α-Actinin 4 Potentiates Myocyte Enhancer Factor-2 Transcription Activity by Antagonizing Histone Deacetylase 7*

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Histone deacetylase 7 (HDAC7) is a member of class IIa HDACs that regulate myocyte enhancer factor-2 (MEF2)-mediated transcription and participate in multiple cellular processes such as T cell apoptosis. We have identified α-actinin 1 and 4 as class IIa HDAC-interacting proteins. The interaction domains are mapped to C terminus of α-actinin 4 and amino acids 72–172 of HDAC7. A point mutation in HDAC7 that disrupts its association with MEF2A also disrupts its association with α-actinin 4, indicating that MEF2A and α-actinin 4 binding sites largely overlap. We have also isolated a novel splice variant of α-actinin 4 that is predominantly localized in the nucleus, a pattern distinct from the full-length α-actinin 4, which is primarily distributed in the cytoplasm and plasma membrane. Using small interfering RNA, chromatin immunoprecipitation, and transient transfection assays, we show that α-actinin 4 potentiates expression of TAF55, a putative MEF2 target gene. Loss of MEF2A interaction correlates with loss of the ability of α-actinin 4 to potentiate TAF55 promoter activity. Ectopic expression of α-actinin 4, but not the mutant defective in MEF2A association, leads to disruption of HDAC7-MEF2A association and enhancement of MEF2-mediated transcription. Taken together, we have identified a novel mechanism by which HDAC7 activity is negatively regulated and uncovered a previously unknown function of α-actinin 4.

The α-actinins are an actin-binding protein family that consists of four members including muscle-specific α-actinin 2 and 3 and the ubiquitously expressed α-actinins 1 and 4 (1). All four proteins share extensive sequence homology with a conserved organization of functional domains that include an N-terminal actin binding domain composed of 2 calponin homology (CH) domains, a central rod domain consisting of four spectrin repeats (SR), 2 EF-hand calcium binding domains, and a C-terminal calmodulin-like domain (2, 3).

Despite intense study, the cellular functions of α-actinin 4 remain unclear. α-Actinin 4 has been shown to bind F actin to modulate cytoskeleton organization and cell motility (4). In addition, another report suggested that α-actinin 4 participates in apoptosis (5). Although α-actinin 4 has been shown to associate with transcription factors, there is no functional data suggesting that it is involved in transcriptional regulation (6, 7). Mutations of α-actinin 4 are linked to familial focal segmental glomerulosclerosis (8) and deletion of α-actinin 4 in mice causes severe glomerular disease (9). These observations suggest that α-actinin 4 plays a role in regulating multiple cellular processes and animal development.

Although predominately localized in the cytoskeleton, α-actinin 4 is also found in the nucleus of certain cell types (4) or translocates into the nucleus in response to extracellular stimuli. For example, treatment with phosphatidylinositol 3-kinase inhibitors or actin depolymerization can lead to nuclear accumulation of α-actinin 4 (4). However, the mechanism by which α-actinin 4 shuttles between the nucleus and the cytoplasm remains unclear, and the functional significance of nuclear α-actinin 4 has not been demonstrated.

Histone deacetylases (HDACs) are well known for their function as epigenetic regulators of the transcription machinery. In mammals, 18 different HDACs have been identified and grouped into three distinct classes based on sequence homology, subcellular localization, and the chemistry of their enzymatic activity. Class II HDACs can be subdivided into class IIa that includes HDAC4, -5, -7, and -9 (will be referred as class II hereafter) and class IIb, HDAC6 and -10 (10). Class II HDACs play a pivotal role in cell differentiation and animal development, partly due to their association with MEF2 transcription factors (10, 11). Class II HDACs are unique in their ability to shuttle between the nucleus and the cytoplasm. Consequently, nucleocytoplasmic shuttling of class II HDACs is an important regulatory mechanism that controls the activity of MEF2 (10, 11). During muscle differentiation and thymocyte development...

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4 The abbreviations used are: CH, calponin homology; SR, spectrin repeats; siRNA, small interfering RNA; HDAC7, histone deacetylase 7; RT, reverse transcription; Y2H, yeast two-hybrid; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; nt, nucleotides; MEF2, myocyte enhancer factor-2.
ment, class II HDACs are sequestered in the cytoplasm (12, 13). As a result, transcriptional repression by HDACs is relieved, leading to up-regulation of MEF2 target genes, such as muscle creatine kinase and Nur77 and subsequent muscle differentiation and thymocyte development, respectively. Thus far, the mechanism accounting for de-repression of MEF2 activity (by class II HDACs) has been largely attributed to the activity of calmodulin kinase and chromosome region maintenance 1 (CRM1) proteins that promote cytoplasmic retention of class II HDACs.

In this study we have identified α-actinin 1 and 4 as HDAC7-interacting proteins and defined their novel function. We also isolated a novel splice variant of α-actinin 4. We demonstrate that α-actinin 4 is capable of interacting with class II HDACs and potentiates transcription activity by MEF2. Taken together, our data support a model in which α-actinin 4 potentiates transcriptional activity of MEF2 in part by antagonizing HDAC7 activity.

EXPERIMENTAL PROCEDURES

Construction of HeLa Yeast Two-hybrid Library—HeLa cell cDNAs were generated by RT-PCR according to manufacturer’s protocol (Invitrogen). cDNAs sized above 1 kilobase were isolated from gels and purified. EcoRI linker was added to both ends of the cDNAs, and the resulting cDNAs were ligated with EcoRI-digested pGAD vector (Stratagene). Ligation reactions were transformed into Escherichia coli (Stratagene). More than 1 × 10^6 independent clones were obtained.

Yeast Two-hybrid Screens—Yeast two-hybrid (Y2H) screens were carried out using the standard lithium acetate method. HeLa Y2H library and pGBT9-HDAC7 (2-533, S178E/S344E/S479E) were co-transformed into yeast strain Y190. Approximately 3 × 10^6 yeast transformants were screened and selected on yeast minimal medium –Leu-Trp-His plates containing 40 mM 3-amino-triazone (Sigma). After 7 days, colonies were picked and confirmed by β-galactosidase assays. For confirmation, plasmids were recovered from yeast and retransformed into yeast along with the bait construct. Positive clones were then subjected to sequencing. In addition to clones encoding α-actinin 1 and 4, we also isolated several cDNA clones encoding C-terminal binding proteins (CtBP) proteins, consistent with previous reports (14). Standard Y2H assays were carried out according our published protocol (15).

RT-PCR—RNA isolation from MCF7, HeLa, and HEK293 cells was performed using the RNeasy Mini RNA isolation kit (Qiagen). All procedures were performed according to the manufacturer’s protocol. DNase I digestion was performed on the RNeasy column following the manufacturer’s suggestions using RNase- and DNase-free DNase I (Qiagen). The isolated RNA was used in a semi-quantitative RT-PCR reaction using the One-Step RT-PCR kit (Invitrogen) according to the manufacturer’s protocol. 200 ng of RNA template was used in each reaction. PCR amplification was repeated for 45 cycles. The final primer concentration in each reaction was 0.2 μM. The primers used are as follows: 5’-GATATGACCCACACCAGGACGAG-3’ (forward), 5’-CCCCCGCTCTGGGTAGTAGG-3’ (reverse, full-length-specific), 5’-GATATGACCCACACCAGGACGAG-3’ (reverse, isoform-specific), and 5’-GGGTTTGGTACATTGTTTGGTACGTG-3’ (reverse, both full-length and isoform).

Plasmid Construction—Full-length cDNA of actinin α4 was generated by PCR using a ZAP-expressed phage vector (a generous gift from Dr. Hirohashi) as a template. α-Actinin 4 (isoform) cDNA was PCR-amplified using Y2H clone as a template. Truncated and deleted α-actinin 4 cDNAs were PCR-amplified using full-length α-actinin 4 or its isoform as a template. The cDNAs were cloned into pCMX-PL1-HA or CMX-PL1-FLAG vectors (16) digested with EcoRI and Nhel site. For the glutathione S-transferase (GST) constructs, α-actinin 4 was PCR-amplified and subcloned into pGEX-4T vector. MEF2 expression plasmids and MEF2 reporter construct have been previously described (17). HDAC7 point mutation and deletions were generated by PCR reactions. TAF55 reporter constructs were gifts from Dr. Cheng-Ming Chiang (18).

Antibodies—α-Actinin 4 antiserum was generated using GST-α-actinin 4 (isoform) fusion proteins. Antibodies were purified by sequential purification through GST and GST-α-actinin 4 affinity columns. Anti-HA, anti-MEF2, and anti-FLAG antibodies were purchased from Santa Cruz and Sigma. HDAC7 antibodies were generated with peptide encompassing amino acids 115–129 of mouse HDAC7. HDAC7 antibodies were purified from peptide affinity chromatography (Affinity BioReagents). Anti-HDAC7 antibodies did not cross-react with HDAC4 or HDAC5 (data not shown).

GST Pulldown Assays—GST fusion proteins GST-α-actinin 4 and GST-HDAC7 were expressed in E. coli DH5α strain, affinity-purified, and immobilized on glutathione-Sepharose 4B beads. GST pulldown assays were carried out with purified, immobilized GST fusion proteins incubated with whole cell extracts expressing FLAG- or HA-tagged proteins. Binding reactions were carried out at 4 °C for 1 h with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 2 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) followed by extensive washes. The bound fractions were separated on SDS-PAGE gel and subjected to immunoblotting with anti-HA or anti-FLAG antibodies. For pulldown assays, 10% of the input is shown.

Immunoprecipitation—Cells were grown on 10-cm plates and transfected with appropriate plasmid (10 μg of total DNA) with Lipofectamine 2000 (Invitrogen). After 48 h, the cells were washed in 1 × PBS and resuspended in NETN buffer with protease inhibitors. After incubating on ice for 2 h, the lysed cells were centrifuged at 4 °C at 10,000 RPM for 10 min; supernatant was collected and kept at −80 °C. These lysates were incubated with appropriate antibody for 4 h at 4 °C, and then protein A/G beads were incubated with pre-clear whole cell extracts for 2 h at 4 °C. The immunopellets were washed 3–4 times followed by Western blots probed with the appropriate antibody. Ten percent of the input is shown on Western analyses.

Confocal Microscopy—Transfected cells were fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature and permeabilized in PBS with the addition of 0.1% Triton X-100 and 10% goat serum for 10 min. The cells were washed 3 times with PBS and incubated in a PBS, goat serum (10%) plus 0.1% Tween 20 solution (ABB) for 60 min. Incubation with primary antibodies was carried out for 120 min in ABB.
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The cells were washed 3 times in PBS, and the secondary antibodies were added for 30–60 min in the dark at room temperature in ABB. Coverslips were mounted to slides using Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole; H-1200, Vector Laboratories, Inc.).

For endogenous HDAC7, images of Alexa Fluor 488 were collected using a 488-nm excitation light from an argon laser, a 488-nm dichroic mirror, and 500–550-nm band pass barrier filter. All 4',6-diamidino-2-phenylindole-stained nuclear images were collected using a Coherent Mira-F-V5-XW-220 (Verdi 5W) Ti-Sapphire laser tuned at 750-nm, a 700-nm dichroic mirror, and a 390–465-nm band pass barrier filter. The primary antibodies used were purified α-HDAC7 rabbit polyclonal (Affinity BioReagents) and α-FLAG mouse monoclonal antibodies (Sigma). The secondary antibodies were used from Molecular Probes (α-mouse or α-rabbit Alexa Fluor 488, α-rabbit Alexa Fluor 594, and mouse Alexa Fluor 594).

Transient Transfection and Luciferase Assay—Transient transfections and luciferase assays were performed in 48-well culture plates. HeLa and CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg of streptomycin sulfate at 37 °C in 5% CO₂. Cells were transfected with a muscle creatine kinase promoter-containing reporter construct (17) and CMX-β-gal expression plasmids. Liptofectamine 2000 was used as a transfection reagent. The amount of DNA was kept constant by the addition of pCMX vector. After 5 h the medium was replaced, and the cells were harvested after 48 h of transfection and luciferase assay, and β-galactosidase activity was measured by using the luciferase assay system (Promega). Each reaction was performed in triplicate. Results shown indicate luciferase activity normalized to β-galactosidase levels. Transient transfection assays for TAF55 were carried out using as reporter constructs as described (18).

siRNA Transfection and RT-PCR—α-Actinin 4 and control siRNA Smart Pool was purchased from Dharmacon and dissolved in 1× siRNA buffer to a final concentration of siRNA 20 pmol/μl. The siRNA sequences are: target 1, 5'-CAACGAGUCAUAGUGAGUA-3' (sense) and 5'-UACUAUCAGUCUGUGUC-3' (antisense); target 2, 5'-UGGAAGUGCAGAUAUACAA-3' (sense) and 5'-UAAUUCUACAGCCCAUCCUG-3' (antisense); target 3, 5'-GAGACGCGGCUACACGGCA-3' (sense) and 5'-AUGACGUUGACCCGUC-3' (antisense).
(antisense); target 4, 5'-AACAAUAGCCCGCUUGUGA-3' (sense) and 5'-UCAGAGCCGUAUGGUGU-3' (antisense). For transfection, siRNA (200 pmol/well) and a 5-μl mixture of Lipofectamine 2000 and Lipofectamine plus reagent (Invitrogen) were re-suspended in Opti-MEM and transfected to HeLa cells (6-well plate, 70% confluent) for 24 h. Transfection medium was then replaced by Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (without penicillin/streptomycin). The cells were collected 48 h post-transfection respect.

Total RNA extraction was prepared using the RNeasy commercial kit (Qiagen). Semi quantitative RT-PCR was performed using the SuperScript One step RT-PCR kit with Platinum Taq. 500 ng of RNA was used for each reaction. The primers used for RT-PCR to examine the effects of α-actinin 4 siRNA are: TAF55, 5'-GAAGGAGCATGATCTGCTGT-3' (forward) and 5'-GAAGTACACCATCTGACA-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5'-TACAAGCATCTGACA-3' (forward) and 5'-AGGTTCCGCTTTCGAGCTT-3' (reverse). The primers to detect expression of the full-length and isoform of α-actinin 4 are described above. The intensity of the signals was quantified using the VERSADOC 3000 (Bio-Rad).

**RESULTS**

To identify proteins interacting with HDAC7, we generated an HDAC7 mutant (S178E/S344E/S479E) fused with the yeast Gal4 DNA binding domain for use in a yeast two-hybrid screen. The resulting cDNA coding 14-3-3 proteins would be isolated. The resulting construct, pGBT9-HDAC7 (1-533, S178E/S344E/S479E), was co-transformed with yeast two-hybrid library to isolate interacting clones. Of 20 clones sequenced, 3 clones encoded human α-actinin 1, and 3 clones encoded human α-actinin 4. Interestingly, one of the α-actinin 4 clones encoded an α-actinin 4 variant containing an internal deletion from nt 263–1433 (accession number NM_004924). This deletion resulted in the loss of amino acids 89–478 of α-actinin 4 that contains part of calponin homology 1 (CH1), all of the CH2 domains, and spectrin homology repeats 1 and 2 (Fig. 1A).

To verify that the cloned α-actinin 4 (isoform) cDNA was generated from mRNA, we carried out RT-PCR to examine the presence of this spliced isoform. Human α-actinin 4 is predicted to consist of 21 exons. Based on the isolated cDNA sequence, exons 3–12 are spliced out in the isoform. Primers that anneal to full-length α-actinin 4 mRNA (lanes 1, 3, and 5) or its isoform (lanes 2, 4, and 6) were used for RT-PCR using RNA prepared from MCF-7, HeLa, and HEK293 cells (Fig. 1B). We found that MCF-7, HeLa, and HEK293 cells express both full-length α-actinin 4 and the spliced isoform. A full-length or isoform HA-α-actinin 4 and its splice variant were used to co-transform yeast. Y2H assays are described under "Experimental Procedures." -Fold activation of the reporter activity is shown. B, cell extracts prepared from HeLa and CV-1 cells were used for immuno-precipitation using α-actinin 4 antibody, anti-α-actinin 4 antibody followed by Western blotting with anti-α-actinin 4 antibodies (lanes 3 and 5, 10% input is shown; lanes 2 and 6, pre-immune serum; lanes 3 and 7, anti-HDAC7 antibody, lanes 4 and 8, anti-α-actinin 4 antibody. Full-length α-actinin 4 is shown. C, control, C, HEK293 cells were co-transfected either with HA-HDAC7 alone or with FLAG-α-actinin 1, FLAG-α-actinin 4, or FLAG-α-actinin 4 (isoform). Forty-eight hours after transfection whole cell extracts were prepared and resolved by SDS-PAGE and probed with either anti-α-actinin 4 or anti-HA antibodies. Whole cell extracts were incubated with Flag M2, and bound fractions were separated by SDS-PAGE and visualized by immunoblotting (IB) with anti-α-actinin 4 antibody. IP, immunoprecipitation.
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A

HDAC7

α-actinin 4 association

-  
+  
-  
+  

B

α-actinin 4 association

HDAC7

-  
+  
-  
+  

C

Input
GST
753-911
832-911
644-839
753-759
644-759
644-911

D

GST
Input
2-254
2-875
2-254
500-938
72-172
156-248

E

Input
GST
GST-HDAC7
(72-172)
Input
GST
GST-HDAC7
(1K-172)

F

Input
GST
GST-HDAC7
(72-172)
Input
GST
GST-HDAC7
(1K-172)

G

HDAC1
HDAC4
HDAC5
HDAC7

Input
5 6 7 8 pulldown
absence of transfected plasmid, in addition to the full-length α-actinin 4, anti-α-actinin 4 antibodies detected another minor protein species, which migrated faster than full-length α-actinin 4 (lanes 1 marked with an asterisk). In cell lysates expressing HA-α-actinin 4 isoform or full-length, we detected both endogenous and transfected isoform (lane 2) and full-length proteins (lane 3). As expected, Western blotting with anti-HA antibodies detected α-actinin 4 isoform (lane 5) and the full-length protein (lane 6). These data indicate that the cloned α-actinin 4 (isoform) comigrates with the endogenous α-actinin 4 (lanes 1 and 2), indicating that the isoform is endogenously expressed in HeLa cells. Furthermore, the low expression of α-actinin 4 (isoform) protein observed is consistent with our RT-PCR data, demonstrating that full-length α-actinin 4 protein is expressed in excess to the isoform in HeLa cells.

Because HDAC7 (S178E/S344E/S479E) was used as bait to screen for HDAC7-interacting proteins, we tested whether α-actinin 4 is capable of interacting with wild-type HDAC7 by Y2H assays. We found that wild-type and mutant HDAC7 (S178E/S344E/S479E) interacted equally well with α-actinin 4 (Fig. 2A). To investigate whether endogenous α-actinin 4 associates with HDAC7, we carried out co-immunoprecipitation with extracts prepared from HeLa and CV-1 cells and found that endogenous HDAC7 and α-actinin 4 associate in these cells (Fig. 2B). We then tested the interaction of other α-actinins and HDAC7 by co-immunoprecipitation experiments. HA-HDAC7 expression plasmid was co-transfected with expression plasmids encoding FLAG-α-actinin 1, FLAG-α-actinin 4, or FLAG-α-actinin 4 (isoform) into HeLa cells. Immunoprecipitation and immunoblotting were carried out using anti-FLAG and anti-HA antibodies. As shown in Fig. 2C, HA-HDAC7 was immunoprecipitated in the presence of α-actinin 1 and α-actinin 4. Furthermore, both full-length and α-actinin 4 (isoform) were co-precipitated. These data indicate that HDAC7 is capable of interacting with both α-actinin 1 and 4 in mammalian cells.

To understand the molecular basis of the interaction between HDAC7 and α-actinin 4, we mapped the interaction domains by yeast two-hybrid and GST pulldown assays. Deletion and truncation fragments of HDAC7 fused with yeast Gal4 DNA binding domain (pGBT9-HDAC7) were co-transformed into yeast with yeast Gal4 activation domain fused with α-actinin 4 (pGAD-α-actinin 4). β-Galactosidase assays were carried out to examine the interaction. We found that HDAC7 fragments containing amino acids 2–172 and 72–254 were sufficient to interact with α-actinin 4, suggesting a minimal interaction domain localized within amino acids 72–172 of HDAC7 (Fig. 3A). The observation that both α-actinin 4 and its isoform interact with HDAC7 suggests that amino acids 644–911 are sufficient for the association with HDAC7 (Fig. 1A). To further map the interaction, we generated deletion and truncation constructs from amino acids 644 of full-length α-actinin 4. Fig. 3B shows that amino acids 832–911 of α-actinin 4 were sufficient to interact with HDAC7.

GST-α-actinin 4 (isoform) and GST-HDAC7 (72–172) fusion protein expression plasmids were generated and transformed into bacteria for protein expression. Immobilized GST-α-actinin 4 fusion proteins were incubated with whole cell extracts containing expressed FLAG-HDAC7. Retained fractions were subjected to Western blot analysis. Fig. 3C demonstrates that, consistent with the results from yeast two-hybrid assays, amino acids 832–911 of α-actinin 4 were capable of binding HDAC7. In reverse assays, immobilized GST-HDAC7 deletion and truncation proteins were incubated with extracts containing HA-α-actinin 4 followed by Western blotting with anti-HA antibodies. We found that amino acids 72–172 of HDAC7 bound α-actinin 4 in GST pulldown assays (Fig. 3D). To test whether region 832–911 was the only HDAC7 interaction domain, we generated expression plasmids HA-α-actinin 4 (2–839) and HA-α-actinin 4 (2–449, isoform), both of which lack the mapped HDAC7 interaction domain (Fig. 3B). We found that amino acids 2–839 did not interact with HDAC7 (72–172) (Fig. 3E). Similarly, GST-HDAC7 (72–172) failed to bind amino acids 2–449 of α-actinin 4 (isoform) (Fig. 3F). In summary, in vitro pulldown data were consistent with those of Y2H assays. Our data further indicate that amino acids 832–911 of α-actinin 4 are sufficient for HDAC7 binding and that amino acids 72–172 are the minimal HDAC7-interacting domain.

Amino acids 72–172 of HDAC7 share extensive sequence homology with other members of the class II HDACs, indicating that α-actinin 4 likely associates with other members of class IIa HDACs. To test this possibility, we carried out GST pulldown assays with the class II HDACs. Immobilized GST-α-actinin 4 (isoform) fusion protein was incubated with whole cell extracts expressing HA-tagged HDAC1, -4, -5, and -7 and Western blotting with anti-HA antibodies. Fig. 3G shows that α-actinin 4 interacts with HDAC4 and HDAC5 but not HDAC1. These data indicate that α-actinin 4 interacts specifically with class II HDACs both in vivo and in vitro.

α-Actinin 4 has been detected in the nucleus, cytoplasm, and plasma membrane depending on the cell type and culture conditions (4). The identification of the α-actinin 4 (isoform) raised the possibility that this isoform has a distinct subcellular localization from full-length α-actinin 4. Therefore, confocal microscopy experiments were carried out to examine the subcellular distribution of α-actinin 4 (isoform) in HeLa cells. A FLAG-tagged full-length α-actinin 4 expression plasmid was transfected into HeLa cells, and immunostaining was carried out.
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out using anti-FLAG and anti-HDAC7 antibodies to probe transfected α-actinin 4 and endogenous HDAC7, respectively. Fig. 4A shows that full-length α-actinin 4 was distributed throughout the whole cell including the plasma membrane and colocalized with α-actinin 4 (panels a, d, and e). In untransfected HeLa cells HDAC7 was primarily localized in the nucleus (panels c, e, and f). In transfected cells, however, a significant fraction of HDAC7 was re-distributed to the cytoplasm (panel c). We also examined the subcellular localization of α-actinin 4 (isoform). To our surprise, FLAG-α-actinin 4 (isoform) was found predominately to localize in the nucleus of HeLa cells (Fig. 4B). We also observed significant colocalization of transfected α-actinin 4 (isoform) and endogenous HDAC7. These data demonstrate that the subcellular localization of α-actinin 4 (isoform) is very different from that of the full-length α-actinin 4 and that overexpression of the full-length α-actinin 4 can sequester endogenous HDAC7 from its normal localization.

Class II HDACs associate with and repress transcription by MEF2 (10, 11, 17). It is possible that α-actinin 4 modulates transcriptional regulation by MEF2 through its association with class II HDACs. To test this possibility, we performed transient transfection assays using a reporter plasmid harboring a MEF2 binding site fused to the firefly luciferase gene (17). A constant amount of HDAC7 expression plasmid was co-transfected with the reporter construct and increasing amounts of full-length α-actinin 4 or its isoform. Fig. 5A shows that in CV-1 cells, overexpression of HDAC7 inhibits the transcription activity of the reporter gene (lanes 1 and 2). However, co-expression of full-length α-actinin 4 (lanes 3–5), isoform (lanes 6–8), or α-actinin 1 (lanes 9–11) with HDAC7 was capable of relieving inhibition by HDAC7 in a dosage-dependent manner. Similarly, we observed relief of HDAC7-mediated transcription repression by both full-length α-actinin 4 and its isoform in HeLa cells (Fig. 5B).

We also observed a slight activation of the reporter activity at the highest concentration of α-actinin 4 (Fig. 5, lanes 5 and 8), suggesting α-actinin 4 is capable of potentiating MEF2 activity. To test this possibility, we co-transfected α-actinin 4 and MEF2C expression plasmids along with the reporter construct in the absence of exogenous HDAC7 expression plasmid. Fig. 6A shows that in CV-1 cells, overexpression of MEF2C (lane 2), full-length α-actinin 4 (lane 3), or α-actinin 4 (isoform) (lane 5) enhanced the transcriptional activation by the MEF2 reporter construct. When α-actinin 4 (lane 4) or its isoform (lane 6) was cotransfected with MEF2C expression plasmid, MEF2C and α-actinin 4 had an additive effect on the reporter activity. Similar results were obtained in
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HeLa cells (Fig. 6B). We further tested whether the HDAC7 interaction domain was essential for α-actinin 4 to activate the MEF2 reporter. Fig. 6C demonstrates that in CV-1 cells co-transfection of α-actinin 4 (isoform) activated the MEF2 reporter construct, whereas a mutant lacking the HDAC7 interaction domain (2–449) did not. A similar trend was observed in HeLa cells (Fig. 6D). These data suggest that the same domain of α-actinin 4 is critical for HDAC7 interaction and transcription activation of a MEF2 reporter construct.

To verify whether our findings apply to endogenous MEF2 target genes, we examined whether α-actinin 4 is capable of modulating expression of TAF55 by siRNA knockdown experiments. TAF55 has been proposed to be a MEF2 target gene (18). To test whether expression of endogenous TAF55 is regulated by α-actinin 4, we introduced siRNA against α-actinin 4 or a control siRNA into HeLa cells and measured expression of TAF55 by RT-PCR. Fig. 7A demonstrates that knockdown of endogenous α-actinin 4, but not the control siRNA, significantly decreased the expression of TAF55. As a control, glyceraldehyde-3-phosphate dehydrogenase expression was included. To test whether α-actinin 4 associates with TAF55 promoter, we carried out ChIP assays. Fig. 7B demonstrates that MEF2, HDAC7, and α-actinin 4 associate with the DNA sequence flanking nt −264 to +7 upstream of the transcription start site of the TAF55 promoter (top panel). As a control, MEF2, HDAC7, and α-actinin 4 do not associate with nt −2898 to −2720 upstream of the transcription start site (bottom panel). We further investigated whether MEF2, α-actinin 4, and HDAC7 regulate TAF55 expression by transient transfection assays. A reporter construct harboring TAF55 basal promoter including the putative MEF2 binding site was co-transfected with MEF2C, HDAC7, and/or α-actinin 4 into HeLa cells. As a control, a deletion construct in which the MEF2 binding site was removed was included for parallel analyses. Fig. 7C shows that MEF2C, HDAC7, and/or α-actinin 4 did not have effects on the reporter activity of the control construct (lanes 1–10). In contrast, whereas MEF2C and α-actinin 4 potentiated the reporter activity (lanes 12, 14–20), HDAC7 inhibited the reporter activity (lanes 11 and 13). Taken together, these results indicate that MEF2C, HDAC7, and α-actinin 4 control TAF55 expression through association with its promoter.

Two possible mechanisms account for the ability of α-actinin 4 to relieve HDAC7-mediated repression on MEF2 reporter activity.

First, α-actinin 4 sequesters HDAC7 without interacting with MEF2. Alternatively, α-actinin 4 may independently interact with HDAC7 and MEF2. As such, α-actinin 4 might compete with HDAC7 for binding to MEF2, thereby disrupting the interaction of HDAC7 with MEF2. To test whether α-actinin 4 interacts with MEF2, GST pulldown assays were carried out. Whole cell extracts expressing FLAG-MEF2A were incubated with GST-α-actinin 4. As a control, whole cell extracts expressing HA-HDAC7 were included. Fig. 8A shows that GST-α-actinin 4 was capable of interacting with HDAC7 and MEF2A in vitro. The MEF2A interaction domain was mapped to amino acids 1–449 of α-actinin 4 (Fig. 8, B and C), whereas α-actinin 4 interacts with amino acids 2–86 of MEF2 (Fig. 8D).

To further investigate the molecular basis for the interaction between α-actinin 4 and HDAC7, we generated HDAC7 mutations within amino acids 72–172 of HDAC7, a region highly conserved in HDAC4 and HDAC5 (Fig. 8E). Three mutants, Δ79–100, L112A, and Δ158–178, were constructed and used to test their associations with α-actinin 4. Fig. 8F demonstrates that whereas L112A lost its interaction with α-actinin 4, Δ79–100 and Δ158–178 remained strongly associated (top panel). We further investigate whether these mutants were still capable of interacting with MEF2. Surprisingly, similar to α-actinin 4.
α-Actinin 4 interacts with MEF2A and HDAC7. A, α-actinin 4 interacts with MEF2 in vitro. GST pulldown assays were carried out by GST-α-actinin 4 incubation with cell extracts expressing HA-HDAC7 or FLAG-MEF2A. Western blotting were performed using anti-HA (lanes 1–3) and anti-FLAG antibodies (lanes 4–6). isoform. B, mapping of the MEF2A binding domain. C, α-actinin 4 and MEF2A interact in HeLa cells. WCE, whole cell extracts. IP, immunoprecipitation. D, α-actinin 4 (isoform) interacts with amino acids 1–86 of MEF2A. 15% of the input for α-actinin 4 (isoform) are shown. E, a sequence alignment of HDAC4, HDAC5, and HDAC7. mHDAC, mouse HDAC; hHDAC, human HDAC. F, isolation of HDAC7 mutant defective in its interaction with α-actinin 4. MEF2A and α-actinin 4 binding sites largely overlap. Note that HDAC7 (L112A) specifically loses its interaction with MEF2A and α-actinin 4 but not 14-3-3ε. WT, wild type.
was co-precipitated (lanes 2 and 3 and lanes 5 and 6). In contrast, α-actinin 4 (Δ441–479) did not have any effect on the association between HDAC7 and α-actinin 4. These data indicate that α-actinin 4 has an inhibitory effect on the association of MEF2 with HDAC7 and that MEF2 and/or HDAC7 interaction domain is critical for this inhibitory activity.

DISCUSSION

Increasing evidence has indicated that some cytoskeletal proteins, including the focal adhesion Lim-domain protein Trip6 not only act in the cytoplasm but may also be active in nuclear events such as transcriptional regulation (20). In this study we demonstrate that α-actinin 4, a well characterized actin-binding protein involved in cross-linking actin filaments (21), is capable of potentiating transcription activation by MEF2C. We have also isolated a splice variant of α-actinin 4 that is predominantly localized in the nucleus of HeLa cells. Taken together, our data support a model in which α-actinin 4 potentiates transcriptional regulation by MEF2 transcription factors.

Several lines of evidence strongly support the notion that α-actinin 4 is a transcriptional co-regulator. First, transient transfection data indicate that α-actinin 4 potentiates transcriptional activity by MEF2. Second, ectopic expression of α-actinin 4 decreases the interaction of MEF2A and HDAC7. Third, knockdown of α-actinin 4 decreases expression of TAF55. Fourth, MEF2C, α-actinin 4, and HDAC7 associate with the TAF55 promoter. These observations suggest that nuclear α-actinin 4 is capable of modulating the activity of a subset of transcription factors. Therefore, how nucleo-cytoplasmic shuttling of α-actinin 4 is regulated becomes an important issue. Although α-actinin 4 does not contain any distinct nuclear localization signal, previous studies have demonstrated that α-actinin 4 is capable of localizing in the nucleus depending on cell type and extracellular signals (4). The observation that α-actinin 4 (isoform) is predominantly localized in the nucleus suggests that amino acids 89–478 of the full-length α-actinin 4 have a negative effect on nuclear localization. Alternatively, it is possible that nuclear localization of the α-actinin 4 (isoform) is simply due to diffusion because of its small size. It has been previously shown that treatment with a phosphatidylinositol 3-kinase inhibitor promoted nuclear accumulation of α-actinin 4 (4). Along with our findings, these data raise the possibility that phosphatidylinositol 3-kinase phosphorylates and controls the subcellular localization of α-actinin 4, thus regulating transcription and possibly other nuclear processes. Further investigation will be necessary to elucidate the
Roles of α-Actinin 4 in Transcriptional Regulation

Our current data indicate that transcriptional regulation by MEF2 involves a protein-protein interaction network among MEF2, HDAC7, and α-actinin 4. Mapping the interaction domains indicates that α-actinin 4 binds to amino acids 2–86 of MEF2A (Figs. 8, B–D), which is highly conserved among MEF2 transcription factors. Notably, our published data indicated that HDAC7 binds to amino acids 1–86 of MEF2A (17), suggesting that MEF2 cannot bind HDAC7 and α-actinin 4 simultaneously. Indeed, we demonstrate that α-actinin 4 competes with HDAC7 for binding to MEF2A (Figs. 9, E and F). Similarly, MEF2 and α-actinin 4 binding sites on HDAC7 largely overlap (Fig. 8E). Therefore, HDAC7 is unlikely to directly associate with MEF2 and α-actinin 4 simultaneously. Our data indicate that the ability of α-actinin 4 to potentiate MEF2 correlates with its ability to interact with MEF2 and/or HDAC7, suggesting a competition model in which MEF2 may directly recruit α-actinin 4 to displace HDAC7 from MEF2. Alternatively, HDAC7 may recruit α-actinin in response to stimuli followed by association of α-actinin 4 with MEF2 and activation of transcription. Further investigation will be required to distinguish between these two possibilities.

Overexpression and mutations of α-actinin 4 are linked to human cancers and kidney diseases, respectively (4, 8, 9). Although the precise roles of α-actinin 4 in tumorigenesis are controversial, α-actinin 4 has been proposed to play a role in cell motility and cancer invasion (4, 22–25). Our findings that HDACs and the α-actinin family are likely significant. Future experiments will investigate the functional significance of the interaction between class II HDACs and the α-actinins during muscle differentiation.

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