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Interaction of myocyte enhancer factor 2 (MEF2) with a mitogen-activated protein kinase, ERK5/BMK1

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ABSTRACT

Myocyte enhancer factor 2 (MEF2) has been implicated in the complex hierarchical regulation of muscle-specific gene expression and differentiation. While the MyoD family members are able to initiate the skeletal muscle differentiation program, whether MEF2 is sufficient in directing skeletal muscle differentiation is still controversial. Furthermore, how MEF2 transactivates its target genes is not fully understood. It has been suggested that the interactions of MEF2 with other factors modify its transcriptional activity. Therefore, the identification of MEF2-interacting factors may be important in understanding the mechanism by which MEF2 activates its target genes. In this study, a mitogen-activated protein kinase (MAP kinase), ERK5/BMK1 was found to interact with MEF2 in a yeast two hybrid screen. The interaction was confirmed by a glutathione *S*-transferase-pull down assay and a co-immunoprecipitation study indicating that endogenous ERK5 and MEF2 interact with each other *in vivo*. The interacting domain of MEF2 was mapped to the N-terminus which contains the highly conserved MADS and MEF2 domains. Functionally, ERK5/BMK1 was able to phosphorylate MEF2 *in vitro*. Furthermore, when cotransfected with ERK5/BMK1, the transactivation capacity of MEF2 was enhanced. These results suggest that the functions of MEF2 could be regulated through ERK5/BMK1.

INTRODUCTION

Molecular mechanisms regulating skeletal muscle development have been extensively studied (reviewed in 1). Proteins of the basic helix–loop–helix (bHLH) myogenic regulators (MyoD, MRF4, Myf5 and myogenin) have been demonstrated as key regulators of myogenesis with the ability to activate the skeletal muscle differentiation program (reviewed in 1). Conversely, relatively little is known about smooth and cardiac muscle development. The presence of myocyte enhancer factor 2 (MEF2) sites in the regulatory regions of many muscle-specific genes that are expressed in skeletal, cardiac and smooth muscle cells, suggests that it may be a common component of the differentiation programs in all three muscle lineages (reviewed in 2). In fact, by generating a loss-of-function of the *D-MEF2* gene in *Drosophila* embryos, undetectable levels of D-MEF2 protein and mRNA correlated with the failure of differentiation of visceral, somatic and cardiac muscle (3,4). Additionally, microinjection of a dominant-negative form of MEF2A inhibited the myogenic conversion of C3H10T1/2 fibroblasts by MyoD and also the differentiation of C2C12 myoblasts (5). Moreover, in mice homozygous for a null mutation of MEF2C, cardiac morphogenesis was inhibited (6).

MEF2 was originally characterized as having a DNA-binding activity recognizing an A/T-rich element in the promoters of many skeletal muscle-specific genes (7). There are four MEF2 genes, referred to as *MEF2A*, *-B*, *-C* and *-D*, identified in vertebrate species while there is a single gene, *D-MEF2*, found in *Drosophila* (reviewed in 2,8; 9,10). MEF2 factors belong to a superfamily of transcription factors that have a highly conserved region termed the MADS domain, named after the yeast *MCM1*, plant homeotic genes *agamous* and *deficiens* and human serum response factor (SRF). The MADS domain is located at the N-terminus (the first 56 amino acids) of MEF2. Immediately following the MADS domain, there is a MEF2 domain (29 amino acids) which is unique to MEF2 factors. One feature of many MADS domain proteins is their propensity to make homo- and heterotypic protein–protein interactions which modulate their activity. For example, MCM1 acts in concert with the α1 repressor or α2 activator to repress or activate cell-type specific genes in α-type yeast, respectively. Also, modulation of SRF by ternary complex factors contributes to the transcriptional activation of the c-*fos* gene through the serum response element (reviewed in 8 and therein). Both MADS and MEF2 domains of MEF2C have been shown to be responsible for DNA binding and

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protein–protein interactions (11). The transactivation domain of these proteins has been mapped to the C-terminus of MEF2A, MEF2C and MEF2D (11–13).

While the MyoD family is a key regulator of skeletal myogenesis, whether MEF2 itself is sufficient in directing skeletal muscle differentiation is still controversial (2,14). How MEF2 transactivates its target genes is not clear. There are suggestions that the transcriptional activity of MEF2 is modified by the recruitment of other factors. For example, MEF2 has been shown to bind to the bHLH region of myogenin and cooperatively activate expression of muscle-specific genes (15). Furthermore, direct association of p300/CBP [CREB (cAMP-responsive element binding protein) binding protein] and the MADS domain of MEF2C was shown to potentiate transcriptional activity of MEF2C (16). Recently, MEF2C was shown to interact with p38 (a member of the MAP kinase family) and the phosphorylation of MEF2C by p38 enhanced its transcription activity (17). Since all of these examples require protein–protein interactions to regulate MEF2 activity, the identification of other MEF2-interacting factors may be important in fully understanding the mechanism by which MEF2 activates its target genes.

The yeast two hybrid system has proven to be a powerful tool in identifying protein–protein interactions and subsequently to isolate cDNAs encoding proteins that interact with a protein of interest (reviewed in 18). In this study, we screened a smooth muscle cDNA library using the yeast two hybrid system and identified several MEF2-interacting proteins. One of them carries a partial cDNA encoding a rat homologue of human ERK5 (or BMK1) which was cloned independently by two groups and was shown to belong to the MAP kinase family by sequence analysis (19,20). MEF2 proteins were found to co-immunoprecipitate with ERK5 *in vivo*. The MADS and MEF2 domains of MEF2 were sufficient to mediate the interaction. We found that ERK5/BMK1 was capable of phosphorylating MEF2D and MEF2C *in vitro*. Functionally, the transcriptional activity of MEF2C and MEF2A was enhanced when co-expressed with ERK5/BMK1 while the transcriptional activity of MEF2D was not affected.

MATERIALS AND METHODS

Accession nos

DDBJ/EMBL/GenBank accession nos are AJ005424 for ERK5/BMK1 and AJ005425 for MEF2D.

Cloning of MEF2D

Total RNAs were extracted from the hearts of neonatal Wistar rats using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. First strand cDNAs were synthesized using reverse transcriptase (Superscript II, Gibco-BRL) and oligo(dT) as primers. *MEF2D*-specific synthetic oligonucleotides (5′-CTCT-CTGGCACTAAGGACCC-3′ and 5′-ACCATCACTTTAATG-TCCAAGTATC-3′) were designed according to the published nucleotide sequence of mouse *MEF2D* and used to amplify *MEF2D* cDNA by the polymerase chain reaction (PCR). The PCR products were cloned into pBluescript-SK(+) (Stratagene) and both the sense and anti-sense strands were sequenced by the dideoxy chain termination method (accession no. AJ005425).

Construction of a rat aorta cDNA library and yeast two hybrid screening

A neonatal rat aorta cDNA library was constructed using λ-ACT vectors as described previously (21,22). The yeast two hybrid system including yeast strain Y190 and Y187, pAS1-CYH, pSE1111 (SNF1) and λ-ACT vectors was a generous gift of S. Elledge (Department of Biochemistry and Human and Molecular Genetics, Baylor College of Medicine, TX). Library screening was described in detail in the report by Durfee *et al.* (21). Briefly, pAS1-CYH vector harboring a fragment of rat *MEF2D* cDNA encoding amino acids 19–167 (pAS1-CYH-MEF2D) was used to transform Y190. pAS1-CYH-MEF2D-bearing Y190 yeast were then transformed with the constructed library and plated on selection medium without tryptophan, leucine and histidine and with 25 mM 3-amino 1,2,4-triazole (3-AT) (Sigma). Histidine-positive (His^+) colonies were then assayed for β-galactosidase (Lac-Z) activity. To determine the specificity of interactions, colonies positive for both reporters, His^+ and Lac-Z^+, were plated on a 20 mM cycloheximide (Sigma)-containing plate to select against pAS1-CYH-bearing yeast and obtain cDNA-containing Y190 yeast. cDNA-containing Y190 yeast were then mated with Y187-containing partial p53, lamin C (Clontech), SNF1 or MEF2D fused with the GAL4-DNA binding domain. The resulting diploids were plated on selection medium without tryptophan, leucine and histidine and with 25 mM 3-AT and the His and Lac-Z activity were determined. cDNAs encoding proteins which interacted with MEF2D but not with the p53, lamin C and SNF1 were defined as capable of interacting with MEF2D specifically.

Glutathione *S*-transferase (GST)-pull down assay

Partial cDNAs encoding amino acids 1–86 of MEF2D, 87–507 of MEF2D, 1–86 of MEF2C and 87–465 of MEF2C, and full length cDNA of MEF2D and MEF2C were cloned into pGex-2T vector (Pharmacia) and transformed into *Escherichia coli* strain, BL21(DE3)-pLys-S (Novagen) to produce GST fusion proteins as instructed (Pharmacia). ERK5 (2 μg) in pBluescriptSK(–) (kindly provided by J. E. Dixon; 20) was linearized with *Sal*I and *in vitro* translated and labeled with [^{35}S]methionine in a 50 μl reaction according to the manufacturer's instructions (Promega). Ten microliters of *in vitro* translated ERK5 products were incubated with 90 μl binding buffer (50 mM Tris–HCl pH 7.6/150 mM NaCl/5 mM EDTA/0.1% NP-40/protease inhibitors aprotinin, leupeptin, pepstatin A, 10 μg/ml each) and 2–3 μg of GST or GST–MEF2 fusion proteins immobilized on agarose beads. The mixtures were shaken for 1 h at room temperature. The beads were then washed three times with binding buffer and six times with washing buffer (50 mM Tris–HCl pH 7.6/450 mM NaCl/5 mM EDTA/1% NP-40). Proteins retained on beads were boiled in 10 μl of SDS sample buffer (New England Biolabs) and subjected to 7.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The presence of labeled ERK5 proteins was detected by autoradiography.

Co-immunoprecipitation between MEF2 and ERK5/BMK1

Immunoprecipitation and immunoblotting for endogenous MEF2 and ERK5 were performed in primary culture of rat myotubes. Hind leg muscles of 2-day-old rats were aseptically collected, minced and digested overnight with 50 mg/ml trypsin

(Worthington Biochemical Corp.). The muscles were dispersed by pipetting. Collagenase (Worthington Biochemical Corp.) was pre-warmed at 37°C and added to a final concentration of 0.1%. After 30 min incubation at 37°C, cells were collected by centrifugation, washed once and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Cells were preplated for 1 h to decrease fibroblast content, remaining myoblasts were seeded onto gelatin-coated 100 mm dishes. Confluent monolayers of myoblasts were induced to form myotubes by switching medium to DMEM with 5% horse serum for 3 days. Prominent contracting myotubes were collected for immunoprecipitation. Goat anti-ERK5 (20 µl; SC-1284, Santa Cruz, CA), or 2 µl of rabbit anti-MEF2A or anti-MEF2D antiserum (kindly provided by R. Prywes, Columbia University, New York) were added to the lysate prepared from a 100 mm plate. The mixtures were shaken gently at 4°C for 1 h. Thirty microliters of 20% protein A-conjugated agarose beads (for MEF2) (Pharmacia) or 20 µl of protein G-plus agarose beads (Santa Cruz) were used to collect the immune complexes which were then washed and boiled in 40 µl SDS buffer. Anti-MEF2 (1 µl) and anti-ERK5 (20 µl) precipitate and 40 µg of myotubes lysate were analysed on 10% SDS–PAGE. Proteins were then transferred to nitrocellulose and probed with anti-ERK5, anti-MEF2A or anti-MEF2D antiserum.

In vitro kinase assay

ERK5 was cloned into the pREP10 vector (Invitrogen) and tagged with a hemagglutinin (HA) epitope, YPYDVPDYA. The resulting construct, pREP10-HA-ERK5, was used to transfect COS-7 cells cultured in α-MEM with 10% FCS in 60 mm plates. Forty-eight hours after transfection, cells were treated with or without 200 mM H_2O_2 for 5 min and lysed with 0.5 ml of TEN buffer (40 mM Tris–HCl, pH 7.5/10 mM EDTA/150 mM NaCl) plus 0.1% Triton X-100. Monoclonal anti-HA antibodies (4 µg) (BABCO, CA) were added into the cell extract. After 1 h of shaking at 4°C, the immune complexes were precipitated with 20 µl protein G-plus agarose beads (Santa Cruz) and washed with phosphate-buffered saline (PBS)/1% Triton X-100 six times. MEF2D and MEF2C (23) cDNAs were subcloned into the pET15b vector (Novagen) with a 6× histidine (6×His) tag fused at the N-terminus, expressed in *E.coli* strain BL21(DE3)-pLysS, and purified with nickel-conjugated beads according to the manufacturer's instructions (Novagen). Purified 6×His-MEF2D (2 µg) or 6×His-MEF2C (1 µg) were added into a reaction mixture containing immunoprecipitated ERK5, kinase assay buffer (10 mM $MgCl_2$/150 mM NaCl/10 mM Tris–HCl, pH 7.4/0.1% DTT/protease inhibitors aprotinin, leupeptin, pepstatin A, 10 µg/ml each), and 2 µCi of [γ-^{32}P]ATP. The mixture was shaken at 30°C for 20 min. Phosphorylation of MEF2 proteins was analyzed by SDS–PAGE and autoradiography.

Cell culture and reporter assays

HeLa cells were cultured in DMEM supplemented with 10% FCS. For the reporter assay, 1 µg of an appropriate chloramphenicol acetyltransferase (CAT) reporter and 0.5 µg of pCMV-luciferase, which served as an internal control for transfection efficiency, were transfected into HeLa cells (35 mm plates) at 60% confluence by calcium phosphate coprecipitation. An additional 0.5 µg of pREP10-HA-MEF2D, pMT2-MEF2A or pMT2-MEF2C (23), and/or 1 µg of pREP10-HA-ERK5 were co-transfected. Empty vector pREP10-HA was used to keep the total amount of DNA constant for each transfection. There were three CAT reporter constructs used in this study: the embryonic myosin heavy chain (MHC) promoter fused with CAT (PE102CAT), two copies of the muscle creatine kinase MEF2-binding sites inserted in a concatemerized orientation at the –102 position of MHC promoter (PE102CAT-2×MEF2), or two copies of mutated MEF2-binding sites inserted at the –102 position of MHC promoter (PE102CAT-2×MEF2mt) (23). Twenty-four hours after transfection, cells were serum-starved for 24 h and then treated with 10% FCS for 4 h before harvest. An aliquot of 0.5 ml of TEN buffer (40 mM Tris–HCl pH 7.5/10 mM EDTA/150 mM NaCl) was used to harvest the cells. The CAT assay was performed as described (24) with minor modifications. After five rounds of freeze–thaw, 100 µl of centrifuged cell extract was added to 150 µl of reagent mixture (final concentration: 1 mM chloramphenicol/80 mM Tris–HCl, pH 7.5) plus 1 µCi of ^{3}H-labeled acetyl-coenzyme A (NEN). After 30 min of incubation at 37°C, 5 ml of organic scintillation fluid was carefully overlaid and samples were incubated for 1 h before being quantitated by liquid scintillation counting. Transfection efficiency was measured by luciferase assay (25). The CAT activities were normalized by transfection efficiency. The relative CAT activities were calculated based on the activity of sample transfected with PE102CAT-2×MEF2 (as 1-fold).

RESULTS

ERK5/BMK1 interacts with the N-terminus of MEF2D in a yeast two hybrid screen

A neonatal rat aorta cDNA library was constructed using λ-ACT vectors. The phage library, which has 2.6 million independent recombinants was converted into a plasmid library for yeast two hybrid screening (21,22). Each cDNA was fused to the GAL4-activation domain. Three constructs containing the N-terminal region of rat MEF2D were prepared for library screening. As shown in Table 1, the construct covering the complete MADS and MEF2 domain (Fig. 1a) of MEF2D (amino acids 1–175) autoactivated the Lac-Z reporter in yeast strain Y190. While amino acids 10–105 resulting from further deletion at both the N- and C-terminus still activated the reporter, amino acids 19–167 (named as pAS1-CYH-MEF2D) abolished the autoactivation activity. Moreover, the bHLH region of myogenin, which is known to interact with the MADS domain (15), can still mildly interact with amino acids 19–167 of MEF2D (data not shown). This result suggested that pAS1-CYH-MEF2D may retain at least part of the protein–protein interaction ability. pAS1-CYH-MEF2D was then used to screen the library. 1.7 million recombinants were screened and >50 cDNAs were found to encode proteins interacting specifically with the N-terminus of MEF2D (Table 1). One of the clones carries a partial cDNA (accession no. AJ005424) encoding a rat homologue of human ERK5/BMK1, which was cloned independently by two groups (19,20). It has been shown by sequence analysis that ERK5/BMK1 belongs to the MAP kinase family (19,20). A full length ERK5/BMK1 cDNA was kindly provided by J. E. Dixon and was used for the following studies.

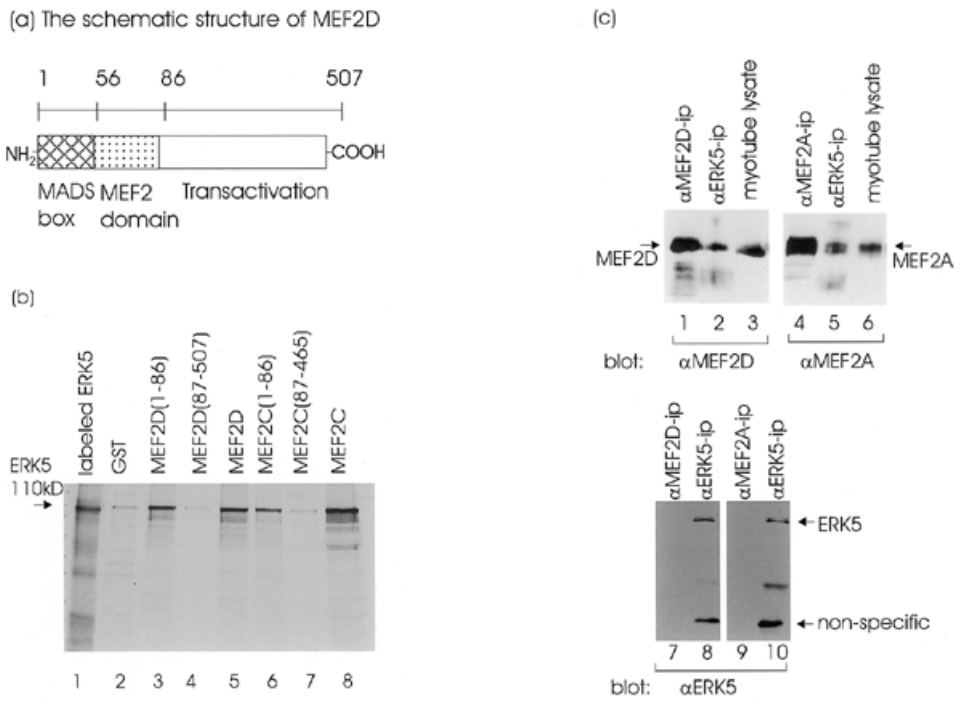

Figure 1. ERK5 interacts with MEF2 *in vitro* and *in vivo*. (**a**) The schematic structure of MEF2D. (**b**) ERK5 associates with MEF2 through the N-terminal region which contains the MADS and MEF2 domains. Ten microliters of ^{35}S-labeled ERK5 (lane 1) were mixed with 2–3 µg of GST (lane 2) or MEF2–GST (lanes 3–8) fusion proteins immobilized on GSH-agarose beads. After intensive washing, retained proteins were analyzed by SDS–PAGE and autoradiography. The arrow indicates the position of 110 kDa ERK5. (**c**) MEF2 co-immunoprecipitates with ERK5. Anti-MEF2D (αMEF2D-ip), anti-MEF2A (αMEF2A-ip) and anti-ERK5 (αERK5-ip) immunoprecipitates obtained from the lysate of rat myotubes were subjected to 10% SDS–PAGE analysis, transferred to nitrocellulose membrane and blotted with anti-MEF2D (blot: αMEF2D, lanes 1–3), anti-MEF2A (blot: αMEF2A, lanes 4–6) and anti-ERK5 (blot: αERK5, lanes 7–10) antiserum. The arrows indicate the positions of MEF2D, MEF2A and ERK5.

Table 1. Results of the yeast two hybrid screening using partial MEF2D

pAS1-CYH construct containing MEF2D	Autoactivation of Lac-Z reporter	Recombinants screened	Number of His$^+$ and Lac-Z$^+$	Interaction specific to MEF2D
Amino acids 1–175	yes	N.A.	N.A.	N.A.
Amino acids 10–105	yes	N.A.	N.A.	N.A.
Amino acids 19–167	no	1.7 million	247	>50

The top two constructs (amino acids 1–175 and 10–105) activated the Lac-Z reporter in Y-190 and therefore were not used in the library screening. N.A., not assayed.

The N-terminus of MEF2 mediates the interaction between MEF2 and ERK5 *in vitro*

Both MADS and MEF2 domains of MEF2C have been shown to be responsible for DNA binding and protein–protein interactions (11). A GST-pull down assay was performed to verify that the N-terminus of MEF2 is sufficient to mediate the interaction and to determine whether full length MEF2 interacts with ERK5/BMK1. *In vitro* translated and ^{35}S-labeled ERK5/BMK1 products (Fig. 1b, lane 1) were mixed with immobilized GST or GST–MEF2 fusion proteins. After extensive washing, ERK5/BMK1 proteins were retained by GST–MEF2D (amino acids 1–86), GST–MEF2D (full length), GST–MEF2C (amino acids 1–86) and GST–MEF2C (full length) fusion proteins (Fig. 1b, lanes 3, 5, 6 and 8, respectively), but not by GST, GST–MEF2D (amino acids 87–507) and GST–MEF2C (amino acids 87–465) (Fig. 1b, lanes 2, 4 and 7, respectively). This result showed that the N-terminus of MEF2 (amino acids 1–86) was sufficient for the interaction and full length MEF2 was able to interact with ERK5/BMK1 *in vitro*.

MEF2 co-immunoprecipitates with ERK5 *in vivo*

To investigate whether ERK5/BMK1 does interact with MEF2 *in vivo*, anti-ERK5, anti-MEF2D and anti-MEF2A antiserum were used to immunoprecipitate ERK5, MEF2D and MEF2A and their associated proteins from the lysate of primary rat myotube cultures. The precipitates were analyzed by SDS–PAGE, then

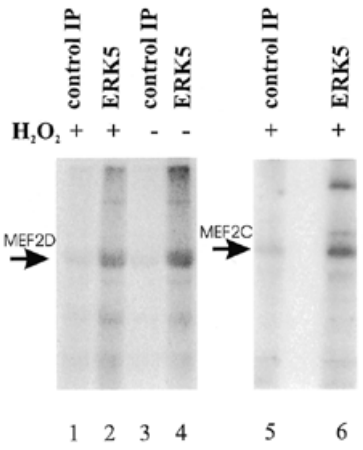

Figure 2. Recombinant MEF2D and MEF2C are phosphorylated by ERK5 *in vitro*. Recombinant MEF2D proteins were incubated with anti-HA immunoprecipitate (IP) from COS without transfection and with H_2O_2 treatment (lane 1); anti-HA IP from COS with ERK5 (HA-tagged) transfection and with H_2O_2 treated (lane 2); anti-HA IP from COS without transfection (lane 3); anti-HA IP from COS with ERK5 transfection (lane 4). Recombinant MEF2C proteins were incubated with anti-HA IP from COS without transfection and with H_2O_2 treatment (lane 5); anti-HA IP from COS with ERK5 transfection and with H_2O_2 treated (lane 6). Arrows indicate the positions of MEF2D and MEF2C.

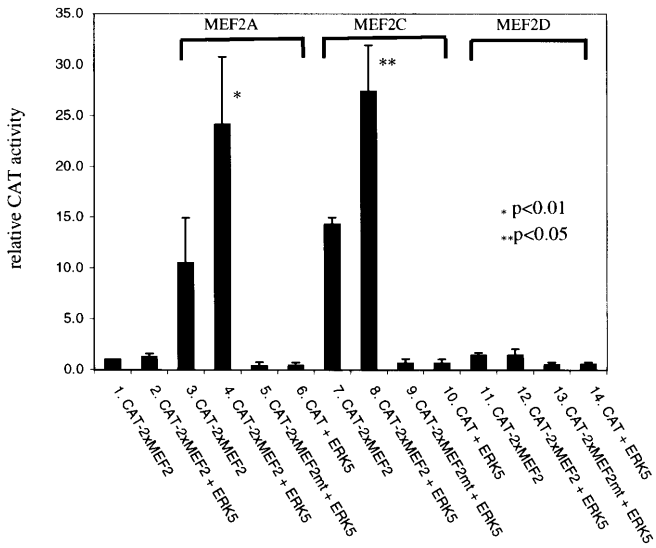

Figure 3. ERK5/BMK1 enhances the transcriptional activity of MEF2A and MEF2C. HeLa cells were transfected with CAT reporters (PE102CAT, PE102CAT-2×MEF2 or PE102CAT-2×MEF2mt), MEF2 and/or ERK5 as indicated. Twenty-four hours after transfection, cells were serum-starved for 24 h and then treated with 10% FCS for 4 h before harvest. CAT activity from cells transfected with PE102CAT-2×MEF2 was defined as 1-fold (lane 1). Results were shown as mean ± standard deviation obtained from three independent experiments.

transferred to nitrocellulose and blotted with anti-ERK5, anti-MEF2D or anti-MEF2A antiserum. The MEF2D, MEF2A and ERK5 proteins are expressed in myotubes since they were detected in the immunoprecipitate prepared from myotube lysate using specific antiserum (Fig. 1c: lanes 1, 4, 8 and 10, respectively). Also, MEF2D and MEF2A were detectable in the myotube lysate (lanes 3 and 6, respectively). Both MEF2A and MEF2D were detected in the anti-ERK5 precipitate (lanes 2 and 5, respectively). However, ERK5 was not detectable in the anti-MEF2A or anti-MEF2D precipitate (lanes 7 and 9). These results indicate that endogenous ERK5 proteins associate with endogenous MEF2D and MEF2A proteins and suggest a functional role for the interaction.

ERK5/BMK1 phosphorylates MEF2D and MEF2C *in vitro*

Phosphorylation of MEF2C has been shown to enhance its DNA-binding and its transactivation activity (17,26). To explore the functional aspects of the MEF2D–ERK5/BMK1 interaction, we next determined whether ERK5/BMK1 is able to phosphorylate MEF2D *in vitro*. Since the MADS and MEF2 domains are homologous among the four MEF2 proteins in vertebrate species and are believed to mediate protein–protein interactions, we also tested whether MEF2C can be phosphorylated by ERK5/BMK1. HA-tagged ERK5/BMK1 proteins were immunoprecipitated from transfected COS cells treated with or without H_2O_2 which has been shown to activate ERK5/BMK1 (27). 6×His-tagged MEF2D and MEF2C recombinant proteins were incubated with anti-HA immunoprecipitate from COS cells in the presence of [γ-^{32}P]ATP. As shown in Figure 2, anti-HA immunoprecipitate prepared from COS cells transfected with pREP10-HA-ERK5 did phosphorylate MEF2D and MEF2C (Fig. 2, lanes 2 and 6, respectively), while anti-HA immunoprecipitate from untransfected cells did not (Fig. 2, lanes 1 and 5). Note that ERK5/BMK1 immunoprecipitate prepared from COS cells in the presence of serum and without treatment of H_2O_2 can still phosphorylate MEF2D (Fig. 2, lane 4). This result suggests that ERK5/BMK1 could also be activated by serum.

The transcriptional activity of MEF2A and MEF2C, but not MEF2D is enhanced by ERK5/BMK1

Phosphorylation of MEF2C by a MAP kinase, p38, was shown to enhance the transcriptional activity of MEF2C (17). Whether transcriptional activities of MEF2A, MEF2C and MEF2D increase in response to activation by ERK5/BMK1 was determined by a co-transfection assay. MEF2, ERK5/BMK1 and PE102CAT, PE102CAT-2×MEF2, or PE102CAT-2×MEF2mt were transfected into HeLa cells. Since the above *in vitro* phosphorylation result suggested that serum activates ERK5/BMK1, cells were incubated with serum to activate ERK5/BMK1. HeLa cells were transfected as indicated in Figure 3. We found that overexpression of MEF2A and MEF2C can activate the PE102CAT-2×MEF2 reporter 10–14-fold (Fig. 3, lanes 3 and 7) over the control, which has only the PE102CAT-2×MEF2 reporter transfected (lane 1), whereas MEF2D did not activate the reporter (lane 11). Transfection of ERK5 alone did not activate the PE102CAT-2×MEF2 (lane 2). However, co-transfection of ERK5 further enhanced the activity of MEF2A and MEF2C by ~2-fold (lanes 4 and 8) but had no effect on MEF2D (lane 12). The induction of the PE102CAT-2×MEF2 reporter was dependent on the activity of MEF2A and MEF2C since there was no induction of PE102CAT and PE102CAT-2×MEF2-mt reporter (lanes 5, 6, 9 and 10).

DISCUSSION

Skeletal muscle differentiation is regulated by the bHLH myogenic regulators, MyoD, MRF4, Myf5 and myogenin (reviewed in 1). Overexpression of any one of these bHLH myogenic factors was able to initiate myogenesis in a variety of non-muscle cell types (reviewed in 28). Although MEF2 was indispensable for the development of visceral, somatic and cardiac muscle in *Drosophila* (3), most reports so far indicate that overexpression of MEF2 is insufficient to convert fibroblasts into differentiated muscle cells (2). It is possible that the functions of MEF2 are regulated through protein–protein interaction and/or post-translational modification. Regulation of activity of MEF2 through protein–protein interactions has been reported. For example, MEF2C has been shown to bind to the bHLH region of myogenin and cooperatively activate expression of muscle-specific genes (15). In addition, MEF2 associates with the ligand-bound thyroid hormone receptor and synergistically activates the α-cardiac myosin heavy-chain gene (29). p300/CBP binds to the MADS domain of MEF2C and potentiates the transcriptional activity of MEF2C (16). With regard to the role of post-translational modification in the regulation of MEF2, casein kinase II phosphorylates MEF2C and increases its DNA binding activity (26). A recent study searching for p38-interacting proteins by the yeast two hybrid screen led to the finding that p38 interacted with, phosphorylated, and activated MEF2C (17).

In this study, we found that ERK5/BMK1 interacted with amino acids 19–167 of MEF2D in a yeast two hybrid screen. Since the N-terminus of MEF2D contains the MADS and MEF2 domains which are homologous among the four MEF2 proteins, the N-terminus of MEF2 may mediate the interaction and therefore other MEF2 proteins may also interact with ERK5/BMK1 proteins. Indeed, the N-terminus of MEF2D and MEF2C was sufficient to interact with ERK5/BMK1 and the full length of MEF2D and MEF2C proteins were capable of interacting with ERK5/BMK1 (Fig. 1b). Furthermore, a co-immunoprecipitation study showed that endogenous MEF2D and MEF2A proteins were present in the anti-ERK5 immunoprecipitate, although ERK5 proteins were not detectable in the anti-MEF2 immunoprecipitate (Fig. 1c). There are several possible reasons for the failure of observing ERK5 in the anti-MEF2 immunoprecipitate. Firstly, it is possible that the amount of ERK5 associated with MEF2 is below the detection limit of anti-ERK5 antiserum since the absolute amount of ERK5 in myotubes could be much less than that of MEF2 and, therefore, anti-MEF2 immunoprecipitate may contain only a small portion of the ERK5-associating MEF2. Our observation that MEF2D and MEF2A proteins, but not ERK5, can be detected in the myotube lysate (data not shown) also supports this notion. Secondly, it is also possible that MEF2:ERK5 interaction masks the epitope on MEF2 recognized by the anti-MEF2 antiserum. Nevertheless, the co-immunoprecipitation results show that endogenous MEF2D and MEF2A interact with endogenous ERK5 *in vivo*.

A functional role for the MEF2–ERK5 interaction was tested by determining whether MEF2D and MEF2C can be phosphorylated and activated by ERK5/BMK1. Although both the recombinant MEF2D and MEF2C proteins were found to be phosphorylated by ERK5/BMK1 *in vitro* (Fig. 2), the transcriptional activity of MEF2C was enhanced by ERK5/BMK1 in HeLa cells; however, the activity of MEF2D was not affected (Fig. 3). MEF2A was included in the CAT-reporter assay and found to act similarly to MEF2C. It has been reported previously that the endogenous MEF2D expressed in myoblasts (30) and also in HeLa cells (23) is transcriptionally inactive, supporting the idea that MEF2D may be regulated differently compared with the other MEF2 proteins. In addition, overexpression of MEF2D weakly stimulates a reporter gene with oligomerized MEF2 sites whereas MEF2C and MEF2A potently activate transcription (23 and Fig. 3). Therefore, the transcriptional activity of MEF2D may be subject to a regulatory mechanism different from the one governing the regulation of MEF2A and MEF2C.

Since two library screening approaches (17 and our data) indicate that MEF2 interacts with two members of the MAP kinase family, MEF2 could be an important physiological target of ERK5/BMK1 and p38 MAP kinases. In this study, ERK5/BMK1 was isolated from a library made from aorta, which contains mainly smooth muscle cells. Indeed, Abe *et al*. (27) detected the expression of endogenous ERK5/BMK1 in cultured rat vascular smooth muscle cells. Moreover, the addition of H_2O_2 and sorbital to cultured cells stimulated the activity of ERK5/BMK1. Recently, Abe *et al*. (31) also showed that c-*Src* is required for oxidative stress-mediated activation of BMK1. Therefore, MEF2 could be a candidate for mediation of stress signals from activated ERK5/BMK1 in vascular smooth muscle cells.

Phosphorylation at Ser59 of MEF2C (26) and at Ser103 of SRF (32), a member of the MADS-domain family, were shown to increase their DNA-binding affinity *in vitro*. During the preparation of this manuscript, Kato *et al*. (33) reported that ERK5/BMK1 regulated serum-induced expression of c-*Jun* through MEF2C. While the direct association between ERK5/BMK1 and MEF2 was not addressed by Kato *et al*., our study shows that MEF2 proteins physically interact with and are phosphorylated by ERK5/BMK1. In two reports (17,33), Ser387 of MEF2C was phosphorylated by p38 or ERK5/BMK1, respectively. Furthermore, this phosphorylation was critical for the activation of MEF2C. Interestingly, this Ser387 is conserved between MEF2C and MEF2A, but not MEF2D which is unresponsive to ERK5/BMK1 (33). Therefore, the results of our reporter assays suggest that both MEF2A and MEF2C could respond to ERK5/BMK1 via phosphorylation of this conserved serine, while phosphorylation of MEF2D at an unknown site by ERK5/BMK1 is insufficient for activation.

In summary, our data indicate that ERK5/BMK1 can target several members of the MEF2 family through the highly conserved N-terminus of MEF2 and this interaction can result in phosphorylation and modulation of the transactivation properties of MEF2A and MEF2C.

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REFERENCES

1 Olson,E.N. (1990) *Genes Dev.*, **4**, 1454–1461.
2 Olson,E.N., Perry,M. and Schulz,R.A. (1995) *Dev. Biol.*, **172**, 2–14.
3 Lilly,B., Zhao,B., Ranganayakulu,G., Paterson,B.M., Schulz,R.A. and Olson,E.N. (1995) *Science*, **267**, 688–693.
4 Bour,B.A., O'Brien,M.A., Lockwood,W.L., Goldstein,E.S., Bodmer,R., Taghert,P.H., Abmayr,S.M. and Nguyen,H.T. (1995) *Genes Dev.*, **9**, 730–741.
5 Ornatsky,O.I., Andreucci,J.J. and McDermott,J.C. (1997) *J. Biol. Chem.*, **272**, 33271–33278.

6 Lin,Q., Schwarz,J., Bucana,C. and Olson,E.N. (1997) *Science*, **276**, 1404–1407.
7 Gossett,L.A., Kelvin,D.J., Sternberg,E.A. and Olson,E.N. (1989) *Mol. Cell. Biol*., **9**, 5022–5033.
8 Shore,P. and Sharrocks,A.D. (1995) *Eur. J. Biochem*., **229**, 1–13.
9 Nguyen,H.T., Bodmer,R., Abmayr,S.M., McDermott,J.C. and Spoerel,N.A. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 7520–7524.
10 Lilly,B., Galewsky,S., Firulli,A.B., Schulz,R.A. and Olson,E.N. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 5662–5666.
11 Molkentin,J.D., Black,B.L., Martin,J.F. and Olson,E.N. (1996) *Mol. Cell. Biol*., **16**, 2627–2636.
12 Wong,M.W., Pisegna,M., Lu,M.F., Leibham,D. and Perry,M. (1994) *Dev. Biol*., **166**, 683–695.
13 Martin,J.F., Miano,J.M., Hustad,C.M., Copeland,N.G., Jenkins,N.A. and Olson,E.N. (1994) *Mol. Cell. Biol*., **14**, 1647–1656.
14 Kaushal,S., Schneider,J.W., Nadal-Ginard,B. and Mahdavi,V. (1994) *Science*, **266**, 1236–1240.
15 Molkentin,J.D., Black,B.L., Martin,J.F. and Olson,E.N. (1995) *Cell*, **83**, 1125–1136.
16 Sartorelli,V., Huang,J., Hamamori,Y. and Kedes,L. (1997) *Mol. Cell. Biol*., **17**, 1010–1026.
17 Han,J., Jiang,Y., Li,Z., Kravchenko,V.V. and Ulevitch,R.J. (1997) *Nature*, **386**, 296–299.
18 Fields,S. and Sternglanz,R. (1994) *Trends Genet*., **10**, 286–292.
19 Lee,J.D., Ulevitch,R.J. and Han,J. (1995) *Biochem. Biophys. Res. Commun*., **213**, 715–724.
20 Zhou,G., Bao,Z.Q. and Dixon,J.E. (1995) *J. Biol. Chem*., **270**, 12665–12669.
21 Durfee,T., Becherer,K., Chen,P.L., Yeh,S.H., Yang,Y., Kilburn,A.E., Lee,W.H. and Elledge,S.J. (1993) *Genes Dev*., **7**, 555–569.
22 Elledge,S.J., Mulligan,J.T., Ramer,S.W., Spottswood,M., Davis and RW (1991) *Proc. Natl Acad. Sci USA*, **88**, 1731–1735.
23 Ornatsky,O.I. and McDermott,J.C. (1996) *J. Biol. Chem*., **271**, 24927–24933.
24 Sleigh,M.J. (1986) *Anal. Biochem*., **156**, 251–256.
25 Elsholtz,H.P., Lew,A.M., Albert,P.R. and Sundmark,V.C. (1991) *J. Biol. Chem*., **266**, 22919–22925.
26 Molkentin,J.D., Li,L. and Olson,E.N. (1996) *J. Biol. Chem*., **271**, 17199–17204.
27 Abe,J., Kusuhara,M., Ulevitch,R.J., Berk,B.C. and Lee,J.D. (1996) *J. Biol. Chem*., **271**, 16586–16590.
28 Edmondson,D.G. and Olson,E.N. (1993) *J. Biol. Chem*., **268**, 755–758.
29 Lee,Y., Nadal-Ginard,B., Mahdavi,V. and Izumo,S. (1997) *Mol. Cell. Biol*., **17**, 2745–2755.
30 Breitbart,R.E., Liang,C.S., Smoot,L.B., Laheru,D.A., Mahdavi,V. and Nadal-Ginard,B. (1993) *Development*, **118**, 1095–1106.
31 Abe,J., Takahashi,M., Ishida,M., Lee,J.D. and Berk,B.C. (1997) *J. Biol. Chem*., **272**, 20389–20394.
32 Rivera,V.M., Miranti,C.K., Misra,R.P., Ginty,D.D., Chen,R.H., Blenis,J. and Greenberg,M.E. (1993) *Mol. Cell. Biol*., **13**, 6260–6273.
33 Kato,Y., Kravchenko,V.V., Tapping,R.I., Han,J., Ulevitch,R.J. and Lee,J.D. (1997) *EMBO J*., **16**, 7054–7066.