Members of the myocyte enhancer factor-2 (MEF2) family of transcription factors activate muscle gene expression by binding an A/T-rich DNA sequence in the control regions of muscle-specific genes. There are four MEF2 factors in vertebrates, MEF2A-D, which share homology in an amino-terminal MADS domain and an adjacent region known as the MEF2 domain, that together mediate DNA binding and dimerization. We show that serine 59 located between the MADS and MEF2 domains of MEF2C is phosphorylated in vivo and can be phosphorylated in vitro by casein kinase-II (CKII). Phosphorylation of this site enhanced the DNA binding and transcriptional activity of MEF2C by increasing its DNA binding activity 5-fold. In vivo 32P labeling experiments showed that serine 59 is the only phosphorylation site in the MADS and MEF2 domains. Mutagenesis of this serine to an aspartic acid resulted in an increase in DNA binding and transcriptional activity of MEF2C comparable to that observed when this site was phosphorylated, suggesting that phosphorylation augments DNA binding activity by introducing negative charge. This phosphorylation site, which corresponds to a CKII recognition site, is conserved in all known MEF2 factors in organisms ranging from flies to humans, consistent with its importance for the functions of MEF2C.

The recent elucidation of the crystal structure of the SRF core DNA-binding domain revealed a novel structural unit that mediates DNA sequence recognition, protein dimerization, and interaction with accessory factors (17, see also Ref. 18). The DNA-binding region of SRF is comprised of three structural domains. The MADS domain encompasses a coiled-coil that interacts with DNA and a central β-sheet region involved in dimerization and DNA binding. Immediately C-terminal to the MADS domain of SRF is a nonconserved region that is involved in dimerization, but does not contact DNA. Because of the extensive amino acid homology between the MADS domains of SRF and MEF2 factors, it is likely that the DNA-binding regions of these factors adopt similar conformations.

While studying the DNA binding properties of MEF2, we observed that the bacterially-expressed protein bound DNA weakly, but that incubation with rabbit reticulocyte lysate significantly enhanced DNA binding activity. This suggested that MEF2 was subjected to a post-translational modification event. Here we show that MEF2C is phosphorylated in vivo at a site that lies between the MADS and MEF2 domains. Phosphorylation of this site augments DNA binding and transcriptional activity of MEF2C by increasing its DNA binding activity. This site corresponds to a CKII recognition site and can be phosphorylated by CKII in vitro. This site is conserved in all known members of the MEF2 family, consistent with its importance for MEF2 function.

Materials and Methods

Expression of MEF2C in Bacteria—The region of the mouse MEF2C cDNA (4) encoding amino acids 1-105 was cloned into the bacterial expression vector pET28a (Novagen, Madison, WI) by polymerase chain reaction. The 5' and 3' primers contained engineered Ncol- and Xhol sites, respectively. The polymerase chain reaction product was digested with these enzymes and cloned into the Ncol-Xhol sites of pET28a to allow for a COOH-terminal MEF2C fusion containing six histidine residues. Peptide production in Escherichia coli and isolation were performed as recommended by the Histidine gene fusion kit (Novagen). After purification, the integrity of the wild-type and mutant fusion proteins was confirmed by SDS-PAGE followed by Coomassie staining. Both fusion proteins migrated as a single band with molecular mass determined to be greater than 95% pure.

Mutagenesis—Mutations were introduced into the MEF2C expression vector (4) by rolling-circle polymerase chain reaction mutagenesis as described previously (19). Minor variations in this procedure as well as the polymerase chain reaction conditions were as described previously (20). After rolling-circle mutagenesis, a 1.4-kilobase pair fragment containing the mutated region was excised by digestion with HindIII.
and XbaI, subcloned into pCDNA1/amp (Invitrogen), and sequenced. Each mutant protein was tested for integrity by in vitro transcription-translation (TNT kit, Promega Corp., Madison, WI) in the presence of [35S]methionine followed by analysis on SDS-PAGE.

**DNA Binding Assays**—The DNA binding properties of each mutant MEF2C protein were analyzed by electrophoretic mobility shift assay (EMSA) with 2 μl of an in vitro transcription-translation reaction. The translated protein was incubated with 40,000 cpm of a 32P-labeled, double-stranded oligonucleotide corresponding to the consensus MEF2 binding site from the muscle creatine kinase (MCK) enhancer (11) in the presence of 1 μg of poly(dI-dC):(dI-dC) for 10 min at room temperature. The EMSA buffer and electrophoresis conditions were as described previously (21). For CKII treatment of bacterially expressed MEF2C 1–105 protein used in the EMSA, 1 ng was incubated with or without 1 unit of CKII (Promega Corp.) at 37 °C for 10 min in the presence of ATP and CKII buffer (Promega Corp.). The treated protein was then incubated with [32P]labeled MCK digo for 30 min. One nanogram of bacterially expressed MEF2C 1–105 protein was also incubated with 2 μl of rabbit reticulocyte lysate or 2 μl of heat-inactivated rabbit reticulocyte lysate in a volume of 10 μl in the presence of 1 × gel shift buffer (21). For quantitation of EMSA bands the gels were subjected to phosphorimager analysis.

**Transient Transfection Assays**—The activity of each MEF2C mutant was assessed in a transient transfection assay with the MEF2-dependent reporter pE102MEF2x2CAT, which contains two MEF2 sites from the MCK enhancer upstream of the minimal promoter from the embryonic-MHC gene (7). Transfections were performed in 10T1/2 cells grown in Dulbecco's modified Eagle's medium with high glucose and L-glutamine and 10% fetal bovine serum. Ten micrograms of the pE102MEF2x2CAT reporter construct was used along with 5 μg of the MEF2C test construct and 1 μg of pRSV/JGAL to control for transfection efficiency. The cells were grown to 60% confluence in 6-cm plates and was then transfected by calcium-phosphate precipitation for 16 h, washed, and harvested 48 h later. The cells were then scraped into 70 μl of 250 mM Tris-HCl, pH 7.5, sonicated, and centrifuged to pellet debris. Five microliters of this supernatant was then used to perform chloramphenicol acetyltransferase (CAT) assays. CAT assays and subsequent thin layer chromatography were performed as described (22).

In vitro and in vivo phosphorylation assays—Wild-type and mutant histidine-tagged MEF2C 1–105 proteins produced in bacteria were tested for their ability to be phosphorylated in vitro with [γ-32P]ATP and purified CKIIa (Promega Corp.). One unit of CKIIa was incubated with 100 ng of either wild-type or mutant protein in the presence of CKII labeling buffer (Promega Corp.) and 1 μM ATP, 1 μM spermidine, 1 μl of [γ-32P]ATP (4500 Ci/mmol) in a reaction volume of 2 μl at 37 °C for 15 min. Two microliters of these reactions were then boiled in SDS sample buffer and subjected to SDS-PAGE.

In vivo phosphorylation of the 1–105 MEF2C wild-type or mutant peptide was assessed in COS cells by transfection with the mammalian expression vector pCDNA1 containing DNA encoding amino acids 1–105 of MEF2C with the 7-amino acid Flag tag (Kodak IBI) fused at its COOH terminus. The Flag tag was then used for immunoprecipitation of the in vivo labeled peptide. Conditions for in vivo labeling with both [35S]methionine and [32P]orthophosphate were as described previously (23).

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was performed on bacterially expressed MEF2C 1–105 following phosphorylation in vitro with purified CKII and [γ-32P]ATP, as described previously (23). Unlabeled phosphoamino acid standards were mixed with the MEF2C hydrolysate prior to separation by thin-layer chromatography and were visualized subsequently with Ninhydrin.

**RESULTS**

**Phosphorylation of the MEF2 Domain of MEF2C in Vivo**—The MADS and MEF2 domains, which are contained within the first 86 residues at the NH2 termini of the MEF2 factors, are sufficient for DNA binding and dimerization (reviewed in Ref. 18). While studying the DNA binding properties of MEF2C, we observed that the DNA-binding domain (residues 1–105) expressed in bacteria bound DNA less efficiently than the same region of MEF2C translated in a rabbit reticulocyte lysate. We therefore considered the possibility that the DNA-binding domain of MEF2C might be modified by the reticulocyte lysate so as to increase its DNA binding activity. To test this, we compared the DNA binding activity of bacterially expressed MEF2C incubated with either native rabbit reticulocyte lysate or heat-inactivated lysate. Indeed, incubation of the bacterially expressed MEF2C protein with the native rabbit reticulocyte lysate increased its DNA binding activity, whereas there was no effect with the heat-inactivated lysate (Fig. 1, lanes 1 and 2). Quantitation of this effect revealed a 5-fold increase in the DNA binding activity of MEF2C in the presence of lysate compared to control.

The most likely mechanism for post-translational augmentation of MEF2C DNA binding activity would be through phosphorylation. To determine whether the MADS or MEF2 domains of MEF2C might be phosphorylated, we transfected COS cells with an expression vector encoding amino acids 1–105 of MEF2C fused to a Flag epitope tag at the carboxyl terminus. As shown in Fig. 2A, lane 1, incubation of cells with [32P]orthophosphate resulted in labeling of the MEF2C dele-
tion mutant protein. The labeled protein migrated as a single band of ~15 kDa. These results demonstrated that the region of sequence corresponding to the MADS and MEF2 domains of MEF2C is phosphorylated in vivo.

We examined the region between amino acids 1 and 105 for potential phosphorylation sites. Amino acids 59 and 60 appeared to correspond to potential consensus sites for CKII (Fig. 2B), which phosphorylates serines and threonines flanked by acidic residues (24). To determine whether these residues were phosphorylated in vivo, we created a mutant (1–105/ST59,60DD) in which these residues were replaced with aspartic acids. This mutant was transfected into COS cells which were incubated in the presence of [32P]orthophosphate. However, in contrast to the wild-type 1–105 peptide, no 32P incorporation was detected in the mutant 1–105 peptide (lane 2). Labeling with [35S]methionine showed that the mutant protein was expressed at a similar level to the wild-type protein (Fig. 2A, lanes 3 and 4). These results suggested that Ser-59 and/or Thr-60 were the only residues within the MADS and MEF2 domains of MEF2C that were phosphorylated in vivo.

Phosphorylation of MEF2C by CKII—To test whether Ser-59 or Thr-60 were capable of being phosphorylated by CKII, we expressed MEF2C deletion mutant 1–105 in bacteria and incubated the purified fusion protein in vitro with purified CKII and [γ-32P]ATP. As shown in Fig. 3A, the MEF2C protein was efficiently phosphorylated by CKII, whereas no 32P labeling was detected in the mutant 1–105/ST59,60DD. In addition, incubation of MEF2C 1–105 with activated PKC showed no phosphorylation, suggesting that this site is not subject to nonspecific phosphorylation (data not shown).

To determine which of the above residues was phosphorylated by CKII, we performed phosphoamino acid analysis on the mutant 1–105 phosphorylated in vitro by CKII. Only phosphoserine was detected (Fig. 3B). We conclude that Ser-59 is the only site for CKII phosphorylation in the MADS and MEF2 domains of MEF2C. This residue and the nearby acidic residues that comprise the CKII consensus are conserved in the four vertebrate MEF2 gene products (1–7), as well as in MEF2 proteins from Drosophila (25, 26), X. laevis (27, 28), and C. elegans.²

² M. Kraus, personal communication.
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To confirm that the increased DNA binding activity in the presence of CKII was due to phosphorylation of the MEF2C DNA-binding domain, we tested the effect of CKII on the DNA binding activity of the mutant 1–105/ST59,60CR, in which Ser-59 and Thr-60 were replaced with cysteine and arginine, respectively (Fig. 1B). This bacterially expressed mutant protein bound DNA comparably to unphosphorylated MEF2C 1–105, but in contrast to wild-type MEF2C 1–105 (lanes 1 and 2), this mutant protein failed to show an increase in DNA binding activity in the presence of CKII (lanes 3 and 4). These results demonstrate that in vitro, CKII can augment the DNA binding activity of the MEF2C deletion protein by phosphorylation of this identified site.

To determine whether phosphorylation effected DNA binding activity by introducing negative charge, we compared the DNA binding activity of wild-type MEF2C 1–105 with the mutant 1–105/ST59,60DD. This mutant contains negatively charged aspartic acid residues in place of Ser-59 and Thr-60 and might be expected to mimic the effect of phosphorylation on the peptide. When translated in rabbit reticulocyte lysate, this mutant showed DNA binding activity comparable to that of MEF2C 1–105 (Fig. 1C, lanes 1 and 2). This suggests that the presence of a negative charge in this region is required for high-affinity DNA binding. This observation also suggests that phosphorylation of this site is stoichiometric and that the 5-fold increase in DNA binding is maximal given that the acidic substitution mutant has the same DNA binding activity as the wild-type phosphorylated protein. We also compared the DNA binding activity of wild-type MEF2C 1–105 and mutant 1–105/ST59,60CR from reticulocyte lysate. This mutant protein bound DNA less effectively than the wild-type protein, consistent with its inability to be phosphorylated by lysate (Fig. 1C, lanes 1 and 3) and hence a lack in negative charge at Ser-59. Furthermore, treatment of the above in vitro translation products with calf intestinal phosphatase reduced the DNA binding activity of wild-type MEF2C to a level comparable to that of the nonphosphorylated mutant ST59,60CR (Fig. 1C, lanes 4 and 6).

In contrast, mutant ST59,60DD failed to show a reduction in DNA binding activity in response to calf intestinal phosphatase treatment (lane 5). Together, these results confirm that the rabbit reticulocyte lysate increased the DNA binding activity of MEF2C by catalyzing its phosphorylation and they indicate that phosphorylation enhances DNA binding activity by introducing negative charge.

Because the above experiments focused on the DNA binding domain of MEF2C, encompassed in residues 1–105, we also tested whether the DNA binding activity of the full-length protein was influenced by phosphorylation at Ser-59. Indeed, mutant ST59,60DD showed enhanced DNA binding activity relative to full-length wild-type MEF2C (Fig. 4). Mutant SS90 showed a similar increase in DNA binding, consistent with the identification of Ser-59 as the site for phosphorylation by phosphoamino acid analysis. Mutants SS90A and ST59,60CR bound DNA less efficiently than the wild-type protein (Fig. 4), consistent with their lack of negative charge at position 59 and their inability to be phosphorylated. These results demonstrate that phosphorylation at amino acid 59 affects the DNA binding properties of full-length MEF2C and the 1–105 deletion mutant to a similar extent.

The CKII Site Potentiates Transcriptional Activity of MEF2C—To determine whether the phosphorylation site in the DNA-binding domain of MEF2C influenced the transcriptional activity of the full-length protein, we compared putative CKII site mutants for their abilities to transactivate the MEF2-dependent reporter, pE102MEF2x2CAT. This reporter contains two tandem copies of the MCK MEF2 site upstream of the embryonic MHC promoter. As shown in Fig. 5, the nonphosphorylated mutant SS9A showed only about 20% of the activity of the full-length wild-type protein. In contrast, mutant SS9D, which contains a negative charge at position 59 and exhibits DNA binding activity comparable to the wild-type protein, showed transcriptional activity similar to that of wild-type MEF2C. Western blots were performed on extracts from transiently transfected 10T1/2 cells expressing each of these full-length mutant MEF2C proteins and wild-type MEF2C. The wild-type and mutant proteins were expressed at comparable levels suggesting that the varying degrees of transactivation were not a result of differences in protein stabilities (data not shown). These results are consistent with the conclusion that negative charge introduced by phosphorylation of Ser-59 augments transcriptional activity and that this results from a parallel increase in DNA binding activity.

**DISCUSSION**

Our results demonstrate that Ser-59 in the DNA-binding domain of MEF2C is phosphorylated in vivo, which enhances the DNA binding and transcriptional activity of MEF2C. The importance of this site for MEF2 function is supported by the fact that it is conserved in all known members of the MEF2 family.

The evidence demonstrates that MEF2 is phosphorylated in vivo at Ser-59 in a region that corresponds to a consensus CKII site. We have shown that this site is efficiently phosphorylated by CKII in vitro but not by protein kinase C. However, it is often difficult to definitively establish whether or not an identified site is indeed phosphorylated by a specific kinase in vivo. The evidence presented here suggests that CKII is likely to mediate the phosphorylation of MEF2 but it is also formally possible that another kinase may act on this site in vivo. The consensus site for CKII is (S/T)XX(D/E) in which a serine or threonine residue is flanked by acidic residues, most notably at the third position COOH-terminal (24). However, CKII can also recognize a site in which an acidic residue is placed at the second and fourth position COOH-terminal to the serine or threonine (29, 30). The site present in all MEF2 factors is of the
amino acid sequence STDMD in which the phosphorylated serine residue is flanked by acidic residues in the second and fourth positions.

The phosphorylation of Ser-59 introduces a negative charge immediately COOH-terminal to the dimerization motif of MEF2. In principle, it is possible that phosphorylation enhances DNA binding activity by affecting the efficiency of subunit dimerization. However, we have detected no effect of phosphorylation on dimerization (9), which supports a mechanism whereby phosphorylation of Ser-59 induces a conformational change in the DNA-binding domain of MEF2 that favors interaction with DNA.

It was also of interest to determine if phosphorylation of Ser-59 had an affect on MEF2's ability to interact with myogenin/E12 heterodimers since we have previously shown that the MADS-box/MEF2 region of MEF2 proteins are capable of physically interacting with the myogenic basic helix-loop-helix proteins to activate myogenesis (8). Analysis of the mutants ST59,60DD and ST59,60CR demonstrated no difference in their abilities to interact with myogenin/E12 heterodimers or to synergize with myogenin to induce muscle gene transcription. (8). Thus, the primary effect of phosphorylation of Ser-59 seems to be to enhance DNA binding activity, resulting in an increase in transcriptional activity of MEF2.

Mutagenesis of Ser-59 in MEF2C to nonphosphorylatable amino acids eliminated all detectable phosphorylation of the MADS and MEF2 domains in transfected COS cells (Fig. 1A) and C2 muscle cells. Because Ser-59 and Thr-60 both correspond to the CKII phosphorylation consensus, we initially created mutants ST59-60DD and ST59-60CR in which both sites were altered to circumvent the possibility that the endogenous kinase might shift its preference between the two residues. However, mutagenesis of only the serine residue to an aspartic acid had the same affect on DNA binding activity and transactivation as the double aspartic acid mutant. We conclude therefore that under the conditions of our assays Ser-59 is the only major site for phosphorylation within the first 105 residues of MEF2C. However, it should also be pointed out that within the COOH-terminal transcription activation domains, there are several phosphorylation sites that have not yet been mapped.

It has previously been shown that SRF is phosphorylated on one or more serines located at positions 77, 79, 83, and 85, which are positioned approximately 60 residues NH2-terminal one or more serines located at positions 77, 79, 83, and 85, several phosphorylation sites that have not yet been mapped. However, it should also be pointed out that within the COOH-terminal transcription activation domains, there are several phosphorylation sites that have not yet been mapped.

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Fig. 5. Effects of phosphorylation mutations on MEF2C transcriptional activity. 10T1/2 cells were transfected with expression vectors encoding full-length wild-type MEF2C or the indicated mutants along with the reporter gene pE102MEF2x2CAT. Forty-eight hours later cells were harvested and CAT activity was determined. Values are expressed as the level of CAT activity relative to that in cells transfected with wild-type MEF2C. Similar results were obtained in five independent experiments.
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