ERK1/2-Dependent Contractile Protein Expression in Vascular Smooth Muscle Cells

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Abstract—In vivo, vascular smooth muscle (VSM) cells change their contractile phenotype toward a more proliferative phenotype during the pathogenesis of vascular diseases. Because these dedifferentiated VSM cells may gradually regain contractile functions, we aimed to identify signaling pathways that result in an increased expression of contractile proteins in VSM cells. In vitro, serum and thrombin induced a reversible upregulation of smooth muscle myosin heavy-chain (SM-MHC) in cultured neonatal rat VSM cells. Cotransfection of a SM-MHC–promoter chloramphenicol acetyltransferase–construct with dominant-negative N17Ras or N17Raf or treatment with the mitogen-activated/ERK-activating kinase (MEK) inhibitor PD 98059 concentration dependently decreased the serum- or thrombin-induced SM-MHC promoter activity. Consistently, the serum- or thrombin-induced phosphorylation of MEK and extracellular signal-regulated kinase 1/2 (ERK1/2) coincided with a MEK-dependent nuclear accumulation of phosphorylated ERK1/2 and subsequent nuclear phosphorylation of the transcription factors c-myc and Elk-1. A 5′-deletion analysis of cis-elements within the SM-MHC promoter demonstrated that a conserved region (nucleotide −1346 to −1102) was required for both cell type–specific expression and serum- or thrombin-induced upregulation of the SM-MHC promoter in VSM cells. Within this region, 2 CArG-boxes, a GC-rich element, and a CTF/NF-1 site are critical positively acting cis-elements for the serum- or thrombin-induced upregulation of SM-MHC. We conclude that the serum- or thrombin-induced differentiation requires an intact Ras/Raf/MEK/ERK signaling cascade, nuclear translocation of activated ERK1/2, phosphorylation of transcription factors, and several cis-elements within the SM-MHC promoter.

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Key Words: atherosclerosis ■ contractile function ■ gene expression ■ phosphorylation ■ signal transduction

Contrary to skeletal or cardiac muscle cells, vascular smooth muscle (VSM) cells are not terminally differentiated. Pathological conditions such as hyperlipidemia or hypertension promote proliferation and a decrease in contractile protein expression.1,2 However, dedifferentiated VSM cells have been shown to regain contractile functions within 3 to 6 months after vascular injury.3

In vitro, receptor-mediated signaling pathways may lead to differentiation in VSM cells. Although previous studies showed that the Gβγ-subunit–induced activation of the Ras/Raf/MEK signaling molecule sequence led to a biphasic extracellular signal-related kinase 1/2 (ERK1/2) activity that is essential for the differentiation, the question remained, how does activated ERK1/2 induce transcription of contractile proteins in neonatal VSM cells.4

The 5′-promoter sequences of the rat smooth muscle myosin heavy-chain (SM-MHC) gene has recently been cloned and partially sequenced.5 The distal domain contains several consensus sequences, including 2 CC(A/T-rich)GG elements, also referred to as CArG boxes. CArG boxes represent the core sequence of the serum response element (SRE), a promoter segment that mediates the transcriptional response of many cellular genes to growth factor stimulation.6 Within the c-fos promoter, SREs contain further motifs to stabilize a ternary complex which is composed of one or several serum response factor (SRF) proteins and SRF accessory proteins (eg, Elk-1). Hereby, the latter are being recruited to the SRE through SRF-mediated protein-protein interactions.7 SRF and Elk-1 have been reported to be direct nuclear effectors for ERK1/2.8,9 Because Ras, Raf, and mitogen-activated/ERK-activating kinase (MEK) are members of a signaling pathway that links signals from activated growth factor receptors to MAP kinases, complex formation at CArG-boxes within the SM-MHC promoter may be mediated via the MAP kinase pathway.10,11

In the present study, we aimed to describe the signal transduction pathway leading to enhanced SM-MHC transcription in VSM cells. Our results demonstrate that the serum- or thrombin-dependent phenotypic modulation is bidirectional and requires nuclear translocation of activated ERK1/2, with subsequent phosphorylation of transcription factors. The analysis of regulatory domains within the SM-
MHC promoter revealed that both cell type specificity and serum- or thrombin-induced regulation of the SM-MHC transcription rely on the same cis-acting elements.

**Methods**

**Materials**

Culture media and trypsin were purchased from Invitrogen. Fetal calf serum and PBS were obtained from Biochrom. Radiochemicals were from NEN Life Science Products. Unless otherwise stated, antibodies were from New England Biolabs (NEB). All other reagents were obtained from Sigma.

**Cell Culture, Transient Transfections, and Reporter Assays**

Primary cultures of VSM cells from newborn rats were established as previously described. Before serum or thrombin application, cells were maintained in serum-free conditions for 48 hours. The transcriptional regulation of SM-1/SM-2 was assessed with a chloramphenicol acetyltransferase (CAT) reporter gene expressed under the control of the SM-MHC promoter as described. In addition, several mutations within the context of pCAT-1346 wild type (CArG-1 mut, NF-1 mut, GC-rich mut, CArG-2 mut) were applied. Transient transfections were performed in triplicates. CAT activities were normalized to the protein concentration of each sample. Transfection of a promoterless CAT construct served as a baseline indicator, allowing all other promoter constructs to be expressed relative to the promoterless activity. All CAT activities (mean±SEM) represent at least 3 independent experiments, with each setting tested in triplicate per experiment.

**Immunohistochemistry**

VSM cells were fixed at room temperature for 30 minutes in 3.7% formaldehyde/TBS. After blocking with 3% BSA in TBS-T (TBS including 0.1% Triton X-100), cells were incubated overnight with phospho-ERK1/2-, phospho-Elk-1-, or phospho-c-myc-specific antisera (1:200 in 3% BSA/TBS-T, NEB) at 4°C followed by another overnight incubation with fluorescein 5-isothiocyanate–conjugated pig anti-rabbit IgG (Dako, 1:10). Fluorescence imaging was performed with a monochromator and a cooled charged coupled device (CCD) camera (TILL-Photonics) connected to an inverted epifluorescence microscope (Carl Zeiss).

**Immunoblotting Procedures**

Whole VSM cells or isolated nuclei were directly lysed in Laemmli buffer containing 10 mmol/L dithiothreitol. Proteins were separated on 10% polyacrylamide, electroblotted, and probed with affinity-purified polyclonal anti-phospho-ERK1/2, anti-phospho-MEK, anti-ERK1/2, or anti-MEK antibodies (NEB). Primary antibodies were detected with a horseradish peroxidase–coupled secondary antibody (1:2000, NEB) by using a chemiluminescent substrate (Lumiglo, NEB).

**RNase Protection Assay**

RNA isolation, generation of DNA templates, and hybridization conditions were described previously. In brief, 10 μg of total RNA was hybridized with radiolabeled probes overnight at 42°C. Nonhybridized fragments were digested with RNase A/T1. The remaining protected fragments were separated by denaturing (8% urea) polyacrylamide gel electrophoresis and were exposed to Amersham Hyperfilm at −80°C for 2 to 24 hours.

**Results**

**Serum- or Thrombin-Induced SM-MHC Expression in VSM Cells**

We have previously shown that fetal calf serum enhances the expression of contractile proteins in cultured neonatal rat VSM cells. Here, RNase protection assays revealed that SM-1 transcripts at day 6 are up to 15-fold more abundant in serum-treated VSM cells compared with serum-starved controls, and subsequent serum withdrawal reduced the steady-state SM-1/SM-2 expression to almost baseline levels within 4 days (Figure 1). A second exposure to serum increased SM-1/SM-2 expression again in VSM cells. High or low expression levels of SM-1/SM-2 followed media changes for at least 4 consecutive cycles, and comparable data were obtained when either smooth muscle α-actin mRNA expression levels were analyzed or thrombin as a single serum component was used as stimulus (data not shown). Consistently, the expression of both SM-1 and SM-2 protein isoforms of SM-MHC was upregulated in the presence of serum (or thrombin; data not shown) within 4 to 6 days, as detected by immunoblot analysis of whole-cell lysates (Figure 2). Agonist withdrawal reduced the SM-1/2 expression within 3 days to 25% of the expression level in the presence of either serum or thrombin. This rather short half-life of the SM-MHC protein underlines the dynamics of plasticity in these cultured cells. The continuous presence of either serum or thrombin is thus required to induce and maintain a differentiated phenotype in cultured neonatal VSM cells.

**Ras/Raf/MEK/ERK Is Required for SM-MHC Promoter Activation**

To characterize signaling pathways leading to increased transcription of contractile proteins in VSM cells, we used a CAT reporter gene construct expressed under the control of the −1346 nucleotide promoter region of the SM-MHC gene (pCAT-1346). Serum or thrombin treatment of transiently...
Transfected VSM cells increased the CAT-activity by ~6-fold and 2-fold, respectively, compared with that of unstimulated controls (data not shown). Thus, increases in SM-1/SM-2 mRNA result from transcriptional activation. Co-expression of pCAT-1346 with dominant-negative N17-Ras or N17-Raf concentration-dependently reduced the serum-stimulated CAT activity (Figure 3A). Conversely, previous studies demonstrated that coexpression of the Raf C-terminus increased CAT activity ~2-fold in the absence of agonists (data not shown). To directly demonstrate an activation of MEK and ERK, whole-cell lysates of serum- or thrombin-stimulated VSM cells were probed with phospho-specific anti-MEK and anti-ERK1/2 antibodies. Figure 3B depicts the temporal pattern of the serum induced MEK- and ERK-phosphorylation in VSM cells. Pretreating VSM cells with the MEK-inhibitor PD 98059 concentration-dependently inhibited the serum-stimulated phosphorylation of ERK1/2, as shown in Figure 3C. Similar data were obtained with UO126, another MEK-inhibitor, and with thrombin as a stimulus (data not shown). To analyze whether ERK1/2 phosphorylation is a necessary intermediate in the serum-induced SM-MHC promoter activation, transiently transfected VSM cells were treated with increasing concentrations of PD 98059 before serum stimulation. As shown in Figure 3D, PD 98059 concentration-dependently reduced the serum-induced SM-MHC promoter activity. Thus, the serum- or thrombin-dependent activation of the Ras/Raf/MEK/ERK signaling cascade is a prerequisite for the SM-1/SM-2 expression.

Figure 3. Serum-induced SM-MHC promoter activation requires the Ras/Raf/MEK/ERK signaling cascade. A, Serum-starved VSM cells were transfected with a ~1346 nucleotide SM-MHC promoter-CAT fusion construct (pCAT-1346) and then stimulated with 10% serum for another 48 hours. To test for participation of Ras/Raf in the serum-mediated SM-MHC promoter induction, VSM cells were cotransfected with 0.5 (0.25) μg/well pCAT-1346 and the indicated amounts (in μg) of dominant-negative N17-Ras (black bars) or N17-Raf (gray bars) expression constructs. Bars represent mean CAT activities±SEM of at least 3 independent transfection experiments. B, Whole-cell lysates of serum-stimulated VSM cells were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and probed with a polyclonal antiserum detecting both SM-1 (204 kDa) and SM-2 (200 kDa) isoforms of SM-MHC. Densitometric scans were averaged from 3 similar experiments. Bar graphs show mean±SEM–fold increase over control at day 0.

ERK-Dependent Nuclear Phosphorylation of Elk-1 and c-myc
If ERK1/2 is involved in transcriptional activation of the SM-MHC gene, serum- or thrombin treatment of VSM cells should result in a nuclear translocation of activated ERK1/2. In serum-starved VSM cells, immunofluorescence analysis demonstrated a diffuse and weak cytoplasmic staining of phosphorylated ERK1/2 (Figure 4A). In contrast, intense nuclear staining was evident in thrombin-treated cells. Cellular ERK translocation, initiated as early as 5 minutes after stimulation, peaked after ~15 minutes and was maintained for at least 1 hour (data not shown). Furthermore, pretreatment with 20 μmol/L PD 98059 prevented the nuclear appearance of phosphorylated ERK1/2 (data not shown). To further evaluate whether ERK1/2 is involved in phosphorylation of transcription factors, nuclear staining of phosphorylated Elk-1 and c-myc was determined by applying the respective phospho-specific antisera. A weak and diffuse staining pattern was detected in serum-starved VSM cells (Figure 4B). In contrast, intense nuclear staining of phosphorylated Elk-1 and c-myc was evident in thrombin-treated cells. Cellular ERK translocation, initiated as early as 5 minutes after stimulation, peaked after ~15 minutes and was maintained for at least 1 hour (data not shown). Furthermore, pretreatment with 20 μmol/L PD 98059 prevented the nuclear appearance of phosphorylated ERK1/2 (data not shown). To further evaluate whether ERK1/2 is involved in phosphorylation of transcription factors, nuclear staining of phosphorylated Elk-1 and c-myc was determined by applying the respective phospho-specific antisera. A weak and diffuse staining pattern was detected in serum-starved VSM cells (Figure 4B). In contrast, intense nuclear staining of phosphorylated Elk-1 and c-myc was evident in thrombin-treated cells.
polyacrylamide and separated on 10% sodium dodecyl sulfate. VSM cells were isolated, normalized for their protein content, and after serum starvation, nuclei of thrombin (2 U/mL)-stimulated control cells. Pictures are representative of 3 experiments. B, identical exposure times for thrombin-treated and untreated fluorescence imaging was performed with a cooled CCD camera at an activity level 30-fold over control cells. Similar data were obtained when cells were deprived into a robust nuclear signal on thrombin stimulation, which lasted for at least 60 minutes, as phosphorylation of Elk-1 became detectable within 1 minute after stimulation, which lasted for at least 60 minutes, as shown by immunoblotting of isolated nuclei (Figure 4B). Taken together, these data suggest that the thrombin- or serum-induced transcription of SM-MHC requires activation and subsequent nuclear translocation of activated ERK1/2 to phosphorylate nuclear transcription factors.

SM-MHC Promoter Contains Several Positively Acting cis-Elements

To identify putative transcription factor binding sites within the SM-MHC promoter that are required for the serum- or thrombin-induced increase in transcriptional activity, CAT-activities of 5'-deletion constructs were analyzed. Transient transfections of SM-MHC promoter constructs in VSM cells revealed that pCAT-1346, which contains 4 putative transcription factor binding sites, was the most active construct after serum stimulation, with an activity level 30-fold over promoterless pCAT-basic (Figure 5, black bars) or 6-fold over untreated serum-free controls (gray bars). The addition of further 5'-flanking promoter DNA sequence (pCAT-3443) reduced the transcriptional activity to 18-fold over promoterless pCAT-basic. The pCAT-1182 construct, lacking CArG box 2 and the GC-rich element, yielded an activity of 21-fold over promoterless pCAT-basic. When CArG box 2, the GC-rich element, the NF-1 binding site, and the CArG box 1 were deleted (pCAT-1102), transcriptional activity decreased to 5-fold over promoterless pCAT-basic. Further 5'-truncation revealed no significant reduction because the activity of pCAT-562 was found to be approximately equal (4-fold over promoterless pCAT-basic) to that of the pCAT-1102 construct. All deletion constructs were also tested for activity in Swiss-3T3 fibroblasts (Figure 5, open bars). Transient transfection in Swiss-3T3 fibroblasts revealed that the used constructs retained their cell type-specific activity because they were only minimally active relative to VSM cells, ranging <3-fold over promoterless pCAT-basic.

To determine whether the transcriptional activity of the 5'-deletion constructs corresponds to functional motifs of putative cis-elements, pCAT-1346 SM-MHC-CAT-constructs harboring mutations within selected cis-elements were transiently transfected in VSM cells and tested for functional activity. As shown in Figure 6 (black bars), mutations of the conserved 5'-CC doublets of CArG-box 2 or CArG-box 1 resulted in a 55% or 70% decrease in transcriptional activity compared with pCAT-1346 wild type, respectively. A mutation within the core element of the NF-1 binding site also reduced the transcriptional activity by 73% compared with that of pCAT-1346. Mutations within the GC-rich element again reduced transcriptional activity by 80%, indicating that all 4 cis-elements are of importance for the serum-induced promoter activation. When transfected into Swiss-3T3 fibroblasts (Figure 6, open bars), none of the mutated cis-elements resulted in a significant gain or loss of transcriptional activity. These data demonstrate that specific cis-acting promoter elements provide a means not only for cell type-specific expression but also for the upregulation in response to serum-induced signaling pathways.

Thrombin and Serum Require Identical Positively Acting cis-Elements for Maximal Transcriptional Activity

Because we identified thrombin as a serum component that induces VSM redifferentiation, we analyzed CAT-activities of transiently transfected SM-MHC-CAT-fusion constructs with or without subsequent thrombin stimulation. As shown in Figure 7, transcriptional activity of pCAT-1346 after thrombin stimulation was 15-fold over promoterless pCAT-basic and 6-fold over unstimulated controls. (Note the difference in CAT activity between serum and thrombin stimulation). Deletion of CArG-box 2 and the GC-rich domain (pCAT-1182) reduced transcriptional activity by 40% compared with the complete pCAT-1346 construct. Further deletion of all 4 putative binding domains, including the CArG-box 1 (pCAT-1102), reduced the thrombin-induced transcriptional activity by 73%. As in serum-stimulated VSM cells, constructs harboring mutations within CArG-box 2 or CArG-box 1 resulted in a 60% and 75% decrease, respect-
tively, in transcriptional activity compared with pCAT-1346 wild type (Figure 7). Transfection of the same constructs in Swiss-3T3 fibroblasts and subsequent stimulation of an endogenously expressed PAR-1 receptor failed to enhance the SM-MHC promoter activity, confirming the cell type–specific transcriptional regulation of the SM-MHC promoter constructs (data not shown). In summary, serum and thrombin induce a transcriptional activation of the SM-MHC promoter via identical cis-elements.

**Discussion**

VSM cells respond to a wide array of humoral stimuli through G protein–coupled receptors and receptor tyrosine kinases. Membrane receptors in turn activate discrete intermediate signal transduction pathways, which ultimately alter inducible gene expression within the nucleus. However, the mechanisms whereby intracellular signaling cascades mediate alterations in smooth muscle cell gene expression are incompletely understood. In the present study, it is demonstrated that a continuous presence of serum or thrombin and an intact Ras/Raf/MEK/ERK signaling cascade with a sustained ERK phosphorylation kinetic are required for increasing and maintaining the contractile protein expression in neonatal rat vascular smooth muscle cells. Interestingly, cell-specific and serum- or thrombin-induced expression of SM-MHC appear to require the same cis-elements within the SM-MHC promoter.

The analysis of 5′-deletion constructs revealed that the highly conserved region in between nucleotides −1321 to −1095 of the rat SM-MHC promoter is required for maximal serum- or thrombin-induced activity of transiently transfected promoter constructs. This is in agreement with work by other

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**Figure 5.** Serum-induced expression of SM-MHC promoter CAT constructs in neonatal rat smooth muscle cells and in Swiss-3T3 fibroblasts. A series of 5′-end deletion constructs of the SM-MHC promoter subcloned into a CAT reporter vector were transiently transfected into neonatal VSM cells (filled bars) or Swiss-3T3 fibroblasts (open bars). The organization of each construct with respect to putative cis-elements is depicted. After transfection and serum starvation for 48 hours, cells were either left untreated (gray bars) or reexposed to serum (black and open bars) for another 48 hours. The CAT activity of each construct (±SEM) is depicted relative to the baseline CAT activity of a promoterless pCAT-basic construct. An SV40 promoter–driven CAT construct served as a positive control.

**Figure 6.** Mutational analysis of potential cis-elements within the rat SM-MHC promoter. VSM cells and Swiss-3T3 fibroblasts were transiently transfected with either wild-type pCAT-1346 or several altered SM-MHC constructs that were mutated within the context of pCAT-1346 (see Methods). The relative CAT-activities in serum-free medium (gray bars) or after serum stimulation for VSM cells (black bars) and Swiss-3T3 fibroblasts (open bars) are depicted as mean±SEM.

**Figure 7.** Thrombin-induced expression of SM-MHC promoter CAT constructs. Serum-starved VSM cells transiently transfected with the SM-MHC promoter constructs were either left untreated (gray bars) or stimulated with thrombin (2 U/mL) for 48 hours (black bars). CAT activities are depicted as mean±SEM.
laboratories focusing on the cell type–specific expression of SM-MHC. A detailed analysis of SM-MHC promoter constructs harboring mutations within the core binding sites of transactivating factors showed marked discrepancies with regard to their respective promoter activities. Madsen et al demonstrated that a mutation within the highly conserved NF-1 binding site was without any impact on the cell type–specific promoter activity, and most strikingly, a mutation within the GC-rich domain induced a promoter activity comparable to the SV40 promoter–driven control plasmid. In contrast, we observed a 50% to 80% drop of serum-induced promoter activity when any of the 4 cis-elements within the conserved region was either deleted or mutated. Although many factors such as differences in cell type (adult versus neonatal VSMC), phenotypic state, and grade of confluence may explain these discrepancies, it is difficult to reconcile these data. In addition, it is mandatory to verify that the endogenous SM-MHC gene is transcribed under similar culture conditions. In our hands, RNase protection assays and Western blots revealed that the endogenous SM-MHC gene is abundantly transcribed and later translated into protein under those exact culture conditions used for the promoter analysis. Thus, cell type–specific and serum- or thrombin-induced expression of SM-MHC appear to require the same cis-elements within the SM-MHC promoter, although the functional importance is different.

There is almost consensus about the importance of CArG-boxes in smooth muscle–specific gene expression. The CArG motif (CC(A/T-rich)GG) was initially described as the core sequence of the SRE within early-response genes such as c-fos and serves as the binding site for one of the MADS box transcription factors, namely, the SRF. CArG-boxes have been shown to direct developmental and tissue-specific expression of many muscle-specific genes, and almost all of the smooth muscle differentiation marker genes contain at least 2 CArG or CArG-like boxes that are required for cell-specific expression. Mutations within either one of the 2 CArG-boxes reduced not only the cell-specific expression, but also the serum- or thrombin-induced SM-MHC promoter activity in VSM cells. Thus, the question arises as to how such a common promoter element and an almost ubiquitously expressed transcription factor may confer cell-specific and/or agonist-inducible transcriptional control. Mack et al proposed that unique CArG-flanking sequences distinguish smooth muscle–selective gene expression and that there is SRF-dependent binding of a smooth muscle cell–selective multiprotein complex to the CArG-containing regions of the promoter. However, the assignment of such proteins is still lacking.

Cell type–specific gene expression is established through the combinatorial action of DNA-binding transcription factors, but is also frequently modulated by transcriptional coactivators that communicate between transactivators and the basal transcriptional complex formed on the promoter near the transcription initiation site. Typical members of this transcriptional coactivator family of proteins, the protein p300 and the cAMP-response element binding protein (CBP), interact with components of the basal transcriptional apparatus (eg, TFII B and TATA-binding protein) and diverse enhancer-binding proteins. The importance of such protein-protein interactions in transcriptional control of SM-MHC has been shown by Wada et al, focusing on the importance of the zinc finger transcription factor GATA-6. The authors demonstrated that coexpression of the rat SM-MHC promoter construct and GATA-6 in COS7 cells results in an agonist-independent activation of the promoter activity, which is further increased by additional p300 expression by 5-fold above the levels observed with either GATA-6 or p300 alone. Interestingly, Kitabayashi et al demonstrated that changes in the phosphorylation status of p300 correlate with the agonist-induced differentiation in embryonal carcinoma F9 cells. They speculated that differently phosphorylated forms of p300 may reflect a dual role for this protein in positive and negative regulation of transcription. Because the regulation of SM-MHC transcription appears to involve complex protein-protein and protein-DNA interactions, alterations in the phosphorylation status of SRF-associated proteins may thus play an important role in this scenario.

Interestingly, transcription factors such as Elk-1 and c-myc became phosphorylated within less than 3 minutes after serum stimulation in VSM cells, whereas increased SM-MHC transcription was delayed for several hours. Taking into account the delayed kinetics and the cell type specificity of contractile protein expression, it is tempting to propose the following hypothesis: serum- or thrombin-induced and Ras/Raf/MEK/ERK-dependent nuclear protein phosphorylation induces a cell type–specific de novo synthesis of as-yet-unidentified transcription factors and/or transcriptional coactivators. These proteins in turn become phosphorylated through sustained ERK1/2 activity and finally build up a transcription initiation complex that is required for transcription of contractile proteins. The following evidence supports this concept.

It has been demonstrated that in a fibroblast cell line (CCL39) thrombin induces a biphasic ERK1/2 phosphorylation kinetic and subsequent nuclear translocation of ERK1/2. Our data, however, revealed no thrombin-induced activity of a transiently transfected SM-MHC promoter construct in Swiss 3T3 fibroblasts, indicating that a sustained ERK1/2 phosphorylation is not sufficient to promote the SM-MHC transcription in an inadequate cellular context. On the other hand, in VSM cells the MEK-inhibitor PD 98059, given after the first phase of serum- or thrombin-induced ERK1/2 phosphorylation, abolished the second-phase ERK1/2 phosphorylation and prevented the SM-MHC transcription (data not shown), corroborating the concept that even within the appropriate cellular background, there is a strict requirement for a sustained ERK1/2 phosphorylation.

**Perspectives**

In summary, we have shown that the serum- or thrombin-induced transcription of SM-MHC requires an intact Ras/Raf/MEK/ERK signaling cascade with sustained ERK1/2 activity and phosphorylation of nuclear proteins. Because multiple cis-elements within the promoter are required for a serum- or thrombin-induced increase in SM-MHC expression, a multi-protein complex, including as-yet-ill-defined components, appears to be required for contractile protein expression in
VSM cells. Future work should be directed toward elucidating the nature and regulation of these unknown components.

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