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Fibroblast Growth Factor Inhibits MRF4 Activity Independently of the Phosphorylation Status of a Conserved Threonine Residue within the DNA-Binding Domain

SERGE HARDY,† YANFENG KONG, AND STEPHEN F. KONIECZNY*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392

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MRF4 is a member of the muscle-specific basic helix-loop-helix transcription factor family that also includes MyoD, myogenin, and Myf-5. Each of these proteins, when overexpressed in fibroblasts, converts the cells to differentiated muscle fibers that express several skeletal muscle genes, such as those for α-actin, muscle creatine kinase, and troponin I. Despite the fact that MRF4 functions as a positive transcriptional regulator, the MRF4 protein is subject to negative regulation by a variety of agents, most notably by exposure of cells to purified growth factors, such as basic fibroblast growth factor (bFGF). In an effort to establish whether bFGF inhibits MRF4 activity through specific posttranslational modifications, we examined whether MRF4 exists in vivo as a phosphoprotein and whether the phosphorylation status of the protein regulates its activity. Our results indicate that MRF4 is phosphorylated predominantly on serine residues, with weak phosphorylation occurring on threonine residues. Both cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) phosphorylate MRF4 in vitro as well as in vivo, and the overexpression of each kinase inhibits MRF4 activity and thus blocks terminal differentiation. PKC-directed phosphorylation of a conserved threonine residue (T-99) situated within the DNA-binding domain inhibits MRF4 from binding in vitro to specific DNA targets. However, although T-99 itself is essential for myogenic activity, our studies demonstrate that the phosphorylation status of T-99 does not play a major role in regulating MRF4 activity in vivo, since PKA, PKC, and bFGF inhibit the activity of MRF4 proteins in which the identified PKA and PKC sites have been mutated. We suggest that the negative regulation of MRF4 imposed by bFGF does not involve a direct modification of the protein at the identified PKA and PKC sites but instead may involve the modification of specific coregulators that interact with this muscle regulatory factor.

The superfamily of basic helix-loop-helix (bHLH) transcription factors regulates a wide variety of developmental processes in organisms ranging from lower eukaryotes to mammals (24). Included in this large protein family are the four muscle-specific bHLH factors known as MRF4 (6, 36, 42), myogenin (15, 54), Myf-5 (7), and MyoD (14). The genes encoding these factors are expressed very early in embryogenesis, at a time when muscle lineage decisions are established (19). The expression of any one factor in a variety of non-muscle cells leads to the production of differentiated muscle fibers that transcriptionally activate muscle-specific genes, such as those encoding the contractile proteins (8, 12, 13, 55). The activation of contractile protein genes requires the formation of a bHLH heterodimer complex consisting of a single muscle regulatory factor and one copy of a widely expressed bHLH protein, such as E12, E47, or HEB (4, 11, 21, 26, 31). This heterodimer complex binds to the nucleotide consensus sequence CANNTG (E box), which is found within the promoters and enhancers regulating most contractile protein genes. The DNA binding, protein dimerization, and transcriptional activation domains of MRF4, myogenin, Myf-5, and MyoD have been identified and partially characterized, making this protein family an excellent model system for studying the complex regulatory events that control tissue-specific gene expression (for reviews, see references 17, 41, and 50).

Although the muscle regulatory factors positively activate the myogenic program, the activity of MRF4 and the other factors is tightly regulated through a growth factor-mediated signal transduction cascade. MRF4-expressing cells maintained in medium containing either a low serum concentration or no serum rapidly fuse and transcriptionally activate several muscle-specific genes, including those for M-creatine kinase, myosin heavy chain, and troponin I (42, 55). Differentiation is inhibited, however, when the serum-free medium is supplemented with fetal bovine serum or supplemented with purified growth factors, such as basic fibroblast growth factor (bFGF) or transforming growth factor beta (TGF-β) (42, 55). Inhibition of MRF4 activity occurs despite the constitutive expression and nuclear localization of the MRF4 protein in cells maintained under either growth factor-supplemented or growth factor-depleted culture conditions. A similar inhibition of activity also is observed when the muscle regulatory factors are coexpressed in cells containing high levels of several growth control gene products, including the c-Myc (37), c-Jun (3, 28), and E1a (5) proteins. The molecular mechanisms through which growth factors and oncoproteins inhibit MRF4, myogenin, Myf-5, and MyoD activities remain largely unknown.

Understanding how the muscle regulatory factors are inhibited in cells exposed to growth factors or to oncogenes is crucial to elucidating the mechanisms by which these factors regulate skeletal myogenesis. Many studies have shown that different regulatory pathways can control the activities of the bHLH factors. As an example, overexpression of the helix-loop-helix protein Id (2), which lacks a basic domain, inhibits MyoD activity by competing for dimerization with E proteins (23). Transcription factor c-Jun also

* Corresponding author.
† Present address: Laboratoire de Biologie et Génétique du Développement, Universite de Rennes, 35042 Rennes Cedex, France.

inhibits muscle-specific gene expression through a direct protein-protein interaction with muscle regulatory factors (3) as well as through competition for putative coregulators of the bHLH proteins (28). Thus, growth factors may inhibit the activities of the myogenic bHLH factors by inducing the expression of any one of these negatively acting partners. Indeed, cells exposed to fetal bovine serum or to bFGF express increased levels of both Id and c-Jun. Consistent with this model of negative regulation, cells exposed to fetal bovine serum express muscle regulatory factors that do not bind to muscle-specific genes (9). However, this mode of negative regulation does not apply to all situations in which differentiation is inhibited, since TGF-β blocks myogenin activity through a mechanism that is independent of DNA binding (9, 34). Thus, several different pathways likely are involved in regulating the activities of MRF4, MyoD, myogenin, and Myf-5 under various growth-promoting conditions.

A second mode of negative regulation may involve direct posttranslational modifications of the bHLH factors, since MyoD and myogenin exist in vivo as phosphoproteins (10, 46). In addition, the activation of several cellular kinases, including cyclic AMP-dependent protein kinase (PKA) (20, 29, 53) and protein kinase C (PKC) (49, 56), has been implicated in the negative regulation of skeletal muscle development. The inhibition of serine-threonine phosphatases also blocks muscle cells from differentiating (25), suggesting that the phosphorylation status of the bHLH proteins may play a critical role in regulating their activities. In agreement with this model, Li et al. (30) demonstrated that myogenin activity is repressed in cells overexpressing the catalytic subunit of PKC and that PKC directly phosphorylates the protein on several sites, including a conserved threonine residue (amino acid 87) that is situated within the myogenin basic domain. Phosphorylation of T-87 in vitro inhibits myogenin from binding to DNA, demonstrating that the phosphorylation status of this single amino acid in myogenin can regulate the DNA binding activity of the protein. Since phosphorylation of this conserved residue occurs in cells exposed to bFGF, the mechanism by which bFGF inhibits muscle cell differentiation may involve a PKC-dependent pathway, ultimately leading to the direct phosphorylation of myogenin within the basic domain. Whether the growth factor signal transduction pathways that regulate MRF4, MyoD, and Myf-5 activities operate via identical or different mechanisms remains to be determined.

As a first step in characterizing MRF4 protein modifications in cells exposed to growth factors, we set out to establish whether MRF4 exists as a phosphoprotein and whether the phosphorylation status of the protein regulates its activity. Our results indicate that MRF4 is phosphorylated predominantly on serine residues, with weak phosphorylation occurring on threonine residues. Both PKA and PKC phosphorylate MRF4 in vitro as well as in vivo, and the overexpression of these kinases inhibits MRF4 activity and thus blocks terminal differentiation. Although the in vitro PKC-directed phosphorylation of MRF4 inhibits the protein from binding to an E-box target, mutational analysis of the identified PKA and PKC sites suggests that the in vivo phosphorylation of MRF4 at these residues has little effect on the activity of the protein. Thus, inhibition by PKA and PKC phosphorylation does not involve phosphorylation of the basic domain but rather involves secondary pathways that nonetheless appear muscle specific. Our results indicate that MRF4 and myogenin are regulated via different mechanisms and that the negative regulation of MRF4 imposed by growth factors does not involve a direct modification of the protein at the PKA and PKC sites but rather may affect specific coregulators that interact with this muscle regulatory factor.

MATERIALS AND METHODS

Cell culture and DNA transfections. C3H10T1/2 (10T1/2) cells were maintained in growth medium containing Eagle basal medium (GIBCO) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml). C3H10T1/2-MRF4-7 (10T1/2-MRF4) cells, which constitutively express a rat MRF4 cDNA, were maintained as myoblasts in growth medium supplemented with 15% fetal bovine serum and 400 μg of G418 (GIBCO) per ml as described previously (42). These cells were induced to differentiate by changing the medium to ITS, a serum-free medium consisting of Dulbecco modified Eagle medium (DMEM; GIBCO) with a low glucose concentration and 5 μg of insulin, 5 μg of transferrin, and 5 ng of selenium per ml (55).

For transient DNA transfections, 10T1/2 cells were plated at 8×10^5/100-mm dish. On the following day, DNA calcium precipitates that consisted of 5 μg of either expression plasmid pEM-MRF4 or control expression plasmid EMSV (see below) plus 5 μg of a chloramphenicol acetyltransferase (CAT) reporter gene were added to the dishes. When PKA or PKC gene constructs were tested, 2 or 5 μg of pEM-MRF4 (with the α-actin CAT or 4R-TKCAT gene, respectively) plus 5 μg of the PKA or PKC expression vector were added to the DNA precipitates. After 4 h, the cells were subjected to a 2-min osmotic shock in serum-free medium containing 20% glycerol and then fed growth medium containing 15% fetal bovine serum. On the following day, the medium was changed to ITS, and the cells were incubated for an additional 48 h. In some instances, cells were fed ITS medium supplemented with 5 ng of bFGF per ml or 5 ng of TGF-β per ml (R&D Products) as described previously (48, 54). For the in vivo labelling studies (see below), T75 flasks were used, and the cell numbers and DNA concentrations were scaled up appropriately. Cultures then were processed either for immunoprecipitations or immunocytochemistry or harvested for CAT assays. For immunocytochemistry, cultures were rinsed twice in cold phosphate-buffered saline and fixed in a 20:2:1 solution of 70% ethanol-formalin-acetic acid for 1 min at 4°C. After incubation with antimyosin mouse monoclonal antibody MF-20 (1), a biotinylated mouse secondary antibody was added. Immunoreactivity was visualized by use of Vectastain ABC reagent (Vector Laboratories, Inc.). CAT assays were performed as described by Gorman (18), and the percentage of acetylated chloramphenicol was determined by liquid scintillation counting. The amount of extract used for each assay was normalized to the protein content of each sample. A minimum of three independent transfections were performed for each experimental group.

Gene constructions. Eukaryotic expression vector pEMscribeα2 (EMSV) utilizes the Moloney sarcoma virus long terminal repeat to drive the expression of cloned cDNAs (14, 27). pEM-MRF4 represents the EMSV vector containing the rat MRF4 cDNA (42). Target genes for *trans*-activation assays include α-actin CAT and 4R-TKCAT. α-actin CAT is the human cardiac α-actin reporter gene LK359CAT (33, 38) containing 359 bp of the α-actin promoter ligated to the CAT gene. 4R-TKCAT contains four tandem copies of the muscle creatine kinase enhancer right E-box site cloned into the

thymidine kinase CAT reporter gene (51). A β-actin CAT gene (16) containing the chicken β-actin promoter also was used as a positive control. Plasmid MT-CEVneo was used for expression of the catalytic subunit of PKA (35). This plasmid contains the C subunit of PKA in an expression vector that utilizes the metallothionein 1 promoter. PKC-7 contains amino acids 302 to 672 of bovine PKC-α cloned into the expression vector pCDM8, containing the cytomegalovirus promoter and enhancer (22). PKC-7 lacks the regulatory domain of PKC and therefore is constitutively active. In some instances, the expression plasmid SRα-PKAC (39), which similarly produces a constitutively active PKC-α catalytic domain, also was tested.

MRF4 protein was expressed in *Escherichia coli* by ligating an *Eco*RI-*Xba*I fragment of MRF4 cDNA, containing amino acids 1 to 242, in frame to a modified pGEX-2T (44) expression plasmid containing an altered multiple cloning site and the glutathione-S-transferase (GST) protein. For some studies, we used full-length MRF4 cDNA in which three arginine codons in the basic region (amino acids 103 to 105) were changed to different arginine codons to increase the amount of protein produced by the bacteria. GST-MRF4 deletions were generated by subcloning *Eco*RI-*Xba*I fragments, derived from published GAL4-MRF4 clones (33), in frame into plasmid pGEX. GST-E12 was produced by ligating the *Eco*RI insert from E12R (40) into plasmid pGEX-1 (44). All GST fusion proteins were purified by chromatography on glutathione-agarose beads and quantified by Coomassie blue staining.

MRF4 point mutations (MRF4-A89, MRF4-A99, MRF4-D99, MRF4-A220/221, and MRF4-D220/221) were generated by use of specific oligonucleotide primers and an Amersham oligonucleotide mutagenesis kit. Mutations were produced with single-stranded DNA obtained from the appropriate pBluescript (Stratagene) clones, after which the mutated cDNAs were subcloned into either eukaryotic expression vector EMSV or prokaryotic expression vector pGEX-2T. All mutations and subsequent reading frames were verified by dideoxynucleotide DNA sequencing.

In vitro kinase assays. In vitro PKA and PKC phosphorylation assays were performed by standard procedures. In brief, 0.5 μg of the GST protein or the appropriate GST-MRF4 protein was used for each reaction. For PKA, GST protein was incubated with 5 U of PKA (catalytic subunit; Sigma P-2645) in 10 mM Tris (pH 7.5)–5 mM magnesium chloride–1 mM calcium chloride–40 μM ATP–40 μCi of γ-[^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) for 20 min at 30°C in a 25-μl reaction mixture. In vitro PKC assays were similarly performed with 0.5 μg of GST protein and 80 μg of phosphatidylserine per ml–10 mM Tris (pH 7.5)–10 mM $MgCl_2$–0.5 mM $CaCl_2$–40 μM ATP–40 μCi of [γ-^{32}P]ATP–1 μl of purified rat brain PKC (specific activity, 4.6 pmol/min/μl, for a histone substrate) (kindly provided by C. Ashendel) for 20 min at 30°C. For direct analysis of phosphorylation, reactions were terminated by the addition of 2× sodium dodecyl sulfate (SDS) sample buffer, and samples were subsequently resolved on SDS–12% polyacrylamide gels.

Electrophoretic mobility shift assays. Oligonucleotide probes containing the troponin I enhancer E-box (32) were generated by labelling one DNA strand with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham). Unincorporated [γ-^{32}P]ATP was removed by passing the reaction mixtures through Sephadex G-50 spin columns. The single-stranded labelled DNA was mixed with a 10-fold molar excess of the unlabelled complementary oligonucleotide strand, and the mixture was heated to 100°C for 5 min and allowed to anneal at room temperature to generate double-stranded oligonucleotides.

Electrophoretic mobility shift assays were conducted essentially as described previously (31). In brief, 0.2 μg of GST, GST-MRF4, GST-E12, or a combination of GST-MRF4 and GST-E12 was heated to 37°C for 15 min and then allowed to cool at room temperature. The protein was incubated with the end-labelled probe for 20 min at room temperature in binding buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 50 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, and 100 μg of poly(dI-dC) per ml. The binding reaction mixture subsequently was loaded onto a 5% polyacrylamide gel and electrophoresed at room temperature. After electrophoresis, the gel was fixed in 10% methanol–10% acetic acid, vacuum dried, and exposed to X-ray film. In some instances, the GST-MRF4 protein was phosphorylated by PKC in the presence or absence of 10 mM ATP prior to being mixed with the GST-E12 protein as described above.

In vivo labelling and immunoprecipitations. 10T1/2 or 10T1/2-MRF4 cells were rinsed in DMEM lacking methionine or rinsed in modified Eagle medium lacking sodium phosphate for labelling with [^{35}S]methionine or with $^{32}P_i$, respectively. Following 1 h of incubation, the cells were refed the same medium containing either 0.2 mCi of [^{35}S]methionine per ml or 0.8 mCi of $^{32}P_i$ per ml. After 4 h, the cells were rinsed in ice-cold saline and harvested by use of a rubber policeman. The cells were pelleted at 4°C in a clinical centrifuge, and the supernatant was discarded. The cell pellet was resuspended in 1 ml of RIPA buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 0.1% Triton X-100) containing 2 μg each of aprotinin, pepstatin, and leupeptin per ml plus 50 mM NaF and 1 mM Na_3VO_4 (phosphatase inhibitors), and the suspension was vortexed and incubated on ice for 30 min. After a brief centrifugation, the supernatant was placed into a 1.5-ml screw-cap tube, and 5 μl of preimmune anti-MRF4 serum and 50 μl of staphylococcal A (Sigma) were added. The tube was rocked for 1 h and then subjected to centrifugation. The cleared supernatant was removed to a new tube, 10 μl of anti-MRF4 serum (45) was added along with 50 μl of protein A-agarose (Schleicher & Schuell), and the mixture was rocked for 2 h. After centrifugation, the pellet was rinsed three times in RIPA buffer and then resuspended in 2× SDS loading buffer. After 5 min of boiling, the immunoprecipitated protein was electrophoresed through an SDS–12% polyacrylamide gel, and the gel was dried without fixation and exposed to X-ray film.

Phosphoamino acid analysis. The in vitro- or in vivo-labelled protein was excised from the dried gel, and the gel slice was placed in a 1.5-ml screw-cap tube containing 500 μl of freshly prepared 50 mM ammonium bicarbonate. After rehydration for 10 min, the gel slice was homogenized in the 1.5-ml tube by use of a disposable plastic pestle (Kontes) and transferred to a new tube. After the original tube and pestle were rinsed two times each with 250 μl of 50 mM ammonium bicarbonate, the solutions were combined. Fifty microliters of β-mercaptoethanol and 10 μl of 10% SDS were added to the 1-ml sample, which was then vortexed, boiled for 5 min, and rocked overnight at room temperature. After a brief centrifugation, the supernatant was transferred to a new tube, which was then placed on ice, 2 μl of 10-mg/ml RNase A and 250 μl of 100% trichloroacetic acid were added and mixed, and the mixture was incubated on ice for 90 min. Following 10 min of centrifugation, the supernatant was

discarded, and the pellet was rinsed once in 500 μl of ethanol and air dried for 30 min.

The precipitated pellet then was resuspended in 200 μl of 5.7 N HCl, and the tube was purged with nitrogen gas prior to replacement of the cap. The sample was incubated at 110°C for 90 min, centrifuged, and transferred to a new tube. After the supernatant was dried under vacuum, the pellet was resuspended in 5 μl of distilled water containing phosphoamino acid standards (Sigma). Two microliters of the sample was spotted onto a thin-layer chromatography (TLC) plate and electrophoresed in the first dimension in pH 1.9 buffer consisting of 80% formic acid, glacial acetic acid, and water (50:156:1,794 [vol/vol/vol]) for 30 min at 1,600 V. After the TLC plate was dried, the sample was electrophoresed in the second dimension in pH 3.5 buffer consisting of glacial acetic acid, pyridine, and water (100:10:1,890 [vol/vol/vol]) for 20 min at 1,300 V. The TLC plate then was dried, sprayed with 0.25% ninhydrin to detect the phosphoamino acid standards, and exposed to X-ray film.

Phosphopeptide mapping. The ^{32}P-labelled protein was isolated from an SDS gel, homogenized, precipitated with trichloroacetic acid, and air dried as described above for the phosphoamino acid analyses. The protein then was oxidized at 4°C for 60 min in 100 μl of performic acid. After the addition of 400 μl of water, the sample was frozen at −80°C for 1 h and then dried in a speed vacuum concentrator for 2 h. The pellet was resuspended in 50 μl of 50 mM ammonium bicarbonate; to this suspension was added 10 μl of trypsin (sequencing grade; Boehringer) at 1 μg/μl, and the mixture was incubated at 37°C overnight. On the following day, 10 μl of trypsin was again added, and the mixture was incubated for 2 to 4 h at 37°C. After the addition of 400 μl of water, the mixture was dried in a speed vacuum concentrator for 2 h. The addition of water and then drying were repeated one more time, the pellet was resuspended in 400 μl of pH 1.9 buffer, and the suspension was vortexed, transferred to a new tube, and dried for 2 h as above. The final pellet was resuspended in 5 μl of pH 1.9 buffer, and 2 μl (typically ~300 cpm) was applied to a TLC plate along with appropriate marker dyes (ε-DNP lysine and xylene cyanol). Electrophoresis in the first dimension was performed with pH 1.9 buffer for 35 min at 1,000 V. After the plate was dried, the sample was subjected to chromatography in the second dimension with phosphochromatography buffer consisting of *n*-butanol, pyridine, glacial acetic acid, and water (75:50:15:60 [vol/vol]) for 4 h. The TLC plate then was dried and exposed to X-ray film.

RESULTS

MRF4 is phosphorylated in vivo. The transcriptional activity of the MRF4 protein is negatively regulated in cells exposed to high concentrations of serum or to purified growth factors, such as bFGF and TGF-β (42, 55). Inhibition by these agents suggests that MRF4 may be controlled posttranslationally by specific phosphorylation events. As an initial step in examining potential MRF4 protein modifications, 10T1/2-MRF4 cells (42) were maintained as undifferentiated myoblasts or were induced to differentiate into myofibers. After incubation of myoblast and myofiber cultures with [^{35}S]methionine or with $^{32}P_i$, the cells were lysed and newly labelled MRF4 protein was immunoprecipitated with an MRF4-specific polyclonal antibody (45). As expected, MRF4 was not detected in control, nontransfected 10T1/2 cells, but the ~33-kDa [^{35}S]labelled protein was observed in 10T1/2-MRF4 myoblasts and in 10T1/2-MRF4 myofibers (Fig. 1A). An identical MRF4 band also was detected in the $^{32}P_i$-labelled cells, establishing that MRF4 exists as a phosphorylated transcription factor in both myoblast and myofiber cultures. Two-dimensional phosphoamino acid analysis of the in vivo-labelled protein revealed that MRF4 was phosphorylated predominantly on serine residues (Fig. 1B), although with extended exposures a faint signal corresponding to phosphothreonine also was detected. Phosphorylation of tyrosine residues was not observed under any culture conditions.

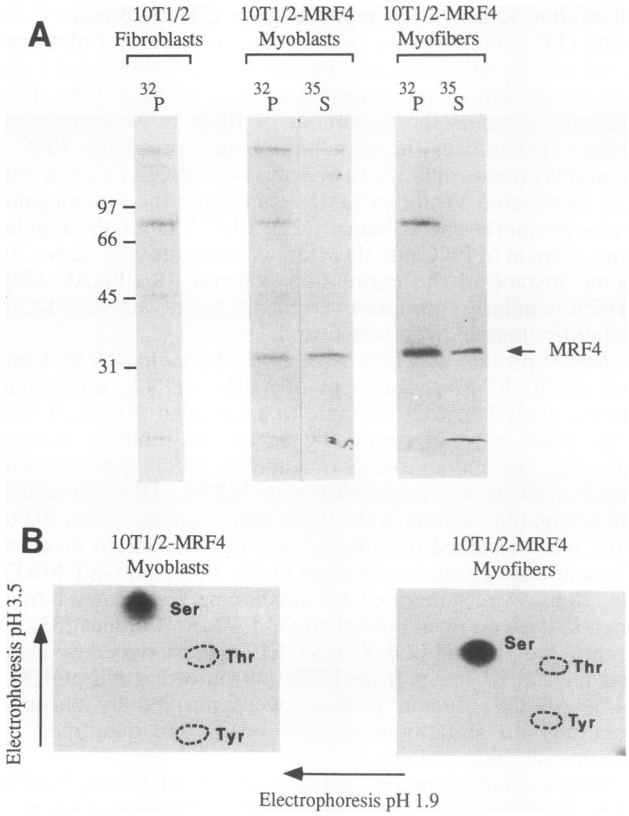

FIG. 1. MRF4 is phosphorylated in myoblasts and in myofibers. (A) Control 10T1/2 fibroblasts as well as 10T1/2-MRF4 myoblast and myofiber cultures were labelled with [^{35}S]methionine or with $^{32}P_i$. After being labelled, the cells were lysed, the 33-kDa MRF4 protein was immunoprecipitated, and the products were resolved by 12% SDS-polyacrylamide gel electrophoresis. The positions of protein standards and the MRF4 protein are indicated. (B) The $^{32}P_i$-labelled MRF4 protein from panel A was subjected to phosphoamino acid analysis and electrophoresed in two dimensions. The positions of phosphoserine, phosphothreonine, and phosphotyrosine residues are shown.

MRF4 is negatively regulated by PKA and PKC. Of the known serine-threonine protein kinases, PKA and PKC are the most extensively characterized. These kinases have been implicated in regulating various aspects of muscle development, including the inhibition of skeletal muscle terminal differentiation events (20, 56). To begin examining whether PKA and PKC regulate MRF4 directly, we measured MRF4 activity in cells expressing constitutively active PKA and PKC isoforms (see Materials and Methods for details). As shown in Table 1, in 10T1/2 cells cotransfected with an MRF4 expression vector, the expression of an α-actin CAT reporter gene was activated. The expression of MRF4 also

TABLE 1. Repression of MRF4 activity by PKA and PKC[a]

Protein kinase	% Expression of:		
	α-Actin CAT	Myosin (as staining)	4R-TKCAT
None	100	100	100
PKA	23	25	33
None	100	100	100
PKC	12	21	17

[a] 10T1/2 cells were cotransfected with pEM-MRF4 and either the α-actin CAT or the 4R-TKCAT reporter gene in the presence or absence of the PKA and PKC expression plasmids (see Materials and Methods for details). Relative expression levels for the CAT reporter genes as well as for the endogenous skeletal myosin genes are given.

converted the cells to a myogenic phenotype, leading to the formation of myofibers and the expression of the endogenous sarcomeric myosin heavy-chain genes (33, 42, 55). Interestingly, cotransfection of 10T1/2 cells with the PKA expression vector decreased MRF4-induced α-actin CAT and myosin expression approximately 75% (Table 1). Similarly, overexpression of the constitutively active PKC protein inhibited MRF4 activity (Table 1), whereas cotransfection of the PKA or PKC expression plasmid into 10T1/2 cells had no effect on the basal level of α-actin CAT expression (data not shown). These results suggest that specific intracellular phosphorylation events via PKA and/or PKC pathways negatively regulate the activity of the MRF4 protein.

Interpretation of the data given above is complicated by the fact that the promoters and enhancers regulating the expression of the contractile protein genes, such as α-actin, contain multiple regulatory elements, in addition to E boxes, that are required for muscle-specific activation (32, 43). Thus, expression of the constitutively active PKA and PKC proteins may inhibit α-actin and myosin expression not by directly repressing MRF4 activity but rather by repressing the activities of other enhancer binding proteins that have a critical role in the regulation of these muscle-specific genes. For examination of this possibility a minimal reporter gene, 4R-TKCAT (51), was tested. 4R-TKCAT contains four copies of the right E-box site derived from the muscle creatine kinase enhancer ligated 5′ to the nonspecific viral thymidine kinase promoter. In the absence of MRF4, 4R-TKCAT is expressed at a low basal level that is not affected by cotransfection of the PKA or PKC expression plasmid (data not shown). In the presence of MRF4, however, 4R-TKCAT is expressed to relatively high levels that are subject to repression by both PKA and PKC (Table 1), confirming that the inhibition of muscle differentiation by these protein kinases involves the inhibition of MRF4 and E-box-dependent transcription complexes. Thus, it is likely that PKA and PKC inhibit MRF4 activity by directly phosphorylating the MRF4 protein or by phosphorylating MRF4-associated factors that may be required to elicit a proper E-box-dependent muscle-specific transcription response.

MRF4 serves as an in vitro substrate for PKA and PKC. The ability of PKA and PKC to inhibit MRF4 activity suggests that these kinases may directly phosphorylate the MRF4 protein. For testing of this hypothesis, GST fusion proteins were generated by using the MRF4 cDNA. The *E. coli*-produced proteins then were subjected to in vitro kinase assays with PKA or PKC. As shown in Fig. 2A, GST-MRF4 served as a substrate for both PKA and PKC, whereas the control GST protein remained unphosphorylated. Phosphoamino acid analyses of the labelled MRF4 protein revealed that PKA exclusively phosphorylated serine residues, whereas PKC phosphorylated serine and threonine residues (Fig. 2A). As expected, PKA and PKC did not phosphorylate tyrosine residues.

Analysis of the primary MRF4 amino acid sequence (42) revealed several potential PKA and PKC sites that were distributed throughout the protein. For identification of each phosphorylation site, tryptic phosphopeptide mapping studies were performed with in vitro-labelled GST-MRF4 proteins. As shown in Fig. 2B, phosphorylation of MRF4 by PKA, coupled with tryptic peptide digestion, produced two labelled peptides (1 and 3) that were distinguishable by two-dimensional analysis. Tryptic digestion of the full-length MRF4 protein labelled with PKC also produced two major phosphopeptides (1 and 2a) plus a third phosphopeptide (2b) that exhibited variable intensities in different experiments (see below). Phosphopeptide 1 corresponds to peptide 1 obtained by PKA phosphorylation, whereas phosphopeptide 2a represents a unique PKC phosphorylation target. Since PKA phosphorylated only serine residues in MRF4, while PKC phosphorylates serine and threonine residues (Fig. 2A), peptides 1 and 3 must contain phosphoserine residues, while peptide 2a minimally contains a phosphothreonine residue (see below).

Identification of the precise MRF4 amino acid targets for PKA and PKC phosphorylation. For identification of the serine and threonine residues that serve as targets for PKA and PKC phosphorylation, site-directed mutagenesis was performed on the MRF4 protein, and phosphorylated proteins were subjected to tryptic peptide analysis. Using a series of GST-MRF4 deletions, coupled with in vitro kinase assays and tryptic digestions (data not shown), we found that the PKA-labelled peptide (1) likely represents MRF4 residues LSSIVDSISSEER (amino acids 219 to 231), while the minor PKA-labelled peptide (3) represents tryptic peptide fragment SAPTDR spanning amino acids 89 to 94. Similarly, tryptic peptide analysis of labelled GST-MRF4 deletions demonstrated that PKC-labelled peptide 2a, which contains a phosphothreonine residue, consists of the sequence AATLR, representing amino acids 97 to 101, while the variable peptide (2b) (Fig. 2B) is produced via partial tryptic digestion of this region, producing the larger peptide AATLRER (see below). Analysis of the in vitro kinase assay results, coupled with the MRF4 primary amino acid sequence data, strongly suggested that the targets for PKA phosphorylation are serines 89, 220, and 221, while the targets for PKC phosphorylation are threonine 99 and serines 220 and 221.

For confirmation of each PKA target, serines 89, 220, and 221 were converted to alanine residues by in vitro mutagenesis. As shown in Fig. 3A, PKA phosphorylation of GST-MRF4-A89, coupled with tryptic digestion, resulted in the appearance of peptide 1 but not peptide 3. Similarly, PKA phosphorylation and tryptic digestion of GST-MRF4-A220/221 produced the expected peptide 3 fragment but not peptide 1. Thus, the major in vitro PKA target in MRF4 is located at serines 220 and 221, while a second PKA site is present at serine 89 (Fig. 3C). Similar strategies also were used to map the precise locations of the serine and threonine residues that were phosphorylated in vitro by PKC. As expected, mutagenesis of T-99 to A-99 and then phosphorylation by PKC produced only peptide 1, whereas PKC phosphorylation of GST-MRF4-A220/221, coupled with tryptic digestion, generated only peptides 2a and 2b (Fig. 3B), confirming that threonine 99 and serines 220 and 221

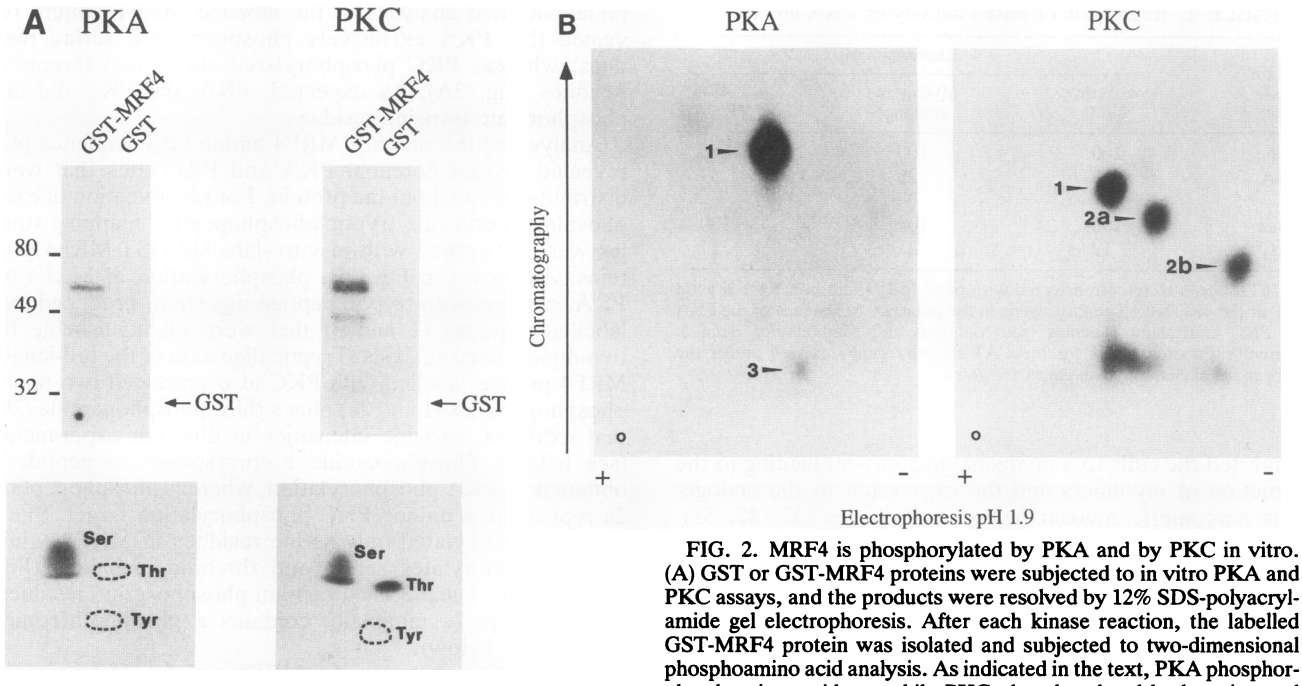

FIG. 2. MRF4 is phosphorylated by PKA and by PKC in vitro. (A) GST or GST-MRF4 proteins were subjected to in vitro PKA and PKC assays, and the products were resolved by 12% SDS-polyacrylamide gel electrophoresis. After each kinase reaction, the labelled GST-MRF4 protein was isolated and subjected to two-dimensional phosphoamino acid analysis. As indicated in the text, PKA phosphorylated serine residues, while PKC phosphorylated both serine and threonine residues, within the MRF4 protein. Numbers at left indicate sizes in kilodaltons. (B) PKA phosphorylation of GST-MRF4 and then tryptic digestion produced one major peptide (1) and a minor peptide (3), while PKC phosphorylation of the same protein produced peptide 1 and a second peptide (2a). Peptide 2b represents partial tryptic digestion of peptide 2a (see the text for details).

serve as substrates for PKC phosphorylation in vitro. These studies demonstrate that PKA and PKC phosphorylate a common site in the carboxyl region of MRF4 as well as two different sites located within the basic domain of the protein (Fig. 3C).

Transcriptional and DNA binding activities of individual MRF4 mutants. In an effort to establish the role that each potential PKA and PKC phosphorylation site plays in regulating MRF4 activity, we examined the ability of the wild-type MRF4 protein and various point mutants to convert 10T1/2 fibroblasts to differentiated muscle fibers. As discussed earlier, MRF4 efficiently activates cotransfected α-actin CAT reporter genes as well as endogenous contractile protein genes, such as myosin heavy-chain genes. Mutation of the basic-region PKA site (MRF4-A89) or mutation of the carboxyl-region PKA and PKC sites to either alanine or aspartic acid residues (MRF4-A220/221; MRF4-D220/221) had no significant effect on the transcriptional activity of the MRF4 protein, as measured by α-actin CAT and myosin expression (Fig. 4). In contrast, mutation of threonine 99, which serves as an in vitro PKC phosphorylation site, had a dramatic effect on MRF4 activity. MRF4-A99 and MRF4-D99 both exhibited reduced transcriptional activity, with MRF4-D99 being completely inactive in its ability to convert cells to a myogenic phenotype (Fig. 4). Immunocytochemistry analysis with MRF4-specific antibodies revealed that both MRF4-A99 and MRF4-D99 were expressed to high levels and efficiently translocated to the nucleus (data not shown). Thus, although MRF4-A99 and MRF4-D99 are deficient in their ability to function as muscle regulatory factors, this deficiency does not involve alterations in protein stability or alterations in nuclear localization.

To establish how mutations of T-99 inhibit MRF4 activity, we next examined the DNA binding properties of the mutant MRF4 proteins. Various GST fusion proteins were incubated with an E-box oligonucleotide probe and electrophoresed through a nondenaturing polyacrylamide gel as described previously (31). When used at limiting protein concentrations, the GST, GST-MRF4, and GST-E12 proteins did not bind to the E-box oligonucleotide (Fig. 5A). Incubation of GST-MRF4 plus GST-E12 with the E-box oligonucleotide, however, produced a major shifted complex that was inhibited efficiently through competition by excess wild-type E-box oligonucleotides but not by oligonucleotides encoding a mutated E-box sequence (Fig. 5A). Analysis of the DNA binding activities of individual MRF4 mutants revealed that GST-MRF4-A89, GST-MRF4-A99, and GST-MRF4-A220/221 bound to the E-box sequence when incubated with the E12 protein (Fig. 5B), although GST-MRF4-A99 bound less efficiently to DNA than the wild-type protein. In contrast, GST-MRF4-D99 did not bind in this assay, even though GST-MRF4-D99 efficiently dimerizes with E12 (32a). Identical results were obtained when the muscle regulatory factors were produced by in vitro translation reactions and analyzed by gel mobility shift assays (data not shown). Our results suggest, therefore, that mutation of threonine 99 to aspartic acid dramatically inhibits the activity of the MRF4 protein and that this inhibition is due in part to an inability of the protein to bind to the E-box sequence, despite dimerization with E12 and translocation to the nucleus.

As discussed above, T-99 is required for the muscle-specific activity of MRF4 and also serves as an in vitro substrate for PKC phosphorylation. Mutation of this threonine residue to aspartic acid interferes with DNA binding, suggesting that the addition of a negative charge to the basic region inhibits MRF4 activity by altering the DNA binding characteristics of this transcription factor. For examination of whether phosphorylated MRF4 proteins exhibit altered

DNA binding activities, GST-MRF4 proteins were phosphorylated in vitro by PKC, mixed with GST-E12 proteins and E-box oligonucleotides, and then subjected to mobility shift assays as described above. Incubation of GST-MRF4-A220/221 or GST-MRF4-A99 with PKC (minus ATP) had no significant effect on the ability of the protein to bind to the targeted E-box oligonucleotide (Fig. 6). DNA binding was reduced approximately 60%, however, when GST-MRF4-A220/221 was preincubated with PKC plus ATP, demonstrating that phosphorylation of threonine 99 reduces the binding of MRF4 to DNA. A similar reduction in DNA binding was observed when myogenin was phosphorylated in vitro by PKC (Fig. 6) (30). When this experiment was repeated with GST-MRF4-A99, however, no inhibition of DNA binding was detected, despite the ability of PKC to phosphorylate serines 220 and 221 in this mutant protein (Fig. 3B). Thus, in these in vitro experiments, the inhibition of DNA binding of MRF4 was due entirely to the phosphorylation of T-99, which is located in the conserved basic region of this muscle regulatory factor.

PKA and PKC phosphorylate MRF4 in vivo. The importance of the phosphorylation status of T-99 and the ability of MRF4 to be phosphorylated by PKA and by PKC in vitro suggest that the MRF4 protein may be regulated in vivo through similar phosphorylation mechanisms. For establishment of whether the identified in vitro PKA and PKC sites are utilized in vivo, 10T1/2 cells were cotransfected with expression vectors containing MRF4 and the constitutively active PKA or PKC cDNA. After incubation of the cells with $^{32}P_i$, the MRF4 protein was immunoprecipitated and subjected to trypsin digestion. As shown in Fig. 7, control MRF4-transfected 10T1/2 cells revealed only a limited amount of MRF4 phosphorylation within peptide 1, whereas the majority of labelling occurred within a large amino-terminal peptide. Unfortunately, this tryptic peptide, which appeared as two smears on the TLC plate, did not resolve well because of its large size (83 amino acids) and hydrophobic properties. Cotransfection of 10T1/2 cells with MRF4 and PKA, however, generated a large increase in the phosphorylation of peptides 1 and 3 compared with the level of phosphorylation obtained with the control cells (Fig. 7). Similarly, overexpression of the constitutively active PKC construct produced an increase in the phosphorylation of peptide 1, but surprisingly, no increase was detected for peptide 2a. In both instances, only previously identified peptides from the in vitro kinase assays were detected with the in vivo assays, confirming that PKA and PKC phosphorylate a limited number of residues within the MRF4 protein. These results demonstrate that, in vivo, PKA phosphorylates MRF4 serines 89, 220, and 221, while PKC phosphorylates serines 220 and 221. PKC expression did not lead to the phosphorylation of threonine 99 in 10T1/2 cells, suggesting that threonine 99 is not readily phosphorylated in vivo under these conditions.

Overexpression of PKA and PKC inhibits MRF4 activity through indirect pathways. The ability of MRF4 to be phosphorylated by PKA and PKC suggests that these phosphorylation events may play a role in regulating MRF4 activity. We therefore examined whether the overexpression of PKA or PKC inhibited the transcriptional activity of MRF4 mutants. For these studies, 10T1/2 cells were cotransfected with the α-actin CAT or 4R-TKCAT reporter gene along with either the PKA or the PKC expression vector. As discussed previously, wild-type MRF4 protein activity was inhibited approximately 75% in cells overexpressing PKA. Interestingly, a similar level of inhibition also occurred when MRF4-A89, MRF4-A99, and MRF4-A220/221 were tested in these assays. In all cases, α-actin CAT and 4R-TKCAT expression was repressed to the same extent as that detected for the wild-type MRF4 protein (Fig. 8). Since PKA phosphorylates serines 89, 220, and 221 in vivo, the individual phosphorylation of these residues cannot account for the observed PKA-dependent repression. Instead, PKA inhibition must involve secondary mechanisms that impinge upon MRF4 activity (see Discussion).

Similar results were obtained when 10T1/2 cells were cotransfected with the PKC expression vector and each MRF4 mutant protein. Again, the overexpression of PKC led to the repression of wild-type MRF4 activity (Fig. 8). Additionally, PKC overexpression inhibited the transcriptional activity of MRF4-A220/221, suggesting that phosphorylation of this in vivo PKC site is not a prerequisite for the PKC-dependent inhibition of MRF4. PKC also inhibited the transcriptional activity of MRF4-A99, demonstrating that although T-99 is crucial for MRF4 activity, phosphorylation of T-99 by PKC is not the primary mechanism by which PKC inhibits in vivo MRF4 activity. We conclude that the ability of PKA and PKC to inhibit MRF4 activity must be due to an indirect mechanism and not to the specific phosphorylation of serines 89, 220, and 221 or of threonine 99 (see Discussion).

bFGF and TGF-β also inhibit MRF4 activity via a mechanism that is independent of threonine 99 phosphorylation. Muscle differentiation and MRF4 activity are inhibited in cells exposed to high concentrations of serum or to purified growth factors, such as bFGF or TGF-β. We have demonstrated in this study that MRF4 activity also is inhibited by the overexpression of PKA or PKC, although the PKA- or PKC-dependent inhibition does not require the direct phosphorylation of the MRF4 protein on specific PKA or PKC target amino acid residues. For examination of whether MRF4 is similarly inhibited via an indirect mechanism by bFGF and TGF-β, 10T1/2 cells were transfected with the MRF4 expression vector and then maintained in differentiation medium either supplemented or not supplemented with bFGF or TGF-β. Following $^{32}P_i$ labelling and immunoprecipitation, the MRF4 protein was subjected to phosphoamino acid analysis as described above. As expected, MRF4 protein isolated from cells maintained in differentiation medium or from cells cotransfected with the PKC expression vector exhibited predominantly phosphoserine residues and only a very weak phosphothreonine signal, confirming that T-99 is not significantly phosphorylated in 10T1/2 cells maintained under these conditions (Fig. 9). Phosphoserine also was the predominantly labelled amino acid detected in MRF4 protein isolated from cells maintained with bFGF or TGF-β, although an extremely small increase in the phosphothreonine signal was detected with bFGF. Tryptic digests of $^{32}P_i$-labelled MRF4 from each group revealed that peptide 1 but not peptide 2a was phosphorylated in cells maintained in the presence of bFGF or in the presence of TGF-β (data not shown), confirming that the phosphorylation of T-99 is not required to inhibit the cells from differentiating.

For examination of whether serines 89, 220, and 221 or threonine 99 is essential for the bFGF-dependent inhibition of MRF4 activity, each MRF4 mutant protein was tested for activity in the absence or presence of bFGF. As expected, bFGF increased the activity of the control non-muscle β-actin reporter gene (Fig. 10). Conversely, bFGF treatment led to the repression of α-actin CAT, 4R-TKCAT, and myosin expression when the wild-type MRF4 protein was

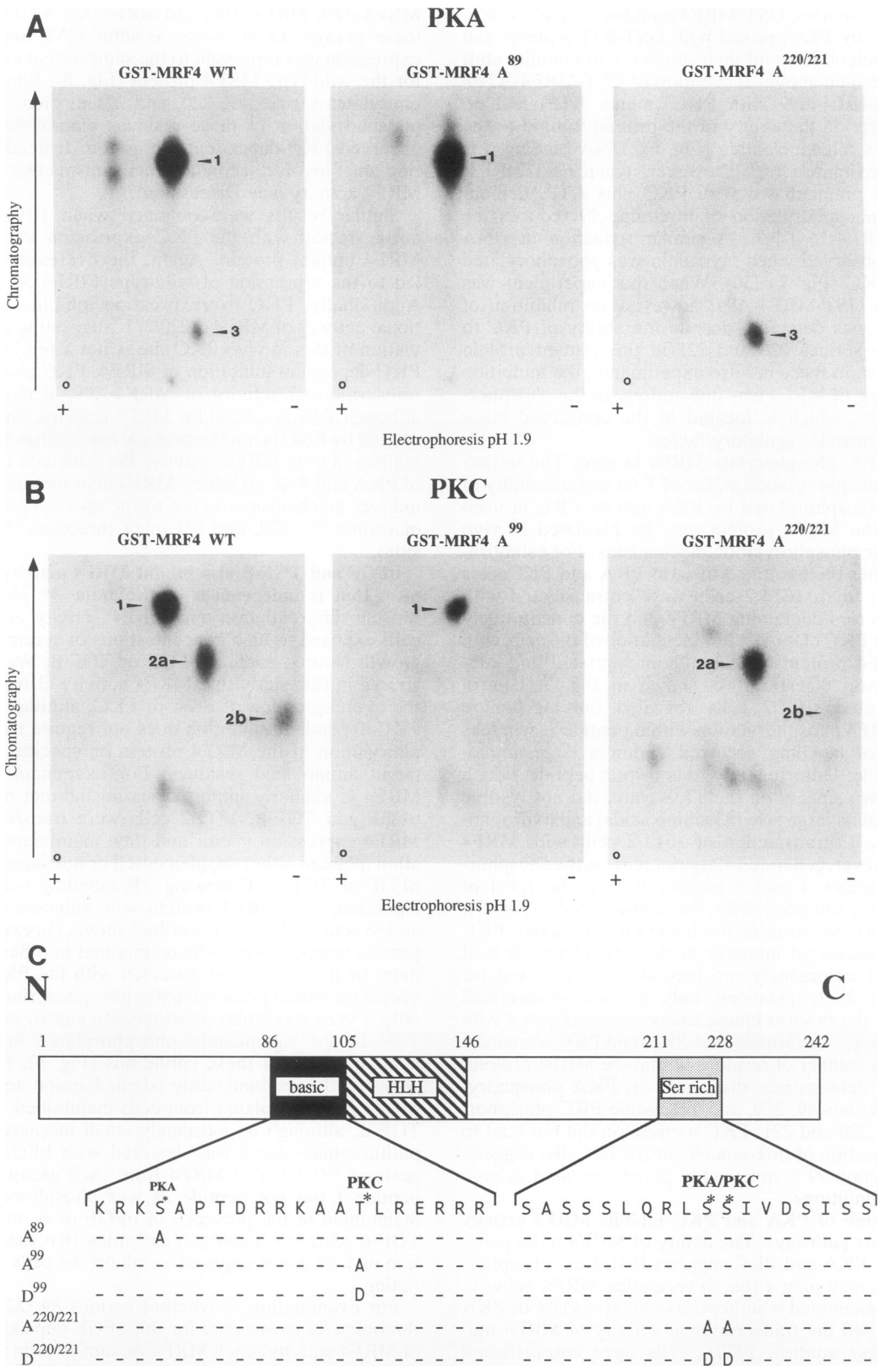

FIG. 3. Identification of the in vitro PKA and PKC sites in MRF4. GST-MRF4 proteins containing specific point mutations were treated in vitro with PKA or PKC, and the labelled proteins were subjected to tryptic peptide analysis. (A) PKA phosphorylation of GST-MRF4-A89 produced only peptide 1, while PKA phosphorylation of GST-MRF4-A220/221 produced only peptide 3. (B) PKC phosphorylation of GST-MRF4-A99 produced only peptide 1, while phosphorylation of GST-MRF4-A220/221 produced only peptides 2a and 2b. (C) Summary of the in vitro PKA and PKC phosphorylation targets in MRF4. As indicated by the smaller font size, serine 89 is a relatively minor PKA site in vitro. Numbers at the top are amino acids. WT, wild type.

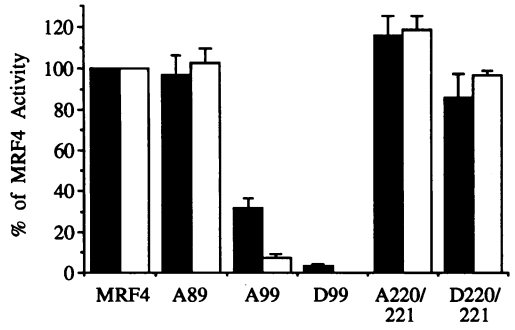

FIG. 4. Transcriptional activities of MRF4 and various point mutants. 10T1/2 cells were cotransfected with α-actin CAT and pEM-MRF4 or different MRF4 point mutants and then induced to differentiate as described in Materials and Methods. The cells were assayed for myofiber formation and expression of the endogenous myosin heavy-chain genes (myosin staining [□]) or assayed for CAT activity (α-actin CAT [■]). All values were normalized to those obtained with the wild-type MRF4 protein. Error bars indicate the standard error of the mean. Only mutations of threonine 99 (A99 and D99) produced a significant decrease in the transcriptional activity of MRF4.

tested. Identical inhibition of MRF4-A89, MRF4-A99, and MRF4-A220/221 activities occurred when the cells were placed in bFGF-containing medium. These results demonstrate that although bFGF inhibits the E-box-dependent activity of MRF4, this inhibition does not require the phosphorylation of serines 89, 220, and 221 or the phosphorylation of threonine 99. Instead, the bFGF signal transduction pathway must operate through other residues within the MRF4 protein or through additional factors that may interact with MRF4 to yield a muscle-specific transcription response.

DISCUSSION

In this study, we have shown that the muscle regulatory factor MRF4 exists as a phosphoprotein and that the overexpression of the serine-threonine protein kinase PKA or PKC inhibits MRF4 activity, as measured by the conversion of 10T1/2 cells to differentiated muscle fibers and by the *trans*-activation of muscle-specific and E-box-containing reporter genes. Although MRF4 is phosphorylated by PKA and PKC, phosphorylation of the PKA and PKC target residues appears to play a minor role in regulating the in vivo activity of the MRF4 protein. Mutation of the amino- and carboxyl-region PKA sites, as well as the carboxyl-region PKC site, does not abolish the repression of MRF4 activity by PKA or PKC. Mutational analysis, however, did confirm the importance of a conserved threonine residue (T-99) that is present within the basic domain of MRF4 and is also found at identical positions within the basic regions of the muscle regulatory factors MyoD, myogenin, and Myf-5 (8, 13). Mutation of T-99 to aspartic acid completely inhibits MRF4 activity by preventing the protein from binding to an E-box oligonucleotide. The addition of a negative charge to T-99 via in vitro PKC phosphorylation similarly produces a protein that exhibits an impaired ability to bind to an E-box

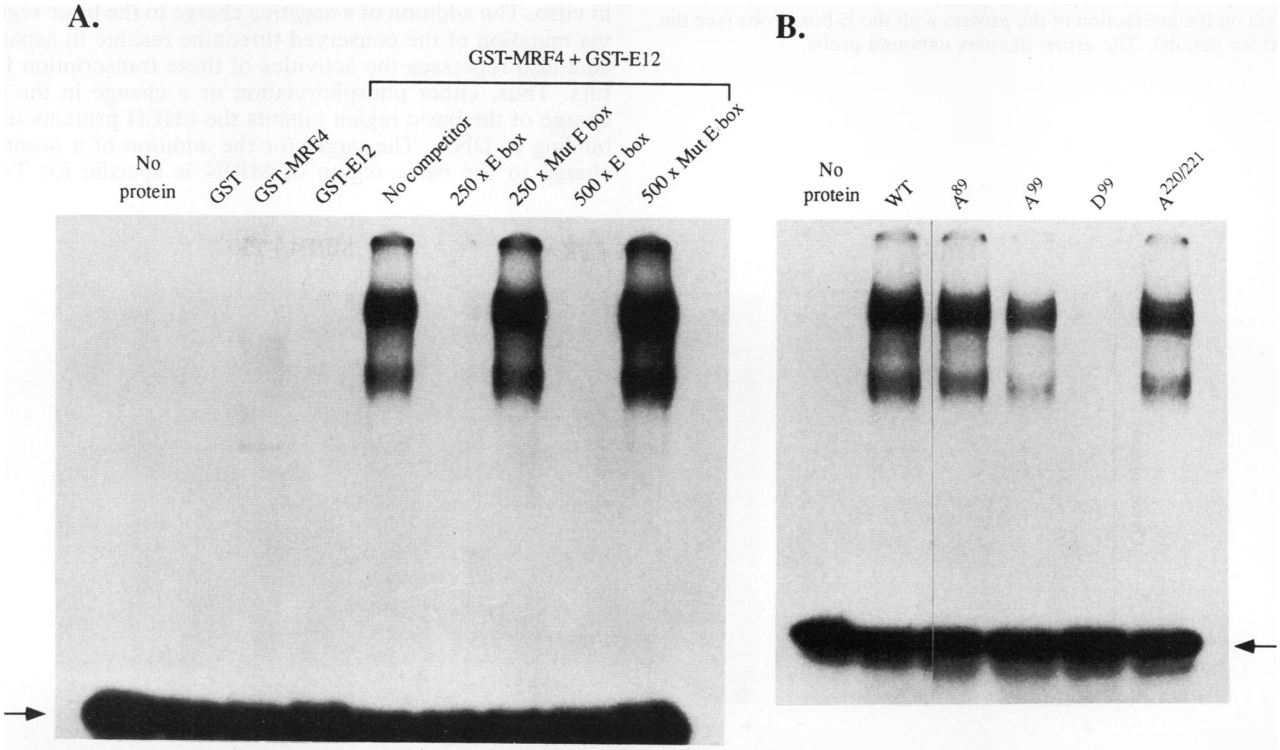

FIG. 5. Mutation of threonine 99 to aspartic acid leads to a loss of DNA binding activity. (A) GST, GST-MRF4, and GST-E12 proteins were incubated with a ^{32}P-labelled E-box oligonucleotide and electrophoresed through a 5% nondenaturing polyacrylamide gel. These proteins alone bound very poorly to the E-box probe, while a mixture of GST-MRF4 and GST-E12 proteins bound specifically to the E-box probe. This binding was inhibited through competition by the wild-type E-box sequence but not by a mutant (Mut) E-box sequence. (B) Individual MRF4 point mutants were incubated with GST-E12 and an E-box oligonucleotide as described for panel A. GST-MRF4-D99 was unable to bind to the E-box probe under these conditions. WT, wild type. Arrows denote unbound probe.

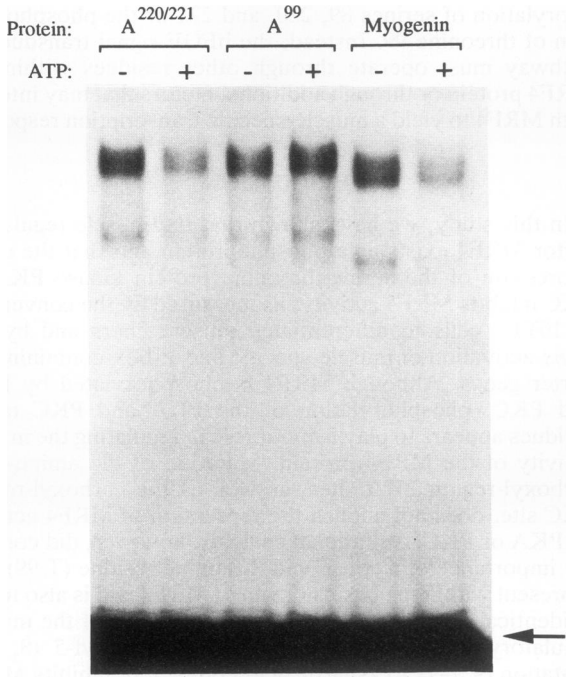

FIG. 6. Phosphorylation of threonine 99 by PKC inhibits MRF4 binding to an E-box site. GST-MRF4-A220/221, GST-MRF4-A99, and GST-myogenin were incubated with PKC in the absence (−) or presence (+) of ATP and then incubated with GST-E12 plus an E-box oligonucleotide probe. Phosphorylation of GST-MRF4-A220/221 and GST-myogenin inhibited the proteins from binding to the E-box probe, whereas phosphorylation of GST-MRF4-A99 had no effect on the interaction of the protein with the E-box probe (see the text for details). The arrow denotes unbound probe.

target. The significance of inhibiting MRF4 activity in vitro via the phosphorylation of T-99 is unclear, however, since phosphorylation of this conserved residue does not occur to any measurable extent in cells overexpressing PKC or in cells blocked from differentiating by exposure to serum, bFGF, or TGF-β. Taken together, our results suggest that the mechanisms by which these agents inhibit MRF4 activity do not involve the phosphorylation of serines 89, 220, and 221 or threonine 99 but rather involve additional muscle-specific pathways.

The ability of PKA to inhibit the activity of MRF4 agrees with the results of recent studies demonstrating that myogenin, MyoD, and Myf-5 also are inhibited by this serine-threonine protein kinase (29, 53). Mutation of the PKA sites of myogenin had little effect on the activity of the myogenin protein. The same phenomenon was found for MRF4, since MRF4 proteins containing mutations of serine 89 or serines 220 and 221 exhibited wild-type activity that was repressed in cells overexpressing PKA. These results demonstrate that PKA inhibits MRF4 and myogenin activities through indirect mechanisms that do not necessarily involve the direct phosphorylation of these transcription factors (29). Nonetheless, both factors are phosphorylated in vivo on their respective PKA sites, suggesting that phosphorylation of the proteins may be essential to full regulation of the activities of the muscle regulatory factors.

Several lines of evidence suggest that phosphorylation of the muscle regulatory factors by PKC also can lead to inhibition of activity. PKC-dependent phosphorylation of the conserved threonine within the basic region inhibits MRF4 and myogenin (30) from binding to an E-box element in vitro. The addition of a negative charge to the basic region via mutation of the conserved threonine residue to aspartic acid also represses the activities of these transcription factors. Thus, either phosphorylation or a change in the net charge of the basic region inhibits the bHLH proteins from binding to DNA. The target for the addition of a negative charge to the basic region of MRF4 is specific for T-99,

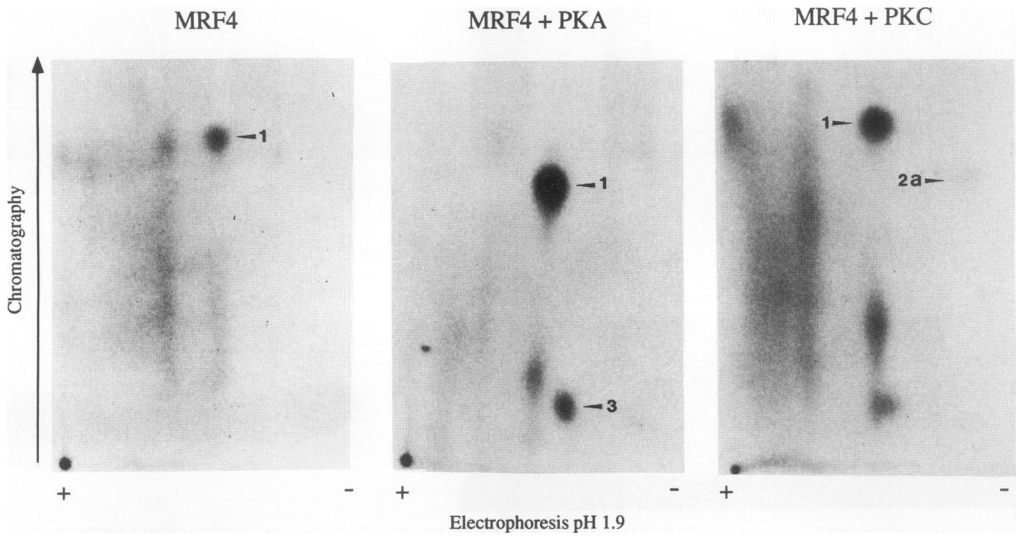

FIG. 7. Cotransfection of 10T1/2 cells with pEM-MRF4 and the PKA or PKC expression plasmid produces an increase in the phosphorylation of the MRF4 protein. 10T1/2 cells were transfected with pEM-MRF4 or with pEM-MRF4 plus the PKA or PKC expression plasmid. After being labelled with $^{32}P_i$, the MRF4 protein was immunoprecipitated and subjected to tryptic digestion. In control cells, MRF4 was weakly phosphorylated within peptide 1, whereas the phosphorylation of peptides 1 and 3 was increased when PKA was overexpressed. Overexpression of PKC also led to an increase in the phosphorylation of peptide 1 but not to an increase in the phosphorylation of peptide 2a.

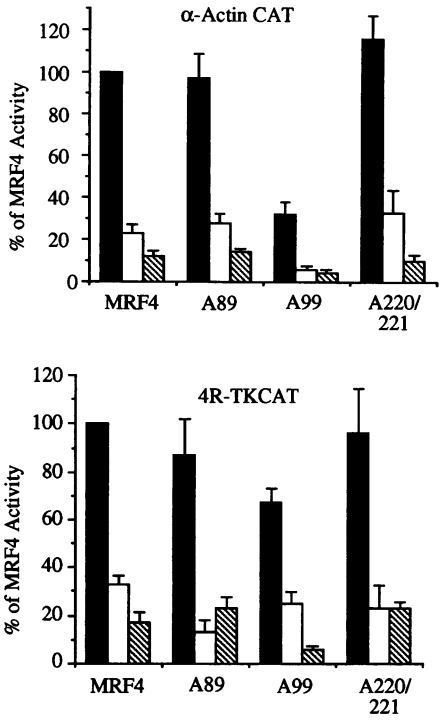

FIG. 8. Overexpression of PKA (□) and PKC (▨) inhibits the transcriptional activity of MRF4 and individual MRF4 point mutants. 10T1/2 cells were cotransfected with pEM-MRF4 or with a corresponding MRF4 mutant and with the PKA or PKC expression plasmid as indicated. Error bars indicate the standard error of the mean. In all cases, the expression of PKA and PKC repressed MRF4 activity, as measured by the reduction in α-actin CAT and 4R-TKCAT expression. ■, control.

however, since mutation of lysine 96 to glutamic acid does not affect the DNA binding or transcriptional activity of the MRF4 protein (unpublished results).

The suggestion that the muscle regulatory factors are negatively inhibited by PKC and by growth factors acting through a PKC-dependent signal transduction pathway represents a very elegant model for controlling the activity of this protein family. Phosphorylation of the conserved threonine residue within the basic regions of MRF4, myogenin, Myf-5, and MyoD could account for why the factors are inactive in proliferating myoblasts or why the factors are inactive in cells maintained with purified growth factors, such as bFGF or TGF-β. In support of these results, Li et al. (30) demonstrated that the overexpression of PKC in COS-1 cells leads to the phosphorylation of threonine 87 in myogenin and thus abolishes myogenin activity. Interestingly, experiments in our laboratory failed to detect the phosphorylation of MRF4 T-99 in 10T1/2 cells overexpressing PKC, even though PKC inhibited MRF4 from activating the myogenic program. Cells exposed to serum, bFGF, or TGF-β also expressed an MRF4 protein that was not significantly phosphorylated on T-99. In addition, the transcriptional activity of MRF4-A99 was repressed by exposure of the cells to bFGF, confirming that bFGF does not inhibit MRF4 activity via phosphorylation of this conserved threonine residue.

In these current studies, a conscious effort was made to perform all experiments with the 10T1/2 cell line, since these cells exhibit the full range of responses to overexpression of the muscle regulatory factors (14). The previously reported myogenin in vivo labelling experiments were carried out with COS-1 cells (30). Since COS-1 cells are not converted to a myogenic phenotype in response to overexpression of the muscle regulatory factors (52, 55), the results obtained with COS-1 cells versus 10T1/2 cells should be compared cautiously. The high degree of labelling obtained by overexpression of PKC in COS-1 cells, for example, is likely due to a much higher PKC content in these cells than in 10T1/2 cells, even though the PKC level in 10T1/2 cells is sufficient to block differentiation. Whether myogenin threonine 87 is phosphorylated in 10T1/2 cells overexpressing PKC or in 10T1/2 cells exposed to bFGF remains to be determined. In this regard, phosphorylation of the conserved threonine residue for any of the bHLH muscle regulatory factors has not been detected under normal growth conditions, i.e., in proliferating myoblasts (30). Given these differences, it is important to note that although MRF4, myogenin, Myf-5, and MyoD proteins are conserved within the bHLH domain, they exhibit a striking diversity in their amino and carboxyl regions. The transcriptional activation domains of MRF4 and myogenin or MyoD, for example, impart specificity to each muscle regulatory factor with respect to targeted reporter genes (12, 33), establishing that these factors exhibit unique characteristics that are not shared by the entire protein family. Thus, as suggested by our studies and those of Li et al. (30), myogenin and MRF4 may be regulated by growth factors or by specific protein kinases, such as PKA

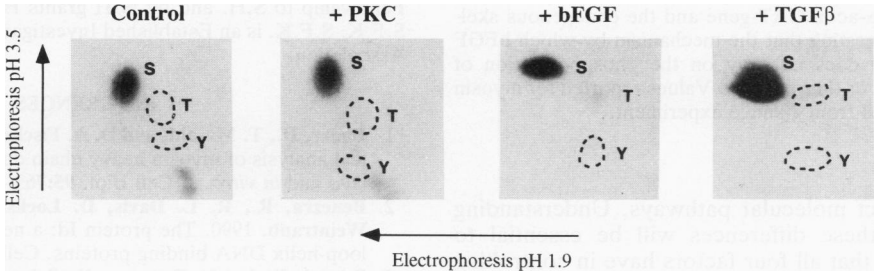

FIG. 9. PKC, bFGF, and TGF-β inhibit MRF4 activity independently of the phosphorylation of threonine 99. 10T1/2 cells transfected with pEM-MRF4 or with pEM-MRF4 plus the PKC expression vector were induced to differentiate in the absence (control and + PKC) or presence of 5 ng of bFGF or TGF-β per ml. After incubation of the cells in $^{32}P_i$, the MRF4 protein was immunoprecipitated and subjected to two-dimensional phosphoamino acid analysis. In each case, the phosphorylation status of threonine 99 was not significantly altered, although the activity of the MRF4 protein was inhibited by PKC, bFGF, and TGF-β. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) residues are shown.

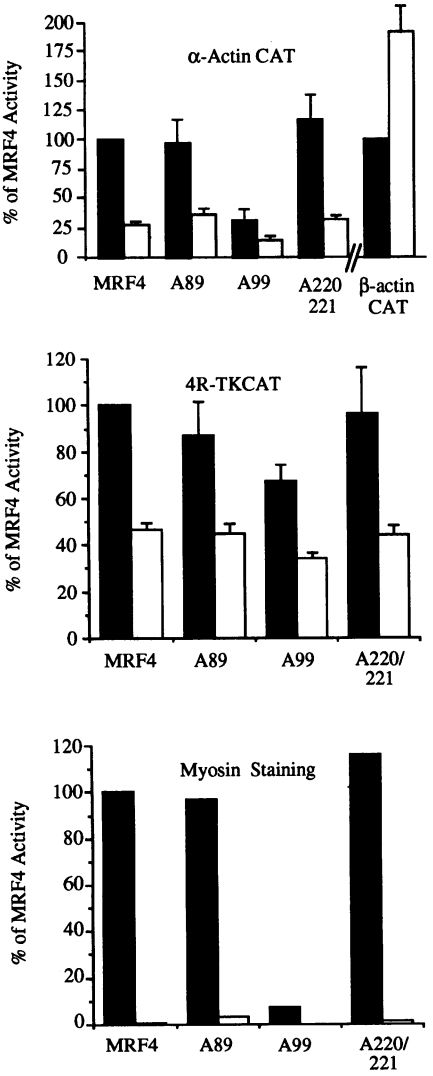

FIG. 10. bFGF inhibits MRF4 proteins from converting 10T1/2 cells to differentiated myofibers and from *trans*-activating the expression of α-actin CAT and 4R-TKCAT. pEM-MRF4 and various point mutants were cotransfected with α-actin CAT or with 4R-TKCAT into 10T1/2 cells, which were then induced to differentiate in the presence (□) or absence (■) of bFGF. Error bars indicate the standard error of the mean. Note that for β-actin CAT, the values with bFGF were compared with the values without bFGF, which were set to 100%. Although bFGF increased the expression of the control β-actin CAT gene, it inhibited the MRF4-induced expression of the muscle-specific α-actin CAT gene and the endogenous skeletal myosin genes, suggesting that the mechanism by which bFGF inhibits MRF4 activity does not rely on the phosphorylation of serines 89, 220, and 221 or threonine 99. Values reported for myosin expression were derived from a single experiment.

and PKC, via distinct molecular pathways. Understanding the importance of these differences will be essential to elucidating the roles that all four factors have in controlling skeletal muscle development.

How then is the inhibition of MRF4 activity by growth factors or by overexpression of PKA and PKC manifested if these agents do not lead to the phosphorylation of the conserved threonine residue in the MRF4 protein? The signal transduction pathways that are operative under these conditions must involve additional mechanisms that remain muscle specific, since they target MRF4 and E-box-dependent transcription. Several possibilities as to the locations at which protein kinases or bFGF operate to inhibit MRF4 activity can be envisioned. We have demonstrated, for example, that MRF4 dimerizes with E2A or a related protein prior to binding to DNA (31). Thus, bFGF could regulate the expression or activity of E2A gene products via a posttranslational mechanism. The fact that E12 also is phosphorylated in vitro by PKC (unpublished data) leaves open the possibility that partners for MRF4 are subject to intracellular kinase and phosphatase regulation. Alternatively, the bFGF signal transduction pathway could modulate the expression of negative regulators of the muscle regulatory factors, such as Id, c-Fos, or c-Jun. The bFGF block of MRF4 activity also could involve the phosphorylation of additional serines within the MRF4 protein. Several MRF4 phosphorylation targets that are not located within the basic domain and that are not PKA or PKC sites have been identified (unpublished data). The significance of these phosphorylation targets must await further analysis. Finally, the bFGF signal transduction pathway could negatively regulate the expression or activity of specific MRF4 coregulators that may interact with the conserved threonine residue within the basic region of MRF4. Recent studies by Tapscott et al. (47) support the idea that 10T1/2 cells express specific coregulators that are essential for the muscle regulatory factors to function as transcriptional activators. How the activities of these proteins are regulated remains unknown. Thus, in each of these cases, understanding the signal transduction mechanisms that negatively and positively control the activities of the muscle regulatory factors will be essential in providing a critical view of how skeletal myogenesis is regulated throughout development.

ACKNOWLEDGMENTS

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