2646–2654 *Nucleic Acids Research, 1999, Vol. 27, No. 13* *© 1999 Oxford University Press*

Post-translational control of the MEF2A transcriptional regulatory protein

Olga I. Ornatsky[1], David M. Cox[1,2], Padma Tangirala[1], John J. Andreucci[1], Zoë A. Quinn[1,2], Jeffrey L. Wrana[3], Ron Prywes[4], Yie-Teh Yu[5] and John C. McDermott[1,2,*]

[1]Department of Biology, York University, Toronto, Ontario M3J 1P3, Canada, [2]Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK, [3]Program in Developmental Biology and Division of Gastroenterology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, [4]Department of Biological Sciences, Columbia University, New York, NY 10027, USA and [5]Division of Cardiology, Department of Medicine and Biochemistry, Vanderbilt University, Nashville, TN 37232, USA

Received March 26, 1999; Revised and Accepted May 6, 1999

ABSTRACT

Myocyte enhancer factor 2 (MEF2) transcriptional regulatory proteins are key regulators of muscle-specific gene expression and also play a general role in the cellular response to growth factors, cytokines and environmental stressors. To identify signaling pathway components that might mediate these events, the potential role of MAP kinase and PKC signaling in the modulation of MEF2A phosphorylation and transcriptional activity were therefore studied. In transient transfection reporter assays, activated p38 MAP kinase potently increased MEF2A *trans*-activating potential, PKCδ and ε isotypes enhanced MEF2A transactivation to a lesser extent, while the ERK1/2 and JNK/SAPK pathways were without effect. A GAL4-based assay system showed that p38 MAP kinase and PKCδ target the MEF2A transactivation domain. We also observed an increase in p38 MAP kinase activity in congruence with the increase in MEF2A expression in differentiating primary muscle cells. COS cells overexpressing MEF2A alone or with one of the kinases were metabolically labeled with [^{32}P]orthophosphate and MEF2A was immunoprecipitated using specific anti-MEF2A antibodies. MEF2A from cells co-transfected with activated p38 MAP kinase showed a decreased electrophoretic mobility due to phosphorylation. Subsequent phosphopeptide mapping and phosphoamino acid analysis indicated the appearance of several phoshopeptides due to p38 MAP kinase activation of MEF2A which were due to phosphorylation on serine and threonine residues. These studies position MEF2A as a nuclear target for the p38 MAP kinase signaling pathway.

INTRODUCTION

Post-translational modification of transcription factors by targeted phosphorylation is proving to be a regulatory paradigm in the control of gene expression (1). Myocyte enhancer factor 2 (MEF2) transcriptional regulatory proteins play a requisite role in the activation of muscle-specific gene expression and in the processes of cardiac and skeletal muscle differentiation (2–8). However, despite considerable understanding of the transcriptional role of MEF2 proteins in the activation of muscle genes, the regulation of these factors by cellular signaling pathways is only just beginning to be understood. Recent studies have suggested that expression of the MEF2 proteins and MEF2 DNA binding activity are not always correlated with the activation of MEF2 site-dependent target genes, an observation supporting the idea that post-translational modification of these proteins by signaling pathways is an important regulatory step controlling their capacity to activate transcription (9).

The MEF2 proteins have, to date, been implicated as nuclear targets for signaling cascades in response to serum stimulation and some cellular stressors (10–12). Amino acid sequence analysis of the MEF2 proteins indicates that they contain numerous putative phosphorylation sites for the PKC and MAP kinase classes of signaling molecules. Indeed, Han *et al.* found that in monocytic cells the response to the microbial pathogen lipopolysaccharide led to enhanced transactivation capacity of MEF2C through p38 MAP kinase catalyzed phosphorylation (11). MEF2 factors have also been implicated in receiving signals from heterotrimeric G protein-linked signaling pathways (12) and the ERK5/BMK1 pathway (13,14).

The PKC family of serine-threonine kinases participate in vital cellular processes such as growth and differentiation, acting as components of several signal transduction cascades initiated by ligand stimulation of transmembrane receptor tyrosine kinases by growth factors (15,16). This large gene family gives rise to multiple isozymes that are classified as conventional cPKCs (α, β and γ), novel nPKCs (ε, δ, η and θ) and atypical aPKCs (ζ, μ and λ). Tissue distribution of PKCs seems to indicate that while some isozymes are ubiquitiously expressed and may play a 'housekeeping' role, others display a more restricted

*To whom correspondence should be addressed at: Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, UK. Tel: +44 191 374 2779; Fax: +44 191 374 2417; Email: j.c.mcdermott@durham.ac.uk

pattern of tissue-specific expression and may play a role in myotube formation (15,17).

MAP kinase family members are proline-directed serine-threonine kinases that play a pivotal role in the response of cells to extracellular stimuli (reviewed in 18). In response to these signals, the MAP kinases are phosphorylated by a dual specificity MAP kinase kinase on both threonine and tyrosine in a Thr-Xaa-Tyr motif that is situated in a loop close to the active site. This phosphorylation activates the enzymatic activity of the MAP kinases and, in turn, they phosphorylate and modify the activity of various target proteins such as transcription factors, other kinases and cytosolic enzymes. There are three distinct classes of MAP kinases, the ERKs (for extracellular-regulated kinases), the JNK/SAPKs (for c-*jun* N-terminal kinases/stress-activated protein kinases) and the p38 MAP kinases (also known as CSBP, RK and HOG 1) (reviewed in 18).

As key regulators of muscle-specific gene expression the MEF2 proteins stand at an important nodal point in the differentiation of cardiac and skeletal muscle cells, making them potentially strategic targets for regulation by cellular signaling pathways involved in growth and differentiation. Our previous studies have shown that the predominant MEF2 gene expressed at the induction of myogenesis is MEF2A (9). Concomitantly, the majority of protein binding to the MEF2 site in cultured muscle cells is a MEF2A homodimer (9). Also, a dominant-negative form of MEF2A was found to block myogenesis when overexpressed in cultured muscle cells (2). It has also recently been reported that MAP kinase signaling exerts an important effect on the differentiation of cultured muscle cells (19,20). Therefore, in order to begin to identify candidate signaling molecules that transduce signals from cell surface receptors to the nuclear MEF2 proteins in muscle cells, we have studied the ability of MAP kinase and PKC isoforms to phosphorylate and modulate the transcriptional activity of MEF2A.

In this study we show that MEF2A is a phosphoprotein and that p38 MAP kinase and, to a lesser extent, the novel PKC isotypes δ and ε positively augment MEF2A transcriptional activity and phosphorylation. These data point toward a role for p38 MAP kinase and novel PKCs in cooperating with MEF2A to activate MEF2 site-dependent target genes.

MATERIALS AND METHODS

Cell culture, transfections and reporter assays

COS and HeLa cells were cultured to ~60% confluence in DMEM containing 10% fetal bovine serum (FBS). Calcium phosphate–DNA co-precipitation was used to transiently transfect cells with various DNA expression vectors and reporter genes (21). For reporter assays the appropriate combination of reporter and expression plasmids was transfected and cells were glycerol shocked after 16 h and harvested after 2 days. Each plate of cells was transfected with 10 μg of the CAT reporter construct and 3 μg of pSV β-galactosidase, which served as an internal control for transfection efficiency. For the overexpression studies 5 μg of each expression vector was transfected [pMT2-MEF2A, pTB-PKCα, β, δ, ε and θ, pMT3-p38 MAP kinase, pcDNA-MKK6b(E), pCMV5 Erk2.1 His6, pMM9 MEK (ΔN3/ED), pcDNA3-HA-SEK and pMT2 HA-p46SAPKβ] or the corresponding empty expression vectors alone as a control (see figure legends for details on individual experiments). Total DNA transfected was standardized at 30 μg for 100 mm dishes (or half of the above amounts for 60 mm dishes when used). Cell extracts were prepared and CAT activity was determined as previously reported (21). The reporter genes used comprised two copies of the muscle creatine kinase (MCK) MEF2 sites, inserted in a concatemerized orientation at the –102 position of the embryonic myosin heavy chain promoter in plasmid pE102CAT. Where indicated, stock 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma) was dissolved in absolute ethanol and used at a concentration of 800 nM. Short-term (1 h) treatment of cells with TPA activates PKC (22). For serum stimulation cells were co-transfected with pMT2-MEF2A, pMT2-MEF2C or pMT2 and the reporter gene pE102CAT 2×MEF2 in growth medium (DMEM with 10% FBS) for 24 h, which was then replaced by serum-free medium with incubation for a further 24 h. Some cultures then received fresh serum-free medium (open bars) while others received DMEM with 10% FBS and cells were collected for β-galactosidase and CAT assays 24 h later. Relative CAT activity levels were normalized to β-galactosidase expression.

Gal4 one-hybrid assays

COS or HeLa cells seeded at ~60% confluence were transfected with a total amount of 15 μg of DNA. Aliquots of 5 μg of the reporter plasmid, $(GAL4)_5$LUC, and 5 μg of the GAL4MEF2A expression vector were used in these assays. We used two different Gal4-MEF2A fusions shown previously to contain the MEF2A transactivation domains (Gal4-MEF2A 274–373 and Gal4-MEF2A 299–373 (23). The empty vector PSG424, which contains only the DNA binding domain of yeast GAL4, was used as the control (24). Where appropriate, GAL4MEF2A was co-transfected with various expression vectors [pTB-PKCδ and ε and pMT3-p38 MAP kinase with constitutively active pcDNA3-MKK6b(E)]. To control for the transfection efficiency, 2 μg of pSV-β galactosidase was used. Where indicated, the polyamide imidazole p38 MAP kinase inhibitor SB203580 (Calbiochem) in DMSO was added to the cultured cell medium to a final concentration of 1 μM; DMSO was added to the controls. Twenty four hours after transfection the cells were fed with fresh medium and harvested after another 48 h. Cell extracts were then analyzed for β-galactosidase and luciferase activity as previously described (2,9).

Immunoprecipitation and immunoblotting

Under identical experimental conditions in which we have observed functional modulation of MEF2A activity by p38 MAP kinase and PKCs, COS cells transfected with expression vectors were lysed and MEF2A was immunoprecipitated with anti-MEF2A antibody followed by adsorption to protein A–Sepharose beads (Pharmacia) as previously described (9). Immunoprecipitates were resolved by 8% SDS–PAGE and analyzed by western blotting as described previously (2,9). For the phosphatase treatment of immunoprecipitated complexes, C2C12 myotubes were cultured in growth (10% FBS in DMEM) or differentiation [5% horse serum (HS) in DMEM] medium and immunoprecipitation was done using anti-MEF2A antisera as described above. After washing, the beads were suspended in calf intestinal phosphatase (CIP) buffer (100 mM Tris, pH 8.0, 100 mM NaCl and 5 mM $MgCl_2$) with 1 μl/tube of CIP enzyme (10 U/μl; New England Biolabs) and

incubated for 20 min at 37°C with gentle shaking. Beads were then washed and boiled in SDS–PAGE buffer for immunoblotting. The anti-MEF2A antibody has been extensively characterized previously (2,9,10).

p38 MAP kinase activity assay

Myoblasts (primary rat and C2C12) were grown on 100 mm gelatin-coated plates. The rat primary myoblasts were cultured in DMEM supplemented with 10% FBS and 5% HS; C2C12 were cultured in DMEM supplemented with 10% FBS. The rat primary myoblast cultures were prepared by overnight cold trypsinization at 50 μg/μl trypsin (Worthington Enzymes), and collagenase digestion at 37°C in 0.1% collagenase. Cells were preplated for 1 h to deplete fibroblasts and then seeded on gelatin-covered tissue culture dishes.

In order for differentiation to occur, the medium was changed to DMEM supplemented with 5% HS. The kinase assay was carried out using a p38 MAP Kinase Assay Kit (New England Biolabs). For collection, the cells were washed with phosphate-buffered saline and incubated for 5 min in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF). The cells were transfered to a microcentrifuge tube, then sonicated four times for 5 s each time and pelleted in a microcentrifuge tube. The p38 MAP kinase antibody was added to 200 μg of total protein as suggested in the manufacturer's instructions and the total volume was made up to 200 μl with lysis buffer. This was incubated with gentle rocking overnight at 4°C. Aliquots of 10 μl of 50% protein A–Sepharose beads were added for 1.5 h with gentle rocking at 4°C. Samples were then microcentrifuged for 30 s at 4°C. The pellet was washed twice with 500 μl of lysis buffer. The pellet was washed twice with 500 μl of kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). The pellet was then suspended in 50 μl of kinase buffer with 200 μM ATP and 2 μg of ATF-2 fusion protein. This was incubated at 30°C for 30 min. The reaction was then terminated with 25 μl of 3× SDS sample buffer (187.5 mM Tris–HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.3% w/v bromophenol blue). The samples were then boiled for 5 min and 30 μl was loaded on a 10% SDS–PAGE gel, transferred to nitrocellulose and probed with anti-phospho-ATF-2 (Thr-71) antibody. Where appropriate, MT were treated for 30 min with 30 μg/ml of anisomycin (an activator of p38 MAP kinase).

Metabolic labeling

COS cells co-transfected with expression vectors (as indicated in the figure legends) were metabolically labeled with 1 mCi/ml [^{32}P]orthophosphate for 2 h. Labeled MEF2A was immunoprecipitated from cell lysates and resolved by SDS–PAGE as described above. Dried gels were visualized by autoradiography.

Phophopeptide mapping and phosphoamino acid analysis

Tryptic peptide mapping was performed as described (25). Briefly, labeled proteins were excised from nitrocellulose membranes after immunoblotting, eluted and precipitated. Precipitates were dried, resuspended and digested with trypsin. Repeated lyophilization was performed to remove salt from the digests. The lyophilized peptides were resuspended in electrophoresis buffer at pH 1.9 and loaded onto TLC plates for two-dimensional peptide mapping. For phosphoamino acid analysis, tryptic peptides were lyophilized and hydrolyzed in HCl. The partial acid hydrolyzates were mixed with phosphoserine, phosphothreonine and phosphotyrosine standards (Sigma) and subjected to two-dimensional electrophoresis with the first dimension at pH 1.9 and the second at pH 3.5. The TLC plates were visualized first by phosphorimaging, then by autoradiography; the unlabeled phosphoamino acid standards were detected by spraying the plate with 0.25% ninhydrin and incubating at 65°C.

RESULTS

MEF2A exists in a phosphorylated form in myogenic cells and its activity is modulated by serum stimulation

Initially we tested the capacity of MEF2A and MEF2C to respond to serum stimulation by assessing whether overexpressed MEF2A or MEF2C could activate the previously characterized MCK enhancer MEF2 site more potently after serum stimulation (Fig. 1A and B). The results of these experiments show that serum stimulation caused an increase in the induction of reporter activity in the presence of overexpressed MEF2A and MEF2C (Fig. 1B). Cells transfected with the vector plasmid pMT2 showed no activation. These data are similar to the data reported by Han and Prywes for serum stimulation of the c-*jun* promoter through the MEF2D protein (10).

Since the premise of our experiments was the idea that MEF2A protein is post-translationally modified by kinase catalyzed phosphorylation, we first wanted to ascertain whether MEF2A exists in a phosphorylated form *in vivo*. The initial approach that we took was to immunoprecipitate MEF2A protein from cultured skeletal muscle cells. By immunoblotting we could detect at least two forms of MEF2A in the immunoprecipitate (Fig 1C). Moreover, if the MEF2A immunoprecipitate was treated with a phosphatase prior to immunoblotting, the low mobility, high molecular weight form of MEF2A was eliminated (Fig 1C), indicating that a significant proportion of the endogenous MEF2A in myotubes is post-translationally modified by phosphorylation. These preliminary observations suggesting that MEF2A activity and phosphorylation status might be modulated by growth- and differentiation-specific signals prompted us to further explore whether MEF2A activity can be modified by various signaling pathway components.

Effects of cPKC isoforms on MEF2A and MEF2C transcriptional activity

We reasoned that if MEF2 activity is dependent on post-translational modification by phosphorylation and there are putative PKC sites on MEF2A, then overexpression of MEF2A with different PKC isotypes in reporter assays might identify a functional regulator of MEF2 activity. In order to assess these possibilities we overexpressed the conventional protein kinase C isotypes, PKCα and PKCβ, with MEF2A in cultures maintained in serum-containing medium without TPA and cultures acutely treated for 1 h with 800 nM TPA before harvesting for CAT assay. As can be seen from Figure 2A and B, both PKCα and PKCβ had no effect on relative CAT activity when co-transfected with either MEF2A or MEF2C, whether TPA was present or not in the culture medium. In these experiments, the

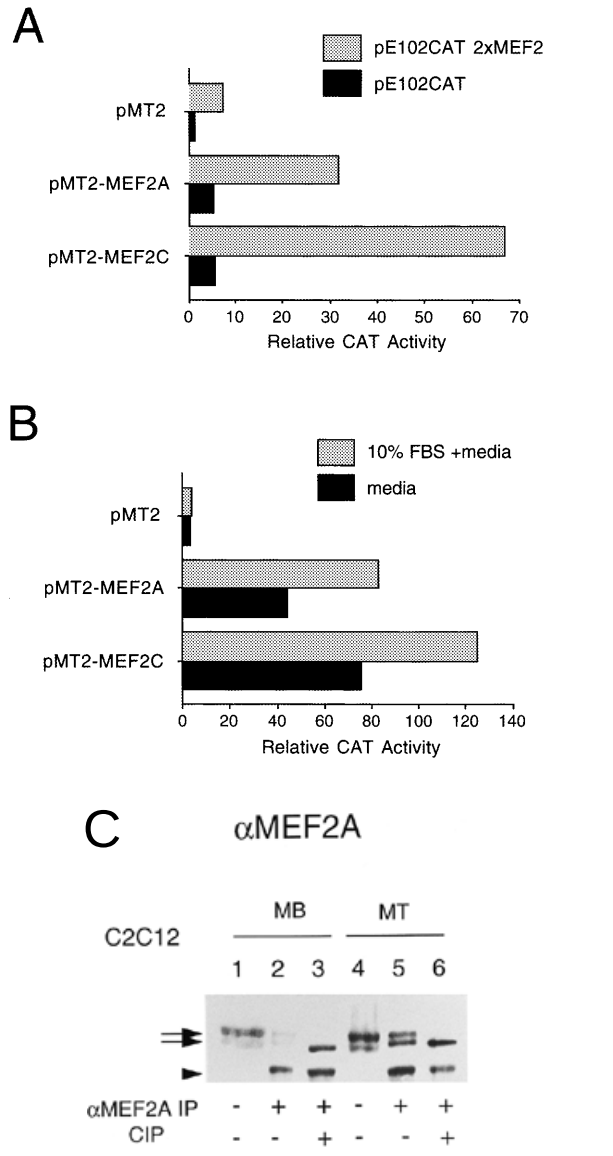

Figure 1. MEF2A is a phosphoprotein and its activity is modulated by serum stimulation. (**A**) In order to confirm that MEF2A stimulates MEF2 site-dependent activation of transcription we co-transfected wild-type MEF2A (pMT2-MEF2A) or MEF2C (pMT2-MEF2C) with a MEF2-responsive CAT reporter gene (pE102CAT 2×MEF2) or the corresponding vector without the MEF2 sites attached (pE102CAT) into HeLa cells. Each data point is the mean of triplicate samples; SEM was not greater than 11% of the mean values. (**B**) To determine if MEF2A/MEF2C site-dependent activation of transcription could be stimulated by serum, we transfected wild-type MEF2A or MEF2C with a MEF2-responsive CAT reporter construct (pE102CAT 2×MEF2) with (10% FBS + media) and without (media) serum stimulation (see Materials and Methods for details) into HeLa cells. Each data point is the mean of triplicate samples; SEM was not greater than 7% of the mean values. (**C**) To assess whether MEF2A is present in muscle cells as a phosphoprotein, we immunoprecipitated endogenous MEF2A from C2C12 myoblasts and myotubes. These immunoprecipitates were incubated with CIP (+) or inactivated CIP (–) prior to immunoblotting with the anti-MEF2A antisera. Lanes 1 and 4, detection of MEF2A in C2C12 myoblasts and myotubes; lanes 2 and 5, detection of MEF2A in MEF2A immunoprecipitates from C2C12 myoblasts and myotubes; lanes 3 and 6, the effect of CIP treatment of C2C12 myoblast and myotube anti-MEF2A immunoprecipitates (see Materials and Methods for experimental details). The arrows indicate MEF2A protein. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody.

empty expression vectors *per se* did not alter the activity of MEF2A in these cells. These experiments were repeated at least three times and the same result was observed in each experiment.

nPKC isoforms enhance MEF2A transcriptional activity

We next proceeded to ascertain whether there were effects of three closely related nPKC isoforms on MEF2A transactivation activity since their regulation and substrate specificity has been shown to be different to the cPKCs (reviewed in 15). PKCδ and PKCε are present in most mammalian tissues and cell lines, while PKCθ is predominantly found in skeletal muscle (26). As is evident from Figure 2C and D, the reporter activity was greater when MEF2A was co-expressed with either PKCδ or PKCε, compared to these factors alone. The muscle-restricted isoform PKCθ and also the aPKCζ had no effect on MEF2A (data not shown). We have previously reported that MEF2A is quite ubiquitously expressed and that HeLa and COS cells (used in these studies) contain some endogenous MEF2A (9). Activation of endogenous MEF2 is likely the reason why some effects are observed in the reporter assays described above when PKCδ was expressed with the MEF2-responsive reporter gene.

nPKC isotypes target the transactivation domain of MEF2A

We next attempted to determine if the nPKC effects that we observed on the wild-type MEF2A protein were mediated by the MEF2A transactivation domain. The MEF2A DNA binding, dimerization and transactivation domains have been well characterized in terms of their location within the MEF2 proteins (23). This information allowed us to use expression constructs encoding the MEF2A transactivation domain fused to the GAL4 DNA binding domain (GAL4MEF2A 299–373), the rationale being that such a molecule would allow us to study the effects of the kinases on the activation domain of MEF2A without complication from the effects of other endogenous interacting proteins or changes in DNA binding affinity. Therefore, to assess activation of the Gal4-MEF2A 299–373 protein, COS and HeLa cells were transfected with a luciferase reporter gene containing five copies of the GAL4 binding site upstream of a minimal promoter. Transfection studies with GAL4MEF2A 299–373 co-transfected with the PKC isotypes confirmed that there was no increase in the activity of MEF2A when overexpressed with PKCα, whereas PKCδ enhanced the transactivation capacity of GAL4MEF2A (Fig. 3). Having assessed the effects of various PKC family members on MEF2A activity we next attempted to analyze whether the MAP kinase pathways could activate MEF2A.

MEF2A is activated by the p38 but not by the ERK1/2 or JNK/SAPK MAP kinase pathways

Since MEF2A contains several putative MAP kinase phosphorylation sites we wanted to assess whether any MAP kinase isoforms could functionally modify MEF2A transctivation capacity. To do these experiments we transfected MEF2A along with one MAP kinase isoform (p38, Erk1/2 or JNK/SAPK) and constitutively activated forms of their respective MAP kinase kinase upstream regulators. Three independent experiments were carried out for each combination of expression vectors and we observed that while the ERK1/2 and JNK/SAPK pathways had no effect on MEF2A (data not shown),

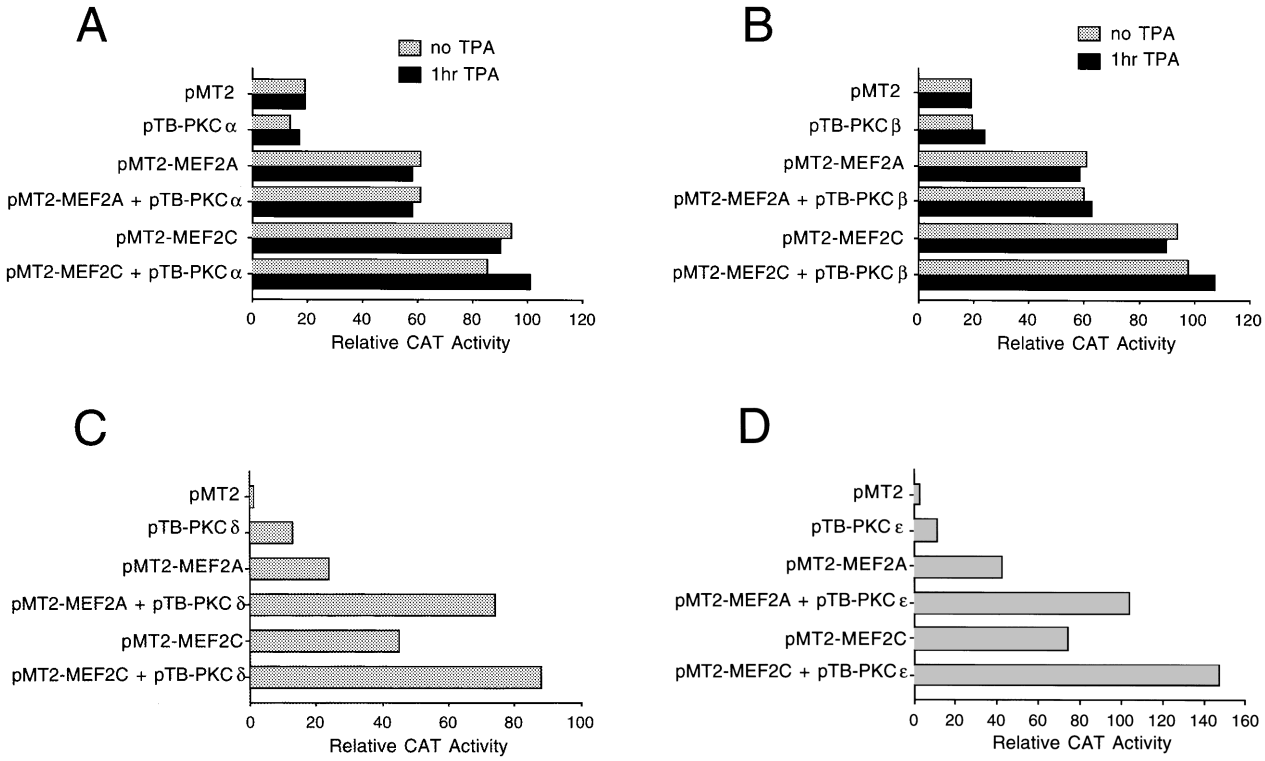

Figure 2. Effects of conventional and novel PKC isoforms on MEF2A and MEF2C transcriptional activity. We performed standard transfections in HeLa cells with the MEF2-responsive reporter gene (pE102CAT 2×MEF2), co-transfected with either pMT2-MEF2A or pMT2-MEF2C alone or with one PKC expression vector [pTB-PKCα (**A**); pTB-PKCβ (**B**); pTB-PKCδ (**C**); pTB-PKCε (**D**)] in order to test whether overexpression of the PKC isoform modulates MEF2 activity. The empty expression vector (pMT2) did not activate the MEF2-responsive reporter gene and the basal reporter gene (pe102CAT) was not affected by these treatments (data not shown). These experiments were performed at least three times with two different DNA preparations. Each data point is the mean of triplicate samples; SEM was not greater than 9% of the mean values.

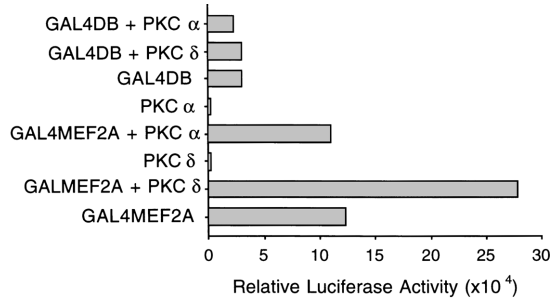

Figure 3. nPKC isotypes target the transactivation domain of MEF2A. The transcriptional activation domain of MEF2A was fused to the DNA binding domain of GAL4 (Gal4MEF2A, amino acids 299–373). This GAL4MEF2A construct was expressed in transient transfection assays in COS cells with pTB-PKCα or pTB-PKCδ. The reporter plasmid contains five copies of the GAL4 binding site linked to the adenovirus E1B promoter and the firefly luciferase gene. Each data point is the mean of triplicate samples.

p38 MAP kinase potentiated the ability of MEF2A to activate transcription of a MEF2 site-dependent reporter gene (Fig. 4A).

The MEF2A transactivation domain is targeted by p38 MAP kinase

To independently confirm that the effects we observed on wild-type MEF2A protein were mediated by the kinase modulating the MEF2A transactivation domain, we used the GAL4-based one-hybrid system to assess this interaction (as described above).

Activation of the p38 MAP kinase was achieved by co-transfection with a constitutively active form of its upstream activator MKK6b(E). We observed a large increase in GAL4-MEF2A (GAL4-MEF2A 299–373 or GAL4-MEF2A 274–373) activity when p38 MAP kinase was transfected in the presence of MKK6b(E) (Fig. 4B). We also observed an increase in activity when MKK6b(E) was transfected without p38 MAP kinase, suggesting that MKK6b(E) could be activating the endogenous p38 MAP kinase in the transfected cells.

In order to determine if the increased activity of GAL4-MEF2A induced by MKK6b(E) in the absence of over-expressed p38 MAP kinase was due to activation of the endogenous form of p38 MAP kinase, transfection studies were done using a specific p38 MAP kinase inhibitor, SB203580 (27). When the cells were treated with this inhibitor, it was observed that the level of activation of GAL4-MEF2A by MKK6b(E) and also by MKK6b(E)/p38 MAP kinase was negated (Fig. 4C). This indicates that MKK6b(E) can activate MEF2A by activating the endogenous p38 MAP kinase.

p38 MAP kinase and PKC-dependent phosphorylation of MEF2A

To further characterize the effects of p38 MAP kinase and PKC on MEF2A at the structural rather than functional level,

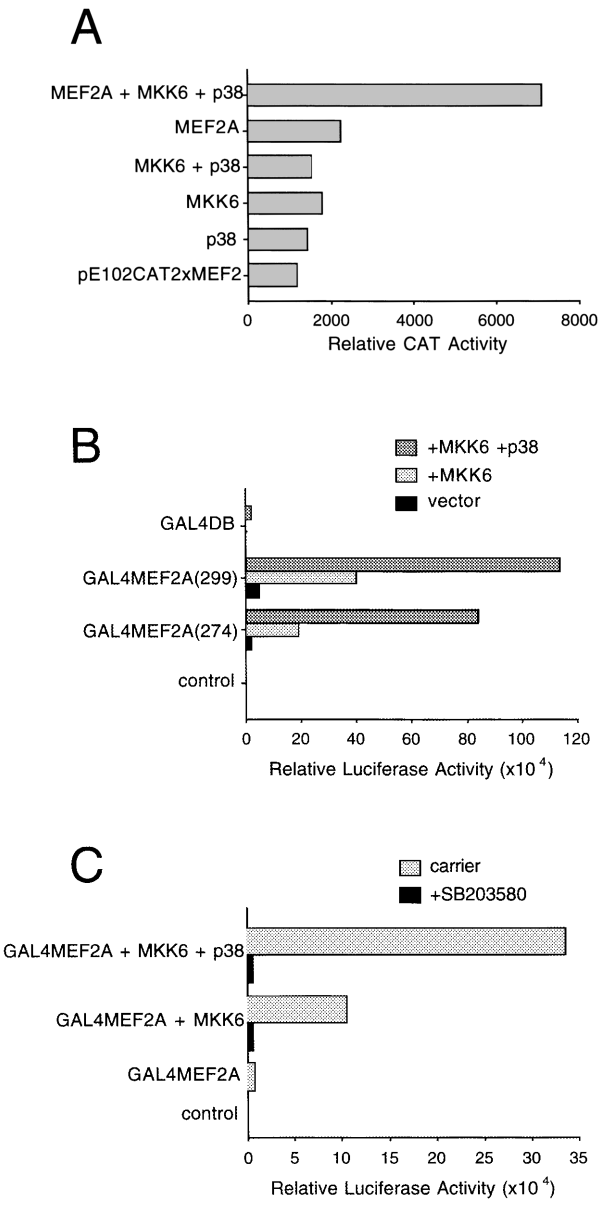

Figure 4. Wild-type MEF2A is activated by p38 MAP kinase through its C-terminal transactivation domain. (**A**) We performed standard transfections into HeLa cells with the MEF2-responsive reporter gene (pE102CAT 2×MEF2), co-transfected with either pMT2-MEF2A alone or with a p38 MAP kinase expression vector (pMT3-p38 MAP kinase) and its upstream activator [PCDNA3-MKK6b(E)], in order to test whether co-expression with p38 MAP kinase modulates MEF2A activity. Representative data from four experiments are shown. Each data point is the mean of triplicate samples; SEM was not greater than 7% of the mean values. (**B**) The transcriptional activation domain of MEF2A was fused to the DNA binding domain of GAL4 (GAL4MEF2A 299–373 or GAL4MEF2A 273–373). These GAL4MEF2A fusions were co-expressed in COS cells with either pMT3-p38 MAP kinase, pMT3-p38 MAP kinase and PCDNA3-MKK6b(E) or the vector alone in transient transfection assays. The reporter plasmid contains five copies of the GAL4 binding site linked to the adenovirus E1B promoter and the firefly luciferase gene. These experiments were performed three times. Each data point is the mean of triplicate samples. (**C**) In order to assess if the effect of MKK6b(E) on MEF2A was mediated through endogenous p38 MAP kinase a GAL4 assay [as described above using GAL4MEF2A 299–373, p38-pMT3-HA and PCDNA3-MKK6b(E) in COS cells] was carried out in conjunction with treatment of the cells with or without 1 μM SB203580, a specific p38 MAP kinase inhibitor.

we used *in vivo* metabolic labeling in conjunction with phosphopeptide mapping and phosphoamino acid composition analysis to determine whether MEF2A phoshorylation status is regulated by the PKC and p38 MAP kinases. To do this we immunoprecipitated transiently expressed MEF2A from cells labeled with [^{32}P]phosphate using anti-MEF2A antibodies. In cells expressing MEF2A alone, basal phoshorylation of the protein was low (Fig. 5A). When cells were co-transfected with p38 MAP kinase and its upstream activator, MKK6b(E), an increase in MEF2A phoshorylation was observed (Fig. 5A). This change in the phoshorylation status of the protein was associated with the appearance of a slower migrating form of MEF2A as shown by western analysis, a common feature of hyperphosphorylated proteins. This altered migration of MEF2A due to p38 MAP kinase phoshorylation was confirmed by treating the cells with a specific p38 MAP kinase inhibitor (SB203580) which negated this mobility change and decreased the overall phoshorylation of the protein (data not shown). These data therefore indicate that overexpression of MEF2A with p38 MAP kinase leads to a change in mobility of MEF2A protein. The mobility of the hyperphosphorylated MEF2A due to p38 MAP kinase activation is similar to the hyperphosophorylated band that disappears with CIP treatment of immunoprecipitated MEF2A from myogenic cells (Fig. 1C). Therefore these data indicate that the *in vivo* modification of MEF2A in myogenic cells is mimicked by p38 MAP kinase expression.

Tryptic phosphopeptide mapping of MEF2A immunoprecipitated from ^{32}P-labeled cells revealed the appearance of several major phosphopeptides due to p38 MAP kinase co-expression (Fig. 5B). PKC co-expression yielded a different phosphopeptide map to the control and p38 MAP kinase-treated MEF2A. Phosphoamino acid analysis revealed that phosphorylation due to p38 MAP kinase co-expression occurred predominantly on serine residues with lesser but detectable amounts of phosphothreonine, while PKC co-expression resulted in a lesser, but detectable, increase in serine phosphorylation (Fig. 5C). These studies demonstrate that MEF2A is phosphorylated on serine by novel PKCs and serine and threonine residues within several tryptic peptides in response to p38 MAP kinase. These data, coupled with the functional data from reporter assays, suggest that MEF2A is a downstream component of the p38 MAP kinase signal transduction pathway.

p38 MAP kinase activity in myogenic cells

Since we have shown that MEF2A is the predominant component of the MEF2 DNA binding complex in differentiating muscle cells (9) and p38 MAP kinase can functionally modify MEF2A in reporter assays (this study), we wanted to determine if p38 MAP kinase is active in differentiating muscle cells, in which MEF2A is abundantly expressed (9). Assessment of p38 MAP kinase activity in muscle cells might thus indicate whether the p38/MEF2 interaction is a physiologically relevant one for the activation of muscle-specific gene expression. Using an immunoprecipitation/kinase assay to assess p38 MAP kinase activity in cultured muscle cells (see Materials and Methods), we observed that p38 MAP kinase is expressed and active in differentiating primary rat myotubes and C2C12 immortalized muscle cells in culture (Fig. 6). Also, these data indicate that the amount of p38 MAP kinase activity is increased in the transition from mononuclear primary myoblasts

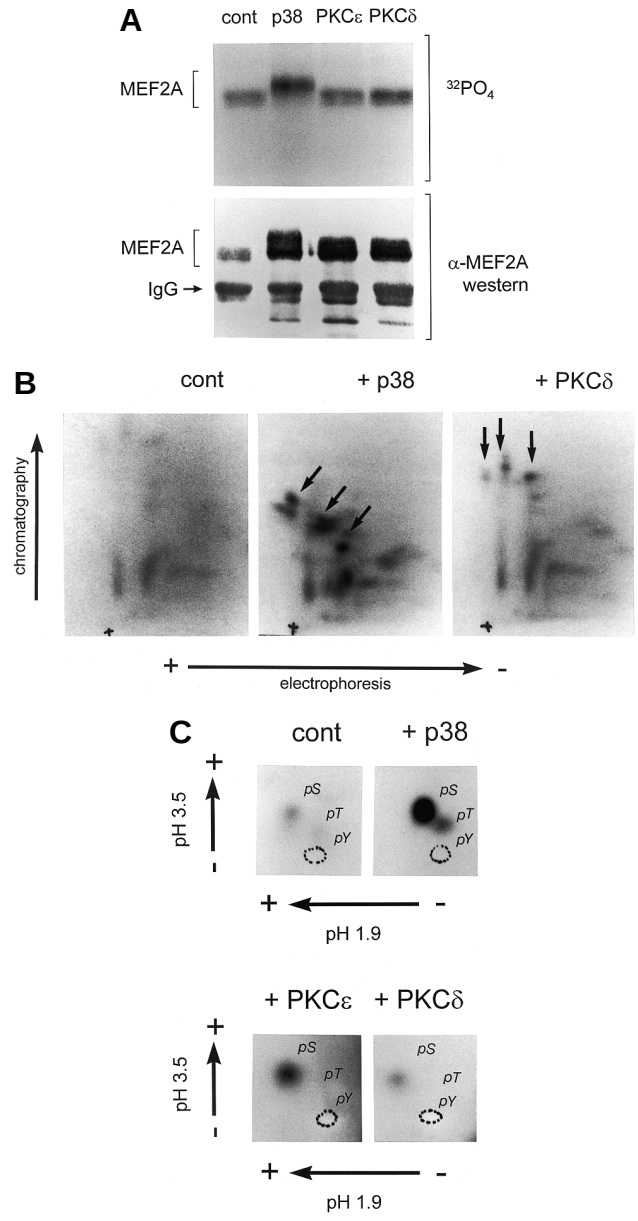

Figure 5. p38 Map kinase and PKC-dependent phosphorylation of MEF2A. (**A**) COS cells were transfected with pMT2-MEF2A in combination with pMT2 alone (control), p38-pMT3-HA/PCDNA3-MKK6b(E) (p38), pTB-PKCδ (PKCδ) or pTB-PKCε (PKCε). Cultures were labeled with [^{32}P]phosphate (at 1 mCi/ml) for 4 h. MEF2A protein was immunoprecipitated and visualized by immunoblotting with anti-MEF2A polyclonal antibody (lower panel) and analyzed by SDS–PAGE and autoradiography (upper panel). (**B**) Phosphopeptide analysis of *in vivo* labeled MEF2A. COS cells were transfected with pMT2-MEF2A in combination with pMT2 (control), pMT3-p38 MAP kinase/PCDNA3-MKK6b(E) (p38) or pTB-PKCδ (PKCδ). Cultures were labeled with [^{32}P]phosphate (at 1 mCi/ml) for 2 h. MEF2A protein was immunoprecipitated with anti-MEF2A antibody and visualized by autoradiography. The band corresponding to MEF2A was excised and digested with trypsin. The peptide fragments were separated on a thin-layer chromatography plate in the first dimension by electrophoresis in pH 1.9 buffer and in the second dimension by chromatography. The radiolabeled peptides were visualized by autoradiography. (**C**) Phosphoamino acid analysis of *in vivo* labeled MEF2A. Transfections were carried out as described in (A) above. Partial acid hydrolysis products of gel-purified MEF2A were resolved by two-dimensional electrophoresis in pH 1.9 and pH 3.5 buffers. pS, pT and pY indicate the relative migration of phosphoserine, phosphothreonine and phosphotyrosine, respectively.

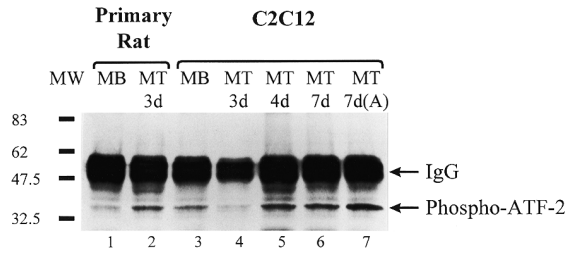

Figure 6. p38 Map kinase activity in myogenic cells. (**A**) An immunoprecipitation/kinase assay was used to determine the activation of p38 MAP kinase activity in C2C12 and primary rat muscle cells. MB, myoblasts; MT, myotubes; 3d, 4d and 7d refer to the number of days in differentiation medium; 7d(A) refers to the treatment of 7d myotubes with anisomycin, a potent activator of p38 MAP kinase (see Materials and Methods for details). The amount of phospho-ATF-2 detected is an indication of p38 MAP kinase activity in the samples.

to differentiated multinuclear myotubes (Fig. 6). Subsequent studies will therefore be aimed at the physiological relevance of the p38 MAP kinase–MEF2A interaction for the activation of muscle gene expression and differentiation.

DISCUSSION

These studies position MEF2A as a recipient of p38 MAP kinase signaling. Activation of the p38 MAP kinase pathway results in a dramatic modification of MEF2A phosphorylation and transactivation status. Whether p38 MAP kinase fulfils a general role in the activation of other members of the MADS superfamily of DNA binding proteins or is restricted to subsets such as the MEF2 proteins remains to be determined. Since MEF2A is an important regulator of muscle gene expression and has also been implicated as a nuclear target for pathways leading to the activation of 'immediate early genes' such as c-*jun* (10–12,28,29), knowledge of the extracellular signals converging on these proteins may shed light on key pathways involved in cellular pathways responsible for growth, differentiation and response to various stressors.

Our observations lead us to propose a model in which MEF2A is a target of p38 MAP kinase catalyzed phosphorylation. The targeting of p38 MAP kinase to MEF2A is mediated by the C-terminal activation domain since GAL4-MEF2A fusion proteins containing the C-terminus (amino acids 299–373) are responsive to the kinase, indicating that the phospoacceptor sites and the protein recognition motif are contained in this region. Targeting motifs of the ERK1/2/MAP kinases to substrate molecules have been characterized (28), but to date there is no evidence concerning the peptide motif on MEF2 targeted by p38 MAP kinase. How might phosphorylation function to regulate MEF2A activity? Analysis of the MEF2A molecule by deletion analysis has revealed that the C-terminus contains two transactivation domains (TADs) and, based on our data, phosphorylation in the C-terminus of the protein by p38 MAP kinase potentiates the ability of MEF2A to activate transcription. Therefore one possibility is that phosphorylation of the region containing the TADs alters the interaction of MEF2A with other molecules, either adaptor proteins or components of the basal transcription machinery. By regulating these interactions phosphorylation could directly modulate the efficiency

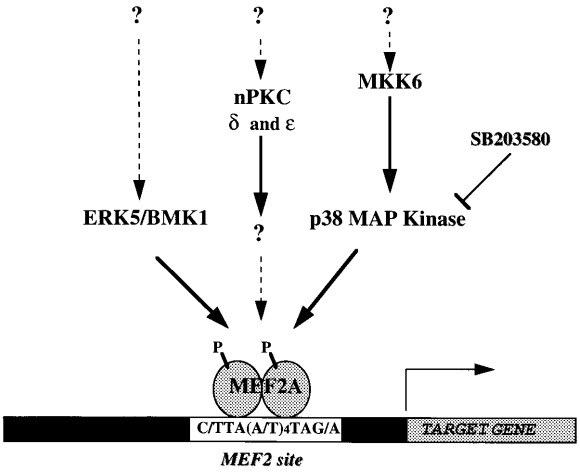

Figure 7. Schematic representation of protein kinase cascades leading to activation of MEF2A transcriptional regulatory proteins. nPKC, novel PKC; SB203580, p38MAP kinase inhibitor; P, presence of phosphorylated serine/threonine. The ERK5/MEF2A interaction is reported by Yang *et al.* (14). Dashed arrows with a question mark indicate speculative interactions; solid arrows indicate proven interactions.

and/or the stability of pre-initiation complex assembly, and thus transcriptional activation, by MEF2A.

Synergistic effects of different signaling pathways targeting the MEF2A protein could add another level of complexity to MEF2A regulation. Given that we have recently reported effects of ERK5/BMK1 (14) on MEF2A activation and that the present study shows that novel PKC isotypes and p38 MAP kinase also target MEF2A (see model in Fig. 7), it is possible that multiple pathways converge on this protein at the same time. In support of this idea is our observation that phosphopeptide mapping of MEF2A indicates that different peptides are targeted by PKC and MAP kinase. Whether multiplex signaling by different pathways leads to any synergy in the activation of MEF2A remains to be determined.

One further consideration is whether the MEF2 proteins are involved in the assembly of a ternary complex for transcriptional activation. For example, the related MADS domain protein, serum response factor (SRF), requires the phopshorylation of an accesory protein (Elk1) for full responsiveness to MAP kinase signalling (29,30). Even though MEF2A is phosphorylated by p38 MAP kinase activation this does not preclude the possibility of recruitment of other components to a ternary complex. It will therefore be important to identify whether p38 MAP kinase-dependent activation of MEF2A requires the assembly of a macromolecular complex in order to activate differentiation and stress-responsive MEF2 target genes.

Based on initial reports the different MAP kinase pathways have been characterized as 'mitogen-responsive', as is the case of the ERKs, and those that respond to cellular stresses, such as the p38 and JNK MAP kinases (reviewed in 18). More recent data has clouded this delineation because some stimuli activate both mitogen and stress-responsive MAP kinases. For example, the ERK5 molecule, while belonging to the ERK class based on its sequence, responds to some stress stimuli (18). It has been claimed that MAP kinase pathways could fulfil a dual role during muscle differentiation, being mitogenic at the initial stage of myogenesis and promoting differentiation at a later stage in the differentation program (19). Clearly, when one considers the number of distinct MAP kinase isoforms, the potential for crosstalk and varying substrate specificities of the kinases is considerable (31). Moreover, the arrangement of a network of many regulatory molecules that likely differs in composition and stoichiometry depending on the cell type suggests a mechanism by which cells can respond in an extremely complex fashion to a variety of extracellular stimuli. Therefore, although the p38 MAP kinase pathway has been characterized previously as responding to cellular stressors, it is not unreasonable to envisage differentiation-specific signals being propagated through the p38 MAP kinase pathway to the MEF2 factors during myogenesis. A recent study has implicated p38 MAP kinase in the differentiation of L8 muscle cells (32). In this study treatment of cells with a specific p38 MAP kinase inhibitor (SB203580) impaired the differentiation program. However, the concentration of SB203580 used in these experiments (20 μM) was considerably in excess of the IC_{50} data reported for p38α MAP kinase (0.04 μM) by Kumar *et al.* (27) and does raise the possibility that other related kinases might be inhibited at this concentration. It will also be ultimately important to determine whether there are other key p38 MAP kinase substrates in skeletal muscle apart from MEF2. In the study by Zetser *et al.* (32) MEF2C was implicated as the p38 substrate in L8 cells but we have previously shown that MEF2A is the predominant MEF2 in differentiating primary and C2C12 muscle cells while MEF2C is expressed after differentiation has taken place (9,21). Therefore we would contend that MEF2A is the more likely p38 MAP kinase substrate during the muscle differentiation program, although this remains to be addressed.

Phosphorylation and phosphoamino acid analysis of p38 MAP kinase catalyzed phosphorylation of MEF2C demonstrated that MEF2C is phosphorylated on threonine in response to p38 MAP kinase and that Thr293 and Thr300 are required for this responsiveness (11). Even though the analogous residues in the sequence of MEF2A are conserved it appears from our analysis that phosphorylation of MEF2A by p38 MAP kinase occurs predominantly on serine as opposed to threonine residues, implicating different or additional phosphoacceptor sites for the p38 MAP kinase catalyzed phosphorylation of MEF2A. In agreement with our data, Zhao *et al.* (33) recently published a report showing that MEF2A is a substrate for p38 MAP kinase. Using *in vitro* kinase assays of purified bacterially expressed MEF2 and p38 MAP kinase proteins they showed that Thr312, Thr319, Ser355, Ser453 and Ser479 on MEF2A are phosphorylated by p38 MAP kinase. However, mutation of these residues in functional assays using Gal4-MEF2A fusions led them to the conclusion that Thr312 and Thr319 are the key p38 MAP kinase targeted phosphorylation sites *in vivo*. In our *in vivo* phosphoamino acid analysis studies (Fig. 5) we also observed a significant phosphorylation of MEF2A on serine in response to p38 MAP kinase, begging the question as to whether the phosphorylated serine residues are important functional modulators of MEF2A activity in some cell types. However, in our GAL4-MEF2 299–373 chimera Thr319, Thr312 and Ser355 are present and this region is responsive to p38 MAP kinase. Zhao *et al.* (33) also showed that p38 Map kinase is the most potent kinase for MEF2A

compared to the other MEF2s and this is consistent with our observations that p38 catalyzed phosphorylation has a more potent effect on MEF2A activity than on MEF2C (data not shown). Further studies will be required to determine which residues on MEF2A are phosphorylated *in vivo* in cell types in which MEF2 activity is critical for gene expression, such as skeletal or cardiac myocytes.

The MAP kinase pathways have been implicated in the differentiation of muscle cells, but, so far, there has been little evidence concerning the nuclear targets for these effects. The results presented here show that the MEF2A protein, which is the predominant MEF2 gene expressed in differentiating muscle cells, is subject to post-translational modification by p38 MAP kinase. Phoshorylation of MEF2A on serine and threonine residues leads to a functional modulation in the capacity of MEF2A to activate transcription. Thus, with increasing insight into the regulation of MEF2 regulatory proteins by the MAP kinase and PKC signaling pathways, we are now poised to address the physiological role of these signals for muscle-specific gene expression and muscle differentiation.

ACKNOWLEDGEMENTS

We thank A. Bedard for providing advice and A. Ware and J. Moscat for PKC expression constructs for these studies. We also thank J. Hassell, J. Woodgett and J. Han for providing various MAP kinase reagents. This work was supported, in part, by grants from The Medical Research Council of Canada and The Wellcome Trust, UK to J.C.M.

REFERENCES

1. Karin,M. (1994) *Curr. Opin. Cell Biol.*, **6**, 415–424.
2. Ornatsky,O.I., Andreucci,J.J. and McDermott,J.C. (1997) *J. Biol. Chem.*, **272**, 33271–33278.
3. Olson,E.N., Perry,M. and Schulz,R.A. (1995) *Dev. Biol.*, **172**, 2–14.
4. Gossett,L.A., Kelvin,D.J., Sternberg,E.A. and Olson,E.N. (1992) *Mol. Cell. Biol.*, **9**, 5022–5033.
5. Yee,S. and Rigby,P.W.J. (1993) *Genes Dev.*, **7**, 1277–1289.
6. Bour,R.A., O'Brien,M.A., Lockwood,W.L., Goldstein,E.S., Bodmer,R., Taghett,P.H., Abmayr,S.M. and Nguyen,H.T. (1995) *Genes Dev.*, **9**, 730–741.
7. Lilly,B., Zhao,B., Ranganayakulu,G., Paterson,B., Schulz,R.A. and Olson,E.N. (1995) *Science*, **267**, 688–693.
8. Lin,Q., Schwarz,J., Bucana,C. and Olson,E.N. (1997) *Science*, **276**, 1404–1407.
9. Ornatsky,O.I. and McDermott,J.C. (1996) *J. Biol. Chem.*, **271**, 24927–24933.
10. Han,T.H. and Prywes,R. (1995) *Mol. Cell. Biol.*, **15**, 2907–2915.
11. Han,J., Jiang,Y., Li,Z., Kravchenko,V.V. and Ulevitch,R.J. (1997) *Nature*, **386**, 296–299.
12. Coso,O.A, Montaner,S., Fromm,C., Lacal,J.C., Prywes,R., Teramoto,H. and Gutkind,J.S. (1997) *J. Biol. Chem.*, **272**, 20691–20697.
13. Kato,Y., Kravchenko,V.V., Tapping,R.I., Han,J., Ulevitch,R.J. and Lee,J.D. (1997) *EMBO J.*, **16**, 7054–7066.
14. Yang,C.-C., Ornatsky,O.I., McDermott,J.C., Cruz,A.F. and Prody,C. (1998) *Nucleic Acids Res.*, **26**, 4771–4777.
15. Hug,H. and Sarre,T.F. (1993) *Biochem. J.*, **291**, 329–343.
16. Newton,A.C. (1997) *Curr. Opin. Cell Biol.*, **9**, 161–167.
17. David,J.D., Fraser,C.R. and Perrot,D. (1990) *Dev. Biol.*, **139**, 89–99.
18. Robinson,M.J. and Cobb,M.H. (1997) *Curr. Opin. Cell Biol.*, **9**, 180–186.
19. Bennett,A.M. and Tonks,N.K. (1997) *Science*, **278**, 1288–1291.
20. Gredinger,E., Gerber,A.N., Tamir,Y., Tapscott,S.J. and Bengal,E. (1998) *J. Biol. Chem.*, **273**, 10436–10444.
21. McDermott,J.C., Cardoso,M.C., Yu,Y.-T., Andres,V., Leifer,D., Krainc,D., Lipton,S.A. and Nadal-Ginard,B. (1993) *Mol. Cell. Biol.*, **13**, 2564–2577.
22. Ryves,W.J., Evans,A.T., Olivier,A.R., Parker,P.J. and Evans,F.J. (1991) *FEBS Lett.*, **288**, 5–8.
23. Yu,Y.-T. (1996) *J. Biol. Chem.*, **271**, 24675–24683.
24. Sadowski,I. (1989) *Nucleic Acids Res.*, **17**, 7539–7542.
25. Boyle,W.J., van der Geer,P. and Hunter,T. (1991) *Methods Enzymol.*, **201**, 110–149.
26. Osada,S.I., Mizuno,K., Saido,T.C., Suzuki,K., Kuroki,T. and Ohno,S. (1992) *Mol. Cell. Biol.*, **12**, 3930–3938.
27. Kumar,S., McDonnell,P.C., Gum,R.J., Hand,A.T., Lee,J.C. and Young,P.R. (1997) *Biochem. Biophys. Res. Commun.*, **235**, 533–538.
28. Liu,S., Liu,P., Borras,A., Chatila,T. and Speck,S.H. (1997) *EMBO J.*, **16**, 143–153.
29. Yang,S.H, Yates,P.R., Whitmarsh,A.J., Davis,R.J. and Sharrocks,A.D. (1998) *Mol. Cell. Biol.*, **18**, 710–720.
30. Treisman,R. (1994) *Curr. Opin. Genet. Dev.*, **4**, 96–101.
31. Frost,J.A., Steen,H., Shapiro,P., Lewis,T., Ahn,N., Shaw,P.E. and Cobb,M.H. (1997) *EMBO J.*, **16**, 6426–6438.
32. Zetser,A., Gredinger,E. and Bengal,E. (1999) *J. Biol. Chem.*, **274**, 5193–5200.
33. Zhao,M., New,L., Kravchenko,V.V., Kato,Y., Gram,H., di Padova,F., Olson,E.N., Ulevitch,R.J. and Han,J. (1999) *Mol. Cell. Biol.*, **19**, 21–30.