The transcriptional corepressor MITR is a signal-responsive inhibitor of myogenesis

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Activation of muscle-specific genes by members of the myocyte enhancer factor 2 (MEF2) and MyoD families of transcription factors is coupled to histone acetylation and is inhibited by class II histone deacetylases (HDACs) 4 and 5, which interact with MEF2. The ability of HDAC4 and -5 to inhibit MEF2 is blocked by phosphorylation of these HDACs at two conserved serine residues, which creates docking sites for the intracellular chaperone protein 14-3-3. When bound to 14-3-3, HDACs are released from MEF2 and transported to the cytoplasm, thereby allowing MEF2 to stimulate muscle-specific gene expression. MEF2-interacting transcription repressor (MITR) shares homology with the amino-terminal regions of HDAC4 and -5, but lacks an HDAC catalytic domain. Despite the absence of intrinsic HDAC activity, MITR acts as a potent inhibitor of MEF2-dependent transcription. Paradoxically, however, MITR has minimal inhibitory effects on the skeletal muscle differentiation program. We show that a substitution mutant of MITR containing alanine in place of two serine residues, Ser-218 and Ser-448, acts as a potent repressor of myogenesis. Our findings indicate that promyogenic signals antagonize the inhibitory action of MITR by targeting these serines for phosphorylation. Phosphorylation of Ser-218 and Ser-448 stimulates binding of 14-3-3 to MITR, disrupts MEF2:MITR interactions, and alters the nuclear distribution of MITR. These results reveal a role for MITR as a signal-dependent regulator of muscle differentiation.

The MyoD and MEF2 families of transcription factors are key regulators of muscle gene expression (reviewed in ref. 1). Members of the MyoD family share homology in a basic helix-loop-helix motif that mediates binding to the E-box consensus sequence (CANNTG) in the control regions of muscle-specific genes (reviewed in ref. 2). The four myocyte enhancer factor 2 (MEF2) factors MEF2A, MEF2B, MEF2C, and MEF2D belong to the MADS (MCM1, Agamous, Deficiens, Serum response factor) box superfamily of transcription factors and bind an A/T-rich sequence associated with muscle genes. Myogenic basic helix-loop-helix and MEF2 factors interact through their DNA binding domains to establish a specific transcriptional code for muscle gene activation.

Recent studies have revealed the importance of chromatin remodeling in the activation of skeletal muscle gene expression by myogenic basic helix-loop-helix and MEF2 factors. A central mechanism for chromatin remodeling involves changes in nucleosomal histone acetylation (reviewed in ref. 3). The acetylation of histones, catalyzed by histone acetyltransferases, results in chromatin relaxation and transcriptional activation. Histone acetyltransferase activity is intrinsic to numerous transcriptional coactivators, such as p300 and CBP, which interact with myogenic basic helix-loop-helix and MEF2 factors and are required for skeletal muscle differentiation (4–7).

The gene-activating functions of histone acetyltransferases are antagonized by histone deacetylases (HDACs), which catalyze the deacetylation of histones, resulting in transcriptional repression. HDACs are categorized into two general classes based on size, sequence homology and protein–protein interactions (reviewed in ref. 8). The Class I HDACs, HDAC1, HDAC2, HDAC3, and HDAC8, are expressed ubiquitously, whereas the Class II HDACs, HDAC4, HDAC5, HDAC6, and HCDAC7, are expressed predominantly in heart, brain, and skeletal muscle. Class II HDACs are also distinguished by an amino-terminal extension that mediates interaction with MEF2 and consequent repression of MEF2 target genes and myogenesis (9–13).

Consistent with its ability to inhibit MEF2 activity and muscle differentiation, HDAC5 is localized to the nucleus of undifferentiated myoblasts and is translocated to the cytoplasm concomitant with activation of the muscle differentiation program (14). Calcium/calmodulin-dependent protein kinase (CaMK) signaling, which stimulates myogenesis (13), phosphorylates two sites in the amino-terminal extension of HDAC5 (14), resulting in association with the intracellular chaperone protein 14-3-3. Interaction of phospho-HDAC5 with 14-3-3 leads to disruption of MEF2:HDAC complexes and subsequent export of HDAC5 from the nucleus (14, 15).

MEF2-interacting transcription repressor (MITR), also called HDAC-related protein (HDRP), shares high homology with the amino-terminal extensions of HDAC4 and -5 and interacts with MEF2, but it lacks an HDAC catalytic domain (16, 17). Nevertheless, MITR inhibits activation of MEF2-dependent reporter genes by recruiting other HDACs and the carboxyl-terminal binding protein corepressor (16–18).

In the present study, we investigated the regulation and function of MITR during skeletal muscle differentiation. Our results demonstrate that MITR is an inhibitor of skeletal myogenesis. However, the ability of MITR to block muscle differentiation depends on its phosphorylation status. Phosphorylation of two serine residues, Ser-218 and Ser-448, inactivates MITR by promoting 14-3-3-dependent release from MEF2 and consequent relocalization of MITR within the nucleus. These results suggest that MITR plays an important role as a transcriptional regulator of muscle differentiation and intranuclear sensor of signals that govern the myogenic program.

Materials and Methods

RNA Isolation and Analysis. Semiquantitative reverse transcription-PCR was performed as described (13). Northern blot analysis was performed by using an adult mouse tissue RNA blot (CLONTECH) and a probe derived from the full-length coding region of mouse MITR (18). For RNA *in situ* hybridization on paraffin sections, riboprobes corresponding to the 3′ untranslated region of mouse MITR were synthesized (Maxiscript kit; Ambion, Austin, TX) and hybridized with embryo sections as previously described (19).

Cell Culture and Transfections. COS, 10T1/2, and C2 cells were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin. COS and 10T1/2 fibroblasts were transfected by using Fugene 6 reagent (Roche Molecular Biochemicals), and C2 myoblasts were transfected by using Lipo-

Abbreviations: MEF2, myocyte enhancer factor-2; CaMK, calcium/calmodulin-dependent protein kinase; HDAC, histone deacetylase; MITR, MEF2-interacting transcription repressor.

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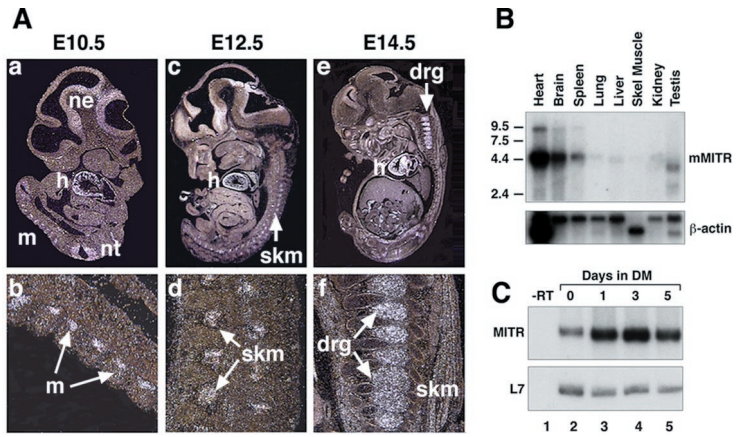

Fig. 1. Expression of mouse MITR mRNA. (*A*) RNA *in situ* hybridization of E10.5, E12.5, and E14.5 mouse embryo sagittal sections. Dark-field images reveal MITR expression (white signal) during mouse development. At E10.5, MITR expression predominates in the developing heart (h). MITR expression is also evident in the neuroepithelium (ne) of the brain, the neural tube (nt), and somite myotomes (m). At E12.5, MITR expression is found in the intervertebral skeletal muscle (skm). At E14.5, prominent expression of MITR was detected in dorsal root ganglia (drg) and adjacent skeletal muscle (skm). High magnification images of myotomes, intervertebral muscle, and dorsal root ganglia are shown (*b*, *d*, and *f*). (*B*) Northern blot analysis of MITR transcripts in adult mouse tissues (*Top*). The blot was hybridized with a β-actin probe to control for RNA loading (*Bottom*). (*C*) RNA was isolated from undifferentiated C2 myoblasts in growth medium (0) or C2 cells that had been exposed to differentiation medium (DM) for 1, 3, or 5 days. Semiquantitative reverse transcription-PCR was performed using primers specific for MITR (*Top*) or L7 as a control for cDNA template abundance (*Bottom*). A sample from a cDNA synthesis reaction lacking reverse transcriptase (RT) was used as a control for genomic DNA contamination.

fectamine PLUS reagent (GIBCO/BRL). For myogenic differentiation of C2 and MyoD-transfected 10T1/2 cells, growth medium was replaced with DMEM containing 2% horse serum.

Plasmids. Expression constructs for MITR, HDAC4, HDAC5, and constitutively active CaMKI have been described previously (14, 18, 20, 21). Full-length MITR was fused to green fluorescent protein (GFP) in the pEGFP-C1 expression plasmid (CLONTECH). Myc-tagged MITR:HDAC fusion proteins were generated in the pcDNA3.1 plasmid (Invitrogen) by fusing either amino acids 2–482 of HDAC1 (MITR:HDAC1), amino acids 610- 1084 of human HDAC4 (MITR:HDAC4C), or amino acids 646-1122 of human HDAC5 (MITR:HDAC5C) to the carboxyl terminus of MITR. MITRΔMEF lacks the MEF2 binding domain of MITR between amino acids 135 and 152. Site-directed mutagenesis was performed by using the QuikChange kit (Strategene). Deletion constructs were generated by PCR using PFU Turbo polymerase (Stratagene).

Coimmunoprecipitation and Immunoblotting. Immunoprecipitation experiments were performed as previously described (15) by using anti-FLAG affinity resin (Sigma) or protein A-Sepharose (Zymed)-bound anti-Myc Ab (rabbit polyclonal, A-14; Santa Cruz). Precipitated proteins were separated by SDS/PAGE, transferred to PVDF membranes, and immunoblotted with either anti-Myc Ab (rabbit polyclonal, A-14 or mouse monoclonal, 9E10; Santa Cruz), pan anti-14-3-3 Ab (mouse monoclonal, H-8; Santa Cruz Biotechnology), or an anti-FLAG Ab (mouse monoclonal, M2; Sigma). Proteins were visualized with a chemiluminescence system (Santa Cruz Biotechnology).

Indirect Immunofluorescence and Analysis of GFP Fusion Proteins. COS and 10T1/2 cells were grown on glass coverslips, fixed in 10% Formalin, and stained in PBS containing 3% BSA and 0.1% Nonidet P-40. Primary antibodies were against FLAG (mouse monoclonal, M2; Sigma) and Myc (rabbit polyclonal, A-14; Santa Cruz). Primary and secondary (Vector Laboratories) Abs were used at a dilution of 1:200. To determine the localization of MITR:GFP fusion proteins in transiently transfected C2 cells, cells growing on glass coverslips were washed with PBS, fixed with 10% Formalin, and mounted on slides using Vectashield (Vector Laboratories).

Results

Expression of Mouse MITR mRNA. As an initial step toward investigating the potential involvement of MITR in muscle development, we examined its expression during mouse embryogenesis by *in situ* hybridization. MITR transcripts were detected specifically in heart, skeletal muscle, and neural lineages as early as E10.5 (Fig. 1*A a* and *b*). At that stage, MITR expression was observed throughout the developing atrial and ventricular chambers of the heart and in skeletal muscle within the somite myotomes. Transcripts were also seen in the neural tube and neuroepithelium of the developing brain. This localized expression pattern continued at E12.5 (Fig. 1*A c* and *d*) and E14.5, when expression in the dorsal root ganglia also became apparent (Fig. 1*A e* and *f*).

Northern blot analysis of RNA from adult mouse tissues showed that MITR transcripts were expressed at high levels in the heart, in which a prominent transcript of ≈4.4 kb and a less abundant transcript of ≈9 kb were detected (Fig. 1*B*). MITR transcripts were also abundant in brain and spleen, but were detected at only very low levels in lung, liver, skeletal muscle, and kidney. A transcript of ≈3.5 kb was detected in testis. This tissue distribution appears to differ from that of *Xenopus* MITR (16), which is expressed at extremely low levels in all adult tissues, and human MITR, which is expressed at comparable levels in heart, brain, and skeletal muscle and at very low levels in other tissues (17).

MITR transcripts were present in undifferentiated C2 myoblasts (Fig. 1*C*, lane 2), and were elevated during differentiation into myotubes (lanes 3–5). This increase in expression is consistent with prior results demonstrating that *Xenopus* MITR expression increases as somite myotomes mature (16).

Failure of MITR to Block Skeletal Myogenesis. Ectopic expression of MyoD is sufficient to convert nonmuscle cells into skeletal muscle, as measured by the formation of multinucleated myotubes that express myosin heavy chain. Previously, we showed that coexpression of either HDAC4 or -5 with MyoD resulted in repression of the muscle differentiation program (13). Given

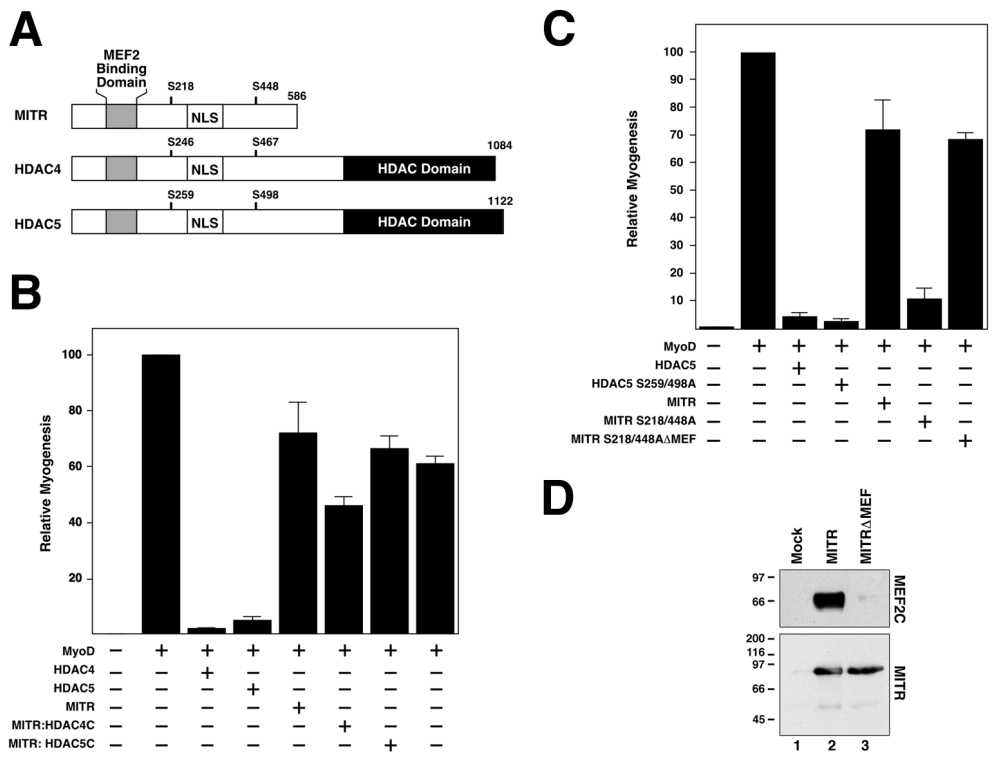

Fig. 2. Regulation of skeletal muscle differentiation by MITR. (*A*) Schematic depictions of MITR, HDAC4, and HDAC5. Each repressor contains a conserved MEF2 binding domain and a nuclear localization signal (NLS), but MITR lacks a carboxyl-terminal catalytic domain (HDAC Domain). The conserved regulatory serines are shown. (*B* and *C*) 10T1/2 fibroblasts were cotransfected with expression vectors for MyoD and the indicated MITR proteins (0.5 μg each). The MITR:HDAC fusion proteins are described in *Materials and Methods*. MITR S218/448A contains alanines in place of Ser-218 and Ser-448. ΔMEF indicates that amino acids 135–152 in the MEF2 binding domain of MITR have been removed. Cells were transferred to differentiation medium 2 days posttransfection and stained with anti-myosin Abs after 4 additional days in culture. Myogenesis is depicted as the percentage of myosin-positive cells in MITR.

that MITR, like HDAC4 and -5, inhibits MEF2-dependent transcription (16–18) and MEF2 cooperates with MyoD to drive muscle differentiation (2), we examined the potential of MITR to antagonize MyoD-dependent myogenesis. Consistent with our prior findings (13), HDAC4 and -5 potently inhibited MyoD-dependent conversion of 10T1/2 fibroblasts into muscle (Fig. 2*B*). However, despite being expressed at comparable levels to these HDACs (data not shown), MITR failed to efficiently block myogenesis.

Catalytically inactive mutants of HDAC4 and -5 exhibit impaired antimyogenic activity (13). Thus, we reasoned that the inability of MITR to block muscle formation could be due to a lack of intrinsic HDAC activity. To test this hypothesis, we generated chimeric MITR proteins containing the HDAC domains of either HDAC1, -4, or -5 fused to the carboxyl terminus of MITR. However, these MITR chimeras also failed to efficiently block myogenesis (Fig. 2*B*). These findings suggest that the amino-terminal region of MITR is functionally distinct from those of HDAC4 and -5, despite the high homology between these proteins.

Identification of Regulatory Serines in MITR. CaMK signaling stimulates myogenesis by phosphorylating HDAC4 and -5 at two conserved serine residues in their amino-terminal extensions (14). Phosphorylation of these sites promotes binding of 14-3-3, which disrupts MEF2:HDAC complexes and stimulates HDAC nuclear export (15). An HDAC5 mutant containing alanines in place of these serines inhibits myogenesis more efficiently than the wild-type protein because it is resistant to CaMK signaling (Fig. 2*C*) (14). These two CaMK phosphorylation sites are conserved in MITR at amino acids 218 and 448 (Fig. 2*A*).

We hypothesized that substitution of Ser-218 and Ser-448 with unphosphorylatable amino acids might enhance the ability of MITR to inhibit myogenesis. We therefore generated an MITR mutant in which these residues were converted to alanine. Strikingly, this mutant, MITR S218/448A, acted as a potent repressor of MyoD-dependent skeletal myogenesis (Fig. 2*C*). Inhibition of myogenesis by the S218/448A mutant depended on association with MEF2, because a double-alanine mutant containing a deletion of 18 amino acids required for MEF2 binding (S218/448A ΔMEF2) failed to efficiently block myogenesis (Fig. 2 *C* and *D*). These results suggest that phosphorylation of Ser-218 and Ser-448 in MITR antagonizes its antimyogenic activity.

Phosphorylation of MITR Stimulates Binding to 14-3-3 and Disrupts MITR:MEF2 Interactions. One explanation for the inability of MITR to inhibit myogenesis and the serine-to-alanine mutations to confer antimyogenic activity to MITR would be that the wild-type protein is phosphorylated at Ser-218 and Ser-448 during muscle differentiation. By analogy to HDAC5, this would be predicted to recruit 14-3-3 to MITR and prevent its association with MEF2 (12, 15). To address this possibility, we performed coimmunoprecipitation experiments to determine whether MITR associates with 14-3-3. Wild-type MITR associated with endogenous 14-3-3 in transfected COS cells (Fig. 3*A*, lane 1), and CaMK signaling markedly enhanced this association (lane 2). Likewise, the single-alanine substitution mutants, S218A and S448A, efficiently associated with 14-3-3 in response to CaMK (lanes 3–6). In contrast, there was no detectable binding of the S218/448A mutant to 14-3-3 in either the absence or presence of CaMK signaling (lanes 7 and 8). Of note, the binding of 14-3-3

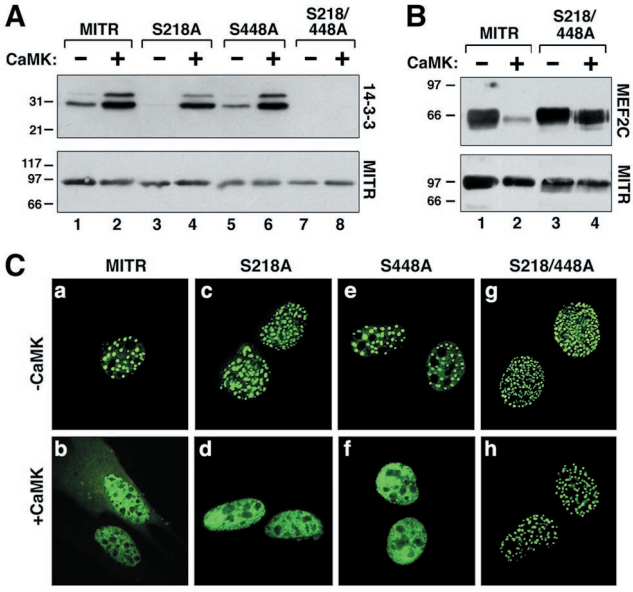

Fig. 3. Signal-dependent association of MITR with 14-3-3. (*A*) COS cells were transfected with expression vectors for the indicated Myc-tagged versions of MITR in the absence or presence of a plasmid for activated CaMKI (1 μg). To compensate for CaMK-mediated increases in expression from the cytomegalovirus-driven expression plasmids, cells receiving CaMKI were transfected with 0.5 μg of MITR plasmid, compared with 1 μg in those lacking CaMKI. Ectopic MITR was immunoprecipitated from cell lysates with an anti-Myc Ab and associated endogenous 14-3-3 was detected by immunoblotting with a pan anti-14-3-3 Ab (upper panel). The membrane was reprobed with anti-Myc Ab to reveal MITR (*Bottom*). (*B*) COS cells were transfected with expression vectors for FLAG-tagged derivatives of the indicated MITR protein and Myc-tagged MEF2C in the absence or presence of a plasmid for activated CaMKI (1 μg), as in *A*. FLAG-tagged proteins were immunoprecipitated from cell lysates and associated MEF2C was measured by immunoblotting with anti-Myc Ab (*Upper*). The membrane was reprobed with anti-FLAG Ab to reveal MITR (*Bottom*). (*C*) C2 cells were transfected with expression vectors encoding the indicated MITR:GFP fusion protein in the absence or presence of a constitutively active hemagglutinin-tagged version of CaMKI (0.5 μg each). After 24 h, cells were fixed and subjected to indirect immunofluorescence with an anti-hemagglutinin primary Ab and a Texas red-conjugated secondary Ab, and GFP fluorescence was analyzed. All photographs were taken at ×64 under oil immersion.

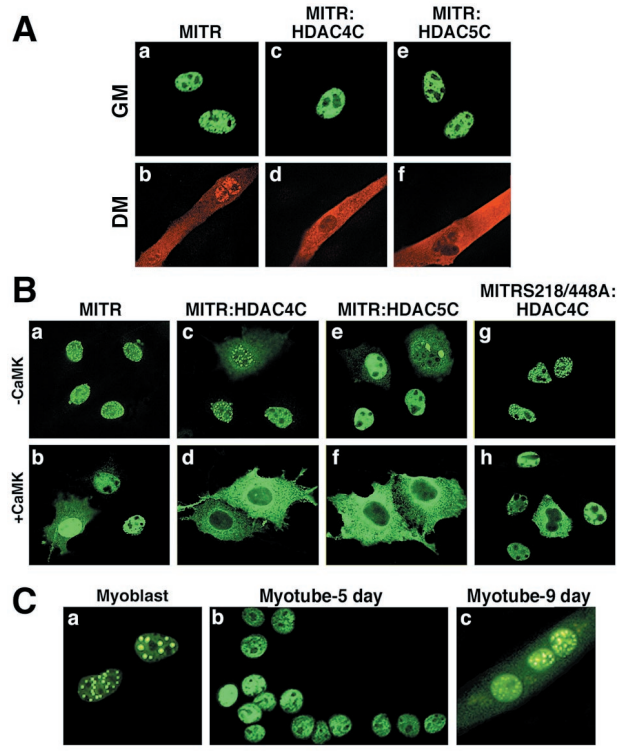

Fig. 4. Promyogenic signals alter the subnuclear distribution of MITR. (*A*) 10T1/2 fibroblasts were cotransfected with expression plasmids for MyoD and Myc-tagged versions of the indicated MITR protein (0.5 μg each) (see *Materials and Methods*). Cells were maintained in growth medium (GM) for 2 additional days and either fixed with Formalin or transferred to differentiation medium (DM) for 4 additional days before fixation. The subcellular distribution of MITR was determined by indirect immunofluorescence using an anti-Myc primary Ab and either fluorescein-GM- or Texas red-DM-conjugated secondary antibodies. Myogenic differentiation of MITR-positive cells was confirmed by staining for myosin heavy chain (data not shown). (*B*) COS cells were transfected with plasmids encoding Myc-tagged versions of wild-type MITR or the indicated MITR fusion protein in the absence or presence of a plasmid for constitutively active CaMKI (0.5 μg each). MITR was detected by immunofluorescence using an anti-Myc Ab and a fluorescein-conjugated secondary Ab. (*C*) C2 skeletal myoblasts were transfected with an expression vector for an MITR:GFP fusion protein (1 μg). Two days after transfection, cells were shifted to differentiation medium for the indicated number of days (*b* and *c*). Localization of MITR in myoblasts (*a*) was determined 1 day posttransfection in cells at 50% confluence.

to MITR detected in the absence of CaMK signaling (lane 1) appears to be the result of basal phosphorylation at Ser-218, because disruption of this site blocked CaMK-independent binding of 14-3-3 to MITR (lane 3). These results demonstrate that MITR associates with 14-3-3 and that this binding depends on phosphorylation of MITR at Ser-218 and Ser-448.

We next determined the role of these sites in regulating the association of MITR with MEF2. As shown in Fig. 3*B*, both wild-type MITR and the S218/448A mutant efficiently coimmunoprecipitated with MEF2C in the absence of CaMK signaling (lanes 1 and 3). In the presence of activated CaMK, the amount of MEF2C associated with MITR was significantly reduced (lane 2), whereas the level bound to the S218/448A mutant was largely unaltered (lane 4). These findings suggest that CaMK signaling stimulates phosphorylation of Ser-218 and Ser-448, leading to 14-3-3-mediated disruption of MEF2:MITR complexes and activation of downstream target genes.

Phosphorylation of Ser-218 and Ser-448 Alters the Nuclear Distribution of MITR. In light of the ability of CaMK to stimulate nuclear export of HDAC4 and -5, we examined the consequences of CaMK signaling on the subcellular distribution of MITR. MITR was localized to discrete nuclear structures in undifferentiated C2 myoblasts (Fig. 3*Ca*). In the presence of activated CaMK, MITR remained nuclear in ≈90% of CaMK-expressing cells, although it was no longer localized in a punctate pattern, but was distributed diffusely throughout the nucleus (Fig. 3*Cb*). In the remaining cells, increased cytoplasmic MITR staining, often in a punctate pattern, was detected as a result of CaMK expression (data not shown).

In the absence of CaMK signaling, the single-alanine mutants, S218A and S448A, and the double-alanine mutant, S218/448A, were localized to nuclear speckles (Fig. 3*C c*, *e*, and *g*). In the presence of activated CaMK, the single-alanine mutants were expressed throughout the nucleus in a pattern that was indistinguishable from wild-type MITR (Fig. 3*C d* and *f*), whereas the S218/448A mutant remained localized to discrete nuclear speckles (Fig. 3*Ch*). These results further suggest that both Ser-218 and Ser-448 of MITR serve as targets for CaMK signaling, and that phosphorylation of either site is required to relocalize MITR within the nucleus, presumably through a 14-3-3-dependent mechanism.

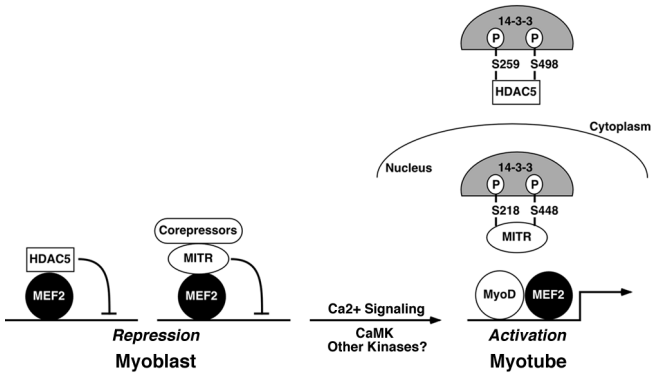

Fig. 5. A model for signal-dependent regulation of myogenesis by MITR and HDAC5. MITR and HDAC5 repress muscle-specific genes by association with MEF2. Repression of MEF2 activity by MITR requires association with corepressors, including carboxyl-terminal binding proteins and HDACs. In response to CaMK signaling, MITR and HDAC5 are phosphorylated at two serines, resulting in binding to 14-3-3 and disruption of MEF2 interactions. HDAC5 is subsequently translocated to the cytoplasm by virtue of a carboxyl-terminal nuclear export sequence. MITR remains in the nucleus, but with altered subnuclear distribution.

MITR Is a Target for Promyogenic Signals. The finding that substitution of Ser-218 and Ser-448 with alanine converted MITR into a potent repressor of myogenesis (Fig. 2*C*) suggested that promyogenic signals might counteract the inhibitory action of MITR by targeting these sites for phosphorylation. To address this hypothesis, we examined the subcellular localization of MITR during muscle differentiation. Initial experiments were performed in 10T1/2 fibroblasts ectopically expressing MyoD. As in C2 cells, MITR was localized to discrete nuclear bodies in proliferating, undifferentiated 10T1/2 cells expressing MyoD (Fig. 4*Aa*). However, MITR was found throughout the nuclei of 10T1/2 cells that had been converted into skeletal myotubes (Fig. 4*Ab*). Increased cytoplasmic staining of MITR was also evident in myotubes.

To address the potential roles of Ser-218 and Ser-448 in the relocalization of MITR during myogenic conversion of 10T1/2 fibroblasts, we used MITR fusion proteins containing carboxyl-terminal sequences from either HDAC4 or -5. Due to the presence of an HDAC-derived nuclear export sequence, these chimeric proteins undergo CaMK-dependent nuclear export (Fig. 4*B c–f*) (unpublished results). Nuclear export of the MITR fusion proteins is also dependent on phosphorylation of Ser-218 and Ser-448 of MITR (Fig. 4*B g* and *h*). As such, nuclear exclusion of these fusion proteins serves as an indirect readout of MITR phosphorylation status.

In proliferating, undifferentiated 10T1/2 cells, the MITR:HDAC chimeric proteins were exclusively nuclear (Fig. 4*A c* and *e*). However, in differentiated myotubes, these proteins were largely cytoplasmic, mimicking the localization pattern observed in CaMK-expressing cells (Fig. 4 *Bd* and *Bf*). Because the cytoplasmic localization of the MITR:HDAC fusion proteins depends on phosphorylation of Ser-218 and/or Ser-448 (Fig. 4*Bh*), the results suggest that these serines are subject to inducible phosphorylation during muscle differentiation.

We next examined the localization of wild-type MITR in differentiated C2 myotubes. In contrast to what was observed in undifferentiated myoblasts (Fig. 4*Ca*), MITR was found throughout the nuclei of myotubes exposed to differentiation medium for 5 days (Fig. 4*Cb*), mimicking the effect of CaMK signaling on MITR localization. Surprisingly, MITR relocalized to discrete nuclear bodies in older myotubes exposed to differentiation medium for 9 days (Fig. 4*Cc*). These findings suggest that Ser-218 and Ser-448 of MITR serve as targets for promyogenic signaling pathways and that activation of these pathways may occur transiently. The sensitivity of MITR to promyogenic signals likely explains the paradoxical findings showing that MITR can block MEF2 activity in undifferentiated cells, but fails to inhibit skeletal myogenesis.

Discussion

The results of this study demonstrate that the MITR corepressor can function as a potent inhibitor of skeletal myogenesis. However, the antimyogenic activity of MITR is under strict signal-dependent control. Promyogenic signals appear to antagonize the inhibitory action of MITR by targeting Ser-218 and Ser-448 for phosphorylation, thereby creating docking sites for the intracellular chaperone 14-3-3. Binding of 14-3-3 to MITR promotes myogenesis by disrupting MEF2:MITR complexes and altering the subnuclear distribution of the repressor, freeing MEF2 to activate target genes involved in myogenesis. These results support a role for MITR as a signal-dependent regulator of muscle differentiation. A model consistent with our results is shown in Fig. 5.

MITR: Unique or Overlapping Functions with Class II HDACs? Our results suggest remarkable similarity between MITR and class II HDACs in terms of their function and the mechanisms by which they are regulated. Indeed, like HDAC4 and -5, MITR is a repressor of MEF2 activity and of skeletal myogenesis. Furthermore, by analogy with HDAC4 and -5, MITR contains two regulatory serine residues that are targets for CaMK signaling, and phosphorylation-dependent binding of 14-3-3 to these sites disrupts MEF2:MITR interactions. These results suggest that MITR and class II HDACs may serve similar functions *in vivo*. However, the distinct subcellular localization of MITR compared with HDAC4 and -5 suggests that this repressor may also perform functions that distinguish it from HDACs.

HDAC5 shuttles from the nucleus to the cytoplasm when myoblasts are triggered to differentiate (14). In contrast, in response to myogenic signaling, MITR moves from discrete nuclear foci to an even distribution throughout the nucleus. The inability of MITR to shuttle to the cytoplasm is likely due to the absence of a carboxyl-terminal nuclear export sequence, which we have identified in class II HDACs (unpublished results). The retention of MITR in the nuclei of differentiated myotubes suggests that this repressor may perform MEF2-independent functions in the nuclear compartment.

It is possible that under certain conditions, MITR contains a *cis*-acting catalytic domain and thereby functions and/or is regulated in a unique manner. Indeed, analysis of human genome sequence reveals the presence of coding region for a putative HDAC domain ≈50 kb downstream of the MITR sequence on chromosome 7, and this putative MITR HDAC domain possesses the class II HDAC-specific nuclear export sequence (Val-X-X-X-X-X-Leu-X-Val) (unpublished results). Notably, a fraction of MITR becomes localized to the cytoplasm of differentiated C2 myotubes. The mechanism responsible for this relocation from the nucleus remains to be determined. One possibility is that MITR is exported to the cytoplasm in a complex with HDAC4 or -5, with which it interacts (18). Alternatively, binding of 14-3-3 to MITR may block nuclear entry of the repressor by disrupting its association with the nuclear import factor, importin α (23).

MITR is localized to discrete nuclear speckles in undifferentiated cells. The precise composition and function of these nuclear domains remains unclear. In this regard, a recent study described the existence of novel nuclear structures, termed matrix-associated deacetylase bodies, composed of multiple proteins, including class II HDACs and the SMRT corepressor (24). Because MITR is capable of associating with class II HDACs (18), it is possible that MITR is also targeted to

matrix-associated deacetylases. However, in our studies, MITR consistently exhibited a different pattern of nuclear localization than class II HDACs.

Differential Inhibition of Myogenesis by Class II HDACs and MITR. Previously, we showed that an HDAC5 mutant containing alanine in place of Ser-259 and Ser-498 is resistant to CaMK signaling and acts as a potent repressor of skeletal myogenesis (14). However, wild-type HDAC5 also inhibits myogenesis, albeit not as efficiently as the serine-to-alanine mutant. Consistent with this, we show here that the MITR S218/448A mutant efficiently inhibits skeletal muscle development. Paradoxically, however, wild-type MITR has only minimal antimyogenic activity, and we were unable to confer antimyogenic activity to MITR by appending a deacetylase domain to its carboxyl terminus. A possible explanation for the differing abilities of wild-type HDAC5 and MITR to block myogenesis is that MITR is more sensitive than HDAC5 to promyogenic signaling pathways that target the regulatory serines for phosphorylation.

Signal-Dependent Phosphorylation of MITR. Our results demonstrate that MITR is a CaMK-responsive transcriptional repressor. However, the existence of distinct MITR kinases with substrate specificity similar to CaMK remains possible. Regardless of the identity of this kinase, in the myogenic lineage, it would need to be subject to stringent signal-dependent control, because inappropriate engagement of this enzyme in proliferating myoblasts would likely result in premature stimulation of MEF2 target genes and precocious muscle differentiation.

Class II HDACs and MITR become phosphorylated and disengage from MEF2 when skeletal myoblasts are triggered to differentiate by removal of mitogens from the extracellular environment. Thus, a unique feature of the HDAC/MITR kinase is that it is activated under conditions normally associated with down-regulation of signaling pathways. Consistent with this mode of regulation, myoblast differentiation is accompanied by increased intracellular calcium and elevated CaMK activity (22, 25).

It also remains possible that an HDAC/MITR-specific phosphatase is negatively regulated by promyogenic signals. Indeed, inactivation of this phosphatase would be predicted to result in a net increase in phosphorylation of these repressors, with subsequent binding to 14-3-3, dissociation from MEF2, and activation of MEF2 target genes.

In summary, our results suggest a role for MITR in the regulation of skeletal myogenesis. Like MEF2, MITR is also expressed in the heart and brain, raising the possibility that the findings presented here may apply to diverse biological processes, including cardiac growth and neuronal functions. Resolution of these issues awaits analysis of MITR-deficient mice.

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