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Calcium Regulates Transcriptional Repression of Myocyte Enhancer Factor 2 by Histone Deacetylase 4*

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The myocyte enhancer factor 2 (MEF2) consists of a family of transcription factors that play important roles in a number of physiological processes from muscle cell differentiation to neuronal survival and T cell apoptosis. MEF2 has been reported to be associated with several distinct repressors including Cabin1(cain), MEF2-interacting transcriptional repressor (MITR), and HDAC4. It has been previously shown that Cabin1 is associated with MEF2 in a calcium-sensitive manner; activated calmodulin binds to Cabin1 and releases it from MEF2. However, it was not known whether the binding of HDAC4 and MITR to MEF2 is also regulated by calcium. We report that HDAC4 and MITR contain calmodulin-binding domains that overlap with their MEF2-binding domains. Binding of calmodulin to HDAC4 leads to its dissociation from MEF2, relieving MEF2 from the transcriptional repression by HDAC4. Together, HDAC4, MITR, and Cabin1 constitute a family of calcium-sensitive transcriptional repressors of MEF2.

Histone acetylation and deacetylation are known to play critical roles in chromatin remodeling and gene expression (1, 2). The mechanisms of regulation of transcription by histone acetylation and deacetylation are highly conserved from yeast to man, and they are mediated by two distinct types of enzymes, histone acetyltransferases (HAT)[1] and histone deacetylases (HDACs) (3, 4). Coactivators such as p300 and CBP, for example, possess intrinsic HAT activity, increasing the accessibility of their associated promoter to the transcription machinery (5, 6). Corepressors, on the other hand, recruit histone deacetylases (HDACs), repressing transcription (7–10). To date, eight isoforms of HDACs have been identified in mammals. They have been divided into two classes based on their homology with yeast homologs. Class I enzymes including HDAC1, -2, -3, and -8 are related to yeast Rpd3p and Class II enzymes including HDAC4, -5, -6, and -7 are related to yeast Hda1p (11–18). Of the Class I HDACs, HDAC1 and HDAC2 have been known to associate with corepressors (mSin3A and NRD/NuRD), which serve as adaptor molecules to mediate the complex formation between DNA-binding transcription factors and HDACs (7, 10, 19, 20). Unique among the known HDACs are HDAC4 and HDAC5, which have been shown to directly bind to the transcription factor myocyte enhancer factor-2 (MEF2) and to repress its transcriptional activity (21–24).

MEF2 was first identified as a transcription factor that participates in the transcription of genes involved in muscle differentiation (25). Four isoforms of MEF2 are known; they are designated MEF2A–D. Subsequent studies of the MEF2 family of proteins, however, have extended the physiological functions of MEF2 to non-muscle cells (26–28). In particular, MEF2D has been shown to play a key role in T cell receptor (TCR)-mediated apoptosis during thymic negative selection. It mediates calcium-dependent transcription of Nur77, a key transcription factor involved in TCR-mediated apoptosis of thymocytes (29, 30). MEF2D has been shown to bind to two calcium-responsive DNA elements in the Nur77 promoter and to mediate the calcium-dependent induction of Nur77 (31).

In the absence of TCR signaling, the expression of Nur77 is suppressed. We recently identified Cabin1/cain (32, 33) as a transcriptional repressor of MEF2 for the silencing of the Nur77 promoter in the absence of calcium signaling (34). Cabin1 binds to the MADS/MEF domain of MEF2 and represses transcriptional activity of MEF2 by recruiting a histone deacetylase complex consisting of mSin3A/HDAC1 and -2 on the MEF2-responsive DNA elements of Nur77 promoter.[2] Cabin1 dissociates from MEF2 in a calcium-dependent manner (34). The regulation of the Cabin1-MEF2 interaction by calcium and calmodulin represents the first case in which calcium/calmodulin regulates transcription by directly displacing a histone deacetylase-corepressor complex from a transcription factor. Recently, two additional repressors, HDAC4 and MEF2-interacting transcriptional repressor (MITR), have been shown to associate with MEF2 and repress its transcriptional activity (21, 35). However, it was not known whether the repression of MEF2 by HDAC4 and MITR is subjected to regulation by calcium. Nor was it clear whether the interaction between MEF2 and HDAC4 is relevant in regulating Nur77 expression. Herein we report that HDAC4 is a calmodulin-binding protein and that its association with MEF2 is regulated by calcium and calmodulin. Similar to Cabin1, HDAC4 binds to the N-terminal MADS/MEF domain of MEF2D and competes with p300 for the binding to MEF2. These results suggest that MEF2 is regulated by a family of calcium-dependent HDAC or HDAC-containing corepressor complexes.

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[1] The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase, MEF, myocyte enhancer factor; TCR, T cell receptor; MITR, MEF2-interacting transcriptional repressor; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate.

[2] H.-D. Youn and J. O. Liu, unpublished results.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat T cells and DO11.10 T-hybridoma cells were grown in RPMI medium supplemented with 10% serum, 2 mM glutamine, and 50 units/ml streptomycin/penicillin.

Plasmids—The mammalian expression vectors for the full-length and an N-terminal deletion mutant of HDAC4 were subcloned into pBJ5 vector as described previously (16). Both HDAC4 constructs were tagged with a Flag epitope at the C termini. The mammalian expression vector for HDAC4-(155–220) was subcloned into the pCS2+MT vector by inserting the corresponding polymerase chain reaction fragment into the *Eco*RI/*Xho*I sites. The bacterial expression vector (pGEX-HDAC4-(155–220)) was constructed by inserting the polymerase chain reaction fragment into the pGEX-KG vector at the *Bam*HI and *Xho*I sites. Recombinant GST-HDAC4-(155–220) was purified from *Escherichia coli* DH5α harboring pGEX-HDAC4-(155–220) by conventional glutathione affinity chromatography. Other plasmids used in this study were described previously (34).

Immunoprecipitation—T cells were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). The lysate was incubated with appropriate antibodies and protein A/G beads for 2 h before the beads were washed three times with lysis buffer. Immunoprecipitates were subjected to SDS-PAGE followed by Western blot with appropriate antibodies. Anti-HDAC4 antibodies were prepared as described previously (16); anti-MEF2 antibody was provided by R. Prywes (Columbia University); anti-Flag antibody was purchased from Sigma; and anti-c-myc antibody was from Babco.

CaM-Sepharose Pull-down Assay—CaM-Sepharose was incubated with either cell lysates containing Flag-tagged HDAC4 or recombinant GST-HDAC4-(155–220) in the presence of either $CaCl_2$ (2 mM) or EGTA (10 mM) for 2 h, washed three times with lysis buffer. Precipitates were subjected to SDS-PAGE followed by Western blot with anti-Flag antibodies or anti-GST antibodies.

Reporter Gene Assay—Jurkat cells were transfected by electroporation (250 V, 950 microfarads) with 10 μg of expression vectors, 2 μg of LacZ, 2.5 μg of luciferase reporter gene plasmids. After a 24-h incubation, transfected cells were treated with 40 nM PMA and 1 μM ionomycin overnight. Luciferase activity was measured and normalized to β-galactosidase activity.

In Vitro Transcription/Translation—To prepare the ^{35}S-labeled MEF2D *in vitro*, 1 μg of pSG-MEF2 vector in which the MEF2 expression is under the control of the T7 promoter was sequentially reacted with T7 RNA polymerase and reticulocyte mixture per manufacturer's instructions (Novagen).

RESULTS

HDAC4 Is a Calmodulin-binding Protein—We have previously shown that the minimal MEF2-binding domain of Cabin1 contains an overlapping CaM-binding domain, accounting for the regulation of Cabin1-MEF2 interaction by calcium and calmodulin (34). As HDAC4 has also been reported to bind to the N-terminal MADS/MEF2 domain of MEF2 and repress the MEF2 transcriptional activity (21, 23), we wondered whether the interaction between HDAC4 and MEF2 is regulated by calcium in a similar manner. The minimal MEF2-binding domain of HDAC4 was reported to lie between amino acid residues 155 and 220. Sequence alignment of minimal MEF2-binding domain of HDAC4 with that of Cabin1 revealed that this domain also possesses features similar to other CaM-binding domains with a hydrophobic residue flanked by a basic region toward the N terminus and a predicted α-helix toward the C terminus (Fig. 1*A*).

To determine whether HDAC4 is a CaM-binding protein, Do11.10 T cell lysates containing Flag-epitope-tagged HDAC4 were incubated with CaM-Sepharose beads in the presence of either EGTA or $CaCl_2$. Flag-HDAC4 is bound to CaM-Sepharose beads only in the presence of Ca^{2+}, but not in its absence (Fig. 1*B*). The same protein did not bind to control Sepharose either in the presence or absence of calcium. To assess whether the binding of Flag-HDAC4 to CaM-Sepharose is direct, we investigated the interaction between recombinant GST-HDAC4-(155–220) and CaM-Sepharose. Recombinant GST-HDAC4-(155–220), which spans at the MEF2-binding domain

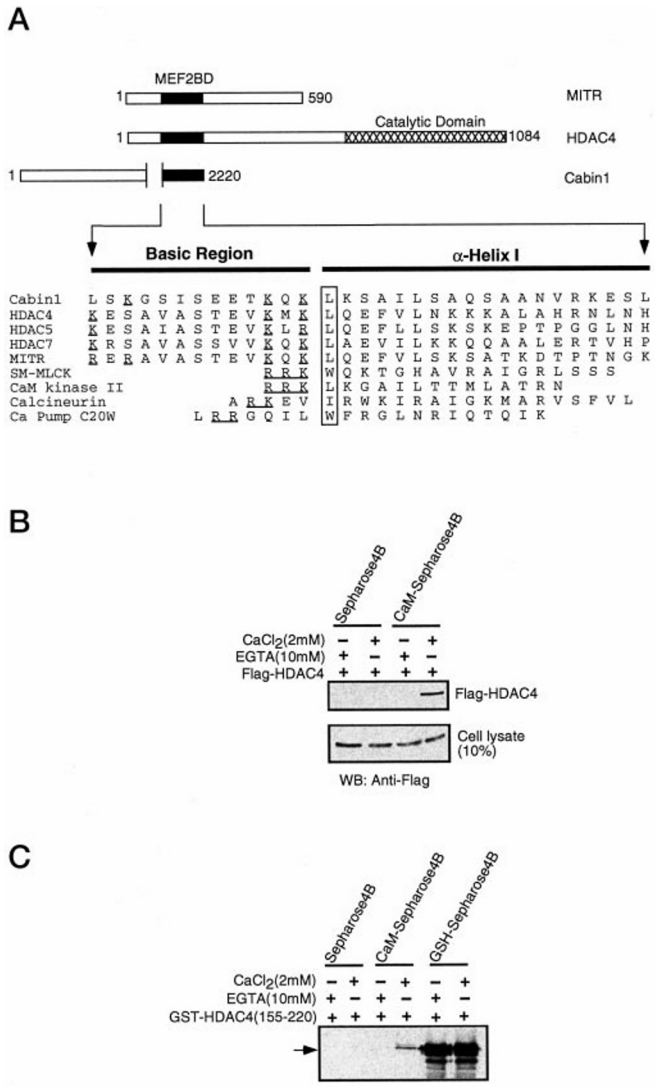

FIG. 1. **HDAC4 is a CaM-binding protein.** *A*, sequence comparison of the MEF2-binding domains (MEF2BD) of HDAC4, Cabin1, MITR, and other known CaM-binding proteins (50). *B*, HDAC4 binds to CaM-Sepharose. 10 μg of pBJ5-HDAC4 encoding Flag-tagged HDAC4 was transfected into DO11.10 cells. Cell lysates were prepared 24 h after transfection and were incubated with CaM-Sepharose in the presence of either 10 mM EGTA or 2 mM $CaCl_2$ for 2 h. *C*, MEF2-binding domain of HDAC4 overlaps its CaM-binding domain. Recombinant GST-HDAC4-(155–220) protein, spanning at the MEF2-binding domain of HDAC4, was incubated with CaM-Sepharose in the presence of either 10 mM EGTA or 2 mM $CaCl_2$ for 2 h. Precipitates from both *B* and *C* are subjected to SDS-PAGE, followed by Western blot using anti-Flag antibody (*B*) and anti-GST antibody (*C*), respectively.

of HDAC4, bound to CaM-Sepharose beads in the presence of Ca^{2+} (Fig. 1*C*), suggesting that HDAC4 is a CaM-binding protein and that the CaM-binding domain of HDAC4 overlaps the MEF2-binding domain of HDAC4.

Calcium and Calmodulin Compete for HDAC4 against MEF2D—Similar to Cabin1, the minimal CaM-binding domain of HDAC4 lies within the MEF2-binding region, suggesting that binding of CaM to HDAC4 and that of MEF2 is mutually exclusive. To test this possibility, we generated ^{35}S-MEF2D by *in vitro* transcription and translation, expressed Flag-HDAC4 in Jurkat T cells, and examined the binding of Flag-HDAC4 to MEF2D by coimmunoprecipitation in the presence or absence of Ca^{2+}. ^{35}S-MEF2D was immunoprecipitated with Flag-HDAC4 by an anti-Flag antibody in the presence of EGTA. The presence of Ca^{2+} significantly decreased the binding, presumably through competition from CaM in the Jurkat cell lysates

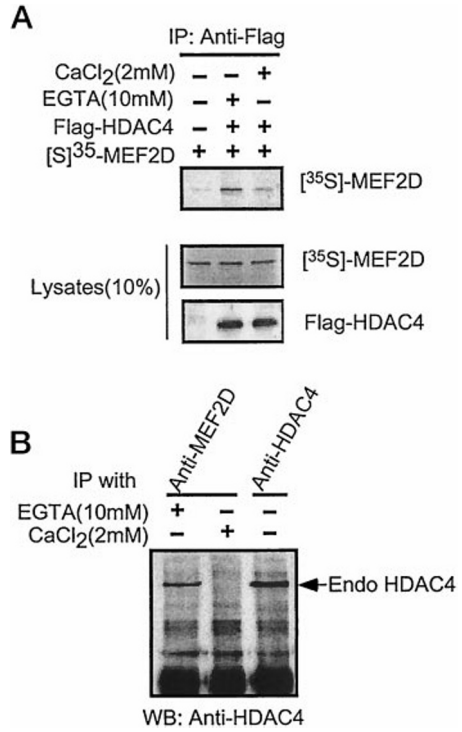

FIG. 2. **Binding of HDAC4 to MEF2D is regulated by calcium.** *A*, lysates containing Flag-tagged HDAC4 were incubated with *in vitro* transcribed/translated product of ^{35}S-MEF2D in the presence of either $CaCl_2$ or EGTA for 2 h. After immunoprecipitation of HDAC4 with anti-Flag antibodies, the bound ^{35}S-MEF2D was visualized by autoradiography. *B*, coimmunoprecipitation of endogenous MEF2D and HDAC4. T cell lysates were incubated with anti-MEF2D antibodies. The immunoprecipitates were subjected to SDS-PAGE followed by Western blot with anti-HDAC4 antibodies.

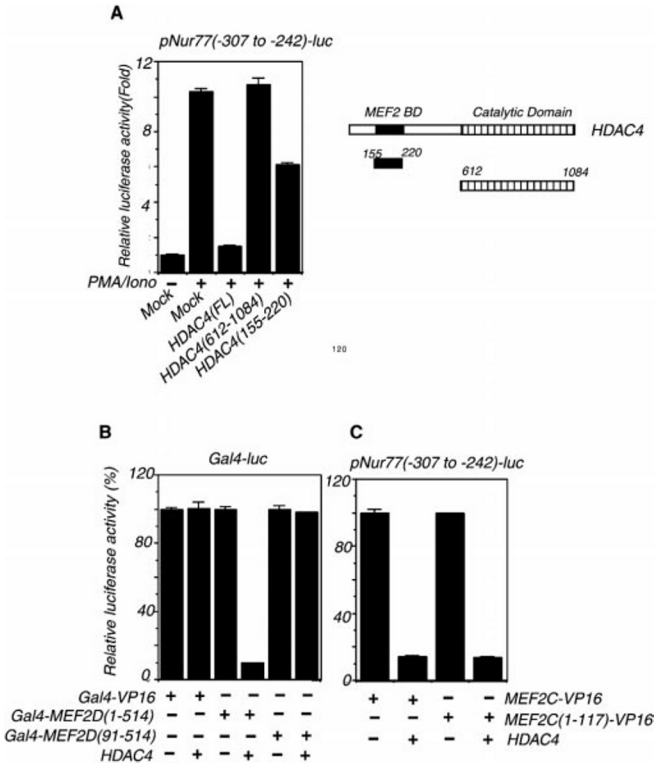

FIG. 3. **HDAC4 represses MEF2D through its MADS/MEF DNA-binding domain.** Jurkat T cells were transfected with the following expression vectors. *A*, HDAC4 deletion mutants along with pNur77 (−307 to −242)-luciferase reporter gene; *B*, pBJ5-HDAC4 with Gal4-VP16, Gal4-MEF2-(1–514), or Gal4-MEF2D-(92–514) along with pG5-luc reporter gene; *C*, pBJ5-HDAC4 with either MEF2C-VP16 or MEF2C-(1–117)-VP16 along with pNur77 (−302 to −242)-luciferase reporter gene. For *A*, transfected cells were allowed to recover for 24 h before they were treated with 40 nM PMA and 1 μM ionomycin for an additional 12 h. Luciferase activity was normalized to β-galactosidase activity.

(Fig. 2*A*). The effect of calcium on the interaction between HDAC4 and MEF2D was further confirmed with endogenous proteins. Thus, endogenous MEF2D from DO11.10 T cell lysates coimmunoprecipitated with HDAC4 only in the presence of EGTA (Fig. 2*B*), consistent with the notion that activated CaM abrogates the interaction between HDAC4 and MEF2D, as has been observed for the interaction between Cabin1 and MEF2D (34).

HDAC4 Represses MEF2 Transcriptional Activity on the Nur77 Promoter—It has been shown that HDAC4 inhibits the activation of the minimal MEF2-responsive element of the c-Jun promoter and Gal4-HDAC4 represses the activation of a Gal4-responsive reporter gene (21, 23). As the two MEF2-binding sites are the primary Ca^{2+}-responsive elements in the Nur77 promoter, it was most likely that HDAC4 also represses transcription from the Nur77 promoter. When Jurkat T cells were transfected with HDAC4 along with the Nur77 (−1148)-luciferase reporter gene, which contains an 1148-base pair Nur77 promoter fragment, HDAC4 inhibited the reporter gene in response to stimulation by PMA and ionomycin (data not shown). It has been known that two MEF2-responsive elements reside within the fragment spanning nucleotides −307 to −242 (31). We determined if HDAC4 repressed the MEF2-mediated activation of Nur77 using pNur77 (−307 to −242)-luciferase reporter gene. As expected, full-length HDAC4 inhibited the MEF2-responsive luciferase activity in response to treatment with PMA and ionomycin (Fig. 3*A*).

To investigate which domain of HDAC4 is involved in the repression of MEF2 transcriptional activity, we generated deletion mutants of HDAC4 and determined their ability to inhibit the Nur77 (−307 to −242)-luciferase reporter gene activation. The deletion mutant of HDAC4 lacking the N-terminal MEF2-binding domain (HDAC4-(612–1048)) no longer inhibited the Nur77 reporter gene activation. In contrast, the minimal MEF2-binding domain of HDAC4 (HDAC4-(155–220)) was capable of inhibiting the minimal Nur77 reporter gene activation, albeit with a lower potency than the full-length of HDAC4 (Fig. 3*A*). These results suggest that both the N-terminal MEF2-binding domain and the C-terminal catalytic domain of HDAC4 are required for the full repression of MEF2 and that binding of the minimal MEF2-binding domain to MEF2 can down-regulate its transcriptional activity independent of HDAC activity.

It has been shown that HDAC4 binds to the N-terminal MADS/MEF domain of MEF2 (21). We further verified that the MADS/MEF domain of MEF2 is necessary for the repression of MEF2 by HDAC4 using fusion proteins between either the Gal4 DNA-binding domain or the VP16 activation domain and various fragments of MEF2D. Although the activation of the Gal4-dependent luciferase reporter gene by Gal4-MEF2D fusion protein was inhibited by overexpression of HDAC4, that mediated by either Gal4-VP16 or Gal4-MEF2D-(91–514) that lacked the MADS/MEF domain of MEF2D was resistant to coexpression of HDAC4. These results indicated that the N-terminal MADS/MEF domain is necessary for inhibition of MEF2 transcriptional activity by HDAC4. Furthermore, activation of the Nur77 reporter gene by fusion proteins between either the full-length MEF2C and MADS/MEF domain of MEF2C-(1–117) or the transactivation domain of VP16 was inhibited by HDAC4 (Fig. 3*C*), suggesting that the MADS/MEF

domain of MEF2 is sufficient to mediate its repression by HDAC4.

HDAC4 Competes with p300 for the Binding to MEF2—The coactivator p300 has been shown to bind to the MADS domain of MEF2 (36). The same domain has also been reported to bind to HDAC4 (21, 23). These observations raised the possibility that HDAC4 may compete with p300 for MEF2 binding. We have found that both the N- and the C-terminal domains of p300 bind to MEF2D with the C-terminal domain having a higher affinity for MEF2D than that of N-terminal domain.[2] We thus used the C-terminal domain of p300 to determine whether the minimal MEF2-binding domain of HDAC4 competed with p300 for binding to MEF2. DO11.10 cells were transfected with Flag-p300-(1737–2414), Flag-HDAC4, and c-myc-MEF2D, respectively. Cells transfected with each plasmid were divided into two aliquots and lysed in a lysis buffer containing either $CaCl_2$ or EGTA. The cell lysates containing c-myc-MEF2D were incubated with those containing Flag-HDAC4 for 1 h in the presence of $CaCl_2$ or EGTA, followed by addition of cell lysates containing Flag-p300-(1737–2414). After a 2-h incubation, c-myc-MEF2D was immunoprecipitated with anti-c-myc antibodies. As shown in Fig. 4*A*, there was little binding between p300 and MEF2D in the presence of EGTA. In the presence of calcium, however, a dramatic increase was observed in the amounts of p300 bound to MEF2D (Fig. 4*A*). Together with the demonstration the HDAC4 is bound to MEF2D only in the presence of EGTA (Fig. 2*A*), these results are consistent with the competition between HDAC4 and p300 for binding to MEF2D.

As p300 and HDAC4 have opposite effects on MEF2 transcriptional activity, we turned to the Nur77 reporter gene to assess the competition between p300 and HDAC4 for MEF2 *in vivo*. When Jurkat cells were transfected with pNur77 (−307 to −242)-luciferase reporter gene and increasing amounts of p300, p300 increased the Nur77 reporter gene activity in a dose-dependent fashion (Fig. 4*B*). Coexpression of HDAC4 led to a dose-dependent cancellation of the enhancement of the reporter gene activity by p300. Thus, the p300 HAT and HDAC4 are capable of turning on and off the Nur77 promoter by mutual competition for MEF2 binding. The presence of a CaM-binding domain in HDAC4 confers calcium dependence to the competitive binding of HDAC4 and Cabin1 to MEF2, which is likely to be partly responsible for the silencing and activation of Nur77 and other MEF2 target genes in the absence and presence of calcium signaling, respectively.

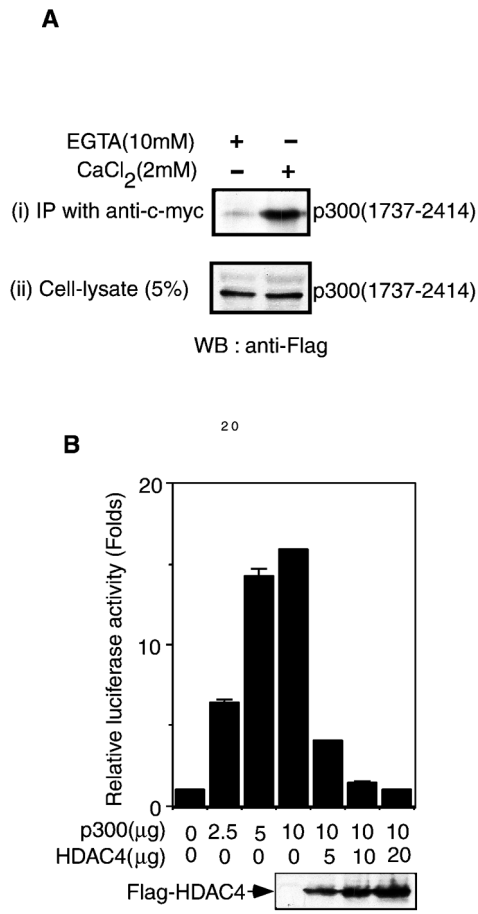

FIG. 4. **HDAC4 competes with p300 for MEF2D binding.** *A*, calcium regulates association between MEF2D and p300. T cells were transfected with expression vectors for Flag-HDAC4, Flag-p300-(1737–2414), or c-myc-MEF2D. Each of the transfected cell population was lysed in a lysis buffer containing either 2 mM $CaCl_2$ or 10 mM EGTA. Lysates containing Flag-p300-(1737–2414) and c-myc-MEF2D were incubated in the presence of either $CaCl_2$ or EGTA for 1 h, before Flag-HDAC4 cell lysates were added, and incubated for an additional 2 h. c-myc-MEF2D was immunoprecipitated with a c-myc antibody and probed with anti-Flag antibodies for the detection of Flag-p300-(1737–2414). *B*, HDAC4 represses p300-mediated activation of MEF2D. Jurkat T cells were transfected with different combinations of expression vectors for MEF2D, p300, and HDAC4 along with the pNur77 (−307 to −242)-luciferase reporter plasmid. Luciferase activity was normalized to β-galactosidase activity.

DISCUSSION

TCR-mediated expression of members of the Nur77 family plays a key role in thymocyte apoptosis (37). MEF2 has been shown to mediate calcium-dependent transcriptional activation of Nur77 (31). That MEF2 is constitutively bound to the promoter of Nur77 regardless of calcium status in cells has implicated the existence of MEF2 repressors that keeps MEF2 in an inactive state. We recently identified Cabin1 as a transcriptional corepressor of MEF2 (34). Furthermore, the repression of MEF2 by Cabin1 is sensitive to calcium signaling. In the presence of calcium, Cabin1 is released from MEF2, enabling its transcriptional activity. In addition to Cabin1, three other MEF2-specific transcriptional corepressors, HDAC4, HDAC5, and MITR, were recently reported. Like Cabin1, HDAC4, HDAC5, and MITR were shown to bind to MEF2 and inhibit its transcriptional activity. Although transcriptional repression of MEF2 by HDAC5 has recently been reported to be regulated by CaM kinase IV (22), it was not known whether the transcriptional repression by HDAC4 and MITR is also sensitive to calcium.

Through sequence comparison, we identified a putative CaM-binding domain with the minimal MEF2-binding domain of HDAC4 as well as that of MITR (Fig. 1*A*). We showed that HDAC4 is a CaM-binding protein. Furthermore, the binding of CaM to HDAC4 releases it from MEF2, offering a mechanism of derepression of MEF2 by calcium and CaM. Although MITR has not been subjected to the same scrutiny, its association with MEF2 is likely to be regulated in the same fashion given that a CaM-binding domain is also found within its MEF2-interacting domain. The same is likely to be true for HDAC5 given its significant similarity to HDAC4 in general and the MEF2-binding domain in particular. Thus, at least four MEF2 corepressors have been identified, and they appear to be functionally redundant. However, the ways they recruit histone deacetylase activity for chromatin remodeling are clearly different. Whereas Cabin1 recruits HDAC1 and -2 through the corepressor mSin3, MITR directly recruits HDAC1/2. The interaction of HDAC4 or HDAC5 and MEF2 is most direct among all four corepressors of MEF2 with a MEF2-binding domain and the HDAC catalytic domain residing within the same polypeptide. These alternative modes of HDAC recruitment

have been seen for other corepressors. Similar to Cabin1, MAD and N-CoR indirectly recruit HDAC1/2 through mSin3. Unlike Cabin1 but similar to MITR, both Rb and TGIF directly recruit HDAC1/2 (38, 39). Each of these corepressors is specific for a distinct transcription factor. It remains unclear why four different repressors exist for MEF2. One explanation may be that each one is optimized for silencing a different region of the promoter when anchored to the chromatin through interaction with MEF2. It is imaginable that the Cabin1-mSin3-HDAC complex may be optimal for silencing chromatin located further away from the MEF2-binding site than MITR and HDAC4/HDAC5. In addition, these corepressors may be expressed at different levels both temporally and spatially during and after development. In fact, Cabin1 has been found to be expressed predominantly in thymus, whereas HDAC4 has been found to be expressed predominantly in muscle and brain (12, 16, 23).

One common feature of all known transcriptional corepressors is their ability to recruit histone deacetylases to remodel the chromatin into a transcriptionally incompetent state. HDAC activity has been shown to be crucial for transcriptional repression. It follows that deletion of the domain necessary for recruiting histone deacetylase activity would render a repressor inactive. It is thus somewhat surprising that the minimal MEF2-binding domain of HDAC4, which lacks histone deacetylase catalytic domain, is still able to inhibit MEF2-dependent activation of the minimal Nur77 promoter (Fig. 3) (23). The HDAC-independent inhibition of MEF2 by HDAC5 has also been reported (24). These results can now be explained by the mutually exclusive binding of HDAC4 and the coactivator p300 as they both bind to the N-terminal MADS/MEF domain of MEF2. In this respect, HDAC4 is similar to TGIF, a Smad repressor, which has also been shown to compete with p300 for the binding to Smad (40). Thus, HDAC4 represses MEF2 by a "two-hit" mechanism, one through its HDAC activity and the other through competition for MEF2 against the coactivator p300. We have found that the Cabin1 also operates through the "two-hit" mechanism.[3]

As an important second messenger, calcium is known to regulate the transcription of a large number of genes. Given the role of chromatin remodeling by HDACs in the repression of transcription, it is not surprising that association of HDACs or HDAC-containing repression complexes with a transcription factor is regulated by calcium signaling. We have previously shown that the repression of MEF2 by Cabin1 is regulated by calcium and calmodulin. In this manuscript, we demonstrated that HDAC4 is a CaM-binding protein itself and that its association with MEF2 is regulated by calcium and CaM. It is likely that the interaction between MITR and MEF2 is regulated by calcium and CaM in a similar fashion. Furthermore, the presence of the putative CaM-binding domain in two other Class II HDACs, HDAC5 and HDAC7, suggests that they are also likely to be bound to and regulated by CaM. Together, HDAC4, HDAC5, and HDAC7, along with Cabin1 and MITR, represent an emerging family of transcriptional repressors that provides a link between calcium signaling and chromatin remodeling.

[3] H.-D. Youn, T. A. Chatila, and J. O. Liu, unpublished results.

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