The phosphorylation status of the myocyte enhancer factor 2 (MEF2) transcriptional regulator is a critical determinant of its tissue-specific functions. However, due to the complexity of its phosphorylation pattern in vivo, a systematic inventory of MEF2A phosphorylation sites in mammalian cells has been difficult to obtain. We employed modern affinity purification techniques, combined with mass spectrometry, to identify several novel MEF2A phosphoacceptor sites. These include an evolutionarily conserved KSP motif, which we show is important in regulating the stability and function of MEF2A. Also, an indirect pathway in which a protein kinase casein kinase 2 (CK2) phosphoacceptor site is phosphorylated by activation of p38 MAPK signaling was documented. Together, these findings identify several novel aspects of MEF2 regulation that may prove important in the control of gene expression in neuronal and muscle cells.

Myocyte enhancer factor 2 (MEF2) is a transcriptional regulatory complex mediating diverse cellular functions in neurons, skeletal muscle, and cardiac muscle. It is now well established that MEF2 plays a role in the differentiation of these cell types as well as functioning in a protective role against neuronal apoptosis.

To respond to diverse developmental and physiological cues, MEF2 is structurally organized to receive and respond to multiple signals from several intracellular signaling pathways. In this regard, the best characterized is the p38 MAPK-MEF2 axis, in mammals, and T cells. It is now well established that MEF2 regulation is intrinsically governed by MEF2 phosphorylation status, which we undertook to systematically document MEF2 phosphorylation patterns in mammalian cells; previous phosphopptide mapping studies used in vitro phosphorylated MEF2 protein. To accomplish this we used several state-of-the-art mass spectrometric techniques to detect phosphorylation sites from MEF2 expressed in mammalian cells. To this end, we have made use of a mammalian tandem affinity purification (TAP) method for low-abundance nuclear transcription factors that allows purification to homogeneity and provides amounts compatible with mass spectrometric analysis of phosphorylation sites.

In these studies, we have identified two important and novel aspects of MEF2 regulation. One is a highly conserved phosphoacceptor motif that regulates MEF2 stability and function. The second is an indirect pathway of MEF2 regulation by p38 MAPK mediated by the CK2 holoenzyme. These studies on MEF2 regulation thus identify novel aspects of functional MEF2 regulation that will serve to modulate MEF2 controlled gene expression in a variety of cell types.

**Experimental Procedures**

**Materials**—Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich. DNA-modifying enzymes were purchased from New England Biolabs (NEB).

**Cell Culture and Transfections**—COS7 and HeLa cell cultures were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing penicillin, streptomycin (Invitrogen), and 10% fetal bovine serum (Atlanta Biologicals). Transfections were performed using the calcium phosphate precipitation method.

**His-tagged Protein Purification**—The coding region for human MEF2A was cloned into the EcoRI site of pCDNA 3.1/His (Invitrogen). The coding region for the N-terminal His-MEF2A was then subcloned into the pET1 site of pCDNA4/TO/His (Invitrogen). Cells were transfected with 30 μg of DNA per 100-mm dish. Typically, 20 plates of cells were used for a single purification. A standard manufacturer’s protocol was used for purification using Ni-agarose resin (Qiagen).

**Tandem Affinity Purification**—The coding region for human MEF2A was ligated into the EcoRI site of pCDNA4/TO/TAP (described in Ref. 17). Cells were transfected with 30 μg of DNA per 100-mm dish. Typically, 5 plates of cells were used for a single purification. The details of mammalian TAP-tagged protein purification are explained in Ref. 17. Briefly, cells were lysed by freeze/thawing. The lysate was passed over IgG resin (Sigma), and the beads were washed. Tagged proteins were eluted by cleaving with TEV proteinase (Invitrogen) then supplementing with Ca2+ and passed over calmodulin resin (Stratagene) for a second round of purification. Proteins were finally eluted using either 2 mM EGTA or SDS sample buffer and analyzed by SDS-PAGE. Proteins were visualized using Gelcode Blue (Pierce).

**In-gel Trypsin Digest**—Protein bands were excised, cut into 1 mm pieces, and washed 3 times with 50% acetonitrile/25 mM ammonium bicarbonate.
bicarbonate for 15 min with shaking. Gel pieces were incubated with 50 mM ammonium bicarbonate + 10 mM dithiothreitol for 30 min at 50 °C, washed with acetonitrile, then incubated with 50 mM ammonium bicarbonate + 55 mM iodoacetamide (freshly made) for 20 min in the dark. The gel pieces were washed with acetonitrile, air dried, and rehydrated with 12.5 nL/g trypsin (Promega, sequencing grade) in 50 mM ammonium bicarbonate, then incubated at 37 °C overnight. Peptides were extracted once using 3% formic acid, 1 min at 80 °C, followed by 20 min of shaking and 1 min of centrifugation.

**Mass Spectrometry**—Peptides were concentrated prior to analysis using µZipTips™ (Millipore) according to the manufacturer's protocol. Peptides were eluted directly onto a steel target using 10 mg/ml α-cyano-4-hydroxycinnamic acid in 65% acetonitrile/0.3% trifluoroacetic acid. To purify phosphopeptides, metal-chelating ZipTips™ (Millipore) were used with copper(II)-sulfate according to the manufacturer's instructions. Peptide fingerprinting was performed on a Voyager DE-STR (Applied Biosystems) using positive ion reflector mode under optimized conditions. Fragmentation analysis was performed using a nanospray ion source on a prototype Q-Star instrument (Sciex). The instrument was optimized and calibrated daily.

**Western Blot Assay**—The MEF2A antibody was generated in rabbits using full-length His-MEF2A purified from bacterial cells. The serum was purified against an immobilized MEF2A column (On-B-activated agarose, Amersham Biosciences) and IgG was concentrated using a affinity beads. The proteins were stained by Coomassie Blue staining. The gel pieces were washed with acetonitrile, air dried, and rehydrated with 12.5 nL/g trypsin (Promega, sequencing grade) in 50 mM ammonium bicarbonate, then incubated at 37 °C overnight. Peptides were extracted once using 3% formic acid, 1 min at 80 °C, followed by 20 min of shaking and 1 min of centrifugation.

**Expression and Purification of MEF2A from Mammalian Cell Culture**—Two purification strategies, His₆ and TAP, were employed for obtaining suitable material to probe the phosphorylation status of MEF2A in mammalian cell culture. The His₆-MEF2A was expressed in HeLa followed by purification using Ni²⁺-affinity beads. This strategy was capable of purifying amounts of MEF2A that could be visualized using Coomassie Blue staining (Fig. 1A). More importantly, the purified MEF2A was found in two distinct bands, indicating that a fraction of it had been post-translationally modified. This pattern of mobility is in agreement with previous detection of MEF2A from HeLa (18), COS7, and muscle cell cultures (19) by Western immunoblotting, and is in sharp contrast to the single band obtained when MEF2A is purified from bacterial cells (data not shown). Purified MEF2A protein was subsequently used to characterize its endogenous phosphorylation status.

To investigate the effect of p38 MAPK on the phosphorylation status of MEF2A, we employed an additional purification strategy utilizing the TAP scheme. TAP-MEF2A was expressed in COS7 cells in the absence or presence of expression vectors for p38 MAPK and a constitutively active upstream regulator, MKK6. The TAP scheme purified MEF2A to near homogeneity, allowing us to use Coomassie Blue staining to reveal the sizable shift in mobility of MEF2A caused by p38 MAPK (Fig. 1B). This shift has previously only been detected by immunoblotting or radiolabelling. This purification was used to probe for novel p38 phosphorylation sites in MEF2A.

**Identification of MEF2A**—Proteins were identified by either tryptic mass fingerprinting or CID fragmentation. The MALDITOF spectrum for the tryptic digest of MEF2A (Fig. 2A) contains a number of peaks whose masses match the expected mass of peptides from MEF2A. This confidently identifies the protein as MEF2A. Further confirmation was obtained, using a nanospray-QStar instrument, by CID fragmentation of selected peptides. The fragmentation products reveal short sequences and characteristic ions (Fig. 2B) that could only be derived from specific MEF2A peptides. These two types of analyses were subsequently used for identification of phosphopeptides.

We used a broad range of techniques to characterize the phosphorylation status of MEF2A in mammalian cell culture. This included comparative analysis between tryptic mass fin-
gerprints, characteristic 80 Da shifts in mass caused by phosphorylation, CID fragmentation, immobilized metal-affinity capture of phosphopeptides, and sequence homology. The details of these analyses will be discussed below.

A Novel, Endogenous Phosphorylation Site in MEF2A—The MALDI-TOF spectra for tryptic mass fingerprints of His₆-MEF2A expressed in mammalian cells were very similar to MEF2A expressed in bacterial cells with one notable exception. A peak at mass 1437.69 Da, corresponding to the amino acids 255–269 (SPPPPGGGNLGMNSR), was significantly lower when MEF2A was expressed and purified from mammalian cells (Fig. 2A), whereas it was prominent in MEF2A expressed in bacterial cells. This prompted us to investigate what was the cause of this difference. A post-translational modification, such as phosphorylation, was a likely candidate; however, no peak was detected at 80, 160, or 240 Da higher in mass. Careful inspection of the region around 2000 m/z revealed two peptides separated by 79.94 Da. This suggests that at least a fraction of the peptide is phosphorylated. D, the phosphorylated peptide was confirmed by sequencing using CID fragmentation. The phosphorylated residue is identified as Ser-255.

**Fig. 2.** Identification of MEF2A and a novel phosphorylation site. A, tryptic peptide mass fingerprint of His₆-MEF2A identifies the presence of MEF2A and heat shock cognate 71 kDa. B, the identification of MEF2A was confirmed by sequencing several peptides using CID fragmentation. C, careful inspection of the region around 2000 m/z reveals two peptides separated by 79.94 Da. This suggests that at least a fraction of the peptide is phosphorylated. D, the phosphorylated peptide was confirmed by sequencing using CID fragmentation. The phosphorylated residue is identified as Ser-255.

**Fig. 3.** Sequence comparison between MEF2A Ser-255 region and numerous MEF2 proteins. The sequence KSPPP is conserved across species and MEF2 isoforms. This suggests that phosphorylation of Ser-255 is important for the function of MEF2 proteins.
binding domain (Gal4DBD lane 1) (diation. COS7 cells were transfected with vectors coding for GAL4 DNA
on cells transfected with these expression vectors indicates
GAL4-MEF2A (S255A) mutation. A Western blot for MEF2A
lower than that of either the wild type GAL4-MEF2A or the
C

FIG. 4. Phosphorylation of Ser-255 targets MEF2A for degra-
dation. COS7 cells were transfected with vectors coding for GAL4 DNA
binding domain (Gal4DBD) (lane 1), wild type GAL4DBD-MEF2A (lane 2), or GAL4DBD-MEF2A with serine mutated to either aspartic acid
(lane 3) or alanine (lane 4). Cells were harvested, and RNA and protein
were isolated. A, Northern blot analysis of total RNA using a MEF2A-
specific probe (top row) and glyceraldehyde 3-phosphate dehydroge-
nase-specific probe (second row). B, Western blot analysis for MEF2A. Proteolytic fragment of GAL4-MEF2A. C, GALA luciferase reporter
gene assay. Data are expressed relative to the β-galactosidase corrected
luciferase activity for GAL4DBD and are the mean ± S.E. (n = 6).

Identification of Novel p38 MAPK Sites in MEF2A—Next, we
re-visited the p38 MAPK-catalyzed phosphorylation of MEF2A,
because p38 MAPK is a potent physiological activator of MEF2
transactivation potential (10, 11). Thr-312 and Thr-319 are
known phosphorylation sites important for the increased tran-
scriptional activation of MEF2A by p38 MAPK. Ser-453 and
Ser-479 are phosphorylated in vitro but were not important
functionally (11). However, previous results indicate that the in
vivo phosphorylation of MEF2A by p38 MAPK is possibly more
complicated than previously reported (10). A two-dimensional
phosphopeptide map of MEF2A expressed in COS7 cells with
p38 MAPK and MKK6 showed a very complex pattern of phos-
phorylation (10) that was different from phosphorylation data
derived from in vitro analysis (11). Because this could have
important regulatory consequences, we have used mass spec-
trometry to further dissect and characterize p38 MAPK phos-
phorylation of MEF2A in mammalian cells.

The tryp tic mass fingerprint of MEF2A, co-expressed with
p38 MAPK and MKK6, was compared with the lower-mobility
band from cells expressing MEF2A alone (Fig. 4). This simple
comparison reveals a number of phosphorylated peptides, the
evidence for which will now be considered individually.

The peptide of mass 4223.05 (MEF2A 190–233, note that the
fourth carbon isotope, 4226.06, is labeled in Fig. 5) has one
methionine residue and two potential p38 MAPK phosphory-
lization sites. Methionine residues are often partially oxidized
due to exposure to air. In the p38 MAPK-treated sample there
is a peak at a mass 80 Da higher, indicating phosphorylation.
This peak is not present in the sample from cells expressing
MEF2A alone. Furthermore, the phosphorylated and non-phos-
horylated peptides both show evidence of an oxidized form at
a mass 16 Da higher from each respective peak. This confirms
that this peptide is the phosphorylated form of MEF2A
190–233.

A similar analysis reveals a phosphorylated peptide at mass
2714.32 (MEF2A 475–498). Again, this peptide is 80 Da higher
than the non-phosphorylated peptide and is absent from the
non-treated sample. Interestingly, in the sample from cells
expressing MEF2A alone, the non-phosphorylated peptide is
also absent. Occasionally, phosphopeptides lose their phos-
hate group, either in solution or during ionization. Because
this peptide is actually a missed tryptic cleavage of Arg-492,
there is the possibility that phosphorylation of this peptide
interfered with the trypsin digestion, prior to the loss of
phosphate.

The MEF2A 95–114 peptide has a mass of 2247.99 whereas
its phosphorylated form is found at 2327.96. The intensity of
the latter peak is quite high compared with the former peak.
However, it is difficult to quantify these peptides based on peak
intensity, as the energy required to ionize a phosphopeptide
differs from that of the MALDI source used for
peptide mass fingerprinting. This form of ionization was
particularly suitable for detecting the MEF2A phosphopeptide

decreased full-length protein levels for GAL4-MEF2A (S255D)
and evidence of degradation products (Fig. 4B). A Northern blot
on these samples demonstrates that transcript expression lev-
eels can not account for the differences in GAL4-MEF2A protein
levels indicated in Fig. 4B, thus indicating that the decreased
protein levels for GAL4-MEF2A (S255D) are likely due to in-
stability of the protein. This is further supported by the detec-
tion of at least one large proteolytic fragment by Western
analysis (Fig. 4B).

Phosphorylation of Ser-255 Targets MEF2A for Degradation—Because Ser-255 is in the MEF2 C-terminal transactiva-
tion domain, we assessed the function at this site in a GAL4-
based transactivation response assay by generating mutations
of Ser-255 in a GAL4-MEF2A (residues 99–507) fusion protein.
These include Ser-255 to aspartic acid (S255D) and Ser-255 to
alanine (S255A) mutations. A GALA luciferase assay system in
COS7 cells was used to determine the activity of these proteins
(Fig. 4C). The activity of the S255D mutation was markedly
lower than that of either the wild type GAL4-MEF2A or the
GAL4-MEF2A (S255A) mutation. A Western blot for MEF2A
on cells transfected with these expression vectors indicates

![image](https://via.placeholder.com/150)

**Fig. 4.** Phosphorylation of Ser-255 targets MEF2A for degradation. COS7 cells were transfected with vectors coding for GAL4 DNA binding domain (Gal4DBD) (lane 1), wild type GAL4DBD-MEF2A (lane 2), or GAL4DBD-MEF2A with serine mutated to either aspartic acid (lane 3) or alanine (lane 4). Cells were harvested, and RNA and protein were isolated. A, Northern blot analysis of total RNA using a MEF2A-specific probe (top row) and glyceraldehyde 3-phosphate dehydrogenase-specific probe (second row). B, Western blot analysis for MEF2A. Proteolytic fragment of GAL4-MEF2A. C, GALA luciferase reporter gene assay. Data are expressed relative to the β-galactosidase corrected luciferase activity for GAL4DBD and are the mean ± S.E. (n = 6).
404–413 (doubly charged m/z of 617.28). Subsequent CID fragmentation of this peptide confirmed the presence of phosphorylation (loss of H₃PO₄ from the precursor ion and several fragment ions) at Ser-408 (Fig. 6).

A p38 MAPK-induced Phosphopeptide with no MAPK Consensus—Of particular interest is the MEF2A peptide 283–300. This region of MEF2A is alternatively spliced, and this particular splice variant is abundant in muscle and nerve cells. Phosphorylation of this peptide is indicated by the 80 Da shift in mass to 2182.95; however, this peptide does not contain a proline-directed MAPK consensus site (Fig. 7A). Further evidence that this peptide is phosphorylated is provided by the oxidation of the two methionine residues in this peptide. The phosphorylated peptide shows evidence of two oxidized methionine residues, in agreement with the two oxidized methionine residues seen for the non-phosphorylated peptide. Additional evidence that at least a fraction of this peptide is phosphorylated is provided by the use of a copper immobilized metal-affinity capture purification. MEF2A tryptic peptides were fractionated using a copper(II)-sulfate-treated metal chelating ZipTip™. Phosphorylated peptides have a higher affinity for Cu²⁺ than non-phosphorylated peptides. The selectively bound peptides were eluted, concentrated on a C-18 ZipTip™, and analyzed by MALDI-TOF. The presence of the m/z 2182.92 peptide in this eluate confirms the phosphorylation status of the MEF2A 283–300 peptide (Fig. 7B). Sequence analysis of this peptide reveals a strong consensus motif that is targeted by protein kinase CK2. Because phosphorylation of this peptide is dependent on p38 MAPK activation, cross-talk between p38 and protein kinase CK2 in MEF2A phosphorylation is implied by these data (see “Discussion”).

DISCUSSION

In these studies we have used state-of-the-art mass spectrometric techniques to assess the in vivo phosphorylation pattern of the MEF2A transcriptional regulator. This approach has yielded novel information concerning the regulation of MEF2: first, the identification of a novel phosphoacceptor site that...
regulates MEF2 stability; second, the identification of previously uncharacterized p38 MAPK phosphoacceptor sites; and third, the discovery of a protein kinase CK2 consensus phosphoacceptor site that requires p38 MAPK activity to be phosphorylated. These studies constitute several novel aspects of MEF2 regulation by reversible phosphorylation.

Phosphorylation of Ser-255 in MEF2A by endogenous kinases is detected in several cell types (C2C12, COS, and HeLa), and this phosphorylation is independent of the activity of p38 MAPK (Fig. 5). This sequence is highly conserved in members of the MEF2 family across numerous species, highlighting the importance of this site in the regulation of MEF2 proteins. The conserved sequence consists of a KSP motif, which is a known phosphoacceptor site for several kinases. This includes MAPK members (ERK1, ERK2, and stress-activated protein kinase) (20, 21), cdc2-like kinases (including CDK5) (22, 23), and glycogen synthase kinase 3 (24). The presence of these proteins in both muscle and nerve cells is suggestive of a link between these pathways and the functional consequences of phosphorylation of this site in MEF2.

The extracellular-regulated kinases (ERK1 and ERK2) are abundant in nerve cells (25) and have been implicated in the myogenic program (26–30). The activity of ERK is biphasic, with higher activity in myoblasts and later stage myotubes (26, 28). In the early stages of the myogenic program, ERK has an inhibitory effect on the expression of myogenic regulators and muscle-specific proteins (28, 29). In later stages of differentiation, ERK appears to enhance the formation of mature myotubes (26, 27) and induce hypertrophy (30). Thus, phosphorylation-induced degradation of MEF2 proteins by ERK would explain the early inhibitory effect that ERK has on the myogenic program.

The CDK5 protein is found predominantly in nervous tissue, although it has also been detected in muscle and other non-neural tissues. It has been implicated in migration, actin dynamics, microtubule transport, cell adhesion, axon guidance, synaptic structure and plasticity, membrane transport, and myogenesis (31). In muscle cells CDK5 can be detected throughout the myogenic program. The level of expression and activity of CDK5 is increased during myogenesis. In addition, proliferating myoblasts show a predominantly cytoplasmic localization of CDK5, whereas differentiating cells have an increase in nuclear levels of CDK5 (32). The possibility exists that CDK5 has a role in regulating the activity of MEF2 proteins by direct phosphorylation, although how this would pertain to regulation of MEF2 by degradation would require further investigation.

The ubiquitous phosphorylation of Ser-255, coupled with its role in the degradation of MEF2, points tantalizingly toward a role for glycogen synthase kinase 3. Originally thought of as an enzyme solely involved in glycogen metabolism, this ubiquitous kinase is now known to be involved in numerous cellular signaling functions (33). Overexpression of active glycogen synthase kinase 3 induces apoptosis in neuronal cells by phosphorylation of downstream targets (34). In addition, glycogen synthase kinase 3 is known to phosphorylate several proteins, which are subsequently targeted for degradation (35, 36). It is interesting to note that MEF2 has recently been identified as protecting neuronal cells from apoptosis (2, 37, 38) and that it is also targeted for degradation by phosphorylation (39).
Whether Ser-255 is the targeted residue that links MEF2 degradation and neuronal apoptosis will be of great interest.

MEF2A has been convincingly identified as a target for p38 MAPK. Phosphorylation of MEF2A by p38 MAPK strongly increases the transcriptional activity of MEF2A, and this increase in activity has been mapped to a couple of key residues (Thr-312 and Thr-319) (10, 11). However, in vivo phosphorylation of MEF2A by p38 MAPK produces a complex pattern of phosphorylation that cannot be entirely explained by these two key residues. The possibility, therefore, remains that other sites within MEF2A are involved in p38 MAPK signaling. Although the tryptic phosphopeptide containing Thr-312 and Thr-319 was too large to detect by mass spectrometry, the data from this study conclusively identify several new phosphorylation sites, induced by p38 MAPK activity (Fig. 8).

Most notable among these p38 MAPK-induced phosphorylation sites is the confirmation of phosphorylation at a consensus protein kinase CK2 site, Ser-289. This region of MEF2A (and the homologous region of MEF2C) is known to be alternatively spliced in different tissues (40, 41), suggesting a possible tissue-specific functional role for this region. Its sequence is highly suggestive of a protein kinase CK2 site ((S/T)XX(D/E)) that, until now, had not been confirmed as a phosphorylation site. The data presented here demonstrates that Ser-289 is likely a target of protein kinase CK2, but more interestingly, it suggests a link between p38 MAPK activity and the phosphorylation of MEF2A by protein kinase CK2. In fact, a previous report indicates that the CK2 holoenzyme (α and β subunits) can be activated specifically by p38 MAPK (42), indicating the possibility that CK2 and p38 MAPK may cooperatively dock with MEF2A.

MEF2 proteins are involved in a number of different cellular processes including proliferation, differentiation, apoptosis, and hypertrophy. These disparate roles for MEF2 are partly explained by the regulated activation of specific signaling pathways. Here we provide conclusive evidence for the phosphorylation of MEF2A by a number of signal-dependant kinases. These data concerning reversible phosphorylation of MEF2A will be a fundamental aspect of understanding the diverse function of MEF2 proteins.

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Phosphorylation Motifs Regulating the Stability and Function of Myocyte Enhancer Factor 2A

David M. Cox, Min Du, Michaela Marback, Eric C. C. Yang, Joseph Chan, K. W. Michael Siu and John C. McDermott

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