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Calcineurin-GATA-6 pathway is involved in smooth muscle–specific transcription

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Intracellular calcium is one of the important signals that initiates the myogenic program. The calcium-activated phosphatase calcineurin is necessary for the nuclear import of the nuclear factor of activated T cell (NFAT) family members, which interact with zinc finger GATA transcription factors. Whereas GATA-6 plays a role in the maintenance of the differentiated phenotype in vascular smooth muscle cells (VSMCs), it is unknown whether the calcineurin pathway is associated with GATA-6 and plays a role in the differentiation of VSMCs. The smooth muscle–myosin heavy chain (Sm-MHC) gene is a downstream target of GATA-6, and provides a highly specific marker for differentiated VSMCs. Using immunoprecipitation Western blotting, we showed that NFATc1 interacted with GATA-6. Consistent with this, NFATc1 further potentiated GATA-6–activated Sm-MHC transcription. Induction of VSMCs to the quiescent phenotype caused nuclear translocation of NFATc1. In differentiated VSMCs, blockage of calcineurin down-regulated the amount of GATA-6-DNA binding as well as the expression of Sm-MHC and its transcriptional activity. These findings demonstrate that the calcineurin pathway is associated with GATA-6 and is required for the maintenance of the differentiated phenotype in VSMCs.

Introduction

In response to vascular injury, vascular smooth muscle cells (VSMCs)* undergo a dedifferentiation process referred to as phenotypic modulation, become proliferative, and act as a major component of thickened arterial intima. Thus, determining the molecular mechanisms of phenotypic modulation is important for understanding mechanisms of vascular disease such as atherosclerosis and restenosis after angioplasty. Phenotype modulation is associated with changes in the expression patterns of contractile proteins. For example, the expression of smooth muscle–myosin heavy chain (Sm-MHC), a well-characterized smooth muscle–specific contractile protein (Owens, 1995), decreases or is even absent in proliferating or dedifferentiated VSMCs (Nagai et al., 1989; Kuro-o et al., 1991; White et al., 1993). Therefore, in addition to the usefulness of Sm-MHC as a highly specific marker for the smooth muscle cell lineage, this protein is a potentially useful marker for specifying the factors involved in the proliferative/dedifferentiated or quiescent/differentiated SMC phenotype. Our recent study has shown that a zinc finger protein, GATA-6, binds to a GATA consensus motif in the rat Sm-MHC promoter and activates this promoter in a sequence-specific manner (Wada et al., 2000). The mutation of this element decreases the Sm-MHC transcriptional activity in differentiated VSMCs. GATA-6 is expressed in VSMCs and is rapidly down-regulated by mitogen stimulation (Perlman et al., 1998). Overexpression of GATA-6 in proliferating VSMCs induces cell cycle arrest with a concomitant increase in the expression of cyclin-dependent kinase inhibitor (Perlman et al., 1998). These findings demonstrated that GATA-6 is involved in the maintenance of the quiescent phenotype in differentiated VSMCs. However, the relationship of GATA-6 to upstream signaling pathways is unknown.

In the skeletal muscle cell lineage, one of the important signalings that initiates differentiation is intracellular calcium. The total cell calcium increases during skeletal muscle differentiation (Przybylski et al., 1989), whereas a decrease in intracellular calcium inhibits differentiation (Salzberg et al., 1995; Seigneurin-Venin et al., 1996). Recently, Friday et al. (2000) have shown that calcium- and calcineurin-dependent pathways are required for commitment of skeletal muscle cells to differentiation. Calcineurin is a calcium-calmodulin–

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*Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; CysA, cyclosporin A; EMSA, electrophoretic mobility shift assay; NFAT, nuclear factor of activated T cell; RSV, Rous sarcoma virus; Sm-MHC, smooth muscle–myosin heavy chain; VSMC, vascular smooth muscle cell.

Key words: smooth muscle; myosin; transcription; calcineurin; GATA-6

dependent serine-threonine phosphatase that activates transcription of the nuclear factor of activated T cell (NFAT) family, causing their translocation to the nucleus to initiate transcription of cytokines involved in the immune response (Batiuk and Halloran, 1997). The immunosuppressant drugs cyclosporin A (CysA) and FK506 bind the immunophilin cyclophilin, forming a complex that binds the calcineurin catalytic subunit and inhibits calcineurin's ability to activate NFAT transcription factors (Shaw et al., 1995; Loh et al., 1996). Molkentin et al. (1998) have reported that a zinc finger protein GATA-4 interacts and cooperates with the NFAT family to activate the transcription of cardiac-specific genes such as B-type natriuretic peptide gene. These findings prompted us to investigate whether a calcineurin pathway connected with GATA-6 is involved in smooth muscle–specific transcription. The present study investigated the role of the calcineurin-GATA-6 pathway for the smooth muscle–specific transcription in differentiated VSMCs.

Results

GATA-6 interacts with NFATc1

To determine whether GATA-6 and NFATc1 associate in vivo, we performed immunoprecipitation, followed by Western blotting. COS7 cells were transfected with an expression plasmid encoding NFATc1 (pNFATc1) and one encoding wild-type GATA-6 (pwtGATA-6). 48 h later, nuclear extracts derived from the transfected cells were immunoprecipitated with the anti-NFATc1 antibody as a positive control (Fig. 1, lane 1), with normal rabbit IgG as a negative control (Fig. 1, lane 2), or with the anti–GATA-6 antibody (Fig. 1, lane 3). These immunoprecipitates were then subjected to Western blotting using the anti-NFATc1 antibody. As shown in Fig. 1 (top panel, lane 3), an interaction between wild-type GATA-6 and NFATc1 was observed even after extensive washing. To determine whether the interaction between GATA-6 and NFATc1 occurs through the zinc finger domain of GATA-6, COS7 cells were transfected with an expression plasmid encoding mutant GATA-6 defective for the zinc finger domain (pΔZFGATA-6) instead of pwtGATA-6. Nuclear extracts were subjected to immunoprecipitation followed by Western blotting as described above. As shown in Fig. 1 (top panel, lane 6), no interaction was observed between mutant GATA-6 and NFATc1. The anti-NFATc1 antibody was stripped, and then the membrane was reprobed with the anti–GATA-6 antibody. As shown in Fig. 1 (bottom panel), expression levels of wild-type and mutant GATA-6 before immunoprecipitation were similar among all lanes (lanes 1–6). In addition, as shown in Fig. 1 (middle panel), wild-type and mutant GATA-6 were similarly immunoprecipitated by the anti–GATA-6 antibody (lanes 3 and 6). These findings indicate that the zinc finger domain of GATA-6 is required for the interaction with NFATc1.

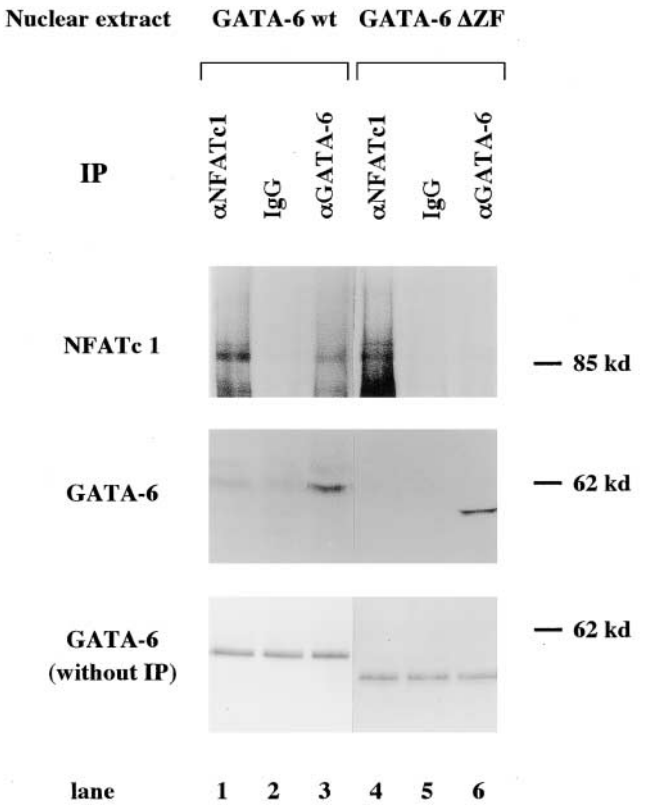

Figure 1. **NFATc1 interacts with GATA-6 through its zinc finger domain.** COS7 cells were cotransfected with 6 μg of pNFATc1 and 6 μg of either pwtGATA-6 or pΔZFGATA-6 as indicated. Nuclear extracts derived from these cells were immunoprecipitated with the anti-NFATc1 antibody (lanes 1 and 4), with control IgG (lanes 2 and 5), and with the anti–GATA-6 antibody (lanes 3 and 6). After electrophoresis and electroblotting, the membrane containing immobilized immunocomplexes was subjected to Western blotting using the anti-NFATc1 antibody (top panel). The anti-NFATc1 antibody was stripped and then the membrane was reprobed with the anti–GATA-6 antibody (middle panel). Nuclear extracts before immunoprecipitation were also subjected to Western blotting using the anti–GATA-6 antibody (bottom panel).

GATA-6 and NFATc1 coactivate the Sm-MHC promoter

To determine whether GATA-6 and NFATc1 functionally cooperate in vivo, we performed transient transfection assays in VSMCs. We cotransfected a chloramphenicol acetyltransferase (CAT) expression vector driven by the 1346-bp Sm-MHC promoter together with a eukaryotic expression plasmid encoding wild-type GATA-6 (pwtGATA-6), alone, or in combination with a vector encoding NFATc1 (pNFATc1). The total amount of DNA was kept constant by addition of pCMVβ-gal. The transfection efficiency was monitored by cotransfected pRSVluc activity. 48 h later, we measured the Sm-MHC reporter activity. As shown in Fig. 2, the coexpression of NFATc1 and wild-type GATA-6 induced a significant elevation of the level of the reporter (lane 5) above the levels observed with either NFATc1 (lane 4) or GATA-6 (lane 2) alone. To determine whether coactivation by GATA-6 and NFATc1 requires the zinc finger domain of GATA-6, we transfected an expression plasmid encoding mutant GATA-6 lacking the zinc finger domain (pΔZFGATA-6) instead of pwtGATA-6. Compatible with the inability of mutant GATA-6 to interact with NFATc1, the coexpression of NFATc1 and mutant GATA-6 did not coactivate the Sm-MHC promoter (lane 6). Therefore, the zinc finger domain of GATA-6 is required for coactivation of the Sm-MHC promoter by GATA-6 and NFATc1 in VSMCs.

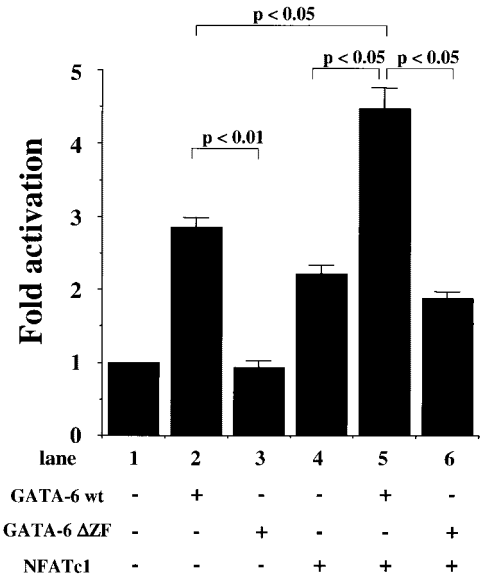

Figure 2. **NFATc1 activates the GATA-6–dependent Sm-MHC transcription.** VSMCs were transfected with 2 μg of a Sm-MHC CAT reporter; 0.1 μg of pRSVluc; 2 μg of either pNFATc1 or pCMVβ-gal; and 2 μg of pwtGATA-6, pΔZFGATA-6, or pCMVβ-gal as indicated. 48 h later, the relative CAT activities (CAT/luc) were determined. The results are expressed as fold activation of the normalized CAT activities (CAT/luc) relative to that produced by cotransfection with the control β-galactosidase expression vector. The data shown are the mean ± standard error of two independent experiments, each performed in duplicate.

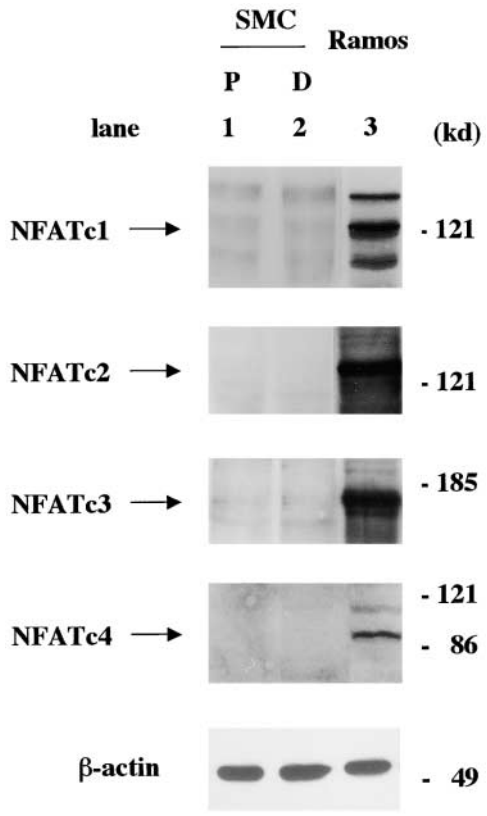

Figure 3. **Endogenous expression of NFATc1–4 in VSMCs.** VSMCs were cultured in proliferation (P) medium (lane 1) or differentiation (D) medium (lanes 2). Lysates from these cells and Ramos cells as a positive control were subjected to Western blotting using the anti-NFATc1 antibody, the anti-NFATc2 antibody, the anti-NFATc3 antibody, the anti-NFATc4 antibody and the anti–β-actin antibody, as indicated.

Induction of the differentiated phenotype causes nuclear translocation of NFATc1

To examine which NFAT family members are expressed in VSMCs, we performed Western blotting using human Burkitt's lymphoma-derived Ramos cells as a positive control. As shown in Fig. 3, Ramos cells abundantly expressed all of NFATc1-4. In contrast, VSMCs expressed a significant amount of NFATc1 and a small amount of NFATc3. NFATc2 and NFATc4 were not detected by our Western blotting. To further examine changes in the subcellular localization of NFATc1 in VSMCs by induction of the differentiated phenotype, immunofluorescence staining was performed. VSMCs were cultured in 5% serum with growth factors to maintain the proliferative phenotype, or in 1% serum without growth factors to induce the differentiated phenotype, in the presence or absence of CysA (0.5 μg/ml). As shown in Fig. 4, NFATc1 was detected in the cytoplasm of nearly all VSMCs of the proliferative phenotype (A). However, induction of these cells to the differentiated phenotype markedly changed this localization and caused the nuclear translocation of NFATc1 (Fig 4 B). This translocation was reversed by CysA (Fig. 4 C) which blocks calcineurin. These findings suggest that calcineurin is activated during VSMC differentiation.

Blockage of calcineurin down-regulates the Sm-MHC transcriptional activity in differentiated VSMCs

We investigated whether calcineurin plays a role in the transcriptional activation of Sm-MHC gene during VSMC differentiation. VSMCs were transfected with a luciferase (luc) gene driven by the Sm-MHC promoter (Fig. 5 A, pwtSm-MHCluc), or that containing the GATA site mutation which abolishes the binding of GATA-6 (Fig. 5 B, pmutSm-MHCluc). VSMCs were then cultured in 5% serum with growth factors to maintain the proliferative phenotype (Fig. 5, A, lanes 1–3, and B, lane 1), or in 1% serum without growth factors to induce the differentiated phenotype (Fig. 5, A, lanes 4–6, and B, lane 2). As shown in Fig. 5 A (lanes 1 and 4), the transcriptional activity of the Sm-MHC promoter increased by induction of the differentiated phenotype. However, as shown in Fig. 5 B (lanes 1 and 2), the mutation of the GATA site within this promoter abolished the activation. This is compatible with our previous report and suggests a role of GATA-6 for VSMC differentiation. To examine the role of calcineurin in the transcriptional activation of the wild-type Sm-MHC promoter, we administered differentiation medium CysA (0.5 μg/ml) or FK506 (1 nM) which blocks calcineurin. As shown in Fig. 5 A (lanes 5 and 6), CysA and FK506 inhibited the activation. However, neither CysA nor FK506 affected the wild-type Sm-MHC promoter activity in proliferation medium (Fig. 5 A, lanes 2 and 3). The β-actin promoter activity did not differ between proliferation medium and differentiation medium (Fig. 5 C, lanes 1 and 4) and was not affected either by CysA (Fig. 5 C, lanes 2 and 5) or by FK506 (Fig. 5 C, lanes 3 and 6). These findings demonstrate that calcineurin activation is required

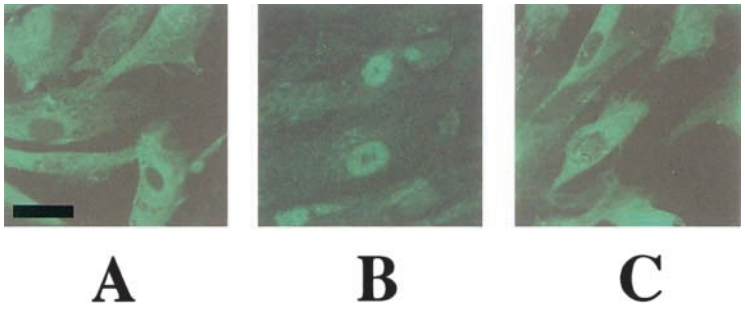

Figure 4. **Induction of the differentiated phenotype of VSMCs translocates NFATc1 into the nucleus in a calcineurin-dependent manner.** VSMCs were cultured in medium with growth factors and 5% serum to maintain the proliferative phenotype (A) or in medium with 1% serum to induce the differentiated phenotype in the presence (C) or absence (B) of CysA (0.5 μg/ml) for 48 h and subjected to immunofluorescence using the anti-NFATc1 antibody. Bar, 20 μm.

for the transcriptional activation of the Sm-MHC gene during VSMC differentiation.

Blockage of calcineurin down-regulates GATA-6-DNA binding in differentiated VSMCs

To determine whether GATA-6-DNA binding in VSMCs is modulated by the cellular phenotype change, and if so, whether calcineurin activation is involved in this change, electrophoretic mobility shift assays (EMSAs) were performed. VSMCs were cultured in 5% serum with growth factors to maintain the proliferative phenotype (Fig. 6, A, lane 1 and C, lane 1), or in 1% serum without growth factors (Fig. 6, A, lanes 2–5, and C, lanes 2 and 3) to induce the differentiated phenotype. Nuclear extracts were probed with a radiolabeled oligonucleotide containing the Sm-MHC GATA site in the presence or absence of competitor DNAs (Fig. 6 A). Competition EMSAs revealed that a retarded band represented specific binding, as evidenced by the fact that it was competed out by an excess of unlabeled wild-type Sm-MHC GATA oligonucleotide (Fig. 6 A, lane 4), but not by the same amount of an oligonucleotide containing the Sm-MHC GATA site with a mutation (Fig. 6 A, lane 5). To further confirm that the retarded band represents an interaction of the probe with GATA-6, we performed a supershift experiment. As shown in Fig. 6 B, the retarded band disappeared with administration of the anti–GATA-6 antibody (lane 3), but not with either the anti–GATA-4 antibody (lane 2) or normal rabbit IgG (lane 1). These data indicate that the retarded band contains the complex that specifically immunoreacts with the anti–GATA-6 antibody. Notably, the amount of GATA-6-DNA binding increased in nuclear extracts from VSMCs with the differentiated phenotype (Fig. 6 A, lane 2), as compared with those from VSMCs with the proliferative phenotype (Fig. 6 A, lane 1). In contrast, the amount of SP-1-DNA binding did not differ between the proliferative and differentiated phenotypes of VSMCs (Fig. 6 C, lanes 1 and 2). To determine whether the up-regulation of GATA-6-DNA binding in VSMCs is calcineurin-dependent, VSMCs were cultured in differentiation medium in the presence or absence of a therapeutic concentration (0.5 μg/ml) of CysA, which blocks calcineurin. The amount of GATA-6-DNA binding in differentiated VSMCs was down-regulated by CysA (Fig. 6 A, lane 3), whereas that of SP-1-DNA binding was not altered by CysA (Fig. 6 C, lane 3). The amount of GATA-6-DNA binding normalized with that of SP-1-DNA binding was quantified and is shown in Fig. 6 D. The relative DNA binding amount of GATA-6 was significantly reduced by CysA (Fig. 6 D, lane 3). These findings sug-

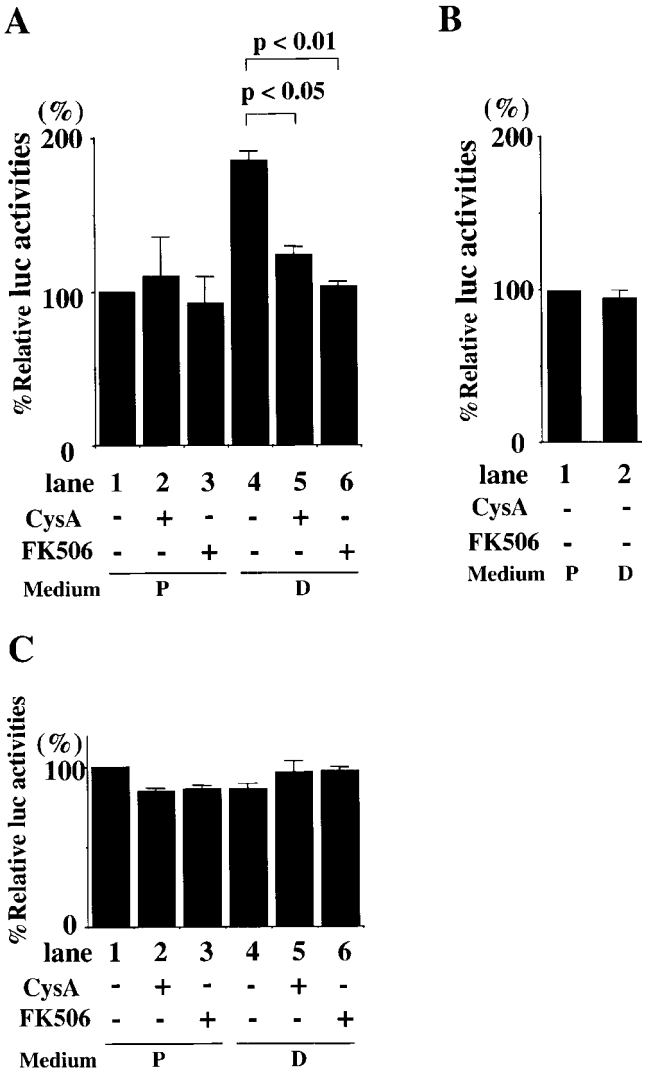

Figure 5. **CysA and FK506 inhibited the activation of the Sm-MHC promoter by the induction of differentiated phenotype in VSMCs.** Two μg of pwtSm-MHCluc (A), pmutSm-MHCluc (B) or pβ-actinluc (C), and 0.1 μg of pRSVCAT were cotransfected into VSMCs which were subsequently cultured in proliferation (P) medium (A and C, lanes 1–3, and B, lane 1) or differentiation (D) medium (A and C, lanes 4–6, and B, lane 2) in the presence of CysA (A and C, lanes 2 and 5, 0.5 μg/ml) or FK506 (A and C, lanes 3 and 6, 1 nM), or in their absence (A and C, lanes 1 and 4, and B, lanes 1 and 2) as indicated. 48 h later, these cells were collected and the relative luc activity (luc/CAT) was determined. The relative luc activity in the proliferative VSMCs was set at 100% in each experiment. The data shown are the mean ± standard error of two independent experiments, each performed in duplicate.

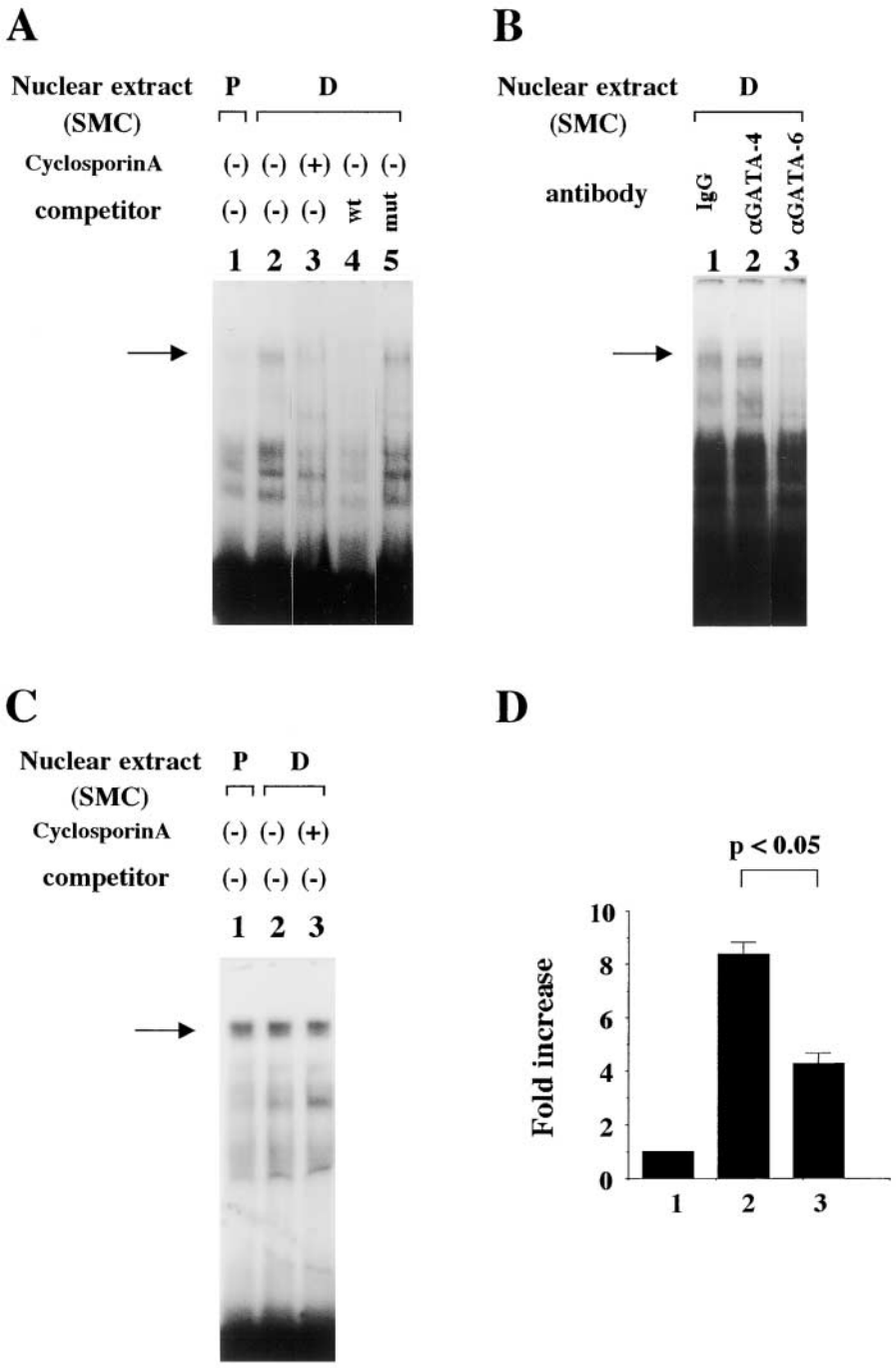

Figure 6. **The amount of GATA-6-DNA binding is up-regulated by induction of the differentiated phenotype.** (A) Nuclear extracts were obtained from VSMCs with proliferative (P) or differentiated (D) phenotype. These extracts were probed with a radiolabeled oligonucleotide containing the Sm-MHC GATA site. Unlabeled competitor DNAs were present at a 100-fold molar excess as indicated: (lane 4) wild-type Sm-MHC GATA (wt); (lane 5) Sm-MHC GATA with a mutation (mut). The arrow indicates the complex corresponding to the GATA-specific interaction between the Sm-MHC GATA site and GATA-6. B, EMSA studies using the Sm-MHC-GATA probe were performed in nuclear extracts from VSMCs with differentiated (D) phenotype. Equal amounts of the anti–GATA-6 antibody (lane 3), the anti–GATA-4 antibody (lane 2), or normal rabbit IgG (lane 1) were added to the binding mixture. (C) EMSA studies using the probe for Sp-1 were performed in nuclear extracts from VSMCs with proliferative (P) or differentiated (D) phenotype as indicated. The arrow indicates the complex corresponding to the interaction between the probe and Sp-1. (D) The amount of GATA-6-DNA binding in A (lanes 1–3) and that of SP-1-DNA binding in C was quantified by densitometry using NIH image 1.61 and the relative DNA binding amount (Sm-MHC GATA/SP-1) was determined. The relative DNA binding amount in the proliferative VSMCs was set at 1.0 in each experiment. Values are the mean ± standard error for three independent experiments.

gest that the up-regulation of GATA-6-DNA binding in VSMCs is, at least in part, calcineurin dependent.

To examine changes in the expression of GATA-6 in VSMCs by induction of the differentiated phenotype, Western blot analysis was performed. As shown in Fig. 7, the expression of GATA-6 was slightly up-regulated by induction of the differentiated phenotype (lane 2) as well as NFATc1. The administration of CysA (lane 3, 0.5 μg/ml) and FK506 (lane 4, 1 nM) did not affect their expression in differentiated VSMCs. Expression levels of GATA-6 and NFATc1 were quantified and normalized with the β-actin expression level. As shown in Fig. 8, the normalized expression levels of GATA-6 and NFATc1 were not significantly reduced by CysA or FK 506 (lanes 3 and 4). Notably, NFATc1 was converted to the high-mobility form after induction of the differentiated phenotype (Fig. 7, lane 2). This conversion was reversed by administration of CysA (Fig. 7, lane 3) and FK506 (Fig. 7, lane 4), which block calcineurin. These findings might suggest that NFATc1 is dephosphorylated by induction of the differentiated phenotype in VSMCs.

Expression of markers for differentiated VSMCs requires a calcineurin pathway

To examine whether a calcineurin pathway is required for the expression of marker proteins for differentiated VSMCs such as Sm-MHC and Sm-α-actin, we performed Western blot analysis. VSMCs were cultured in proliferation medium or

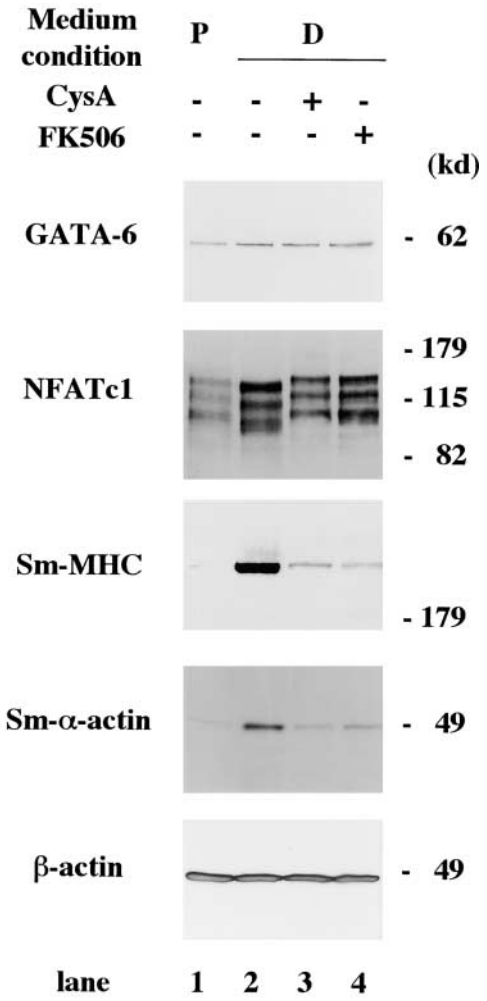

Figure 7. **Endogenous expression of GATA-6, NFATc1, and smooth muscle–specific proteins in VSMCs.** VSMCs were cultured in proliferation (P) medium (lane 1) or differentiation (D) medium (lanes 2–4) in the presence of CysA (lane 3, 0.5 μg/ml) or FK506 (lane 4, 1 nM), or in their absence (lanes 1 and 2). Lysates from these cells were subjected to Western blotting using the anti–GATA-6 antibody, the anti-NFATc1 antibody, the anti–Sm-MHC antibody, the anti–Sm-α-actin antibody, and the anti–β-actin antibody, as indicated.

differentiation medium in the presence or absence of 0.5 μg/ml of CysA and 1 nM of FK506. As shown in Fig. 7, the expression of Sm-MHC and Sm-α-actin were induced during differentiation. CysA and FK506 repressed the endogenous expression of Sm-MHC and Sm-α-actin in VSMCs with the differentiated phenotype. However, the levels of ubiquitously expressed β-actin protein were similar in VSMCs with the proliferative and differentiated phenotypes, and were not altered by CysA or FK506. Relative expression levels of smooth muscle–specific proteins normalized with the β-actin expression level were shown in Fig. 8. Relative expression levels of Sm-MHC and Sm-α-actin were significantly reduced by CysA (Fig. 8, lane 3) and FK506 (Fig. 8, lane 4).

To further examine the role of calcium and calcineurin during VSMC differentiation, we performed immunocytochemical analysis. VSMCs were cultured in proliferation medium (Fig. 9

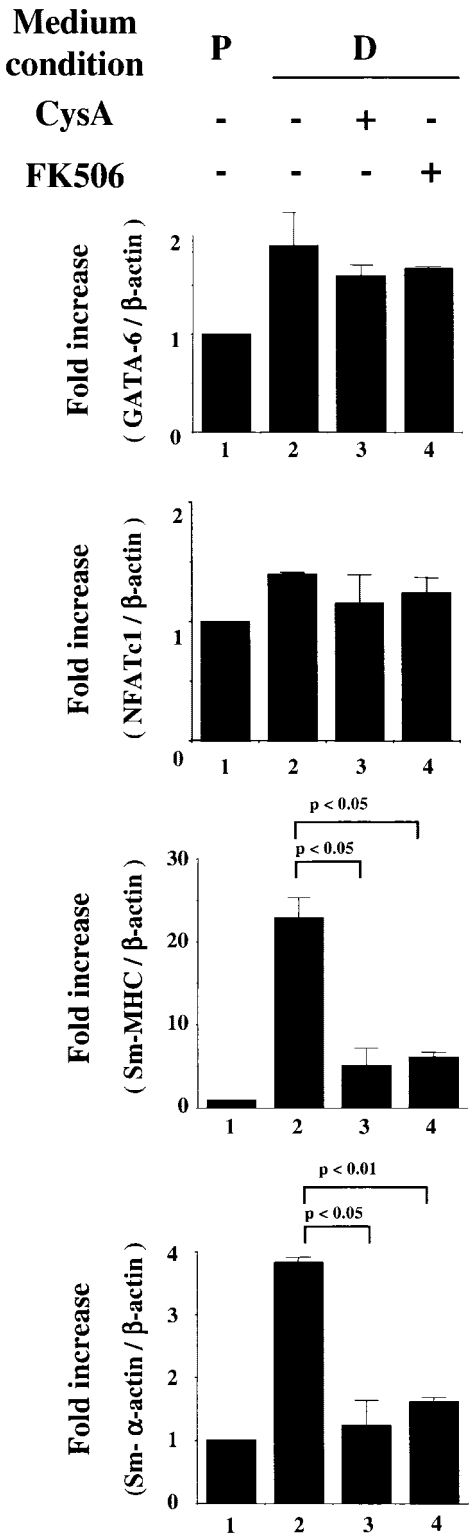

Figure 8. **Quantitative analysis of the endogenous expression of GATA-6, NFATc1, and smooth muscle–specific proteins in VSMCs.** Immunoblots in Fig. 7 were quantified by densitometry using NIH image 1.61 and the relative expression levels of these proteins normalized with β-actin expression were determined. The relative expression level in VSMCs with a proliferative phenotype was set at 1.0. Values are the mean ± standard error for the three independent experiments.

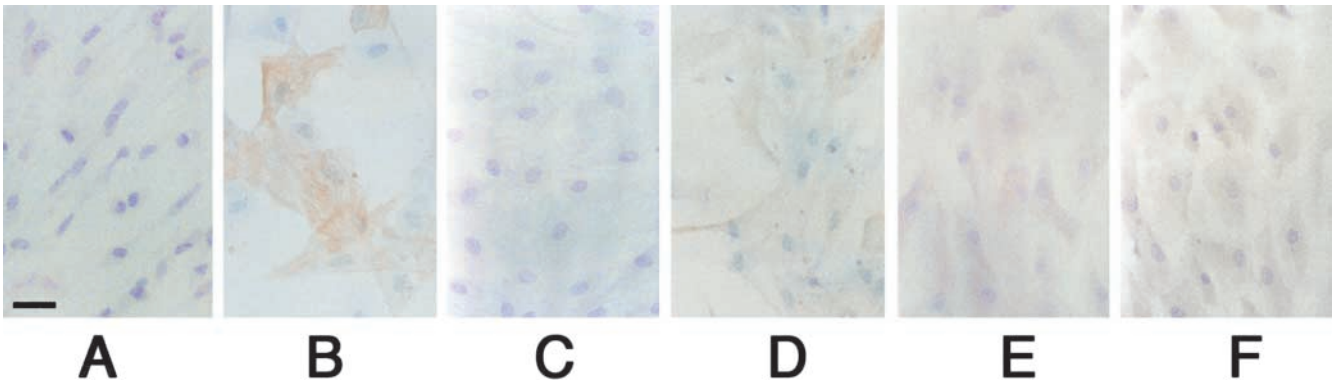

Figure 9. **CysA and FK506 repressed the endogenous expression of the Sm-MHC in differentiated VSMCs.** VSMCs were cultured in proliferation medium (A) or in differentiation medium in the presence of CysA (D, 0.5 μg/ml), FK506 (E, 1 nM) or nifedipine (F, 1 μM), or in their absence (B and C). These cells were subjected to immunostaining with the anti–Sm-MHC antibody. The primary antibody was further incubated with a secondary antibody conjugated with peroxidase (brown signals). The primary antibody was replaced with normal mouse IgG in C. The nuclei were counterstained with hematoxylin. Bar, 20 μm.

A) or in differentiation medium in the presence of CysA (Fig. 9 D), FK506 (Fig. 9 E), or nifedipine (Fig. 9 F), or in their absence (Fig. 9, B and C). These cells were then immunostained with the anti–Sm-MHC antibody. As shown in Fig. 9, brown positive signals indicating Sm-MHC were absent in cells with the proliferative phenotype (A), and prominent in cells with the differentiated phenotype (B). The replacement of the primary antibody with normal mouse IgG abolished the signals (Fig. 9 C). Both CysA (Fig. 9 D) and FK506 (Fig. 9 E) decreased the signals in VSMCs with the differentiated phenotype. In addition, a calcium antagonist, nifedipine, decreased the signals (Fig. 9 F). These findings further support the idea that calcium and calcineurin play a role during VSMC differentiation.

Discussion

The mechanisms that regulate the differentiation of VSMCs and their phenotype modulation are poorly understood. Previous studies have shown that a zinc finger transcription factor, GATA-6, is expressed in VSMCs and is required for the maintenance of their differentiated phenotype (Morrisey et al., 1996, 1997; Narita et al., 1996; Suzuki et al., 1996; Perlman et al., 1998; Wada et al., 2000). The present study investigated whether a calcineurin pathway is connected with GATA-6 and plays a role in the smooth muscle–specific transcription. We showed that GATA-6 interacted with NFATc1, a target transcription factor of calcineurin. GATA-6 and NFATc1 cooperate during the transcription of the Sm-MHC gene. Induction of VSMCs to the quiescent phenotype caused nuclear translocation of NFATc1. In differentiated VSMCs, blockage of calcineurin down-regulated the amount of GATA-6-DNA binding as well as the expression of Sm-MHC and its transcriptional activity.

Calcineurin is a calcium-calmodulin–dependent serine-threonine phosphatase that activates transcription of the NFAT family. The NFAT family comprises at least four members: NFATc (NFATc1), NFATp (NFATc2), NFAT3 (NFATc4), and NFAT4 (NFATc3) (McCaffrey et al., 1993; Northrop et al., 1994; Ho et al., 1995; Hoey et al., 1995; Masuda et al., 1995; Park et al., 1996). These factors bind the consensus DNA sequence through a Rel homology domain (Rooney et al., 1994; Hoey et al., 1995). VSMCs expressed preferentially NFATc1 (Boss et al., 1998) and a small amount of NFATc3, whereas NFATc2 and NFATc4 were not detectable by Western blotting. Although the present study demonstrates the role of NFATc1 in smooth muscle–specific transcription, our results do not rule out possible roles of NFATc2-4 in VSMC differentiation as a recent study reported that vascular development in NFATc3/4–null mice was impaired (Graef et al., 2001). Our results demonstrate that GATA-6 interacted with NFATc1, and that this interaction required the zinc finger domain of GATA-6. Because zinc finger domains are highly conserved among GATA-4/5/6, these results are compatible with those in a previous study that a second zinc finger domain of GATA-4 interacts with NFAT3 (NFATc4). Compatible with the interaction between GATA-6 and NFATc1, these two factors synergistically activated the smooth muscle–specific Sm-MHC promoter. The rat Sm-MHC promoter contains NFATc1 consensus sequences, GGAAAA, at −519/−514 relative to the transcription start site as well as a GATA-6 binding site at sequences −810/−805. However, NFATc1 alone was not sufficient to activate the Sm-MHC promoter in COS cells in which GATA-6 was not expressed (unpublished data). These findings suggest that the binding of NFATc1 to an NFATc1 site within the rat Sm-MHC promoter is weak. At present, the precise mechanism by which NFATc1 activates GATA-6–mediated Sm-MHC transcription is unclear. However, NFATc1 did not affect the Sm-MHC transcriptional activity mediated by a mutant GATA-6 unable to interact with NFATc1. These findings suggest that coactivation of Sm-MHC transcription by GATA-6 and NFATc1 requires the interaction of these two proteins.

Nuclear NFATc1 interacts with zinc finger transcription factors GATA-4 in cardiac myocytes and GATA-6 in VSMCs. We showed that induction of the differentiated phenotype increases the amount of GATA-6-DNA binding in a calcineurin-dependent manner. One of the mechanisms for the up-regulation of GATA-6-DNA binding may be an increase in the quantity of GATA-6 itself. The other possible mechanism is that calcineurin activation modifies GATA-6

posttranslationally as we reported that phosphorylation of GATA-4 increases its DNA binding activity (Morimoto et al., 2000). Further study will be needed to clarify these mechanisms. Interestingly, the transcriptional coactivator p300 interacts not only with GATA-6 but also with NFAT (Gracia-Rodriguez and Rao, 1998). The GATA-6/p300 complex in VSMCs is also up-regulated during induction of the differentiated phenotype (Wada et al., 2000). These findings suggest that p300, together with GATA-6 and NFATc1, form a large complex in differentiated VSMCs, and that this complex plays a role in the maintenance of the differentiated phenotype. Our preliminary data suggest that interaction of GATA-6 with p300 increases the DNA binding activity of GATA factors. However, further studies are needed to clarify the precise relationship between the calcineurin-GATA-6 pathway and p300 during VSMC differentiation.

It has been suggested that calcium- and calcineurin-dependent pathways are required for skeletal myogenesis (Salzberg et al., 1995; Seigneurin-Venin et al., 1996; Friday et al., 2000). Compatible with this suggestion, the expression of endogenous Sm-MHC in differentiated VSMCs was down-regulated by a calcium antagonist, nifedipine. Calcineurin dephosphorylates the NFAT family members, causing their translocation to the nucleus (Batiuk and Halloran, 1997). The present study demonstrated that induction of VSMCs to the differentiated phenotype caused nuclear translocation of NFATc1. A physiological concentration of CysA or FK506, which blocks calcineurin activity, inhibited the endogenous expression of Sm-MHC in differentiated VSMCs. These findings suggest that calcineurin pathway is activated during differentiation of VSMCs and required for smooth muscle–specific Sm-MHC expression. Taken together with the fact that NFATc1 and GATA-6 coactivated the Sm-MHC promoter, it seems that a calcineurin pathway is connected with GATA-6 and plays a role in the maintenance of differentiated phenotype in VSMCs. Further studies regarding the role of the calcineurin pathway in VSMC growth and differentiation in vivo will facilitate the development of new therapeutic strategies for vascular diseases in humans.

Materials and methods

Primary VSMC culture and transfection

Primary human aortic VSMCs were obtained from Kurabo Industries Ltd. and cultured in the medium provided by the manufacturer supplemented with epithelial growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml), insulin (5 μg/ml), and 5% FBS. For induction of the differentiated phenotype, VSMCs (passages 6) were cultured in medium with heparin (30 μg/ml) instead of the above growth factors and with 1% FBS. For transfection, VSMCs (passages 6) were washed twice with serum-free media and then transfected with a total of <5 μg of DNA in 60-mm plates using LipofectAMINE PLUS (Life Technologies, Inc.) according to the manufacturer's recommendation. After a 3-h incubation with DNA-PLUS-LipofectAMIN complex, VSMCs were cultured in media to induce the differentiated or proliferative phenotype in the presence or absence of CysA (0.5 μg/ml) or FK506 (1 nM) for 48 h. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed for luc and CAT assays as described (Hasegawa et al., 1997; Morimoto et al., 2000). Luc and CAT activities were determined in the same cell lysate as described previously (Hasegawa et al., 1997; Morimoto et al., 2000). The relative luc activity was calculated from the ratio of luc minus background to CAT minus background.

Immunocytochemical staining

VSMCs (passages 6) were fixed in 3.0% formaldehyde in PBS for 10 min at room temperature. Immunocytochemical staining for NFATc1 was performed using the indirect immunofluorescense method. Cells were incubated with the anti-NFATc1 monoclonal antibody (cat # sc-7294; Santa Cruz Biotechnology) at a dilution of 1:100. Signals of NFATc1 were detected using the anti–mouse FITC-conjugated secondary antibody (ICN Biomedicals) at a dilution of 1:500 for 45 min.

Immunocytochemical staining was performed by use of the indirect immunoperoxidase method as described previously (Hasegawa et al., 1993). Endogenous Sm-MHC was detected by the anti–Sm-MHC monoclonal antibody (Sigma-Aldrich) (1:250 dilution), followed by peroxidase-conjugated $F(ab')_2$ fragment of goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) (1:500 dilution).

Plasmid constructs

The plasmid construct pSm-MHCCAT consists of the bacterial chloramphenicol acetyltransferase (CAT) cDNA driven by 1346-bp of the rat Sm-MHC gene promoter (Madsen et al., 1996) and was a gift from Dr. Gray K. Owens (University of Virginia, Charlottesville, VA). The firefly luc cDNA driven by 836-bp rat Sm-MHC promoter (pwtSm-MHCluc), and that with mutation of the distal (−810/−805) GATA element (pmutSm-MHCluc) were described previously (Wada et al., 2000). pRSVluc and pRSVCAT contain luc and CAT cDNA, respectively, driven by Rous sarcoma virus (RSV) long terminal repeat sequences (Hasegawa et al., 1997; Morimoto et al., 2000). pβ-actinluc contains luc cDNA driven by avian cytoplasmic β-actin promoter (Hasegawa et al., 1997). The expression plasmids encoding wild-type human GATA-6 (pwtGATA-6) and mutant human GATA-6 which lacks the zinc-finger domain (pΔZFGATA-6) were kindly donated by Dr. Kenneth Walsh (Tufts University, Boston, MA) (Suzuki et al., 1996). Plasmid pCMVβ-gal carries the cytomegalovirus promoter/enhancer fused to β-galactosidase cDNA (Kamei et al., 1996). The murine NFATc1 expression plasmid (pNFATc1) was a generous gift from Dr. Ken-ichi Arai, University of Tokyo, Japan (Pan et al., 1997). Plasmids were purified by anion exchange chromatography (QIAGEN), quantified by the measurement of OD_{260}, and examined on agarose gels stained with ethidium bromide prior to use.

COS7 cell culture and transfection

COS7 cells (African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The cells were washed twice with serum-free medium and then transfected with 6 μg of pNFATc1 and 6 μg of either pwtGATA-6 or pΔZFGATA-6 in 100-mm plates using LipofectAMINE (Life Technologies, Inc.). After a 5-h incubation with DNA-LipofectAMINE complex, the cells were washed twice with serum-free medium and further incubated in the medium with 10% FBS. 48 h later, nuclear extracts were prepared from these cells.

Immunoprecipitation and Western blotting

200 μg of the extracts from COS cells was immunoprecipitated using the mouse monoclonal antibody against NFATc1 (cat # sc-7294; Santa Cruz Biotechnology), the rabbit polyclonal anti–GATA-6 antibody (cat # sc-9055; Santa Cruz Biotechnology) or normal rabbit IgG in low stringency buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% SDS, Nonidet P-40, 1 mM EDTA, 10 mg/ml aprotinin and leupeptin, and 0.5 mg PMSF) for 16 h at 4°C and incubated with protein G (Sigma-Aldrich) beads for 1 h at 4°C. The precipitate was washed four times in the same buffer, resuspended in 20 μl of SDS-lysis buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% SDS,1 mM dithiothreitol), heated to 95°C for 2 min, electrophoresed on an SDS-polyacrylamide gel (8%), transferred to an Immobilon membrane, reacted with the anti-NFATc1 antibody (cat # sc-7294), and subsequently detected using horseradish peroxidase-conjugated anti–mouse IgG (Jackson ImmunoResearch Laboratories). Signals were detected using an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Antibodies were stripped using 2% SDS, 62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol at 60°C for 30 min. The membrane was reprobed with goat polyclonal anti–GATA-6 antibody (cat # sc-7244; Santa Cruz Biotechnology) for 1 h at 25°C. Detection was with horseradish peroxidase-conjugated anti–goat IgG (Jackson ImmunoResearch Laboratories) and enhanced chemiluminescence Western blotting detection system.

Western blotting using the anti–GATA-6 antibody (cat # sc-7244), the anti–Sm-MHC antibody (Sigma-Aldrich), the anti–Sm-α-actin antibody (Daco Laboratories), the anti–β-actin antibody (Sigma-Aldrich), the ant-NFATc1 antibody (cat # sc-7294), the anti-NFATc2 antibody (cat # sc-7296), the anti-NFATc3 antibody (cat # sc-8405) and the anti-NFATc4 antibody (cat # sc-1153) was performed with 25 μg of cell lysates prepared from VSMCs or Ramos cells derived from human Burkitt's lymphoma (cat # sc-2216).

EMSAs

Nuclear extracts were prepared as described previously from VSMCs which were cultured in 5% FBS containing medium with growth factors or

in the 1% low-serum medium without growth factors. Double-stranded oligonucleotides for GATA-6 were designed based on the rat Sm-MHC upstream sequences that contained a GATA motif (−815/−810 relative to the transcription start site). The sequences of the sense strand of these oligonucleotides were as follows: Sm-MHC-GATA, 5′-ACTTTAGGGACGTAATCATCACAGGGAAATCAA-3′; mutSm-MHC-GATA, 5′-ACTTTAGGGACGTAtagATCACAGGGAAATCAA-3′. Oligonucleotides were synthesized by Greiner Inc. and purified by SDS-polyacrylamide gel electrophoresis. Sp-1 consensus double-stranded oligonucleotides were purchased from Santa Cruz Biotechnology (cat # sc-2502).

EMSAs were carried out as described previously (Hasegawa et al., 1997) at 4°C for 20 min in 15-μl reaction mixtures containing 10 μg of nuclear extract, 0.25 ng (>20,000 cpm) of radiolabeled double-stranded oligonucleotide, 500 ng of poly (dI-dC), 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 37.5 mM KCl, and 4% Ficoll 400. For cold competition experiments, a 100-fold molar excess of unlabeled competitor oligonucleotides was included in the binding reaction mixture. Protein-DNA complexes were separated by electrophoresis on 4% nondenaturing polyacrylamide gels in 0.25 × TBE (100 mM Tris, 100 mM boric acid, and 2 mM EDTA) at 4°C.

Statistical analysis

Data are presented as means ± standard error. Statistical comparisons were performed using unpaired two-tailed Student's *t* tests or analysis of variance with Scheffe's test where appropriate, with a probability value <0.05 taken to indicate significance.

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