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Big Mitogen-activated Kinase Regulates Multiple Members of the MEF2 Protein Family*

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Big mitogen-activated protein (MAP) kinase (BMK1), a member of the mammalian MAP kinase family, is activated by growth factors. The activation of BMK1 is required for growth factor-induced cell proliferation and cell cycle progression. We have previously shown that BMK1 regulates c-*jun* gene expression through direct phosphorylation and activation of transcription factor MEF2C. MEF2C belongs to the myocyte enhancer factor 2 (MEF2) protein family, a four-membered family of transcription factors denoted MEF2A, -2B, -2C, and -2D. Here, we demonstrate that, in addition to MEF2C, BMK1 phosphorylates and activates MEF2A and MEF2D but not MEF2B. The blocking of BMK1 signaling inhibits the epidermal growth factor-dependent activation of these three MEF2 transcription factors. The sites phosphorylated by activated BMK1 were mapped to Ser-355, Thr-312, and Thr-319 of MEF2A and Ser-179 of MEF2D both *in vitro* and *in vivo*. Site-directed mutagenesis reveals that the phosphorylation of these sites in MEF2A and MEF2D are necessary for the induction of MEF2A and 2D transactivating activity by either BMK1 or by epidermal growth factor. Taken together, these data demonstrate that, upon growth factor induction, BMK1 directly phosphorylates and activates three members of the MEF2 family of transcription factors thereby inducing MEF2-dependent gene expression.

Mitogen-activated protein (MAP)[1] kinase signal transduction pathways constitute one of the major mechanisms by which extracellular stimuli are converted into specific nuclear responses (1–3). These pathways are considerably evolutionarily conserved, as they have been identified in a diverse group of organisms ranging from *Saccharomyces cerevisiae* to humans (2, 4). In mammalian cells, four families of MAP kinases have been identified, including the extracellular signal-regulated protein kinase (ERK1/2), c-Jun N-terminal kinase, p38 kinase, and big MAP kinase 1 (BMK1/ERK5). Each MAP kinase family is regulated by a distinct signal transduction mechanism that influences specific cellular functions, including cell growth, cell differentiation, programmed cell death, neoplastic transformation, and certain stress-related responses (1, 5–7).

An ever growing number of nuclear targets for the different MAP kinase cascades are being identified and, in most cases, it appears that a specific transcription factor acts as a target for one or a limited subset of MAP kinases (8). For example, MAP kinases have been shown to regulate the activity of c-Jun, CHOP10, c-Myc, ternary complex factor, and MEF2 transcription factors (9–13). MEF2 transcription factors bind as homodimers and heterodimers to MEF2 sites found within the promoters of numerous genes, including muscle-specific genes and the proto-oncogene c-*jun* (14, 15). Interestingly, these four MEF2 proteins exhibit amino acid sequence similarity within their DNA binding and transactivating domains (16).

BMK1, a newer member of the MAP kinase family, was cloned by us and others using distinct approaches (17, 37). We have previously demonstrated that BMK1 is activated by growth factors, oxidative stress, and hyperosmolarity and that BMK1 activity is required for growth factor-induced cell cycle progression through S phase (18–20). Transcription factor MEF2C is a direct substrate for BMK1, and its phosphorylation by BMK1 is involved in serum-induced proto-oncogene c-*jun* expression via the MEF2 site found within c-jun promoter (18). However, the effect of the BMK1 signaling pathway on other MEF2 family members has not yet been explored. In this study, we have investigated the regulation of MEF2A, -2B, and -2D proteins by BMK1. We found that, in addition to MEF2C, BMK1 can specifically phosphorylate and activate MEF2A and -2D but not -2B. The BMK1 phosphorylation sites within MEF2A and MEF2D were subsequently identified both *in vivo* and *in vitro* and shown to be critical for both BMK1 and growth factor-mediated activation of these transcription factors.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—HeLa cells and Chinese hamster ovarian (CHO-K1) cells were maintained in Dulbecco's modified Eagle's me-

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[1] The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; BMK1, big mitogen-activated protein kinase 1; MEK, MAP kinase/ERK kinase; MKK, MAP kinase kinase; MEKK, MAP kinase kinase kinase/ERK kinase kinase; SAPK, stress-activated protein kinase; EGF, epidermal growth factor; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; MEF, myocyte enhancer factor; FCS, fetal calf serum; GAL4(BD), GAL4 DNA binding domain.

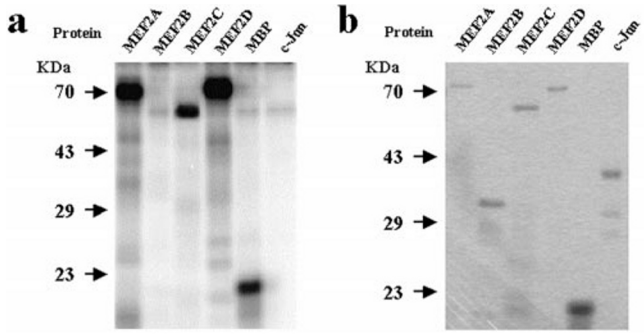

FIG. 1. **BMK1 phosphorylates MEF2A, -2C, and -2D.** *a*, equal amounts of recombinant MEF2A, -2B, -2C, -2D, MBP, and c-jun (1–93) were used as substrates for BMK1 in an *in vitro* protein kinase assay as described. The phosphorylation of these substrates by BMK1 was detected by SDS-PAGE followed by using a PhosphorImager (Molecular Dynamics). *b*, Coomassie Blue staining of MEF2s, MBP, and c-jun (1–93) proteins used in the protein kinase assay.

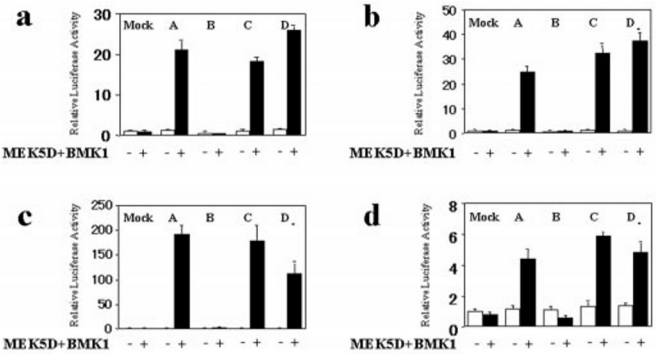

FIG. 2. **BMK1 simulates the transcriptional activity of MEF2A and MEF2D.** *a*, HeLa; *b*, Chinese hamster ovary; *c*, 293; or *d*, PC12 cells were cotransfected with the reporter plasmid pG5ElbLuc (0.2 μg) and a plasmid encoding a given MEF2 isoform fused with GAL4(DB) as indicated. Additionally, control vector pcDNA3 (0.4 μg) or expression plasmids encoding MEK5(D) (0.2 μg) along with BMK1 (0.2 μg) were included in each transfection as indicated at the *bottom* of each *panel*. Cell extracts were prepared 48 h following transfection. The luciferase activities were normalized against cells transfected with pG5ElbLuc and GAL4 reporter plasmid alone, whose value was taken as 1.

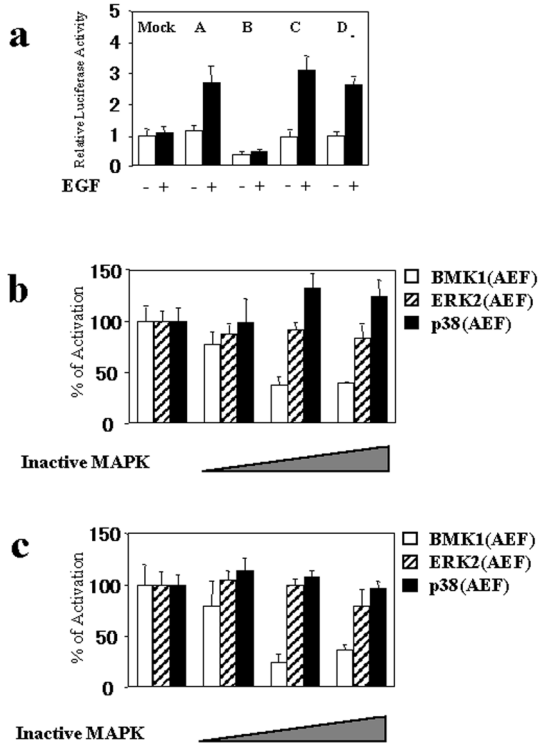

FIG. 3. **BMK1 mediates EGF-induced activation of MEF2A and -2D.** *a*, HeLa cells were transfected with the reporter plasmid pG5ElbLuc (0.4 μg) along with either no MEF plasmid (*Mock*) or plasmids encoding GAL4/MEF2A, -2B, -2C, or -2D (0.4 μg) as indicated. 24 h after transfection, the cells were transferred into serum-free media for an additional 24 h. Then 5 ng/ml EGF was added and the cells were incubated for an additional 6 h. Relative luciferase activities were normalized to non-EGF-treated mock cells whose activity was taken as 1. *b*, HeLa cells were transfected with the reporter plasmid pG5ElbLuc (0.3 μg) and GAL4/MEF2A (0.3 μg) along with various amounts of plasmids encoding the inactive MAP kinases BMK1(AEF), ERK(AEF), or p38(AGF) (0, 0.1, 0.2, or 0.3 μg) as indicated. The total amount of transfected DNA was adjusted to 1.0 μg through the addition of empty vector pcDNA3. Cells were then incubated, treated with EGF, and then assayed as described above. Relative luciferase activities were normalized to cells transfected with pG5ElbLuc and GAL4/MEF2A whose value was taken as 100%. *c*, same as *b*, except that GAL4/MEF2D was used in place of GAL4/MEF2A.

dium (Sigma) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 1% nonessential amino acids (Life Technologies, Inc., Gaithersburg, MD). NIH-3T3 cells were maintained in the same medium but with 10% FCS. RAW264 cells were maintained in RPMI 1640 medium (Sigma) containing 10% FCS, 2 mM glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 1% nonessential amino acids.

MEF2 Constructs—Expression plasmid GAL4-MEF2A(wt) was constructed by fusing the GAL4 DNA binding domain (GAL4(BD)) with the transactivation domain of human MEF2A (amino acids 87–507). GAL4-MEF2B, -MEF2C, and -MEF2D were constructed by fusing GAL4(BD) separately with mouse MEF2B (amino acids 87–349), human MEF2C (amino acids 87–442), and mouse MEF2D1b (amino acids 87–506) as described previously (13, 18, 21). Plasmids carrying point mutations such as MEF2A(S355A), MEF2A(T312A/T319A), MEF2A(S355A, T312A/T319A), MEF2D(S179A), and MEF2D(S430A) were generated as described elsewhere (18, 21). The His-tagged full length MEF2A, MEF2B, MEF2C, MEF2D, MEF2A(S355A), MEF2A(T312A/T319A), MEF2A(S355A, T312A/T319A), MEF2D(S179A), and MEF2D(S430A) were individually cloned into the bacterial expression vector pETM1 as described elsewhere (18, 21).

Preparation of Recombinant Proteins—Escherichia coli BL21(DE3) was transformed with the vector pETM1 with individual cDNAs encoding MEF2A, MEF2B, MEF2C, MEF2D, MEF2A(S355A), MEF2A(T312A/T319A), MEF2A(S355A, T312A/T319A), MEF2D(S179A), and MEF2D(S430A). The transformed bacteria were grown at 37 °C in L broth until the A_{600} reached 0.5, at which time isopropyl-D-thiogalactopyranoside at a final concentration of 1 mM was added for 5 h. Cells were collected by centrifugation at 8,000 × *g* for 10 min, and the bacterial pellet was resuspended in 10 ml of buffer A (30 mM NaCl, 10 mM EDTA, 20 mM Tris-HCl, 2 mM phenylmethylsulfonyl fluoride) for every 100 ml of original bacterial culture. The cell suspension was sonicated, and cellular debris was removed by centrifugation at 10,000 × *g* for 30 min. Recombinant proteins were then purified from the cleared lysate by using a Ni-nitrilotriacetic acid purification system (Qiagen). GST c-jun was purchased from Calbiochem.

Protein Kinase Assays—In vitro kinase assays were carried out at 37 °C for 60 min, using 0.1 μg of recombinant kinase, 1 μg of kinase substrate, 250 μM ATP, and 10 μCi of [γ-^{32}P]ATP in 20 μl of kinase reaction buffer as described previously (18). Reactions were terminated by the addition of Laemmli sample buffer. Reaction products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). $^{32}P_i$-Labeled proteins were visualized by autoradiography.

Phosphoamino Acid Analysis and Phosphopeptide Mapping—These experiments were performed as described previously (18). In brief, recombinant proteins were phosphorylated *in vitro* by BMK1 as described above. Phosphorylated MEF2A and MEF2D proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, visualized by autoradiography, and excised. Eluted proteins were digested with trypsin, and the resulting tryptic peptides were analyzed by thin-layer electrophoresis followed by thin-layer chromatography. In some cases, phosphopeptides were recovered from thin-layer chromatography plates and then analyzed for phosphoamino acid content as described elsewhere (18). Phosphopeptides were visualized and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To detect *in vivo* phosphorylation site(s) in MEF2A and -2D, HeLa cells expressing

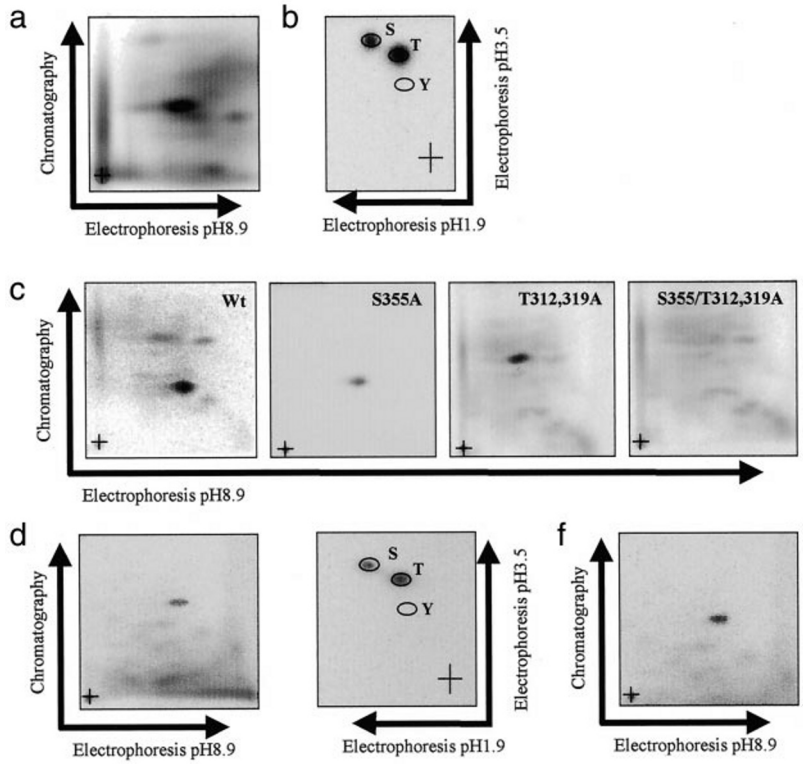

FIG. 4. **BMK1 phosphorylates MEF2A at amino acids Ser-355, Thr-312, and Thr-319.** *a*, phosphopeptide map of MEF2A phosphorylated by BMK1 *in vitro* as described under "Experimental Procedures." *b*, phosphoamino acid analysis of the phosphorylated peptide from *a*. *c*, phosphopeptide mapping of MEF2A, MEF2A(S355A), MEF2A(T312A/T319A), and MEF2A(S355A/T312A, T319A), which have been phosphorylated by BMK1 *in vitro* as indicated. *d*, HeLa cells expressing wild-type GAL4/MEF2A were starved in serum-free media for 24 h and then metabolically labeled with [^{32}P]orthophosphate for 3 h. The cells were then stimulated with 5 ng/ml EGF for 30 min. GAL4 MEF2A protein was immunoprecipitated from the cell lysate using the anti-GAL4 DNA-binding domain monoclonal antibody RK5C1 followed by phosphopeptide mapping. *e*, phosphoamino acid analysis of the phosphorylated peptide from *d*. *f*, phosphopeptide map of a mixture of equal counts of *in vitro* (*a*) and *in vivo* labeled MEF2A (*d*).

GAL4-MEF2A and GAL4-MEF2D proteins were labeled with $^{32}P_i$ for 3 h followed by treatment with or without 50 nM epidermal growth factor (EGF) for 20 min. These MEF2A or -2D proteins were recovered from the cell lysate (from 5×10^6 cells) by immunoprecipitation using 50 μg of anti-GAL4 antibody (Santa Cruz Biotechnology), and the resulting immunoprecipitates were then subjected to peptide mapping analysis as described above.

Reporter Gene Assays—The GAL4-responsive reporter plasmid pG5E1bLuc contains five GAL4 binding sites upstream of a minimal promoter driving a *luciferase* gene. Plasmids encoding a *luciferase* gene driven by the wild-type c-Jun promoter (pJluc), or a c-Jun promoter with a mutated MEF2 site (pJSXluc), were kindly provided by Dr. R. Prywes (22, 23). The reporter plasmid pG5E1bLuc was cotransfected into cells along with a construct encoding the GAL4(BD) fused to MEF2A, MEF2B, MEF2C, MEF2D, MEF2A(S355A), MEF2A(T312A/T319A), MEF2A(S355A, T312A/T319A), MEF2D(S179A), or MEF2D(S430A). In some experiments, the expression vector encoding MKK6b(E), MEK1(E), or MEK5(D) was also included in the transfection mixtures as indicated in the figure legends.

Cells were grown on 35-mm-diameter multiwell plates (Nunc, Naperville, IL) and transiently transfected with 1 μg of total plasmid DNA, using Lipofectamine Plus reagent (Life Technologies, Inc.). A β-galactosidase expression plasmid (pCMV-β-gal; CLONTECH, Palo Alto, CA) was used to determine the transfection efficiency in each transfection. The total amount of DNA for each transfection was kept constant by using the empty vector pcDNA3. After 24 h, the medium was changed to serum-free Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and nonessential amino acids; 24 h after transfection, cell extracts were prepared and the activities of β-galactosidase and luciferase were measured as described previously (18).

RESULTS

MEF2A and MEF2D Are Downstream Targets for BMK1—We have previously shown that the transcription factor MEF2C is phosphorylated and activated by BMK1 in response to serum (18). Because MEF2 family members share amino acid sequence similarity within the DNA binding and transactivating domains, we speculated that other MEF2 family members may be substrates of BMK1. Using recombinant MEF2A, -2B, -2C, and -2D as substrates in *in vitro* protein kinase assays, we found that BMK1 phosphorylated MEF2A, -2C, and -2D but did not phosphorylate MEF2B (Fig. 1). We next investigated the ability of BMK1 to regulate the transactivating activity of these transcription factors in a trans-reporter assay as described previously for MEF2C (18). First, expression vectors encoding MEF2A, -2B, and -2D as fusion proteins containing the GAL4 DNA binding domain (GAL4(BD)) were constructed. These fusion proteins were cotransfected with a GAL4-driven *luciferase* reporter gene to measure the transactivating activity of each transcription factor. In this system, activated BMK1 enhanced the transactivating activity of MEF2A, -2C, and -2D but not MEF2B in a variety of cell types (Fig. 2). Taken together, these results indicate that BMK1 phosphorylates and activates all MEF2 family members with the exception of MEF2B.

EGF Activates MEF2A and MEF2D via BMK1—We previously demonstrated that BMK1 is strongly activated by EGF and that this event leads directly to activation of MEF2C (18, 20). Therefore, we investigated whether MEF2A and MEF2D were also be activated by EGF through BMK1. Using the GAL4 trans-reporter assay, we found that EGF enhanced the transactivating activity of MEF2A, -2C, and -2D about 3-fold but had no effect on the activity of MEF2B (Fig. 3*a*). To ascertain the role of BMK1 pathway in EGF-mediated MEF2A and -2D activation, we expressed enzyme-dead forms of the MAP kinases BMK1 (BMK1(AEF)), ERK2 (ERK2(AEF)), or p38 (p38(APF)) in the trans-reporter assay. The activation of MEF2A and MEF2D by EGF was only inhibited by the enzyme-dead form of BMK1 but not by enzyme-dead forms of ERK2 or p38 MAP kinases (Fig. 3). These results suggest that the EGF-induced activation of MEF2A and MEF2D is specifically mediated by BMK1.

Identification of BMK1-mediated Phosphorylation Sites in MEF2A—We have previously demonstrated that BMK1 regulates the activity of MEF2C by phosphorylating a specific amino acid residue within the transactivating domain of this transcription factor (18). To identify the specific amino acids within MEF2A and MEF2D that are phosphorylated by BMK1, we performed tryptic phosphopeptide mapping on MEF proteins incubated with BMK1 in an *in vitro* protein kinase assay. This analysis on MEF2A revealed one BMK1-phosphorylated

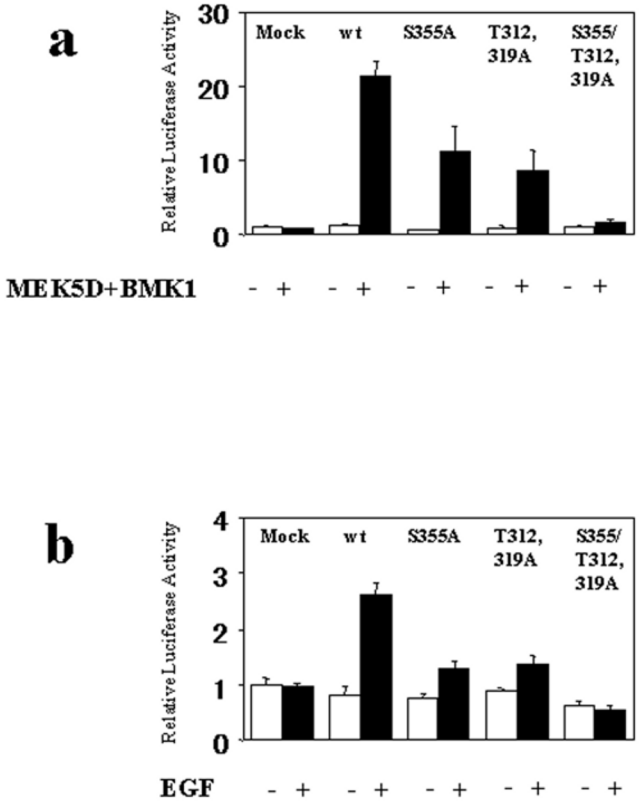

FIG. 5. **BMK1 phosphorylation sites in MEF2A are required for both EGF and BMK1-mediated MEF2A activation.** HeLa cells were transfected with plasmids pG5ElbLuc (0.2 μg) along with empty vector pcDNA3 (*Mock*) or individual plasmids (0.2 μg) encoding MEF2A, MEF2A(S355A), MEF2A(T312A, T319A), or MEF2A(S355A/T312A, T319A) as indicated. *a*, plasmids encoding BMK1 and MEK5(D) were included as indicated. *b*, EGF was used, as indicated, to stimulate the cells as described in legend of Fig. 3. All luciferase activities were normalized to unstimulated mock cells whose value was taken as 1.

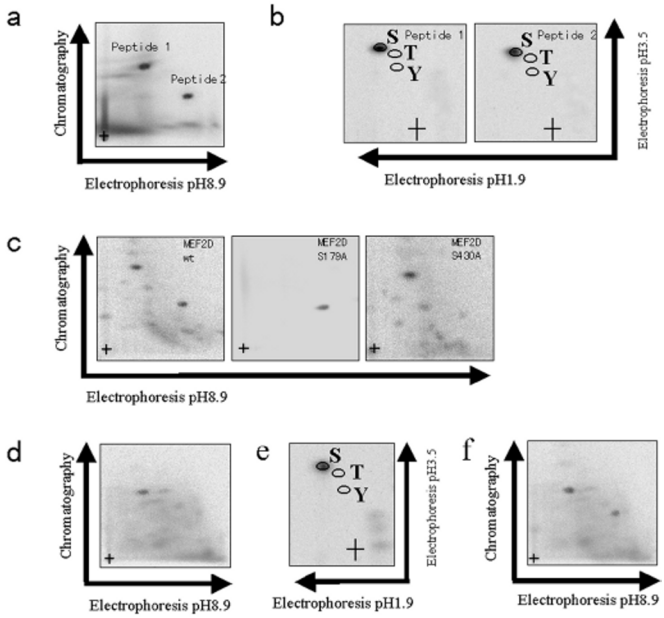

FIG. 6. **BMK1 phosphorylates MEF2D at Ser-179.** *a*, phosphopeptide map of MEF2D that was phosphorylated by BMK1 *in vitro*. *b*, phosphoamino acid analysis of the phosphorylated peptides from *a*. *c*, phosphopeptide mapping of MEF2D, MEF2D(S179A), and MEF2D-(S430A) that have been phosphorylated by BMK1 *in vitro*. *d*, HeLa cells expressing wild-type GAL4/MEF2D were starved in serum-free media for 24 h, metabolically labeled with [^{32}P]orthophosphate for 3 h, and then stimulated with EGF for 30 min. GAL4/MEF2D protein was immunoprecipitated from the cell lysate using the anti-GAL4 DNA binding domain monoclonal antibody RK5C1 followed by phosphopeptide mapping. *e*, phosphoamino acid analysis of the phosphorylated peptide from *d*. *f*, phosphopeptide map of a mixture of equal counts of *in vitro* (*a*) and *in vivo* labeled MEF2D (*d*).

peptide (Fig. 4*a*). Subsequent phosphoamino acid analysis revealed that this phosphopeptide consisted of both phosphoserine and phoshothreonine (Fig. 4*b*). The relative radioactivity between phosphoserine and phosphothreonine was approximately 1:2.2 in this phosphopeptide. After examining the amino acid sequence of MEF2A, only one tryptic peptide contained both Ser-Pro and Thr-Pro amino acid sequences (consensus phosphorylation sites for MAP kinases) at a ratio of 1:2. This peptide comprises amino acids 301–403 of MEF2A and contains one Ser-Pro (Ser-355) and two Thr-Pro (Thr-312 and Thr-319) sites. Interestingly, others have shown that threonines 312/319 as well as serine 355 of MEF2A are phosphorylated by the MAP kinase p38 after stimulation of cells using stress-related agonists. To confirm that these are the sites in MEF2A phosphorylated by BMK1 *in vitro*, mutant forms of MEF2A were constructed with serine 355 and/or threonines 312 and 319 converted to alanines. These mutant forms of MEF2A, denoted MEF2A(S355A), MEF2A (T312A, T319A), and MEF2A(S355A/T312A, T319A), were utilized as substrates in an *in vitro* kinase reaction containing BMK1 followed by tryptic peptide mapping. A single radioactive peptide was observed using MEF2A(S355A) or MEF2A(T312A, T319A) as a substrate. As expected, no radioactive tryptic peptide was detected using MEF2A(S355A/T312A, T319A) as a substrate (Fig. 4*c*). These results indicate that *in vitro* BMK1 phosphorylates MEF2A at Thr-312, Thr-319, and Ser-355.

To investigate whether these sites in MEF2A are phosphorylated in cells following EGF stimulation, GAL4-MEF2A was expressed in HeLa cells labeled with [^{32}P]orthrophosphate and, after treating the cells with 5 ng/ml EGF, GAF-MEF2A protein was immunoprecipitated from the cell lysate. One phosphopeptide (Fig. 4*d*) was detected following tryptic peptide mapping of immunoprecipitated GAL4-MEF2A. Phosphoamino acid analysis revealed that the phosphopeptide contained both phosphoserine and phosphothreonine (Fig. 4*e*) with a ratio of radiolabeling of about 1:2. To confirm that the phosphorylation of MEF2A obtained *in vivo* and *in vitro* is identical, we mixed equal counts of these phosphopeptides followed by two-dimensional phosphopeptide mapping. The identical migration of the two phosphopeptides confirms that cellular MEF2A is phosphorylated at amino acids Thr-312, Thr-319, and Ser-355 upon stimulation with EGF (Fig. 4*f*).

We next examined the role of these phosphorylation sites in regulating MEF2A activity as a result of either BMK1 activation or EGF induction. Mutation of Thr-312, Thr-319, and Ser-355 to alanine in GAL4-MEF2A significantly reduced the BMK1- and EGF-mediated activation of MEF2A (Fig. 5, *a* and *b*). Moreover, the BMK1- or EGF-induced transactivation of MEF2A was completely abrogated by mutating all three phosphorylation sites of MEF2A (Fig. 5, *a* and *b*). These results indicate that BMK1 or EGF mediates transactivation of MEF2A through all three of the phosphorylation sites identified by phosphopeptide mapping.

Identification of Phosphorylation Sites in MEF2D Catalyzed by BMK1—Similar to the analyses performed on MEF2A, the sites of MEF2D phosphorylated by activated BMK1 were determined. Tryptic peptide mapping of recombinant MEF2D, which had been phosphorylated by BMK1 in an *in vitro* protein kinase assay, revealed two phosphopeptides with equal radioactive intensity (Fig. 6*a*). Both phosphopeptides contained only phosphoserine as indicated by phosphoamino acid analysis

(Fig. 6*b*). The R_F and M_r values of these phosphopeptides are 0.45 and 18.9×10^{-4} for peptide 1 and 0.32 and 26.7×10^{-4} for peptide 2, respectively. Sequence analysis reveals that there are 10 predicted tryptic peptides containing Ser-Pro in the transactivating domain of MEF2D. Among them, only two phosphopeptides; one phosphorylated at Ser-179 and the other phosphorylated at both Ser-355 and Ser-404, would produce similar R_F and M_r values observed for peptide 1. To distinguish among these possibilities, we individually mutated each of these serines to alanine and phosphorylated the resulting proteins *in vitro* with BMK1 followed by tryptic peptide mapping. We found that phosphopeptide 1 was absent in the tryptic peptide map of the MEF2D(S179A) mutant (Fig. 6*c*) but not in the tryptic peptide map on the other mutants (data not shown).

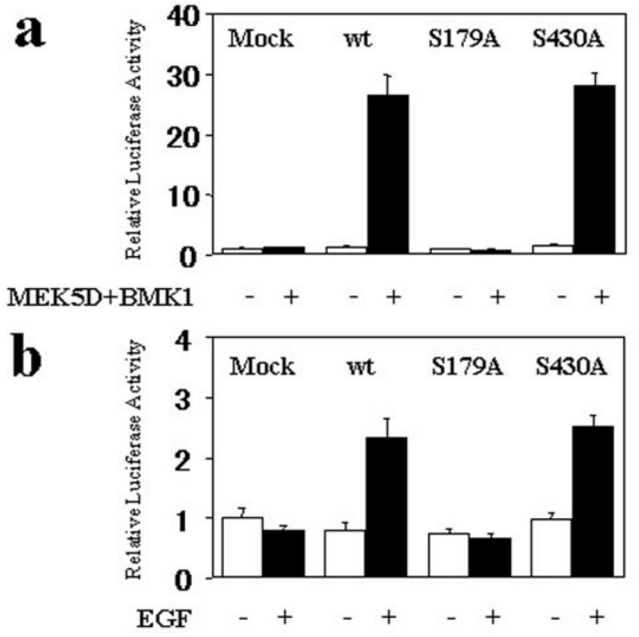

FIG. 7. **The BMK1 phosphorylation site in MEF2A is required for both EGF and BMK1-mediated MEF2D activation.** HeLa cells were transfected with plasmids pG5ElbLuc (0.2 μg) along with empty vector pcDNA3 (*Mock*) or individual plasmids (0.2 μg) encoding MEF2D, MEF2D(S179A), or MEF2D(S430A) as indicated. *a*, plasmids encoding BMK1 and MEK5(D) were included as indicated. *b*, EGF was used, as indicated, to stimulate cells as described in the legend of Fig. 3. All luciferase activities were normalized to unstimulated mock-transfected cells whose value was taken as 1.

Two putative phosphopeptides; one phosphorylated at Ser-430 and the another phosphorylated at both Ser-436 and Ser-457, would produce similar R_F and M_r values observed for peptide 2 (Fig. 6*a*). These three serines in MEF2D were individually mutated to alanine, and these mutant forms of MEF2D, denoted MEF2D(S430A) and MEF2D (S436A/S457A), were utilized as substrates in an *in vitro* kinase reaction containing BMK1 followed by tryptic peptide mapping. We found that phosphopeptide 2 was present in the tryptic peptide map of mutant MEF2D(S436A/S457A) but was absent using the mutant MEF2D(S430A) mutant (Fig. 6*c*). These results indicate that S430 is the site phosphorylated in MEF2D by BMK1 *in vitro*.

To determine the sites phosphorylated in MEF2D in cells activated by EGF, GAL4-MEF2D-expressing HeLa cells were labeled with ^{32}P and these cells were subsequently treated with 5 ng/ml EGF. Tryptic peptide mapping of immunoprecipitated GAL4-MEF2D revealed only one phosphopeptide (Fig. 6*d*) containing phosphoserine (Fig. 6*e*). Tryptic peptide mapping of a mixed sample containing equal counts of *in vitro* and *in vivo* phosphorylated MEF2D revealed a 2:1 ratio between phosphopeptide 1 and phosphopeptide 2 (Fig. 6*f*). These results identify phosphopeptide 1 as the phosphoryated tryptic peptide, and S179 as the site phosphorylated in MEF2D upon EGF treatment of cells.

We next examined the role of these two phosphorylation sites in regulating MEF2D activity in response to BMK1 activation or EGF stimulation. Mutation of S179 to alanine in GAL4-MEF2D alone completely abolished the BMK1- and EGF-mediated activation of MEF2D (Fig. 7, *a* and *b*). In contrast, BMK1- or EGF-induced MEF2D activity was not affected by mutating S430 to alanine (Fig. 7, *a* and *b*). These results indicated that only S179 is involved in regulating MEF2D activity in response to BMK1 and EGF.

Phosphorylation of MEF2A and MEF2D Is Required for MEF2-mediated c-jun Induction—The MEF2 site in the c-jun promoter is important for the activation of c-jun expression in response to various agonists, including EGF (18, 22, 24, 25). It has been shown that MEF2 proteins are involved in serum-induced expression of c-jun through binding of a MEF2 site within the c-jun promoter (18, 22, 23). However, the mechanism by which MEF2A and MEF2D regulate the activity of c-jun has not yet been thoroughly explored. The activation of BMK1 in cells was found to activate the expression of a reporter gene driven by the c-jun promoter (Fig. 8*a*). In contrast, this BMK1-dependent induction of c-jun was abolished using a

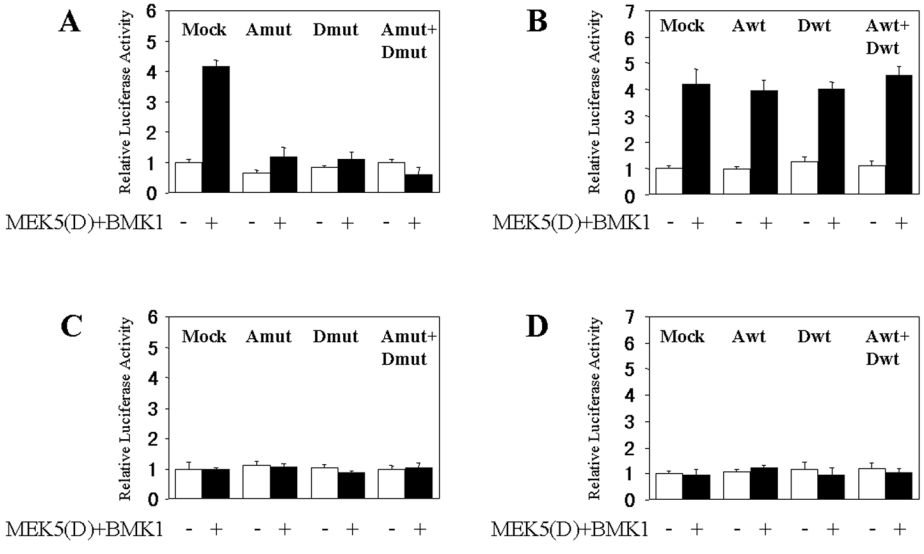

FIG. 8. **BMK1 mediates c-jun induction by phosphorylation of MEF2A and MEF2D.** HeLa cells were transfected with the reporter plasmid pJLuc (0.2 μg) along with empty vector pcDNA3 (*Mock*) or individual expression plasmids (0.2 μg) encoding MEF2A (*Awt*), MEF2A(S355A/T312A, T319A) (*Amut*), MEF2D (*Dwt*), or MEF2D(S179A) (*Dmut*) as indicated. Plasmids encoding BMK1 and MEK5(D) were included as indicated. In *c* and *d*, the reporter plasmid pJXLuc was used instead of pJLuc. All luciferase activities were normalized against unstimulated mock cells whose value was taken as 1.

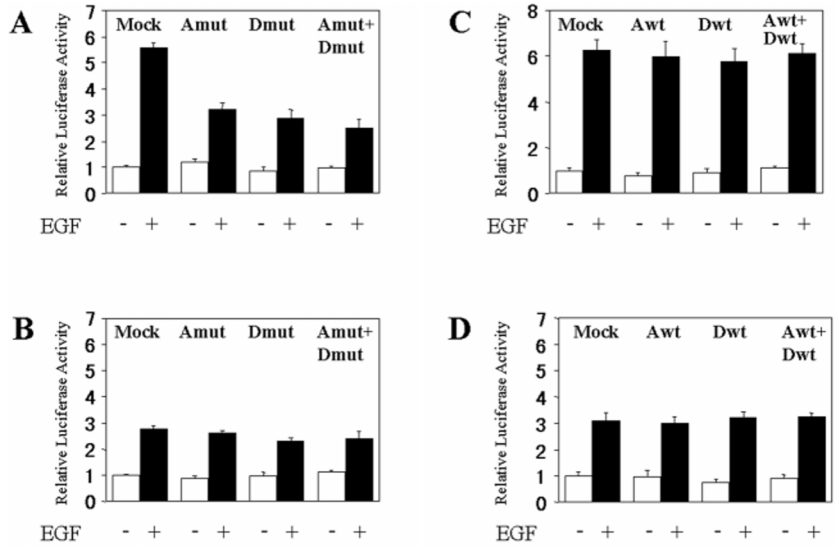

FIG. 9. **EGF-dependent c-jun induction requires BMK1-mediated phosphorylation of MEF2A and MEF2D.** HeLa cells were transfected with the reporter plasmid pJLuc (0.2 μg) along with empty vector pcDNA3 (*Mock*) or individual plasmids (0.2 μg) encoding MEF2A (*Awt*), MEF2A(S355A/T312A, T319A) (*Amut*), MEF2D (*Dwt*), or MEF2D(S179A) (*Dmut*) as indicated. EGF was used, as indicated, to stimulate the cells as described in the legend of Fig. 3. In *c* and *d*, the reporter plasmid pJXLuc was used instead of pJLuc. All luciferase activities were normalized against unstimulated mock cells whose value was taken as 1.

reporter gene driven by a c-jun promoter containing a deletion of the MEF2 binding site (Fig. 8*c*). To determine the contribution of the previously identified MEF2A or MEF2D phosphorylation sites to c-jun expression, the mutants MEF2A (S355A/T312A, T319A) or MEF2D(S179A) were expressed in this reporter assay. The expression of either MEF mutant blocked BMK1-mediated c-jun induction. This effect was specific for the MEF mutations, because expression of wild type MEF2A or MEF2D had no effect on BMK1-dependent c-jun induction (Fig. 8*b*). These data indicate that BMK1 regulates c-jun expression through phosphorylation of specific sites in MEF2A and MEF2D.

Previous studies have implicated MEF2 proteins in EGF-induced c-jun expression (24), however the signaling pathway leading to MEF2 activation remains largely unknown. The elimination of the MEF2 site in the c-jun promoter resulted in a 50% reduction in the EGF-mediated activation of a reporter gene (compare Figs. 9*a* and 9*b*). To determine whether phosphorylation of MEF2A or MEF2D by BMK1 is involved in EGF-dependent c-jun induction, MEF2A(S355A/T312A, T319A) or MEF2D(S179A) was expressed in this reporter assay. The expression of either MEF mutant resulted in a 50% decrease in EGF-mediated c-jun induction (Fig. 9*a*). This decrease was similar to that observed using the MEF2-deleted c-jun promoter (Fig. 9*b*) indicating that expression of mutant MEF2A or MEF2D eliminated the contribution of any MEF to EGF-induced c-jun expression. In contrast, expression of wild type MEF2A or MEF2D had no effect on EGF-dependent c-jun induction (Fig. 9*c*). Together, these results suggest that BMK1-mediated phosphorylation of MEF2A and MEF2D makes a significant contribution to EGF-induced c-jun expression.

DISCUSSION

The MEF2 family members, along with certain specific splice variants, were originally described as muscle-specific DNA binding proteins that recognize MEF2 motifs found within the promoters of many muscle-specific genes (14, 26). Recently, however, several groups have reported MEF2 binding activity and MEF2 proteins in a wide variety of cell types where these proteins appear to play an important role in growth factor- and stress stimulus-induced early gene responses (13, 18, 22). These findings suggest that the expression and function of MEF2 transcription factors is broader than originally thought. All four MEF2 family members can bind MEF2 promoter elements as either homodimers or a combination of heterodimers. Our laboratory has previously determined that BMK1 can

FIG. 10. **Sequence comparison of members of the MEF2 group of transcription factors in the regions phosphorylated by BMK1.** The Pileup program (Wisconsin Genetics Group, Madison, WI) was used for sequence alignment. Gaps introduced into the sequence to optimize alignment are indicated by a *hyphen*. The *numbers* indicate the starting and ending amino acid residues of each MEF2 protein shown. Amino acid residues phosphorylated by BMK1 are indicated by *asterisks*.

modulate MEF2C activity induced by growth factors (18, 20). Herein, the effect of BMK1 on other MEF2 family members was investigated, and we found that, in addition to MEF2C, the transcriptional activity of MEF2A and -2D is also directly upregulated by BMK1-catalyzed phosphorylation. Nonetheless, the biological consequence of BMK1-mediated activation of MEF2 proteins in various cell types and tissues is still largely unknown and needs to be explored.

MEF2A, -2C, and -2D in MEF2 family share amino acid sequence similarity within their transactivating domains, and, in this paper, we have demonstrated that their activity can be modulated by the phosphorylation of these domains by BMK1. Interestingly, our findings, combined with our previous report (18), suggest that the MEF2 sites phosphorylated by BMK1 are not conserved with respect to each other (Fig. 10). These results indicate that the general structural similarity among MEF2 proteins may only contribute to their initial recognition/interaction with BMK1 but that the actual site phosphorylated by BMK1 is determined by other factors. One possibility is that different cellular cofactors orient the kinase domain of BMK1 with different phosphorylation sites of these three MEF proteins. Another possibility is that the more subtle variations in the amino acid sequences of each of the MEFs contribute to the precise site phosphorylated by BMK1.

The MAP kinase p38 has also been shown to regulate the activity of MEF2A and MEF2C (13, 21, 27). In this regard, a conserved p38-docking domain was found in both MEF2A and MEF2C. In comparison, the docking domain of MEF2D is significantly different and has been considered inaccessible by p38 (27). We have shown that BMK1 can interact with all three of the aforementioned MEF proteins Thus, the recognition and

interaction of BMK1 with MEF family members may to be less stringent than that of p38. The other possibility is that BMK1 recognizes domains in these MEFs distinct from that utilized by p38. Moreover, BMK1 has been shown to physically interact with amino acids found within the DNA binding domain of MEF2A and MEF2C (28). However, the functional consequences or existence of this association in MEF2D have not yet been explored. It appears unlikely that this function of p38 acts in concert with BMK1 in the induction of c-jun expression, as the agonists for p38 are largely activators of stress and inflammation and are not mitogenic in nature (13, 29, 30).

In collaboration with B. C. Berk's laboratory, we have previously shown BMK1 is strongly activated by oxidative stress in smooth muscle cells and by shear stress in endothelial cells (19, 31, 32). Interestingly, MEF2 proteins are expressed in these cell types where they have been shown to be involved in vascular development (33, 34). Thus, a possible role for BMK1 in endothelial cell organization and smooth muscle cell differentiation warrants investigation. In addition, because these MEF2 proteins are critical in regulating muscle-specific gene expression, it is worthy to determine if BMK1-mediated signaling is involved in the MEF2-dependent differentiation or proliferation of muscle cells during development.

The proto-oncogene c-*jun* is an immediate early gene expressed in quiescent cells in response to mitogens such as growth factors. The induction of c-jun expression, mediated by mitogens or by signals from G protein-coupled receptors, requires ATF1 (AP-1 like) and MEF2 sites in the promoter of this gene (24, 35). Here, we demonstrate that a MEF2 promoter element is required for the BMK1-mediated activation of c-jun expression as deletion of this element completely abrogates this effect (Fig. 8*a*). The induction of c-jun expression appears to be essential for cell proliferation as microinjection of a deactivating anti-c-jun antibody has been shown to abrogate serum-induced cell cycle progression (36). In addition, previous studies in our laboratory have established that BMK1 activity is required for mitogen-induced cell proliferation and cell cycle progression (20). Taken together, these results indicate that the requirement for BMK1 in mitogen-induced cell cycle progression may be due to its ability to activate MEF transcription factors and thereby induce c-jun expression. As the ras/rac/MEKK1 signal transduction pathway has also been implicated in EGF-induced c-jun expression (24), it will be interesting to determine if this pathway, along with that provided by BMK1, mediates synergistic effects on c-jun activation.

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