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The Coactivator-associated Arginine Methyltransferase Is Necessary for Muscle Differentiation

CARM1 COACTIVATES MYOCYTE ENHANCER FACTOR-2*

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Studies with the myogenic basic helix-loop-helix and MADS box factors suggest that efficient transactivation is dependent on the recruitment of the steroid receptor coactivator (SRC) and the cofactors p300 and p300/CBP-associated factor. SRCs have been demonstrated to recruit CARM1 (coactivator-associated arginine methyltransferase-1), a member of the *S*-adenosyl-L-methionine-dependent PRMT1–5 (protein-arginine *N*-methyltransferase-1–5) family, which catalyzes the methylation of arginine residues. This prompted us to investigate the functional role of CARM1/PRMT4 during skeletal myogenesis. We demonstrate that CARM1 and the SRC cofactor GRIP-1 cooperatively stimulate the activity of myocyte enhancer factor-2C (MEF2C). Moreover, there are direct interactions among MEF2C, GRIP-1, and CARM1. Chromatin immunoprecipitation demonstrated the *in vivo* recruitment of MEF2 and CARM1 to the endogenous muscle creatine kinase promoter in a differentiation-dependent manner. Furthermore, CARM1 is expressed in somites during embryogenesis and in the nuclei of muscle cells. Treatment of myogenic cells with the methylation inhibitor adenosine dialdehyde or *tet*-regulated CARM1 "antisense" expression did not affect expression of MyoD. However, inhibition of CARM1 inhibited differentiation and abrogated the expression of the key transcription factors (myogenin and MEF2) that initiate the differentiation cascade. This work clearly demonstrates that the arginine methyltransferase CARM1 potentiates myogenesis and supports the positive role of arginine methylation in mammalian differentiation.

Nuclear hormone receptors have served as prototypic models of coactivator recruitment (1). One current hypothesis suggests that the steroid receptor coactivator (SRC)[1] class of cofactors (2, 3) function as primary coactivators by binding directly to ligand-regulated transcription factors. Transcriptional activation also involves the subsequent recruitment of secondary coactivators such as p300, p300/CBP-associated factor, and the protein methyltransferases PRMT1 protein-arginine *N*-methyltransferase-1–5 and CARM1/PRMT4 (4–6). CBP/p300 and PCAF can acetylate histones and others components of the transcription complex, which leads to chromatin remodeling and initiation. PRMT1 and CARM1 are members of the *S*-adenosyl-L-methionine-dependent PRMT family, each of which catalyzes the methylation of arginine residues on specific proteins. At least five distinct members have been described, including PRMT1, PRMT2, PRMT3, CARM1/PRMT4, and PRMT5/JBP1 (Refs. 7–10 and references therein). A growing number of proteins, including RNA-binding proteins, contain ω-N^G-monomethylarginine and asymmetric ω-N^G,N^G-dimethylarginine residues. Furthermore, the PRMT family interacts with a range of proteins, including heterogeneous nuclear ribonucleoproteins, Np13 mRNA export protein, poly(A)-binding protein, TIS21, Jak2 receptor tyrosine kinase, SRCs, interferon-α/β receptors, interleukin enhancer-binding factor, etc. Hence, the evidence to date implicates PRMT proteins as essential components of RNA processing and trafficking, receptor-mediated signaling, and transcription (11).

Arginine methylation in proteins is a common post-translational modification. Furthermore, PRMT1, a predominantly nuclear protein, methylates arginine residues in proteins containing RGG and RXR motifs, including fibrillarin (the sclerodoma antigen) (12) and the fragile X mental retardation gene protein (directly involved in fragile X syndrome) (13). Arginine methylation is emerging as an additional layer of control in the regulation of chromatin remodeling and gene expression.

The involvement of SRC-2/GRIP-1 (a cofactor that recruits CARM1/PRMT4) in a functional complex that coactivates myogenic transcription factors (15) prompted us to investigate the expression and functional role of the CARM1 methyltransferase during skeletal muscle differentiation (myogenesis). CARM1 can methylate histone H3 *in vitro*, whereas PRMT1 methylates histone H4 (4). PRMT1 prefers to methylate arginine residues in glycine-rich regions of RNA-binding proteins; in contrast, CARM1 has little or no activity with the substrates preferred by PRMT1. However, PRMT1 and CARM1 function synergistically to transactivate nuclear receptor-mediated gene expression in an SRC-dependent manner (6). However, despite this information, the recent cloning of the *prmt* genes, and the determination of the methyltransferase core crystal structure

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[1] The abbreviations used are: SRC, steroid receptor coactivator; AdoMet, *S*-adenosyl-L-methionine; MEF2, myocyte enhancer factor-2; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; bHLH, basic helix-loop-helix; MHC, myosin heavy chain.

of the PRMT proteins (14), the function of these methyltransferases in differentiation, development, and disease remains obscure.

The PRMT proteins are recruited by C-terminal activation domain-2 of the SRC GRIP-1, whereas p300 interacts with activation domain-1 in the C terminus (5). We report here that the CARM1 and *S*-adenosyl-L-methionine (AdoMet)-dependent methylation are necessary for the latter stages of biochemical and morphological skeletal muscle differentiation and that CARM1 functions as a critical secondary coactivator for myocyte enhancer factor-2 (MEF2)-mediated gene expression in an SRC-2/GRIP-1-dependent manner.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Proliferating CH310T1/2 cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 and 10% fetal calf serum (FCS), respectively. To induce C2C12 cells to differentiate, confluent myoblast culture medium was changed to differentiation medium (DMEM supplemented with 2% horse serum). C2C12 cells normally started to differentiate 48–72 h after the serum withdrawal. Transient transfections were performed with a mixture of two liposomes (DOTAP and DOSPER (Roche Molecular Biochemicals)) as described previously (15). Luciferase activity was assayed using a Luclite kit (Packard Instrument Co.) according to the manufacturer's instructions, and relative luciferase units were measured in a Wallac Trilux 1450 Microbeta luminometer.

Immunostaining of Myocytes and Fluorescence Immunohistochemistry—Cells were fixed and stained as described previously (15). Immunohistochemical analysis was then performed using a monoclonal antibody directed against the fast isoform of the major thick filament protein skeletal myosin heavy chain (clone MY32, Sigma). This procedure is described in detail (15).

For fluorescence immunohistochemistry analysis, C2C12 cells were grown on cover slips held in six-well dishes. Cells were washed with phosphate-buffered saline (PBS) once and then fixed in 100% methanol at −20 °C for 5 min. After fixation, cells were washed once with PBS and then quenched in PBS containing 50 mM NH_4Cl to avoid the deleterious effect of the methanol on the antibodies. Cells were incubated in blocking solution (0.2% fish skin gelatin and 0.2% bovine serum albumin diluted in PBS) at room temperature for 10 min and subsequently treated with primary antibody (1:200) in blocking solution at room temperature for 30 min. Finally, the cells were washed with PBS prior to incubation with BODIFY-conjugated secondary antibodies (diluted 1:2000; Molecular Probes, Inc.) in blocking solution at room temperature for 30 min. Visualization of nuclei was achieved with 4,6-diamidino-2-phenylindole staining (1:2000 dilution in PBS) at room temperature for 10 min after the secondary antibody incubation.

Gal4 and Mammalian Two-hybrid Assays in Pluripotent C3H10T1/2 Cells—C3H10T1/2 cells were passaged into 12-well plates and transfected at 60–80% confluence with 1000 ng of reporter G5E1b-luc and 330 ng of Gal4-MEF2C or the Gal4 DNA-binding domain alone in the presence and absence of GRIP-1 and/or CARM1 by the DOTAP/DOSPER-mediated method (15) in DMEM supplemented with 10% FCS. After 16–24 h, the medium was replaced, and the cells were harvested 24–48 h after transfection for the assay of luciferase activity. Mammalian two-hybrid assays were performed in 12-well plates of JEG-3 cells (60–70% confluence) cotransfected with 1 μg of reporter G5E1b-luc, 0.3 μg of Gal4 chimeras, and 0.3 μg of VP16 chimeras (in the presence and absence of cofactors (GRIP-1 and/or CARM1)) in 1 ml of DMEM containing 5% charcoal-stripped FCS by the DOTAP/DOSPER-mediated procedure as described previously (15). Each experiment represented at least two sets of independent triplicates to overcome the variability inherent in transfections (24).

Construction of Stable Cell Lines Expressing tet/dox-regulated CARM1 Antisense Expression—C2C12 cells were transfected at 40% confluence using the liposome-mediated method as described above for transient transfection. The tetracycline-repressible pUHD (*tet*-off) plasmid (5–10 μg) was transfected into cells grown in a 10-cm Petri dish. After transfection, cells were kept in normal growth medium for 24 h to allow them to recover and for high expression of NEO before starting G418 selection. Monoclonal cell lines were isolated after 14 days of selection in growth medium containing 800 μg/ml G418 (Invitrogen). Monoclonal cell lines expressing transfected constructions were further characterized for response to tetracycline repression by transient transfection of reporter constructs containing luciferase under the regulation of tetracycline response elements in the presence of tetracycline (100 μg/ml) or doxycycline (1 μg/ml). Clones that showed a response to tetracycline were selected and used for the stable transfection of pUHD10.3-CARM1, which expresses antisense CARM1. The stable cloning of the second plasmid (pUHD10.3-CARM1) was similar to that of the first (pUHD), except that hygromycin was used as selection antibiotic instead of G418. After selection in hygromycin for 2–3 weeks, monoclonal cells were picked and passaged in 10-cm dishes for RNA isolation. Clones expressing antisense CARM1 were verified by Northern blotting first and Western blotting later.

GST Pull-down Assays and Western and Immunoprecipitation Analyses—GST and GST fusion proteins were expressed in *Escherichia coli* strain BL21 and purified by glutathione-agarose affinity chromatography as described previously (15). Total RNA from C2C12 myoblasts and myotubes was prepared using the acid/guanidinium/thiocyanate-based method (30). Briefly, cells were harvested in PBS containing 5 mM EDTA and then lysed in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH. 7.0), 0.5% Sarkosyl, and 0.1 M β-mercaptoethanol as described previously (15). Northern blotting and hybridization were performed by standard techniques (15). Nuclear proteins were isolated by resuspending cells in 0.5–2 ml of TM buffer (10 mM Tris (pH 7.4), 2 mM MgCl, and 5 mM dithiothreitol) freshly supplemented with protease inhibitors and incubated on ice for 5–10 min. The cells were spun down at 2000 rpm for 10 min at 4 °C. The supernatant was discarded, and the cells were frozen and thawed twice to break the cell membranes. The broken cells were resuspended in 0.5 ml of TM buffer and incubated on ice for 10 min, followed by spinning at 2000 rpm for 10 min at 4 °C. The supernatant was aspirated for analysis of cytosolic protein, and 0.5 ml of nuclear extract buffer (20 mM Tris, 400 mM KCl, 2 mM EDTA, 2 mM MgCl, 1 mM dithiothreitol, and 10% glycerol) freshly supplemented with protease inhibitors was added to resuspend the cells and then incubated on ice for 60–90 min. The cells were vigorously vortexed every 10 min during this incubation to maximize the extraction efficiency. Cell debris was cleared by centrifugation at 15,000 rpm for 20 min at 4 °C, and the supernatant was dialyzed against PBS at 4 °C to reduce the salt concentration. After dialysis, the protein concentrations were determined by the Bradford dye method. Western and immunoprecipitation analyses were performed as described previously (15).

RESULTS

CARM1 Directly Interacts with MEF2C in Vitro, but Not with MyoD and Myogenin—The SRC GRIP-1 has been demonstrated to recruit the coactivator-associated methyltransferase CARM1/PRMT4, which functions as a secondary cofactor in nuclear receptor-mediated transactivation of gene expression. CARM1 directly interacts with GRIP-1 and acts as a secondary coactivator for transcription in an SRC-dependent manner. Recently, GRIP-1 has been found to be necessary for skeletal muscle differentiation in culture; furthermore, GRIP-1 directly interacts with the MADS (MCM1-agamous deficiens-serum response factor) box factor MEF2C and the basic helix-loop-helix (bHLH) factor myogenin (in an E2A-dependent manner) and increases their activity (15). MEF2 and myogenin function cooperatively to regulate the latter stages of skeletal muscle differentiation and the activation of contractile protein transcription.

Hence, we examined the ability of CARM1 to interact with GRIP-1 (a positive control) and the myogenic factors MEF2C, MyoD, and myogenin. We tested this using a biochemical approach, the *in vitro* GST pull-down assay, to examine potential interactions among CARM1, GRIP-1, MEF2C, MyoD, and myogenin. Glutathione-agarose-immobilized GST-CARM1 was tested for direct interaction with *in vitro* ^{35}S-radiolabeled native GRIP-1, MEF2C, myogenin, and MyoD. We observed, as expected (4), that GRIP-1 interacted with CARM1. Surprisingly, MEF2 interacted with CARM1 directly; however, myogenin and MyoD did not interact with CARM1 (Fig. 1*A*).

To rigorously validate the physical association of CARM1 and MEF2C in an additional assay, CARM1 and MEF2C were translated in a rabbit reticulocyte lysate. We observed that immunoprecipitation of a rabbit reticulocyte lysate programmed with CARM1 and MEF2C in the presence and absence of GRIP-1 with an antibody directed against MEF2C

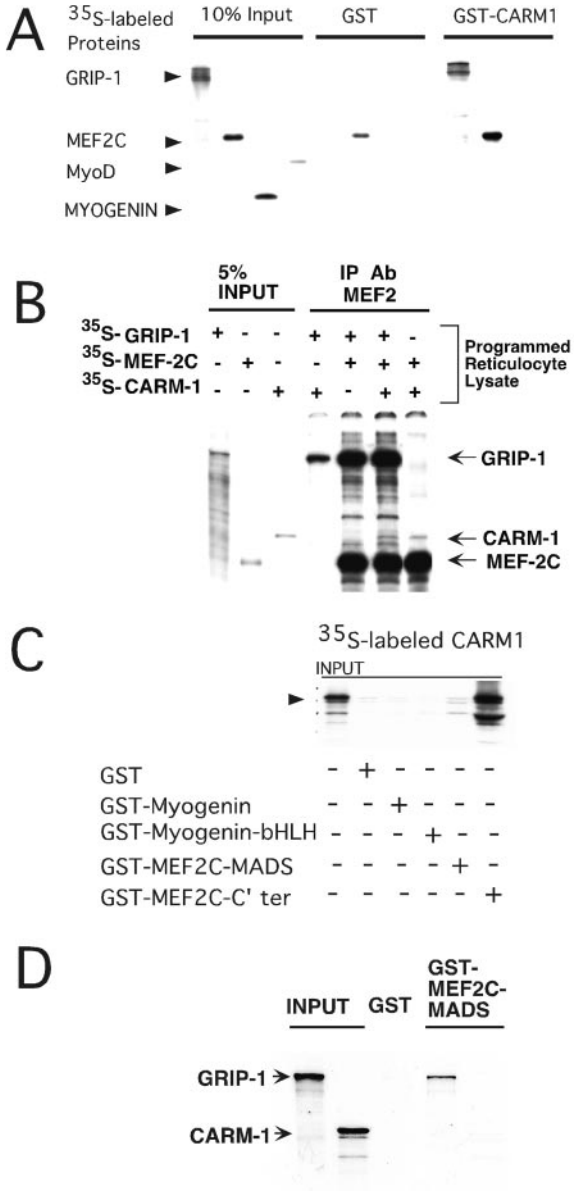


FIG. 1. **MEF2C interacts with GRIP-1.** *A*, glutathione-agarose-immobilized GST and GST-CARM1 proteins were incubated with ^{35}S-radiolabeled full-length GRIP-1, MEF2C, MyoD, or myogenin. The input lanes represent ~10% of the total protein. *B*, shown are the results from co-immunoprecipitation of CARM1 and GRIP-1 with MEF2C. CARM1, GRIP-1, and MEF2C expression plasmids were transcribed/translated in a rabbit reticulocyte lysate and mixed as indicated. Reticulocyte-produced proteins were then immunoprecipitated (*IP*) with anti-MEF2 antibody (*Ab*), and the ^{35}S-labeled proteins were analyzed by SDS-PAGE. CARM1, GRIP-1, and MEF2C were co-immunoprecipitated with anti-MEF2 antibodies. *C*, the MADS domain does not interact with CARM1. Glutathione-agarose-immobilized GST, GST-myogenin, GST-myogenin-bHLH, GST-MEF2C-MADS, and GST-MEF2C-C′ter proteins were incubated with ^{35}S-radiolabeled full-length CARM1. The input lanes represent ~10% of the total protein. *D*, glutathione-agarose-immobilized GST and GST-MEF2C-MADS proteins were incubated with either ^{35}S-radiolabeled GRIP-1 or CARM1. The input lanes represent ~10% of the total protein.

resulted in co-immunoprecipitation of CARM1 (Fig. 1*B*). This demonstrates that these two proteins directly interact. However, the MEF2 interaction with CARM1 was weak compared with the efficient MEF2/GRIP-1 interaction.

We further verified the interaction of CARM1 with MEF2C by examining the ability of GST-myogenin, GST-myogenin-bHLH, GST-MEF2C-MADS-(1–57), and GST-MEF2C-C′ter-(224–465) to interact with *in vitro* ^{35}S-radiolabeled native CARM1. As expected, CARM1 did not interact with GST-bHLH chimeras, but interestingly required the C-terminal region of MEF2C for interaction (Fig. 1*C*). We verified that this was not an artifact of a nonfunctional GST-MADS chimera by demonstrating interaction with GRIP-1 (but not with CARM1) in a GST-pull down assay (Fig. 1*D*). The above experiments demonstrate the interaction among MEF2C, GRIP-1, and CARM1 and are in accordance with GRIP-1-mediated coactivation of MEF2-mediated transcription (15).

CARM1/PRMT4 Directly Interacts with the C-terminal Region of MEF2C—To further support the specificity of the interaction between the carboxyl-terminal region of MEF2C and CARM1, we examined the ability of a number of GST-MEF2C fusion chimeras containing various functional subdomains of MEF2C immobilized on glutathione-agarose beads (*i.e.* native GST-MEF2C-(1–465), GST-MEF2C-(1–57), GST-MEF2C-(58–465), GST-MEF2C-(90–465), GST-MEF2C-(91–223), and GST-MEF2C-(224–465). CARM1 showed a strong interaction with GST-MEF2C (native) relative to GST alone. Deletion of the MADS box (required for the interaction with GRIP-1) or the MEF2 domain did not compromise the interaction with CARM1. This was consistent with the inability of CARM1 to interact with GST-MEF2C-(1–57). Furthermore, CARM1 failed to interact with GST-MEF2C-(91–223), which encodes the N-terminal activation domain of MEF2C. However, CARM1 interacted very efficiently with the C-terminal region of MEF2C between amino acids 224 and 465 (Fig. 2*A*). This segment encodes the C-terminal activation domain of MEF2C between amino acids 247 and 327. The above experiments verify the direct interaction between MEF2C and CARM1.

MEF2C, GRIP-1, and CARM1 Interact in a Cellular Context—To further characterize transcriptional regulation by MEF2C, we investigated whether these transcriptional cofactors interact with MEF2C in a cellular context *in vivo*. Protein/protein interaction assay systems were initially developed in yeast and further refined for the study of protein/protein interactions in transfected mammalian cells. In these experiments, the yeast Gal4 DNA-binding domain was fused to MEF2C and expressed in transfected cells with GRIP-1 linked to the transactivation domain of herpes simplex virus VP16. Because coactivation of MEF2-dependent transcription by GRIP-1 was augmented by the secondary coactivator CARM1, we used a modified mammalian two-hybrid system (Fig. 2*B*) to further investigate the molecular basis of the MEF2/GRIP-1-mediated activation. We examined the effect of CARM1 expression on the interaction between Gal4-MEF2 and VP16-GRIP-1. Significant transactivation of a luciferase reporter gene downstream of Gal4-binding sites fused to the E1b promoter was achieved only when the coexpressed proteins physically interacted (Fig. 2*B*).

Chimeric Gal4-MEF2C regulated transcription of the G5E1b-luc reporter (as previously demonstrated above) (Fig. 2*B*). Gal4-MEF2C alone activated transcription (~10-fold) relative to the Gal4 DNA-binding domain. We then examined the ability of coexpressed VP16-GRIP-1 to enhance reporter gene expression in this mammalian two-hybrid system. We observed that VP16-GRIP-1 effectively and significantly induced transcription by 5–7-fold, resulting in a 50–70-fold increased transactivation of gene expression.

We observed that addition of the CARM1 expression vector further increased transactivation of gene expression by MEF2 in a GRIP-1-dependent manner. In conclusion, the mammalian two-hybrid experiments demonstrate that the MEF2·GRIP-1 complex interacts with CARM1 in the cellular context and suggest that the mechanism of activation involves physical association.

The Coactivator-associated Arginine Methyltransferase

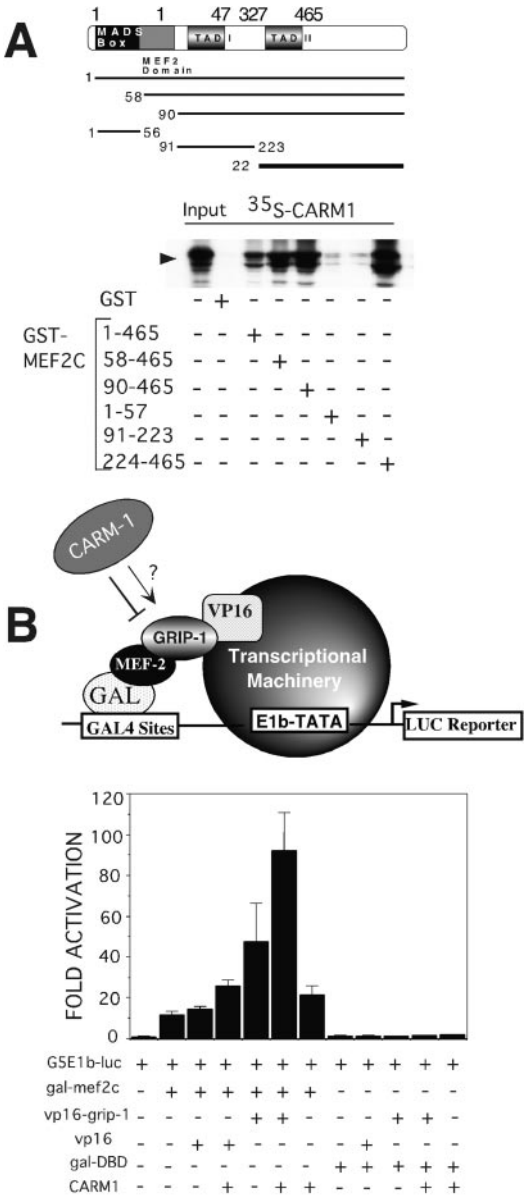


FIG. 2. **The C-terminal region of MEF2C mediates the interaction with CARM1.** *A*, diagrammatic representation showing the MEF2C regulatory domains. Glutathione-agarose-immobilized GST and GST-MEF2C proteins were incubated with ^{35}S-radiolabeled full-length CARM1. Deletion of the C-terminal domain between amino acids 223 and 465 ablated binding to CARM1 *in vitro*. The input lanes represent ~10% of the total protein. *B*, diagrammatic representation of the mammalian two-hybrid assay used to determine the interaction and cooperativity of CARM1, GRIP-1, and MEF2C in a cellular context. CARM1 interacted with MEF2 in a GRIP-1-dependent manner. -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the Gal4 DNA-binding domain alone, arbitrarily set at 1. *TAD I*, N-terminal activation domain; *TAD II*, C-terminal activation domain; *LUC*, luciferase.

CARM1/PRMT4 Coactivates MEF2C-mediated Transcription—The biochemical experiments presented above suggest that CARM1 interacts with MEF2C in complex with the SRC GRIP-1. Furthermore, CARM1 functions as a secondary cofactor to enhance transactivation by nuclear hormone receptors in an SRC-dependent manner (4, 5). Because the SRC cofactor GRIP-1 interacts with and modulates the activity of MEF2C (which cooperates with the myogenic bHLH proteins in the activation of contractile protein gene expression) (15), we examined the ability of CARM1 to regulate MEF2-dependent gene expression.

We stably transfected pluripotent CH310T1/2 cells with an MEF2-dependent luciferase reporter with three tandem copies of the MEF2-binding site upstream of a basal E1b promoter (Fig. 3*A*). This cell line was then transiently transfected with different combinations of MEF2C, GRIP-1, and CARM1. We observed maximal transactivation of gene expression upon cotransfection of MEF2C, GRIP-1, and CARM1. These experiments demonstrate that MEF2C, the SRC GRIP-1, and the secondary cofactor CARM1 synergistically coactivate MEF2C-mediated transactivation of an MEF2-dependent reporter.

The Coactivator-associated Arginine Methyltransferase CARM1 Increases MEF2 Activity in a GRIP-1-dependent Manner—The experiments presented above suggest that the SRC cofactor GRIP-1 and the secondary cofactor CARM1 coactivate MEF2C-mediated transcription. Because MEF2C can activate transcription directly and/or by protein/protein interaction through tethering to other DNA-bound factors, we examined the effect of CARM1 expression on MEF2C-mediated transactivation in the Gal4 hybrid system in the presence and absence of GRIP-1 (Fig. 3*B*). In these assays, the activity of MEF2C is independent of its binding to its cognate DNA motif, the A/T-rich MEF2 site. If CARM1 regulates the transcriptional activity, then the potential of the Gal4-myogenic factor fusions to transactivate gene expression should be greatly increased in this assay (Fig. 3*B*).

We subsequently transfected pluripotent C3H10T1/2 cells with Gal4-MEF2C and the G5E1b-luc reporter in the presence and absence of CARM1 and GRIP-1 expression vectors. G5E1b-luc contains five copies of the Gal4-binding site placed upstream of a minimal E1b promoter. Transfection of Gal-MEF2C alone modestly induced transcription relative to the Gal4 DNA-binding domain; this level of activity was significantly stimulated (5–7-fold) by the simultaneous expression of GRIP-1 and CARM1 (Fig. 3*B*), as was observed with the MEF2-dependent luciferase reporter genes (Fig. 3*A*). CARM1-mediated coactivation was dependent on the presence of GRIP-1; CARM1 expression in the absence of GRIP-1 failed to increase the activity of MEF2C. We similarly observed that CARM1 also mediated coactivation of MEF2A and MEF2D in the presence of GRIP-1 (data not shown).

CARM1 and MEF2 Are Recruited "in Vivo" by the Muscle Creatine Kinase Promoter in a Differentiation-dependent Manner—Chromatin immunoprecipitation is a sensitive technique that enables detection of endogenous transcription factors and cofactors recruited *in vivo* to promoters under different physiological conditions. We employed this procedure to investigate the recruitment of MEF2 and CARM1 to the endogenous MEF2-dependent muscle creatine kinase promoter during skeletal myogenesis. The mouse myogenic C2C12 cell line was used for these studies, as they differentiate well in culture and are very well characterized in terms of MEF2-dependent gene expression. Soluble chromatin was prepared from proliferating (undifferentiated) myoblasts cultured in growth medium (DMEM supplemented with 20% FCS) and from post-mitotic multinucleated myotubes after 72–96 of serum withdrawal in differentiation medium. The samples were immunoprecipitated with antibodies specific to MEF2 and the cofactor CARM1. The presence of specific promoters in the immunoprecipitates was analyzed by PCR using primers designed to amplify the region spanning the MEF2-binding sites in the muscle creatine kinase enhancer. Fig. 3*C* shows that the amount of MEF2 and CARM1 recruited to the muscle creatine kinase promoter increased in material derived from differentiated cells. This result was not influenced by chromatin amounts in the myoblast and myotube inputs confirmed by PCR prior to immunoprecipitation; in fact, the differentiated sample had less material. The soluble chro-

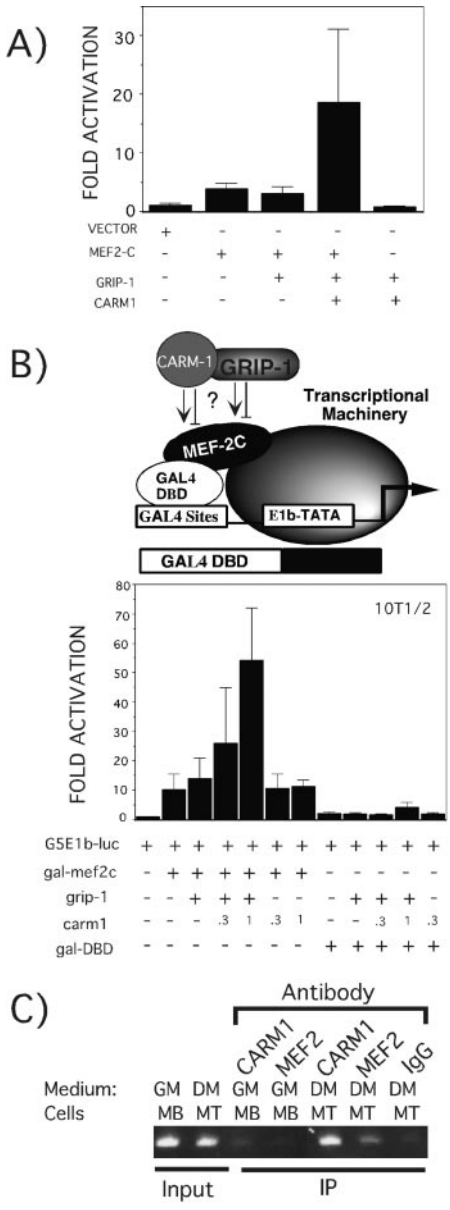


FIG. 3. **CARM1 coactivates MEF2-mediated transcription in an SRC-dependent manner.** *A*, CARM1 coactivates MEF2-mediated transactivation of a stably integrated MEF2-dependent reporter. The luciferase reporter composed of three tandem copies of the MEF2-binding site linked to a basal E1b-luc reporter in pGL3-back was stably transfected into pluripotent C3H10T1/2 cells. Subsequently, this cell line was transiently transfected with different combinations of expression vectors driving MEF2C, GRIP-1, and CARM1 expression. -Fold activation is expressed relative to the native luciferase activity of the stably transfected cell population obtained after transient transfection of the empty expression vector. Activation mediated by vector alone was arbitrarily set at 1. Transfection results were derived from three independent transfections. *B*, CARM1 potentiates the activity of MEF2C in a GRIP-1-dependent manner. A diagrammatic representation of the Gal4 hybrid assay is shown. This assay was used to determine the effect of CARM1 and GRIP-1 expression on MEF2C transcriptional activity. CH310T1/2 cells were transfected with a plasmid encoding Gal4-MEF2C (*i.e.* the yeast Gal4 DNA-binding domain (*DBD*) fused in frame with full-length MEF2C) and a reporter construct in which five copies of the Gal4-binding site are placed upstream of the minimal E1b promoter (*i.e.* G5E1b-luc). *C*, soluble chromatin was prepared from cultures of proliferating myoblasts (*MB*) in growth medium (*GM*) and from post-mitotic multinucleated myotubes (*MT*) in differentiation medium (*DM*). Samples were immunoprecipitated (*IP*) with antibodies specific to MEF2 and CARM1. The myotube material was also treated with rabbit IgG to control for nonspecific precipitation of chromatin. Immunoprecipitated material was analyzed by PCR using primers designed to amplify sequences spanning the MEF2-binding sites in the muscle creatine kinase enhancer as described by Lu *et al.* (31).

matin was also treated with rabbit IgG (nonimmune) as a check against nonspecific precipitation. These data demonstrate differentiation-dependent CARM1 recruitment to the well characterized MEF2-dependent muscle creatine kinase promoter and provide *in vivo* evidence for CARM1 function during skeletal myogenesis.

CARM1 Is Expressed during Mouse Embryogenesis—Expression of *carm1* during mouse embryo development was studied by whole-mount *in situ* hybridization. Antisense RNA probes were prepared from *carm1* cDNA spanning the 3′-coding region and the 3′-untranslated region that is specific to the *carm1* gene so as to exclude possible cross-reactivity with other *prmt* sequences. At ~8.25 days postcoitus, CARM1 expression was observed in the fore- and hindbrain, neural fold, somites, and posterior lateral plate (Fig. 4*A*, *panels i* and *ii*). At ~8.75 days postcoitus, CARM1 expression was prominent in the neural tube and somites (Fig. 4*B*, *panels i* and *ii*). The pattern of CARM1 expression was consistent with the early expression of MEF2 proteins in muscle precursor cells and non-myogenic lineages during mouse embryogenesis (16).

CARM1 mRNA and Protein Are Expressed during Skeletal Muscle Differentiation—CARM1 is expressed in the skeletal muscle precursors (*i.e.* somites) during mouse embryogenesis and, in cultured cells, modulates the activity of MEF2C, a critical factor in the activation of contractile protein transcription. Hence, we examined the expression and functional role of CARM1 in muscle differentiation (myogenesis), an ideal paradigm for analysis of the determinative events that govern the cells' decision to divide or differentiate.

Proliferating C2C12 myoblasts were induced to differentiate biochemically and morphologically into post-mitotic multinucleated myotubes by serum withdrawal in culture over a 72-h period. The transition from a non-muscle phenotype to a contractile phenotype is associated with the activation/expression of (i) the *myoD* gene family (*myoD*, myogenin, *myf-5*, and *MRF-4*), (ii) the MEF2 MADS box proteins, and (iii) a structurally diverse group of genes that encode a functional sarcomere responsible for contraction. The differentiation process is orchestrated and dependent on the cooperative interaction between myogenin and MEF2 (17, 18).

Total RNA was isolated from proliferating myoblasts and post-mitotic myotubes after 72 h of serum withdrawal and examined by Northern blot analysis. The CARM1 mRNA was constitutively expressed in proliferating myoblasts and post-mitotic differentiated multinucleated myotubes that have acquired a muscle-specific phenotype (Fig. 4*C*). The hybridization signal corresponded to the expected transcript size of ~3.8 kb.

Total lysate and nuclear extract were isolated from proliferating myoblasts, confluent myoblasts, and myotubes after 3 days of serum withdrawal. Western analysis of total lysates with the polyclonal antibody to mouse CARM1 demonstrated that the CARM1 protein was expressed in myoblasts and myotubes (Fig. 4*D*). However, Western analysis of nuclear extracts demonstrated that CARM1 protein levels in the nucleus increased during the differentiation process, reaching a peak in the post-mitotic myotube cells.

In conclusion, the CARM1 mRNA and protein are expressed during skeletal muscle differentiation in culture. The expression pattern of CARM1 and its function as a secondary coactivator through association with the SRC cofactor GRIP-1 (which interacts with steroid receptors and MEF2) were consistent with its function as an MEF2 cofactor.

CARM1 Is Expressed in the Nucleus during Skeletal Muscle Differentiation—The CARM1 mRNA and protein are abundantly expressed during the differentiation of mouse muscle cells in culture. To detect and analyze the subcellular localiza-

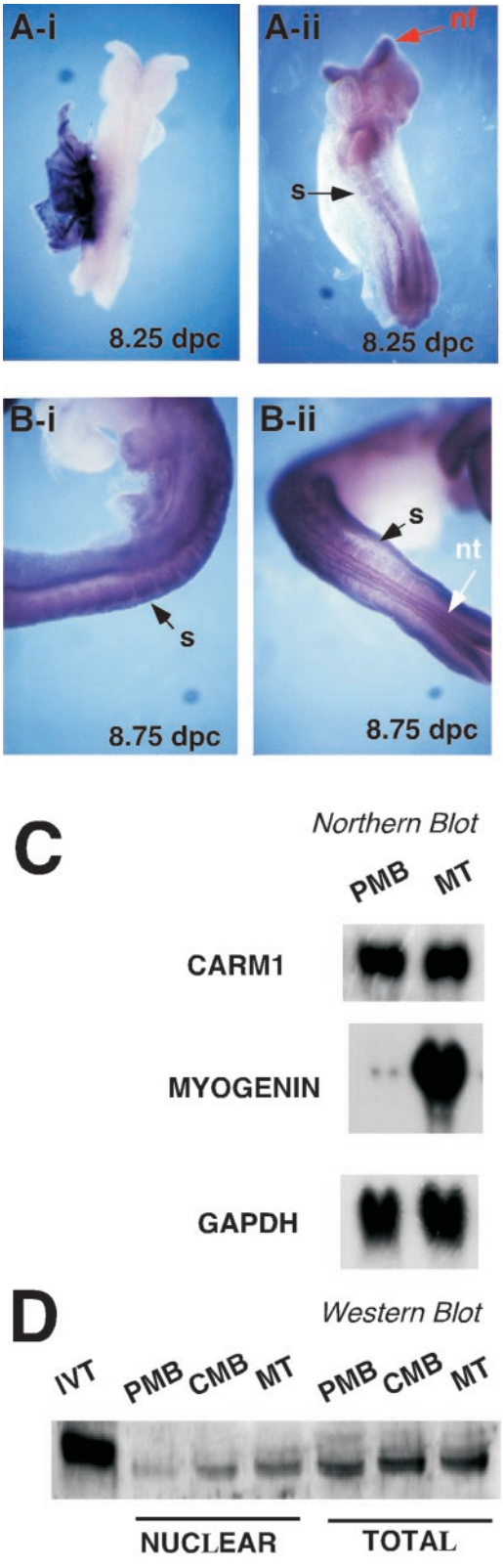


FIG. 4. **Analysis of CARM1 expression during mouse embryogenesis by whole-mount *in situ* hybridization.** *A* and *B*, we isolated embryos at 8.25 and 8.75 days postcoitus from the outbred mouse strain CD1. They were staged by standard morphological criteria. Expression of CARM1 during mouse embryo development was studied by whole-mount in *situ* hybridization. Antisense and sense RNA probes were prepared from *carm1*, but not containing any poly(A) sequences, so as to exclude possible cross-reactivity with other *prmt* sequences. The digoxigenin-labeled CARM1 riboprobes were hybridized to whole mouse embryos isolated at 8.25 days postcoitus (*dpc*) (*A*, *panels i* and *ii*), and 8.75 days postcoitus (*B*, *panels i* and *ii*). *nf*, neural fold; *s*, somite; *nt*, neural tube. *C*, CARM1 mRNA is constitutively expressed during skeletal muscle differentiation. Total RNA was isolated from proliferating myoblasts (*PMB*; ~50% confluent propagated in growth medium) and from differentiated myotubes after 72 h of serum withdrawal (*MT*; propagated in differentiation medium). After blotting, RNA was probed with ^{32}P-radiolabeled cDNA encoding CARM1, myogenin, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The induction of myogenin mRNA indicated that these cells had undergone terminal differentiation. *D*, shown are the results from Western analysis of CARM1 levels during skeletal muscle differentiation and *in vitro* translated (*IVT*) Western control. Total cellular lysate and nuclear extracts were isolated from proliferating myoblasts (*PMB*), confluent myoblasts (*CMB*), and myotubes after 3 days of serum withdrawal (*MT*). Myoblasts were cultured in growth medium, and myotubes were cultured in differentiation medium.

tion pattern of the steroid receptor secondary coactivator CARM1, we conducted immunofluorescence staining using polyclonal anti-CARM1 antibody (catalog number 07-0780, Upstate Biotechnology, Inc.). CARM1 was endogenously expressed in low to high confluent proliferating C2C12 myoblasts grown in high serum (Fig. 5, *A* and *C*). The majority of staining was in the cell nucleus, but there was also significant staining observed in the cytoplasm. The staining pattern in the nucleoplasm was extranucleolar and appeared in prominent nuclear dots in a punctate pattern. After 72 h of serum withdrawal and induction of the differentiation program, the CARM1 staining was almost exclusively localized to the nucleus (Fig. 5, *E* and *F*), with little (if any) staining in the cytoplasm.

The Methyltransferase Inhibitor Adenosine Dialdehyde Blocks Terminal Muscle Differentiation—To delineate the functional role of the protein-arginine methyltransferase CARM1 in myogenesis and to identify the signal pathway that this secondary cofactor regulates, we examined the effect of treatment with the methylation inhibitor adenosine dialdehyde on differentiation. This treatment leads to an accumulation of methyl-accepting substrates/proteins in hypomethylated states. Adenosine dialdehyde is an inhibitor of *S*-adenosylhomocysteine hydrolase and can elevate the cellular levels of *S*-adenosylhomocysteine, the product inhibitor of methyltransferases, utilizing AdoMet as the methyl donor.

C2 muscle cells were cultured in growth medium and then cultured in differentiation medium for 72 h. Mitogen withdrawal activated the differentiation program. These cells were compared with cells cultured for 72 in differentiation medium supplemented with 20 μM adenosine dialdehyde (Fig. 6*A*). The effect of methyltransferase inhibition at the biochemical level was assayed by Western analysis of treated (adenosine dialdehyde) and untreated cells cultured in differentiation medium for 72 h. We examined the effect of adenosine dialdehyde treatment on factors involved in determination (*e.g.* MyoD), differentiation (myogenin and MEF2), and contraction (myosin heavy chain (MHC)). Furthermore, we assayed GRIP-1 expression; this SRC cofactor is necessary for skeletal muscle differentiation and interacts with MEF2 and the methyltransferase CARM1 during steroid receptor-mediated transactivation.

We isolated nuclear extracts from C2 cells cultured in differentiation medium in the presence and absence of adenosine dialdehyde. Western analysis was performed with antibodies against MyoD, myogenin, MEF2, GRIP-1, and MHC. Immunoblot analysis (Fig. 6*A*) demonstrated that adenosine dialdehyde treatment had no effect on either MyoD or GRIP-1 expression levels. However, myogenin and MEF2 protein levels were ablated and dramatically reduced, respectively, after adenosine dialdehyde treatment (Fig. 6*A*). Finally, we examined a later step in the differentiation program, *i.e.* the expression of structural proteins that encode a functional sarcomere and are responsible for the contractile phenotype. Immunoblot analysis with the monoclonal

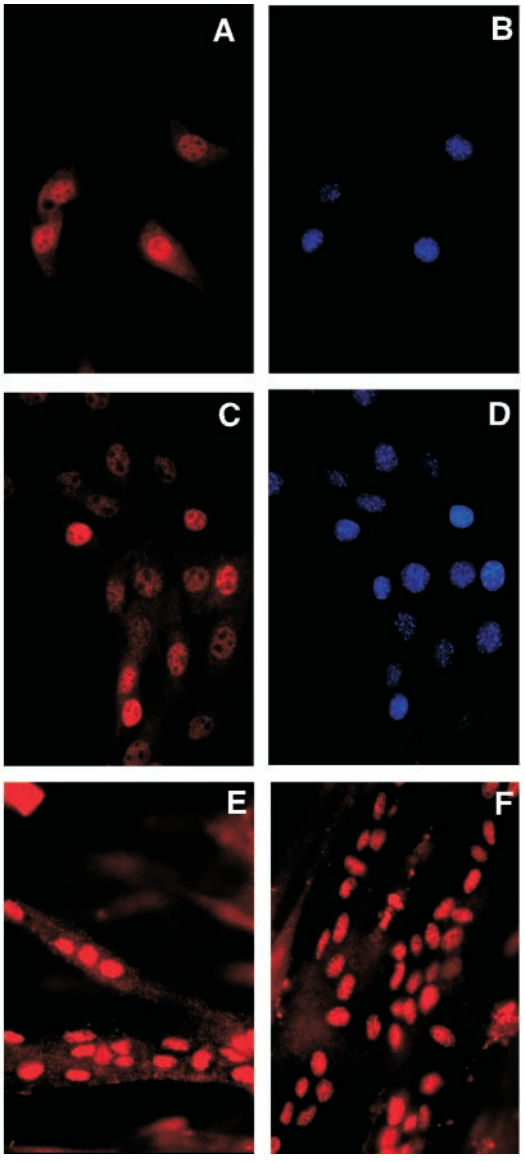
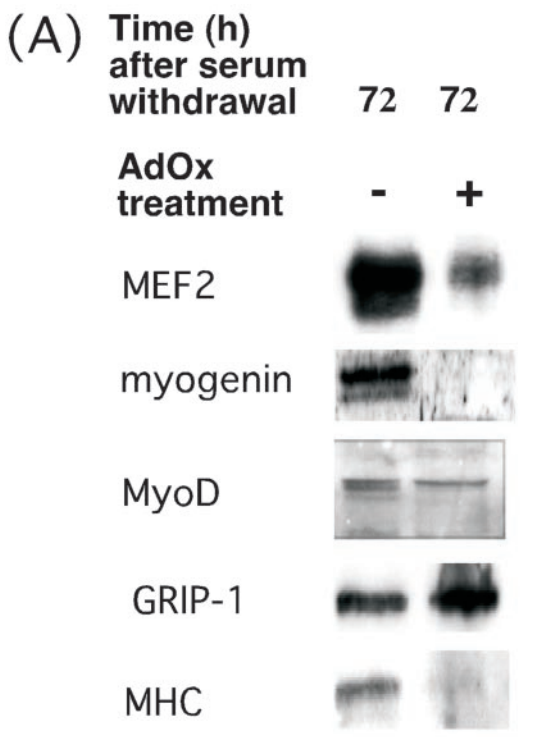


FIG. 5. **CARM1 progressively localizes to the nuclei of muscle cells during differentiation.** *A* and *C*, CARM1 expression (*red*) in increasingly confluent proliferating C2C12 myoblasts grown in growth medium; *E* and *F*, CARM1 in differentiated myotubes after 72 h of serum withdrawal (*i.e.* cultured in differentiation medium); *B* and *D*, 4,6-diamidino-2-phenylindole-stained nucleus (*blue*). C2 cells were grown on cover slides held in six-well dishes. CARM1 immunostaining (*red*) of fixed cells was performed with the purified antibody against CARM1 (residues 595–608 of mouse CARM1). Cells were washed and incubated with the secondary antibody (Texas Red-conjugated goat anti-rabbit IgG; 1:2000).



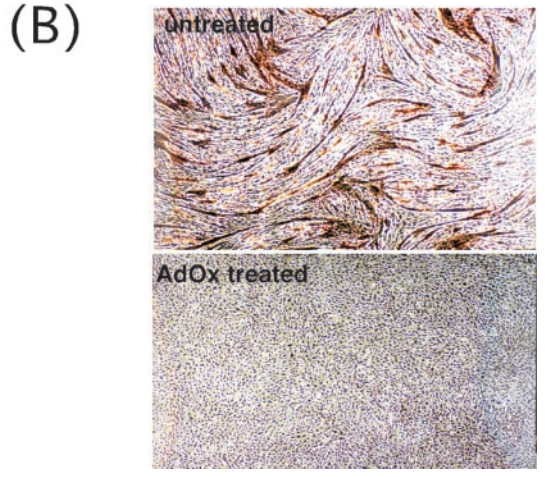


FIG. 6. *A*, Western analysis of total lysates from both normal C2C12 and adenosine dialdehyde-treated cells after 72 h of serum withdrawal in the presence and absence of 20 μM adenosine dialdehyde (*AdOx*). Proteins were detected by Western blotting with 30 μg of nuclear proteins from each sample using a rabbit antibody to MEF2 (C-21, Santa Cruz Biotechnology) that is generated against MEF2A, but that cross-reacts with mouse/human MEF2A/C and MEF2D. GRIP-1 proteins were detected by Western blotting with 30 μg of nuclear proteins from each sample using a monoclonal antibody to human GRIP-1/TIF-2 (T73620, Transduction Laboratories) that cross-reacts with the mouse species. *B*, immunostaining of untreated and treated (20 μM adenosine dialdehyde) C2C12 cells with a monoclonal antibody directed against the fast isoform of the major thick filament protein skeletal MHC after 72 h of culture in differentiation medium. Immunohistochemistry revealed that adenosine dialdehyde treatment compromised the activation of contractile protein gene(s) and formation of post-mitotic multinucleated myotubes. MHC-positive cells are stained *red*, and nuclei are counterstained *blue* by Mayer's hematoxylin solution.

antibody directed against the fast isoform of the major thick filament protein skeletal MHC demonstrated that treated cells lack the major thick filament sarcomeric protein.

Subsequently, the extent of differentiation was assayed by immunostaining with the monoclonal antibody directed against the fast isoform of skeletal MHC. MHC is associated with the contractile phenotype and was expressed during the later stages of muscle differentiation (Fig. 6*B*). The immunohistochemistry results dramatically demonstrated that the inhibitor ablated morphological differentiation of proliferating myoblasts into multinucleated myotubes. This suggests that hypomethylation of proteins significantly compromises (i) morphological differentiation and formation of post-mitotic myotubes and (ii) the activation of contractile protein gene(s). In summary, adenosine dialdehyde treatment revealed that AdoMet-dependent methylation is necessary for (i) morphological differentiation, (ii) activation of essential transcription factors, and (iii) activation of contractile protein gene(s).

CARM1 Antisense RNA Expression Inhibits Skeletal Muscle

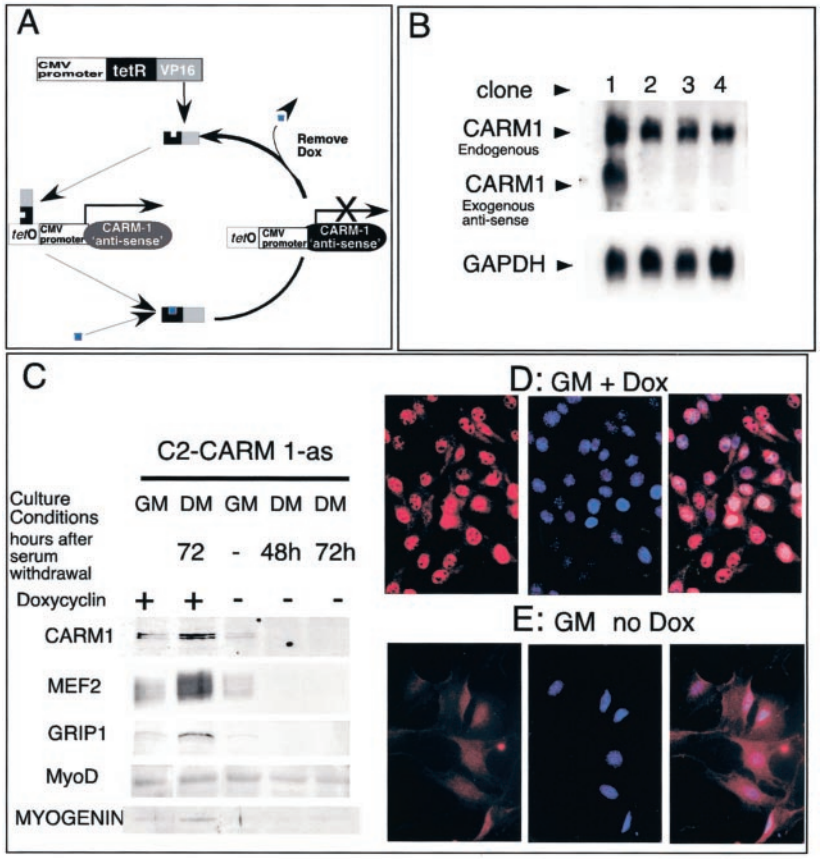


FIG. 7. *A*, schematic displaying the essential features of tet-off-regulated gene expression; *B*, CARM1 Northern analysis of stably transfected independent clones and of the cell line displaying tet-off-inducible antisense CARM1; *C*, Western analysis of CARM1, MEF2, GRIP-1, MyoD, and myogenin levels in the antisense CARM1 (*CARM 1-as*)-transfected cells in the presence and absence of doxycycline; *D* and *E*, immunofluorescence and 4,6-diamidino-2-phenylindole analysis of CARM1 expression in proliferating myoblasts from C2C12 muscle cells transfected with *tet*-regulated antisense CARM1 in the presence (*D*) and absence (*E*) of doxycycline (*Dox*). *CMV*, cytomegalovirus; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GM*, growth medium; *DM*, differentiation medium.

Cell Differentiation—To delineate the specific functional role of the CARM1 protein-arginine methyltransferase in myogenesis, we proceeded to use the tetracycline-off system to express CARM1 antisense RNA in myogenic cells. We initially transfected proliferating C2C12 myoblasts with a plasmid encoding the pTet-off tetracycline-regulated transactivator, pUHD.*Neo*^{r}, which expresses a chimeric fusion protein of amino acids 1–207 of *tetR* and the C-terminal 130 amino acids of the herpes simplex virus activation domain. This converts *tetR* from a transcriptional repressor to a tetracycline-regulated transactivator that binds to the *tet* operator (*tetO*) sequences in the absence of tetracycline (Fig. 7*A*). Following G418 selection, resistant clones were selected and subsequently screened by transient transfection with a tetracycline-regulated luciferase reporter. Cell clones that exhibited a high degree of regulated expression of the reporter and still retained the ability to differentiate in culture were selected and subsequently transfected with a tetracycline-dependent cytomegalovirus promoter (pUHD.10.3-*hyg*^{r}) driving CARM1 antisense expression. Hygromycin-resistant clones were selected and subsequently screened for a low/zero background of CARM1 antisense expression in the presence of doxycycline/tetracycline, potential to differentiate, and high inducibility and expression of CARM1 antisense RNA after tetracycline withdrawal (Fig. 7*B*).

C2-CARM1 antisense muscle cells were cultured in growth medium in the presence and absence of doxycycline and then cultured in differentiation medium in the presence and absence of doxycycline for 48–72 h (Fig. 7*C*). We isolated nuclear extracts from C2 cells cultured in differentiation medium in the presence and absence of doxycycline. Western analysis was performed with antibodies against MyoD, myogenin, MEF2, GRIP-1, and CARM1. Immunoblot analysis (Fig. 7*C*) demonstrated that serum withdrawal in the presence of doxycycline resulted in the expected biochemical changes associated with muscle differentiation in culture, *i.e.* increases in MEF2, myogenin, GRIP-1, and CARM1. MyoD expression levels remained constitutive.

Serum withdrawal in the absence of doxycycline induced CARM1 antisense RNA expression and reduced CARM1 protein expression dramatically (Fig. 7*C*). This was substantiated and highlighted after immunofluorescence analysis of myoblasts cultured in growth medium in the presence (Fig. 7*D*) and absence (Fig. 7*E*) of doxycycline. As expected, CARM1 was expressed in the nucleus in myoblasts cultured in the presence of doxycycline. However, in the absence of doxycycline, the CARM1 protein was expressed in a diffuse cytoplasmic manner at reduced levels in myoblasts (Fig. 7, *C* and *E*).

In the absence of doxycycline after 48 and 72 h of serum withdrawal, the induction of CARM1 antisense expression completely abrogated the induction of the CARM1, MEF2, GRIP-1, and myogenin proteins. In contrast, MyoD expression was unaffected by the cellular knockout of CARM1 expression. This clearly demonstrates that CARM1 expression is necessary and potentiates muscle differentiation in a positive manner. In summary, CARM1 antisense expression reveals that the coactivator-associated arginine methyltransferase activity is necessary for the induction of critical transcription factors and cofactors associated with terminal differentiation.

DISCUSSION

This study demonstrates that CARM1 is necessary for skeletal muscle differentiation. Furthermore, we show that CARM1 functions as a secondary cofactor for MEF2-mediated transcription in an SRC-2/GRIP-1-dependent manner in the regulation of muscle-specific gene expression (Fig. 8). Consistent with these functions, we observed abundant CARM1 expression in muscle cells and in skeletal muscle precursors during embryogenesis. CARM1 function and AdoMet-mediated

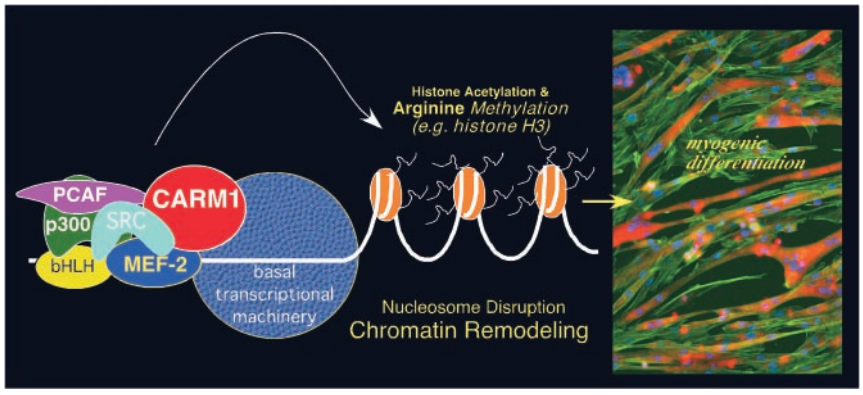


FIG. 8. **Schematic summarizing the role of CARM1 in the activation of MEF2-dependent transcription during myogenic differentiation.**

methylation are critical for myogenesis and suggest that arginine methylation potentiates differentiation. Our observations are consistent with (i) GRIP-1-mediated coactivation of MEF2-dependent transcription (15), (ii) direct interaction between MEF2 and GRIP-1 (15), (iii) SRC-dependent recruitment of CARM1 during nuclear hormone receptor-mediated transcription, and (iv) MEF2 expression during mouse embryogenesis (16).

SRC and p300 coactivate several different classes of activators, *e.g.* NF-κB (2, 19) and the myogenic bHLH and MADS box factors. For example, CBP/p300 and GRIP-1 are coactivators for MyoD and MEF2 during myogenic differentiation (summarized in Fig. 8) (15, 20–23). CARM1 was previously shown to function as a secondary coactivator for nuclear hormone receptors (4, 5), *i.e.* CARM1 is recruited indirectly by nuclear receptors through its interaction with GRIP-1. Our data suggest that CARM1 also functions a secondary cofactor for GRIP-1 in the nuclear receptor-*independent* functions of this coactivator.

We tested the hypothesis that MEF2 primary and secondary cofactors are necessary for skeletal myogenesis by chemical-mediated inhibition of methyltransferase activity and *tet*-regulated antisense RNA-mediated inhibition of CARM1 expression. This provided both a "blunt" and "directed" approach to investigating methyltransferase function in skeletal muscle. The blunt approach involved using the methyltransferase inhibitor adenosine dialdehyde, which inhibits all AdoMet-dependent methyltransferases. Adenosine dialdehyde treatment blocks morphological and biochemical muscle differentiation, which is concordant with (i) the data demonstrating arginine methyltransferase-mediated coactivation of MEF2-dependent transcription/activity in an SRC-2/GRIP-1-dependent manner and (ii) the critical role of MEF2 in contractile protein transcription and differentiation. These data suggest that an accumulation of methyl-accepting substrates in hypomethylated states results in a block in the signal transduction pathways that induce differentiation. Furthermore, it has been demonstrated that the majority of hypomethylated substrates after adenosine dialdehyde treatment are substrates of the arginine-specific methyltransferase PRMT1, which prefers substrates rich in glycine and arginine. These substrates include many proteins involved in RNA binding/processing, signal transduction, and transcription. Whether the effect of adenosine dialdehyde on skeletal myogenesis was due to the specific inhibition of CARM1 or other AdoMet-dependent methyltransferases cannot be discerned from these experiments. This question was addressed by a more targeted approach involving tet-off-inducible CARM1 antisense RNA expression as a "tool" to selectively inhibit CARM1 expression and function. CARM1 antisense expression abrogates the expression/induction of CARM1, MEF2, GRIP-1, and myogenin; these proteins are crucial regulators of skeletal muscle differentiation.

The blunt and targeted approaches to the ablation of CARM1 activity in muscle cells highlighted that inhibition of myogenesis occurred downstream of MyoD expression (RNA and protein). It seems plausible that CARM1 activity may be necessary for the downstream functions of MyoD and the activation of the MyoD-dependent signal cascade. Future studies will examine targets of CARM1 activity in muscle cells and the effects of CARM1 on MyoD-mediated transcription.

CARM1 is expressed in the neural fold, neural tube, and somites in 8.25–8.75-day postcoitus embryos. The spatial and temporal expression pattern of CARM1 shows similarity to that of MEF2 expression during mouse embryogenesis. For example, MEF2A, MEF2C, and MEF2D are detected in somites, and MEF2A is expressed in the brain and neural fold. The similarities in the spatiotemporal expression patterns of MEF2 and CARM1 are consistent with the function of the methyltransferase as a cofactor of MEF2. Expression of CARM1 in somites, which are the precursors to the skeletal muscle lineage, is also concordant with abundant expression of CARM1 mRNA and protein in muscle cells in culture. Furthermore, the early expression of CARM1 in somites during embryogenesis suggests that the secondary cofactor has a functional role in the hierarchical cascade of skeletal muscle differentiation during embryogenesis.

The MEF2 family is encoded by four independent genes, *mef2a*, *mef2b*, *mef2c*, and *mef2d* (28). Skeletal muscle differentiation and the activation of myogenic specific gene expression involve the concerted action of bHLH factors (*e.g.* MyoD and myogenin) and the MADS protein MEF2 (18, 24–27). The recruitment of CARM1 during MEF2-dependent activation of muscle creatine kinase expression and its function as an MEF2 cofactor suggest that arginine methylation of histone (*e.g.* histone H3) and non-histone (*e.g.* MyoD, myogenin, and MEF2) targets potentiates the process of differentiation. Current studies are focused on identifying the specific targets of the methyltransferases in skeletal muscle cells.

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The Coactivator-associated Arginine Methyltransferase Is Necessary for Muscle Differentiation: CARM1 COACTIVATES MYOCYTE ENHANCER FACTOR-2

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