Mechanism for Nucleocytoplasmic Shuttling of Histone Deacetylase 7*

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Here we show that HDAC7, a member of the class II histone deacetylases, specifically targets several members of myocyte enhancer factors, MEF2A, -2C, and -2D, and inhibits their transcriptional activity. Furthermore, we demonstrate that DNA-bound MEF2C is capable of recruiting HDAC7, demonstrating that the HDAC7-dependent repression of transcription is not due to the inhibition of the MEF2 DNA binding activity. The data also suggest that the promoter bound MEF2 is potentially capable of remodeling adjacent nucleosomes via the recruitment of HDAC7. We have also observed a nucleocytoplasmic shuttling of HDAC7 and dissected the mechanism involved. In NIH3T3 cells, HDAC7 was primarily localized in the cytoplasm, essentially due to an active CRM1-dependent export of the protein from the nucleus. Interestingly, in HeLa cells, HDAC7 was predominantly nuclear. In these cells we could restore the cytoplasmic localization of HDAC7 by expressing CaMK I. This CaMK I-induced nuclear export of HDAC7 was abolished when three critical serines, Ser-178, Ser-344, and Ser-479, of HDAC7 were mutated. We show that these serines are involved in the direct interaction of HDAC7 with 14-3-3. Mutations of these serine residues weakened the association with 14-3-3 and dramatically enhanced the repression activity of HDAC7 in NIH3T3 cells, but not in HeLa cells. Data presented in this work clearly show that the signal dependent subcellular localization of HDAC7 is essential in controlling its activities. The data also show that the cellular concentration of factors such as 14-3-3, CaMK I, and other yet unknown molecules may determine the subcellular localization of an individual HDAC member in a cell type and HDAC-specific manner.

Regulation of gene expression in eukaryotic cells is achieved through the recruitment of promoter-specific transcription factors and the basal transcription apparatus associated with the reorganization of chromatin on the promoter. Recent studies strongly suggest that the amino-terminal tail of histones play a critical role in the chromatin structure and hence, the regulation of gene expression (1–5). The amino-terminal tails of histones are targets for several modifications including methylation, phosphorylation, and acetylation. While histone acetylation at specific lysines is associated with transcriptional activation, deacetylated histones are found in transcriptionally silent regions. The acetylation status of the histone tails is determined by the interplay between histone acetyltransferases and histone deacetylases (HDACs)1. It has been recently suggested that promoter-specific transcription factors recruit either coactivators or corepressors to achieve their positive or negative regulation. Notably, co-activators such as CBP/p300, PCAF, and p160 family proteins possess intrinsic histone acetyltransferase activity and therefore, normally activate transcription (6–10). By contrast, co-repressors such as SMRT, N-CoR, and mSin3A recruit HDACs to repress transcription (11–15). In several cases, physical association between promoter-specific transcription factors and HDACs have been reported (16).

Mammalian class II HDACs include HDAC4, HDAC5, HDAC6, HDAC7, and HDAC9, sizes ranging from 938 to 1215 amino acids (17–23). HDAC4, HDAC5, and HDAC7 constitute a subclass that besides a catalytic domain localized in their carboxy-terminal region, contain an NH₂-terminal non-catalytic region showing certain extent of sequence homology with each other. Among these three HDACs, HDAC7 contains the most divergent sequence in its amino terminus. This region of HDAC7 possesses, however, three short regions containing several myocyte enhancer factors, MEF2A, -2C, and -2D, and inhibits their transcriptional activity. Furthermore, we demonstrate that DNA-bound MEF2C is capable of recruiting HDAC7, demonstrating that the HDAC7-dependent repression of transcription is not due to the inhibition of the MEF2 DNA binding activity. The data also suggest that the promoter bound MEF2 is potentially capable of remodeling adjacent nucleosomes via the recruitment of HDAC7. We have also observed a nucleocytoplasmic shuttling of HDAC7 and dissected the mechanism involved. In NIH3T3 cells, HDAC7 was primarily localized in the cytoplasm, essentially due to an active CRM1-dependent export of the protein from the nucleus. Interestingly, in HeLa cells, HDAC7 was predominantly nuclear. In these cells we could restore the cytoplasmic localization of HDAC7 by expressing CaMK I. This CaMK I-induced nuclear export of HDAC7 was abolished when three critical serines, Ser-178, Ser-344, and Ser-479, of HDAC7 were mutated. We show that these serines are involved in the direct interaction of HDAC7 with 14-3-3. Mutations of these serine residues weakened the association with 14-3-3 and dramatically enhanced the repression activity of HDAC7 in NIH3T3 cells, but not in HeLa cells. Data presented in this work clearly show that the signal dependent subcellular localization of HDAC7 is essential in controlling its activities. The data also show that the cellular concentration of factors such as 14-3-3, CaMK I, and other yet unknown molecules may determine the subcellular localization of an individual HDAC member in a cell type and HDAC-specific manner.

1 The abbreviations used are: HDAC, histone deacetylase; SMRT, silencing mediator for retinoid and thyroid hormone receptors; N-CoR, nuclear receptor co-repressor; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; YFP, yellow fluorescent protein; TK, thymidine kinase; MCK, muscle creatin kinase.
The MEF2 proteins constitute a family of transcription factors involved in the regulation of multiple cellular processes including muscle differentiation, T cell apoptosis, and the survival of neurons (25–29). Four MEF2 proteins (MEF2A-D) have been identified and they bind to their cognate DNA sequence as homodimers or heterodimers. Both positive and negative regulators have been shown to influence the transcriptional activity of MEF2 (30). The coactivator, p300 protein, appears to regulate MEF2 activity through direct association with NFAT (31, 32). In contrast, class II histone deacetylases, HDAC4 and HDAC5, repress MEF2 activity through direct physical interaction (18, 33–36). In addition, a number of other transcription factors including MyoD have been shown to play a role in regulating MEF2 activity through direct association (37–41). During muscle differentiation, MEF2 proteins activate the expression of many muscle-specific genes. Recent reports suggest that nuclear export of HDAC4 and HDAC5 may be involved in this process (18, 34–36, 42, 43). The subcellular localization of both HDAC4 and HDAC5 are regulated through a carboxy-terminal nuclear export sequence and a direct association with MEF2 responsive genes. We then show that the nucleocytoplasmic shuttling of HDAC7 is intimately linked to the control of its activity and moreover, our data indicate that the presence and the abundance of molecules such as 14-3-3 and CaMk I can account for the different subcellular localization of HDAC7 in various cell types.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The plasmids pCMX, pCMX-GAL4 DBD, pCMX-mHDAC7-HA, pH1100-TK-luc have been described (20). Standard polymerase chain reaction amplifications and subsequent subclonings were employed to generate pCMX NH2-terminal FLAG and HA epitope-tagged and GAL4 fusion MEF2A, MEF2C, and MEF2D constructs. GST-HDAC7 and CMX-VP16-HDAC7 fusion constructs were generated by inserting HDAC7 polymerase chain reaction fragments into vector pGEX4T-1 and CMX-VP16, respectively. Site-directed mutagenesis was carried out using the QuickChange kit according to the manufacturer’s protocol (Stratagene). GST-MEF2A constructs have been previously described (35). The plasmids pCMX, pCMX-GAL4 DBD, pCMX-YFP-mHDAC5, pCMX-YFP-mHDAC7, and pH1100-TK-luc have been described (20).

Co-immunoprecipitation—For co-immunoprecipitations, 293 cells on 15 × 15-mm plates were transfected with 15 μg of the appropriate plasmids using Targefect F1 (Targeting Systems, San Diego, CA). The cells were harvested after 48 h, washed with 1 × PBS, and resuspended in 2 ml of 50 μl Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% Triton, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. The cells were then sonicated and centrifuged for 15 min at 13,000 rpm. The supernatant was kept at −80 °C. Whole cell extract. After precipitation by incubation with A/G-agarose (Santa Cruz), immunoprecipitations were carried out using M2-agarose beads (Sigma) and proceeded for 2 h at 4 °C. After washing 3–4 times with resuspension buffer, the samples were boiled in SDS loading buffer, separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with the appropriate antibodies.

RESULTS

Association of HDAC7 with MEF2s in Vivo—Two class II HDACs, HDAC4 and HDAC5, have been previously shown to interact with members of the MEF2 transcription factors. Since HDAC7 shows certain degrees of sequence homology with these two deacetylases, we wondered whether it is also capable of interacting with MEF2 members. To test this hypothesis, we carried out co-immunoprecipitation experiments. Expression vectors containing HA-tagged-HDAC7 (HA-HDAC7) and FLAG-tagged-MEF2A, -C, and -D (FLAG-MEF2A, -C, and -D) sites was used to transfect HeLa cells (33). Co-transfections were carried out with 200 ng of a MEF2A expression vector alone or together with the indicated amounts of HDAC7 expression vectors. 100 ng of a β-galactosidase expression vector were also used in each transfection. 24 h post-transfection, the luciferase activity was measured and normalized with respect to that of β-galactosidase.

Green Fluorescence and Immunofluorescence Microscopy—For in situ immunofluorescence analysis, transfected cells were fixed in 4% paraformaldehyde in PBS for 5 min at room temperature and permeabilized by the addition of 0.1% Triton X-100 for 1 min. The cells were washed three times with PBS and incubated in a PBS/milk solution for 30 min. Incubation with primary antibodies was carried out overnight at 4 °C in the PBS/milk solution. The cells were washed three times in PBS at room temperature for 5 min incubated with the PBS/milk solution for 15 min before the addition of the secondary antibodies. The incubation was carried out for 45 min at 37 °C and the cells were washed and counterstained with Hoechst 33258. Slides were then mounted using an anti-fading solution. The preparations were observed under an epifluorescence microscope (Zeiss Axiohot) equipped with a 100 W mercury. Numeric image acquisitions were realized using a cooled CCD camera (C4880 Hamamatsu) mounted on the epifluorescence microscope. Three micrograms of CaMk I and 1 μg of YFP-HDAC7 were co-transfected into HeLa cells to detect the effect of CaMk I on the subcellular localization of HDAC7. For statistical analysis, a total of 100 cells were examined.

In Vitro Interaction Assays—Glutathione S-transferase (GST) fusion protein was expressed in Escherichia coli DH5α strain and affinity purified by glutathione-Sepharose 4B beads. In vitro pull-down assays were carried out by incubating GST fusion proteins with 35S-labeled MEF2s and HDACs generated in vitro (36). 35S-Labeled proteins were generated using in vitro TNT (Promega). After extensive washes, protein sample buffer was added to retained fractions, boiled, and then separated on 7.5% SDS-PAGE. GST-MEF2 fusion proteins were produced in BL21 E. coli transformed with the pGEX-5X-3 plasmid (Amersham Pharmacia Biotech), encoding GST alone, or GST fused to various domains of MEF2A protein. 35S-Labeled HDAC7 and HDAC4 proteins were produced in rabbit reticulocyte lysate from pcDNA plasmids using the TNT transcription/translation kit and [35S]methionine (Amersham Pharmacia Biotech). The pull-down assays were performed as described by Lemercier et al. (33).

Electrophoresis Mobility Shift Assays—MEF2C synthesized in vitro was incubated with a 32P-labeled probe containing a MEF2C-binding site derived from the MCK promoter containing the sense strand sequence 5′-CCCTCTAAATATCACCTT-3′. DNA–protein binding was conducted in a reaction mixture containing 20 mM Hepes, pH 7.4, 50 mM KCl, 1 mM β-mercaptoethanol, and 10% glycerol. After 30 min of incubation at 25 °C, the purified GST fusion proteins were added followed by an additional 30 min of incubation. The final reaction mixtures were loaded into a 6% polyacrylamide gel (29:2:0.8) nondenaturing gel followed by gel electrophoresis in 1 × TBE buffer. After electrophoresis, the gel was stained and subjected to autoradiography.

Co-immunoprecipitation—For co-immunoprecipitations, 293 cells on 15 × 15-cm plates were transfected with 15 μg of the appropriate plasmids using Targefect F1 (Targeting Systems, San Diego, CA). The cells were harvested after 48 h, washed with 1 × PBS, and resuspended in 2 ml of 50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% Triton, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. The cells were then sonicated and centrifuged for 15 min at 13,000 rpm. The supernatant was kept at −80 °C. Whole cell extract. After precipitation by incubation with A/G-agarose (Santa Cruz), immunoprecipitations were carried out using M2-agarose beads (Sigma) and proceeded for 2 h at 4 °C. After washing 3–4 times with resuspension buffer, the samples were boiled in SDS loading buffer, separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with the appropriate antibodies.

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were used to express these proteins either alone or together in 293 cells. Whole cell extracts were prepared and incubated with M2-agarose beads. After extensive washes, retained immunocomplexes were resolved on SDS-PAGE followed by Western blot analyses, and probed with either anti-FLAG (lanes 1–8) or anti-HA (lanes 17–24) antibodies. As shown in Fig. 1A, the expression level of HA-HDAC7 was comparable in each transfected sample (lanes 2 and 6–8). HA-HDAC7 can be co-precipitated only in the presence of MEF2A, -2C, and -2D (lanes 14–16). As a control, M2-agarose beads were unable to co-precipitate HA-HDAC7 in cell extracts containing only HA-HDAC7 (lane 2). To confirm these results, we carried out in situ co-localization experiments. Yellow fluorescence protein (YFP)-tagged HDAC7 and MEF2A were co-transfected into HeLa cells. After 24 h, the localization of both proteins was monitored using an anti-MEF2 antibody. YFP fluorescence and MEF2 immunofluorescence were recorded and shown in the indicated panels. DNA panel shows nuclei of cells present in the field. C, HDAC7 interacts with MEF2C in mammalian two-hybrid. Gal4-MEF2 and VP-HDAC7 were transfected into CV-1 cells for mammalian two-hybrid assays. The VP-HDAC7 constructs were generated by fusing polymerase chain reaction fragments of HAC7 with VP16. The amino acids of HDAC7 corresponding to each fragment are indicated.
bodies in HeLa cells. MEF2A was shown to completely co-localize
with HDAC7 (Fig. 1B).

To map the MEF2 interaction domain of HDAC7, we used
mammalian two-hybrid assays in CV-1 cells. The cells were
transfected with expression vectors encoding Gal4-MEF2s and
a series of VP16-HDAC7 fusion proteins and a luciferase re-
porter construct containing multiple copies of yeast Gal4-bind-
ing site cloned upstream from the TK promoter. An internal
control plasmid constitutively expressing the /H9252-galactosidase
gene was also used. In contrast to SMRT, which binds to the
decaylase catalytic domain of class II HDACs, MEF2s asso-
ciated with HDAC7 through the amino-terminal of HDAC7
(Fig. 1C, lanes 10, 18, and 26). The MEF2s-interacting domain
was further mapped to amino acid 72–172 of HDAC7 (Fig. 1C,
lanes 13, 21, and 29). Interestingly, this region is conserved
among class II HDACs, HDAC4, -5, and -7. Other HDAC7
fragments could not interact with MEF2. Taken together, we
conclude that amino acids 72–172 of HDAC7 were sufficient to
interact with MEF2 in vivo.

Association of HDAC7 with MEF2s in Vitro—GST pull-down
experiments were carried out to test whether HDAC7 could
interact directly with MEF2s. GST-HDAC7 fusion peptides
containing overlapping fragments were produced in bacteria
and purified. 35S-Labeled MEF2A, -C, and -D were synthe-
sized in vitro and incubated with GST-HDAC7-coupled glutathione
beads. Bound fractions were extensively washed and resolved
on SDS-PAGE followed by autoradiography (Fig. 2A). Consistent
with the mammalian two-hybrid data, amino acids 72–172
FIG. 3. HDAC7 represses MEF2s activity. A reporter plasmid containing four copies of Gal4-binding sites cloned upstream of thymidine kinase promoter and directing the expression of luciferase was used to monitor the transactivation capacity of Gal4-MEF2C in transient transfection assays. A, HDAC4, HDAC5, and HDAC7 inhibit transcription activity of MEF2C in CV-1 cells. B, mutants that are defective in HDAC catalytic activity still inhibit MEF2 activity. The point mutations in HDAC domain are as follows: D692A represents the replacement of aspartate 692 by alanine; D694A represents the replacement of aspartate 694 by alanine, and D692A/D694A represents the replacement of both aspartate 692 and 694 by alanines. C, HDAC4, HDAC5, and HDAC7 inhibit MEF2C in MCF7 cells. Please note that the activity of Gal4-MEF2C is similar to Gal4 alone (lanes 1 and 5). D, HDAC4, HDAC5, and HDAC7 inhibit MEF2C activity in NIH3T3 cells. Please note that Gal4-MEF2C activity is 3–4-fold higher than Gal4 alone (lanes 1 and 5). E, HDAC7 represses MEF2s activity in Hep G2 cells. Please note that Gal4-MEF2s activity is at least 5-fold higher than Gal4 alone (lanes 1, 4, 7, and 10). F, repression of MEF2A transcriptional activity by HDAC7 in HeLa cells. 100% represents mean values of three independent experiments performed in the absence of HDAC7 expression. G, SMRT and N-CoR inhibit MEF2C activity. Transient transfection experiments were carried out in CV-1 cells. Full-length mouse SMRT and N-CoR expression vectors were included to determine their effects on MEF2C activity.
of HDAC7 were found sufficient to interact with MEF2 (lanes 6, 13, and 20).

To test whether HDAC7 could bind DNA-bound MEF2, electrophoretic mobility shift assays were conducted. In vitro translated MEF2C was incubated with a [32P]-labeled probe containing the MEF2-binding site derived from MCK (muscle creatin kinase) promoter and the formation of the MEF2C-DNA complex was monitored. Consistent with previous reports, MEF2C bound to this sequence, resulting in the formation of a stable protein-DNA complex (Fig. 2B, lane 2). As a control, incubation with a mock translation lysate did not lead to the formation of such a complex (lane 1). Furthermore, the addition of the GST protein alone did not have an effect on the DNA binding activity of MEF2C nor the mobility of the MEF2C-DNA complex (lane 3). Consistent with our pull-down and mammalian two-hybrid assays, addition of a GST-HDAC7 fusion protein spanning amino acids 72–172 resulted in the formation of a larger complex (lane 4), indicating the interaction of this region of HDAC7 with the MEF2C-DNA complex. Furthermore, GST fusion proteins containing other regions of HDAC7 did not modify the DNA binding activity of MEF2C nor the mobility of the MEF2C-DNA complex (lanes 5 and 6). Based on these results, we conclude that MEF2C can bind DNA and HDAC7 simultaneously and that binding of HDAC7 does not interfere with the MEF2C DNA binding activity.

To determine the minimal HDAC7 interaction domain within MEF2s, we carried out an additional set of GST pull-down assays. [35S]-Labeled HDAC4 was also included as a control (Fig. 2C, lane 7). We found that the affinity of HDAC7 for MEF2A was comparable to that of HDAC4 for this protein (lanes 1 and 7). We further mapped the HDAC7 interacting domain to the first 86 amino acids of MEF2A (lane 2). Taken together, we conclude that amino acids 72–172 of HDAC7 interacts directly with amino acids 1–86 of MEF2s.

Class II HDACs Down-regulate MEF2s Transcriptional Activity—Based on the interaction data, we reasoned that the recruitment of HDAC7 by MEF2 could be a way to counteract the transactivator function of MEF2 members. To confirm this hypothesis, we fused MEF2C to the DNA-binding domain of Gal4. A reporter plasmid containing four copies of the Gal4-binding sites cloned upstream of the thymidine kinase promoter and directing the expression of luciferase was used to monitor the transactivator capacity of MEF2C in this assay.

While the expression of HDACs modestly activated the basal transcription of the reporter from 3–5-fold (Fig. 3A, lanes 1–7), the expression of HDAC7 significantly repressed MEF2 activity (lane 14). Furthermore, class I HDACs including HDAC1, HDAC2, and HDAC3 did not significantly affect MEF2C activity (lanes 9–11). In this assay, HDAC4 and HDAC5 repressed the MEF2C activity to the same extent as HDAC7 (lanes 12 and 13). We have previously shown that the amino-terminal non-catalytic domain of HDAC7 possessed a strong repressor activity (20) since HDAC7 containing point mutations D692A, D694A, and D692A/D694A that abrogated its HDAC activity could repress transcription efficiently (51). Accordingly, we reasoned that these mutants would also be able to repress MEF2 activity through the amino-terminal repressor domain. As shown in Fig. 3B, these mutants efficiently repressed MEF2C activity albeit with slightly lower efficiency than the wild-type HDAC7. The difference is presumably due to the loss of their catalytic activity. These results further suggested that MEF2C binding does not interfere with the amino-terminal repression activity of HDAC7. In these assays the repression of MEF2 activity was independent of the cell type used since we obtained the same results in other cell types such as MCF7 and NIH3T3 (Fig. 3, C and D). We next extended these experiments to

**Fig. 4.** 14-3-3e interacts directly with HDAC7. A, schematic diagram of HDAC7 domains. Three highly conserved regions at the amino-terminal region among class II HDACs are depicted. B, 14-3-3 interacts with HDAC7 in mammalian cells. HA-HDAC7 and FLAG-14-3-3e were transfected either alone or together into 293 cells. After 48 h, whole cell extracts were prepared, resolved on SDS-PAGE followed by Western blot analyses, and probed with either anti-FLAG (lanes 1–4) or anti-HA (lanes 5–8) antibodies. Whole cell extracts were incubated with M2-agarose beads and bound fractions were washed and resolved on SDS-PAGE followed by Western blot analyses probed with anti-FLAG (lanes 9–12) or anti-HA (lanes 13–16) antibodies. C, 14-3-3e directly interacts with HDAC7 in vitro. D, serine residues 178, 344, and 479 of HDAC7 are critical for 14-3-3 binding. Lanes 1–8, one-twentieth of the input is loaded. Lanes 9–16, pull-down fractions of wild-type of mutant HDAC7s transfected whole cell extracts. A histogram is used to measure the % of retained/input. The retained fraction of wild-type HDAC7 is used as 100%.
FIG. 5. Subcellular localization of the wild-type and mutant HDAC7s. A, the nuclear export of HDAC7 in NIH3T3 cells is inhibited by LMB, an inhibitor specific for Crm1-mediated nuclear export. A, mock treatment; B, 1 h after treatment with leptomycin B. B, the number of cells with nuclear or cytoplasmic HDAC7 at different time points after LMB treatment. Please note that by 1 h (lanes 10–12), all the cells contain nuclear HDAC7. C, subcellular localization of the wild-type and mutant HDAC7s in NIH3T3 cells. Mutations of putative phosphorylated serines...
examine the effect of HDAC7 on MEF2A and MEF2D. We found that HDAC7 also repressed the activity of MEF2A and MEF2D in a dose-dependent manner (Fig. 3E). Interestingly, however, we noticed that the capability of MEF2 proteins to activate transcription depends on the cell type used. The transactivation property of MEF2 proteins was much lower in CV-1 and MCF-7 cells than that in NIH3T3 and Hep G2 cells.

MEF2 has been shown to regulate the expression of the gene encoding MCK (52). We tested whether HDAC7 can also repress the expression of a MCK promoter-reporter gene in a MEF2A-dependent manner. MEF2A was co-transfected with a reporter construct containing two copies of minimal MEF2-binding sites from MCK promoter cloned upstream of rat skeletal muscle MHC embryonic promoter. As shown in Fig. 3F, MEF2A activated the MCK promoter to high levels and HDAC7 efficiently repressed this MEF2A-dependent transcription.

We have previously shown that HDAC7 can associate with the corepressors pSMRT and N-CoR. Accordingly, we reasoned that SMRT and N-CoR might also be involved in the regulation of MEF2 activity through their association with endogenous class II HDACs. Indeed, overexpression of SMRT and N-CoR inhibited transcriptional activity by MEF2C in transient transfection assays (Fig. 3G). In summary, class II HDACs are able to interact with and inhibit the transcriptional activity of MEF2A, -C, and -D.

**Association of 14-3-3 Proteins and HDAC7—**During our initial characterization of HDAC7, we identified three regions showing high degree of sequence homology with the amino-terminal non-catalytic domain of HDAC4 and -5 (Fig. 4A) (20). These regions of HDAC4 and HDAC5 have recently been shown to bind 14-3-3 proteins. These findings strongly suggested that HDAC7 like HDAC4 and -5, is capable of interaction with the 14-3-3 proteins. To test this possibility, cells were co-transfected with FLAG-tagged 14-3-3 and HA-HDAC7 followed by immunoprecipitation and Western blot analyses. HA-HDAC7 and FLAG-14-3-3 were efficiently co-precipitated, indicating the association of 14-3-3 and HDAC7 in vivo (Fig. 4B, lane 8). We further tested whether this association could be reproduced in vitro using a nuclear extract pull-down approach. Nuclear extracts prepared from HDAC7-HA expressing cells were incubated with purified GST-14-3-3 fusion protein. After extensive washes, retained fractions were subjected to SDS-PAGE and Western blot analysis using anti-HA antibodies. As expected, GST-14-3-3 fusion protein interacted efficiently with HDAC7 in vitro (Fig. 4C, lane 3) while the GST protein alone did not bind HDAC7 under the same conditions (lane 2).

The phosphorylated serines of the 14-3-3 ligands are believed to play an important role in the interaction between the two proteins. To know whether HDAC7 is a ligand for the 14-3-3 protein, we determined the role of three critical serine residues of HDAC7 in the interaction with 14-3-3. Serines 178, 344, and 479 of HDAC7 were mutated into alanine individually or in combination. These mutants were employed to test their ability to interact with 14-3-3. As shown in Fig. 4D, mutation of serine 178 to alanine reduces 14-3-3 binding (compare lanes 2 and 10), indicating that serine 178 has a role in the interaction with 14-3-3. Interestingly, mutants S344A and S479A were less effective in 14-3-3 binding than the S178A mutant (compare lanes 3, 4, and 11, and 12, respectively). A protein containing a triple mutation, S178A/S344A/S479A, completely lost its ability to bind 14-3-3 (compare lanes 8 and 16).

**Nuclear Export of HDAC7 Is Mediated by 14-3-3 and Crm1—**It is now clearly established that 14-3-3, in cooperation with Crm1, are involved in active export of substrate proteins from the nucleus (53–55). Binding of 14-3-3 to their ligands represents a critical step in this process. To test whether Crm1 mediates nuclear export of HDAC7, we first transfected NIH3T3 cells with YFP-tagged HDAC7 and treated the cells with leptomycin B (LMB), a specific CRM1 inhibitor (56). The number of cells with nuclear HDAC7 was scored at different time points after LMB treatment. As shown in Fig. 5A (panels a and b), we found that treatment of LMB increased the number of cells with nuclear HDAC7. By 1 h, all the cells contained nuclear HDAC7 (Fig. 5B). The results suggest that LMB efficiently inhibits the nuclear export of HDAC7 and that Crm1 mediates nuclear export of HDAC7.

We then wanted to know if 14-3-3s are involved in the Crm1-mediated export of HDAC7 from the nucleus. We argued that if 14-3-3s participate in this process, HDAC7 containing serines changed to alanines should no longer bind 14-3-3 proteins and should localize in the nucleus. Wild-type and mutants YFP-HDAC7 were expressed in NIH3T3 cells and the subcellular localization of the protein was monitored. As shown in Fig. 5, C and D, while the majority of cells expressing wild-type HDAC7 localized in the cytoplasm, increasing numbers of cells expressing HDAC7 serine mutants showed an accumulation of the protein in the nucleus. Interestingly, we found a preferential nuclear localization of mutant S178A compared with mutants S344A or S479A (Fig. 5D, lanes 4, 7, and 10). Furthermore, nuclear export of wild-type and mutants HDAC7 correlated very well with the ability to bind 14-3-3 (Fig. 4D and 5, C and D). This conclusion was most evident for the triple mutation S178A/S344A/S479A. Indeed, in more than 95% of cells expressing this mutant, the protein was found in the nucleus. We also examined the cellular localization of wild-type and mutants HDAC7 in HeLa cells. Surprisingly, in contrast to NIH3T3 cells, wild-type HDAC7 localized primarily in the nucleus of HeLa cells and serine mutations did not significantly affect this subcellular localization of HDAC7 (Fig. 5E).

**HDAC7 Triple Mutations Enhances Repression—**Since nuclear localization is a prerequisite for HDAC7 to repress MEF2 activity, we reasoned that serine mutants should possess better repression activity than wild-type HDAC7 in NIH3T3 cells but not in HeLa cells. To test this hypothesis, transient transfection assays were conducted. As shown in Fig. 6A, while single and double mutations had a moderate effect on the MEF2C activity, the HDAC7 containing the triple mutation dramatically enhanced the repressive effect of HDAC7 on MEF2C activity (Fig. 6A). Consistent with the subcellular localization data, the repressive effect of mutant S178A was stronger than that of S344A or S479A (lanes 3–5). Similarly, double mutations S178A/S344A and S178A/S479A possessed a better repression activity than the S344A/S479A mutant (lanes 6–8). Since wild-type HDAC7 is primarily nuclear in HeLa cells, we predicted that serine mutants would not have any effect on the repression activity of HDAC7. Indeed, serine mutants did not further repress MEF2 activity in these cells (Fig. 6B).
HDAC5 (45, 46). To test whether the cellular distribution of HDAC7 is also subject to similar regulation, we co-transfected YFP-HDAC7 and CaMK I into HeLa cells, where HDAC7 was essentially nuclear. The subcellular localization of HDAC7 was examined using fluorescence microscopy and the number of cells with nuclear or cytoplasmic HDAC7 was scored (Fig. 7A).

In the absence of CaMK I, HDAC7 primarily localized in the nucleus of HeLa cells (Fig. 7B). Interestingly, the number of cells with cytoplasmic HDAC7 was dramatically increased in the presence of CaMK I. We also tested whether the localization of the HDAC7 triple mutant, S178A/S344A/S479A, was affected by CaMK I expression. The cellular localization of the mutant HDAC7 was not altered by CaMK I expression.

Our data and those recently published (45, 47) strongly suggest that the subcellular localization of both HDAC5 and HDAC7 are controlled by a mechanism involving CaMK I and 14-3-3. However, a more complex mechanism should be envisaged to explain the subcellular localization of these two class II HDACs.
HDACs. Indeed, we found that in MCF7 and Hep G2 cells, while HDAC5 was predominantly localized in the nucleus of cells from both cell lines, HDAC7 was nuclear in MCF7, but was essentially pancellular with a slight increase in Hep G2 cells (Fig. 7C). This result suggests that even though the 14-3-3-binding sites of both HDACs are similar, the subcellular localization of HDAC5 and HDAC7 would probably involve additional unknown mechanisms.

**DISCUSSION**

**HDAC7 Modulates MEF2 Activity through Direct Physical Association**—The transcriptional activity of MEF2 proteins has been shown to be developmental- and cell type-dependent, which involve post-translational modification of MEF2 proteins as well as their combinatorial association with tissue- and developmental stage-specific positive and negative factors (30). Together with recent reports (18, 33–35), our data demonstrates that class II HDACs represent an additional level of regulation in the control of MEF2s activity. Accordingly, the expression and the subcellular localization of class II HDACs in different cell types may account, at least in part, for cell-type- and signal-dependent regulation of MEF2s activity. Moreover, the relationship between HDAC7 subcellular localization and its ability to respond to 14-3-3 and CaMKI establishes a link between regulatory signals and MEF2-dependent control of transcription.

**Transcriptional Repression of MEF2 by HDAC7**—It has been shown that the amino acids 1–86 of MEF2s containing the MADS box and MEF2 domains are sufficient and essential for sequence-specific DNA binding and dimerization (57). The fact that HDAC7 interacts with this region raises the question of whether HDAC7 binding interferes with the MEF2s DNA binding activity. Using electrophoretic mobility shift assays, we demonstrated that HDAC7 was able to bind to the DNA-MEF2C complex, suggesting that different interaction surfaces are responsible for DNA binding and dimerization, and HDACs association. Indeed, mutational and structural studies demonstrate that the region that contacts DNA differs from the region responsible for HDAC4 binding (42, 58). We conclude that HDAC7 represses transcription activity of MEF2 through its associated repressor activity. During preparation of our article, a recent report also demonstrated that HDAC7 interacts with MEF2 proteins and inhibits their activity (59).

The MEF2 interaction surface of HDAC7 was mapped to amino acids 72–172 of the protein, a region conserved among three class II HDACs: HDAC4, HDAC5, and HDAC7. The fact that all these proteins share the ability to interact and repress MEF2 members suggest that differential expression of these class II HDACs may be the only way to achieve a functional specificity. However, analysis of the expression of HDAC4, -5, and -7 showed that all the three genes are expressed in myoblasts as well as in myotubes in C2C7 cell line. These observations highlight the importance of the subcellular localization of these proteins in the control of their nuclear activity. Accordingly, we investigated the mechanism involved in the control of the subcellular localization of HDAC7.

**Association of 14-3-3 and Class II HDACs**—Molecular and structural studies indicated that the optimal binding motifs for 14-3-3 are RX(Ar/R/S)/+pS(LEAM)/P and R(S/Ar)/(+pS(LEAM)/P, where Ar denotes aromatic residues and + denotes basic residue (54, 60, 61). In both cases, phosphorylated serines play an essential role in 14-3-3 binding. In this study, we have identified three highly conserved regions in the amino-terminal of HDAC4, -5, and -7 that contain putative 14-3-3-binding sites. These sequences closely resemble, but do not perfectly match the consensus 14-3-3-binding site. The binding of HDAC7 to 14-3-3 appeared to be dependent on the serine residues Ser-178, Ser-344, and Ser-479, since the replacement of these residues by alanine reduced 14-3-3 binding by HDAC7. Interestingly, all three 14-3-3-binding sites within each HDAC are not equivalent. Ser-178 in the 14-3-3-binding site, KRTA/AV/pSEP, was the most critical for 14-3-3 binding in both HDAC4 and HDAC7 (47). Indeed, the inactivation of this site had a stronger effect that than of the two others (Fig. 5, C and D). Sequence comparison revealed that among the three sites, the NH2-terminal one presented the best homology to the consensus binding motif. Nevertheless, other
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sites also contributed to the binding of 14-3-3 by HDAC7 since the binding of HDAC7 by 14-3-3 was completely abolished only when all three motifs were inactivated. Similar results have also been observed for HDAC4 (47). Taken together, these results suggest that these three serines are the primary regulatory sites for nuclear export of class II HDACs. It is also interesting to note that class II HDACs are the only molecules identified so far that contain more than two 14-3-3-binding sites.

Mechanism Controlling Nucleocytoplasmic Shuttling of HDAC5 and HDAC7—The observation of differential subcellular localization of HDAC7 in several cell lines suggested that the machinery controlling nuclear export of HDAC7 was differentially expressed in different cell types. One obvious factor involved in this phenomenon is the level of the expression of CaMK I/V, that when overexpressed, promotes nuclear export of HDAC7 in HeLa cells (Fig. 7, A and B). A similar mechanism has also been demonstrated to mediate the nuclear export of HDAC5 (46). This observation clearly demonstrates class distinction between class I and class II HDACs. Surprisingly, we found that the subcellular distribution of HDAC5 and HDAC7 was differentially regulated in different cell types. For example, in CV-1 and MCF7 cells, both HDAC5 and HDAC7 are found in the nucleus (Fig. 7C and Ref. 20), while in Hep G2 cells, HDAC5 is predominantly accumulated in the nucleus and HDAC7 appears to be pancellular. This differential localization may relate to their ability to associate with the repressor complex and suggests that HDAC5 and HDAC7 are not interchangeable. These results further suggest that an additional, yet to be identified regulator(s) other than CaMK I/V, participates in the decision of the localization of HDAC5 and HDAC7. Moreover, others have also shown that while HDAC5 is primarily nuclear, HDAC4 predominantly localizes in the cytoplasm of COS cells (45). These observations might explain why cells contain three closely related HDACs. This fine regulatory mechanism will allow cells to achieve specificity and selectivity when responding to extracellular signals. In the literature, there are implications regarding the nature of these signals. Indeed, constitutive activation of the Ras-MAPK signal transduction pathway results in the increased nuclear localization of HDAC4 (62). Furthermore, ERK1/2 can be co-immunoprecipitated with HDAC4. It will be of interest to see whether HDAC7 can be induced by ET-1, a member of the endothelin family that possesses vasoconstrictive activity (63). Although HDAC7 can be induced by ET-1, a member of the endothelin family, it is not known whether the phosphorylation state of these serines is regulated individually by different kinases or a single kinase is capable of phophorylating all three sites. Although the immunoprecipitated CaMK IV from transfected cells can phosphorylate HDAC4 and HDAC5 in vitro (46), additional direct evidence will be essential to conclude that CaMK IV is the bona fide kinase for the serine residues of the class II HDACs. It is also important to know which phosphatase, either nuclear or cytoplasmic, is responsible for the dephosphorylation of class II HDACs. So far, this issue remains open. A recent report suggested that cytoplasmic HDAC7 that is not bound to HDAC3 is enzymatically inactive (64). However, cytoplasmic functions of HDAC7 cannot be formally ruled out. If so, what are the important cytoplasmic targets of HDAC7? A hint with this respect could come from the fact that it has been recently suggested that HDAC7 may participate in the degradation of ETA, a membrane-bound G protein (63).

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