Novel Phosphorylation Target in the Serum Response Factor MADS Box Regulates α-Actin Transcription†

Dinakar Iyer,‡ Narasimhaswamy Belaguli,§ Martin Flück,|| Brian G. Rowan,⊥,¶ Lei Wei,⊥ Nancy L. Weigel,⊥ Frank W. Booth,# Henry F. Epstein,⊥,▽ Robert J. Schwartz,○,⊥,@ and Ashok Balasubramanyam*,‡,⊥

Departments of Medicine (Divisions of Endocrinology and Cardiovascular Science), Surgery, Molecular and Cellular Biology and Neurology, and the Center for Cardiovascular Development, Baylor College of Medicine, Houston, Texas 77030, Anatomical Institute, University of Bern, Bern, Switzerland, and Department of Veterinary Biomedical Science, University of Missouri, Columbia, Missouri 65211

Received February 19, 2003; Revised Manuscript Received April 14, 2003

ABSTRACT: Serum response factor (SRF) is a phosphoprotein that regulates skeletal and cardiac α-actin gene transcription. Myotonic dystrophy protein kinase (DMPK), a muscle- and neuron-restricted kinase, enhanced SRF-mediated promoter activity of the skeletal and cardiac α-actin genes in C2C12 myoblasts as well as in nonmyogenic cells. DMPK phosphorylated SRF in vitro in the αI coil of the DNA-binding domain in the MADS box, a highly conserved region required for DNA binding, dimerization, and co-activator interaction in COS and CV1 cells. Threonine 159 in the MADS box αI coil was a specific phosphorylation target in vitro as well as in vivo of both DMPK and protein kinase C-α. Substitution of threonine 159 with the nonphosphorylatable residue alanine markedly diminished activation of the cardiac α-actin promoter in the presence of kinase, while its substitution with aspartic acid, to introduce a negative charge and mimic phosphorylation, restored activation completely. Phosphorylation of the MADS box may constitute a novel mechanism for regulation of SRF-dependent actin gene transcription.

Serum response factor (SRF), a M_r 67 transcription factor, is essential for both early mesodermal development (*1*) and late myogenic differentiation (*2*, *3*) and plays a key role in the expression of genes containing serum response elements (SREs) in their promoter regions (*4*). The skeletal and cardiac α-actins are muscle-specific genes regulated by SRF (*5*, *6*), and their developmental expression correlates with myogenic differentiation (*7*). The mechanism whereby SRF regulates the developmental expression of the α-actins is likely to be complex, involving differing levels of associated myogenic accessory factors and posttranslational modifications of SRF.

SRF consists of an amino-terminal regulatory domain (aa 1−142), a domain required for dimerization and DNA-binding termed the MADS box (aa 142−171), and a carboxyl-terminal transactivation domain (aa 266−508) (*8*). The MADS box structure has been highly conserved through evolution and comprises the DNA-binding and part of the dimerization domains in SRF-like transcription factors (*8*). SRF is regulated by several intracellular signaling pathways, including those involving the small GTPase RhoA (*2*). At least six broadly expressed kinases (pp90^{rsk}, casein kinase II, protein kinase A, Ca^{2+}/calmodulin kinase, DNA-dependent protein kinase, and MAPKAP-kinase 2) are known to phosphorylate SRF in its amino- or carboxyl-terminal domains, with variable functional consequences (*9*−*18*). The physiologic importance of these modifications remains unsettled and has been investigated largely in the context of SRF's regulation of the c-*fos* gene. Phosphorylation-dependent mechanisms that regulate SRF's effects on the transcription of genes required for myogenic differentiation have been less well-studied.

By virtue of its homology to the family of Rho-activated ser/thr kinases (*19*), restriction to cells of muscle and neuronal lineage (*20*), and association with defects in myogenesis (*21*), myotonic dystrophy protein kinase (DMPK) is a candidate for an upstream regulator of SRF. Evidence for a role of DMPK in myogenic differentiation include data in which (a) overexpression of DMPK in BC3H1 cells induces a skeletal muscle phenotype (*22*); (b) DMPK transgenic mice develop a mild form of hypertrophic cardiomyopathy (*23*), and DMPK knock-out mice develop late-onset myopathy (*24*); and (c) patients with congenital myotonic dystrophy have immature myocytes (*25*) and reduced expression of DMPK (*26*). We therefore investigated the functional effect of DMPK on SRF-mediated transactivation of the skeletal and cardiac α-actin genes and whether SRF is a regulatable target of this novel kinase. The results with DMPK revealed a phosphorylation consensus motif in the SRF MADS box that is also favored by protein kinase C (PKC). Since isotypes

† This work was supported by NIH-K08, Juvenile Diabetes Foundation Career Development Award, Chao Scholars Award, and Methodist Hospital Foundation Grant (A.B.) and NIH Grants PO1-HL49953, RO1-HL64856 (R.J.S.), CA57539 (N.L.W.), and AR19393 (F.B.)

* Corresponding author. Ph: (713) 798-8654. Fax: (713) 798-3810. E-mail: ashokb@bcm.tmc.edu.

‡ Division of Endocrinology, Baylor College of Medicine.

§ Department of Surgery, Baylor College of Medicine.

|| University of Bern.

⊥ Department of Molecular and Cellular Biology, Baylor College of Medicine.

University of Missouri.

¶ Present address: Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH 43614.

▽ Department of Neurology, Baylor College of Medicine.

○ Division of Cardiovascular Science, Baylor College of Medicine.

@ Center for Cardiovascular Development, Baylor College of Medicine.

10.1021/bi030045n CCC: $25.00
Published on Web 05/24/2003

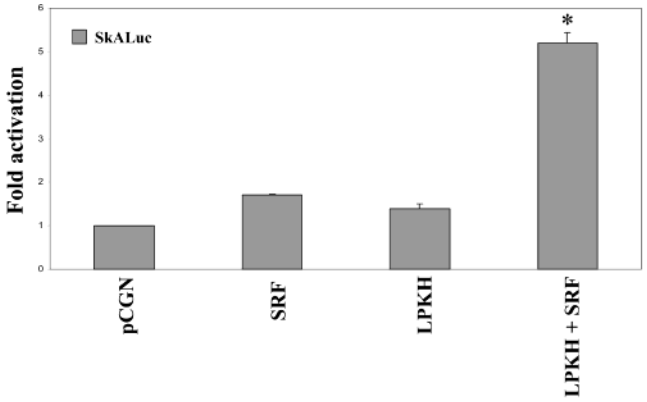

FIGURE 1: DMPK synergizes with SRF to activate the skeletal α-actin promoter in C2C12 cells. Relative luciferase activity after transient cotransfection of pGL2-SkA-Luc and the indicated plasmids. Data are representative of two to three experiments, each performed in triplicate and displayed ±SE; basal activation of the SkA promoter was measured by mock transfection of the vector (pCGN) alone and given a value of 1 (*P < 0.01 as compared to SRF).

of PKC are known to regulate myogenic differentiation (*27*), we also assessed whether they could target the same SRF site in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Kinase Constructs. LPKHT (for leucine rich domain−protein kinase domain−α-helical coiled-coil domain−transmembrane domain) is the complete 629 aa product of the human myotonic dystrophy DM1 gene (*28*); LPKH is a truncated 550 aa protein lacking the C-terminal transmembrane domain (Figure 2). pcDNA3-LPKHT was a gift of Dr. B. Perryman, University of Colorado. pcDNA3-Flag-LPKH-$(His)_6$ was made by removing a T7-Tag epitope-LPKH-$(His)_6$ construct previously generated in pMFH2/Gal4 (a gift of Dr. J. Bonventre, Massachusetts General Hospital) and cloning it into the EcoRI site of pcDNA3. pCG-LPKH has the T7-Tag epitope (Novagen, Madison, WI) N-terminal to LPKH cloned into the KpnI site of pCG. The ATP-binding site (kinase-deficient) mutants pCG-LPKHT-K100R and pCG-LPKH-K100R were derived from pcDNA3-DMPK-K100R and pcDNA3-myc-DMPK-K100R (gifts of Dr. R. Korneluk, University of Ottawa), by cloning the cDNA inserts into the KpnI site of pCG. Constructs of PKCϵ-epitope tagged PKC isoforms (PKCα, βII, δ, ϵ, and dominant negative PKCα) in the expression vector MTH (*29*−*31*) were gifts of Dr. S. Yuspa (National Cancer Institute, Bethesda, MD).

SRF Constructs. pCGN-HA-SRF contains the full-length human SRF cDNA downstream of the *Haemophilus influenzae* hemagglutinin (HA) epitope (Figure 1). SRFpm1, which contains three substitutions in the MADS box DNA-binding domain (Arg143Ile, Lys145Ala, and Leu146Gly) that permit SRF dimerization but diminish SRF−DNA binding affinity (*8*), was a gift of Dr. R. Prywes, Columbia University. SRFpm1 was cloned into pCGN-HA to make pCGN-HA-SRFpm1. Full-length and partial human wild-type SRF (*32*) and triple mutant SRF were also expressed as glutathione-*S*-transferase (GST) fusion proteins in pGEX-4T3 (Amersham Pharmacia, Piscataway, NJ). pCGN-Nkx2.5 and pCGN-GATA4 were made as described (*33*).

Luciferase Reporter Constructs. The chicken skeletal α-actin (SkA) promoter from nt −394 to +24 (Figure 2, ref

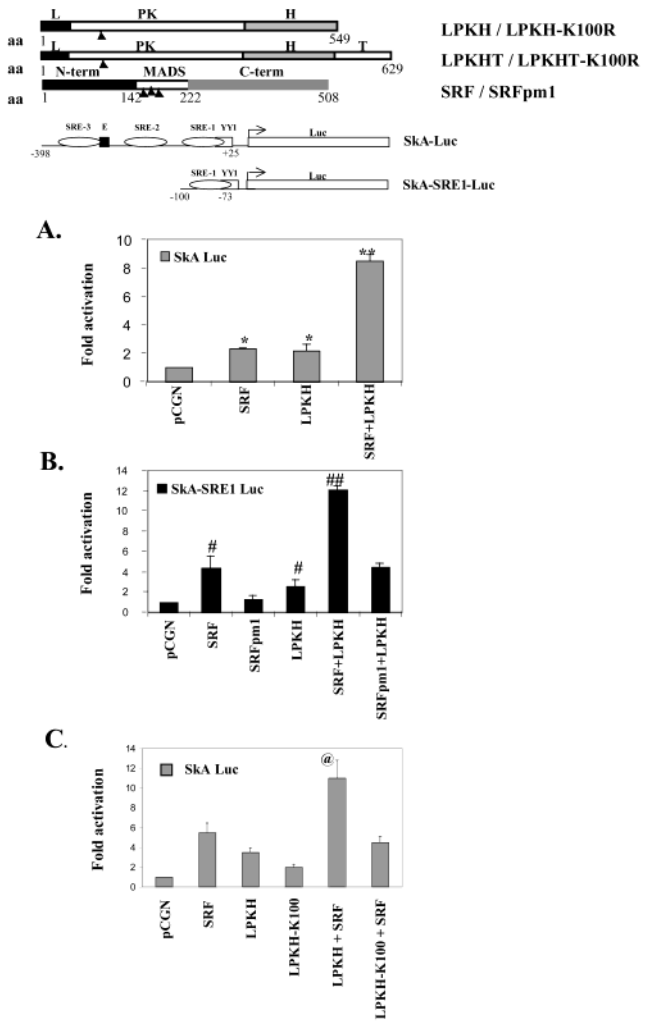

FIGURE 2: DMPK activates the skeletal α-actin promoter via SRF in COS/CV1 cells. At the top are schematic representations of relevant constructs. Single arrowheads beneath LPKH and LPKHT indicate single amino acid substitution in the K100R (kinase deficient) mutants. Three arrowheads beneath SRF indicate amino acid substitutions producing the SRFpm1 (SRE binding deficient) mutant. (A−C) Relative luciferase activity after transient cotransfection of pGL2-SkA-Luc (A and C) or SkA-SRE-1-luc (B) and the indicated plasmids. Data are representative of two to three experiments, each performed in triplicate and displayed ±SE; basal activation of the SkA promoter was measured by mock transfection of the vector (pCGN) alone and given a value of 1. (In panel A: *P < 0.05 as compared to pCGN; **P < 0.01 as compared to SRF and LPKH. In panel B: $^{\#}P$ < 0.05 as compared to pCGN; $^{\#\#}P$ < 0.01 as compared to SRF, LPKH, and SRFpm1 + LPKH. In panel C: $^{@}P$ < 0.05 as compared to SRF, LPKH, and LPKH-K100R + SRF.)

34) was cloned into pGL2 (Promega, Madison, WI). SkA-SRE-1 (provided by Dr. K. Walsh, Tufts University), which contains only the most proximal SkA SRE (nt −100 to −73, Figure 2), was cloned in pGL2. pGL2-C-Act-luc contains the region from −315 to +15 of the avian cardiac α-actin promoter (*35*).

Antibodies. T7-Tag (anti- Flag epitope) was obtained from Novagen, 12CA5 (anti-HA epitope) from Roche (Indianapolis, IN), and the PKC-ϵ-tag antibody from Invitrogen/Gibco Life Sciences (Carlsbad, CA). Anti-SRF peptide and anti-threonine 159-phospho-specific antibodies were raised against peptides representing aa 152−169 of SRF: DNKLRRYT-TFSKRKTGIM and DNKLRRYT(-PO_4)TFSKRKTGIM

(Bethyl Laboratories, Montgomery, TX). They were affinity-purified before use.

SRF Mutagenesis. SRF MADS box αI helix potential phosphorylation site mutants were made by PCR mutagenesis with the following primers (mutations in lower case): for threonine 159 to alanine, 5′-GCTGCGGCGCTACgCGAC-CTTCAGCA G-3′; for threonine 160 to alanine, 5′-GCG-GCGCTA CACGgCCTTCAGCAAGAG-3′; for serine 162 to alanine, 5′-CTACACGACCTTCgcCAAGAGG AAGACG-3′; for triple mutant (threonine 159 to alanine/threonine 160 to alanine/serine 162 to alanine), 5′-AACAAGCTGCG-GCGCTAcgCGgCCTTCgcCAAGAGGAAG-3′; and for threonine 159 to aspartic acid, 5′-GCTGCGGCGCTAc-gatACCTTCAGCAAG-3′.

Transfections/Transcription Assays/Immunoblotting. C2C12, Sol8, COS, and CV1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cells were transfected using either DEAE-dextran with glycerol shock for expression of DMPK or PKC isoforms in immunoprecipitation-kinase assays, or lipofectamine (Gibco-BRL) for expression of recombinant proteins in transcription assays. For transcription experiments, cells were transfected at 50% confluence with a standard amount of the appropriate luciferase reporter plasmid (0.25 or 0.5 μg) and varying amounts of test plasmids, balanced with parent expression vector plasmid (pCGN) in a total of 1.25 μg. The plasmids were mixed with 4 μL of lipofectamine per 60 mm plate and applied to the cells according to the manufacturer's instructions. Cell extracts were harvested after 48 h and assayed using the Luicferase Assay System (Promega). Luminescence was measured using a luminometer (Monolight 2010, Analytic Luminescence Laboratory, Sparks, MD). Luminescence data are normalized for total cellular protein concentration and reported as mean or representative values of two to five independent transfection experiments, each performed in triplicate. Immunoblotting was performed after resolving proteins by SDS−PAGE and transferring to Immobilon-P membranes (Millipore, Bedford, MA), using enhanced chemiluminescence with the appropriate antibodies and the Supersignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

In vitro Kinase Assays of SRF Phosphorylation. COS cells were transfected with 5 μg of pcDNA3-Flag-LPKH-$(His)_6$ or pcDNA3 vector. After 60 h, the cells were scraped in 500 μL/100 mm plate kinase lysis buffer (50 mM Tris-Cl (pH 7.5), 500 mM NaCl, 0.5% Triton X-100, 2 mM sodium vanadate, 50 mM NaF, protease inhibitor cocktail (Complete Protease Inhibitor Tablet without EDTA, Roche)). Recombinant LPKH was purified from the lysate at 4 °C through a Histrap nickel column (Amersham, Piscataway, NJ), followed by immunoprecipitation with preimmune rabbit serum, and finally by immunoprecipitation with T7-tag antibody and 50% protein A Sepharose. Kinase assay was performed for 30 min at 30 °C in a 50 μL reaction containing purified, immunoprecipitated LPKH attached to protein A sepharose beads, 20 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 50 μM ATP, 10 μCi γ^{32}P-ATP (4000 Ci/mmol), and 4 μg of various GST-SRF proteins. The reaction was terminated by addition of 50 μL of 2× SDS−PAGE sample buffer (50 mM Tris-Cl (pH 6.8), 10 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heating at 95 °C for 5 min.

For in vitro phosphorylation experiments with PKC, constructs of PKC isoforms or their vector control plasmid MTH were transfected into COS cells. After 60 h, the cells were treated with 100 mM phorbol 12-myristate 13-acetate (PMA) for 45 min prior to cell lysis. The cells were then scraped in lysis buffer as described above for DMPK. Recombinant PKC proteins were purified from the lysates at 4 °C by immunoprecipitation with preimmune rabbit serum and then by immunoprecipitation with the PKC-ϵ-tag antibody and 50% protein A Sepharose. Kinase assay was performed exactly as described above for DMPK, using bacterial SRF 1−171 as substrate.

HPLC Phosphopeptide Mapping of SRF. After SDS−PAGE and Western transfer of proteins in the in vitro kinase reaction with DMPK, the nitrocellulose membranes were stained with Ponceau S and briefly exposed to X-ray film. Pieces of membrane corresponding to radiolabeled SRF fusion proteins were excised, destained in 1 mL of 200 mM NaOH for 1 min in siliconized microfuge tubes, and washed with H_2O and then with 0.5% poly(vinylpyrrolidone)-40 in 100 mM acetic acid before the unbound sites were blocked for 1 h at 37 °C in the same solution. The membrane pieces were washed with H_2O, rinsed with 5% acetonitrile/100 mM $NaHCO_3$, and incubated for 24 h at 37 °C with acetylated trypsin from bovine pancreas (ICN, Costa Mesa, CA) at a final concentration of 0.4 mg/mL in the same solution. The released peptides were harvested in a final volume of approximately 300 μL. The efficiency of ^{32}P-peptide release was 88−95%. The tryptic phosphopeptides were acidified with 1% trifluoroacetic acid and separated by reverse phase HPLC using a Beckman model 406 system (Beckman, Fullerton, CA), with System Gold software and a 3.9 × 300 mm C18 column (Waters, Milford, MA), at a flow rate of 1 mL/min with the following gradients: 0−5 min, 98% solution A, 2% solution B; 5−35 min, 2−75% B; and 35−45 min, 75−98% B (solution A = 0.05% TFA in H_2O; solution B = 0.05% TFA in 80% acetonitrile). The eluted fractions were monitored by detecting radioemission on-line with a flow-one/β detector (Packard, Meriden, CT). Peptide sequencing was performed as previously described (*36*). Sequencing was performed in a Model 477A Protein Sequencer with an in-line 120A PTH-Analyzer (Applied Biosystems, Foster City, CA) using optimized cycles. 90% methanol containing phosphoric acid was used to extract the cleaved amino acids. After conversion, 50% of the sample was transferred to the HPLC for PTH-amino acid identification, and the other 50% was collected in a fraction collector for scintillation counting.

In Vitro and in Vivo Phosphorylation of Threonine 159, Detected by Anti-Threonine 159-Phospho Specific Antibody. For in vitro phosphorylation experiments, LPKH and PKC-α constructs or their vector controls were transfected into COS cells, and the recombinant proteins were purified as described above. The recombinant kinases or mock transfection IP products were placed in in vitro kinase reactions using SRF 1−171 as substrate. The kinase reactions were carried out as described above, with the exceptions that 1 mM ATP was used, and γ^{32}P-ATP was excluded. The reaction products were subjected to SDS−PAGE and immunoblotted using anti-SRF peptide antibody or the anti-threonine 159-phospho specific antibody.

For in vivo phosphorylation experiments, LPKH and PKC-α constructs or their vector controls were cotransfected with HA-tagged full-length SRF (wtSRF) or triple mutant SRF (tmSRF) into CV1 cells. Cells transfected with PKC constructs or the MTH vector were treated with 100 mM PMA or vehicle for 45 min prior to lysis. Recombinant SRF proteins were immunoprecipitated using 12CA5 antibody, subjected to SDS−PAGE, and immunoblotted using anti-SRF peptide antibody or anti-threonine 159-phospho specific antibody.

SRF-DNA Binding. Nuclear extracts of COS cells transfected with pCGN vector, full-length wtSRF, or the corresponding threonine 159 alanine/aspartate mutants were prepared (*37*). Double-stranded cardiac α-actin complete promoter region nt −315 to −58 was used as probe (*38*). Five μg of nuclear protein was incubated at RT for 30 min with 1 μg of poly(dI/dC), 4 μL of 5× binding buffer (10 mM Tris-Cl (pH7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) and 50 000 cpm of ^{32}P-end-labeled probe in a total volume of 20 μL. DNA−protein complexes were resolved by nondenaturing 4% PAGE.

RESULTS

DMPK and SRF Synergize to Activate the Skeletal α-Actin Promoter in C2C12 Myoblasts. We first examined the effects of DMPK on the full-length skeletal α-actin (SkA) promoter in replicating C2C12 myoblasts, a cell line with endogenous expression of DMPK. Transient transfection of SRF modestly increased transcription of the SkA-Luc reporter gene, as measured by luciferase assay (Figure 1). The addition of recombinant LPKH (C-terminal truncated DMPK—see Experimental Procedures) enhanced transcription 5-fold over basal. Similar data were obtained using Sol8 myoblasts. These results suggested that DMPK could modulate the transcriptional activity of SRF in myogenic cells. To explore the mechanisms underlying the effect, we performed subsequent experiments in COS or CV1 cells. These heterologous cell lines were utilized to overcome problems of low transfection efficiency in the myoblast lines and to avoid interference from endogenous DMPK in experiments designed to probe the mechanism of the kinase effect.

DMPK Activates the Cardiac and Skeletal α-Actin Promoters via SRF in COS and CV1 Cells. Transfection of COS or CV1 cells with SRF alone led to a 3-fold increase over basal activity of the SkA promoter (Figure 2A). Transfection of LPKH alone also resulted in a 3-fold increase in promoter activity, but cotransfection of SRF and LPKH resulted in a synergistic 9-fold increase. Since the most proximal SRE in the SkA promoter (SRE−1) is sufficient to confer approximately 90% of the SRF-mediated transcriptional effect (*5*), we examined the effects of DMPK and SRF on a minimal SkA-SRE-1 promoter construct (Figure 2B). SRF alone activated SkA-SRE-1-Luc 5-fold, while the mutant SRFpm1 diminished this activation by over 60%. Cotransfection of SRF and LPKH resulted in a synergistic 12-fold increase, while substituting SRFpm1 (an SRF−DNA binding-defective mutant) for SRF reduced the effect by 60%. LPKH-K100R, a kinase-deficient form of LPKH, eliminated the LPKH effect (Figure 2C). Similar results were obtained using the full-length DMPK constructs LPKHT and LPKHT-K100R (data not shown). These results confirmed the synergism between DMPK and SRF in regulating SkA promoter activity and indicated that the effect of DMPK is due to its kinase activity.

Next, the effect of LPKH on the full-length cardiac α-actin (C-Act) promoter was assayed. Again, SRF and LPKH each activated the C-Act promoter 3−4-fold, but the combination activated the promoter 10-fold (Figure 3A). Further synergism was seen in the presence of Nkx2.5, a cardiac homeodomain factor known to synergize with SRF to provide strong cotranscriptional activation of C-Act (*32*), and GATA4, a zinc-finger protein that interacts with SRF to coactivate SRE-containing promoters (*39*) (Figure 3B). The combination of SRF, Nkx2.5, and GATA4 increased C-Act promoter activity about 25-fold over basal, and the addition of LPKH increased transcriptional activity further to 55-fold. The effects of both these combinations were reduced by 60% by substituting SRFpm1 for SRF. A lesser degree of synergistic activation was also seen with the combination of LPKH, Nkx2.5, and GATA4, possibly because of endogenous cellular SRF.

DMPK Phosphorylates SRF in Vitro. We asked if SRF could be a direct phosphorylation target of DMPK. Full-length histidine-tagged SRF (1−508) was phosphorylated in vitro in the presence of LPKH (Figure 4A, lane 1). We then attempted to map the region of SRF targeted by DMPK. His-tagged truncations of the SRF protein did not express well in bacteria, so we used glutathione-*S*-transferase (GST)-tagged fusion proteins. GST-SRF 1−171 containing the amino-terminal region and the MADS box (50 kDa; Figure 4B, lane 4) and GST-SRF 142−171 containing the MADS box alone (33 kDa; Figure 4B, lane 3) were strongly phosphorylated by LPKH. In full-length and C-terminal-truncated versions of SRF, the presence of the GST moiety appeared to inhibit phosphorylation (data not shown), perhaps because folding of these proteins places the GST in a position that blocks the phosphorylation site. Therefore, using these GST-fusion constructs, we could not eliminate the possibility that the portion of SRF C-terminal to aa 171 is also phosphorylated by LPKH—however, in subsequent experiments we focused on the N-terminal/MADS box region (aa 1−171) that is clearly phosphorylated.

Both His-tagged full-length SRF and GST-SRF 1−171 were phosphorylated in the presence of the immunoprecipitated product of the mock (vector) transfection (Figure 4A, lane 2; Figure 4B, lane 1), although significantly less than in the presence of LPKH (Figure 4A, lane 1; Figure 4B, lane 4). Partial phosphorylation of mock-treated SRF was a consistent finding that led us to perform phosphopeptide mapping of this band in parallel with that of the DMPK-treated band in all subsequent experiments. GST itself was not phosphorylated by LPKH (Figure 4B, lane 2). Under the conditions of the in vitro kinase assay, the molar efficiency of phosphate transfer was 45 mmol Pi/mol GST-SRF 1−171 in the presence of LPKH, comparable to the efficiency of phosphate transfer to myelin basic protein (*40*). These results suggested that DMPK targets the SRF MADS box in vitro.

Threonine 159 in the MADS Box αI Coil Is a Prominent in Vitro Target of DMPK. Tryptic phosphopeptide maps of wild-type and mutant forms of SRF 1−171 (obtained after in vitro phosphorylation of SRF 1−171 by purified LPKH or the IP product of mock transfection) were compared to

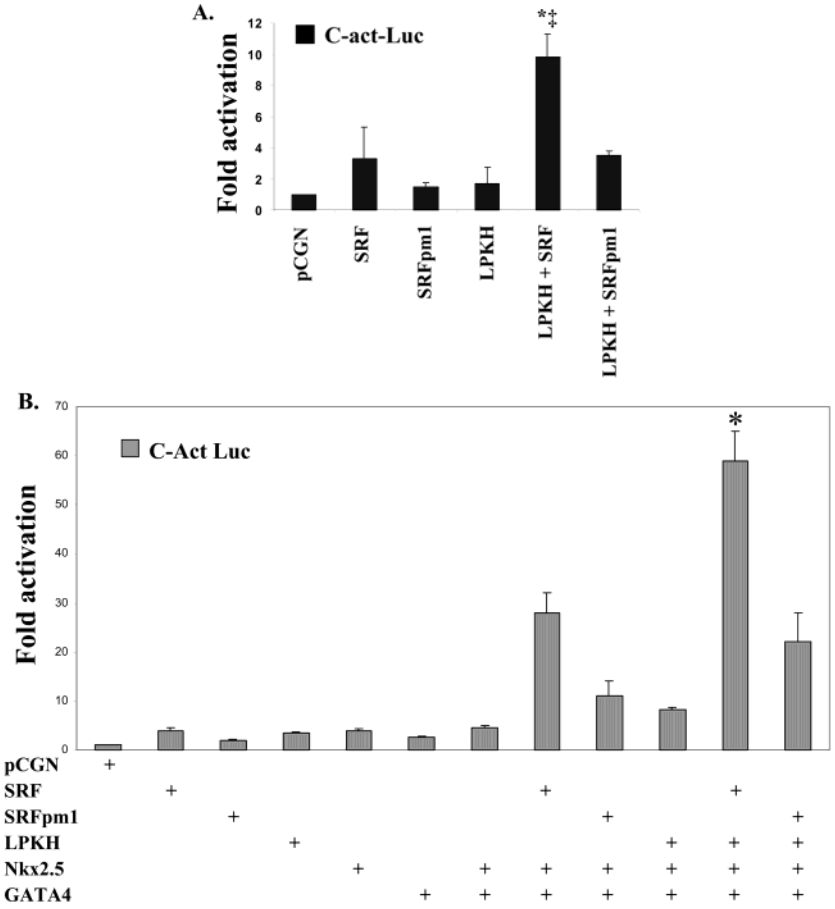

FIGURE 3: DMPK facilitates cardiac α-actin promoter activity via SRF in COS cells. (A) Relative luciferase activity after transient cotransfection of pGL2-C-Act-Luc and the indicated plasmids. Data are representative of two to three experiments, each performed in triplicate and displayed ±SD; basal activation of the C-Act promoter was measured by mock transfection of the vector (pCGN) alone and given a value of 1. (*P < 0.01 as compared to SRF and LPKH + SRFpm1; ‡P < 0.001 as compared to LPKH.) (B) Relative luciferase activity after transient cotransfection of pGL2-C-Act-Luc and the indicated combinations of plasmids; basal activation of the C-Act promoter was measured by mock transfection of the vector (pCGN) alone and given a value of 1. (*P < 0.05 as compared to SRF + Nkx-2.5 + GATA-4 and SRFpm1 + LPKH + Nkx-2.5 + GATA-4.)

identify the residues phosphorylated in vitro by DMPK (Figure 4C). Wild-type SRF 1−171 revealed two small (peaks A and B) and one prominent (peak C) phosphopeptide peaks that were specific to the LPKH treatment, as well as a nonspecific peak (peak D) that was seen in both LPKH- and mock-treated conditions (slide I). Because the fragment aa 142−171 (containing the MADS box αI coil) was phosphorylated (Figure 4B, lane 3), we mutated the three candidate serine/threonine residues within the tryptic peptide. Substitution of the nonphosphorylatable amino acid alanine for threonine 160 (T160A) had no effect on the pattern of phosphorylation (slide II), although the absolute amount of radioactivity recovered was higher in this case. In contrast, mutation of threonine 159 (T159A) removed peaks A and C (slide III). Finally, substitution of alanine for threonine 159, threonine 160, and serine 162 (T159A, T160A, and S162A, or triple mutant) eliminated all LPKH-dependent phosphorylation (slide IV). These data suggested that peaks A and C contain a peptide that is phosphorylated on threonine 159 and that peak B contains a peptide that is phosphorylated on serine 162. The peptide yield in peak C (but not in peaks A and B) was sufficient to permit direct protein sequencing and detection of the phosphoamino acid. As expected, threonine 159 was phosphorylated in the peptide in peak C (Figure 4D). The peptide was the result of incomplete tryptic digestion, presumably because the negative charge of the phosphate inhibited cleavage at the arginine residue. Thus, peak B likely contains a peptide with serine 162 phosphorylated, and peak A is perhaps the diphosphorylated peptide. Alternatively, peak A may contain the limited tryptic digest tyrosine 158—lysine 163 with monophosphorylated threonine 159. We concluded that threonine 159 clearly is phosphorylated by DMPK in vitro and that serine 162 may be an additional target. Hence, we focused on the function and in vivo status of threonine 159 as a key phosphorylation target of DMPK and other kinases.

PKC Isoforms Also Phosphorylate SRF 1−171 in Vitro. Inspection of the amino acid sequence of the SRF MADS box αI coil peptide targeted by DMPK revealed a good consensus motif for PKC (RRYTTFSKRK), which preferentially phosphorylates serine or threonine residues surrounded by basic amino acids. Since PKC is involved in myogenic regulation (*27*, *41*−*44*), we asked if four isotypes (PKCα, βII, δ and ϵ)—which have previously been shown to be differentially expressed during myogenic proliferation and differentiation (*42*)—could phosphorylate SRF 1−171 in vitro (Figure 5). PKCα strongly phosphorylated SRF 1−171 and PKCβII, and PKCδ did so to a lesser extent, while SRF phosphorylation did not rise above background level with PKCϵ. The specificity of the PKCα effect was demonstrated

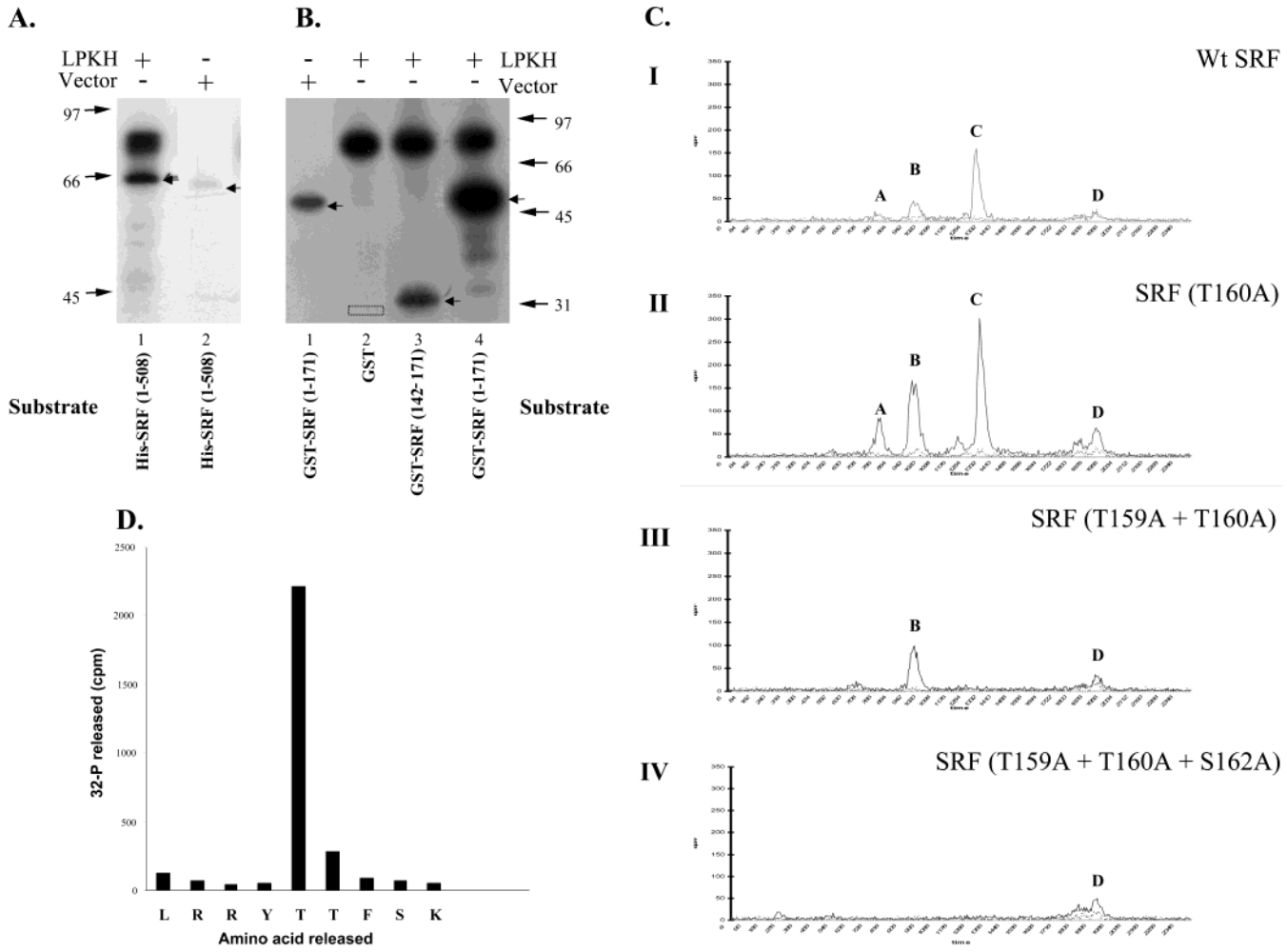

FIGURE 4: DMPK phosphorylates SRF in vitro. (A and B) In vitro kinase reactions were carried out as described in Experimental Procedures, using either recombinant LPKH purified from transiently transfected COS cells or the purified product of mock-transfected cells (as indicated above the lanes), and the substrates indicated below each lane. Panels A and B show autoradiographs of SDS−PAGE after electrophoresis of the reaction products. The small arrows in panel A, lanes 1 and 2 and in panel B, lanes 1, 3, and 4 indicate the position of recombinant SRF proteins as identified by Coomassie staining. In panel A, lane 1, and in panel B, lanes 2−4, the cluster of radiolabeled bands at 80−72 kDa represents autophosphorylated species of LPKH. In panel B, lane 2, the dotted area represents the position of GST protein as identified by Coomassie staining. GST = glutathione-*S*-transferase. (C) HPLC-phosphopeptide mapping of wild-type SRF 1−171 or various MADS box mutants (alanine substitutions for threonine 159, threonine 160, or serine 162) after in vitro kinase assay. Tryptic peptide digests of radiolabeled, phosphorylated GST-SRF 1−171 proteins were separated by HPLC as described in Experimental Procedures. Each panel shows a pair of phosphopeptide maps, from the kinase reaction in the presence of recombinant LPKH purified from transiently transfected COS cells (solid line) and from the reaction in the presence of the purified product of mock-transfected cells (dashed line). The SRF 1−171 protein represented in each panel is as follows: slide I, wt SRF; slide II, SRF with alanine substituted for threonine 160 (T160A); slide III, SRF with alanines substituted for threonine 159 and threonine 160 (SRF T159A + T160A); and slide IV, SRF with alanines substituted for threonine 159, threonine 160, and serine 162 (SRF T159A + T160A + S162A, or triple mutant SRF). (D) ^{32}P cpms released with each cycle of Edman degradation of the peptide in peak C from panel C, slide I. The sharp spike of cpms in the 5th cycle from the N-terminus corresponds to threonine 159. The identity of this residue was confirmed by sequencing.

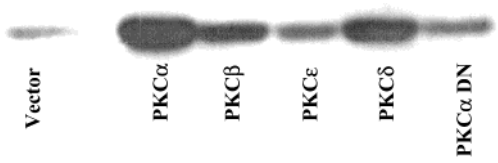

FIGURE 5: In vitro phosphorylation of SRF 1−171 by the indicated recombinant PKC isoforms purified from transiently transfected COS cells or the IP product of cells transfected with empty vector. PKCαDN = dominant negative mutant of PKCα.

by the ability of dominant negative PKCα to suppress the phosphorylation of the substrate to background levels.

Threonine 159 in the MADS Box Is Phosphorylated in Vivo by both DMPK and PKCa. To examine the in vivo relevance of threonine 159 as a target for kinases regulating myogenesis, we developed a threonine 159-phospho specific antibody. First, we utilized the affinity-purified antibody in conjunction with in vitro kinase assays to determine its specificity (Figure 6A). The anti-threonine 159-PO_4 specific antibody detected only the SRF 1−171 peptides that had been exposed to LPKH or PKCα but not those that had been exposed to the immunoprecipitation products from the respective vector control transfections. (Of note, the lack of 159-PO_4 specific antibody reactivity in the absence of LPKH cotransfection also indicated that the background phosphorylation previously observed with mock (vector) transfection in the in vitro phosphorylation experiments (Figure 4A, lane 2; Figure 4B, lane 1) could not have been due to phospho-

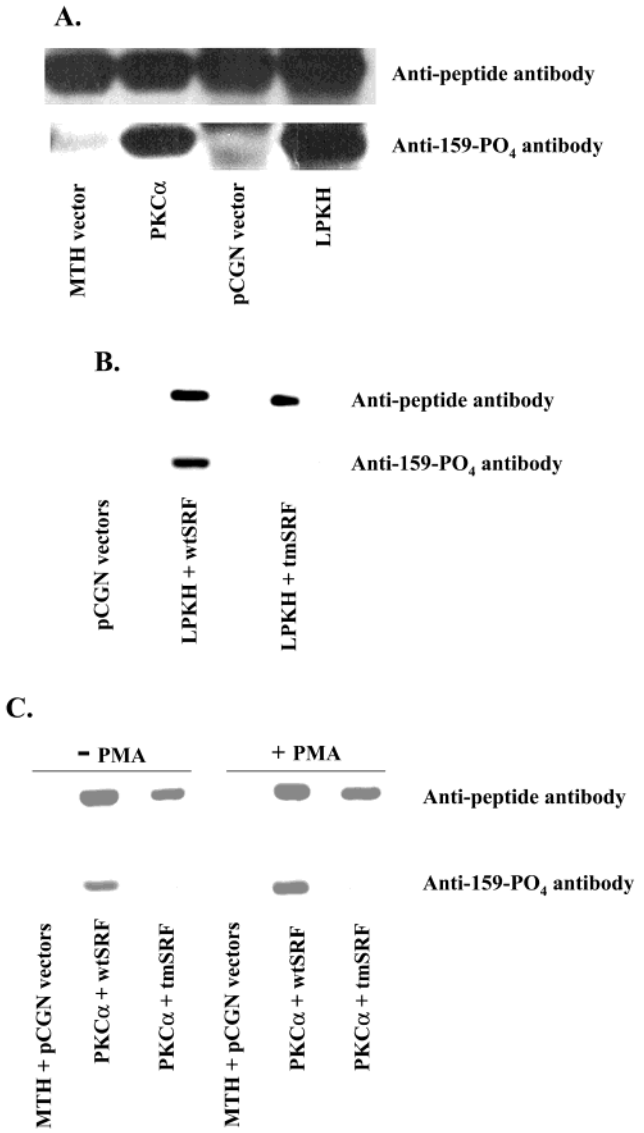

FIGURE 6: Phospho-specific antibody reveals phosphorylation of threonine 159 in the MADS box occurs both in vitro and in vivo. (A) In vitro kinase reactions were carried out in the presence of cold ATP, using recombinant LPKH or PKCα purified from transiently transfected COS cells or the purified products of cells transfected with the appropriate vector (pCGN for LPHK; MTH for PKCα), with SRF 1−171 as substrate. The reaction products were subjected to SDS−PAGE and immunoblotted with either anti-SRF-peptide antibody (top panel) or anti-SRF-threonine 159-phospho antibody (bottom panel). (B) COS cells cotransfected with the indicated plasmids and the HA-epitope tagged SRF proteins were immunoprecipitated by 12CA5 antibody. The immunoprecipitates were subjected to SDS−PAGE and immunoblotted with either anti-SRF-peptide antibody (top panel) or anti-SRF-threonine 159-phospho antibody (bottom panel). (C) COS cells were cotransfected with the indicated plasmids, treated with or without PMA, and the HA-epitope tagged SRF proteins immunoprecipitated by 12CA5 antibody as described in Experimental Procedures. The immunoprecipitates were subjected to SDS−PAGE and immunoblotted with either anti-SRF-peptide antibody (top panel) or anti-SRF-threonine 159-phospho antibody (bottom panel).

rylation of threonine 159.) We next used the anti-159-PO_4 antibody to demonstrate that threonine 159 is targeted in vivo, using the cotransfection protocol described in Experimental Procedures (Figure 6B,C). The results clearly showed that threonine 159 is phosphorylated in the full-length wild-type SRF in vivo, and the specificity of this effect was revealed by the absence of a signal when the triple mutant full-length SRF, in which all three potential phosphorylation sites in the αI coil are replaced by alanine, was the in vivo substrate. In the experiment with PKCα, treatment with PMA also increased the immunoblot signal intensity of the 159-phosphorylated wild-type SRF, suggesting that phorbol ester-induced activation of the kinase increased threonine 159 phosphorylation.

Effects of Alanine and Aspartic Acid Mutants of Threonine 159 Are Consistent with an Activating Effect of Threonine 159 Phosphorylation on α-Actin Gene Transcription. To assess the functional significance in vivo of the phosphorylation of threonine 159, we measured the effects of alanine or aspartic acid substitutions of this residue on SRF-mediated cardiac α-actin gene transcription, using full-length SRF constructs (Figure 7A). In this experiment, 13-fold activation was seen with LPKH in the presence of wt SRF. Substitution of alanine for threonine 159 in full-length SRF decreased C-Act promoter activity by 60%, while substitution with aspartic acid, which carries a positive charge and mimics phosphorylation, restored transcriptional activity to the level seen with LPKH + wt SRF. Similar results were obtained when the SRF proteins were coexpressed not with the kinase but with the C-Act coactivators Nkx2.5 and GATA4. In this instance, C-Act promoter activity was decreased in the presence of SRF T159A and increased significantly in the presence of SRF T159D. Similar trends were observed when the SRF proteins were expressed alone, but the fold changes were too small to attain statistical significance (data not shown).

Since threonine 159 lies within the region of the MADS box αI coil that interacts with the major groove of the serum response element DNA (*45*), we investigated if mimicking phosphorylation of threonine 159 could alter the binding characteristics of SRF with the α-actin promoters, using gel mobility retardation assays (Figure 7B). Neither the alanine nor the aspartic acid substitution of threonine 159 altered the interactions of of SRF with the cardiac α-actin promoter significantly compared to wt SRF. Similar results were obtained with the skeletal α-actin promoter (data not shown). Hence, modulation of SRF-DNA binding characteristics is unlikely to be the mechanism whereby threonine 159 phosphorylation enhances α-actin gene transcription.

DISCUSSION

These data demonstrate the novel finding that phosphorylation of the SRF MADS box αI coil—specifically, of threonine 159 in the DNA binding domain—enhances transcription of the skeletal and cardiac α-actin genes in heterologous cells. Threonine 159 is a target of at least two kinases involved in myogenic regulation—DMPK and PKCα. The in vivo effect of phosphorylating threonine 159 (as inferred from its aspartic acid substitution) is to enhance cardiac α-actin promoter activity, while that of abrogating its phosphorylation (as inferred from its alanine substitution) is to diminish this activity, consistent with the positive effect of DMPK on SRF-mediated α-actin transcription. The results also demonstrate that the transcriptional effect of DMPK can synergize with those of other known SRF regulators and coactivators such as Nkx2.5 and GATA4 in the context of the cardiac α-actin promoter.

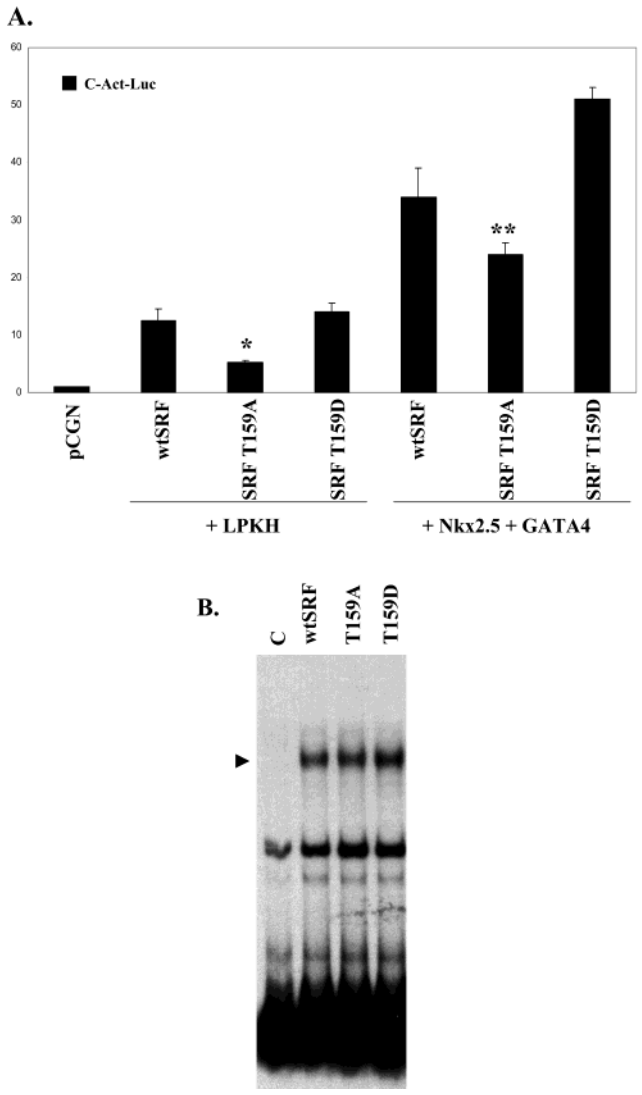

FIGURE 7: Mutation of threonine 159 in the SRF MADS box alters cardiac α-actin promoter activity. (A) Relative luciferase activity after transient transfection of pGL2-C-Act-Luc and the indicated combinations of plasmids in CV1 cells. Basal activation of the C-Act-Luc promoter is measured by mock transfection of the vector (pCGN) alone and given a value of 1. Data are representative of two experiments, each performed in triplicate and displayed ±SD. The figure is a composite of two sets of data. (*$P < 0.05$ for LPKH + SRF T159A as compared to LPKH + wt SRF and LPKH + SRF T159D; **$P < 0.01$ for Nkx2.5 + GATA4 + SRF T159A as compared to Nkx2.5 + GATA4 + SRF T159D.) (B) Binding affinity of wt SRF or threonine 159 mutants to the cardiac actin promoter. Gel mobility retardation assays were performed with nuclear extracts of cells transfected with the indicated plasmid constructs. C = vector control. Arrowhead indicates the SRF:DNA complex.

SRF plays a mandatory role in myogenic differentiation (*3*). Strong evidence suggests that SRF acts as a platform to interact with other regulatory proteins and alter the regulation of muscle specific gene programs (*32*, *35*, *39*). The SRF MADS box αI coil region is crucial for interactions with several transcriptional coactivators, including Nkx2.5 and GATA4. Basic residues in the αI coil are essential for the interaction of SRF with its cognate SREs (*17*). SRF facilitates the recruitment of Nkx2.5 to both high affinity (SREs 2 and 3) and intermediate strength (SREs 1 and 4) binding sites on the cardiac α-actin promoter (*32*, *35*, *46*). SRF and Nkx2.5 also interact directly through the basic region of the SRF αI coil and the N-terminus/helix I and helix II regions of the Nkx2.5 homeodomain (*32*, *47*). SRE1 is also a site of interaction between the MADS box αI coil and the C-terminal zinc finger of GATA4 (*33*). Thus, the MADS box αI coil contains essential sites of interaction with both Nkx2.5 and GATA4, as well as an N-terminal extension that wraps around the DNA to interact with the minor groove of the SRE. We have previously demonstrated the combinatorial synergy of these interactions toward cardiac α-actin promoter activation in heterologous cells (*33*), and the present results demonstrate the added effect of DMPK.

The mechanism whereby threonine 159 phosphorylation regulates α-actin transcription may involve the assembly of specific proteins that interact with the MADS box at either the αI or the αII coils and play important roles in determining myogenic differentiation. Nkx2.5/SRF complexes foster cooperative transactivation complex formation on the cardiac α-actin promoter in 10T1/2 fibroblasts (*46*) and CV1 cells (*33*). Possibly, during early cardiogenesis, paired-related homeodomain genes such as Prx1/Phox-1/MHox or Prx2/S8 (*48*) form nonproductive inhibitory complexes with SRF on α-actin promoter SREs. Such reciprocal interactions could be affected by the phosphorylation state of threonine 159.

Modulation of α-actin expression by DMPK is consistent with its role in skeletal and cardiac muscle differentiation (*22*−*24*, *26*). SRF is expressed early post-replication and during myoblast fusion, while the actin genes are expressed relatively late, during myoblast differentiation (*3*). DMPK mRNA expression rises still later in myogenesis, peaking in C2C12 myoblasts 72−96 h after onset of differentiation (*49*). DMPK expression patterns are thus close to that of mrf-4, and DMPK might play a similar role in the maturation of myotubes. DMPK may also be important for muscle repair, consistent with the phenotype of the DM-1 knockout mouse (*24*). The mild phenotypes of the knockout and transgenic mice suggest the presence of DMPK-like molecules with redundant function that regulate SRF during myogenesis. In this regard, we have recently shown that the RhoA effector $p160^{ROCK}$, which is highly homologous to DMPK in its catalytic domain, modulates transcriptional activity of α-actin promoters in chicken and mouse embryos (*50*) and C2C12 myoblasts (*2*).

SRF is thought to be constitutively present at its cognate SREs, and it has been suggested that physiologically relevant modifications would have to occur in the nucleus (*8*). DMPK is apparently located exclusively in the myocyte cytosol (*20*), so how could it regulate SRF function? In replicating myoblasts, SRF is expressed at low levels predominantly in the cytosol (*3*). During the process of myoblast fusion, SRF appears at higher levels in the cytosol and along filopodia and becomes predominantly nuclear only after fusion, in postreplicative myoblasts and differentiated myotubes. SRF can be detected in both nuclear and cytosolic fractions of skeletal myocytes (*7*), and its regulated translocation between cytosolic and nuclear compartments is important for gene expression in smooth muscle (*51*). Thus, phosphorylation of SRF by DMPK could occur in a cytosolic location, with subsequent translocation of activated SRF to the nucleus, as has been established in other signaling pathways (e.g., the JAK−STAT pathway following cytokine receptor activation (*52*) and PKA-mediated phosphorylation and activation of

Nkx2.1 for transcription of the surfactant protein-A gene (*53*)).

PKC was originally implicated in myogenic gene regulation as a repressor of myogenic differentiation. Activated PKCα can phosphorylate a conserved site in the DNA-binding domain of myogenin and repress the myogenic differentiation program (*41*) while enhancing the activities of growth factor-stimulated pathways that converge on the c-*fos* SRE to promote myoblast proliferation (*44*). However, recent investigations reveal a more complex role for the various PKC isotypes in the regulation of both myoblast proliferation and terminal myogenic differentiation. Capiati et al. have shown a biphasic pattern of PKC expression in chick embryo myogenesis, wherein total PKC catalytic activity is high during myoblast proliferation, declines as myoblasts elongate and fuse, and increases again in differentiated myotubes. These changes are associated with differential isotype-specific expression patterns. PKCα expression is highest during myoblast proliferation and declines during differentiation, whereas PKC*β*, *δ*, and *ϵ* show a reciprocal pattern (*27*, *42*, *43*). Other effects, such as the localization of the PKC isoforms and their intracellular binding proteins, may be involved in a complex network regulating PKC isotype activity. The mechanism whereby PKCα, an isotype associated with inhibition of myogenic differentiation, phosphorylates a site that results in enhanced transcription of a gene associated with terminal myogenic differentiation, remains to be explained. It is possible that the phosphorylation signals that regulate SRF-mediated myogenic differentiation are not simply determined through the activation of specific kinase pathways but require a switching mechanism that directs SRF from a myoblast proliferation program to one of differentiation by exposing specific phosphorylation sites to the appropriate kinases. Although PKCα expression may be low during myogenic differentiation, its catalytic activity toward threonine 159 is likely still present during this phase of myogenesis. Alternatively, the predominant PKC isotypes responsible for phosphorylating threonine 159 and enhancing α-actin transcription during myogenesis may be PKC*β* or *δ*, which are known to be upregulated during differentiation (*42*). Finally, we have demonstrated this effect of PKCα in nonmuscle cells, so it remains to be seen if threonine 159 phosphorylation, and its functional consequences, are similar in the context of replicating myoblasts.

These data demonstrate the key role of phosphoregulation of the DNA binding region in the MADS box αI coil through at least one residue, threonine 159 (but also perhaps others, e.g., serine 162), in modulating SRF's transcriptional activity toward the actin genes. The signaling pathways that regulate this mechanism remain to be elucidated, and are likely to be multiple, since at least two distinct kinases can phosphorylate threonine 159 in vivo. Ongoing investigations will reveal whether phosphorylation of threonine 159 or other sites in the MADS box αI coil also regulate the expression of SRF-dependent proliferative genes such as c-*fos*, if the same mechanism governs DMPK- or PKC-mediated actin gene regulation in the context of myogenic cells, whether nuclear complex formation with known or novel coactivators participate in the transcriptional effect, and whether other kinases can perform a similar regulatory function.

ACKNOWLEDGMENT

We thank P. Porter, R. Narayanan, J. Yeung, and M. Sharma for technical assistance; J. Waring for K100R constructs; and J. Avruch, B. Perryman, A. Rajan, and B.V.V. Prasad for helpful discussions.

REFERENCES

1. Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998) *EMBO J. 17*, 6289−99.
2. Wei, L., Zhou, W., Croissant, J. D., Johansen, F. E., Prywes, R., Balasubramanyam, A., and Schwartz, R. J. (1998) *J. Biol. Chem. 273*, 30287−94.
3. Croissant, J. D., Kim, J. H., Eichele, G., Goering, L., Lough, J., Prywes, R., and Schwartz, R. J. (1996) *Dev. Biol. 177*, 250−64.
4. Treisman, R. (1990) *Semin. Cancer Biol. 1*, 47−58.
5. Chow, K. L., and Schwartz, R. J. (1990) *Mol. Cell Biol. 10*, 528−38.
6. Gauthier-Rouviere, C., Vandromme, M., Tuil, D., Lautredou, N., Morris, M., Soulez, M., Kahn, A., Fernandez, A., and Lamb, N. (1996) *Mol. Biol. Cell 7*, 719−29.
7. Fluck, M., Carson, J. A., Schwartz, R. J., and Booth, F. W. (1999) *J. Appl. Physiol. 86*, 1793−9.
8. Johansen, F. E., and Prywes, R. (1995) *Biochim. Biophys. Acta 1242*, 1−10.
9. Gauthier-Rouviere, C., Basset, M., Blanchard, J. M., Cavadore, J. C., Fernandez, A., and Lamb, N. J. (1991) *EMBO J. 10*, 2921−30.
10. Heidenreich, O., Neininger, A., Schratt, G., Zinck, R., Cahill, M. A., Engel, K., Kotlyarov, A., Kraft, R., Kostka, S., Gaestel, M., and Nordheim, A. (1999) *J. Biol. Chem. 274*, 14434−43.
11. Janknecht, R., Hipskind, R. A., Houthaeve, T., Nordheim, A., and Stunnenberg, H. G. (1992) *EMBO J. 11*, 1045−54.
12. Liu, S. H., Ma, J. T., Yueh, A. Y., Lees-Miller, S. P., Anderson, C. W., and Ng, S. Y. (1993) *J. Biol. Chem. 268*, 21147−54.
13. Manak, J. R., de Bisschop, N., Kris, R. M., and Prywes, R. (1990) *Genes Dev. 4*, 955−67.
14. Manak, J. R., and Prywes, R. (1991) *Mol. Cell Biol. 11*, 3652−9.
15. Marais, R. M., Hsuan, J. J., McGuigan, C., Wynne, J., and Treisman, R. (1992) *EMBO J. 11*, 97−105.
16. Miranti, C. K., Ginty, D. D., Huang, G., Chatila, T., and Greenberg, M. E. (1995) *Mol. Cell Biol. 15*, 3672−84.
17. Sharrocks, A. D., Gille, H., and Shaw, P. E. (1993) *Mol. Cell Biol. 13*, 123−32.
18. Shore, P., and Sharrocks, A. D. (1994) *Mol. Cell Biol. 14*, 3283−91.
19. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) *EMBO J. 15*, 1885−93.
20. Lam, L. T., Pham, Y. C., Nguyen, T. M., and Morris, G. E. (2000) *Hum. Mol. Genet. 9*, 2167−73.
21. Brook, J. D., McCurrach, M. E., Harley, H. G., Buckler, A. J., Church, D., Aburatani, H., Hunter, K., Stanton, V. P., Thirion, J. P., Hudson, T., Sohn, R., Zemelman, B., Snell, R. G., Rundle, S. A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P. S., Shaw, D. J., and Housman, D. E. (1992) *Cell 68*, 799−808.
22. Bush, E. W., Taft, C. S., Meixell, G. E., and Perryman, M. B. (1996) *J. Biol. Chem. 271*, 548−52.
23. Jansen, G., Groenen, P. J., Bachner, D., Jap, P. H., Coerwinkel, M., Oerlemans, F., van den Broek, W., Gohlsch, B., Pette, D., Plomp, J. J., Molenaar, P. C., Nederhoff, M. G., van Echteld, C. J., Dekker, M., Berns, A., Hameister, H., and Wieringa, B. (1996) *Nat. Genet. 13*, 316−24.
24. Reddy, S., Smith, D. B., Rich, M. M., Leferovich, J. M., Reilly, P., Davis, B. M., Tran, K., Rayburn, H., Bronson, R., Cros, D., Balice-Gordon, R. J., and Housman, D. (1996) *Nat. Genet. 13*, 325−35.
25. Soussi-Yanicostas, N., Chevallay, M., Laurent-Winter, C., Tome, F. M., Fardeau, M., and Butler-Browne, G. S. (1991) *Neuromuscul. Disord. 1*, 103−11.
26. Lam, L. T., Furling, D., Nguyen-thi, M., Butler-Browne, G., and Morris, G. E. (2001) in *The 3rd International Myotonic Dystrophy Conference*, Kyoto, Japan.
27. Capiati, D. A., Tellez-Inon, M. T, and Boland, R. L. (1999) *Mol. Cell. Endocrinol. 153*, 39−45.

28. Mahadevan, M. S., Amemiya, C., Jansen, G., Sabourin, L., Baird, S., Neville, C. E., Wormskamp, N., Segers, B., Batzer, M., Lamerdin, J., de Jong, P., Wieringa, B., and Korneluk, R. G. (1993) *Hum. Mol. Genet. 2*, 299−304.
29. Mischak, H., Pierce, J. H., Goodnight, J., Kazanietz, M. G., Blumberg, P. M., and Mushinski, J. F. (1993) *J. Biol. Chem. 268*, 20110−5.
30. Olah, Z., Lehel, C., Jakab, G., and Anderson, W. B. (1994) *Anal. Biochem 221*, 94−102.
31. Rosson, D., O'Brien, T. G., Kampherstein, J. A., Szallasi, Z., Bogi, K., Blumberg, P. M., and Mullin, J. M. (1997) *J. Biol. Chem. 272*, 14950−3.
32. Chen, C. Y., and Schwartz, R. J. (1996) *Mol. Cell Biol. 16*, 6372−84.
33. Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R. J. (1998) *Mol. Cell Biol. 18*, 3405−15.
34. Lee, T. C., Zhang, Y., and Schwartz, R. J. (1994) *Oncogene 9*, 1047−52.
35. Chen, C. Y., Croissant, J., Majesky, M., Topouzis, S., McQuinn, T., Frankovsky, M. J., and Schwartz, R. J. (1996) *Dev. Genet. 19*, 119−30.
36. Anderson, A. E., Adams, J. P., Qian, Y., Cook, R. G., Pfaffinger, P. J., and Sweatt, J. D. (2000) *J. Biol. Chem. 275*, 5337−46.
37. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res. 11*, 1475−89.
38. French, B. A., Chow, K. L., Olson, E. N., and Schwartz, R. J. (1991) *Mol. Cell Biol. 11*, 2439−50.
39. Belaguli, N. S., Sepulveda, J. L., Nigam, V., Charron, F., Nemer, M., and Schwartz, R. J. (2000) *Mol. Cell Biol. 20*, 7550−8.
40. Dunne, P. W., Walch, E. T., and Epstein, H. F. (1994) *Biochemistry 33*, 10809−14.
41. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992) *Cell 71*, 1181−94.
42. Capiati, D. A., Limbozzi, F., Tellez-Inon, M. T., and Boland, R. L. (1999) *J. Cell. Biochem. 74*, 292−300.
43. Capiati, D. A., Vazquez, G., Tellez-Inon, M. T., and Boland, R. L. (2000) *Cell Prolif. 33*, 307−15.
44. Soh, J.-W., Lee, E. H., Prywes, R., and Weinstein, I. B. (1999) *Mol. Cell Biol. 19*, 1313−24.
45. Pellegrini, L., Tan, S., and Richmond, T. J. (1995) *Nature 376*, 490−8.
46. Chen, C. Y., and Schwartz, R. J. (1997) *Mol. Endocrinol. 11*, 812−22.
47. Biesiada, E., Hamamori, Y., Kedes, L., and Sartorelli, V. (1999) *Mol. Cell Biol. 19*, 2577−84.
48. Leussink, B., Brouwer, A., el Khattabi, M., Poelmann, R. E., Gittenberger-de Groot, A. C., and Meijlink, F. (1995) *Mech. Dev. 52*, 51−64.
49. Sabourin, L. A., Tamai, K., Narang, M. A., and Korneluk, R. G. (1997) *J. Biol. Chem. 272*, 29626−35.
50. Wei, L., Roberts, W., Wang, L., Yamada, M., Zhang, S., Zhao, Z., Rivkees, S. A., Schwartz, R. J., and Imanaka-Yoshida, K. (2001) *Development 128*, 2953−62.
51. Camoretti-Mercado, B., Liu, H. W., Halayko, A. J., Forsythe, S. M., Kyle, J. W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J. E., Patel, N. M., Hershenson, M. B., Fuchs, E., Sinha, S., Miano, J. M., Parmacek, M. S., Burkhardt, J. K., and Solway, J. (2000) *J. Biol. Chem. 275*, 30387−93.
52. Darnell, J. E., Jr. (1997) *Science 277*, 1630−5.
53. Li, J., Gao, E., and Mendelson, C. R. (1998) *J. Biol. Chem. 273*, 4592−600.

BI030045N