Direct Interaction of Ca\(^{2+}\)/Calmodulin Inhibits Histone Deacetylase 5 Repressor Core Binding to Myocyte Enhancer Factor 2*

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Myocyte enhancer factor 2 (MEF2) proteins play a pivotal role in the differentiation of cardiac and skeletal muscle cells. MEF2 factors are regulated by histone deacetylase enzymes such as histone deacetylase 5 (HDAC5). HDAC5 in turn is responsive to Ca\(^{2+}\) signaling mediated by the intracellular calcium sensor calmodulin. Here a combination of proteolytic fragmentation, matrix-assisted laser desorption ionization mass spectrometry, Edman degradation, circular dichroism, gel filtration, and surface plasmon resonance studies is utilized to define and characterize a stable core domain of HDAC5 and to examine its interactions with MEF2a and calmodulin. Results from real time binding experiments provide evidence for direct interaction of Ca\(^{2+}\)/calmodulin with HDAC5 inhibiting MEF2a association with this enzyme.

In eukaryotes, transcription occurs in the nucleus where the DNA template is packaged in chromatin. DNA is wrapped tightly around histone proteins in nucleosomes that are arranged in a chromatin higher order structure (1–9). The organization of DNA in chromatin is thought to act as a barrier to transcription causing gene repression. Two alterations of chromatin have been implicated in transcription regulation: remodeling by ATP-dependent factors such as SWI/SNF, RSC, NRD, or NURF (4); and post-translational modification of the histone N-terminal tails including phosphorylation, methylation, ubiquitination, and acetylation (5). Among these, the acetylation modification has been recognized as a major contributor to transcription regulation (6) and is maintained by the dynamic interplay of histone acetylase and deacetylase (HDAC) enzymes. Acetylation of histone tails by histone acetylases is thought to create a chromatin structure accessible to the transcription machinery (1, 7), and conversely, hypoacetylated chromatin, the product of HDACs, is often associated with transcriptionally silent DNA (8).

Histone deacetylases have attracted considerable attention recently, due to findings that compounds blocking these enzymes can reactivate gene expression (9), inhibit growth and survival of tumor cells (10), and increase the life span of *Drosophila* (11). To date, 17 HDAC isoforms were described in humans, which are divided into classes based on sequence homology to yeast enzymes and association with DNA-binding proteins. Class I HDACs 1–3 and 8 are similar to Rpd3 from yeast (12–15). Class III enzymes (7 human genes), with yeast Sir2 as prototype, appear to be unique in their dependence on NAD\(^{-}\) cofactor (16). Class II HDACs 4–7, 9, and 10 show similarity to yeast Hda1 (17–22) and contain a conserved C-terminal catalytic domain, with the exception of HDAC6 which has two functional deacetylase domains arranged in tandem (22). Furthermore, HDACs 4, 5, 7, and 9 contain a conserved N-terminal region that shows similarity to a co-repressor protein first isolated from *Xenopus laevis* named MITR (MEF2 interacting transcription repressor, see Ref. 24). These class II members, like MITR, bind directly to myocyte enhancer factor 2 (MEF2) transcription factors and repress their transcriptional activity (25–28).

MEF2 transcription factors have an essential role in the myogenesis and morphogenesis of cardiac and skeletal muscle cells (29). MEF2 factors specifically recognize the control regions of the majority of muscle-specific genes, as well as nerve-specific and other unrelated genes (30, 31). In vertebrates, the MEF2 family comprises the members MEF2a–d, each having several splicing variants. Together, the factors show more than 85% identity in an 86-amino acid core that is sufficient for specific DNA binding and dimerization (32). MEF2 proteins belong to the MADS box superfamily of transcription factors characterized by strong sequence homology in a 58-amino acid DNA binding domain of this MADS domain is N-terminal in MEF2 proteins and immediately followed by a conserved 28-amino acid MEF2 domain that determines homo- and heterodimerization products of MEF2 family members and excludes heterodimerization with other MADS box transcription factors (33, 34). Recently, this laboratory reported the crystal structure of MEF2a core encompassing amino acids 2–78 bound to cognate DNA at 1.5 Å resolution (35). The structure revealed the DNA binding interactions and showed that the MEF domain adopts a configuration distinct from the respective regions in other MADS box proteins, see Ref. 24). These class II members, like MITR, bind directly to myocyte enhancer factor 2 (MEF2) transcription factors and repress their transcriptional activity (25–28).

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The abbreviations used are: HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; DTT, dithiothreitol; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CaM, calmodulin; CaMK, calcium/calmodulin-dependent protein kinase; MALDI, matrix-assisted laser desorption ionization; NHS, N-hydroxysuccinimide; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; MCK, muscle creatine kinase.
Ca\(^{2+}\)/Calmodulin Inhibits HDAC5 Binding to MEF2a

The responsiveness to CaMK mediated activation mapped to the MADS/MEF domains of MEF2a, which were unphosphorylated, however (36). Instead, the targets of CaMK activity were HDAC4 and HDAC5. Phosphorylation of these proteins leads to the disruption of the MEF2a-HDAC complex and results in activation of MEF2-controlled genes (26). Phosphorylated HDAC was found to bind the chaperone protein 14-3-3, resulting in translocation of phospho-HDAC into the cytosol (34, 35). Consistent with a role in regulation of MEF2, class II HDACs are expressed primarily in tissues where MEF2 levels are highest (heart, skeletal muscle, and brain). CaMK function is triggered by calmodulin, which in turn is activated by increased Ca\(^{2+}\) levels in the cell, thus linking MEF2 function to Ca\(^{2+}\) signaling (29). Recently, however, a more direct role of Ca\(^{2+}\)/calmodulin in the Ca\(^{2+}\)-dependent regulation of MEF2-controlled gene expression was suggested based on the observation that HDAC4 protein is retained on calmodulin-conjugated resin in the presence of Ca\(^{2+}\) and that a putative calmodulin-binding motif exists in the N-terminal region of HDAC4 (42).

In this report, we demonstrate that class II enzyme HDAC5 directly associates with calmodulin in a Ca\(^{2+}\)-dependent manner. By using a combination of biochemical and biophysical techniques, we define a stable core of HDAC5 that binds to MEF2a and calmodulin with high affinity. The dissociation constants of the interaction with MEF2a on the one hand and calmodulin on the other hand are determined by real time binding experiments. Experimental support for overlapping MEF2a and calmodulin-binding sites in HDAC5 is provided. By using purified proteins, we show for the first time that direct interaction of Ca\(^{2+}\)/calmodulin in the Ca\(^{2+}\)-dependent regulation of MEF2a.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Human HDAC5 repressor core polypeptides were expressed as fusion proteins containing a His\(_6\) tag at the C terminus. Genes encoding for amino acids 140–308 (Rpr,L) and 140–227 (Rpr,S), respectively, of human HDAC5 (17) with a starting methionine added were cloned into a pet28a plasmid (Novagen) using the NcoI and HindIII sites and expressed in Escherichia coli BL21(DE3). Pellets were resuspended in ice-cold Buffer T (25 mM Bis-Tris, 100 mM NaCl, 10 mM imidazole, pH 7.0) and passed through a cell crusher. Cleared lysate was loaded on a Ni2+-NTA resin (Qiagen) and washed with Buffer T containing 600 mM NaCl. Bound protein was eluted with an imidazole gradient to 200 mM, concentrated into Buffer P (25 mM BisTris, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, pH 6.0), and applied to a Poros HS column (Perseptive Biosystems). Rpr,L as well as Rpr,S eluted in a single peak around 400 mM NaCl. During purification of Rpr,L, a major proteolytic breakdown product ("SmFr," see "Results") was eluted at 300 mM NaCl and could be pooled separately. Repressor core proteins were finally passed through a Superdex 200 HR column (Amersham Biosciences) and concentrated up to 14 mg/ml for storage. Protein concentrations were determined by UV absorption at 280 nm assuming an extinction coefficient of 13,800 M\(^{-1}\) cm\(^{-1}\).

Protein degradation of the purified protein revealed that the N-terminal methionine had been quantitatively removed. The resulting protein encompassing amino acids alanine 2 to lysine 149 is termed full-length CaM throughout the text.

For biotinylation, purified CaM protein was incubated with NHS-LC-biotin (Pierce) following a procedure described by Billingays et al. (46) modified such that an equimolar ratio of NHS-LC-biotin to protein was chosen rather than an excess of 14 to 1 (46). Thus, on average the attachment of one biotin to each CaM molecule was achieved as verified by MALDI analysis.

MEF2a core protein (amino acids 2–86 of full-length MEF2a) encompassing the MADS box and the MEF domain was expressed and purified following published procedures (35). This core protein is termed MEF2a throughout the text. All expressed proteins (Rpr,L, Rpr,S, Rpr,S(L187G), CaM, and MEF2a) were purified to homogeneity as verified by Coomassie staining, N-terminal sequencing, and mass spectrometric analysis.

Circular Dichroism Spectroscopy—CD spectra were acquired using a J710 spectropolarimeter (Jasco) equipped with a thermoelectric temperature controller and interfaced to a personal computer. Stock solutions of protein samples were prepared in a 25 mM BisTris buffer, pH 6.0, containing 100 mM KF. Protein solutions of 10 μg/ml or less were used to obtain the data. The CD spectra were measured at a bandwidth of 2 nm, with a step size of 0.5 nm with a 4 s averaging time per point in a 1.0-cm cuvette. Spectra were signal averaged by adding three scans, baseline corrected, and smoothed using the software provided by Jasco.

Tryptic Fragmentation Experiments—Protease digestion of HDAC5 repressor core proteins was performed by treating 1 mg/ml solution of protein with trypsin from bovine pancreas (Roche Applied Science) in Preproteinase Buffer (50 mM NaCl, 50 mM Tris, pH 8.0) at a fixed protein to protease mass ratio at 24 °C in a 1 ml reaction volume. At serial time points, 55-μL aliquots were taken from the reaction. A fraction (5 μL) of these aliquots was immediately flash-frozen in liquid nitrogen and stored for mass spectrometric analysis. The remaining 50 μL were transferred into prepared tubes containing reducing protein gel loading dye and flash-frozen for SDS-PAGE analysis. Reactions were carried out several times showing the reproducibility of the proteolytic fragmentation pattern by using this simple freezing procedure. Digests of calmodulin or purified calmodulin-repressor core complex, respectively, were performed in a similar fashion, with the exception that CaCl\(_2\) was added to the reaction buffer to a final concentration of 2 mM.

Proteolytic fragments were separated by SDS-PAGE (18%) and visualized by staining with Coomassie Brilliant Blue. Protein gels were blotted on a polyvinylidene difluoride membrane, and prominent bands were subjected to N-terminal sequencing by Edman degradation. Mass spectrometry of the proteolytic mixtures was performed using a Vesta Voyager Biospectrometry Workstation (Perseptive Biosystems). Sina-pinic acid in 67% acetonitrile, 0.03% trifluoroacetic acid was used as a matrix. Data were analyzed with the Data Explorer software package 4.0 (Applied Biosystems). In ambiguous cases, where Edman degradation yielded several overlapping sequences for the N terminus, further purification of the proteolytic fragments was performed by reversed phase chromatography on a Nucleosil 300-5 C8 column (Macherey-Nagel) applying a trifluoroacetic acid/acetonitrile gradient. Fractions containing enriched peptide species were lyophilized, resuspended in 30% acetonitrile containing 0.1% trifluoroacetic acid, and used directly for N-terminal sequencing and MALDI analysis.

Tryptic fragments were assigned according to their experimental average masses and taking into account the N-terminal sequences utilizing the FindPept tool of the Expasy package (www.expasy.ch). Band intensities of full-length calmodulin on SDS-PAGE gels displaying tryptic fragments of CaM or CaM-Rpr,S complex, respectively, were recorded on an Alpha Imager 2200 Documentation and Analysis System device (Alpha Innotech Corp.), using the spot densitometry module of the ZetaGen package Image Master 2000 version 5.1, and normalized to the respective band intensity of the undigested sample.

Electrophoretic Mobility Shift Assay (EMSA)—A 60-mer DNA oligonucleotide duplex matching the muscle creatine kinase (MCK) promoter region (47) centered on the Alpha Imager 2200 Documentation and Analysis System device (Alpha Innotech Corp.), using the spot densitometry module of the ZetaGen package Image Master 2000 version 5.1, and normalized to the respective band intensity of the undigested sample.

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\[
\text{NcoI} \quad 140 \\
\text{Hin} \quad 280 \\
\]
Calmodulin Inhibits HDAC5 Binding to MEF2a

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**Real Time Binding Studies by Surface Plasmon Resonance (SPR)**

**Measurement Using BIAcore**—Binding experiments were performed on a BIAcore 1000 biosensor system (Amersham Biosciences AB) at 20 °C. MEF2a protein (15–25 µl of 1 µM polypeptide in 10 mM sodium acetate, pH 6.5) was coupled through its amino groups to the sensor surface of a CM5 biosensor chip (Amersham Biosciences) using N-hydroxysuccinimide/ethyl-N-(dimethylaminopropyl)carbodiimide chemistry as recommended by the manufacturer. After immobilizing 1200 resonance units of MEF2a, remaining activated groups on the sensor surface were inactivated with 1 mM ethanolamine. For control experiments, one sensor surface was treated as above in the absence of MEF2a protein.

Interaction experiments between MEF2a and MCK promoter DNA (as above) were carried out with DNA concentrations ranging from 0.5 to 100 nM at a constant flow rate of 10 µl/min using Buffer M (12.5 mM HEPES-Na, pH 7.2, 50 mM NaCl, 5 mM MgCl₂, 2 mM DTT) as running buffer. Between injections, the sensor chip was regenerated with 50 µl of Buffer M containing 500 mM NaCl. Binding experiments of HDAC5 Rpr-L protein to MEF2a were carried out in Buffer R1 (25 mM BisTris, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, pH 7.0) with protein concentrations ranging from 2 to 35 nM. Rpr-S has a lower theoretical pI (8.0) as compared with Rpr-L (9.95), and its titration curve shows a plateau in the dissociation constant at 9.85. To measure the interaction between calmodulin and repressor core protein, 1500 resonance units of MEF2a, remaining activated groups on the sensor surface were inactivated with 1 mM ethanolamine. For control experiments, one sensor surface was treated as above in the absence of MEF2a protein.

To measure the interaction between calmodulin and repressor core protein, 1500 resonance units of biotinylated calmodulin were immobilized on a streptavidin-coated SA5 chip (Amersham Biosciences). A control was run where streptavidin was conjugated to the sensor surface without calmodulin. An uncoated streptavidin surface was used as a control. Measurements were performed in Buffer R (10 mM BisTris, 250 mM NaCl, 1 mM CaCl₂, pH 6.2) at a flow rate of 20 µl/min in a range of 2–50 nM Rpr-S. The sensor surface was regenerated with buffer containing 500 mM NaCl and 5 mM EDTA instead of CaCl₂. Rpr(SL187G) mutant protein was measured similarly, however, in the concentration range of 50–400 nM.

Non-specific binding of protein or DNA to the respective control surfaces was not detectable under the conditions of our experiments. Data were evaluated using the BIACore evaluation software package (Amersham Biosciences AB). Kinetic constants were obtained by linear regression and quantified with software Advanced Image Data Analyzer (Raytest Isotopenmessgeräte GmbH). The ratio of protein-DNA complex [PD] to free DNA [D] was obtained from the band intensity data and plotted against the concentration of free protein [P].

**Analytical Size Exclusion Chromatography of CaM-RprC Complexes**—Rpr-L or Rpr-S, respectively, was mixed with an excess (less than 10%) of CaM (assuming ε₂₈₀ = 2560 M⁻¹ cm⁻¹ for calmodulin) and incubated on ice (1 h) in a 1-ml volume at a total protein concentration of 2 mg/ml in Buffer C (25 mM BisTris, 100 mM NaCl, 1 mM DTT, 1 mM CaCl₂, pH 6.0). The sample was passed through an S200 HR size exclusion column pre-equilibrated in Buffer C at a flow rate of 0.25 ml/min with fractions collected at 1-min intervals. Peaks containing CalM-Rpr complex were identified by 18% SDS-PAGE and pooled separately from fractions containing excess CaM. Pooled complex was re-chromatographed to yield a single symmetric peak in the A₂₈₀ trace. The complex was concentrated to 0.5 ml, dialyzed extensively against Buffer E (10 mM BisTris, 100 mM NaCl, 1 mM DTT, 2 mM EGTA, pH 6.0), and passed through the S200 HR column pre-equilibrated in Buffer E. The split peak profiles were analyzed for protein content by SDS-PAGE. Unbound calmodulin and HDAC5 repressor core proteins were passed through the S200 HR column in Buffer C under the same conditions as above for comparison.

**CaCl₂, pH 6.0.** The sample was passed through an S200 HR size exclusion column pre-equilibrated in Buffer C at a flow rate of 0.25 ml/min with fractions collected at 1-min intervals. Peaks containing CaM-Rpr complex were identified by 18% SDS-PAGE and pooled separately from fractions containing excess CaM. Pooled complex was re-chromatographed to yield a single symmetric peak in the A₂₈₀ trace. The complex was concentrated to 0.5 ml, dialyzed extensively against Buffer E (10 mM BisTris, 100 mM NaCl, 1 mM DTT, 2 mM EGTA, pH 6.0), and passed through the S200 HR column pre-equilibrated in Buffer E. The split peak profiles were analyzed for protein content by SDS-PAGE. Unbound calmodulin and HDAC5 repressor core proteins were passed through the S200 HR column in Buffer C under the same conditions as above for comparison.
**RESULTS**

**HDAC5 Core Encompassing the Proposed MEF2a and CaM Binding Regions**—HDAC5 contains a C-terminal catalytic domain and an N-terminal extension that mediate binding to MEF2 proteins (17, 19, 23) (Fig. 1A). The N-terminal part of the protein is highly homologous to the non-catalytic transcriptional co-repressor MITR (24, 48) and is termed “repressor” in this study. It has a glutamine-rich stretch close to its N terminus (Q in Fig. 1A) followed by a domain (A in Fig. 1A) that contains the 18-amino acid stretch identified as crucial for HDAC5 repressor core polypeptides RprcL(−)(Fig. 1A) and RprcS(−)(Fig. 1A)) that interacts with MEF2 centered around the 100% conserved segments 148–168 in Fig. 1A(−). The occurrence of this proteolytic fragment, resulting from cleavage of RprcL by endogenous protease present in the E. coli lysate between segments 14,506–14,606 in Fig. 1A(−) was separated from ful-length RprcL by ion exchange chromatography (Fig. 2, Tr4a) and could be separated from full-length RprcL by ion exchange chromatography (Fig. 2, Tr4a and Tr4b) and EDTA did not reduce the amount of SmFr in the preparation.

**Mass spectroscopic analysis of RprcL proteolytic fragments**

| Fragment | Peptide | Measured mass | Calculated mass | Missed sites |
|----------|---------|---------------|----------------|--------------|
| Tr0 | M140E L E Q...AERR277 | 15,963 | 15,963.1 | 23 |
| Tr1a | M140E L E Q...VRSR260 | 14,850 | 14,853.8 | 20 |
| Tr1b | M140E L E Q...LKVR266 | 14,606 | 14,610.5 | 19 |
| Tr2 | M140E L E Q...PLRK256 | 13,510 | 13,514.2 | 17 |
| Tr3 | M140E L E Q...QHPR212 | 8772 | 8774.0 | 13 |
| Tr4a | M140E L E Q...LKVR266 | 7764 | 7761.7 | 7 |
| Tr4b | M140E L E Q...PLRK256 | 6668 | 6665.4 | 10 |
| Tr5a | 146Q R E Q...LILR168 | 6168 | 6168.1 | 11 |
| Tr5b | M140E L E Q...VKLR186 | 5905 | 5908.7 | 10 |
| Tr6a | 171E K S K...QHPK212 | 4642 | 4642.3 | 6 |
| Tr6b | 256K T A S...FKKR296 | 4765 | 4767.6 | 13 |
| Tr6c | M140E L E Q...NKEK172 | 4412 | 4408.0 | 7 |
| Tr7a | 195S E P...LKVR266 | 3912 | 3908.4 | 5 |
| Tr7b | 171E K S K...QHPK212 | 3898 | 3898.5 | 6 |
| Tr8a | 146Q R E Q...LILR168 | 2996 | 2993.4 | 4 |
| Tr8b | 213C W G A...PSYK239 | 2790 | 2791.0 | 0 |
| Tr8c | 146Q R E Q...LILR168 | 2710 | 2709.1 | 3 |

**Fig. 3. Circular dichroism of purified proteins.** Spectra of HDAC5 repressor core polypeptides RprcL(−) and RprcS(−) were subtracted to yield the difference spectrum marked Δ(−)(--). The CD spectrum of a purified breakdown product (SmFr, see under “Results”) of RprcL encompassing its C-terminal half is superimposed (−−). The CD signal (−) of calmodulin was recorded with 2 mM CaCl² added to the sample. The spectrum of RprcS(−) mutant protein is identical to that of RprcS wild type (not shown).

around 12 kDa on an SDS gel accounting for between 30 and 50% of total protein. This small fragment (termed “SmFr”) could be separated from full-length RprcL by ion exchange chromatography (Fig. 2, Tr4a and Tr4b) and EDTA did not reduce the amount of SmFr in the preparation. The occurrence of this proteolytic fragment, resulting from cleavage of RprcL by endogenous protease present in the E. coli lysate between segments A and B (Fig. 1A), indicated that the region between the boundaries of these conserved segments is exposed.
We investigated the folding state of RprL by CD spectroscopy (Fig. 3). The molar ellipticity of RprL exhibits a shoulder at 222 nm, consistent with α-helical secondary structure content. A pronounced minimum at 203 nm is observed, indicative of a significant proportion of random coil in the protein. Interestingly, the spectrum of purified SmFr, which encompasses the C-terminal part of RprL, is also that of a random coil (Fig. 3). This implies that the region contained within SmFr may also be unstructured in the context of RprL, explaining the comparatively large random coil content of RprL. If so, the secondary structure elements accounting for the CD signal amplitude at 222 nm would mainly be located in the N-terminal portion of the protein.

To verify this, we probed RprL by performing limited proteolytic fragmentation using trypsin from bovine pancreas. Limited proteolysis is a powerful tool for the identification of boundaries of stable domains within proteins for structural (50, 51) or folding (52, 53) studies. The potential trypsin cleavage sites are clustered in the N-terminal and C-terminal portion of RprL (Fig. 1B); tryptic fragmentation could therefore be expected to provide information about the relative stabilities of these regions of the protein. The results of a proteolytic fragmentation of RprL at a 500 to 1 protein to trypsin mass ratio is shown in Fig. 2 (lanes 4–7) and depicted schematically in Fig. 1B, with corresponding masses of tryptic fragments as determined by MALDI listed in Table I. RprL was trypsinized leaving no full-length protein detectable after 30 min. Cleavage occurred at the onset of the reaction mainly in the C-terminal region of RprL at amino acids Arg-277, Arg-268, Arg-266, and Lys-256, leading to tryptic fragments Tr0, Tr1a, Tr1b, and Tr2 (Fig. 1B and Fig. 2). Of these fragments, only Tr1a, Tr1b, and Tr2 persisted until 30 min in the digest, and then also disappeared completely. Cleavage occurred at Lys-212 as well, with fragment Tr3 appearing after 10 min in the digest. Further proteolysis occurred in the central region of the protein, between amino acids Lys-184 and Lys-196, leading to progressively shorter fragments (Tr5a,Tr6a). The only tryptic fragment derived from the most C-terminal part of the protein that was stable for a significant time corresponded to a stretch encompassing amino acids Lys-256 to Arg-296 of RprL (Tr6b). Fragments Tr4a and 4b, which are derived from Tr1b and Tr2 and cleaved after Lys-194, appeared at 10 min. At 60 min, they were only weakly detectable by MALDI due to further degradation resulting in fragment Tr8b, which is resistant to cleavage by trypsin as it lacks lysine or arginine amino acids (Table I).

At the N terminus, three tryptic sites were targeted by the protease, leading to truncations after amino acids Arg-145, Arg-147, and finally Arg-152 of the protein. At 120 min, the reaction mixture consisted mainly of an assortment of N-terminal pieces of the protein ranging from less than 3 (Tr8a and -8c) to 4 kDa (Tr7a and Tr7b) and Tr8b from the central region of RprL (Fig. 1 and Table I). A strong Coomassie band from the 120-min sample migrates around 10 kDa in Fig. 2 (lane 7), which seemed to indicate that comparatively large amounts of Tr3, Tr4a, or Tr4b are still present. However, only Tr3, encompassing amino acids 140–212 of HDAC5, was unambiguously identified in this sample by MALDI and Edman degradation of
the corresponding band. The C-terminal half of RprL was more rapidly proteolyzed than the N-terminal half, without any larger (>3 kDa) fragments derived from the C-terminal part of the protein detected after 120 min (Fig. 1B and Table I).

Taken together, these results suggest that the C-terminal part of RprL containing segment B of HDAC5 (Fig. 1) consists largely of random coil with limited tertiary folding. The N-terminal part, containing segment A with the proposed MEF2 and CaM binding domains (Fig. 1A), on the other hand, contains a better defined tertiary structure, as numerous potential tryptic sites in this region were protected even after 120 min (Table I). We therefore prepared a construct of HDAC5 devoid of the proteolytically sensitive C-terminal portion. We extended the protease-resistant fragment Tr3 (Glu-140 to Lys-212) toward the C terminus by adding a high homology region including the amino acid stretch PKCW with tryptophan 214. This 4-amino acid motif PXWX is well conserved in all class II HDACs containing an MITR-like repressor domain. The resulting protein, RprS, encompasses amino acids 140–227 of HDAC5. The CD spectrum of purified RprS (Fig. 3) has a minimum at 222 nm characteristic for α-helices. The second minimum is shifted to 207 nm and is smaller than in the case of RprL, clearly indicating a lower random coil content and a more compact structure for the shorter polypeptide. The difference spectrum Δλ80 (Fig. 3) of RprL with RprS is virtually superimposable on the spectrum of SmFr, confirming that the C-terminal part of RprL largely accounts for its random coil content.

MEF2a Binding Studies—We analyzed the interaction of RprL and RprS with MEF2a by real time binding studies (Fig. 4). As a control for the activity of purified MEF2a used in our experiments, we determined the binding constant of MEF2a to a DNA oligonucleotide duplex by electrophoretic mobility shift assay (Fig. 4A) and by BIAcore with the protein immobilized on a dextran surface (Fig. 4B). The DNA sequence used was derived from the muscle creatine kinase promoter containing a MEF2-responsive element (see “Experimental Procedures”). EMSA and BIAcore yielded close to identical dissociation constants (KD = 0.5–0.6 nM) for the MEF2a/DNA interaction demonstrating that immobilized MEF2a had retained its activity. Next, the dissociation constants of RprL and RprS were determined by binding the repressor core proteins to immobilized MEF2a over a range of concentrations. The resulting sensorgrams are shown in Fig. 4, C and D. Both polypeptides bound immobilized MEF2a with comparable, high affinity (KD = 6–8 nM) in our experiments, corroborating that the region of HDAC5 important for high affinity interaction with MEF2a is indeed contained within RprS. In contrast, the C-terminal extension present in RprL encompassing segment B of HDAC5 does not contribute significantly to binding MEF2a.

Ca2+/Calmodulin Inhibits HDAC5 Binding to MEF2a

Youn and co-workers (42) had observed that the HDAC4 enzyme is readily retained on calmodulin-conjugated resin in the presence of Ca2+. HDAC5 has a sequence homologous to the CaM binding region proposed for HDAC4 (42). We therefore investigated whether our HDAC5 repressor core constructs also interacted with CaM activated by Ca2+. Fig. 5 shows the results of analytical gel permeation experiments of CaM mixtures with RprL (Fig. 5A) and RprS (Fig. 5B), respectively. In both cases, equimolar addition of CaM shifted the elution peak of the repressor core protein quantitatively to higher molecular weights in the presence of 1 mM CaCl2. SDS-PAGE revealed that this peak contained both CaM and the repressor core protein in a 1:1 ratio as judged by the intensity of the bands. Fractions containing complex could be rechromatographed resulting again in the same symmetric peak profile. Replacing Ca2+ with 2 mM EGTA in sample and running buffer resulted in dissociation of the complex with the components eluting...
individually. The analytical gel filtration experiments therefore confirm that class II deacetylases containing an MITR-like N-terminal domain interact with CaM in a Ca\(^{2+}\)-dependent manner and that the Rpr\(_{cS}\) region of HDAC5 is sufficient for MEF2a and CaM binding.

**Trypsin Digest of Calmodulin-Repressor Core Complex**—The tryptic digestion of Ca\(^{2+}\)-saturated CaM results in cleavage mainly at amino acids Lys-78, Arg-75, and Lys-76, leading to a mixture of tryptic fragments containing the N-terminal EF-hand motifs and the C-terminal EF-hand motifs extended by the respective amino acids of the connecting linker helix (54). Ca\(^{2+}\)/CaM has a dumb-bell shape in solution, with the linker helix connecting the EF-hand containing domains being rather flexible. Upon binding to most cognate peptide motifs, CaM collapses to a globular structure with the “hinge” amino acids (Lys-78 to Ser-82) permitting this rearrangement. Cognate peptide motifs are typically present as \((\text{Lys}-78 \text{ to } \text{Ser}-82)\) permitting this rearrangement. Cognate peptide motifs are typically present as \((\text{Lys}-78 \text{ to } \text{Ser}-82)\) permitting this rearrangement. Cognate peptide motifs are typically present as \((\text{Lys}-78 \text{ to } \text{Ser}-82)\) permitting this rearrangement.

With the aim of detecting tryptic sites on either calmodulin or HDAC5 repressor core that are protected on complex formation, we performed limited tryptic fragmentation of Rpr\(_{cS}\), CaM, and purified Rpr\(_{cS}\)/CaM complex (Figs. 6 and 7). Tryptic fragments identified by Edman degradation and MALDI mass spectrometry are listed in Table II. The tryptic fragmentation under the conditions used in this study (see “Experimental Procedures”) yielded predominantly three C-terminal fragments in the digest (Tr\(_{1C}\), Tr\(_{2Ca}\), and Tr\(_{2Cc}\), compare with Ref. 54). At 120 min, about 50% of full-length CaM was digested at the tryptic to protein ratio indicated (Fig. 6). The MALDI spectrum of the reaction mixture at this time point is shown in the region from 8,000 to 17,000 Da (Fig. 7A, right). The corresponding tryptic cleavage sites are depicted in a CaM model (Fig. 7C, right), and the fragments are listed (Table II).

The tryptic digestion of free or bound Rpr\(_{cS}\) showed a similar pattern as the Rpr\(_{cS}\) digest as far as the repressor core is concerned, with only minor differences detected by SDS-PAGE, MALDI, and Edman degradation. In contrast, CaM was apparently not digested at all, showing no proteolytic breakdown product of CaM even after 120 min (Fig. 6C). MALDI mass spectrometry of the 120-min digest sample (Fig. 7B, right) resulted in only one peak above 8000 Da matching full-length CaM (16,708 Da). The linker helix thus appears to be fully protected upon complex formation with Rpr\(_{cS}\).

Tryptic digestion of Rpr\(_{cS}\) in complex with CaM showed a virtually indistinguishable tryptic pattern for free or bound Rpr\(_{cS}\) on SDS-PAGE (Fig. 6). However, the stability of full-length CaM in complex with Rpr\(_{cS}\) allowed for a more detailed analysis of the MALDI spectra of the digested complex, because all protein mass signals with the exception of the full-length CaM signal (16,706 Da) could be attributed to Rpr\(_{cS}\). Representative MALDI spectra of Rpr\(_{cS}\) and CaM-Rpr\(_{cS}\) complex taken at identical time points (60 min) in the digest are shown (Fig. 7, A and B). Direct comparison of the two spectra make it evident that the two peaks (4995 and 5910 Da) present in the digest of unchanged Rpr\(_{cS}\) are absent in the digest of CaM-Rpr\(_{cS}\).
Ca\(^{2+}\)/Calmodulin Inhibits HDAC5 Binding to MEF2a

These peaks correspond to tryptic fragments Tr13c and Tr15b (Table II), which extend to the tryptic site Arg-186 (Fig. 7C, left). Arginine 186 is apparently not accessible during tryptic fragmentation of the complex, whereas it is readily available if RprcS alone is digested. Although MALDI-MS is not a quantitative tool for detection of proteolytic fragments in a digest, the consistent absence of fragments Tr13c and Tr15b in the MALDI spectra acquired for the purified CaM-RprcS complex suggests that the region centered around RprcS Leu-187 is inaccessible in the complex and that the binding sites for MEF2a and CaM are overlapping. Conversely, tryptic fragments Tr12 and Tr13b (Table II), which extend to Lys-194 (Fig. 7C), still appear in the MALDI spectra, even though lysine 194 is also embedded in the postulated CaM binding domain. The reason may be found in the nature of the HDAC5 CaM-binding site that is different from classical CaM-binding motifs. It does not adhere to conventional 1-5-10 or 1-8-14 rules concerning the positions of key hydrophobic amino acids and is probably more extended (Table III). In fact, certain CaM-binding motifs with extended recognition sequences were recently shown to form hairpins or loop regions rather than a single \(\alpha\)-helix upon complex formation (Table III). Such loop regions could be cleavage sites in the fragmentation experiments.

\(\text{Ca}^{2+}/\text{CaM Competes with MEF2a for Repressor Core Binding—}\)

We investigated the interdependence of HDAC5 repressor core binding to MEF2a and CaM by SPR measurement with BIAcore (Fig. 8). First, the interaction between RprcS and CaM was quantified using biotinylated calmodulin immobilized on a streptavidin-coated SA5 chip surface. RprcS protein was passed over immobilized biotinylated calmodulin at the concentrations indicated, yielding a dissociation constant of 3 ± 0.8 nM for the CaM/RprcS interaction (Fig. 8A). The evolutionary conservation of the central leucine 187 in class II HDACs as well as MITR (Table III) implies a central role of this hydrophobic amino acid for the functionality of HDAC5. Leu-187 is in a region identified as crucial for MEF2a interaction (Fig. 7C). Hydrophobic amino acids are also important for binding Ca\(^{2+}\)/CaM (Table III), and mutating these amino acids in CaM-binding motifs to alanine or glycine has been shown to markedly decrease (5-100-fold) CaM binding affinity (58). We mutated leucine 187 in RprcS to glycine generating the mutant protein RprcS(L187G). Real time binding experiments yielded a \(K_D\) of \(-60\) nM for the interaction of the mutant protein with immobilized CaM (data not shown), which represents a 20-fold decrease in affinity compared with wild type.

Having obtained experimental support for overlapping MEF2a- and CaM-binding sites in RprcS, we proceeded finally to investigate the influence of HDAC5 repressor core binding to CaM on the interaction with MEF2a. MEF2a was immobilized on a dextran surface, and RprcS was passed over the MEF2a-coated surface at a constant concentration (30 nM) with CaM added in a range from 30 nM to 3 \(\mu\)M in the mobile phase (Fig. 8B). We found that CaM inhibits RprcS binding to immobilized MEF2a with increasing concentration. At a molar ratio of 1:100 (RprcS to CaM), repressor core binding to MEF2a was virtually abolished in this experiment.
In the present study, a stable core domain from the N-terminal repressor part of human HDAC5 was defined, and the interaction of this core domain with MEF2a on the one hand and Ca²⁺/CaM on the other hand was analyzed. Both interactions were found to have dissociation constants in the nanomolar range. Experimental support for overlapping MEF2a- and CaM-binding sites was derived from proteolysis in conjunction with MALDI mass spectrometry and mutant data. Furthermore, direct interaction of Ca²⁺/CaM was demon-

| TABLE II |
| Mass spectroscopic analysis of Rpr cS and CaM tryptic fragments |
| Fragment | Peptidea | Rpr cS tryptic fragments | Measured mass | Calculated mass | Missed sites |
| --- | --- | --- | --- | --- | --- |
| Tr10a | 146QREQ...SSPP²²his⁵ | 10,952 | 10,953.5 | 14 |
| Tr10b | 146QREQ...SSPP²²his⁵ | 10,667 | 10,669.0 | 13 |
| Tr11 | M¹⁰ELEQ...QHEK²²² | 8774 | 8774.0 | 13 |
| Tr12 | M¹⁰ELEQ...LLSK¹³⁴ | 6868 | 6867.9 | 11 |
| Tr13a | 195RLQ...SSPP²²his⁵ | 6247 | 6248.0 | 5 |
| Tr13b | 195RLQ...SSPP²²his⁵ | 5955 | 5952.8 | 10 |
| Tr14 | M¹⁰ELEQ...VLR¹³⁴ | 5913 | 5908.7 | 10 |
| Tr15a | 195SKEP...SSPP²²his⁵ | 5022 | 5019.5 | 3 |
| Tr15b | 195SKEP...SSPP²²his⁵ | 4995 | 4993.7 | 9 |
| Tr15c | 195SKEP...SSPP²²his⁵ | 4726 | 4724.0 | 7 |
| | | | | | |
| Fragment | Peptidea | CaM tryptic fragments | Measured mass | Calculated mass | Missed sites |
| --- | --- | --- | --- | --- | --- |
| TrR107 | 2ADQL...AEEL²⁹⁶ | 11,890 | 11,893.1 | 10 |
| Tr2E | 2ADQL...EAFR⁹¹ | 10,153 | 10,156.2 | 8 |
| TrR87 | 2ADQL...EEIR⁸⁷ | 9648 | 9652.6 | 7 |
| Tr2Cc | 76KMK...MTAK¹⁴⁹ | 8532 | 8536.4 | 8 |
| Tr1C | 2ADQL...MMAR⁷⁵ | 8189 | 8190.0 | 4 |
| Tr2Ca | 79DTS...MTAK¹⁴⁹ | 8150 | 8148.9 | 6 |
| Tr3E | 107HMV...MTAK¹⁴⁹ | 4832 | 4833.3 | 2 |
| Tr7 | 2ADQL...LILR¹⁶⁸ | 3906 | 3908.4 | 5 |
| Tr17b | 2ADQL...LILR¹⁶⁸ | 2996 | 2993.4 | 4 |
| Tr17c | 2ADQL...LILR¹⁶⁸ | 2708 | 2709.8 | 3 |
| Tr17d | 2ADQL...LILR¹⁶⁸ | 213CWG...SSPP²²his⁵ | 3113 | 3113.4 | 1 |
| Tr17e | 2ADQL...LILR¹⁶⁸ | 3198 | 3193.7 | 6 |

a Residue numbers are left and right in superscript in accordance with Fig. 6.  
*b* his denotes KLAAALEHHHHHH vector-encoded His₆ tag.  
*c* M stands for N-terminal vector-encoded methionine.  
*d* CaM N-terminal methionine was removed during translation.  
*e* CaM fragment denoted adapted from Ref. 54.

| TABLE III |
| Ca²⁺/Calmodulin Inhibits HDAC5 Binding to MEF2a |
| NCBI accession codes are in parentheses. |
| HDAC/MITR/Cabin |
| HDAC5 (Q9Z2V6) | STEVKKLRQFELLSKSKETPGGLNH |
| HDAC4 (P56524) | STEVKKMQFELLSKSKETPGGLNH |
| HDAC7B (NP_478057) | STEVKKQKQFELLSKSKETPGGLNH |
| MITR (X. l.) (CAB10167) | STEVKKQKQFELLSKSKETPGGLNH |
| Cabin 1 (NP_036427) | STEVKKQKQFELLSKSKETPGGLNH |
| 1-8-14 motifa |
| Type A | Calcineurin A (P16299) | KIEIRNKPRAGKMARVS |
| MLCK (Q9H1R3) | KIEIRNKPRAGKMARVS |
| Type B | Titin (CAA49245) | HTRKKDLNMIAVSAAR |
| Caldesmon (Q05682) | HTRKKDLNMIAVSAAR |
| 1-5-10 motifb |
| Type A | Heat shock, 84 kDa (P08238) | QVANFKRVRKFGSS |
| CaM kinase II (rat) (P11730) | QVANFKRVRKFGSS |
| Divergent CaM-binding motifs |
| CaMKKb | VKLPSATVILVKSMLRKS |
| CaMKKb | VKLPSATVILVKSMLRKS |
| SK2-CaMBDb | (P70604) | (loop) |

a Adapted from Ref. 55.  
b Adapted from Ref. 56.  
c Adapted from Ref. 57.  
d All proteins are from human unless indicated otherwise.

**DISCUSSION**

In the present study, a stable core domain from the N-terminal repressor part of human HDAC5 was defined, and the interaction of this core domain with MEF2a on the one hand and Ca²⁺ activated CaM on the other hand was analyzed. Both interactions were found to have dissociation constants in the nanomolar range. Experimental support for overlapping MEF2a- and CaM-binding sites was derived from proteolysis in conjunction with MALDI mass spectrometry and mutant data. Furthermore, direct interaction of Ca²⁺/CaM was demonstra-
The presence of Ca$^{2+}$/calmodulin inhibits HDAC5 binding to MEF2a. MEF2-controlled myogenic genes that are active in early myocyte development need to be efficiently repressed in the adult muscle cell. The importance of the underlying molecular mechanisms is exemplified by the fact that deregulation of the tight control over expression of myogenic genes or their faulty reactivation, for example, under pathological challenge can lead to severe diseases such as myocardial hypertrophy. The repression functionality has been attributed to class II histone deacetylases such as HDAC5, an enzyme that binds MEF2a transcription factor with low nanomolar affinity. Ca$^{2+}$ signaling in the cell in turn regulates class II HDACs. It is well established that kinases activated by the cellular calcium effector CaM target class II histone deacetylases by phosphorylation of key serine amino acids. This leads to removal of these HDACs from the nucleus by 14-3-3 chaperone-mediated translocation into the cytosol. Our results, and in particular the demonstration that Ca$^{2+}$-activated CaM inhibits HDAC5 binding to MEF2a by direct interaction, argue for a second functionality of CaM in addition to kinase activation within the framework of myogenic control. This CaM dual action is illustrated in Fig. 9. In this scenario, dissociation of HDAC5 from MEF2a is aided by CaM binding directly to the enzyme in the presence of Ca$^{2+}$, thus inhibiting the HDAC5/MEF2a interaction.

CaM interaction with transcription regulators provides a direct mechanism by which transcription can be regulated in a Ca$^{2+}$-dependent manner. For example, Ca$^{2+}$/CaM was demonstrated to inhibit HDAC5 repressor core binding to MEF2a.

![Calmodulin Inhibits HDAC5 Binding to MEF2a](image)

**Fig. 8.** Real time calmodulin binding studies. A, determination of the dissociation constant of CaM and RprS in the presence of Ca$^{2+}$. Repressor core solutions ranging from $2 \times 10^{-10}$ to $5 \times 10^{-8}$ M (boxed on the right) were passed over biotinylated calmodulin (bCaM) immobilized on a streptavidin-coated sensor surface. All curves were used for calculating the $K_D$ value (top right). Curves from one of three experiments are shown. B, CaM inhibits RprS binding to MEF2a. Solutions containing 30 nM repressor core and increasing amounts of CaM (30 nM to 3 μM) were incubated (30 min at room temperature) and passed over immobilized MEF2a as shown schematically on the left. Representative sensorgrams (right) show progressive reduction of binding with increasing CaM concentration.

**Fig. 9.** Ca$^{2+}$/calmodulin dual action activation model. A, binding of HDAC5 to MEF2 factors leads to repression of MEF2-dependent genes through deacetylase activity resulting in chromatin containing hypoacetylated histone proteins (adapted from Ref. 29). B, increased Ca$^{2+}$ levels lead to Ca$^{2+}$/calmodulin-mediated activation of calmodulin-dependent kinase (CaMK), which phosphorylates HDAC5 at specific serine residues. In addition, direct interaction of Ca$^{2+}$/calmodulin with the repressor core releases HDAC5 from MEF2 proteins. Transcriptional adapter factors such as p300 can now bind to MEF2 and acetylate histone protein tails. Binding of the 14-3-3 chaperone protein to phosphorylated deacetylase masks the nuclear localization sequence and exposes the nuclear export signal (40). HDAC5 is subsequently sequestered in the cytosol.
reccurent motifs for peptide domains bound by Ca\(^{2+}\)-activated CaM. These motifs contain key hydrophobic amino acids that are arranged in defined register (Table III). Typically, hydrophobic amino acids spaced 10 or 14 amino acids apart each form key interactions with either the N- or C-terminal lobe of CaM depending on the orientation of calmodulin (parallel or antiparallel) with respect to its binding site. It appears that CaM-binding motifs found in class II HDACs do not conform to these conventional patterns. Our analysis of the HDAC5 repressor core mutant Rpr\(_{S(L87G)}\) shows that the mutant protein binds CaM with markedly reduced affinity compared with wild type, thus corroborating the key importance of this leucine amino acid in CaM interaction. This fully conserved leucine is evidently the central hydrophobic amino acid clamped by one of the two EF-hand containing lobes present in CaM. It is not obvious from the alignment (Table III), however, where the interaction site of the other lobe is located within the HDAC5 primary sequence. Clearly, high resolution structural studies are required to resolve the atomic details of HDAC5 binding to CaM. Complementing these studies with the elucidation of the three-dimensional structure of a MEF2a-HDAC5 repressor complex will allow us to identify further key amino acids that are important for CaM binding but at the same time do not compromise MEF2a interaction. Mutation of these residues in full-length HDAC5 may largely reduce or abolish CaM binding by this protein. Such a structure-based HDAC5 mutant can then be used to verify the functional role of the direct interaction between class II histone deacetylase and calmodulin in living myocytes.

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