Cooperative Transcriptional Activation by the Neurogenic Basic Helix-Loop-Helix Protein MASH1 and Members of the Myocyte Enhancer Factor-2 (MEF2) Family*

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Establishment of skeletal muscle and neural cell types is controlled by families of myogenic and neurogenic basic helix-loop-helix (bHLH) proteins, respectively. Myogenic bHLH proteins have been shown to activate skeletal muscle transcription in collaboration with members of the myocyte enhancer factor-2 (MEF2) family of MADS (MCM1-agamous-deficiens-serum response factor) proteins, whose products bind as homo- and heterodimers to an A/T-rich DNA consensus sequence associated with numerous muscle-specific genes (18). MEF2 proteins are members of the MEF2 family of transcription factors (10). There are four vertebrate mef2 genes, mef2a, mef2b, mef2c, and mef2d (11–17), whose products bind as homo- and heterodimers to an A/T-rich DNA consensus sequence associated with numerous muscle-specific genes (18). MEF2 proteins are members of the MADS (MCM1-Agamous-Deficiens-serum response factor) family of transcription factors. The MADS domain and the adjacent MEF2 domain encompass the first 86 amino acids, and together these domains mediate DNA binding and dimerization (10). Recent studies have shown that members of the MEF2 family interact directly with heterodimers formed between myogenic bHLH proteins and E proteins and that the DNA binding and dimerization domains of these two different classes of transcription factors mediate this interaction (19, 20). In addition to being expressed in differentiated muscle cells, members of the MEF2 family are expressed in the developing brains of vertebrates in specific patterns that correlate with neuronal differentiation (13, 21, 22). Similarly, the formation of central and peripheral neurons in Drosophila has been shown to be controlled by the achaete-scute family of bHLH factors, which are expressed in neuronal precursors and differentiated neurons (1). Two mammalian achaete-scute homologs (MASH) have been identified (3). One of these factors, MASH1, is expressed in subsets of cells in the peripheral and central nervous systems and is required for the formation of the peripheral nervous system during mouse embryogenesis (4–6). Cell-type-specific bHLH proteins like the myogenic and neurogenic factors form heterodimers with a family of ubiquitous bHLH factors known as E proteins, which includes the products of the E2A gene, E12 and E47, HEB, and the Drosophila daughtercless gene product (7–9). These heterodimers bind the E box consensus sequence, CANNTG, which is found in the control regions of numerous muscle-specific genes, as well as other cell type-specific genes (7).

Recent studies suggest that there are parallels between the mechanisms that regulate differentiation in the myogenic and neurogenic lineages (1). Formation of skeletal muscle is controlled by a family of myogenic basic helix-loop-helix (bHLH)1 proteins, MyoD, myogenin, Myf5, and MRF4, which are expressed specifically in skeletal muscle and can activate the complete program for skeletal muscle differentiation when expressed in several non-muscle cell types (2). Similarly, the formation of central and peripheral neurons in Drosophila has been shown to be controlled by the achaete-scute family of bHLH factors, which are expressed in neuronal precursors and differentiated neurons (1). Two mammalian achaete-scute homologs (MASH) have been identified (3). One of these factors, MASH1, is expressed in subsets of cells in the peripheral and central nervous systems and is required for the formation of the peripheral nervous system during mouse embryogenesis (4–6). Cell-type-specific bHLH proteins like the myogenic and neurogenic factors form heterodimers with a family of ubiquitous bHLH factors known as E proteins, which includes the products of the E2A gene, E12 and E47, HEB, and the Drosophila daughtercless gene product (7–9). These heterodimers bind the E box consensus sequence, CANNTG, which is found in the control regions of numerous muscle-specific genes, as well as other cell type-specific genes (7).

Myogenic bHLH proteins activate muscle-specific transcription in collaboration with members of the myocyte-enhancer factor-2 (MEF2) family of transcription factors (10). There are four vertebrate mef2 genes, mef2a, mef2b, mef2c, and mef2d (11–17), whose products bind as homo- and heterodimers to an A/T-rich DNA consensus sequence associated with numerous muscle-specific genes (18). MEF2 proteins are members of the MADS (MCM1-Agamous-Deficiens-serum response factor) family of transcription factors. The MADS domain and the adjacent MEF2 domain encompass the first 86 amino acids, and together these domains mediate DNA binding and dimerization (10). Recent studies have shown that members of the MEF2 family interact directly with heterodimers formed between myogenic bHLH proteins and E proteins and that the DNA binding and dimerization domains of these two different classes of transcription factors mediate this interaction (19, 20). In addition to being expressed in differentiated muscle cells, members of the MEF2 family are expressed in the developing brains of vertebrates in specific patterns that correlate with neuronal differentiation (13, 21, 22). Similarly, the single mef2 gene in Drosophila, D-mef2, is expressed in developing muscle and in the central and peripheral nervous systems (23).

Like differentiating skeletal muscle cells, differentiating neurons exit the cell cycle irreversibly and up-regulate an array of tissue-specific genes. Given the high levels of MEF2 expression in differentiated neurons and the similar roles of bHLH proteins in specifying myogenic and neurogenic cell fates, we examined whether MEF2 factors might act as cofactors for the neurogenic bHLH protein MASH1 in a manner analogous to their interaction with myogenic bHLH factors. Our results show that MEF2 factors interact directly with MASH1/E12 heterodimers to synergistically activate transcription. These results suggest that MEF2 factors are cofactors.

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1 The abbreviations used are: bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; DBD, DNA binding domain; DMEM, Dulbecco’s modification of minimal essential medium; FCS, fetal calf serum; MADS, MCM1-agamous-deficiens-serum response factor; MASH, mammalian achaete-scute homolog; MCK, muscle creatine kinase; NCAM, neural cell adhesion molecule; RA, retinoic acid; PAGE, polyacrylamide gel electrophoresis; VP16, herpes virus virion protein 16; MEF2, myocyte enhancer factor-2.
that positively modulate the transcriptional activities of bHLH proteins in both myogenic and neurogenic lineages.

MATERIALS AND METHODS

Cell Culture and Western Blots—10T1/2 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). P19 embryonal carcinoma cells were maintained in DMEM supplemented with 7.5% calf serum and 2.5% FCS. P19 cells (5 × 10⁵ cells/plate) were aggregated in 60-mm bacterial Petri dishes for 4 days. For cells treated with retinoic acid (RA), RA was added at a concentration of 1 μM for the first 48 h of aggregation. Following aggregation, the P19 cell aggregates were dispersed and replated in 60-mm tissue culture plates and were maintained for an additional 4 days. All cells were fed with fresh medium every 48 h.

Western blots were performed as described previously (24). Briefly, cells were lysed in cracking buffer, and equivalent quantities of total cellular protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Proteins were detected using an enhanced chemiluminescence kit (Amer sham Corp.). The polyclonal rabbit anti-MEF2A antibody was purchased from Santa Cruz Biotechnology. The polyclonal anti-MEF2D (kindly provided by Ron Pryw es, Columbia University) and anti-MEF2C antibodies have been described (24). Monoclonal anti-NCAM and α-tubulin were purchased from Sigma.

Plasmids, Transfections, and CAT Assays—The MEF2 expression plasmids used in this study are described elsewhere (25) and encode the MEF2 factors in plasmid pCDNAI/amp (Invitrogen, Inc.). The full-length MASH1 cDNA was expressed under control of the Rous sarcoma virus long terminal repeat in plasmid pRSVSV40 and was kindly provided by David Anderson (26). The full-length MASH1 and MASH1 bHLH cDNAs were also expressed as Myc-tag fusions in plasmid pCS2MTY (+) (27). No differences in activation or interaction were observed for full-length MASH1 expressed under control of either promoter. The stability and expression of MASH1 and MASH1 bHLH were confirmed in vitro and in vivo using the Myc-tag vectors by in vitro translation and Western blot, respectively (data not shown). The E12 expression plasmid encodes a partial cDNA containing the E12 bHLH region, but lacking a transactivation domain (7). The E-box-dependent reporter, 4RtkCAT, contains four tandem copies of the right E-box from the muscle creatine kinase (MCK) enhancer and has been described previously (28). MASH1/E12 heterodimers have been reported to activate transcription through the MCK E-box (29). The MEF2-dependent reporter plasmid, pE102MEF2x2CAT, which contains two copies of the MCK enhancer MEF2 site upstream of the embryonic myosin heavy chain promoter (12) and the GAL4-dependent reporter plasmid, pG5E1bCAT, have been described previously (20). Plasmids GAL (DBD)-E12 bHLH and GAL(DBD)-MASH1 bHLH encode the DNA binding domain (DBD) of yeast GAL4 (amino acids 1–147) fused to either the bHLH region of E12 or MASH1, respectively. Plasmids 1–117 and 1–117/VP16 encode amino acids 1–117 of MEF2C either alone or fused to the activation domain of the viral activator, VP16.

10T1/2 cells were transfected by calcium phosphate precipitation, and tri-hybrid assays were performed as described previously (20). In each transfection, 10-cm plates were transfected with 5 μg of each plasmid. In transfections in which not all expression plasmids were transfected, 5 μg of the appropriate parental expression plasmid without a cDNA insert were cotransfected such that all transfections received the same amount of DNA and the same amount of each expression plasmid. Lysates for CAT assays were prepared and normalized using standard techniques as described previously (25). Conversion to acetylated forms was analyzed by thin-layer chromatography and quantified by PhosphorImager analysis (Molecular Dynamics, Inc.).

RESULTS

MEF2 Factors Are Up-regulated during Neural Differentiation.—Previous studies of MEF2 gene expression in the developing mouse showed MEF2 transcripts present in the embryonic brain during the time of neural differentiation and development (13, 21, 22). The pattern of MEF2 expression in the developing central nervous system of the mouse overlaps with the expression of MASH1 during embryogenesis (4). Based on these studies, we were interested in whether MEF2 factors were preferentially up-regulated in differentiation of neuronal cells in culture. P19 embryonal carcinoma cells when aggregated in the presence of RA differentiate to form neurons with a few cells resembling other neuronal cell types such as astrocytes and glial cells (30–32). Aggregation of P19 cells in the absence of RA results in differentiation to endodermal or mesodermal fates (30, 31, 33). We were particularly interested in this cell line, since upon aggregation of P19 cells in the presence of RA, MASH1 expression is dramatically up-regulated, and its expression persists until the after the cells are fully differentiated (26).

Fig. 1 shows the results of Western blot analyses performed on extracts isolated from P19 cells that had been differentiated by aggregation in the presence or absence of RA or were undifferentiated. The P19 cell extracts were compared with extracts isolated from C2C12 myotubes and from neonatal mouse brain for the expression of MEF2 proteins and the neural marker, NCAM. Analysis of NCAM expression showed a dramatic up-regulation in P19 cells differentiated in the presence of RA (Fig. 1), confirming the neural phenotype that nearly every cell in the population visually displayed following treatment with RA (data not shown).

Analysis of MEF2 protein expression in P19 cells (Fig. 1) showed that MEF2A was slightly up-regulated in RA-treated cells when compared with untreated cells. MEF2C was dramatically up-regulated upon neuronal differentiation of P19 cells. Blots probed with α-MEF2C antisera displayed almost no detectable MEF2C in undifferentiated or endodermally differentiated P19, whereas MEF2C was present in aggregated RA-treated P19 at levels nearly as high as in fully differentiated myotubes. In contrast, MEF2D was down-regulated upon neural differentiation. This result is consistent with the observation that MEF2D is involved in serum-mediated cell prolifera-
MEF2 Factors Interact with MASH1/E12 Heterodimers—To determine whether MEF2 proteins interact directly with MASH1, we used a tri-hybrid assay in which a CAT reporter gene under control of the GAL4 DNA binding site was transfected into 10T1/2 fibroblasts with expression vectors encoding the bHLH region of E12 fused to the DNA binding domain of GAL4 along with MASH1, MEF2A, MEF2D, or MEF2C (Fig. 2B). A schematic representative of this assay is shown in Fig. 2A. The GAL4-E12 fusion protein, GAL(DBD)-E12 bHLH (GAL4-E12), failed to activate reporter gene expression alone (lane 2) or in the presence of only the MASH1 bHLH (lane 3). Likewise, GAL4-E12 bHLH plus MEF2C alone did not activate the CAT reporter (lane 7). However, when GAL4-E12 was expressed with the MASH1 bHLH and MEF2A, MEF2D, or MEF2C, activation of the reporter occurred (lanes 4–6). This activation ranged from 4- to 8-fold over the level of activation in the presence of GAL4-E12 alone. None of the MEF2 factors could activate transcription of the reporter alone or with GAL4-E12 alone. Only when the MASH1 bHLH, E12 bHLH, and MEF2 factors were all present together did activation occur.

To further analyze the interaction between MEF2 and MASH1/E12 heterodimers, we used a chimeric protein containing amino acids 1–117 of MEF2C, which includes the MADS and MEF2 domains, fused to the strong viral transactivator protein, VP16. We examined the ability of this chimeric protein (1–117/VP16) to interact with heterodimers formed between full-length MASH1 or the bHLH region of MASH1 and the E12 bHLH. As shown in Fig. 2C, 1–117/VP16 interacted with the E12/MASH1 bHLH heterodimer (lane 7), and strong transcriptional activity of the CAT reporter occurred as a result of the potent activation domain of VP16 fused to MEF2. The strong activation provided by the VP16 transactivation domain allows examination of even relatively weak interactions. In spite of this, no interaction was observed between E12 homodimers and 1–117/VP16 (lane 5) or with GAL4-E12 plus 1–117/VP16 alone (lane 3), indicating that MEF2C can only interact with the heterodimer formed between MASH1 and E12 and not with E12 alone. These results also show that the bHLH region of MASH1 is all that is required for heterodimerization with E12 and subsequent interaction with MEF2. However, interaction between full-length MASH1 and MEF2C also occurred. When GAL4-E12 was expressed together with full-length MASH1, we observed a high level of reporter gene expression, since heterodimer formation brings the strong transcriptional activation domain of MASH1 in proximity to the minimal promoter (lane 8). However, strong synergism still occurred when GAL4-E12 was coexpressed with full-length MASH1 plus 1–117/VP16 (lane 9). These results demonstrate that MASH1/E12 heterodimers interact with the first 117 amino acids of MEF2, which contain the DNA binding and dimerization motifs present in the MADS and MEF2 domains.

Next, we wanted to more closely examine the regions within MEF2 required for interaction with MASH1/E12. To do this, we used a tri-hybrid system in which the bHLH region of MASH1 was fused to the DNA binding domain of GAL4 in plasmid GAL (DBD)-MASH1 bHLH (GAL4-MASH1). A schematic representation is shown in Fig. 3A. In addition, plasmids encoding a truncated form of E12 containing its bHLH but lacking a strong activation domain and full-length MEF2C or various mutants of MEF2C were cotransfected. A schematic of the MEF2C mutants analyzed is shown in Fig. 3B. Analysis of the MEF2C deletion mutants showed that regions in both the MADS and MEF2 domains were important for interaction with MASH1/E12 heterodimers (Fig. 3C, lanes 5–7). In particular, deletion of residues 40–57 resulted in a complete loss of interaction with MASH1/E12 (lane 5). Since this region of MEF2 has been shown to be critical for homodimerization of MEF2C (35), we were interested in whether homodimerization was required for MEF2 to interact with MASH1/E12. Mutation of residues L145, 46, which are critical for MEF2 homodimerization (35), had
little or no affect on interaction with MASH1/E12 (lane 9). In fact, none of the mutations which changed only a few amino acid residues had a dramatic affect on the ability of MEF2 to interact with MASH1/E12. Therefore, we favor the idea that it is not a few residues within the MADS and MEF2 domains that are responsible for the interaction but rather that larger areas within those domains form a binding surface that mediates interaction with MASH1/E12.

**MEF2 and MASH1 Can Activate Transcription through Each Other’s Binding Sites**—We also investigated whether MEF2 and MASH1 could interact to activate transcription through the DNA binding site of the other factor (Fig. 4). The heterodimer formed between the bHLH regions of MASH1 and E12 only weakly activated transcription of a CAT reporter linked to tandem copies of the right E-box from the MCK enhancer, because these regions of the proteins lack transcriptional activity (Fig. 4A). The weak activation which occurred was likely due to dimerization of the MASH1 bHLH with endogenous E12, which has a strong transactivation domain. Similarly, full-length MEF2 was unable to activate this reporter gene because the bHLH regions of MASH1 and E12 were expressed together with full-length MEF2, the reporter gene was expressed at high levels (Fig. 4A, lane 5). We interpret these results to indicate that the bHLH heterodimer binds the E-box in the reporter and serves as a platform to recruit MEF2 with its transcriptional activation domain, resulting in CAT expression.

Likewise, the DNA binding region of MEF2C, encoded by amino acids 1–117, was unable to activate a CAT reporter gene linked to two copies of the MEF2 site (Fig. 3B, lane 2), because amino acids 1–117 of MEF2C do not encode an activation domain (35). This reporter also failed to respond to full-length MASH1 containing its strong activation domain in the presence of E12, because the reporter lacks a binding site for bHLH proteins (lane 4). However, when MEF2/1–117 was expressed with full-length MASH1 and E12 bHLH, the reporter gene was expressed at high levels (lane 5). In this case, the DNA binding domain of MEF2 appears to recruit the MASH1/E12 bHLH heterodimer to the DNA, and the MASH1 activation domain activates transcription of the reporter gene.

**DISCUSSION**

The results of this study show that members of the MEF2 family interact with heterodimers formed between the neurogenic bHLH protein, MASH1 and E12. The interaction between MEF2 and MASH1/E12 allows either type of factor to activate transcription through the binding site of the other. Considered together with previous studies (19, 20), these results suggest that interactions between MEF2 factors and cell type-specific
bHLH proteins are important for activation of tissue-specific transcription in both the myogenic and neurogenic cell lineages.

In contrast to the myogenic bHLH factors, which can induce muscle differentiation in transfected non-muscle cells, forced expression of MASH1 does not activate neuronal differentiation (26). We have transfected 10T1/2 cells with MASH1 and MEF2 expression vectors and also have not observed activation of neuronal genes. Likewise, we did not observe conversion to neuronal cell fates in P19 cells which were transfected with MASH1 plus MEF2, but were otherwise untreated with RA. Thus, while MEF2 appears to serve as a cofactor for MASH1, these two proteins alone are insufficient to initiate neurogenesis under the conditions of transfection assays. Whether these two factors require additional coregulators remains to be determined.

While this manuscript was in preparation, Mao and Nadal-Ginard reported that MEF2A could cooperate with MASH1 to activate transcription (36). Our results are in general agreement with theirs, but they differ in two respects. In our experiments, MASH1 and MEF2 were able to activate transcription through each other’s binding sites when only one of the factors was bound to DNA. In contrast, they reported that both factors needed to bind DNA to cooperatively activate transcription. They also used bacterially expressed proteins in vitro and found that MASH1 and MEF2 could interact directly without a need for E12. In our in vivo tri-hybrid assays, we found that MASH1 required E12 to efficiently interact with MEF2 proteins. In addition, while we have primarily focused on MEF2C, our results extend the analysis of the interactions to include MEF2A, MEF2B, and MEF2D.

The high levels of MEF2 expression in neuronal cell lines and in differentiated neurons in vivo suggest that MEF2 is a regulator of neuron-specific gene expression. We have analyzed the control regions of numerous neuronal genes that have been characterized, and we have identified MEF2 consensus sites within several neuronal genes from mammals and Drosophila. In the case of the neuronally restricted Drosophila T5 scute gene, a perfect consensus MEF2 site overlaps the TATA box in the promoter (37). In addition, there are three functional E-boxes in close proximity to the MEF2 site (37–39). The arrangement of the MEF2 site and the E-boxes in the scute gene is strikingly similar to the arrangement of those sites in several muscle-specific promoters where MEF2 and bHLH factors have been shown to collaborate with each other and to activate transcription through each other’s binding sites (25, 40–42). These observations and results of this study, taken together with previous studies of myogenesis (19, 20), suggest that members of the MEF2 family of transcription factors serve as general potentiators of cell type-specific transcription in both myogenic and neurogenic lineages by collaborating with tissue-specific bHLH factors.

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