Association of COOH-terminal-binding Protein (CtBP) and MEF2-interacting Transcription Repressor (MITR) Contributes to Transcriptional Repression of the MEF2 Transcription Factor*

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The class II histone deacetylases (HDACs) 4, 5, and 7 share a common structural organization, with a carboxy-terminal catalytic domain and an amino-terminal extension that mediates interactions with members of the myocyte enhancer factor-2 (MEF2) family of transcription factors. Association of these HDACs with MEF2 factors represses transcription of MEF2 target genes. MEF2-interacting transcription repressor (MITR) shares homology with the amino-terminal extensions of class II HDACs and also acts as a transcriptional repressor, but lacks a histone deacetylase catalytic domain. This suggests that MITR represses transcription by recruiting other corepressors. We show that the amino-terminal regions of MITR and class II HDACs interact with the transcriptional corepressor, COOH-terminal-binding protein (CtBP), through a CtBP-binding motif (P-X-D-L-R) conserved in MITR and HDACs 4, 5, and 7. Mutation of this sequence in MITR abolishes interaction with CtBP and impairs, but does not eliminate, the ability of MITR to inhibit MEF2-dependent transcription. The residual repressive activity of MITR mutants that fail to bind CtBP can be attributed to association with other HDAC family members. These findings reveal CtBP-dependent and -independent mechanisms for transcriptional repression by MITR and show that MITR represses MEF2 activity through recruitment of multicomponent corepressor complexes that include CtBP and HDACs.

Regulation of chromatin structure is a central mechanism for the control of gene expression and modulation of the acetylation state of nucleosomal histones has been revealed as a dynamic mechanism for chromatin remodeling (reviewed in Ref. 1). Acetylation of the amino-terminal tails of core histones by histone acetyltransferases results in chromatin relaxation and transcriptional activation, whereas deacetylation of histones by histone deacetylases (HDACs) is associated with transcriptional repression. Recruitment of histone acetyltransferases and HDACs by DNA-bound transcription factors results in the formation of multiprotein transcription regulatory complexes that confer cell type specificity and signal-dependent regulation to arrays of subordinate genes. There is also evidence that direct acetylation/deacetylation of certain transcription factors provides a mechanism for reversible regulation of transcription.

HDACs can be divided into two classes, I and II, on the basis of size, sequence homology, and formation of distinct complexes. Class I includes HDACs 1, 2, and 3, which are expressed ubiquitously (2–5). Class II includes HDACs 4, 5, 6, and 7, which contain a conserved domain of several hundred amino acids extending amino-terminal from the carboxy-terminal catalytic domain (6–10). Class II HDACs are tissue-restricted, with especially high levels of expression in heart, skeletal muscle, and brain. MEF2-interacting transcription repressor (MITR) (11), also called HDAC-related protein (HDRP) (12), shares homology with the amino-terminal extension of class II HDACs, but lacks a carboxy-terminal catalytic domain.

Class II HDACs and MITR/HDRP have been shown to interact with members of the MEF2 family of MADS (MCM1, Agamous, Deficiens, serum response factor) box transcription factors, resulting in transcriptional repression (7, 8, 11, 13, 14). MEF2 factors play central roles in the control of muscle differentiation and have been implicated in growth factor signaling and apoptotic pathways (reviewed in Ref. 15). Thus, negative regulation of MEF2 activity by association with HDACs may inhibit growth and/or differentiation, depending on cell type and the presence of other corepressors for MEF2 and HDACs.

While HDACs are presumed to inhibit transcription as a consequence of their deacetylase activity, the mechanism whereby MITR represses transcription is unclear. MITR has been shown to interact with HDAC1, which could account for its ability to repress MEF2-dependent transcription (11). Consistent with this conclusion, the HDAC inhibitor trichostatin (TSA) has been reported to partially attenuate transcriptional repression by MITR (11). However, another study reported that TSA had no effect on transcriptional repression by MITR, concluding that its mechanism for repression did not involve other HDACs (12).

Here we report that HDACs 4 and 5 and MITR associate with the transcriptional corepressor COOH-terminal binding protein (CtBP) via a CtBP-binding motif (P-X-D-L-R) conserved in HDACs 4, 5, and 7 and MITR. Mutation of this sequence abolishes association of CtBP with MITR and substantially diminishes, but does not eliminate, the transcriptional repressing transcription factor; TSA, trichostatin; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
Association of CtBP with MITR

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—A mouse 17-day embryo MATCHMAKER cDNA library (CLONTECH) was screened with GALA-HDAC4 bait in the yeast two-hybrid system, as described previously (16). The bait contained amino acids 1–640 of human HDAC4 fused to the GAL4 DNA binding domain. Positive clones were subjected to specificity tests using the GAL4 DNA binding domain alone as bait. Those clones that were specific for interaction with GAL4-HDAC4 bait were sequenced.

**Cloning of Mouse MITR**—A mouse expressed sequence tag encoding MITR (GenBank™ accession number AV118321), identified by searching the data base, was used to screen a mouse embryonic day 10.5 cDNA library (Stratagene) for cDNAs encompassing the complete open reading frame. The deduced open reading frame of mouse MITR is 586 amino acids and shows 94 and 66% identity to human and Xenopus MITR proteins, respectively (11, 12). The nucleotide sequence of mouse MITR was deposited in the data base (GenBank™ accession number AF324492).

**Cell Culture, Plasmids, and Transfections**—10T1/2, COS, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mm l-glutamine, and penicillin-streptomycin. Transfections were performed using the lipid-based reagent Fugene 6 (Roche Molecular Biochemicals) and cells growing at a density of 5–10×10^6 cells/35-mm dish. Epitope-tagged derivatives of CtBP, MITR, HDAC4, and HDAC5, containing amino-terminal FLAG or Myc tags, were generated using the pcDNA3.1 expression vector (Invitrogen). Expression plasmids for carboxy-terminal FLAG-tagged HDAC1 (pBD) and HDAC9 (pcDNA6) were kindly provided by Dr. Stuart Schreiber (Harvard). For GALA-dependent reporter assays, 10T1/2 cells were cotransfected with a luciferase reporter plasmid under control of four GALA DNA binding sites and the thymidine kinase (tk) promoter, pM1-based expression vectors encoding MITR or MITR(DL-AS) fused to the GALA DNA binding domain, and a CMV-lacZ (Invitrogen) plasmid to normalize for variable transfection efficiency. For MEF2-dependent transcription assays, 10T1/2 cells were transfected with a luciferase reporter plasmid encoding MITR or MITR(DL-AS) fused to the GAL4 DNA binding domain alone as bait. Those clones that were specific for interaction with GAL4-HDAC4 bait were sequenced.

**RESULTS**

**Interaction between HDAC4 and CtBP in the Yeast Two-hybrid System**—HDACs 4, 5, and 7 are bipartite, with an amino-terminal region that contains a MEF2-binding motif, and a carboxy-terminal catalytic region (Fig. 1A). In previous studies, we (13) and others (7, 8, 11, 14) showed that association of HDACs 4, 5, and 7 with MEF2 is mediated by a conserved 18-amino acid motif near their amino-termini. This motif is also conserved in MITR/HDRP, which lacks a catalytic domain.

To further investigate the functions of the amino-terminal extension of class II HDACs and MITR, we performed yeast two-hybrid screens using amino acids 1–640 of HDAC4 fused to the GAL4 DNA binding domain as bait (Fig. 1A). Two independent cDNA clones encoded the entire 441-amino acid CtBP1 protein; the other encoded amino acids 25–441. These CtBP1-GAL4 activation domain fusion proteins interacted specifically with the HDAC4 bait and not with the GALA DNA binding domain alone (see “Experimental Procedures”).

Because CtBP has been shown to interact with the consensus motif P-X-D-L-S/R (20, 21), we scanned the 640-amino acid region of HDAC4 used as bait and identified a potential CtBP-binding motif between residues 43–46 and data not shown). Residues 1–201 of MITR (GST-MITR-(1–201)) contained the CtBP-binding motif shown in black.

![Diagram 1](image)

**Fig. 1. Schematic diagrams of class II HDACs and MITR.** A, schematic diagrams of MITR and class II HDACs. B, amino acid sequences of human (h) and mouse (m) HDACs and MITR with the CtBP-binding motif shown in black.
derivatives of HDAC4, HDAC5, or MITR (1 vector encoding FLAG-tagged CtBP and the indicated Myc-tagged and in vivo 10% of the input [35S]methionine-labeled CtBP was applied directly to resolved by SDS-PAGE and analyzed by autoradiography. In After washing and recovery of the beads, associated proteins were onine-labeled CtBP, as described under “Experimental Procedures.” The positions of molecular weight markers are indicated to the left hand panel. The regions of MITR and HDAC5 responsible for recruitment of CtBP were also assayed by coimmunoprecipitation assays in transfected 293T cells. As observed in the GST binding assays, the interaction of MITR with CtBP required the CtBP-binding motif between residues 23 and 27 and was abolished by the DL-AS mutation of this motif (Fig. 2D). In contrast, mutation of the corresponding region of HDAC5 (mutant HDAC5-(EL-AS)) did not prevent its association of CtBP (Fig. 2E). This result suggested that HDAC5 contained additional CtBP-binding motifs. Indeed, a deletion mutant containing residues 121–664, which lacked the CtBP consensus binding motif at the analogous position to that in MITR, also bound CtBP (Fig. 2E), indicating the presence of an additional CtBP-binding motif in this region of the protein. We conclude that CtBP is specifically recruited to MITR through interaction with the P-X-D-L-R motif, which is also conserved in the amino-terminal region of class II HDACs. However, additional regions of HDAC5 can also recruit CtBP.

Association of CtBP with MITR—To investigate the potential significance of CtBP binding to MITR, we compared the repressive activity of wild-type MITR and MITR mutant DL-AS, which failed to bind CtBP. As shown in Fig. 3A, wild-type MITR fused to the GAL4 DNA binding domain potently repressed transcription of a GAL4-dependent luciferase reporter linked to the highly active thymidine kinase promoter. GAL4-MITR(DL-AS) also repressed this reporter, but the level of repression was reduced by at least 50% over a 30-fold range of expression plasmid. Wild-type and mutant forms of MITR were expressed at comparable levels in transfected cells (Fig. 2D).

Because MITR has been shown to repress MEF2-dependent transcription (11), we compared the abilities of wild-type MITR and MITR(DL-AS) to repress activation of a luciferase reporter linked to three tandem copies of the MEF2 consensus sequence. Consistent with the results obtained with GAL4-MITR fusions, the MITR(DL-AS) mutant was compromised in its ability to repress transcription, although it clearly retained repressive activity (Fig. 3B).

Association of MITR with Class I and Class II HDACs—The ability of MITR(DL-AS) to repress transcription in the absence of CtBP binding suggests the existence of redundant, CtBP-independent mechanisms for MITR-mediated repression. Consistent with this, MITR has been shown to interact with HDAC1 (11). Coimmunoprecipitation experiments were performed to determine whether MITR(DL-AS) retained the capacity to bind HDAC1. As shown in Fig. 4A, both wild-type MITR and MITR(DL-AS) efficiently associated with HDAC1 in transfected with expression vectors (1 µg each) for the indicated HDAC constructs (left-hand panels, FLAG-tagged; right-hand panels, Myc-tagged) and CtBP (left-hand panels, Myc-tagged; right-hand panels, FLAG-tagged). HDAC5 was immunoprecipitated from cell lysates, and coimmunoprecipitating CtBP was detected by immunoblotting (upper panels). The membranes were reprobed with the appropriate antibodies to reveal total immunoprecipitated HDAC5 (lower panel).
were transiently cotransfected with a GAL4-dependent luciferase reporter plasmid, pMH100-tk-luc (0.4 μg), expression vectors (10–300 ng) for either GAL4-MITR or GAL4-MITR(DL-AS), and a CMV-lacZ control plasmid (0.1 μg). Forty-eight hours later, cells were harvested and luciferase, and β-galactosidase activities were measured under conditions of linearity with respect to time and extract concentration. The luciferase values were normalized to β-galactosidase activity to control for differences in transfection efficiency. A, 10T1/2 fibroblasts were transiently cotransfected with a luciferase reporter plasmid driven by three copies of a MEF2 binding site, 3×MEF2-luciferase (0.3 μg), and expression vectors for MEFC2 (0.3 μg), and either MITR (0.1 μg) or MITR(DL-AS) (0.1 μg). Luciferase activity in cell extracts was measured 48 h post-transfection as described above. For A and B, luciferase activity is depicted as -fold repression of the reporter gene in cells expressing GAL4-MITR or GAL4-MITR(DL-AS) relative to activity in cells lacking ectopic MITR. Values represent the mean ± S.D. from at least two separate experiments.

**DISCUSSION**

The results of this study demonstrate that MITR and class II HDACs share homology in an amino-terminal CtBP-binding motif that recruits CtBP to enhance transcriptional repression. Mutations in this CtBP-binding motif that disrupt CtBP binding diminish the ability of MITR to repress transcription, but such mutants retain substantial repressive activity. Thus, we conclude that recruitment of CtBP by MITR contributes to transcriptional repression. However, maximal inhibition of transcription by MITR, as well as class II HDACs, likely involves the cooperative action of CtBP and deacetylase activity provided in trans by associated HDACs. Indeed, we show that MITR is able to physically associate with several different class I and II HDACs.

CtBP1, first identified as a cellular protein that bound to the carboxyl-terminal region of the E1a oncoprotein (18, 19), acts as a transcriptional corepressor through association with the consensus sequence P-X-D-L-S/R, found in a variety of transcription factors (20–28). CtBP1 and the closely related CtBP2 are expressed in a wide range of tissues. The precise mechanism for transcriptional repression by CtBP is unclear. Other corepressors, such as NcoR and SMRT, repress transcription by recruiting HDACs (9, 10). CtBP1 has also been shown to interact with HDAC1 (27), and TSA has been shown to partially relieve repression by CtBP. However, other studies have found that promoters repressed by CtBP are insensitive to TSA (29), suggesting the existence of HDAC-independent mechanisms for repression by CtBP.

The results of the present study provide the first demonstration of a direct interaction between CtBP and class II HDACs. Class II HDACs can physically associate with class I HDACs, and both types of HDACs can interact with CtBP. Thus, it is clear that each of these proteins has the capacity to serve as a bridge to the other, with the potential to generate repression complexes that are linked to DNA-bound transcription factors such as MEF2.

**In contrast to the ubiquitous expression of CtBP, MITR and class II HDACs are expressed predominantly in heart, skeletal muscle, and brain (6, 30, 31), the same tissues in which MEF2 expression is highest (32, 33). While CtBP played only a partial role in MITR-mediated transcriptional repression in our assays, in other cell backgrounds, its contribution to the repres-
lossive activity of MTR could be greater, if for example other trans-acting HDACs were expressed at lower levels or were less active due to intracellular signals (13).

It is becoming increasingly clear that HDACs associate with a complex array of cell-specific and ubiquitous transcriptional activators and repressors to control gene expression of large sets of downstream genes. In this regard, a role for these multiprotein complexes in the regulation of cell differentiation is strongly supported by our recent demonstration that class II HDACs, including HDAC4 and HDAC5, potently repress the transcriptional program required for skeletal muscle development (34, 35). This inhibitory action of HDAC is absolutely active due to intracellular signals (13).

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