Like the full-length histone deacetylase (HDAC) 4, its amino terminus (amino acids 1–208) without the carboxyl-terminal binding protein; MITR, MEF2-interacting transcriptional repression activity. To whom correspondence should be addressed: Dept. of Biochemistry, Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong, Tel.: 852-2358-8704; Fax: 852-2358-1552; E-mail: bcgwu@ust.hk.

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Functional Characterization of an Amino-terminal Region of HDAC4 That Possesses MEF2 Binding and Transcriptional Repressive Activity*

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Some of the key steps during myogenesis and are useful in cultures (1). Genes and are involved in regulating each other’s expression with each other to activate synergistically muscle-specific Myogenic regulatory factors and MEF2s can physically interact for the repressive effect. The amino terminus of HDAC4 can associate with the DNA-bound MEF2 in vitro, suggesting that it does not repress MEF2 simply by disrupting the ability of MEF2 to bind DNA. In vivo, MEF2 induces nuclear translocation of both the full-length HDAC4 and HDAC4-(1–208), whereas the nuclear HDAC4 as well as HDAC4-(1–208) are activated and repressed by the class II HDACs (9, 10) in turn specifically sequesters MEF2 to distinct nuclear bodies. In addition, we show that MyoD and HDAC4 functionally antagonize each other to regulate MEF2 activity. Combined with data from others, our data suggest that the full-length HDAC4 can repress MEF2 through multiple independent repressive domains.

Two families of transcription factors, namely the myogenic regulatory factors and the myocyte enhancer factor 2s (MEF2s),1 play key and decisive roles during myogenesis (1–3). Myogenic regulatory factors and MEF2s can physically interact with each other to activate synergistically muscle-specific genes and are involved in regulating each other’s expression (1).

Several myogenic differentiation model systems based on cell cultures (e.g. C2C12 and L6) are available, which recapitulate some of the key steps during myogenesis and are useful in dissecting the signaling pathways controlling myogenesis. In proliferating C2C12 myoblasts, it is known that MyoD and several MEF2 members are already present (4, 5). However, they are functionally repressed to ensure that differentiation does not start precociously (6–8). The underlying repression mechanisms have just begun to be unraveled, which represent one of the major breakthroughs in the study of myogenesis in recent years. Both myogenic regulatory factors and MEF2s are bound and repressed by members of the histone deacetylase family (HDACs) (9, 10). HDACs are thought to repress transcription by removing the acetyl group from histone tails resulting in tighter packaging of nucleosomes and reduced access of transcription factors to regulatory regions of genes (11, 12). In addition to histones, HDACs are also known to deacetylate other cellular proteins including p53, MyoD, and tubulin, which modulate their biological activities ranging from DNA binding to microtubule dynamics (8, 13–15). MyoD is bound and repressed by class I HDACs, whereas MEF2s are directly bound and repressed by class II HDACs (9, 10). Whereas the MADS/MEF2 motifs in MEF2 proteins directly interact with the class II HDACs, conserved motifs in the amino termini of the class II HDACs bind MEF2s (6, 16–18). One of the characteristic features of the class II HDACs is that they shuttle between the nucleus and the cytoplasm under different cellular conditions (9, 10, 12, 19). In the nucleus, class II HDACs mainly localize at nuclear bodies, the distinct punctate/or speckle-like structures with unknown identity (20, 21). For HDAC5 and -7, they are preferentially nuclearly localized in proliferating myoblasts (22, 23). At the start of differentiation in response to the Ca2+/calmodulin-activated protein kinase-mediated signaling pathway, HDAC5/7 dissociate from MEF2s and translocate to the cytoplasm where they are retained by associating with 14-3-3 (22–27). Although HDAC4 is equally effective in repressing MEF2 activities in reporter assays and in inhibiting differentiation of C2C12 cells when stably expressed (6, 16), HDAC4 differs from HDAC5 and -7 in that it is mainly cytoplasmic in proliferating C2C12 and NIH3T3 cells (28–30). At the start of differentiation, HDAC4 translocates to the nucleus (28, 29). The underlying mechanism and its biological significance remain unclear.

Although HDAC4 contains a canonical deacetylase domain at its carboxyl terminus, it is enzymatically inactive on its own and exerts its repressive effect through recruitment of a corepressor complex consisting of HDAC3, SMRT, and N-CoR (31). Interestingly, the amino termini of HDAC4 (aa 1–208), -7 (aa 1–372), and -9 (aa 1–590, also called MEF2-interacting transcriptional repressor or MITR), in the absence of their carboxyl-terminal deacetylase domains, are also capable of repressing MEF2s (17, 23, 32). In the case of MITR, the underlying re-
pression mechanisms are thought to be due to recruitment of either the carboxyl-terminal binding protein (CtBP), a transcription corepressor, or HDAC1 (32, 33). Although a conserved CtBP-binding motif has been found in both MTR and HDAC4, mutations that disrupt CtBP binding to MTR only partially relieve the repressive effect of MTR, suggesting the existence of a second repressive region in the amino termini of MTR and HDAC4. A common identity of this second repressive region in either HDAC4 or MTR remains uncharacterized.

In this report, we looked further into the mechanism by which the amino terminus of HDAC4 (i.e. aa 1–208) represses MEF2.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, DNA Constructs, and Reagents—**HeLa and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% CO2. C2C12 cells were grown in DMEM with 20% fetal bovine serum and antibiotics (also called growth medium) and were induced to differentiate in DMEM containing 2% horse serum and antibiotics (differentiation medium) when cells were near-confluent. 3xMEF2-luc, 5xGal4-luc, Gal4-MEF2C, GFP-HDAC4, and FLAG-HDAC4 were described previously (17, 34). FLAG-MEF2-(1-208) was provided by E. Olson.FLAG-Six1 was constructed by inserting the mouse Six1 cDNA into the Nhel/NotI sites of pCDNA3 containing a cassette encoding the FLAG tag. xp-HDAC4-(1–208) and xp-HDAC4-(119–208) were constructed by inserting the cDNA fragments amplified by PCR into pcDNA3.1HisC (Invitrogen) and verified by sequencing. Antibodies employed in this work included anti-FLAG (M2, Sigma) and anti-Xpress (or Omni-Ab, M21, Santa Cruz Biotechnology). HDAC4-(1–208)-GFPI and GFP-MEF2 were constructed by inserting the corresponding cDNA fragments into pEGFP-N1 and pEGFP-C1, respectively (Invitrogen). Trichostatin A was purchased from Calbiochem.

**Transfection, Cell Lysis, and Luciferase Reporter Assays—**Cells were first transfected with various plasmids using LipofectAMINE Plus reagents (Invitrogen) following the manufacturer’s instruction and cultured in either DMEM with 10% fetal bovine serum for 24–36 h (for HeLa and COS-7) or growth medium for 36 h by following a differentiation medium for another 30 h (for C2C12) prior to harvest. Cells were then lysed in the lysis buffer (50 mM HEPES, pH 7.6, 1% (w/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 100 mM NaF, 20 mM p-nitrophenyl phosphate, 20 mM β-glycerophosphate, 50 μM sodium vanadate, 2 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin), followed by removal of insoluble debris with a bench-top centrifuge at 14,000 rpm for 2 min to obtain whole cell extracts (WCEs). Luminometry (Analytical Luminescence Laboratory, San Diego). Luciferase units were normalized against total protein amount present in each sample determined by the protein assay reagent from Bio-Rad.

**Fluorescent Imaging of Live Cells—**Fluorescent images of live HeLa or C2C12 myoblasts containing GFP fusion proteins were acquired by an Olympus IX70 fluorescent microscope linked to a charge-coupled device digital camera (Spot RT, Diagnostic Instruments Inc.).

**Electrophoretic Mobility Shift Assays—**A double-stranded oligonucleotide probe containing a consensus MEF2 site, 5′-GATGCGTCTA-AAAAATACCCGCCTGC-3′ (sense) (sc-2521, Santa Cruz Biotechnology), was end-labeled with T4 polynucleotide kinase and [α-32P]ATP. Unincorporated ATP was removed by QIAquick spin column (Qiagen). For each binding reaction, 10 μg of HeLa WCE was added to a 20-μl reaction mixture containing ~22,000 cpm of probe, 1 μg of poly(dI-dC), and 20 μg of bovine serum albumin in the binding buffer (15 mM HEPES, pH 7.6, 40 mM KC1, 1 mM EDTA, 1 mM diithiothreitol, 5% glycerol, and without the recombinant GST-HDAC4-(1–208), GST-HDAC4-(119–208), or GST. All the components above except the probe were first pre-mixed on ice for 30 min. Upon addition of the probe, the binding reactions were carried out for another 20 min at room temperature. The samples were loaded onto a 3.75% non-denaturing polyacrylamide gel, and electrophoresis was performed at 200 V for 2 h at 4 °C. The gel was subsequently dried and subjected to autoradiography.

**Co-immunoprecipitation Assays—**COS-7 cells were co-transfected with various plasmids. 36 h after transfection, the cells were cross-linked with 20 μg/ml dithiothreitol/succinimidyldiisocyanate (Pierce) for 5 min followed by lysis in RIPA buffer (25 mM HEPES, pH 7.4, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin). Protein A-Sepharose beads were first preincubated with 50 mg/ml of bovine serum albumin for 30 min to reduce nonspecific binding. 200 μg of extracts were then added along with 2 μg of anti-Xpress antibody for 2 h at 4 °C. After extensive washing with the RIPA buffer, bound proteins were eluted out by boiling and subjected to SDS-PAGE and immunoblotting.

**Full-dose Assays Using Biotinylated Oligonucleotide Probes Containing Either a Wild Type or a Mutant MEF2 Site—**The sense strand of a wild type MEF2 oligonucleotide (5′-AAAAAGGTCTTAAATACCCGCCTGC-3′) (6) was synthesized with a covalently linked 5′ biotin and annealed to its antisense strand to form a double-stranded probe. The matching biotinylated MEF2 oligonucleotide is identical to the wild type except for two base changes (in lowercase letters) in the ME2F2 site: 5′-AAAAAGGTCTTAAATACCCGCCTGC-3′ C.

**RESULTS**

**The Amino-terminal Region (aa 119–208) of HDAC4 Represses MEF2-dependent Transcription—**It was shown previously that an amino-terminal fragment of HDAC4 (i.e. aa 1–208) binds MEF2 and represses MEF2-dependent transcription (17). A smaller fragment (aa 119–208) of HDAC4 was also shown to bind MEF2 (17). However, it is not known whether it still process the MEF2 repressive activity. To confirm the previous result and to further define the region that contains the repressive activity, we performed a reporter assay in HeLa cells using a MEF2-dependent luciferase reporter (3xMEF2-luc). In agreement with previous results, the full-length HDAC4 and HDAC4-(1–208) significantly repressed MEF2-dependent transcription (Fig. 1A) (17). Interestingly, HDAC4-(119–208) also repressed MEF2 in a dose-dependent manner (Fig. 1A). Although HDAC4-(119–208) was less repressive compared with the full-length and HDAC4-(1–208), at a higher dose it could still repress the activity of the MEF2 reporter below the basal level. To test whether HDAC4-(119–208) also repressed the endogenous MEF2, we co-transfected myogenic C2C12 cells with 3xMEF2-luc along with either the full-length HDAC4 or its two amino-terminal derivatives. As shown in Fig. 1B, all three HDAC4 constructs repressed the activity of the endogenous MEF2s to different extent with the full-length HDAC4 being the most repressive (~95%) and HDAC4-(119–208) being the least repressive (~50%). Therefore, our results here demonstrated that HDAC4-(119–208) can repress MEF2 in vivo.

**The Amino-terminal Derivatives of HDAC4 Repress MEF2 Without Interfering with MEF2 DNA Binding—**In the reporter assays above, the repression of MEF2 by HDAC4 or its amino-terminal derivatives could conceivably be caused by a block to

**Multiple Sequence Alignment—**Amino acid sequences of human HDAC4 (SwissProt accession number NP_005465.1), HDAC5 (RefSeq accession number NP_055465.1), HDAC7 (GenBank accession number AF63491.1), and MTR (RefSeq accession number NP_478056.1) were aligned by the ClustalX 1.81 using the GONNET series as the Multiple alignment parameter (35). Similarity calculation of residues was performed using the BLOSUM62 Matrix (36).
either DNA binding or transactivating function of MEF2. To distinguish between the two possible mechanisms, we fused the genes encoding the full-length MEF2C and the yeast Gal4 DNA binding domain (Gal4DBD, aa 1–147) to generate the Gal4-MEF2 fusion protein, and we tested the repressive effect of the full-length HDAC4 and its amino-terminal derivatives on this fusion protein. As the MEF2 DNA binding domain was not required in this assay system, any repression detected would most likely be due to an effect on the transactivating function of MEF2. As shown in Fig. 2A, both the full-length HDAC4 and its amino-terminal derivatives repressed the transcriptional activity of the fusion protein, suggesting that they repress MEF2 by interfering with the transactivating function of MEF2. To prove directly that the amino-terminal derivatives of HDAC4 repress MEF2 without affecting its binding to DNA, we performed electrophoretic mobility shift assays using HeLa whole cell extracts. Our electrophoretic mobility shift assays resolved at least two MEF2-containing bands (Fig. 2B, lane 1), indicating the existence of different MEF2-containing higher order protein complexes. Importantly, both bands shifted upwards (supershift) in the presence of the bacterially expressed HDAC4-(119–208) Represses MEF2.
HDAC4-(119–208) Represses MEF2 by Recruiting Additional HDACs—As HDAC4-(1–208) contains the CtBP-binding motif (33), this could partially account for the repressive effect of HDAC4-(1–208). In contrast, HDAC4-(119–208) was devoid of the CtBP-binding motif and any intrinsic deacetylase domain; this suggested that HDAC4-(119–208) may repress transcription by recruiting additional repressors. We first carried out a co-immunoprecipitation assay to test whether HDAC4-(119–208) forms complexes with other HDACs in vivo. COS-7 cells were co-transfected with various expression constructs as indicated (Fig. 3A). Although we failed to detect stable binding of either HDAC4, HDAC3 (data not shown), or Six1 to HDAC4-(119–208), we could clearly detect binding of HDAC1 to HDAC4-(119–208), suggesting that HDAC4-(119–208) could repress MEF2 by recruiting HDAC1 (Fig. 3A). In addition, we showed that the repressive effect of HDAC4-(119–208) on the MEF2-dependent reporter could be partially relieved by treatment of cells with trichostatin A (TSA) (Fig. 3B). We could clearly detect binding of HDAC1 to HDAC4-(119–208)/MEF2 complex, indicative of HDAC4 interacting with nuclear MEF2 to exert the repressive effect.

MyoD and HDAC4/HDAC4-(1–208)/HDAC4-(119–208) Antagonize Each Other to Regulate MEF2—As both MyoD and HDAC4 interact with MEF2 through the MADS/MEF2 motifs (6, 38), we asked whether MyoD and HDAC4s antagonize each other in regulating MEF2 activity. As seen in Fig. 4A, the repression of MEF2 by HDAC4 and its amino-terminal derivatives could be effectively relieved by MyoD in a dose-dependent manner. Moreover, MyoD was more effective in relieving MEF2 repression by the amino-terminal derivatives than by the full-length HDAC4. The result above indicated that overexpression of MyoD could switch MEF2 from a repressed state to a highly activated state. Interestingly, MyoDT4basic, a MyoD mutant defective in MEF2 binding, failed to de-repress MEF2 as effectively as the wild type MyoD did (Fig. 4B) (39). This suggested that direct MEF2 binding is a prerequisite for MyoD to antagonize HDAC4. To demonstrate further that MyoD and HDAC4 compete to regulate MEF2, we studied the effect of HDAC4 and its amino-terminal derivatives on MyoD-mediated synergistic activation of 3xMEF2-luc. In the absence of HDAC4, addition of MyoD significantly activated the reporter gene activity due to interaction of the transfected MyoD with the endogenous MEF2 (Fig. 4C). In the presence of either HDAC4 or its amino-terminal derivatives, the synergistic effect between MyoD and MEF2 was significantly reduced/abolished (Fig. 4C). Therefore, our data above support the hypothesis that MyoD and HDAC4 antagonize each other to regulate MEF2.

The Amino Terminus of HDAC4 Targets MEF2 to Nuclear Bodies—Although HDAC4 is predominantly localized to the cytoplasm (28–30), MEF2 is mainly a nuclear protein. A question arises as to how cytoplasmic HDAC4 can physically associate with nuclear MEF2 and repress its transcriptional activ-
Several Independent Repression Domains Exist in HDAC4—

As mentioned in the Introduction, several independent repressive domains are already known to exist in HDAC4 and other class II HDACs. They include the amino-terminal CtBP-binding motifs and the carboxyl deacetylase domain that recruits SMRT/N-CoR/HDAC3 (31, 33). In a recent report, two motifs in MITR (aa 390–400 and 400–490) were shown to bind heterochromatin protein 1 (HP1) which recognizes the methylated lysine in histone tails and represses transcription by recruiting additional HDACs and histone methyltransferases (40). Conserved regions in both HDAC4 and -5 (roughly around aa 400–540 before the start of the carboxyl deacetylase domain) were also shown to bind HP1 (40). In addition to these known repressive domains, we demonstrate in this report that a small region in the amino terminus of HDAC4 (aa 119–208), which is physically separate from the CtBP-binding motif (aa 43–57) and without the HP1-binding region and the carboxyl deacetylase domain, is still capable of binding and repressing MEF2. HDAC4-(119–208) specifically associates with HDAC1 which accounts for its repressive activity. This result is further supported by a previous finding that HDAC1 associates with MITR which also contains a region homologous to HDAC4-(119–208) (Fig. 6A) (32, 33). Thus, it appears that the full-length HDAC4 can repress transcription through at least four independent domains: binding to CtBP through aa 43–57, recruitment of HDAC1 through aa 119–208, interaction with HP1 through aa 408–510, and association with HDAC3/SMRT/N-CoR through the carboxyl deacetylase domain (aa 612–1084) (Fig. 6B). Our data also suggest that the four distinct repression domains of HDAC4 cooperate with each other to bring about the most efficient transcription repression, as loss of either two or three repressive domains (in the case of HDAC4-(1–208) and HDAC4-(119–208), respectively) results in gradual decrease of the repressive activity in the truncated HDAC4s (Figs. 1A, 2A, and 4C). Furthermore, we think other class II HDACs may also recruit HDAC1 through a similar mechanism as they all contain motifs at the amino termini that are homologous to aa 119–208 in HDAC4 (Fig. 6A).
shown that the MADS/MEF2 motif of MEF2 is involved in interaction with both MyoD and HDAC4 (6, 38). The question arises as to whether MyoD and HDAC4 compete for MEF2 binding in a mutually exclusive manner or whether both proteins can bind MEF2 simultaneously. Although direct biochemical binding data are lacking (mainly due to weak binding between MEF2 and MyoD/E proteins in vitro), we favor the “mutually exclusive model.” On one hand, the synergistic activation of a MEF2-dependent reporter by MEF2 and MyoD can be effectively abolished by either the full-length HDAC4 or, to a lesser extent, HDAC4-(1–208) and HDAC4-(119–208) (Fig. 4C). On the other hand, only the wild type MyoD, but not a MyoD mutant defective in MEF2 binding, can overcome the repressive effect of either the full-length or the amino-terminal derivatives of HDAC4 in a dose-dependent manner (Fig. 4, A and B). The latter result differs from a previous report (6). The ratio of MyoD over HDAC4 used in the transfection scheme may underlie the difference. In addition, the weaker binding between MEF2 and MyoD/E proteins may partly explain why it is less efficient for MyoD to counteract the repressive effect of HDAC4 on MEF2. Furthermore, the mutually exclusive model was supported by the dynamic nature of class II HDACs during myogenic differentiation. In myoblasts, class II HDACs associate with MEF2 to repress transcription of muscle-specific genes. Although MyoD is present in C2C12 myoblasts, it is in a repressed state itself and is not believed to interact with MEF2 (7). At the beginning of differentiation when calmodulin-activated protein kinase is activated, class II HDACs will be dissociated from MEF2 and shuttled to the cytoplasm (9, 10). This would be the time when MyoD (perhaps together with p300/PCAF) is assembled on MEF2 to synergistically activate the transcription of pro-differentiation genes.

HDAC4 differs from other class II HDACs in that it is mainly cytoplasmic in proliferating C2C12 myoblasts (18, 29). We show that the cytoplasmic HDAC4 can be shuttled to the nucleus by co-transfected MEF2 (Fig. 5A, b), in agreement with previous findings (18, 30). This suggests that MEF2 may be one of the limiting factors in determining the subcellular localization of HDAC4. Due to limited amount of endogenous MEF2 in myoblasts, a large fraction of HDAC4 that cannot associate with MEF2 ends up in the cytoplasm. As MEF2 protein level increases during differentiation (41), MEF2 would be expected
regions in other class II HDACs by ClustalX 1.81. Identical amino acids different repressive domains.

A

to induce nuclear translocation of HDAC4 in differentiated myotubes, which is in agreement with previous findings (28, 29). This could be a unique role played by HDAC4 among class II HDACs to ensure that active MEF2 is repressed again by HDAC4 in myotubes at the end of differentiation process (presumably when calmodulin-activated protein kinase signal is already down-regulated).

From our data in Fig. 5A, it is also interesting to note the intricate interplay between MEF2 and HDAC4 in cells. Whereas MEF2 is the driving force to drag HDAC4 from the cytoplasm into the nucleus (Fig. 5A, b), once in the nucleus, HDAC4 can target MEF2 to distinct clusters of nuclear bodies (Fig. 5A, f). As neither GFP-HDAC4 nor GFP-MEF2 alone localizes at nuclear bodies, our results suggest that another factor(s) may influence the localization of the HDAC4-MEF2 complex in the nucleus. SMRT is one such candidate factor, as it normally localizes at nuclear bodies and also drives cytoplasmic HDAC4 to nuclear bodies when co-expressed with HDAC4 in the cells (21). As HDAC4 was previously found to interact with SMRT through its carboxyl terminus (31), it came as a surprise that aa 1–208 of HDAC4 is crucial for targeting MEF2 to nuclear bodies.

It would be interesting to find out next which adaptor molecule(s) bridges HDAC1 and an (119–208) of HDAC4 and which molecule(s) (in addition to SMRT) specifically targets the HDAC4-MEF2 complex to nuclear bodies. Other issues worthy of consideration in the near future include whether the endogenous MEF2 associates with class II HDACs in the free or DNA-bound form and which of the class II HDACs preferentially binds MEF2 in proliferating myoblasts and mature myotubes.

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