

BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C

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Big MAP kinase 1 (BMK1), also known as ERK5, is a mitogen-activated protein (MAP) kinase member whose biological role is largely undefined. We have shown previously that the activity of BMK1 in rat smooth muscle cells is up-regulated by oxidants. Here, we describe a constitutively active form of the MAP kinase kinase, MEK5(D), which selectively activates BMK1 but not other MAP kinases *in vivo*. Through utilization of MEK5(D), we have determined that a member of the MEF2 transcription factor family, MEF2C, is a protein substrate of BMK1. BMK1 dramatically enhances the transactivation activity of MEF2C by phosphorylating a serine residue at amino acid position 387 in this transcription factor. Serum is also a potent stimulator of BMK1-induced MEF2C phosphorylation, since a dominant-negative form of BMK1 specifically inhibits serum-induced activation of MEF2C. One consequence of MEF2C activation is increased transcription of the *c-jun* gene. Taken together, these results strongly suggest that in some cell types the MEK5/BMK1 MAP kinase signaling pathway regulates serum-induced early gene expression through the transcription factor MEF2C.

Keywords: BMK1/*c-Jun*/MAP kinase/MEF2C/MEK5

Introduction

Mitogen-activated protein (MAP) kinases play an essential role in regulating many critical cellular processes including growth, differentiation, apoptosis and stress-related responses. In this respect, a large diversity of extracellular stimuli activate MAP kinases, including growth factors, hormones, cytokines, antigens and many physical-chemical stimuli such as oxidative stress, heat shock, osmotic imbalance and UV light (Davis, 1993; Cowley *et al.*, 1994; Xia *et al.*, 1995; Johnson *et al.*, 1996; Verheij *et al.*, 1996). These diverse extracellular stimuli initiate signals via receptors or 'sensors' which activate intracellular protein kinase cascades. A central feature of this series of events is activation of members of the MAP kinase family which regulate diverse biological functions by phosphorylating specific target molecules found within the membrane, the cytoplasm or the nucleus (Blenis, 1993; Blumer and Johnson, 1994; Cano and Mahadevan, 1995; Seger and Krebs, 1995; Johnson *et al.*, 1996).

The specificity of cellular responses is imparted by the activation of restricted individual MAP kinases in response to a given stimulus and by the downstream substrate specificity of a given MAP kinase. Transcription factors form one group of MAP kinase substrates which upon phosphorylation acquire increased transactivation activity. Examples of this include regulation of *c-Jun* (Gupta *et al.*, 1995; Raingeaud *et al.*, 1995), CHOP10 (Wang and Ron, 1996), *c-Myc* (Gupta and Davis, 1994) and ternary complex factor (Gille *et al.*, 1992, 1995; Marais *et al.*, 1993).

One recently identified member of the MAP kinase family, ERK5 or BMK1, was discovered independently by two laboratories using distinct experimental strategies (Lee *et al.*, 1995; Zhou *et al.*, 1995). Similarly to ERK1/2, BMK1 has a TEY sequence in its dual phosphorylation site. However, other structural features such as a large C-terminus and a unique loop-12 sequence distinguish BMK1 from ERK1/2 (Lee *et al.*, 1995). The unique structure of BMK1 suggests that its biological function and mechanism of regulation are distinct from those of other MAP kinase family members. However, presently there is little information available to support this contention.

We have shown previously that oxidative stress and hyperosmolarity serve as extracellular stimuli for BMK1 (Abe *et al.*, 1996). A physical interaction between BMK1 and the MAP kinase kinase MEK5 led to the proposal that MEK5 is the upstream regulatory kinase for BMK1 (Zhou *et al.*, 1995). However, this possibility has not been substantiated in cellular activation studies. Here we prove that the BMK1-dependent signaling pathway is regulated by MEK5, whose activation results in increased transactivation activity of a specific transcription factor via BMK1-dependent phosphorylation. First, we have found that in addition to oxidative stress and hyperosmolarity, serum also activates BMK1. Second, we show that MEK5 activates cellular BMK1 activity but not the activity of other members of the MAP kinase family such as ERK2, p38 and JNK1. Third, through selective activation of the cellular BMK1 MAP kinase pathway, we have discovered that MEF2C, a member of the MEF2 family of transcription factors, is a cellular target for BMK1. Finally, we demonstrate that a consequence of MEF2C activation, either by serum treatment or by direct activation of BMK1, is the induced expression of the immediate early response gene *c-jun*. The totality of these findings establishes an important biological role for the BMK1 signaling pathway which is the regulation of serum-induced early gene expression.

Results

Activators of BMK1

To delineate a biologically relevant signaling pathway for BMK1, we initially compared the effects of a series of

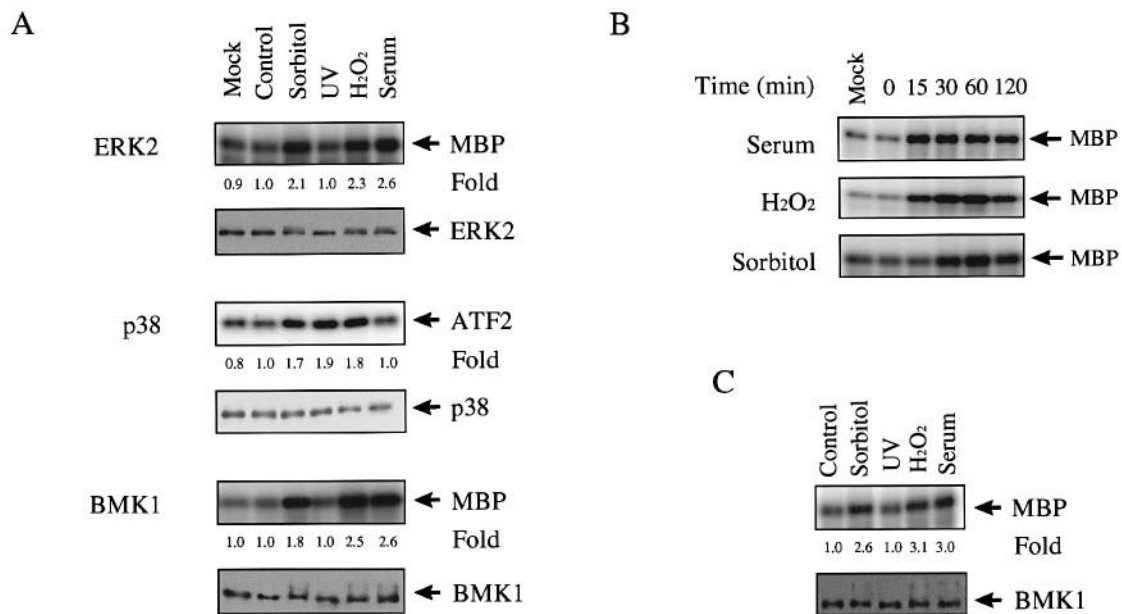


Fig. 1. Activation of BMK1 by extracellular stimuli. (A) CHO-K1 cells were transiently transfected with expression vectors encoding epitope-tagged BMK1, p38 or ERK2, and 24 h after transfection the cells were transferred into serum-free media for an additional 24 h. The serum-starved cells were stimulated for 30 min with nothing (control), 10% serum, 0.4 M sorbitol, 40 J/m² of UV-C or 500 μM H₂O₂ and subsequently collected for protein kinase assays. Kinase activity was measured by an immune complex protein kinase assay using MBP as a substrate for ERK2 and BMK1, or GST-ATF2 as a substrate for p38. The extent of MBP phosphorylation was evaluated by SDS-PAGE followed by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA) as described (Han *et al.*, 1996). The relative activation of the MAP kinases was determined by setting the activities, measured using the PhosphorImager, for control cells at a value of 1.0. (B) A time course of BMK1 activation by serum, H₂O₂ or sorbitol was assessed by measuring BMK1 activity in immune complex protein kinase assays. The duration of the agonist treatment is indicated in the figure. (C) CHO-K1 cells were cultured in serum-free media for 24 h and stimulated as described in (A). Endogenous BMK1 activity was assessed by measuring BMK1 activity in immune complex protein kinase assays. The bottom gels of (A) and (C) show corresponding samples of each immune complex run on SDS-PAGE gels, electrotransferred and then analyzed by Western blotting using the anti-flag antibody M2 (to detect BMK1, p38 and ERK2) or an anti-BMK1 antibody (to detect endogenous BMK1).

extracellular stimuli for their ability to activate BMK1 and two other members of the MAP kinase family, ERK2 and p38. This was accomplished by first stimulating CHO-K1 cells transfected with epitope-tagged constructs of BMK1, ERK2 and p38. As shown in Figure 1A, both BMK1 and ERK2 were activated when CHO-K1 cells were treated with serum, H₂O₂ and sorbitol, whereas neither was activated in response to UV. In contrast, p38 was activated by UV, sorbitol and H₂O₂, but not by serum. Western blot analysis demonstrated that these differential effects were not due to the presence of unequal levels of kinase in the immune complexes. To study further the activation of BMK1, we evaluated its activation kinetics following exposure to serum, sorbitol or H₂O₂. Increased BMK1 activity was detectable 15 min after the addition of serum or H₂O₂ and was maximal after 30 min (Figure 1B). In contrast, BMK1 activation by hyperosmolarity was slower, with an increase in activity observed at 30 min and maximal activity observed at 60 min following addition of sorbitol (Figure 1B). To confirm that endogenous BMK1 is activated, we examined the effects of these extracellular agonists by immunoprecipitating endogenous BMK1 from untransfected cells and performing an *in vitro* kinase assay. We observed that similarly to transfected BMK1, endogenous BMK1 is activated in response to hyperosmolarity, H₂O₂ and serum, but not UV (Figure 1C).

MEK5 is a specific upstream kinase for BMK1

Using the yeast two-hybrid system, Zhou *et al.* have shown that MEK5 specifically interacts with BMK1, leading these authors to propose that MEK5 is the regulatory kinase directly upstream of BMK1 (Zhou *et al.*, 1995). However, whether or not MEK5 activates BMK1 has not been demonstrated formally *in vitro* or *in vivo*. Since MAP kinase kinases are activated by dual phosphorylation within subdomain VIII (Cowley *et al.*, 1994; Han *et al.*, 1996; Raingeaud *et al.*, 1996), and these phosphorylation sites are conserved in MEK5, we examined the effect of introducing permanent negative charges at these sites by constructing MEK5(D) which replaces both Ser313 and Thr317 with aspartic acid. Similar mutations have been made in MEK1, MKK3b and MKK6b to produce constitutively active forms of these MAP kinase kinase family members (Cowley *et al.*, 1994; Han *et al.*, 1996; Raingeaud *et al.*, 1996). The effect of MEK5 activation on BMK1 activity was then assessed by co-expressing MEK5(D) with BMK1 in CHO-K1 cells and measuring BMK1 kinase activity using myelin basic protein (MBP) as a substrate. In this assay, we observed a greatly enhanced phosphorylation of MBP using immune complexes from cells expressing both BMK1 and MEK5(D) but not BMK1 alone. To confirm the specificity of this effect, we utilized a BMK1 mutant containing the sequence AEF in place of the actual TEY dual phosphorylation site. In contrast to wild-type BMK1,

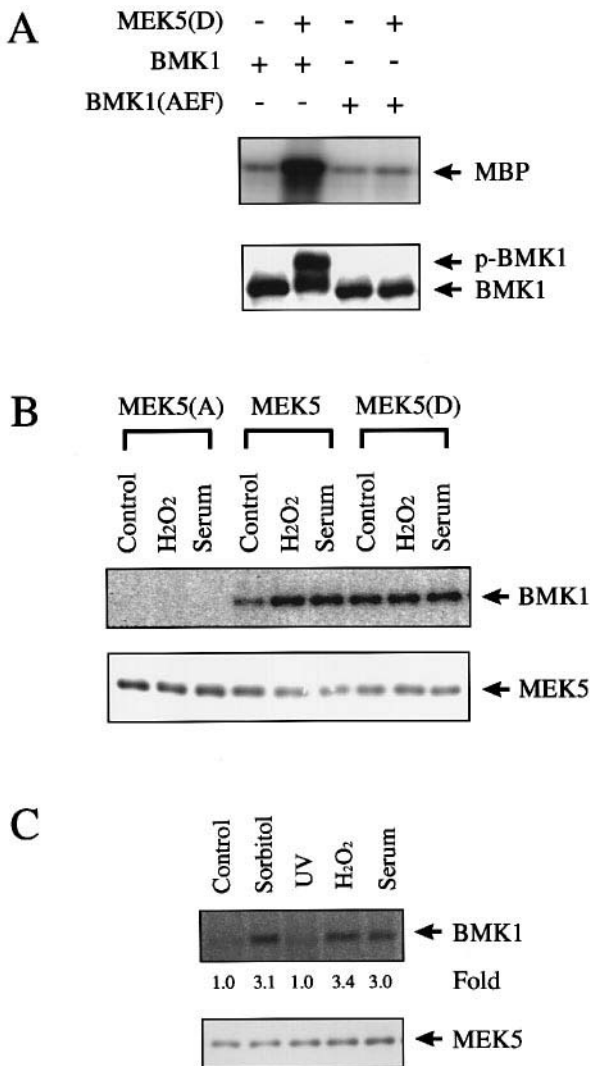


Fig. 2. MEK5 phosphorylates and activates BMK1. (A) Expression vectors encoding either epitope-tagged wild-type BMK1(TEY) or mutated BMK1(AEF) were co-transfected with or without a vector containing constitutively activated MEK5(D). The activity of BMK1 was measured in an immune complex kinase assay as described in Materials and methods using MBP as a substrate. (B) CHO-K1 cells were transfected with expression vector encoding wild-type HA-tagged MEK5, MEK5(A) or MEK5(D). At 24 h after transfection, cells were transferred into serum-free media for an additional 24 h. The serum-starved cells were stimulated for 30 min with nothing (control), 10% serum or 500 μ M H₂O₂ and collected for protein kinase assays. MEK5 kinase activity was measured in an immune complex kinase assay as described in Materials and methods with recombinant BMK1(M) as a substrate. BMK1(M) contains a single amino acid mutation in the ATP-binding site of BMK1 to prevent BMK1 autophosphorylation. (C) CHO-K1 cells were starved in serum-free media for 24 h and stimulated as described in (B). Endogenous MEK5 activity was assessed by measuring MEK5 activity in immune complex protein kinase assays using BMK1(M) as a substrate. The bottom gel of each panel shows corresponding samples of each immune complex run on SDS-PAGE gels, electrotransferred and then analyzed by Western blotting using the anti-flag antibody M2 [to detect BMK1 and BMK1(AEF)], the anti-HA antibody 12CA5 [to detect MEK5, MEK5(D) or MEK5(A)] or anti-MEK5 antibody (to detect endogenous MEK5).

BMK1(AEF) was not activated in CHO-K1 cells when co-transfected with MEK5(D) (Figure 2A). The kinase assays contained similar levels of flag-tagged BMK1, as shown by Western blotting (Figure 2A, lower panel). In

addition, activated BMK1, but not BMK1(AEF) displayed an activation-dependent altered electrophoretic mobility. A similar phenomenon has been observed upon activation of ERK1/2, and results from the phosphorylated protein exhibiting an altered mobility in SDS-PAGE gel (Boulton *et al.*, 1991). Together, these findings demonstrate that MEK5(D) is an activator of BMK1 *in vivo* and that the dual phosphorylation site of BMK1 is required for its activation by MEK5.

Since MEK5 appears to be the natural upstream cellular regulator of BMK1 activity, we reasoned that its activity should be responsive to those agonists that cause BMK1 activation. To test this, we first constructed an enzyme-dead mutant of MEK5, MEK5(A), by replacing the dual phosphorylation sites of MEK5 with alanines. CHO-K1 cells expressing HA-tagged MEK5, MEK5(A) or MEK5(D), were then stimulated with H₂O₂ or serum and the activity of these MEK5s was analyzed in a kinase assay using recombinant BMK1(M) as a substrate. We found that H₂O₂ and serum up-regulated the ability of MEK5 to phosphorylate recombinant BMK1(M) (Figure 2B). In contrast, MEK5(D) exhibited a constitutively high level of this activity, which was unaffected by H₂O₂ and serum. As expected, the inactive MEK5 mutant, MEK5(A), was unable to phosphorylate BMK1. To confirm that the activity of MEK5 is up-regulated by the known BMK1 agonists, we performed similar experiments on untransfected cells and found that endogenous MEK5 is activated by hyperosmolarity, H₂O₂ and serum, but not UV (Figure 2C). To determine if MEK5(A) exerts a dominant-negative effect on BMK1 activity, we tested its ability to interfere with BMK1 activation in response to extracellular stimuli. We observed that MEK5(A) inhibited the H₂O₂, serum- and sorbitol-dependent stimulation of BMK1 in a dose-dependent fashion (Figure 3A, B and C, respectively). This effect appears to be specific for BMK1 since there was no measurable effect on the activity of the MAP kinase ERK2. The specificity of this inhibitory effect was confirmed in this experimental system using a dominant-negative form of MEK1, MEK1(A), which has been shown to be effective in blocking the activation of ERKs by extracellular stimuli (Cowley *et al.*, 1994). As expected, MEK1(A) inhibited ERK2 activation in a dose-dependent fashion, but showed no effect on BMK1 activation in response to any of the three extracellular stimuli tested (Figure 3D, E and F, respectively). Together, these results demonstrate that MEK5 is activated by the same set of extracellular stimuli which activate BMK1, and support the contention that it is MEK5 which is specifically responsible for catalyzing the activation of BMK1.

In order to confirm that BMK1 is a specific substrate for MEK5, we co-transfected MEK5(D) with expression vectors encoding a number of other MAP kinase family members including ERK2, p38 and JNK1. The upstream activators for ERK2, p38 and JNK1 have been well defined by others as MEK1, MKK6b and MEKK1 Δ , respectively (Lange-Carter *et al.*, 1993; Cowley *et al.*, 1994; Minden *et al.*, 1994; Han *et al.*, 1996; Raingeaud *et al.*, 1996). As expected, in co-transfection experiments MEK1(E), MEK6b(E) and MEKK1 Δ specifically activated their appropriate downstream MAP kinases (Figure 4). MEK5(D) specifically activated BMK1 and was unable to activate other MAP kinases, ERK2, p38 and JNK1,

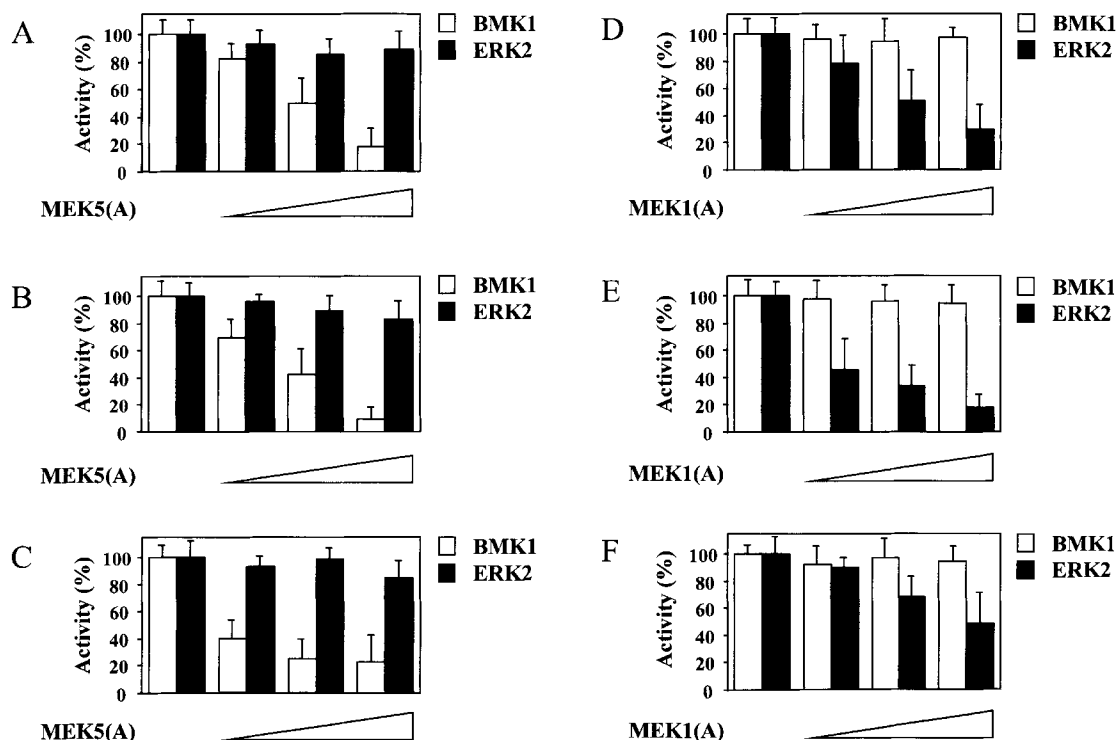


Fig. 3. Dominant-negative MEK5, MEK5(A), can specifically inhibit H_2O_2 -, serum- or hyperosmolarity-mediated activation of BMK1 in cells. CHO-K1 cells were co-transfected with 0.25 μ g of an expression vector encoding either epitope-tagged BMK1 or ERK2 either alone or with increasing amounts (0, 0.125, 0.25 and 0.5 μ g) of MEK5(A) (A–C) or MEK1(A) (D–F) plasmid DNA as described in Materials and methods. The transfected cells were stimulated with 500 μ M H_2O_2 (A and D), 10% serum (B and E) or 0.4 M sorbitol (C and F). The kinase activity of BMK1 or ERK2 was measured in an immune complex protein kinase assay using MBP as the substrate as described in Materials and methods. After subtracting the activity detected in the absence of agonist from all samples, the kinase activity determined for agonist-stimulated cells expressing either BMK1 alone or ERK2 alone was normalized to 100%. Each data point represents the average value from at least three independent experiments.

when co-expressed in cells (Figure 4). The specificity of MEK5(D) toward BMK1, but not other members of the MAP kinase family, supports the concept that the MEK5/BMK1 pathway is distinct from other known MAP kinase pathways. In addition, these findings establish the utility of MEK5(D) as a reagent that can be employed to delineate the biological role of this signal transduction pathway in cellular systems.

A variety of MAP kinases have been reported to be redistributed within the cell upon activation (Lenormand *et al.*, 1993; Cavigelli *et al.*, 1995). Therefore, we examined the subcellular distribution of BMK1 by indirect immunofluorescence microscopy using cells expressing epitope-tagged BMK1. We observed that the majority of BMK1 was present in the cytoplasm and appeared to be excluded from the nucleus (Figure 5A and B). Co-expression of BMK1 with MEK5(D) resulted in the appearance of BMK1 in the nucleus (Figure 5C and D). The apparent translocation of activated BMK1 to the nucleus suggests that upon activation BMK1 may have a role in regulating gene expression via effects on nuclear proteins.

Transcription factor MEF2C is a substrate for BMK1

The transactivation activity of a diverse group of transcription factors is up-regulated by MAP kinase-induced phosphorylation, resulting in altered expression of specific target genes. In order to determine if BMK1 can regulate gene expression by acting on transcription factors, we

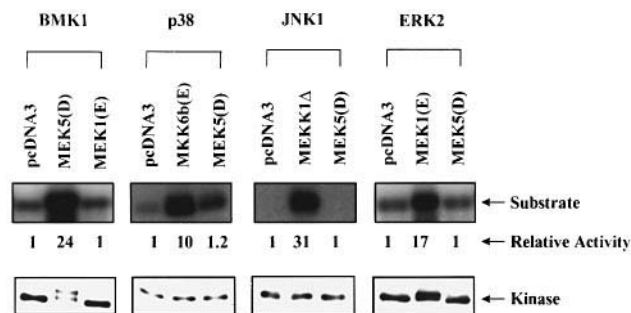


Fig. 4. The constitutively active MAP kinase kinase MEK5(D) selectively activates BMK1. Expression vectors encoding the epitope-tagged MAP kinases ERK2, p38, JNK1 and BMK1 were co-transfected into CHO-K1 cells with control vector pcDNA3 or expression vectors containing MEK5(D), MEK1(E), MKK6b(E) or MEKK1 Δ , as indicated in the figure. The kinase activity of ERK2, p38, JNK1 or BMK1 was determined using immune complex protein kinase assays as described in Materials and methods with MBP as the protein substrate for ERK2 and BMK1, GST-ATF2(1–109) as the substrate for p38, and GST-c-Jun(1–93) as the substrate for JNK1. After quantitating the gel using a phosphorimager, the relative activity for each MAP kinase substrate was determined by normalizing each activity against the corresponding pcDNA3 control vector with a value assigned as 1. The lower panel of the figure shows corresponding samples of each immune complex analyzed for each kinase by Western blotting using the anti-flag antibody M2.

utilized fusion proteins containing the transactivation domain of various transcription factors fused to the DNA-binding domain of the yeast transcription factor GAL4.

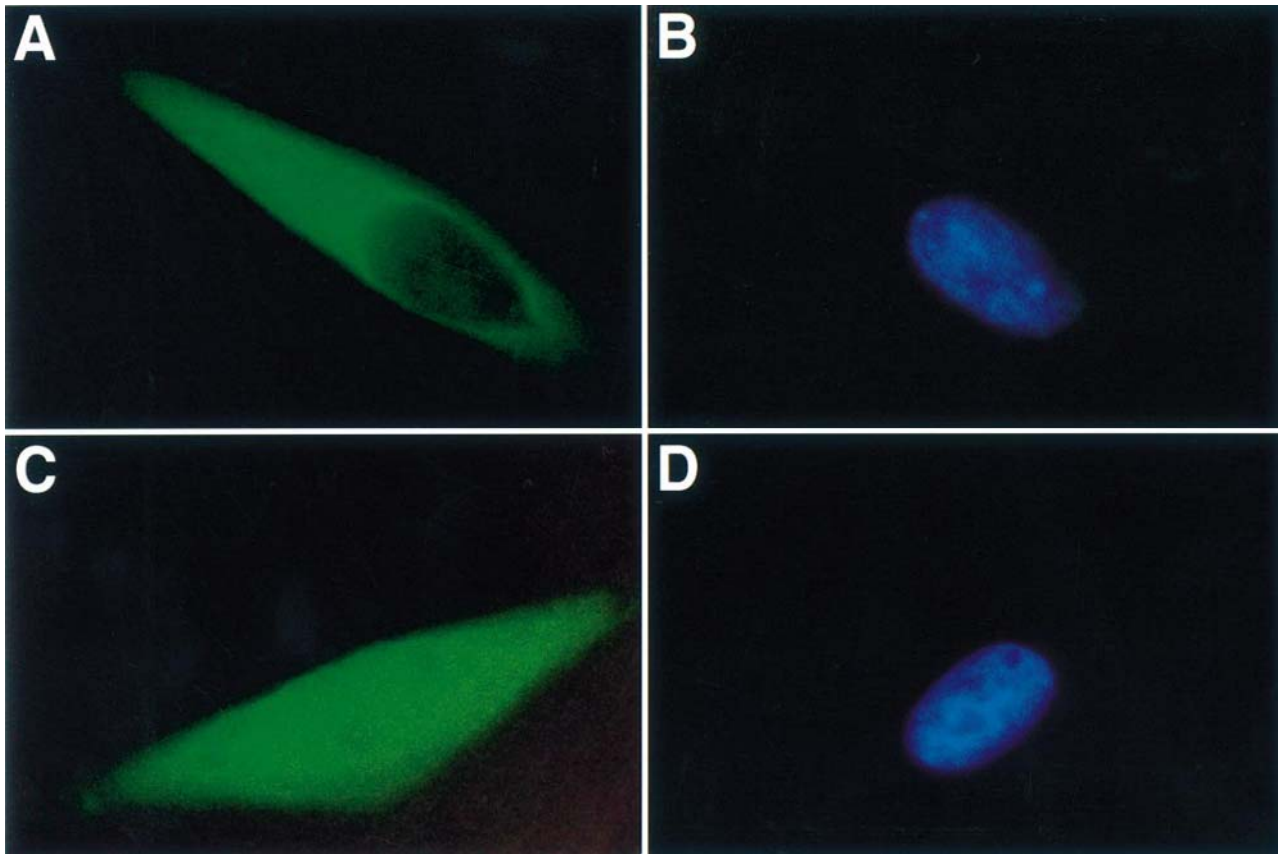


Fig. 5. Subcellular distribution of BMK1. A plasmid encoding epitope-tagged BMK1 kinase was co-transfected into CHO-K1 cells along with either empty expression vector (A and B) or expression vector containing MEK5(D) (C and D). (A) and (C) show cells after immunofluorescence staining for BMK1, while (B) and (D) show the same cells after nuclear staining. Cell staining was performed as described in Materials and methods.

The activity of these transcription factor fusion proteins was then assessed by measuring the luciferase activity from CHO-K1 cells co-transfected with a construct containing five copies of the GAL4-binding site upstream from a luciferase reporter gene. To assess the effectiveness of this system in discriminating between different transcription factor substrates, we utilized a GAL4 fusion of ELK-1, a known downstream target for the ERK MAP kinase pathway (Gille *et al.*, 1992, 1995). As expected, ELK-1-dependent reporter gene expression was enhanced dramatically when ERK2 and MEK1(E) were co-transfected into CHO-K1 cells (Figure 6A). In addition, ERK2/MEK1(E) had no effect on the activity of ATF2, CHOP and MEF2C, transcription factors which are known downstream substrates for the p38 MAP kinase pathway (Gupta *et al.*, 1995; Wang and Ron, 1996; Han *et al.*, 1997). Through testing a panel of transcription factors, which are known targets for MAP kinase family members, we found that BMK1 with MEK5(D) had little or no effect on the activity of transcription factors such as CHOP, ELK-1 or ATF2, but dramatically enhanced MEF2C-dependent reporter gene expression. This enhancement was determined to be about 25-fold in CHO-K1 cells (Figure 6B). MEF2C is a member of the MEF2 transcription factor family which is expressed mainly in skeletal muscle and brain tissue (Leifer *et al.*, 1993a,b; Martin *et al.*, 1993, 1994; McDermott *et al.*, 1994) and shown to be functionally important for the regulation of several muscle-specific genes (Molkentin *et al.*, 1995; Zhao *et al.*, 1995). Neverthe-

less, MEF2 message and protein have also been detected in other tissues and cell types, and additional biological roles for this family of transcription factors have been proposed (Dodou *et al.*, 1995; Han and Prywes, 1995; Han *et al.*, 1997). In this regard, we have observed that the MEK5/BMK1 signal transduction pathway activates MEF2C-dependent reporter gene expression in a variety of cell lines, in addition to CHO cells, including COS-7, NIH-3T3 and C2C12 cells (Figure 6C).

The transactivation activity of MEF2C was recently shown to be regulated by p38-induced phosphorylation (Han *et al.*, 1997). The overlapping effects of two MAP kinases, p38 and BMK1, is not without precedent, since there are reports demonstrating that different MAP kinases share the same transcription factor as their downstream target (Gupta *et al.*, 1995; Whitmarsh *et al.*, 1995; Jiang *et al.*, 1996). Therefore, we compared the kinase activity of BMK1 toward recombinant MEF2C with that of two other MAP kinase family members, ERK2 and p38, in an *in vitro* protein kinase assay. In addition to MEF2C, the assay included equal amounts of recombinant PHAS-1, GST-c-Jun, GST-ATF2 and MBP as MAP kinase substrates. The results revealed that MEF2C is indeed a preferred substrate of BMK1 (Figure 6D). In addition, both ATF2 and MEF2C were preferred substrates for p38. When the amount of phosphorylated MEF2C was quantitated, the activity of BMK1 toward MEF2C was estimated to be ~3-fold higher than p38 and 80-fold higher than ERK2 in relation to their kinase activities for MBP.

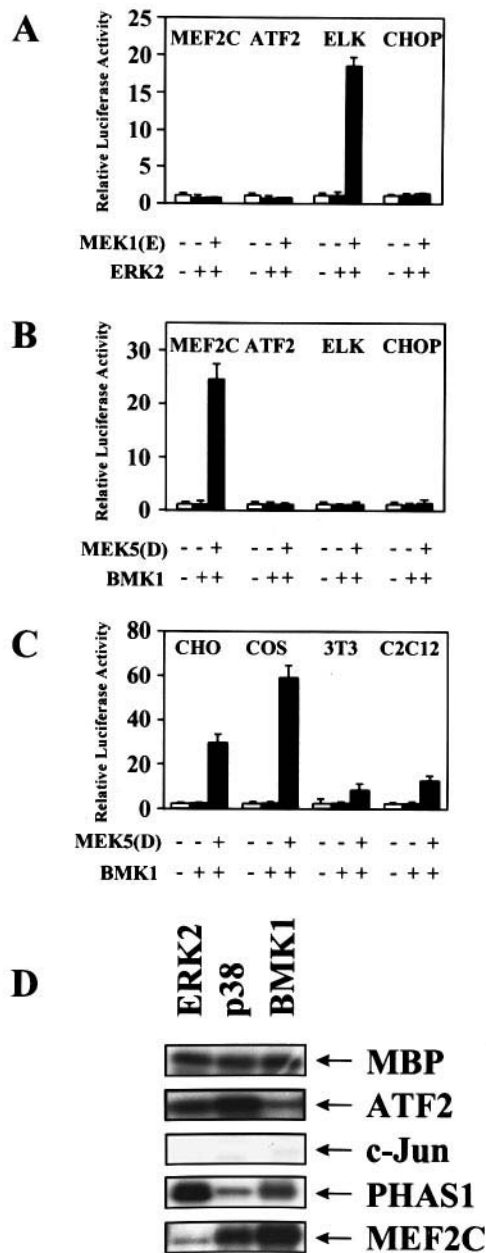


Fig. 6. BMK1 can regulate MEF2C transactivation activity. (A) C2C12 cells were co-transfected with vectors pCMV- β and the reporter plasmid pG5E1bLuc along with GAL4 fusion expression vectors containing either MEF2C, ATF2, ELK-1 or CHOP. To evaluate this system, ERK2 was also included in the above transfection procedure with or without MEK1(E) as indicated. The luciferase activities were normalized against cells transfected with pG5E1bLuc and GAL4 reporter plasmid alone, whose value was taken as 1. (B) Same as (A) except that BMK1 and MEK5(D) was used instead of ERK2 and MEK1(E). (C) CHO-K1, COS7, NIH-3T3 or C2C12 cells were transfected with pCMV- β , pG5E1bLuc and GAL4-MEF2C along with the expression vectors BMK1 or MEK5(D) as indicated in the figure. The luciferase activities were normalized against cells transfected with pG5E1bLuc and GAL4/MEF2C alone, without BMK1, whose value was taken as 1. (D) *In vitro* kinase assays were performed as described in Materials and methods using recombinant ERK2, p38 and BMK1 as the MAP kinases and MBP, GST-ATF2(1-109), GST-c-Jun(1-93), PHAS1 and His-MEF2 as substrates as indicated in the figure.

These data support the conclusion that BMK1 is capable of phosphorylating MEF2C.

We have shown that serum activates BMK1, but is without effect on activation of p38. Thus, serum was used as a stimulus to study BMK1-mediated MEF2C activation specifically without any contribution from p38. Serum has also been shown to stimulate the transcriptional activity of MEF2D, another member of the MEF group of transcription factors (Han and Prywes, 1995). To determine if serum can stimulate MEF2C activity, we measured MEF2C-dependent luciferase reporter gene expression in C2C12 cells treated with serum. Upon exposure to serum, C2C12 cells exhibited a 15-fold induction of MEF2C-dependent luciferase activity (Figure 7A). We have also observed serum induction of MEF2C activation in CHO-K1 and COS-7 cells (not shown). A series of co-expression experiments were performed in order to determine the relative contributions of p38 or BMK1 to MEF2C activation in cells exposed to serum. Co-expression of BMK1 with its constitutive active upstream kinase MEK5(D) resulted in a 23-fold induction of MEF2C-dependent reporter gene activity, while co-expression of p38 with its constitutive active upstream kinase MKK6b(E) resulted in a 5-fold induction (Figure 7A). Serum increased the MEF2C-dependent reporter gene expression in cells expressing p38 MAP kinase components ~3-fold, to a level comparable with that observed with serum treatment alone. In contrast, serum did not increase MEF2C-dependent reporter gene expression in cells co-expressing BMK1 and MEK5(D). Taken together, these results demonstrate that serum directly stimulates MEF2C activity, and suggest that BMK1 and serum-induced stimulation share a common pathway for MEF2C activation.

To show more definitively the involvement of BMK1 in serum-induced MEF2C activation, we added dominant-negative BMK1(AEF) to the serum stimulation assay described above. Introduction of BMK1(AEF) inhibited up to 95% of serum-induced MEF2C-dependent reporter gene expression in a dose-dependent manner. In contrast, p38(AGF), a dominant-negative form of p38 with a mutated dual phosphorylation site, was unable to inhibit the serum stimulation of MEF2C (Figure 7B). Since p38 is involved in MEF2C activation in response to inflammatory stimuli (Han *et al.*, 1997), the ability of a dominant-negative form of BMK1, but not p38, to inhibit serum-induced MEF2C activation demonstrates the strict specificity of the MAP kinase response to different extracellular stimuli even though these kinases appear to share a common downstream substrate. The ability of BMK1(AEF) to inhibit serum-induced activation of MEF2C is specific for this transcription factor, and not due to a non-specific inhibition of the serum response itself, since BMK1(AEF), but not ERK2(AEF), was unable to inhibit serum-induced ELK-1-dependent reporter gene expression (Figure 7C). These results support the contention that BMK1 specifically regulates the transactivation activity of MEF2C in response to serum stimulation.

MEF2C regulates the transcription of a variety of genes through corresponding AT-rich MEF2 promoter elements (McDermott *et al.*, 1994). To evaluate binding of the MEF transcription factor family, electrophoretic mobility shift assays (EMSA) were performed using a consensus oligonucleotide for the MEF2 DNA-binding site. Serum

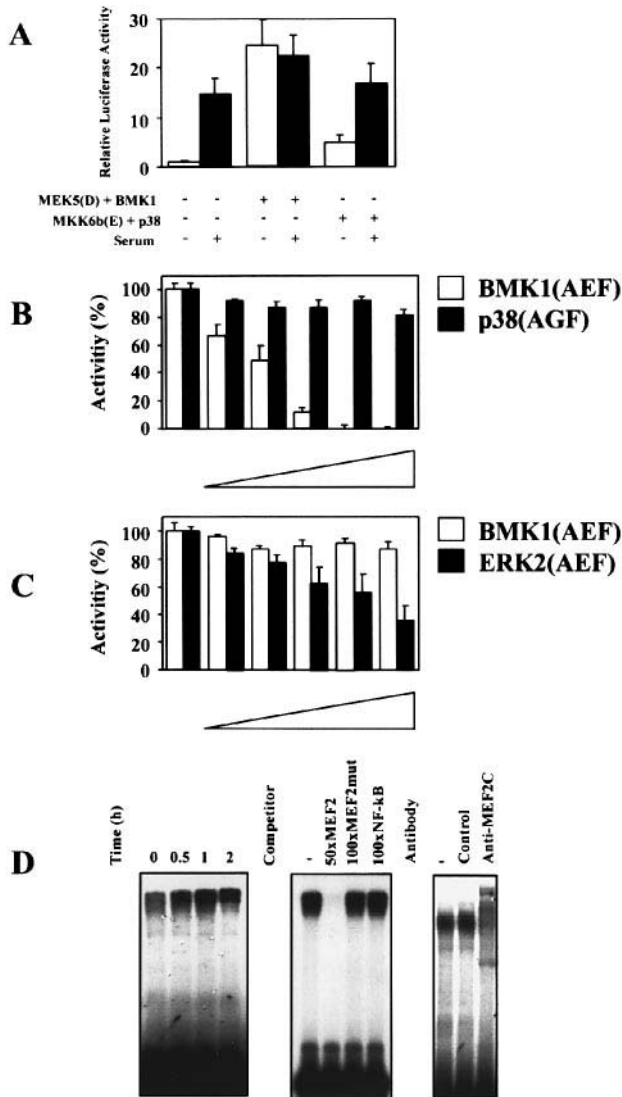


Fig. 7. BMK1 mediates serum-induced MEF2C activation. (A) C2C12 cells were transfected with pCMV- β , pG5E1bLuc and GAL4-MEF2C as described in Figure 5C, along with expression vectors encoding p38, MKK6b(E), BMK1 or MEK5(D) as indicated. After 24 h, the cells were transferred into serum-free media for an additional 24 h. Then 10% serum was added and the cells were incubated for an additional 4 h before harvesting. Relative luciferase activities were normalized to non-serum treated cells not expressing MAP kinases whose activity was taken as 1. (B) C2C12 cells were co-transfected with pCMV- β , pG5E1bLuc and GAL4-MEF2C with increasing amounts of BMK1(AEF) or p38(AGF) expression vector (0, 0.125, 0.175, 0.25, 0.375 and 0.5 μ g) as indicated. Serum stimulation was performed as described in (A). After correcting for transfection efficiency using β -galactosidase, all luciferase activities were normalized against serum-stimulated cells transfected with pG5E1bLuc and GAL4-MEF2C alone, whose activity was taken as 100%. (C) Same as (B) except that GAL4/ELK-1 and ERK2(AEF) were used instead of GAL4-MEF2C and p38(AGF). (D) Serum-starved C2C12 cells were incubated with 10% serum for the times indicated. Nuclear extracts from these cells were prepared and an EMSA assay was performed using a radiolabeled double-stranded oligonucleotide containing a MEF2 DNA-binding site as a probe. Excess unlabeled oligonucleotides containing a MEF2-binding site, a mutant MEF2 site and an unrelated NF- κ B DNA-binding site were used as competitors to determine binding specificity as described in Materials and methods. Control rabbit serum or anti-MEF2C immune serum was used to detect the presence of MEF2C in the complexes.

stimulation of the myoblast cell line C2C12 resulted in a time-dependent enhancement of MEF2 DNA-binding activity which reached a maximum after 1 h (Figure 7D). This binding activity is specific for the MEF2 site since it is competed effectively by an oligonucleotide containing a MEF2 motif but not by oligonucleotides containing either a mutated MEF2 site or an unrelated NF- κ B site (Figure 7D). To determine if MEF2C participates in serum-induced MEF2 DNA-binding activity, we incubated the complexes with an antibody specific to MEF2C. The MEF2C antibody shifted about half of the MEF2 DNA-binding complex, indicating the presence of MEF2C in this complex. However, the inability of the MEF2C antibody to shift all of the complex suggests the involvement of the other members of the MEF2 family.

Ser387 in MEF2C is the regulatory site for serum-induced MEF2C activation through BMK1

The sites in MEF2C phosphorylated by p38 have been shown previously to be Thr293, Thr300 and Ser387 (Han *et al.*, 1997). To identify sites phosphorylated by BMK1, we first treated recombinant MEF2C with either BMK1 or p38 *in vitro* and then performed tryptic phosphopeptide mapping. The peptide maps showed that BMK1 and p38 phosphorylated the same peptides, one containing amino acid residues Thr293 and Thr300 and the other containing Ser387 (Figure 8A). Based on these observations, mutants of the GAL4-MEF2C fusion protein were utilized to determine if these phosphorylation sites play a role in BMK1-mediated MEF2C activation *in vivo*. The MEF2C mutations which were constructed change Thr293 and Thr300 to alanines [MEF2C(T293,300A)], Ser387 to alanine [MEF2C(S387A)] and Ser240 to alanine [MEF2C(S240A)]. Expression of GAL/MEF2C(S387A), in the GAL4 driven luciferase reporter experiment described above, completely abolished the ability of serum or BMK1 to enhance reporter gene expression (Figure 8B and C). In contrast, GAL4/MEF2C(T293, 300A) had no effect on serum- or BMK1-mediated MEF2C activation (Figure 8B and C). We have observed similar results in serum-stimulated CHO-K1 and COS-7 cells transfected with the GAL4/MEF2C mutants (unpublished data). These results indicate that even though BMK1 can phosphorylate amino acids Ser387, Thr293 and Thr300 *in vitro*, only Ser387 is crucial for serum- and BMK1-mediated activation of MEF2C *in vivo*. We were interested in determining if Ser387 was also critical for p38-mediated activation of MEF2C. Therefore, we tested the MEF2C mutants in C2C12 cells co-expressing p38 with one of two dominant active forms of the upstream p38 kinases, MKK3b(E) or MKK6b(E). Surprisingly, GAL4/MEF2C(S387A) displayed identical activation levels to wild-type MEF2C, while GAL4/MEF2C(T293,300A) displayed no activation in response to p38 activation (Figure 8D). These results strongly suggest that although p38 and BMK1 MAP kinases activate MEF2C transactivation, they do so by phosphorylation at distinct sites of this transcription factor *in vivo*.

To confirm that Ser387 in MEF2C is phosphorylated after serum stimulation, we directly evaluated the phosphorylation site(s) of MEF2C after serum treatment of cells. To accomplish this, stably transfected C2C12 cells expressing either GAL4/MEF2C, GAL4/MEF2C(S387A)

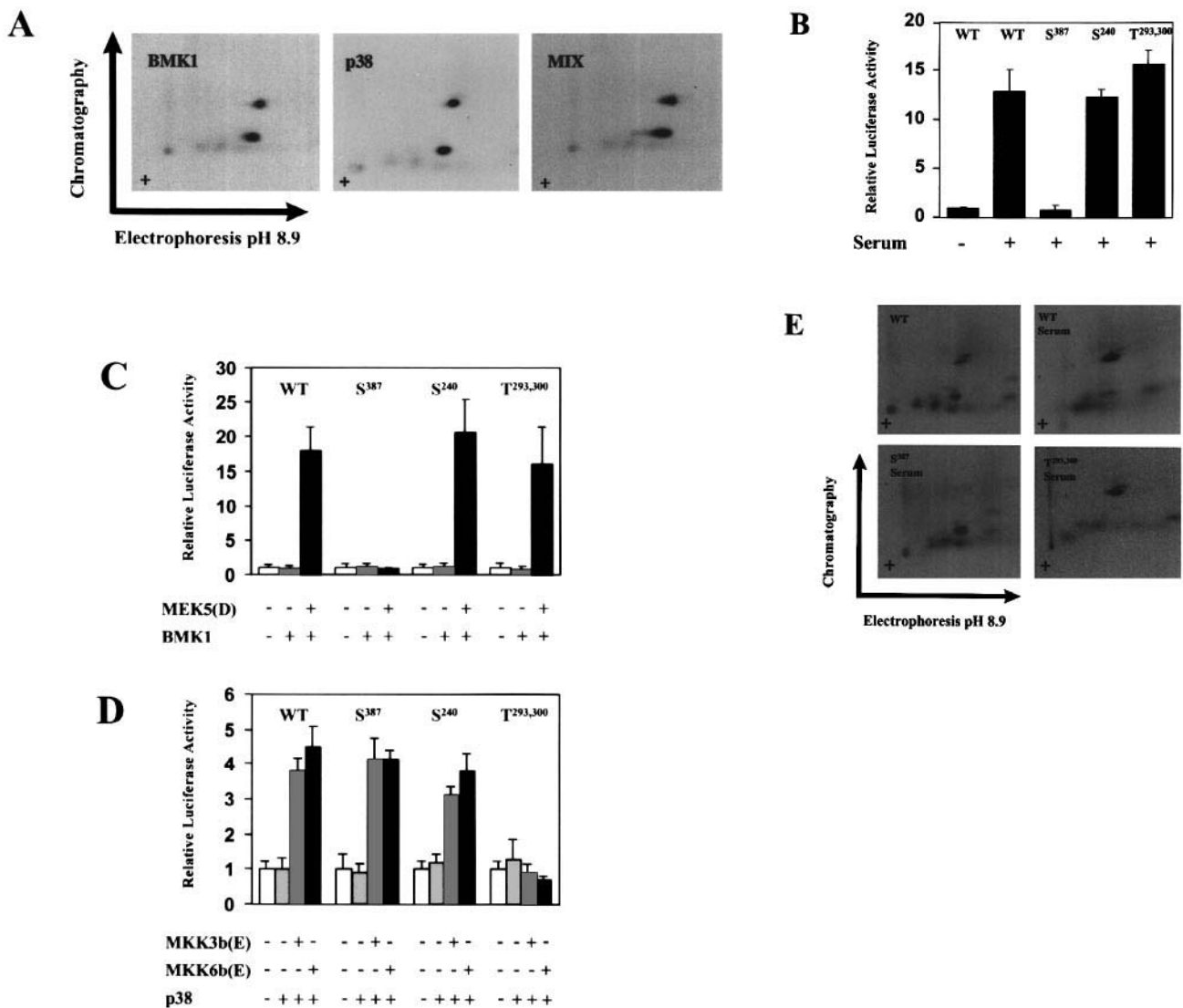


Fig. 8. Serum-induced MEF2C transactivation activity is mediated by BMK1 through phosphorylation of Ser387 on MEF2C. (A) A phosphopeptide map of MEF2C which was phosphorylated by p38 and BMK1 *in vitro* as indicated. A similar map of a 1:1 mixture of p38- and BMK1-mediated *in vitro* phosphorylation of MEF2C is also present. In (B), (C) and (D), C2C12 cells were co-transfected with pCMV- β , pG5E1bLuc and expression vectors containing either wild-type GAL4/MEF2C or the mutants; GAL4/MEF2C(S387A), (S240A) or (T293,300A), as indicated in the figure. The cells were starved as described in the legend of Figure 5. Relative luciferase activities were calculated as described in the legend of Figure 6. In (B), serum was used to stimulate cells for 4 h. In (C), expression vectors for BMK1 or MEK5(D) were included in the transfection as indicated. In (D), expression vectors for p38, MKK6b(E) or MKK3b(E) were included in the transfection as indicated. (E) Stable C2C12 cell lines expressing wild-type GAL4/MEF2C or the mutants GAL4/MEF2C (S387A) or (T293,300A) were starved in serum-free media for 24 h and then metabolically labeled with [³²P]orthophosphate for 3 h. The cells were then stimulated with 10% serum for 30 min as indicated. GAL4 fusion proteins were immunoprecipitated from cell lysates using the anti-GAL4 DNA-binding domain monoclonal antibody RK5C1 followed by phosphopeptide mapping as described in Materials and methods. WT indicates wild-type GAL4/MEF2C.

or GAL4/MEF2C(T293,300) were labeled *in vivo* with [³²P]orthophosphate and stimulated with serum. Tryptic phosphopeptide mapping of wild-type GAL4/MEF2C revealed that inducible phosphorylation occurred only on one peptide, which corresponded exactly in position to the peptide containing Ser387 (Figure 8E). The importance of this phosphorylation was confirmed in studies using the GAL4/MEF2C(S387A) mutant which failed to show inducible phosphorylation of this peptide. In contrast, the peptide isolated from the GAL4/MEF2C(T293,300A) mutant was phosphorylated in response to serum in a manner identical to wild-type GAL4/MEF2C.

The MEK5/BMK1 pathway is involved in serum-induced c-Jun expression

The oncogene *c-jun* is one of many early genes induced by serum (Lamph *et al.*, 1988; Ryder and Nathans, 1988). The promoter of the *c-jun* gene contains a MEF2 site which has been shown to be required for serum-induced c-Jun expression in HeLa cells (Han and Prywes, 1995). Based on our studies, we speculate that BMK1 may be involved in serum-induced c-Jun expression through its regulation of MEF2C activity. To examine this, we utilized two expression plasmid encoding a luciferase gene; one driven by the wild-type c-Jun promoter (pJluc), and the

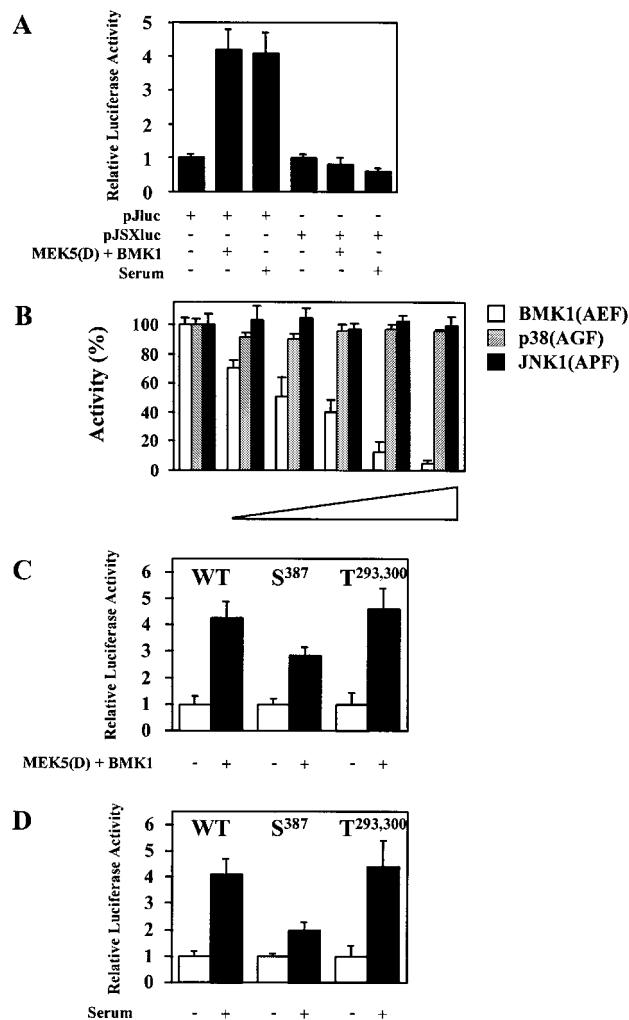


Fig. 9. Phosphorylation of MEF2C increases gene expression driven by the c-Jun promoter. (A) C2C12 cells were transfected with the reporter plasmid pJluc or pJSXluc which encode luciferase genes driven by either the wild-type c-Jun promoter or a c-Jun promoter which contains a mutated MEF2 site, respectively. In addition, these cells were either stimulated with serum or co-transfected with expression vectors encoding either BMK1 and MEK5(D) or p38 and MKK6b(E) as indicated. (B) C2C12 cells were co-transfected with the reporter plasmid pJluc and an expression vector containing MEF2C along with increasing amounts (0, 0.125, 0.175, 0.25, 0.375 and 0.5 μ g) of BMK1 (AEF), p38(AGF) or JNK1(APF) expression vector as indicated. Serum stimulation was performed as described in Figure 6. The luciferase activities were normalized against serum-stimulated cells transfected with the reporter plasmid pJluc and the MEF2C expression vector alone whose value was taken as 100%. (C) and (D) C2C12 cells were transfected with the reporter plasmid pJluc and expression vectors encoding either wild-type MEF2C or the MEF2C mutants (S387A) or (T293,300) as indicated. In addition, these cells were co-transfected with BMK1 and MEK5(D) (C) or treated with serum (D) as indicated.

other driven by a c-Jun promoter containing a mutated MEF2 site (pJSXluc) (Han and Prywes, 1995). Both serum stimulation and expression of activated BMK1 induced luciferase expression driven by the c-Jun promoter (Figure 9A). Mutation of the MEF2 site in the c-Jun promoter abolished the induction of reporter gene expression in C2C12 cells either stimulated with serum or expressing activated BMK1 (Figure 9A). Serum-induced luciferase expression, driven by the c-Jun promoter, was inhibited

dramatically by dominant-negative BMK1(AEF), but not by dominant-negative p38(AGF) or dominant-negative JNK1(APF). These results confirm an important role for BMK1, but not for p38 or JNK1, during serum-induced cellular activation. To determine if the MEF2C transcription factor is responsible for the MEF2 site-dependent activation of the reporter gene, we co-expressed activated BMK1 in C2C12 cells with either wild-type MEF2C, the MEF2C mutant T293,300A or the MEF2C mutant S387A. In this assay, c-Jun promoter-driven luciferase expression was enhanced by activated BMK1 in cells transfected with wild-type MEF2C and the mutant MEF2C(T293,300A) but was severely reduced in cells expressing the mutant MEF2C(S387A) (Figure 9C). We observed identical results in serum-stimulated C2C12 cells expressing these MEF2C constructs (Figure 9D). The inability of the MEF2C mutant S387A to completely inhibit BMK1 or serum-mediated activation of c-Jun-driven luciferase expression is probably due to the presence of endogenous MEF2 family proteins in these cells.

Discussion

Here we provide several lines of evidence which prove that exposure of cells to serum activates BMK1/ERK5, that this activation is dependent on MEK5 and that activated BMK1 phosphorylates the transcription factor MEF2C. The activation of this pathway causes increased transactivation activity of MEF2C, with one consequence being increased expression of the early gene *c-jun*. These data provide the first insight into the regulation and biological consequences of BMK1 activation.

The MAP kinase signaling cascade consists of three protein kinases that act sequentially within one pathway: a MEKK, a MEK and a MAP kinase. Through the use of a constitutively activate form of MEK5, we show that this MAP kinase kinase is specific for BMK1 and has little or no effect on the activation of other members of the MAP kinase family in co-transfection experiments. Although MEK5 and BMK1 represent part of a unique signaling pathway distinct from that used by other MAP kinases, the upstream regulatory kinase of MEK5 has not been identified. Others have shown that MEK5 cannot be phosphorylated by the MEKKs, Raf-1, c-Mos or MEKK1 (English *et al.*, 1996). Studies are currently underway to identify cellular activators of MEK5.

The subcellular distribution of BMK1, revealed by indirect immunofluorescence microscopy, showed that BMK1 was present in the cytoplasm but appeared to be excluded from the nucleus. The activation of BMK1 with MEK5(D), however, resulted in the appearance of BMK1 in the nucleus (Figure 5). This event supports the contention that BMK1 has a role in regulating the activity of nuclear factors.

The MEF2 family members, along with certain specific splice variants, are expressed preferentially in muscle tissue and in the nervous system (Yu *et al.*, 1992; Leifer *et al.*, 1993; Martin *et al.*, 1993, 1994; McDermott *et al.*, 1994). Nevertheless, MEF2 mRNA has also been detected in other tissues and, recently, several groups reported MEF2 binding activity and MEF2 proteins in a wide variety of cell types (Dodou *et al.*, 1995; Han and Prywes, 1995; Han *et al.*, 1997). These findings suggest that the

		*		
MEF2C	N' -HTRHEAGR	S	PVDSLSS-CSSSYDG-C'	417/439
MEF2A	QPRQEMGR	S	PVDSLSS-SSSSYDG	445/467
MEF2D	AVFFAARP	E	PGEGLSSPAGGSYET	453/476
MEF2B	-----		-----	

Fig. 10. Ser387 is conserved in both MEF2C and MEF2A. Sequence comparison of members of the MEF2 group of transcription factors in the region containing the Ser387 residue identified in MEF2C. 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.

expression and function of MEF2 transcription factors is more ubiquitous than originally thought. In support of this, Han *et al.* have shown that the MAP kinase p38 regulates the transactivation activity of MEF2C in macrophage cells induced with Gram-negative bacterial lipopolysaccharide as part of the inflammatory response (Han *et al.*, 1997). Furthermore, this group demonstrates that p38-dependent MEF2C activation occurs through phosphorylation of Ser387, Thr293 and Thr300 in the C-terminal domain of MEF2C. Here, using C2C12, CHO-K1 and COS-7 cells, we have shown that Ser387, and not Thr293 and Thr300, is the target for serum-induced BMK1-dependent MEF2C activation. The different sites in MEF2C utilized by BMK1 and p38 to regulate its activity do not appear to be due to differences in substrate specificity between these two MAP kinases, since the tryptic peptide mapping results presented here show that BMK1 and p38 phosphorylate all three sites in an *in vitro* protein kinase reaction. We speculate that unidentified cofactors may be involved in regulating the phosphorylation of alternative sites in MEF2C. Moreover, we show that in C2C12, CHO-K1 and COS7 cells, p38-dependent activation of MEF2C occurs only through phosphorylation of Thr293 and Thr300, whereas in the RAW 264.7 cell line all three phosphorylation sites are required for p38-dependent activation (Han *et al.*, 1997). Therefore, the putative cofactors suggested to be involved in the regulation of MEF2C activity appear to be cell type specific.

The MADS box transcription factor Rlm1, of *Saccharomyces cerevisiae*, shares sequence homology with the mammalian MEF2 transcription factors (Dodou and Treisman, 1997). The C-terminal domain required for transcriptional activation of Rlm1 has been shown to be a target of the MAP kinase Mpk1, which is the yeast counterpart of mammalian ERK MAP kinases (Watanabe *et al.*, 1995, 1997; Dodou and Treisman, 1997). The MADS box and MEF2 site of Rlm1, which are responsible for DNA binding and dimerization, share sequence homology with mammalian MEF2 members. In contrast, the C-terminal domain of Rlm1, which is regulated by Mpk1, has little or no sequence similarity with mammalian MEF2 proteins. We have shown here that the mammalian MEF2C transcription factor is regulated by BMK1 and p38 through C-terminal phosphorylation sites. Therefore, it appears that the structure and mechanism of regulation of these transcription factors have diverged from yeast to mammals to adopt other MAP kinase pathways, such as BMK1 and p38, probably in order to accommodate the more diverse biological responses found in mammalian systems.

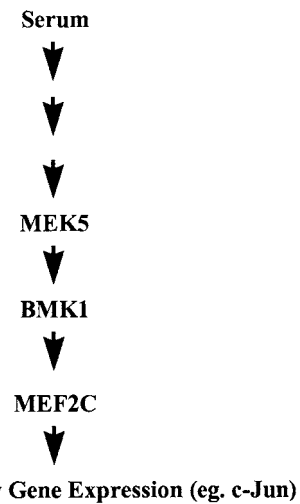


Fig. 11. Proposed model for the BMK1-mediated signal transduction pathway. The BMK1 signaling pathway is activated by serum via activation of MEK5 which phosphorylates and activates BMK1. Activated BMK1 then phosphorylates and activates transcription factor MEF2C which enhances the transcription of immediate early genes such as *c-jun*.

Serum induces the expression of a number of immediate early genes which are essential for cell growth (Carter *et al.*, 1991; Kovary and Bravo, 1991). The expression of one of these genes, *c-jun*, is regulated through a MEF2 promoter element during serum stimulation (Han and Prywes, 1995). In contrast to our results with MEF2C, the transcription factor MEF2D has been shown to be involved in the serum induction of the *c-Jun* promoter through regulation of its binding domain. The C-terminal domain of MEF2D appears to play no role in the serum regulation of *c-Jun* (Han and Prywes, 1995). A sequence comparison between MEF2 family members indicates that only MEF2A, but not MEF2B and MEF2D, has a serine at the position equivalent to Ser387 found in MEF2C (Figure 10). In fact, MEF2D possesses a glutamine residue at this position which is negatively charged and unable to be regulated through phosphorylation. Since the results of our bandshift experiments indicate the presence of other MEF2 family members, we currently are exploring the possibility that MEF2A may also be regulated by BMK1 through phosphorylation of this serine residue. We have analyzed the sequence of a number of immediate early genes and found potential MEF2-binding sites in the promoters of *junB* (DDBJ/EMBL/GenBank accession No. U20734), *fra-1* (DDBJ/EMBL/GenBank accession No. D14493) and *CL100* (DDBJ/EMBL/GenBank accession No. U01669). Although the role of the MEF2 sites in the promoters of these genes still needs to be examined, the implication is that MEF2 proteins may have a broader role in regulating early gene expression than previously has been appreciated.

In summary, we show that serum treatment regulates the activity of the transcription factor MEF2C which subsequently modulates the expression of *c-jun*, a gene required for cell proliferation (Figure 11). Furthermore, we demonstrate that serum-induced MEF2C activation requires the MEK5/BMK1 signaling pathway, but not the p38 signaling pathway which is responsible for MEF2C

activation during the inflammatory response. Thus, the MEF2C transcription factor integrates BMK1 and p38 MAP kinase signaling pathways *in vivo* to coordinate biological responses to different types of extracellular stimuli. More importantly, these studies demonstrate for the first time that a given transcription factor can be regulated by two distinct MAP kinase signaling pathways through the utilization of different phosphorylation sites.

Materials and methods

Reagents

MBP and PHAS1 were purchased from Sigma (St. Louis, MO) and Stratagene (La Jolla, CA), respectively.

Cell culture

CHO-K1, NIH-3T3 and COS-7 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin and 1% non-essential amino acids. C2C12 cells were maintained in the same medium but with 20% FCS.

Immunoprecipitation and protein kinase assay

Cells were solubilized with lysis buffer [20 mM HEPES (pH 7.6), 1% Triton X-100, 137 mM NaCl, 0.1 mM Na₃VO₄, 25 mM β-glycerophosphate, 3 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and the resulting lysate was centrifuged at 15 000 *g* for 15 min at 4°C. Epitope-tagged protein kinases were immunoprecipitated using monoclonal antibodies against the flag epitope, M2 (IBI-Kodak, New Haven, CT), the c-Myc epitope, 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) or the hemagglutinin (HA) epitope, 12CA5 (kindly provided by Dr Ian Wilson, TSRI). Endogenous BMK1 and MEK5 were immunoprecipitated using specific anti-BMK1 antibody (Abe *et al.*, 1996) or anti-MEK5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were bound independently to protein G-Sepharose beads, and protein kinases were then immunoprecipitated by incubation for 16 h at 4°C with the antibody-bound protein G-Sepharose beads. The beads were washed twice with 1 ml of lysis buffer and twice with 0.5 ml of kinase reaction buffer [20 mM HEPES (pH 7.6), 20 mM MgCl₂, 25 mM β-glycerophosphate, 0.1 mM Na₃VO₄ and 2 mM dithiothreitol]. The presence of the epitope-tagged proteins in the immunoprecipitates was confirmed by Western blot analysis using M2, 12CA5 or 9E10 antibody. Kinase assays were performed using MBP or the recombinant proteins BMK1(M), ATF2, c-Jun, PHAS-1 and MEF2C as substrates, and analyzed as described elsewhere (Han *et al.*, 1996; Jiang *et al.*, 1996).

cDNA constructs and expression plasmids

Dominant-negative MAP kinases were created by PCR-based mutagenesis (Han *et al.*, 1996; Jiang *et al.*, 1996) by mutating the dual-phosphorylation site or ATP-binding site as follows: BMK1(AEF) (Thr218 and Tyr220 with alanine and phenylalanine), BMK1(M) (Lys83 with methionine), MEK5(A) (Ser311 and Thr315 with alanine), ERK2(M) (Lys52 with methionine), ERK2(AEF) (Thr183 and Tyr 185 with alanine and phenylalanine) and p38(AGF) (Thr180 and Tyr182 with alanine and phenylalanine). The flag epitope tag, DYKDDDDK, was added to the N-terminus of BMK1 or ERK2 by PCR as described for p38 (Jiang *et al.*, 1996), and the resulting cDNA fusion was cloned into pcDNA3 (Invitrogen, San Diego, CA). Rat MEK5α-1 (English *et al.*, 1996) was cloned into the *Clal* and *HindIII* sites of the vector pCMV5 which introduced three copies of the HA epitope YPYDVPDYAGYPYDVPDYAGSPYDVPDYAAQC at the C-terminus. A PCR-based procedure (Han *et al.*, 1996; Jiang *et al.*, 1996) was used to create a dominant active form of MEK5, MEK5(D), by replacing the phosphorylation sites Ser313 and Thr317 with Asp. The constructs Myc-ERK1, Flag-p38 and Flag-JNK1 cDNA have been described previously (Han *et al.*, 1996; Jiang *et al.*, 1996). Plasmids expressing GAL4 fusion protein GAL4/MEF2C, MEF2C(T293,300A), MEF2C(S387A) and MEF2C(S240A) were constructed as described (Han *et al.*, 1997).

Preparation of recombinant proteins

The DH5α strain of *Escherichia coli* was transformed with the vector pGEX-2X (Pharmacia Biotech, Piscataway, NJ) containing cDNAs

encoding either full-length BMK1 or BMK1(M). The transformed bacteria were grown at 37°C in Luria broth until the optical density was 0.5, at which time isopropyl-β-D-thiogalactopyranoside (1 mM final) was added for 2 h. The cells were collected by centrifuging at 8000 *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 PMSF) for every 100 ml of original bacterial culture. The cell suspension was sonicated, and cellular debris was removed by centrifuging at 100 000 *g* for 30 min. Recombinant BMK1 and BMK1(M) proteins were purified from the cleared lysate using a glutathione-Sepharose 4B column (Pharmacia Biotech, Piscataway, NJ). GST-ATF(1-109), GST-c-Jun(1-93) and His-MEF2C were prepared as described previously (Han *et al.*, 1997).

Reporter gene expression

The GAL4-responsive reporter plasmid pG5E1bLUC contains five GAL4 sites cloned upstream of a minimal promoter driving a luciferase gene (Gupta *et al.*, 1995). 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 (Han and Prywes, 1995). The reporter plasmids pG5E1bLuc, pJluc or pJSXluc were co-transfected into cells along with a construct encoding the GAL4-binding domain fused to either ATF2(1-505) (Gupta *et al.*, 1995), ELK-1(307-428) (Raingeaud *et al.*, 1996), CHOP (Wang and Ron, 1996) or MEF2C(87-441) (Han *et al.*, 1997) transcription factors. Cells were grown on 35 mm multiwell plate (Nunc, Naperville, IL) and transiently transfected with 1 μg of total plasmid DNA using lipofectamine reagent (Gibco BRL, Gaithersburg, MD) in the presence of OPTI-MEM (Gibco BRL). A β-galactosidase expression plasmid called pCMVβ-gal (Clontech, Palo Alto, CA) was employed to control for transfection efficiency. The total amount of DNA for each transfection was kept constant using the empty vector pcDNA3. After 24 h, the medium was changed to serum-free DMEM supplemented with 2 mM glutamine and non-essential amino acids. At 48 h after transfection, the cells were treated with or without stimuli as described in the figure legends. Cell extracts were prepared and the activities of β-galactosidase and luciferase was measured as described elsewhere (Han *et al.*, 1996; Jiang *et al.*, 1996). To correct for transfection efficiency, the luciferase activity was divided by the β-galactosidase activity in every case. The data represent the means and standard deviations of at least three independent transfections.

Phosphopeptide mapping and EMSA

Phosphopeptide mapping and EMSA were performed as described previously (Han *et al.*, 1997). Briefly, for EMSA, nuclear extracts of C2C12 cells treated with or without 10% serum for different times were incubated with a double-stranded, ³²P-labeled oligonucleotide containing a MEF2 DNA-binding site as a probe. An unlabeled MEF2 oligonucleotide, an oligonucleotide with a mutation in the MEF2 site (MEF2mut) (Han and Prywes, 1995) or an unrelated oligonucleotide containing a NF-κB site were used as competitors to determine the binding specificity as described elsewhere (Lee *et al.*, 1993). A control rabbit serum and anti-MEF2C antiserum (Molkentin *et al.*, 1996) were used in the EMSA assay to detect MEF2C in the DNA-binding complex.

Immunocytochemistry

Coverslips were pre-coated with 10 μg/ml fibronectin (Collaborative Biomedical, Bedford, MA) for 16 h at 4°C and then blocked with 1% denatured bovine serum albumin (BSA) for 1 h at 37°C. Transfected CHO-K1 cells were plated directly on the coverslips and allowed to adhere for 5 h at 37°C in serum-free RPMI medium. The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and were then permeabilized with 0.2% Triton X-100 in PBS for 1 min. After washing twice with PBS, the cells were incubated in blocking buffer (PBS + 1% BSA) for 1 h at room temperature. Primary antiserum (anti-BMK1 antibody) (Abe *et al.*, 1996) was applied to the cells at a 1:1000 dilution for 1 h at room temperature. Five μg/ml of the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and a 1:250 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig secondary antibody (Tago Immunological, Burlingame, CA) were applied to the cells in the blocking buffer for 30 min at room temperature. The cells were washed and mounted onto slides with fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) for immunofluorescence analysis. As a control, the transfected cells were stained similarly using pre-immune serum to assess the specificity of the observed immunofluorescence.

Acknowledgements

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