the proximal binding of Sp1. Sp1 is likely activated in the mature SM cells via posttranslational modifications and/or binding of cofactors, because Sp1 expression was unchanged between aorta tissues and AoSMC (data not shown).

Sp1-dependent elements of the CPI-17 promoter are controlled through MAPK pathways but not by PKC or ROCK. Figure 7 shows the promoter activity of the mouse 3-kr or 173-bp reporter gene constructs in AoSMC. Both the 3-kr and 173-bp segments were negatively and positively regulated in response to PDGF (A) and sorbitol (B), respectively. Inhibition of ERK1/2 activity with U0126 eliminated PDGF-induced repression of the 3-kr segment (Fig. 7A). Interestingly, U0126 treatment elevated the 3-kr promoter activity but not mRNA level (Fig. 1C). These results suggest that additional regulatory elements beyond the 3-kr region or potential differences between rat and mouse genes. Sorbitol-induced activation of the 173-bp CPI-17 promoter was eliminated by the inhibition of p38 or JNK (Fig. 7B). Thus the proliferative stimuli and stress signals are converged onto the proximal Sp1-binding region of the CPI-17 promoter, suggesting the involvement of Sp1 in the pathways. Y27632 significantly reduced the promoter activity of the 3-kr segment, but not the 173-bp of the proximal Sp1-binding region, suggesting that a ROCK-dependent enhancer element exists between −3 kb/−173 bp. By contrast, both 3-kr and 173-bp promoter activities were insensitive to calphostin-C (Fig. 7C), indicating that PKC and ROCK regulate the CPI-17 transcription through different response elements. Potentially, the PKC response elements present in the further distal region and/or the intron of the CPI-17 gene.

CPI-17 transcription was independent of KLF4/myocardin/ SRF-mediated SM gene regulation. We tested whether the Sp1/Sp3-dependent CPI-17 transcription is regulated through myocardin pathway and histone acetylation using siRNA knockdown and trichostatin-A (TSA), an inhibitor for histone deacetylases (HDACs). Figure 8A shows qRT-PCR analysis in AoSMC treated with siRNA for myocardin. The siRNA knockdown reduced the extent of the endogenous myocardin mRNA to 50%. This reduction in myocardin resulted in the concomitant reduction in sm22α mRNA but no effect on CPI-17 mRNA (Fig. 8A). PDGF stimulus is known to induce the expression of KLF4, the repressor of myocardin gene. Importantly, KLF4 is a member of Sp1 superfamily, recognizing GC box. The silencing of KLF4 also lower in AoSMC, compared with aorta tissue. These results suggest that Sp1 binding to the proximal GC-box cluster causes remodeling of the chromatin and activates CPI-17 transcription. We further determined the role of Sp1 in CPI-17 transcription using drosophila S2 cells, which lack endogenous Sp1 and Sp3 (Fig. 6C) (8). The mouse CPI-17 173-bp fragment was activated in S2 cells, as the extent of ectopic Sp1 cDNA increased (Fig. 6C). These results strongly suggest the positive regulation of the CPI-17 promoter through the proximal binding of Sp1. Sp1 is likely activated in the mature SM cells via posttranslational modifications and/or binding of cofactors, because Sp1 expression was unchanged between aorta tissues and AoSMC (data not shown).
at SM-gene promoters, and this myocardin-promoted histone acetylation can be mimicked by the inhibition of HDACs with TSA (46). The quantitative ChIP assay showed the prominent Ac-H4 binding in aorta tissues (Fig. 6B). Indeed, histone H4 acetylation was significantly higher in aorta tissues (1.0 ± 0.05; n = 6), compared with AoSMC (0.29 ± 0.02; n = 3; P < 0.05 vs. aorta tissue), in parallel with the CPI-17 expression (Fig. 1A). The expression of sm22α was elevated in AoSMC treated with TSA in parallel to the increase in histone H4 acetylation, whereas CPI-17 expression was unchanged in response to histone acetylation (Fig. 8C). These results suggest that histone H4 acetylation is not the factor determining the CPI-17 promoter activity, because the forced acetylation using the HDAC inhibitor (TSA) did not increase CPI-17 expression.

DISCUSSION

By comparison of rat aorta tissue and the cell culture using a pharmacological approach, we determined the mechanisms controlling CPI-17 transcription in response to stimuli. Agreeing with the previous reports in physiological and pathological studies using arterial tissues (9, 26, 27, 51), the CPI-17 gene promoter in the AoSMC system was positively and negatively regulated in response to proliferative, inflammatory, and excitatory stimuli that alter SM responsiveness. Figure 9 summarizes the proposed model for the reciprocal signaling pathways in CPI-17 transcription. The gene promoter activity is determined through Sp1/Sp3 binding to the GC boxes that cluster at the proximal site.

Proliferative stimulus with PDGF reduces CPI-17 transcription, in parallel with repression of sm22α, through the ERK1/2 pathway (Fig. 9). The transcription of CPI-17 in the AoSMC culture was partially reduced by inhibition of PKC and ROCK. Upon osmotic stress with sorbitol, the transcription was enhanced through JNK/p38 pathways. This stress-induced up-regulation of CPI-17 transcription likely mimics the signaling pathway induced by TGFβ and inflammatory cytokine stimuli. Therefore, PKC, ROCK JNK, and p38 positively regulate the CPI-17 gene promoter. MAPK signaling pathways, ERK1/2, p38, and JNK are converged onto the proximal Sp1/Sp3 binding region. Sp1/Sp3-induced CPI-17 gene transcription is augmented by the adjacent GATA elements. In the mouse CPI-17 promoter, a repressor exists between 273/173-bp region. The 510/235-bp region and GATA-b/c elements likely interfere with the repressor, because the deletion or mutation at these sites causes a reduction in the promoter activity. Furthermore, there are distal enhancer elements responding to PKC and ROCK. Interestingly, these responses to the specific kinases are similar to myocardin-regulated SM genes. For example, ERK1/2 negatively regulates myocardin/SRF signal through Elk-1, KLF4, and Ac-histone pathways (33, 35, 60), whereas ROCK stimulates myocardin and myocardin-related transcription factors, inducing the contractile phenotype (57). Thus CPI-17 expression is synchronized to the SM-specific genes, although the transcription factors regulating the gene promoter are different.

This Sp1/Sp3-induced CPI-17 gene regulation is evolutionally conserved among mouse, rat, and human with some differences in the gene architecture, such as location and number of the regulatory elements. It has been documented
that Sp1 is involved in other vascular smooth muscle (VSM) gene regulation, independent from myocardin pathway. ACLP and CRP2 are positively regulated through Sp1/Sp3 binding (30, 64), although the physiological roles of these gene products in the regulation of VSM tone have yet to be established. The gene promoter of cGMP-dependent kinase I (cGK-I) is also regulated through the proximal GC boxes. Sellak et al. (49) originally reported that the TATA-less promoter of cGK-I gene is activated by the proximal binding of Sp1, whereas KLF4 was later reported to bind to the GC boxes of the cGK-I gene promoter and activate the transcription (67). Moreover, Sp1 was reported to negatively regulate myocardin-driven sm22α gene promoter. For example, at atheroplaques, PDGF stimulation causes an increase in Sp1 expression, leading the repression of sm22α gene promoter (59), and activates KLF4 expression, causing repression of the myocardin gene (11).

CPI-17 expression is low within the neointima (27) as well as in AoSMC stimulated with PDGF without the downregulation of the Sp1 expression. Apparently, PDGF-induced ERK1/2 signal is capable of ceasing the action of Sp1 to the CPI-17 promoter. A question arose: how are Sp1/Sp3 activities in SM cells regulated by ERK1/2, p38 and JNK independently of the myocardin pathway? Importantly, Sp1 and Sp3 are ubiquitously expressed in eukaryotic cells (52), whereas CPI-17 expression is restricted to SM and some others. One possible mechanism is the regulation of Sp1 through the phosphorylation. For example, Sp1 phosphorylation at Thr453, an activation site, declined in AoSMC to 25%, compared with aorta tissue (data not shown). A growing body of evidence indicates that at least 24 Ser/Thr sites of Sp1 phosphorylation sites positively and negatively regulate Sp1 transcription activity, protein stability, and DNA binding (52). Posttranscriptional modification of Sp1 at Thr453 and other sites potentially facilitates CPI-17 expression in SM cells. Another possibility involves SM-specific microRNAs, such as miR143/145 and 223, which regulate SM genes (2, 7, 10, 14). Of particular interest, miR143/145 gene ablation causes the repression of CPI-17 and ROCK1 in SM cells (2). Although CPI-17 mRNA does not include miR143/145 recognition sites, it is possible that SM-specific microRNAs play roles in Sp1/Sp3-induced CPI-17 expression in SM cells. In addition, this study does not rule out the possibility that Sp1 activity is driven by an unidentified SM-specific cofactor, like ubiquitous SRF controlled by SM-specific myocardin. GATA4 and GATA6 are highly expressed in SM cells (56, 65), and may play a role in SM-specific expression of CPI-17 through the proximal GATA motifs in the CPI-17 promoter. The SM-specific activation of CPI-17 promoter deserves further investigation.

Upon G-protein activation, PKC and ROCK are sequentially activated and phosphorylate CPI-17 protein at Thr38. This phosphorylation of CPI-17 and the consequential inhibition of MLCP are necessary for the robust and sustained MLC20 phosphorylation (12). Our finding here suggests that this agonist-induced activation of PKC and ROCK augments CPI-17 signal through increases in the gene expression and the phosphorylation at Thr38. During the embryonic development, CPI-17 expression in arterial SM elevates between E10 and E17, in parallel with an increase in cardiac output (27). Likewise, CPI-17 expression is repressed in embryonic SMs, such as bronchi and intestines, but is enhanced in active SM tissues. Thus it is possible that exposure of immature SM cells to excitatory stimuli triggers CPI-17 expression and establishes the Ca²⁺ sensitization signaling pathway during SM development. This agonist-induced CPI-17 expression reminds us excitation-transcription coupling of myocardin-dependent gene activation in response to depolarization of VSM cells through Ca²⁺, cAMP response element binding protein, and ROCK pathways (3, 57, 58). Recently, Pagiatakis et al. (44) reported that this ROCK-induced myocardin activation is mediated by
CPI-17, although the mechanisms remains unknown. We pre-
sume that Sp1/Sp3-induced CPI-17 expression is a part of 
process in SM cell maturation, adjusting the contractile re-
sponse and the gene expression to stimuli.

The reciprocal regulation through multiple kinase pathways 
determines the response of the CPI-17 promoter to specific 
stimuli. For example, TGFβ/H9252 and TNFα/H9251 are known to activate 
ERK1/2, JNK, and p38 in VSM cells, simultaneously (41, 48). 
In rat AoSMC we used, both agonists increased the CPI-17 
expression, although the extent of the elevation was unique to 
the stimuli. We presume that the balance of reciprocal activi-
Eties of the kinases regulating the CPI-17 promoter (Fig. 1 
C) determines the specific response to each stimulus. The dual 
regulation was also evident in ANG II stimulus. ANG II is 
known to activate PKC/ROCK through G-protein activation 
and JNK/p38 through oxidation pathways in parallel with the 
cross-activation of PDGF-receptor signaling pathway (36). 
Inhibition of negative signal through ERK1/2 enhanced CPI-17 
transcription driven by PKC/ROCK/p38/JNK. Thus, upon 
ANG II stimulus, the positive signal is offset by the negative 
one. A particular side of response may be emphasized when the 
balance is broken in response to pathological stresses.

An increase in CPI-17 expression has been reported in 
hypoxic pulmonary artery (9), diabetic bladder (4), urinary 
obstruction (1), airway inflammation (38, 47), ischemic dam-
age in bladder (23), and subarachnoid hemorrhage (26), which 
have been linked to hyper-responsiveness of SM contraction, 
such as asthma attack and vasospasm, in addition to remodeling. 
Neither the hypoxia-inducible factor-1-response element 
or the NFκB binding element is evident in the mouse CPI-17 
promoter region. Also, NFκB inhibition by Rel-A knockdown 
did not alter the activity. We presume that these increases in 
CPI-17 expression occur through the activation of JNK/p38 
and/or PKC/ROCK pathways. For example, p38 is activated 
under hypoxia, causing the cyclin D1 activity in PC12 cells (6).

In addition, CPI-17 upregulation occurs in pregnant myome-
trium (43) and testosterone-treated renal cortex (51), suggest-
ing an involvement of steroid hormone signaling that activates 
multiple MAPK pathways. By contrast, in intestinal SM, IL-1β 
stimulus induces the downregulation of CPI-17 expression, 
which is linked to a loss of SM tone in inflammatory bowel 
disease (40). Also, CPI-17 is downregulated in rabbit bladder 
in response to urinary obstruction (5) and aged bladder detrusor 
SM (17). Consistently, IL-1β stimulation suppresses CPI-17 
expression in colon SM cell culture (20). The CPI-17 down-

Fig. 8. Kruppel-like factor 4 (KLF4)/myocardin-independent 
extension of CPI-17. qRT-PCR was performed using 
AoSMC treated with small interfering RNA for myocardin 
(A) and KLF4 (B; n = 4). C, value of β-tubulin was used as 
reference. Ctl indicates cells treated with control small inter-
fering RNA. Myocd and sm-A indicate primer sets for myo-
cardin and sm-α-actin, respectively. Quiescent AoSMC was 
treated overnight with 0.2 μM trichostatin-A (TSA) and sub-
jected to immunoblotting (C) and qRT-PCR (D; n = 6). 
***P < 0.05 compared with control.

Fig. 9. A proposed model for the regulation of CPI-17 transcription. Stress-
induced p38/JNK activation enhances Sp1/Sp3 binding to the proximal GC 
boxes that triggers CPI-17 transcription, whereas PDGF-induced ERK1/2 
activation represses the signal. There is a repressor element (R) in mouse and 
human CPI-17 promoters. PKC and ROCK, downstream kinases in G-protein 
signaling pathways, positively regulate the CPI-17 promoter through distal 
enhancer elements (E).
regulation also occurs in IL-10-knockout mice and human patients who suffer from chronic colitis (39). In our study, IL-1β stimulation rather increased CPI-17 expression in AoSMC. We presume that negative signal through ERK1/2 dominates in intestinal SM stimulated with IL-1β, causing CPI-17 downregulation and reduced SM tone. Recently, Ihara et al. (21) reported that CPI-17 expression is unchanged in another colitis model mouse, induced by a 7-day treatment with dextran sodium sulfate, which causes hypercontractility of the circular SM tissue. Likely, the reciprocal regulation of the CPI-17 promoter confers the plasticity in the expression depending on the disease stages. Understanding of the regulatory circuits in CPI-17 transcription through multiple kinase pathways is a key to elucidate an adaptation system of SM tone to pathological challenges.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.I.K. and M.E. performed experiments; J.I.K., M.U., and G.D.Y., and M.E. edited and revised manuscript; J.I.K., M.U., and J.I.K., M.U., G.D.Y., and M.E. edited and revised manuscript; J.I.K., M.U., and M.E. approved final version of manuscript.

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