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Bone Morphogenetic Protein-Induced Msx1 and Msx2 Inhibit Myocardin-Dependent Smooth Muscle Gene Transcription▽

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During the onset and progression of atherosclerosis, the vascular smooth muscle cell (VSMC) phenotype changes from differentiated to dedifferentiated, and in some cases, this change is accompanied by osteogenic transition, resulting in vascular calcification. One characteristic of dedifferentiated VSMCs is the down-regulation of smooth muscle cell (SMC) marker gene expression. Bone morphogenetic proteins (BMPs), which are involved in the induction of osteogenic gene expression, are detected in calcified vasculature. In this study, we found that the BMP2-, BMP4-, and BMP6-induced expression of Msx transcription factors (Msx1 and Msx2) preceded the down-regulation of SMC marker expression in cultured differentiated VSMCs. Either Msx1 or Msx2 markedly reduced the myocardin-dependent promoter activities of SMC marker genes (SM22α and caldesmon). We further investigated interactions between Msx1 and myocardin/serum response factor (SRF)/CArG-box motif (*cis* element for SRF) using coimmunoprecipitation, gel-shift, and chromatin immunoprecipitation assays. Our results showed that Msx1 or Msx2 formed a ternary complex with SRF and myocardin and inhibited the binding of SRF or SRF/myocardin to the CArG-box motif, resulting in inhibition of their transcription.

The phenotypic modulation of vascular smooth muscle cells (VSMCs) from differentiated to dedifferentiated is a critical feature of the onset and progression of the vascular remodeling under conditions such as atherosclerosis, vascular stenosis, and hypertension. During this process, the expression of smooth muscle cell (SMC) markers, such as smooth muscle myosin heavy chain, SM22α, caldesmon (CaD), and calponin, is markedly down-regulated (37, 46). Accumulating evidence suggests that the VSMC-specific expression of these genes is regulated by the coordination of serum response factor (SRF), its associated homeobox protein (Nkx 3.2) and GATA (GATA6) transcription factors (35), or by cysteine-rich LIM-only proteins (CRP1 and CRP2) (9). Recently, the SMC/cardiac myocyte-restricted SRF activator, myocardin (Mycd) was identified as a central regulator of SMC marker expression occurring via the SRF-dependent mechanism (10, 50). In fact, the ectopic expression of Mycd can activate the SMC differentiation program in multilineaged mesenchymal 10T1/2 cells (51). Changes in the expression levels of SRF and Mycd in differentiated and dedifferentiated VSMCs are, however, less significant, indicating that the down-regulation of SMC marker expression during VSMC dedifferentiation cannot be simply explained by the expression levels of these positive transcription factors.

It has been speculated that repressor proteins that interact with SRF and/or signaling pathways may negatively regulate the Mycd/SRF/CArG-mediated transcription. Elk1 (52), Kruppel-like transcription factor 4 (KLF4) (30), HES-related repressor protein 1 (13), and Foxo4 (31) are candidates for such regulators. We previously reported that the IGF-I-stimulated phosphoinositide 3-kinase (PI3-K)/protein kinase B [PKB(Akt)] pathway plays a critical role in maintaining the differentiated phenotype of VSMCs, whereas the coordinated activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase triggered by platelet-derived growth factor-BB, epidermal growth factor family members, basic fibroblast growth factor, or unsaturated lysophosphatidic acids induces the dedifferentiation of VSMCs (17, 18). Based on these findings, we hypothesized that changes in the balance between the strengths of the PI3-K/PKB(Akt) pathway and the two mitogen-activated protein kinase pathways determine the VSMC phenotype. In support of this hypothesis, Liu et al. recently identified one of the downstream targets of the PI3-K/PKB(Akt) pathway in VSMCs as a forkhead transcription factor, Foxo4. Foxo4 interacts with Mycd and represses its transactivation of SMC marker gene transcription, and this inhibition is released by the phosphorylation of Foxo4 by PKB(Akt), followed by its nuclear export (31). The molecular mechanism of the down-regulation of SMC markers at the transcriptional level, however, remains unclear.

Calcification within the vessel wall is one of the progressive features of atherosclerosis (20, 32, 45). Bone morphogenetic protein 2 (BMP2), BMP4, and BMP6 are detected in atherosclerotic lesions that are accompanied by calcification (5, 12, 43). BMP2, in particular, has been shown to up-regulate the osteogenic gene expression in passaged VSMCs (11). These properties of BMP2 are closely associated with the induction of Msx1 and Msx2, transcription factors involved in osteogenic gene expression (11). It is, however, unclear whether the BMPs released from atherosclerotic lesions act solely to induce the

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osteogenic transition from dedifferentiated VSMCs or whether they also act as an autocrine/paracrine factor for the progression of the dedifferentiation of the surrounding intact VSMCs. In this study, we found that BMPs strikingly induce phenotypic modulation of VSMCs, and we further uncovered the molecular mechanism of the BMP-induced down-regulation of SMC marker expression at the transcriptional level. Our results showed that the BMP-induced Msx transcription factors, Msx1 and Msx2, formed a complex with SRF and Mycd and inhibited the binding of SRF or SRF/Mycd to the CArG-box motif located in the promoters of SMC marker genes, resulting in the inhibition of their transcription. This is the first report demonstrating that Msx transcription factors directly interact with both SRF and Mycd and are involved in the negative regulation of SMC gene transcription.

MATERIALS AND METHODS

Reagents and antibodies. Commercially available primary antibodies were as follows: anti-Flag (M2 and F7425) and anti-α-tubulin (DM 1A) antibodies (Sigma); anti-hemagglutinin (anti-HA, 3F10) antibody (Roche Applied Science); anti-Msx1, anti-Msx2, anti-SRF, anti-Mycd, and anti-Myc (9E10) antibodies (Santa Cruz Biotechnology); anti-SM22α antibody (Novocastra Laboratories). Secondary antibodies were conjugated to Alexa 488, Alexa 568, and Alexa 350 (Molecular Probes). The anti-CaD antibody was prepared in our laboratory (16).

Plasmids. The chicken CaD promoter region was isolated from GP2CAT (54) and inserted into the pGL3-Basic vector (Promega) (CaD GP2-Luc). A mutation in the CArG-box motif of the CaD promoter (CaD GP2-Luc CArGmut) was introduced as described elsewhere (54). The promoter region of chicken SM22α (29) was isolated from a chicken genomic library, and the BalI-NdeI fragment was inserted into pGL3 Basic (SM22P-Luc). Mutations in the proximal CArG (pCArG)-box motif, the distal CArG (dCArG)-box motif, or both of them (dpCArGmut) in the SM22α promoter were introduced as follows: pCArG mutation, from CCAAATATGG to CCAAGTATAC; dCArG mutation, from CCTATAAAAGG to CAGATAAAAGT. We used pGL3 control (Promega) as a control luciferase reporter gene carrying the simian virus 40 promoter. The cDNAs of mouse full-length Mycd (GenBank accession number AF384055), Msx1, and Msx2 were amplified by reverse transcriptase-PCR (RT-PCR) and were inserted into the mammalian expression plasmid, pCS2+, with the indicated tags. Deletion mutants of Mycd and Msx1 were constructed by PCR-mediated mutagenesis. Expression plasmids for human SRF and its derivative with point mutations, SRF Pm143-146, were described previously (34, 39). Expression plasmids for the SRF derivatives, SRF PRGI-In206, SRFΔMADS (133 to 266), and MADS (133 to 266), were constructed using PCR-mediated methods. The sequences of these constructs were confirmed.

Cell cultures and transfection. Culture conditions for rat VSMCs were described previously (17, 18). 10T1/2 and Cos7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) for Cos7 cells or Trans IT-LT1 for 10T1/2 cells (Pan Vera Corporation) under 10% fetal calf serum-stimulated conditions, according to the manufacturer's instructions. For analyses of protein-protein interactions in Cos7 cells, the cells were shifted to DMEM with 2% horse serum (HS) 4 h after transfection and cultured for another 20 h. For analyses of promoters, expression of endogenous SMC markers, and chromatin immunoprecipitation (ChIP), 10T1/2 cells were shifted to DMEM–2% HS 4 h after transfection and cultured for 44 h.

Promoter assays. The cell extracts were prepared by passive lysis buffer (Promega) according to the manufacturer's instructions, and then assayed for luciferase activity using the luciferase assay kit (Promega). The relative promoter activity was expressed in luminescence units normalized to the β-galactosidase activity of pSVβ-gal (Promega) in the cell extracts. These assays were performed in triplicate and were repeated at least three times.

Expression of Msx transcription factors and SMC markers. The expression of Msx1, Msx2, and SMC markers, *h*- and *l*-CaDs, SM22α, calponin, and MHC SM2 at the mRNA level was quantified by RT-PCR normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, as described previously (18, 47). In brief, we first estimated the GAPDH mRNA levels in each sample by RT-PCR using differentially diluted single-stranded cDNA mixtures (1×, 0.5×, 0.25×, 0.1×) with different PCR cycles. PCR products were sampled at intervals of two cycles between 18 to 26 cycles and were separated on 1.2% agarose gels. The intensities of the GAPDH cDNA bands stained with SYBR green I (FMC Bioproducts) were determined using a Fluor Imager (Amersham Pharmacia Biotech) at cycle numbers where the intensities were linearly increased. Next, we amplified the target cDNAs using defined amounts of single-stranded cDNA mixtures containing equal amounts of GAPDH cDNA. PCR products of each target cDNA were sampled at intervals of two cycles between 22 to 38 cycles and were quantified as described above. The specific primers for rat Msx1 and Msx2 were as follows: Msx1 sense primer, ATGACTTCTTTGCC ACTCGGTG; Msx1 antisense primer, CTATGTCAGGTGGTACATGCTG; Msx2 sense primer, ATGAGCCCCACCACCTGCCACCC; Msx2 antisense primer, TTAGGATAGATGGTACATGCCATATC. The specific primer sets for the rat SMC markers, rat GAPDH (18, 47), mouse SMC markers (SM22α and MHC-SM2), and mouse GAPDH (7) were described elsewhere. The specific primer sets for mouse calponin and CaD were as follows: calponin sense primer, ATGTCTTCTGCACATTTTAACC; calponin antisense primer, GCTCAAATC TCCGCTCTTG; CaD sense primer, ATGCTTAGCGGATCCGGGTC; CaD antisense primer, GGGCCTGAGAGACTGCCATC. The expression of Msx1, Msx2, *h*- and *l*-CaD, and SM22α at the protein levels was analyzed by immunoblots normalized to the expression of α-tubulin.

Quantitative real-time PCR. The expressions of Msx1, Msx2, SM22α, and *h*- and *l*-CaD in VSMCs cultured under indicated conditions were quantified by 7500 real-time PCR System (Applied Biosystems). Total RNAs were reverse transcribed by using TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer's protocol. Real-time PCR was performed using TaqMan gene expression assays (Applied Biosystems), and the expression levels of respective mRNAs were normalized to the GAPDH mRNA.

Immunocytochemistry. Cells were fixed with 4% formaldehyde for 30 min, permeabilized, and blocked with 0.1% Triton X-100 and 0.2% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. The cells were then incubated with the indicated primary antibodies for 1 h followed by the indicated secondary antibodies with or without Hoechst 33258 for 1 h at room temperature. Fluorescence images were collected using a cooled charge-coupled device camera (Roper Scientific, Tucson, AZ) mounted on an Olympus IX-70 microscope with the appropriate filters and MetaMorph software.

Protein-protein interaction analyses. Whole-cell extracts were prepared from Cos7 cells transfected with the indicated expression plasmids according to methods described elsewhere (49) with some modifications. In brief, cells were incubated for 30 min at 0°C in lysis buffer, and then the salt concentration was decreased to 100 mM KCl and 50 mM NaCl, and cells were further incubated for 30 min. Equal amounts (400 μg of protein) of the cell extracts thus obtained were first treated with control immunoglobulin G (IgG)-bound protein A- or protein G-Sepharose beads for clearance of nonspecific interactions and then incubated with the indicated antibodies for 6 h at 4°C. The immune complexes were collected by incubating with protein A- or protein G-Sepharose beads for 3 h at 4°C. Proteins in the immunoprecipitates were detected by immunoblotting using the indicated antibodies. In vitro translation was performed using the TNT quick coupled transcription/translation systems (Promega) according to the manufacturer's instructions. The indicated in vitro-translated proteins were incubated in the same lysis buffer described above containing 100 mM KCl and 50 mM NaCl for 1 h at 0°C, and the interactions were analyzed by immunoprecipitation followed by immunoblotting using the indicated antibodies according to the procedures described above. Target proteins were detected with a SuperSignal chemiluminescence detection kit (Pierce).

Gel shift assay. A probe containing the proximal CArG-box motif sequence of the chicken SM22α promoter was prepared by annealing the respective sense and antisense synthesized oligonucleotides to form duplex DNA. The sequence of the sense strand of this probe was TTTTCGGAGTCTTTCCCCAAATATG GTGTCTGGGCTGGAA. The probe was labeled by T4 polynucleotide kinase with [^{32}P]ATP. The gel shift assay was carried out using the indicated in vitro-translated proteins as described previously (6).

ChIP assay. ChIP assays were carried out using the ChIP assay kit (Upstate Biotech) according to the manufacturer's protocol with some modifications. DNAs isolated from input chromatin fragments and those from precipitated chromatin fragments by anti-SRF antibody or control IgG were subjected to PCR using primers flanking the proximal CArG-box motif from rat/mouse SM22α promoter and the CArG-box motif from rat/mouse CaD promoter. These primer sequences, which are conserved between rat and mouse SM22α and CaD promoter regions, are as follows: SM22α sense primer, CTGCCCAT AAAAGGTTTTTCCC; SM22α antisense primer, GCCCATGGAAGTCTGCT TGG; CaD sense primer, GCTCTATTTGTGTTCTACAAGAC; CaD antisense primer, GCAGGCTGCCAAAACCAGC.

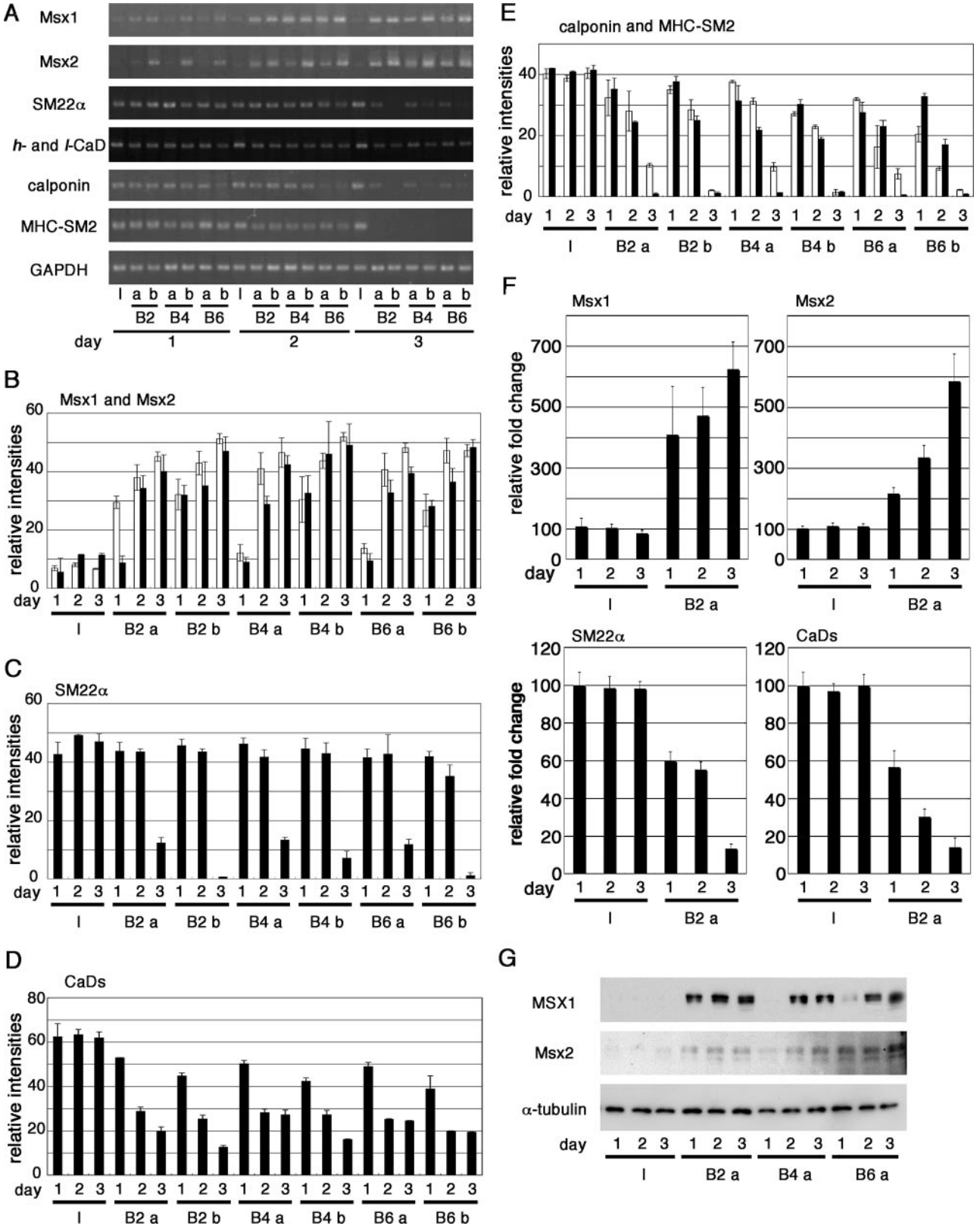

FIG. 1. BMP-induced expression of Msx transcription factors and down-regulation of SMC marker expression in VSMCs. Differentiated VSMCs were stimulated with 10 ng/ml (a) or 100 ng/ml (b) of the indicated BMP family members (B2, BMP2; B4, BMP4; and B6, BMP6) or 2 ng/ml IGF-I (I) for 1 to 3 days. (A) RT-PCR analysis of the expression of Msx1, Msx2, and the indicated SMC marker mRNAs. RT-PCR was performed using RNAs isolated from the indicated VSMC cultures, as described in Materials and Methods. Cycle numbers of the PCRs and the sizes of PCR products are as follows: Msx1, 32, 446 bp; Msx2, 32, 420 bp; SM22α, 26, 461 bp; *h*- and *l*-CaD, 34, 537 bp; calponin, 32, 459 bp;

RESULTS

BMP-induced expression of Msx1 and Msx2 precedes the down-regulation of SMC markers. BMP2, BMP4, and BMP6 are detected in atherosclerotic lesions that are accompanied by calcification (21). We previously developed a novel VSMC culture system in which IGF-I and laminin are critically involved in maintaining the differentiated phenotype of VSMCs, as monitored by cell morphology, ligand-induced contractility, and the expression of SMC markers (17, 18). Here, we examined the effect of these BMPs on the expression of Msx transcription factors (Msx1 and Msx2) and SMC markers using our VSMC culture system (Fig. 1). BMP2, BMP4, and BMP6 induced a strong expression of Msx1 and a moderate one of Msx2 at the mRNA (Fig. 1A, B, and F) and protein (Fig. 1G) levels. Both the Msx1 and Msx2 proteins were expressed within the first day after BMP2 or BMP6 treatment (Fig. 1G), and BMP2 was the most potent for inducing the Msx transcription factors among the BMP family members examined. The down-regulation of the mRNA expression of SMC markers SM22α, *h*- and *l*-CaD, calponin, and MHC-SM2, was apparent 2 to 3 days after BMP treatment. Similar expression profiles of Msx1, Msx2, and SMC markers (SM22α and *h*- and *l*-CaD) in VSMCs treated with IGF-I or BMP2 were confirmed by real-time PCR analysis (Fig. 1F). These results indicate that the expression of Msx1 and Msx2 is stimulated by BMPs and precedes the down-regulation of SMC marker expression.

Msx1 and Msx2 inhibit the Mycd-dependent transcription of SMC marker genes. We further examined the effects of Msx1 and Msx2 on the Mycd-dependent transactivation of the SM22α and CaD promoters. The SM22α and CaD promoters carry two CArG-box motifs (29) and a single CArG-box motif (54), respectively. We constructed reporter genes containing wild-type and mutant promoters of SM22α and CaD and analyzed the Mycd-dependent transactivation of these promoters in 10T1/2 cells. In the mutant SM22α and CaD reporter genes, either or both of the two CArG-box motifs of the SM22α promoter and the sole CArG-box motif of the CaD promoter were mutated. We confirmed that the pCArG box motif of the SM22α promoter was essential for the Mycd-dependent transactivation (Fig. 2A, lanes 1, 2, 5, and 6), but the distal one (dCArG) had only a moderate role (Fig. 2A, lanes 1 to 4). The single CArG-box motif of the CaD promoter was critical for the Mycd-dependent transactivation (Fig. 2B, lanes 1 to 4).

As shown in Fig. 2C and D, exogenous Mycd markedly activated the promoters of SM22α (13-fold activation) and CaD (10-fold activation) (lanes 1 and 2), whereas either Msx1 or Msx2 suppressed these activations in a dose-dependent manner (lanes 3 to 6). The inhibitory effect of Msx1 or Msx2 was more potent for the CaD promoter than for the SM22α promoter. We confirmed the expression of Mycd and its nuclear localization and the similar expression levels of Msx1 and Msx2 and their nuclear localization in 10T1/2 cells using antibodies against their respective tags (data not shown). Although the coexpression of Mycd and SRF activated both promoters, the activation rates were lower than those obtained with Mycd alone (lanes 2 and 7). This may be due to the occupation of the Mycd or the CArG-box motifs in the promoters by excess SRF. In these cases, suppression of the Mycd-dependent promoter activities by Msx1 and Msx2 was low (lanes 8 to 11). Exogenous SRF alone slightly activated the promoters of SM22α (3.5-fold activation) and CaD (2-fold activation), whereas the suppressive effect of Msx1 or Msx2 on the SRF-dependent activation of both promoters was less significant than their suppression of the Mycd-dependent activation (data not shown). The basal promoter activities of SM22α and CaD were slightly suppressed by Msx1 or Msx2 (lanes 1 and lanes 12 to 15). In a control, exogenous Mycd and/or Msx1 or Msx2 had no effect on the simian virus 40 promoter activity (Fig. 2E). These data indicate that Msx transcription factors inhibit the Mycd-dependent transcription of SMC marker genes.

Msx-dependent regulation of the Mycd-induced expression of endogenous SMC markers. Mycd-transfected 10T1/2 cells adopt an SMC lineage, as revealed by the endogenous expression of SMC markers (51). We examined the effects of Msx1 and Msx2 on the Mycd-dependent expression of endogenous SMC markers in 10T1/2 cells. In these cells, Mycd induced the expression of endogenous SMC markers, such as SM22α, calponin, and MHC-SM2, at the mRNA level (Fig. 3A, lane 2), and Msx1 potently inhibited their expressions (lane 3). Compared with Msx1, Msx2 moderately interfered with the Mycd activity (lane 4). In a control, Msx1 or Msx2 alone had no effect on the SMC marker expression in 10T1/2 cells (lanes 5 and 6). The inhibitory effects of Msx1 and Msx2 on the Mycd-induced expression of endogenous SMC markers were also confirmed at the protein level. SM22α protein was expressed in 10T1/2 cells transfected with Mycd, and its expression was reduced by the coexpression of Msx1 or Msx2 (Fig. 3B and C). The SMC-specific CaD isoform, *h*-CaD, which has a molecular mass of 140 to 150 kDa, was not detected in the Mycd-transfected 10T1/2 cells (Fig. 3B). The effect of Mycd with or without Msx1 or Msx2 on the expression of CaD was moderate at the mRNA and protein levels (Fig. 3A and B). Immunocytochemical analysis revealed that 10T1/2 cells transfected with Mycd expressed increased CaD protein, and the coexpression of Mycd with Msx1 or Msx2 suppressed CaD's expression to the basal level (Fig. 3D). We also confirmed by immunocytochemistry that

MHC-SM2, 34, 498 bp; and GAPDH, 24, 575 bp. The mRNAs of the caldesmon isoforms, *h*- and *l*-CaD, are transcribed from the same promoter and are generated by SMC phenotype-dependent alternative splicing; *h*-CaD is specifically distributed in differentiated SMCs, whereas *l*-CaD is in dedifferentiated SMCs and nonmuscle cells. Expression levels of Msx transcription factor and SMC marker mRNAs were normalized to GAPDH mRNA and were quantified (B, C, D, and E). (B) Open and closed bars indicate Msx1 and Msx2 mRNAs, respectively. (E) Open bars, calponin mRNA; closed bars, MHC-SM2 mRNA. (F) The expression profiles of Msx1, Msx2, SM22a, and *h*- and *l*-CaD were confirmed by real-time PCR. Relative abundance of transcripts was presented based on those in VSMCs cultured under IGF-I-stimulated conditions for 1 day, which were set as 100. Each value represents the mean ± standard deviations of results from three independent experiments. (G) Whole-cell lysates from the indicated VSMC cultures were probed with anti-Msx1 and anti-Msx2 antibodies by immunoblot analysis. These results are taken from one representative experiment (of at least three).

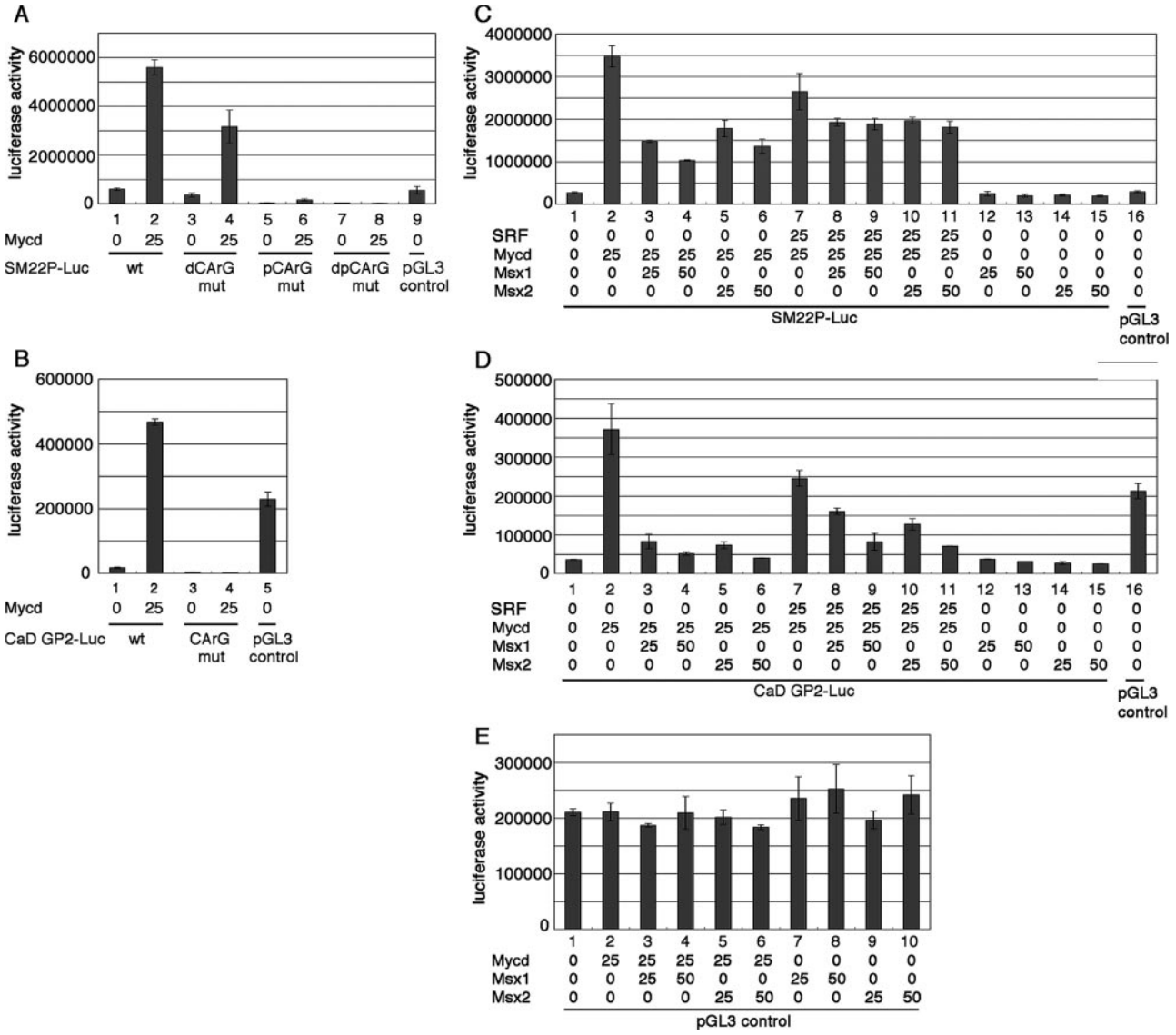

FIG. 2. Effects of Msx1 or Msx2 on the Mycd-dependent activation of the SM22α and CaD promoters. SM22P-Luc (wt, dCArGmut, pCArGmut, or dpCArGmut) (400 ng) (A and C), CaD GP2-Luc (wt or CArGmut) (400 ng) (B and D), pGL3 control (E) (400 ng), pSVβ-gal (200 ng), and empty plasmid were cotransfected into 10T1/2 cells with or without the indicated expression plasmids (25 or 50 ng) (total, 1.0 μg plasmids/well of a 12-well culture plate). The culture conditions of the 10T1/2 cells and the assay procedures are described in Materials and Methods. Relative luciferase activities normalized to the β-galactosidase activity are shown. Each value represents the mean ± standard deviation of results from three independent experiments.

the Mycd-induced expression of endogenous calponin and anti-SM actin proteins was suppressed by Msx1 or Msx2 (data not shown). These results indicate that Msx transcription factors specifically suppress the Mycd-dependent expression of endogenous SMC markers.

Msx transcription factors form a ternary complex with SRF and Mycd. As demonstrated in Fig. 2 and 3, Msx1 and Msx2 potently suppressed the Mycd-induced expression of SMC markers. These results might have arisen from a competition between Mycd/SRF/CArG and the Msx transcription factors. To test this possibility, we examined the physical interactions between Msx1, SRF, and Mycd by a combination of coimmunoprecipitation and immunoblotting using the whole-cell extracts from Cos7 cells coexpressing either HA-tagged Msx1 (HA-Msx1) and Flag-tagged SRF (SRF-Flag) or HA-Msx1 and Flag-tagged Mycd (Flag-Mycd). When tagged SRF, Mycd, or Msx1 was expressed in Cos7 cells, each was localized to the nucleus (Fig. 4A). Immunoblots of the anti-Flag immunoprecipitates showed that Msx1 interacted with SRF (Fig. 4B, lane 1) or Mycd (lane 2), whereas such interactions were not detected in immunoprecipitates with control IgG (Fig. 4B, lanes 3 and 4). The interaction between Msx1 and SRF or Msx1 and Mycd was also confirmed on immunoblots of the anti-HA immunoprecipitates probed with anti-Flag antibody (data not shown).

The interaction between Msx1 and Mycd was stronger than that between Msx1 and SRF (Fig. 4B). To address whether differences in expression efficiencies between SRF and Mycd

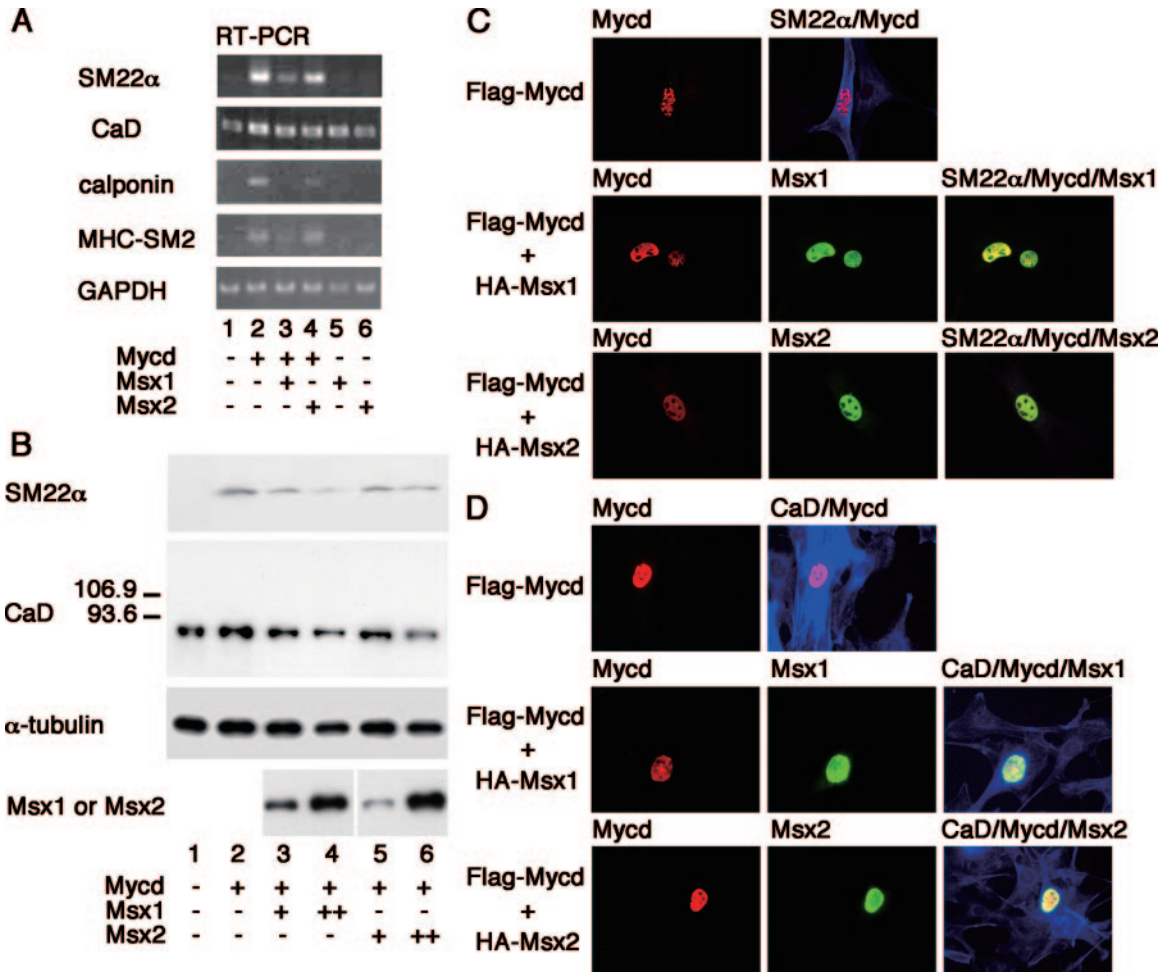

FIG. 3. Inhibition of the Mycd-induced expression of endogenous SMC markers by the coexpression of Msx transcription factors. 10T1/2 cells were transfected with Flag-Mycd (1.0 μg) and/or HA-Msx1 (0.5 μg) or HA-Msx2 (0.5 μg) expression plasmids with (+) or without (−) empty plasmid (total 2 μg plasmids/well of a six-well culture plate) and were cultured as described in Materials and Methods. Expression of the indicated SMC markers was analyzed by RT-PCR (A), immunoblotting (B), and immunocytochemistry (C and D). (A) Cycle numbers of the PCRs and the sizes of PCR products are as follows: SM22α, 28, 329 bp; CaD, 24, 380 bp; calponin, 30, 400 bp; MHC-SM2, 28, 323 bp; GAPDH, 22, 984 bp. (B) Whole-cell lysates of 10T1/2 cells from the indicated cultures were probed with anti-SM22α, anti-CaD, and anti-HA (Msx1 or Msx2) antibodies by immunoblot analysis. In this analysis, 10T1/2 cells were transfected with the indicated expression plasmids as described above except for HA-Msx1 or HA-Msx2 expression plasmid; + and ++ indicate 0.25 μg and 0.5 μg of the respective expression plasmid. (C) Tagged Mycd (red) and Msx transcription factors (green) were stained with anti-Flag and anti-HA antibodies, respectively, and endogenous SM22α and CaD (blue) were stained using their respective antibodies.

might have given rise to the different interactions with Msx1, we confirmed that the anti-Flag antibody precipitated most of the Flag-tagged SRF or Mycd protein from the cell extracts (data not shown). We then analyzed the interactions between Mycd and SRF with or without Msx1. Mycd interacted with Msx1 (Fig. 4C, lane 1) or SRF (lane 2), and SRF/Mycd interacted with Msx1 (lane 3), suggesting that Msx1 forms a ternary complex with both SRF and Mycd. Similar interactions between Mycd, SRF, and Msx2 and the formation of a ternary complex of these transcription factors were also confirmed (data not shown). We further found that endogenous Mycd, SRF, and Msx1 formed a ternary complex in BMP2-stimulated VSMCs (Fig. 4D, lane 2) but not in VSMCs without BMP2 stimulation (Fig. 4D, lane 1). These data indicate that Msx transcription factors form a ternary complex with Mycd and SRF in vivo.

Domain mapping of the interactions between Msx1, SRF, and Mycd. To identify the Msx1-interacting domain in Mycd, we constructed a series of expression plasmids encoding Flag-tagged Mycd deletion mutants and examined their interactions with HA-Msx1 (Fig. 5A and B). We used an in vitro translation system for the interaction analysis because the Mycd deletions examined were not necessarily localized to the nucleus when they were expressed in Cos7 cells (data not shown). Compared with wild-type Mycd (Mycd wt) (lane 2), the N-terminal deletions, MycdΔN51 lacking residues 1 to 51, MycdΔN80 lacking residues 1 to 80, and MycdΔN128 lacking residues 1 to 128, significantly reduced Mycd's ability to interact with Msx1 (lanes 3, 4, and 7). Although a mutant Mycd with the N-terminal basic sequence (residues 98 to 103) deleted (MycdΔNB) fully retained its ability to interact with Msx1 (lane 5), deletion of the central basic sequence (residues 243 to 260) (MycdΔCB) reduced this

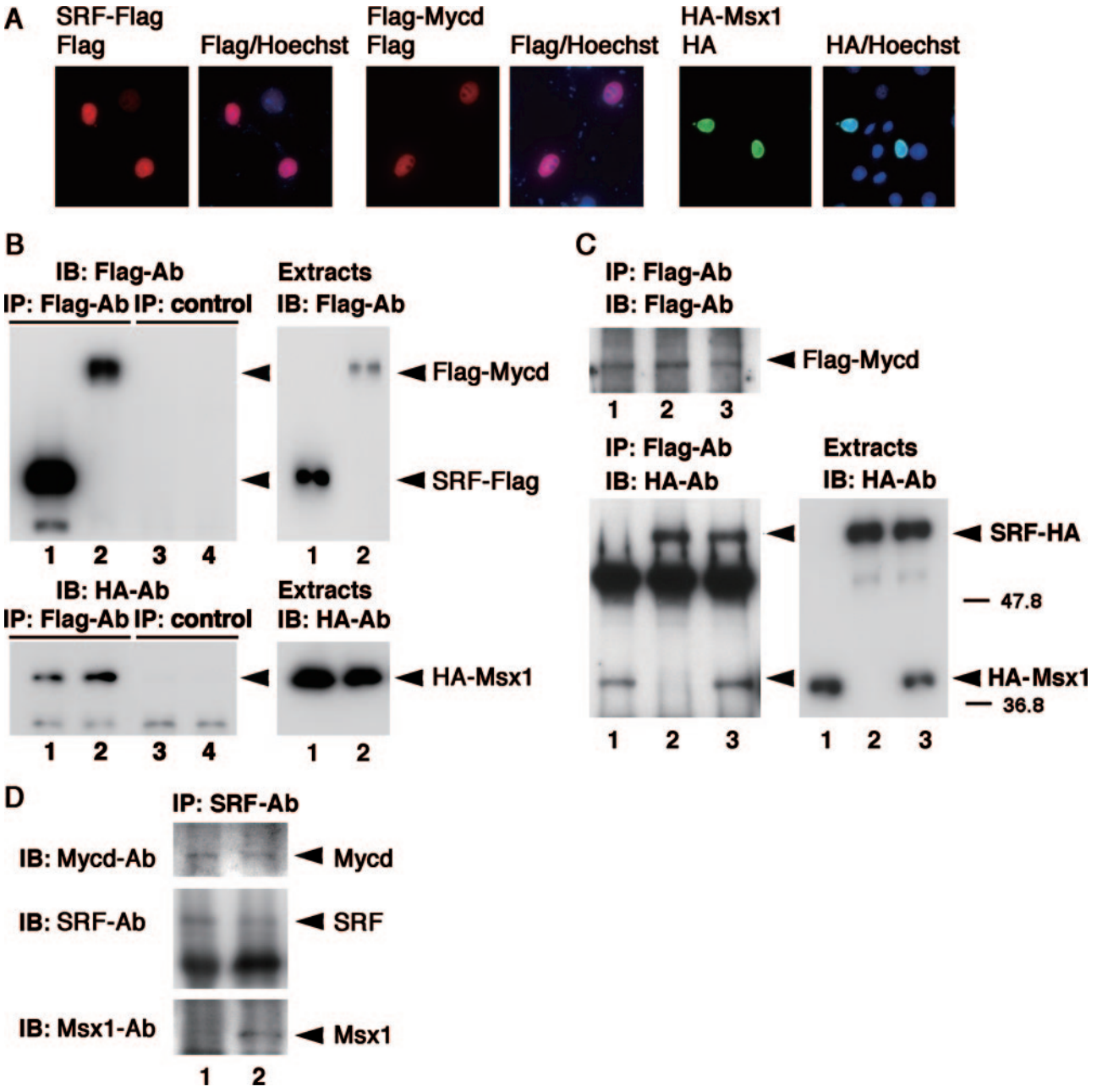

FIG. 4. Interactions between Msx1, SRF, and Mycd. Cos7 cells were transfected with the indicated expression plasmids and were cultured in the presence of 2% HS for 20 h. (A) Tagged SRF (red), Mycd (red), and Msx1 (green) were localized by staining with antibodies against the indicated tags, and the nuclei (blue) were stained with Hoechst 33258. (B) Cos7 cells were cotransfected with expression plasmids for SRF-Flag and HA-Msx1 (lanes 1 and 3) or Flag-Mycd and HA-Msx1 (lanes 2 and 4). The cell extracts from respective transfectants were incubated with anti-Flag antibody (lanes 1 and 2) or control IgG (lanes 3 and 4), and the extracts (right panels) and immunoprecipitates (IP) (left panels) were probed with an anti-Flag antibody (SRF or Mycd) or anti-HA antibody (Msx1) by immunoblot (IB) analysis. (C) Cos7 cells were cotransfected with expression plasmids for Flag-Mycd and HA-Msx1 (lane 1), Flag-Mycd and SRF-HA (lane 2), or Flag-Mycd, SRF-HA, and HA-Msx1 (lane 3). The cell extracts were incubated with anti-Flag antibody (Ab), and the extracts (right panel) and immunoprecipitates (left panels) were probed with an anti-Flag antibody (Mycd) or anti-HA antibody (SRF and/or Msx1) by immunoblot analysis. (D) Endogenous Msx1, SRF, and Mycd formed a ternary complex in VSMCs cultured under BMP2-stimulated conditions. VSMCs were cultured in the presence of IGF-I (2 ng/ml) (lane 1) or BMP2 (10 ng/ml) (lane 2) for 24 h. The cell extracts were incubated with an anti-SRF antibody, and the extracts and immunoprecipitates were probed with the indicated antibodies by immunoblot analysis.

ability (lane 6). MycdΔN128/ΔCB, which lacked residues 1 to 128 and the central basic sequence, completely lost its ability to interact with Msx1 (lane 8). An Mycd deletion mutant lacking the C-terminal region from the central basic region (residues 243 to 935) retained its Msx1-interacting ability (data not shown). These deletions showed the same profiles for interacting with Msx2, and immunoprecipitation with control IgG did not show any interaction (data not shown). These results sug-

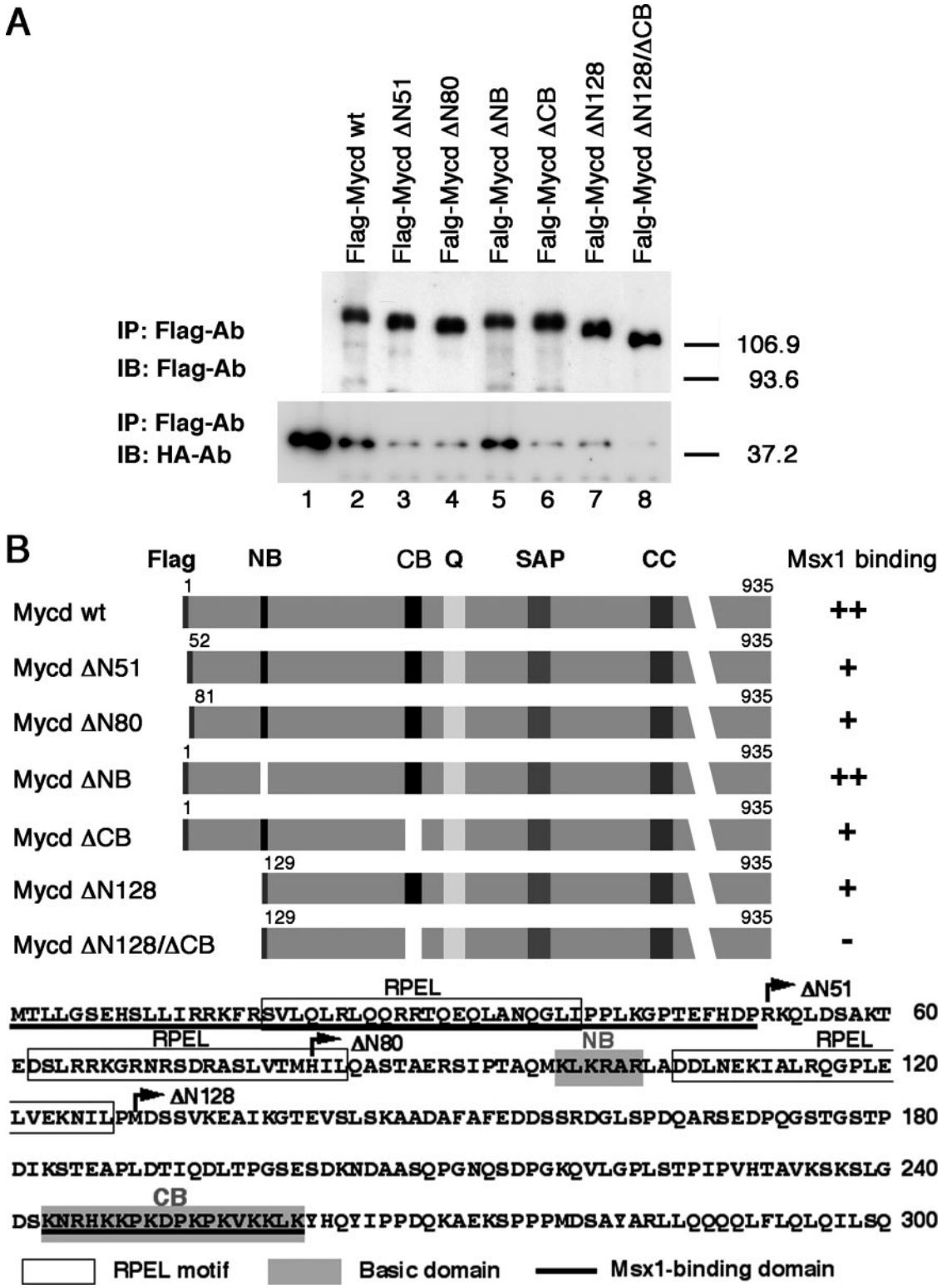

FIG. 5. Domain mapping of Mycd's interaction with Msx1. (A) In vitro-translated Flag-Mycd derivatives, as indicated, were incubated with HA-Msx1, and their interactions were analyzed by immunoprecipitation (IP) using an anti-Flag antibody (Ab) (for the Mycd derivatives) followed by immunoblotting (IB) using the indicated antibodies. Input HA-Msx1 protein is also shown (lane 1). (B) The results from panel A are presented schematically. The binding of the Mycd derivatives to Msx1 are presented as follows: ++, strong binding; +, moderate binding; −, no binding.

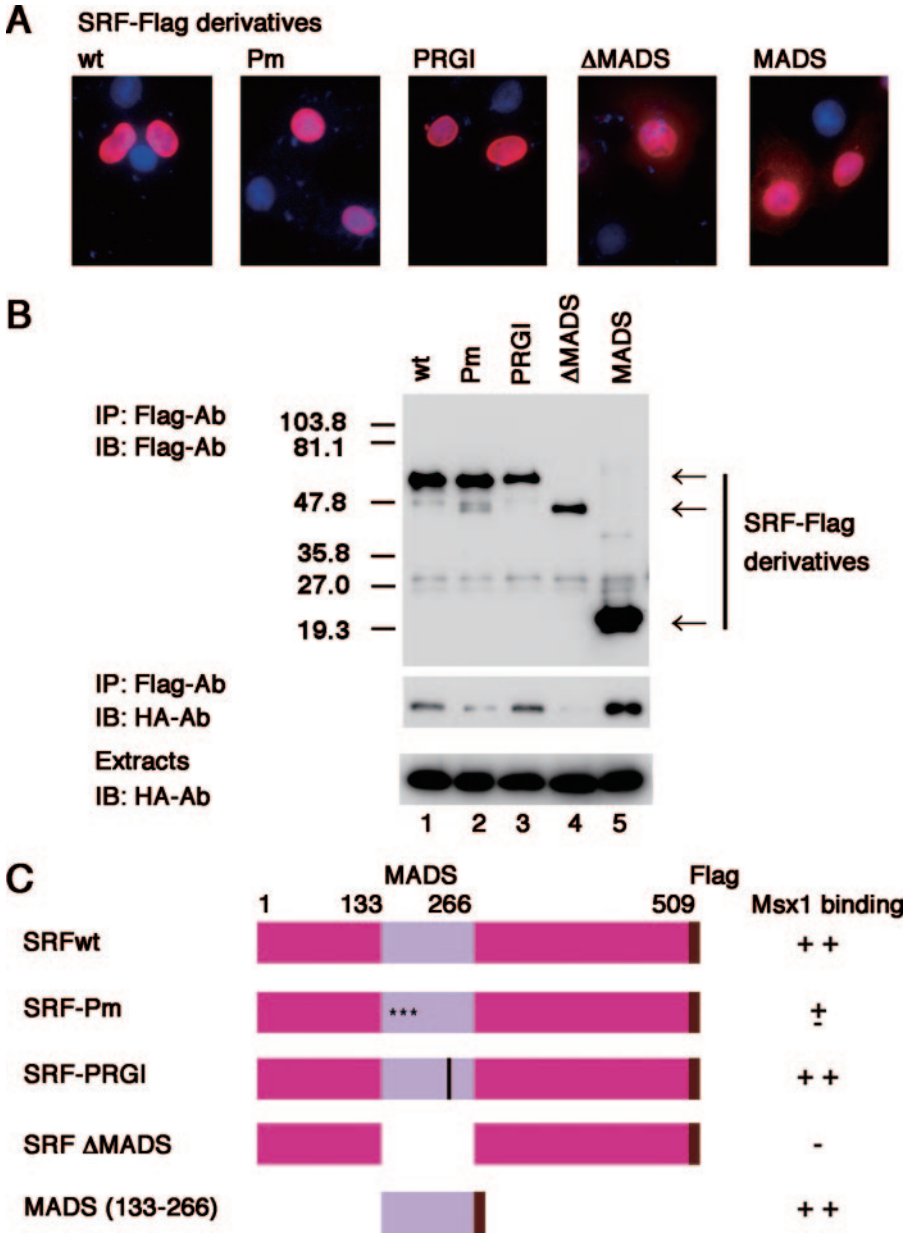

FIG. 6. Domain mapping of SRF's interaction with Msx1. (A) The localization of the SRF derivatives is shown. Cos7 cells were transfected with the indicated expression plasmids for SRF-Flag derivatives [wt, SRFwt; Pm, SRF Pm143-146; PRGI, SRF PRGI-In206; ΔMADS, SRFΔMADS (133-266); MADS, MADS (133-266)] and cultured as described in the legend to Fig. 4. The SRF derivatives (red) and the nuclei (blue) were stained with an anti-Flag antibody and Hoechst 33258, respectively. (B) Identification of the Msx1-interacting domain of SRF. Cos7 cells were cotransfected with each of the indicated expression plasmids for the SRF-Flag derivatives and the HA-Msx1 expression plasmid. Interactions between SRF derivatives and Msx1 were analyzed as described in the legend to Fig. 4B. (C) The results of panel B are presented schematically. The ability of SRF derivatives to bind Msx1 is presented as follows: ++, strong binding; ±, faint binding; −, no binding. IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

gest that the N-terminal short region (amino acids 1 to 51) and the central basic sequence of Mycd are the key regions required for its interaction with Msx1 or Msx2.

We analyzed the interaction between SRF and Msx1 using the following SRF-Flag variants: wild type (SRFwt), a point mutation (SRF Pm143-146) lacking DNA-binding activity (39), a mutation with 4 amino acids inserted into the MADS domain (SRF PRGI-In206) lacking DNA-binding and dimer formation abilities (39), a deletion lacking the MADS domain [SRFΔMADS (133 to 266)], and the MADS domain only [MADS (133 to 266)]. When SRFwt, Pm143-146, and PRGI-In206 were expressed in Cos7 cells, they were localized to the nucleus. Most of the SRFΔMADS (133 to 266) and MADS (133 to 266) was also observed in the nucleus, with faint labeling in the cytoplasm (Fig. 6A). SRFwt, SRF PRGI-In206, and MADS (133 to 266) interacted with Msx1 (Fig. 6B, lanes 1, 3, and 5), but SRFΔMADS (133 to 266) did not (lane 4), indicating that the Msx1-interacting domain of SRF resides in the

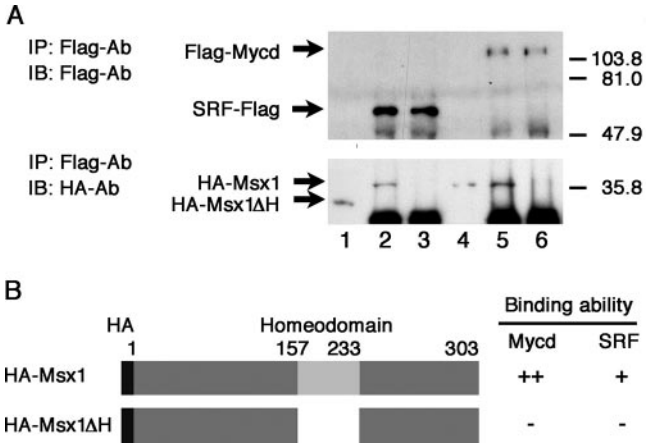

FIG. 7. Domain mapping of Msx1's interaction with SRF or Mycd. (A) In vitro-translated SRF-Flag or Flag-Mycd was incubated with in vitro-translated HA-Msx1 or HA-Msx1ΔH as follows: lane 1, input HA-Msx1ΔH; lane 2, SRF-Flag plus HA-Msx1; lane 3, SRF-Flag plus HA-Msx1ΔH; lane 4, input HA-Msx1; lane 5, Flag-Mycd plus HA-Msx1; lane 6, Flag-Mycd plus HA-Msx1ΔH. Their interactions were analyzed by immunoprecipitation (IP) followed by immunoblotting (IB) as described in the legend to Fig. 5A. Ab, antibody. (B) The results of panel A are presented schematically. The ability of Msx1 derivatives to bind Mycd or SRF is presented as follows: ++, strong binding; +, moderate binding; −, no binding.

MADS domain. The MADS (133 to 266) alone showed a strong Msx1-interacting ability compared with SRFwt or SRF PRGI-In206. Adjusting for the higher expression levels of MADS (133 to 266), the Msx1-interacting ability of MADS (133 to 266) was equivalent to that of SRFwt or SRF PRGI-In206 (lanes 1, 3, and 5), indicating that the other parts of SRF, outside of MADS, do not interfere with its interaction with Msx1. Compared with SRF PRGI-In206 (lane 3), SRF Pm143-146 markedly reduced the Msx1-interacting ability (lane 2). Taken together, these results suggest that residues 143R, 145K, and 146I within the MADS domain of SRF, which are involved in CArG-box motif binding, play a critical role in SRF's interaction with Msx1.

We further analyzed Msx1 to determine its SRF- or Mycd-interacting domain using in vitro-translated HA-Msx1 variants and Flag-tagged SRF or Mycd. Msx1 lacking the homeodomain (Msx1ΔH), which was mainly localized to the cytoplasm of Cos7 and 10T1/2 cells, did not interact with SRF or Mycd (Fig. 7A, lanes 3 and 6). Consistent with the results in Fig. 4B, Msx1 interacted more potently with Mycd than with SRF (lanes 2 and 5). Our results, summarized in Fig. 7B, indicate that the homeodomain of Msx1 is important for its interaction with SRF and Mycd.

Msx1 inhibits the binding of SRF or SRF/Mycd complex with the CArG-box motif. The formation of a complex between SRF or SRF/Mycd and a DNA probe containing the CArG-box motif is reported (49). Our present data (Fig. 2 to 7) raise the possibility that Msx1 disrupts the Mycd/SRF/CArG complex by binding to the SRF/Mycd complex, leading to suppression of the Mycd-dependent transactivation of SMC marker genes. To address this possibility, we carried out gel-shift assays using a sequence of the SM22α promoter containing the pCArG-box motif as a probe and in vitro-translated Myc-tagged SRF (SRF-Myc), HA-Msx1, and Flag-Mycd proteins. SRF alone formed an intense band with the CArG probe (Fig. 8A, lane 1), which was inhibited by the addition of excess amounts of a specific competitor (lane 2) but not of a mutant one (lane 3). This band was supershifted by an anti-Myc antibody (lane 4) but not by a control antibody (lane 5). Notably, the addition of Msx1 protein significantly reduced the SRF-specific band in an Msx1 dose-dependent manner (lanes 6 to 8). Msx1 protein alone did not form any band with this probe (lane 9). These results indicate that Msx1 inhibited the direct interaction between SRF and the pCArG probe. In addition to the band formed by the SRF/CArG interaction, a high-molecular-weight (M_r) band was detected using SRF, Mycd, and the pCArG probe (Fig. 8B, lane 1). This high-M_r band was supershifted by an anti-Flag antibody but not by a control antibody (lanes 2 and 3), indicating that the high-M_r band was a complex of SRF, Mycd, and the pCArG probe. This complex was dose dependently reduced by the addition of increasing amounts of Msx1 protein (lanes 4 to 6). These results, taken together with the promoter assays (Fig. 2) and analysis of endogenous SMC marker expression in 10T1/2 cells (Fig. 3), suggest that Msx1 inhibits formation of the Mycd/SRF/CArG or SRF/CArG complex, resulting in the suppression of SMC-specific transcription.

To investigate whether Msx1 inhibits the interactions between SRF/Mycd and the CArG-box motif in vivo, we performed a ChIP assay. Compared with differentiated VSMCs cultured in the presence of IGF-I, BMP2-stimulated VSMCs, in which the expression of Msx1 was increased but that of SMC marker genes was decreased (Fig. 1), markedly reduced SRF binding to chromatin DNAs containing the CArG-box motifs in the promoter regions of SM22α and CaD genes (Fig. 8C, left panel, lane 3). Similarly, 10T1/2 cells expressing Mycd enhanced bindings of SRF to these chromatin DNAs, whereas these bindings were reduced in the cells coexpressing Mycd and Msx1 (Fig. 8C, right panel, lane 3). In this experiment, chromatin DNAs containing the CArG-box motifs in both promoter regions were not precipitated by control IgG (Fig. 8C, lane 2). These results are in good agreement with the regulation of endogenous SMC marker expression in cultured VSMCs stimulated with BMPs (Fig. 1), 10T1/2 cells expressing either Mycd or Mycd/Msx1 (Fig. 3), and gel-shift assay (Fig. 8A and B).

DISCUSSION

In this study, we demonstrated that BMP triggers the down-regulation of SMC marker gene expression in VSMCs via the induction of Msx transcription factors Msx1 and Msx2, and we demonstrated the molecular mechanism of this process, based on the following findings. (i) BMPs induced the expression of Msx transcription factors prior to the down-regulation of SMC marker expression in VSMCs. (ii) Msx transcription factors suppressed the Mycd-dependent transactivation of the SM22α and CaD promoters and the endogenous expression of SMC markers in 10T1/2 cells. (iii) Msx transcription factors interacted with SRF and Mycd and formed a ternary complex. (iv) The homeodomain of Msx1 was critical for its interaction with Mycd and SRF. We identified both the N-terminal short region (1 to 51 amino acids) and central basic sequence of Mycd and

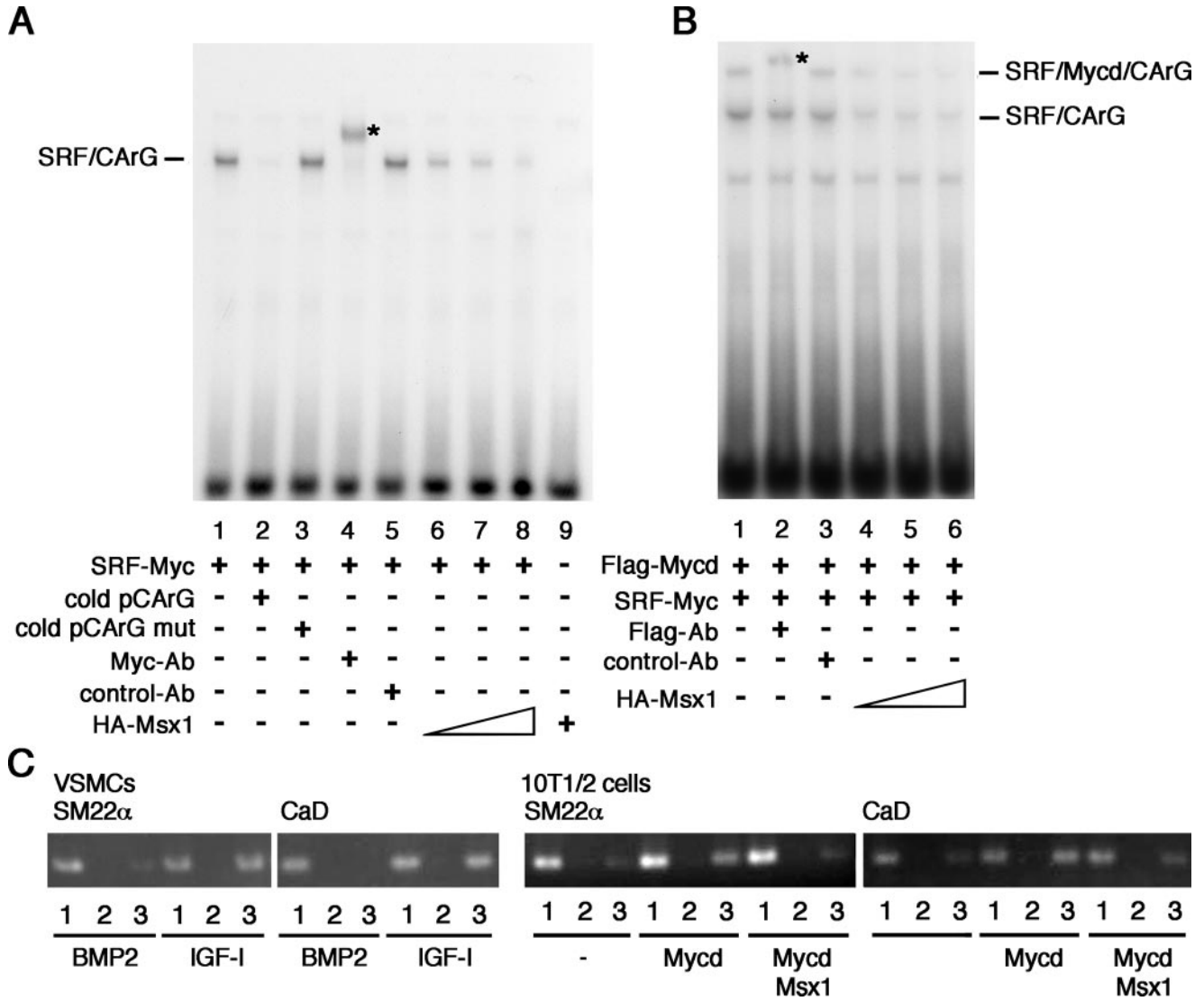

FIG. 8. Msx1 inhibits the binding of SRF or the SRF/Mycd complex to the CArG-box motif. (A and B) The ^{32}P-labeled pCArG-box motif of the SM22α promoter was incubated with the indicated in vitro-translated proteins, antibodies, and/or double-stranded oligonucleotides, and their interactions were analyzed by 5% polyacrylamide gel electrophoresis. (A) Constant amounts of SRF (5 μl of in vitro-translated aliquot) (lanes 1 to 8) or Msx1 (4 μl of in vitro-translated aliquot) (lane 9) were used. One hundred-fold excess amounts of indicated cold competitors were added (lanes 2 and 3). Increasing amounts of Msx1 (2 μl, 4 μl or 8 μl) (lanes 6 and 7) were mixed with SRF (5 μl) (lanes 6 and 7). (B) Constant amounts of Mycd (8 μl of in vitro-translated aliquot) and SRF (4 μl) were used. Increasing amounts of Msx1 (2 μl, 4 μl, or 8 μl) (lanes 4 to 6) were mixed with Mycd/SRF. Arrows and asterisks show the indicated pCArG-box motif/protein complexes and supershifted complexes, respectively. +, present; −, absent. (C) ChIP assays were performed using endogenous proteins associated with extracted chromatin fragments prepared from VSMCs by BMP2 stimulation and differentiated VSMCs cultured in the presence of IGF-I (left panels) or 10T1/2 cells expressing Mycd or Mycd and Msx1 (right panels). The extracted chromatin fragments were immunoprecipitated with control IgG (lane 2) or antibody against SRF (lane 3), and precipitated genomic DNA was analyzed by PCR using primers for the SM22α and CaD promoter regions containing the CArG-box motif. The sizes of PCR products are as follows: rat SM22α promoter, 195 bp; rat CaD promoter, 141 bp; mouse SM22α promoter, 196 bp; mouse CaD promoter, 141 bp. PCR amplification was also performed prior to immunoprecipitation for the input control (lane 1). −, transfection of control vector.

the MADS domain of SRF as the Msx1-interacting domains. In the MADS domain of SRF, a region encompassing 143R, 145K, and 146I, which is essential for CArG-box motif binding, was critical for its interaction with Msx1. (v) Msx1 inhibited the interactions between the CArG-box motif and SRF or the SRF/Mycd complex in gel-shift and ChIP assays. This is the first study demonstrating the inhibition of Mycd activity by the transcription of a homeodomain protein.

BMP-induced expression of Msx1 and Msx2. BMP-induced expression of Msx1 and Msx2 genes at the transcriptional level is partially characterized (4, 23); activation of Msx1 transcription is required for Smad8 and that of the Msx2 gene depends on Smad4 and lymphoid-enhancing factor, respectively. We also confirmed that BMP2 stimulation triggered a sustained activation of Smad1/5/8 for at least 2 days, but IGF-I stimulation did not (data not shown). BMP signaling has been shown

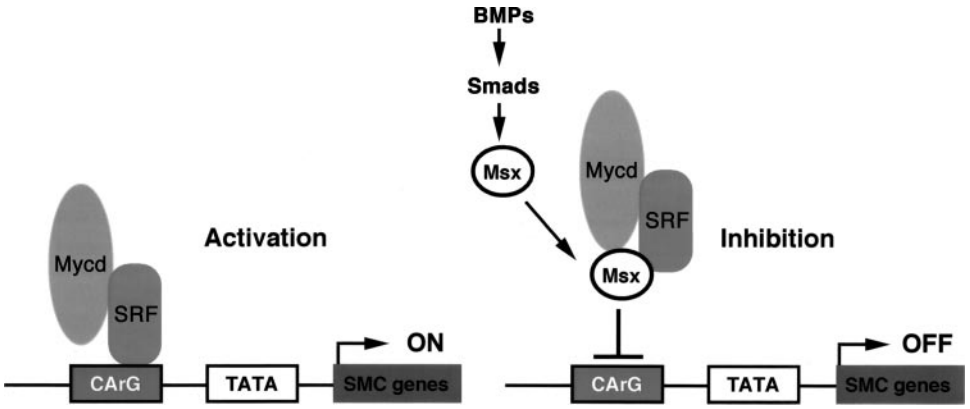

FIG. 9. Model for the inhibition of SRF/Mycd-dependent SMC marker gene transcription by Msx transcription factors.

to enhance the expression of Osterix (Osx) (53) and plasminogen activator inhibitor 1 (55) at posttranscriptional levels. The molecular mechanism of such posttranscriptional events, however, remains to be fully uncovered. It has been recently reported that Msx1 is sumoylated in vivo (15). In our present study, accumulation of in Msx1 protein in response to BMP stimulation was robust compared with that of its mRNA (Fig. 1). It is possible to speculate that the posttranscriptional regulation either via stabilization of Msx1 protein half-life or enhanced translation may be involved in such robust accumulation. Further study is required to address this point. Contrary to Msx transcription factors, there were no significant differences in the expression levels of SRF and Mycd (data not shown) and SRF/Mycd interaction in cultured VSMCs stimulated by IGF-I or BMP2 (Fig. 4D). Hendrix et al. have reported that the expression of Mycd mRNA is significantly decreased within 3 days after arterial injury compared to the uninjured control, but its expression returns to control levels by 7 days after injury (19). This difference in the expression levels of Mycd may be due to distinct assay systems. In our culture system, VSMCs were only stimulated by IGF-I or BMPs. In the arterial injury model, VSMCs may be affected by numerous factors such as blood-originated growth factors and cytokines in addition to a mechanical injury.

BMP-induced phenotypic modulation of VSMCs. Vascular calcification is one of the progressive features of atherosclerosis (32, 45). Based on these reported activities, BMPs are likely conveyers of such vascular remodeling. It has been indeed reported that BMP2, BMP4, and BMP6 are expressed in atherosclerotic lesions and suggested to be involved in vascular calcification (5, 12, 43). Of the BMP family, the role of BMP2 in this process is well studied. In the vasculature, oxidative stress, inflammation, hyperglycemia, and a high-fat diet induce the expression of BMP2 (21). Among the downstream effectors of BMP signaling, three transcription factors, Msx2, Msx1, and Runx/Cbfal, are known to regulate orthotopic tissue mineralization and osteogenic differentiation (1), and their expressions are up-regulated during vascular calcification (11). The roles of Msx2 and Runx/Cbfa1 in vascular calcification are partially characterized. Msx2 induces the up-regulation of Osx, a global transcription factor involved in mineralization and osteoblast differentiation mediated through Runx/Cbfa1 (21). Thus, the activation of Osx via Msx2 and Runx/Cbfa1 is thought to be the most likely cascade leading to vascular calcification. In contrast, the role of Msx1 in vascular calcification has not been uncovered. Most previous studies have focused on the roles of BMPs in osteogenic *trans*-differentiation of VSMCs; however, the effect of BMPs on the VSMC phenotype remains unclear. Msx2 is demonstrated to be a key factor for promoting arterial calcification (11, 44). Considering these findings, our present study suggests that the down-regulation of SMC marker expressions by Msx1/2 might be prerequisite for induction of transcription factors involved in arterial calcification. Recently, King et al. have reported that BMPs reduce the expression of SMC markers in passaged VSMCs exhibiting a dedifferentiated phenotype (24) and proposed that this reduction is associated with the binding of KLF4, which is up-regulated by BMP in VSMCs, to the transforming growth factor (TGF) control element in the promoter regions of SMC marker genes. However, they did not address the molecular mechanism of transcriptional suppression via KLF4/TGF control element. Here, we demonstrated that the BMP-triggered expression of Msx1 and Msx2 induced phenotypic modulation of VSMCs, as revealed by the down-regulation of SMC marker expression, and the molecular mechanism by which these Msx transcription factors repress SMC-specific transcription (Fig. 9). We also addressed the involvement of BMPs/Msx transcription factors in the progression of atherosclerosis. In our preliminary experiment, RT-PCR analysis revealed that the expression of BMP2, BMP6, Msx1, and Msx2 mRNAs was highly up-regulated in advanced human atherosclerotic arteries but not in normal arteries. In contrast, the expressional features of SMC markers were markedly down-regulated (data not shown). These results are partially in accordance with previous reports by Cheng et al. (11) and Tyson et al. (48); the expression of BMP2 and Msx2 is significantly increased in calcified arteries. They suggest that the BMPs/Msx transcription factor-mediated down-regulation of SMC marker expression would occur in atherosclerotic lesions. Our present study suggests a role for BMPs in the progression of atherosclerosis as an autocrine/paracrine factor for phenotypic modulation of VSMCs in addition to their involvement in the osteogenic *trans*-differentiation of VSMCs.

Transcriptional regulation by Msx1 and Msx2. Msx transcription factors are widely expressed in many organs during the development of vertebrate embryos (1). Msx1 and Msx2

are highly expressed in the craniofacial regions and play a role in craniofacial development (41, 42), in which they are known to act as transcriptional repressors (8). They also inhibit the differentiation program of skeletal muscle and other mesenchymal cell types (22). Msx1 suppresses MyoD expression by recruiting a linker histone, histone H1, which induces an inactive chromatin structure, to a key regulatory element in the MyoD promoter, resulting in inhibition of myogenic differentiation (27). Msx1 is also known to elicit transcriptional repression by interacting with general transcription factors (7) or with other homeodomain transcription factors such as Dlxs, Lhx2, and Pax3 (2, 3, 56). We demonstrated that the homeodomain of Msx1 was essential for its binding to Mycd and SRF (Fig. 7) and that Msx1ΔH lost the inhibitory effect on Mycd activity. As a result, neither Msx1 nor Msx2 affected the transcriptional activity of a constitutively active viral promoter. These results suggest that the inhibitory activity of Msx1 depends on its interaction with Mycd and SRF but not on other transcription factors, including general transcription factors.

Differentiated VSMCs predominantly express *h*-CaD, whereas dedifferentiated VSMCs and nonmuscle cells only express *l*-CaD (46). The basal expression of *l*-CaD in 10T1/2 cells might be regulated by a mechanism distinct from the CArG/SRF-dependent one, given that Msx1 or Msx2 suppressed the endogenous expression of *l*-CaD and its transcription, which were enhanced by Mycd, but not *l*-CaD's basal expression and promoter activity (Fig. 3A and B and Fig. 2D).

Brunelli et al. have recently reported that Msx2 also functions as a positive regulator for SMC marker expression in combination with a transcriptional corepressor, necdin, during the SMC-like differentiation of mesoangioblasts (7). Undifferentiated mesoangioblasts are capable of an SMC-like phenotype in response to TGFβ1 stimulation. During this process, TGFβ1 induces the simultaneous up-regulation of necdine and Msx2 followed by the expression of SMC marker genes. Kuwajima et al. have also shown that necdin and Msx2 form a stable complex via MAGE-D1 in C2C12 cells (25). In this case, Mycd would not be involved in transactivation of SMC marker genes because mesoangioblasts do not express Mycd (7). Mycd expression is largely restricted to a cardiovascular lineage. In contrast, Mycd-related transcriptions (MRTF-A and MRTF-B) are ubiquitously expressed in a broad range of embryonic and adult tissues and most culture cells (38). In our preliminary experiments, we have found that Msx1 also interacts with MRTF-A and inhibits the transcriptional activity of MRTF-A (data not shown), suggesting that the regulatory interactions described here may, generally occur in a wide variety of cells as well. Qiu et al. have reported that TGFβ1-induced Smad3 directly binds to Mycd and activates the transcription of SMC marker genes (40). Based on these findings, we speculate that activation of MRTF-A and/or MRTF-B via Smad family members would result in the transcriptional activation of SMC marker genes in combination with counteracting the repressive effect of Msx2 by a ternary complex formation with necdin and MAGE-D1 in mesoangioblasts stimulated with TGFβ1.

Interaction of Msx transcription factors with Mycd and SRF and the molecular mechanism of Msx-mediated transcriptional inhibition. Our present results demonstrated that Msx1 interacted with two regions of Mycd, the N-terminal short region (amino acids 1 to 51) and the central basic sequence (Fig. 5). It has been reported that the region encompassing the central basic sequence and glutamine (Q)-rich domain of Mycd is required for its interaction with SRF (50). The Msx1-interacting site in the central basic sequence of Mycd should be distinct from the SRF-interacting site because the interaction between Mycd and Msx1 did not compete with SRF (Fig. 4). Mycd and MRTFs have a conserved N-terminal domain composed of RPEL repeats that is critical for the Rho-dependent nuclear import of MRTF-A (33). However, Mycd is constitutively located in the nucleus of Cos7 and 10T1/2 cells even under low-serum culture conditions (50) (Fig. 4A). Thus, the critical role of the N-terminal region of Mycd has been obscure. Mycd ΔN128, which lacks the N-terminal region, interacted modestly with Msx1, but its Msx1-interacting ability was weak compared with that of Mycd wt, indicating that the N-terminal region of Mycd is necessary for a stable interaction with Msx1 and exertion of the full inhibitory effect of Msx1 on Mycd activity. Here, we demonstrated a biological role for the N-terminal region of Mycd which is involved in Rho-dependent nuclear import.

Msx1 also interacted with SRF via SRF's MADS domain (Fig. 6). The MADS domain of SRF plays a critical role in its DNA-binding activity and dimer formation (36). SRF PRGI-In206, which cannot form a dimer, interacted with Msx1 as well as SRFwt, whereas SRF Pm143-146, which lacks DNA-binding activity, lost almost all of its Msx1-interacting ability. These results suggest that SRF loses its DNA-binding ability by interacting with Msx1 because the Msx1-interacting site of SRF overlaps with its critical site for DNA binding. This property causes inhibition of the transactivation of SMC marker genes because of Msx1's masking of the interaction between Mycd/SRF and the CArG-box motif. The results of gel-shift and ChIP assays (Fig. 8) strongly support this notion.

VSMCs originate from two different sources. VSMCs in the dorsal aorta are derived from the mesoderm, and those in the aortic arch arteries are from the neural crest (28). Kwang et al. reported that Msx2 was a critical downstream target for Pax3, which represses the expression of Msx2 (26). Pax3-deficient mice showed neural crest defects including aortic arch anomalies, whereas double deficiency of Pax3/Msx2 rescues such defects. In embryonic day 9.5 (E9.5) mouse embryos, the expression of Msx1 gene is detected in the somites as monitored by $Msx1^{nlacZ}$ transgene, whereas such expression is no longer detectable in E10 embryos. In coincidence with the down-regulation of $Msx1^{nlacZ}$ transgene, an early myogenic regulatory factor, Myf5, is expressed (20). These results clearly indicate that Msx1 inhibits myogenic differentiation in vivo. A recent study has demonstrated that VSMCs in the dorsal aorta, in part, originate from the somites (14). In E10.5 mouse embryos, in which Msx1 is not expressed in the somites, anti-SM actin-positive cells derived from the somites are found in the dorsal aorta. In contrast, populations of such anti-SM actin-positive cells are low in E9.5 embryos, while the expression of Msx1 is detected in the somites. Considering these findings, Msx transcription factors would play a role in repression of SMC markers in distinct VSMC progenitors, such as mesoderm/somite-derived cells and neural crest-derived cells, in a mechanism similar to that demonstrated here.

We summarize the molecular mechanism of the Msx-mediated suppression of SMC marker gene transcription combined

with predicted BMP signaling pathways in Fig. 9. A ternary complex of SRF/Mycd/Msx1 cannot access the CArG-box motif because the DNA-binding site of SRF is masked by Msx1 binding to the complex. As a result, the transcription of SMC marker genes is inhibited. The N-terminal Msx1-interacting region of Mycd might function to stabilize such a ternary complex. Some recent studies have reported transcription factors that interfere with the Mycd activity in VSMCs. These include HERP family factors (13), KLF4 (30), Foxo4 (31), and Elk-1 (52). These factors interact with either SRF or Mycd (13, 30, 31) or compete with Mycd for interaction with SRF (52), inhibiting the transactivation of SMC marker gene expression. The inhibitory mode of Msx1 presented here is unique and distinct from its previously identified modes of action as a transcriptional repressor.

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