Role of USF1 phosphorylation on cardiac α-myosin heavy chain promoter activity

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Xiao, Qianxun, Agnes Kenessey, and Kaie Ojamaa. Role of USF1 phosphorylation on cardiac α-myosin heavy chain promoter activity. Am J Physiol Heart Circ Physiol 283: H213–H219, 2002. First published March 28, 2002; 10.1152/ajpheart.01085.2001.—Contractile activity of the cardiac myocyte is required for maintaining cell mass and phenotype, including expression of the cardiac-specific α-myosin heavy chain (α-MHC) gene. An E-box hemodynamic response element (HME) located at position −47 within the α-MHC promoter is both necessary and sufficient to confer contractile responsiveness to the gene and has been shown to bind upstream stimulatory factor-1 (USF1). When studied in spontaneously contracting cardiac myocytes, there is enhanced binding of USF1 to the HME compared with quiescent cells, which correlates with a threefold increase in α-MHC promoter activity. A molecular mechanism by which contractile function modulates α-MHC transcriptional activity may involve signaling via phosphorylation of USF1. The present studies showed that purified rat USF1 was phosphorylated in vitro by protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) but not casein kinase II. Phosphorylated USF1 by either PKC or PKA had increased DNA binding activity to the HME. PKC-mediated phosphorylation also leads to the formation of USF1 multimers as assessed by gel shift assay. Analysis of in vivo phosphorylated nuclear proteins from cultured ventricular myocytes showed that USF1 was phosphorylated, and resolution by two-dimensional gel electrophoresis identified at least two distinct phosphorylated USF1 molecules. These results suggest that endogenous kinases can covalently modify USF1 and provide a potential molecular mechanism by which the contractile stimulus mediates changes in myocyte gene transcription.

ventricular myocytes; contractile activity; protein kinase C; cAMP-dependent protein kinase; E-box

EXPRESSION OF THE cardiac-specific α-myosin heavy chain (α-MHC) gene is regulated by multiple humoral and hemodynamic factors (10, 21, 32). These include the hemodynamic workload imposed on the heart and, specifically, the contractile activity of the ventricular myocyte (12, 23, 25). Our previous studies (24, 38) have identified a hemodynamic responsive element (HME) at position −47 of the α-MHC promoter that contains an E-box motif (CACGTG) that binds upstream stimulatory factor-1 (USF1). Overexpression of cloned rat USF1 increased α-MHC promoter activity in contracting ventricular myocytes but failed to stimulate the promoter in contractile-arrested cardiomyocytes, suggesting that posttranslational modification of USF1 may be required for its enhanced transcriptional activity (38).

USF1 and the related polypeptide USF2 (43 and 44 kDa, respectively) belong to the basic helix-loop-helix leucine zipper family of transcription factors. These nuclear transacting factors are involved in the expression of several tissue-specific and developmentally regulated genes, including cardiac myosin light chain-2 (22), α-MHC (38), ribosomal RNA (8), and cyclin B1 (5). The USFs form homodimers, heterodimers, and multimers and have been shown to interact with the basal transcription factor complex TFIIID (13, 37). Numerous studies (17, 29) have shown that phosphorylation of transcription factors is an effective mechanism by which DNA binding and transcriptional activity can be modulated. Cardiomyocyte nuclear factors MEF2C and GATA4, believed to mediate hypertrophic-induced gene transcription, have been shown to be phosphorylated by casein kinase II and extracellular signal-regulated kinase (ERK), respectively, resulting in altered DNA binding and transcriptional activities (15, 16, 20). Similarly, one study (3) showed how the transcriptional activity of USF1 could be modified by phosphorylation. A recent report (7) showing phosphorylation of USF1 by the stress responsive p38 kinase may have significance in the cardiomyocyte response to stress-induced hypertrophic gene transcription.

Protein phosphorylation has been shown to either enhance DNA binding activity and transcriptional efficacy as described for the muscle-specific MEF2C (20) or to disrupt DNA binding as shown for Max, c-Myc, and c-Myb (2, 26, 29). Phosphorylation of amino acid residues within the prebasic and basic regions of the DNA binding domain of the transcription factors disrupts DNA binding (2), whereas phosphorylation within the leucine zipper region results in stabilization of the multimeric complexes and enhances DNA bind-

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ing and transactivation (17, 34). Rat USF1 contains three protein kinase C (PKC) consensus phosphorylation sites within the leucine zipper domain, which may enhance its DNA binding potential, as well as two sites within the prebasic region that may act to disrupt DNA binding.

Thus the present study was undertaken to determine whether the transcriptional activity of USF1 in cardiomyocytes could be regulated by phosphorylation. In vitro studies with bacterially expressed USF1 showed that exogenous PKC and cAMP-dependent protein kinase (PKA), but not casein kinase II, phosphorylated USF1, enhanced its DNA binding affinity, and promoted multimerization. Phosphoprotein analysis of nuclear extracts from cultured cardiomyocytes showed that USF1 was phosphorylated in vivo, suggesting a potential mechanism of transcriptional regulation.

MATERIALS AND METHODS

Construction of pGEX-rUSF1 expression vector. The pGEX expression system (Pharmacia; Piscataway, NJ) was used to synthesize the glutathione S-transferase (GST)-rUSF1 fusion protein. Full-length rat (r) USF1 cDNA (1.6 kb) that we had previously cloned (38) was ligated in frame into the BamH I-Xho I site of pGEX-4T-3 and verified by DNA sequence analysis. The plasmid (pGEX-rUSF1) was transformed into BL21 Escherichia coli for fusion protein expression according to the manufacturer's instructions (36).

Expression and purification of rUSF1 in bacteria. BL21 E. coli harboring the pGEX-rUSF1 plasmid were grown to A600 ~ 1 and then induced with 0.75 mM isopropyl-β-thiogalactoside at 25°C for an additional 6 h. Cells were collected by centrifugation, resuspended in ice-cold phosphate-buffered saline (50 μl/ml initial culture), and then disrupted by sonication. Triton X-100 was added to 1% final concentration and gently mixed for 30 min. The supernatant containing the GST-rUSF1 fusion protein was recovered by centrifugation at 12,000 g for 10 min at 4°C and then incubated with glutathione Sepharose 4B (50:1), as described by the manufacturer (Pharmacia). The GST-rUSF1 fusion protein was eluted from the Sepharose beads by incubation with glutathione. Alternatively, the Sepharose-bound GST-rUSF1 fusion protein was first digested with thrombin protease to dissociate rUSF1 from GST, and then the purified rUSF1 was eluted from the beads. The eluted proteins were concentrated by ~10-fold using microconcentrators (3 kDa MWCO, Amicon; Beverly, MA), and the protein concentration was determined by Lowry assay.

Western blot analysis. Nuclear extracts were prepared as previously described (24, 38). HeLa cell nuclear extracts (10 μg), GST-rUSF1 fusion protein (2 μg), and thrombin protease-activated rUSF1 (2 μg) were resolved by electrophoresis on 1% sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. The proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad; Hercules, CA) at 250 mA for 2 h, then incubated overnight at 4°C in 3% milk-10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20 (TBST). Rabbit anti-human USF1 antibody (Santa Cruz Biotechnology; Santa Cruz, CA) diluted 1:2,000 in 3% milk-TBST was incubated with the membrane at room temperature for 2 h and then washed extensively before incubation with the secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000 dilution). The signal was developed with the use of a chemiluminescence reagent (Renaissance, DuPont; Boston, MA) and detected by exposure to X-ray film. The same membrane was stripped and reprobed with anti-GST antibody (1:2,000 dilution) and developed with secondary antibody as described above.

In vitro phosphorylation of rUSF1. For in vitro phosphorylation reactions, 5 μg of bacterially expressed rUSF1 protein was added to a 12-μl reaction containing PKC (isoforms α, β, and γ) purified from rat brain (0.24 U) (Upstate Biotechnology; Lake Placid, NY) in buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.5 mM CaCl2, 100 μM/ml phosphatidyl serine, 20 μg/ml phosphoribol 12-myristate 13-acetate (PMA), and 10 μM [γ-32P]ATP (3,000 Ci/mmol). Control reactions did not contain exogenous protein kinase. Reaction conditions for PKA (60 U) (Promega; Madison, WI) contained 40 mM Tris-HCl (pH 7.4), 20 mM Mg-acetate, and 10 μM [γ-32P]ATP. Reactions were resolved by SDS-PAGE.

For analysis of DNA binding activity of phosphorylated rUSF1, phosphorylation reactions were carried out either in the presence or absence of [γ-32P]ATP and used for electrophoretic mobility shift assay (EMSA), as we have previously published (24).

EMSA. A deoxyligonucleotide double-stranded DNA probe (dsHME) used in the EMSA included the α-MHC promoter sequences from −59 to −39 (5′-CGAGCCCAGC-TGTGAATGACG-3′), with the rUSF1 binding sequence underlined (24). The sense DNA strand was 5′-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP to −2 × 106 dpm/pmol, purified over Sephadex G-25 spin columns, and then annealed to 50-fold excess of the unlabeled antisense strand. Bacterially expressed and purified rUSF1 (2 μg) was phosphorylated with either PKA or PKC as described above except that radiolabeled ATP was not used. Parallel reactions were carried out in the absence of the kinases, and equal aliquots of all reactions were compared for DNA binding activity by EMSA using conditions as previously described (24, 38). To measure DNA binding activity of the bacterially expressed rUSF1 in the presence of mammalian cell extract, 2 μg of purified rUSF1 was incubated with either untreated or heat-inactivated (95°C for 10 min) rabbit reticulocyte lysate (1.5 μl) (Promega) and analyzed by EMSA.

Cultures of neonatal rat ventricular myocytes and in vivo analysis of phosphorylation of rUSF1. Neonatal rat ventricular myocytes (NRVM) were plated at ~1.5 × 10^4/cm^2 on collagen-coated 60-mm plates and cultured as previously described (24, 25). After 30 h in culture, the medium was replaced by phosphate-free Dulbecco's modified Eagle's medium (GIBCO-BRL; Gaithersburg, MD) for 10 h, followed by the addition of [32P]orthophosphate (9,000 Ci/mmol; 1 mCi/ml medium) for 4 h. Cells were washed extensively and harvested, and nuclear extracts were prepared as previously described (24). Extract proteins were desalted and concentrated ~10-fold using microconcentrators (Amicon; Beverly, MA).

In a separate series of experiments, NRVM were transiently cotransfected with control plasmid β-galactosidase (pRSVZ) and luciferase reporter plasmids containing either wild-type or mutant α-MHC promoter sequences (~388 to +32) (p388wt) or containing a mutant HME at position ~50 within the context of the ~388/+32 bp region of the promoter (p388A), as we have previously published (24). Minimal promoter constructs containing two copies of either the wild-type or mutant HME element ligated to luciferase (pHMEwt, pHMEΔ) were similarly transiently transfected into NRVM, as previously published (38). After transfection, cells were cultured in serum-free medium with half of the culture plates treated with PMA (2 × 10^-7 M) for 24 h (or 1 h followed by 23 h culture without PMA) before harvest and analysis of luciferase and β-galactosidase (β-gal) activities. Results are
expressed as luciferase luminescence units normalized for β-gal activity in the same volume of cell lysate, as previously described (24, 38). Some cell cultures were treated simultaneously with PMA and staurosporine (10 nM), an inhibitor of PKC activity, for 24 h before harvest.

**Two-dimensional gel electrophoresis.** Two-dimensional (2D) gel electrophoresis used the mini-Protean II 2-D system (Bio-Rad; Hercules, CA). Nuclear proteins (10–20 µg) were solubilized in 2D sample buffer containing 4.75 M urea, 1.0% Triton X-100, 2.5% β-mercaptoethanol, 0.8% 5/8 amphotolites, and 0.2% 3/10 amphotolites (Sigma; St. Louis, MO) and isoelectric focused at 12,000 V·h in a 4% polyacrylamide tube gel containing 9.5 M urea, 2% Triton X-100, 1.6% pH 5–8 amphotolites, and 0.4% pH 3–10 amphotolites. The isofocused proteins were resolved in the second dimension by electrophoresis in an SDS-10% polyacrylamide slab gel, in which an additional lane was used to resolve the same nuclear protein sample only by SDS-PAGE. The resolved 2D and one-dimensional (1D) proteins were then transferred to PVDF membrane (Bio-Rad). The membrane was first exposed to phosphorimage analysis (Bio-Rad) and subsequently used for Western blot analysis with anti-human USF1 antibodies as described above. To increase the resolution of the phosphorylated isoforms of USF1, nuclear extracts (10–15 µg) were isofocused on ReadyStrip IPG pH 4–7 (Bio-Rad) and then subjected to 2D SDS-PAGE electrophoresis before Western blot analysis, as described above.

**RESULTS**

**Characterization of the bacterially expressed rUSF1.** Rat USF1 was synthesized in bacteria as a GST fusion protein and purified by glutathione Sepharose affinity chromatography. Figure 1 shows a Western blot of the fusion protein (GST-rUSF1) as well as the cleaved proteins GST and rUSF1. Analysis with anti-USF1 antibody (Fig. 1, left) recognized the fused protein (~64 kDa) and a protein of ~35 kDa in size that was not recognized by anti-GST antibody (Fig. 1, right), suggesting that rUSF1 had been appropriately cleaved from GST. The anti-GST antibody recognized a 29-kDa protein that was not recognized by the anti-USF1 antibody, indicating that this protein was GST. The smaller apparent molecular mass of rUSF1 produced in bacteria (~35 kDa) may be the result of altered processing of the protein in bacteria compared with its expression in eukaryotic cells, as we and others have previously shown (36, 38).

**In vitro phosphorylation of rUSF1 by PKA and PKC.** To determine whether rUSF1 can be phosphorylated in vitro, the ability of PKC, PKA, and casein kinase II to phosphorylate the protein was examined. As shown in the SDS-PAGE in Fig. 2, both PKA and PKC were able to phosphorylate rUSF1 observed as the ~35 kDa band. Autophosphorylation of PKA occurred as evidenced by the phosphorylated band at ~40 kDa (Fig. 2, left, lanes 1 and 2), whereas autophosphorylation of PKC was not observed (Fig. 2, right). In contrast to PKC and PKA, rUSF1 was not phosphorylated by casein kinase II (data not shown).

**Phosphorylation enhances rUSF1 DNA binding activity.** EMSA was used to determine whether the DNA binding affinity of rUSF1 was enhanced by phosphorylation. Bacterially produced rUSF1 bound to the α-MHC E-box element (HME) (Fig. 3, lane 1) and was prevented from DNA binding by the anti-USF1 antibody (lane 4) similar to rUSF1 derived from cardiomyocyte nuclear extracts, as we have previously published (24). We tested the hypothesis that the DNA binding activity of bacterially expressed rUSF1 may be covalently modified by factors present in a mammalian cell lysate. Therefore, we compared binding of bacterially expressed rUSF1 to the HME sequence in gel shift assays incubated either in the presence of native rabbit reticulocyte lysate or with heat-inactivated lysate. In Fig. 3, DNA binding of rUSF1 incubated with native rabbit reticulocyte lysate (lane 2, +RL) was greater than when incubated with heat-inactivated lysate (lane 3, +ΔRL), suggesting that the mammalian cell lysate modified rUSF1 in a manner leading to an alteration in its DNA binding potential.

We used EMSA to determine whether DNA binding of rUSF1 was altered by phosphorylation in vitro using either PKA or PKC. Compared with nonphosphorylated rUSF1 (Fig. 3, lanes 6 and 8), phosphorylated rUSF1 by either PKA (lane 5) or PKC (lane 7) markedly increased DNA binding (Fig. 3, band I). A higher
molecular mass complex (band II) was also observed with PKC treatment (lane 7), suggesting phosphorylation-mediated multimerization of rUSF1.

Phorbol esters activate α-MHC promoter activity via the hemodynamic E-box element. To determine whether a PKC signaling pathway may be involved in mediating the activation of the α-MHC promoter through the E-box HME enhancer, we treated transiently transfected cultured neonatal rat ventricular myocytes with the phorbol ester PMA, which is known to activate several PKC isoforms, and measured reporter gene activity. As shown in Fig. 4, PMA treatment for 24 h (1-h treatment showed similar results) stimulated α-MHC promoter (p388wt) activity by 3.5-fold, whereas mutation of the HME enhancer (p388Δ) completely obliterated the PMA effect. To further determine whether PMA activation of the α-MHC promoter was mediated by the HME element, we transiently transfected minimal promoter constructs containing two copies of wild-type or mutant HME. As shown in Fig. 4, PMA treatment increased pHMEwt activity by twofold and had no stimulatory effect when the HME enhancer was mutated (pHMEΔ). Simultaneous treatment of the PMA-treated cells with staurosporine completely prevented the PMA-induced increase in wild-type α-MHC promoter activity (data not shown). Together, these data suggest that PKC-mediated signaling may be involved in the activation of the α-MHC promoter by altering the binding activity of the HME-binding protein, USF1.

In vivo phosphorylation of rUSF1. We determined whether phosphorylation of rUSF1 occurred in vivo using spontaneously contracting neonatal cardiomyocytes cultured in the presence of [32P]orthophosphate. The nuclear phosphoproteins were analyzed by two-dimensional gel electrophoresis as shown in Fig. 5. Figure 5A shows the phosphorimage analysis of the nuclear phosphoproteins resolved by 2D analysis (predicted isoelectric point and SDS-PAGE) and 1D SDS-PAGE. Figure 5B shows the same PVDF membrane as in Fig. 5A but analyzed by Western blotting with anti-USF1 antibody. In lane 1D of Fig. 5B, one immunoreactive band at the predicted molecular mass of ~40 kDa was recognized by the anti-USF1 antibody. In contrast, at least two distinct immunoreactive USF1 proteins were resolved at the same molecular mass by 2D analysis as indicated by the arrowhead and the arrow, and these proteins isofocused at the predicted isoelectric point of 5.6.

The corresponding areas on the 2D phosphoprotein gel in Fig. 5A are similarly indicated, supporting the hypothesis that USF1 is phosphorylated in vivo. We further resolved the individual phosphoproteins by isofocusing in a narrow range of pH 4–7, as shown in Fig. 6. Three USF1-immunoreactive proteins were resolved, indicating that phosphorylation of USF1 at two or more sites produced proteins that had distinct resolution patterns by 2D gel electrophoretic analysis. The more highly phosphorylated USF1 molecule, migrating toward the more acidic pH range, was less immunoreactive, suggesting that the amount of this

Fig. 3. Effects of phosphorylation of USF1 on DNA binding activity. Electrophoretic mobility shift assay (EMSA) analysis showing binding activity of bacterially expressed rUSF1 to radiolabeled double-stranded hemodynamic response element (dsHME) probe (lane 1), incubated in the presence of native (lane 2) or heat-inactivated rabbit reticulocyte lysate (lane 3). Inhibition of USF1 binding to HME by incubation with anti-USF1 antibody (lane 4). In vitro phosphorylated rUSF1 by either PKA (lane 5) or PKC (lane 7), and the corresponding control reactions without enzyme (lanes 6 and 8) were analyzed for dsHME binding activity. The protein DNA complexes are indicated by the shifted radiolabeled bands indicated by the arrows at I and II, where the slower migrating band II represents rUSF1 multimers. F designates free radiolabeled dsHME probe. The reaction products were resolved by 6% native PAGE and exposed to X-ray film.

Fig. 4. Effects of phorbol 12-myristate 13-acetate (PMA) treatment of neonatal rat ventricular myocytes (NRVM) in culture on α-myosin heavy chain (α-MHC) promoter activity. Cultured neonatal rat ventricular myocytes were cotransfected with control plasmid β-galactosidase (pRSVβ) and α-MHC promoter/luciferase plasmids, p388wt or p388Δ containing a mutant HME. A minimal promoter construct, pHMEwt, contains two copies of the USF1 DNA binding element ligated to the TATA box of α-MHC promoter; pHMEΔ contains single base substitutions within the two HME elements. PMA treatment for 24 h induced wild-type promoter activities (⁎ P ≤ 0.05) but did not activate promoters containing mutant HME. Promoter activities expressed as luciferase normalized for β-galactosidase (Luciferase/β-gal). Control conditions were cell culture in serum-free media.
phosphoprotein was less (Fig. 6). Using densitometric analysis to quantify the immunoreactive spots developed on X-ray film, we compared USF1 from nuclear extracts from six separate preparations of myocyte cultures that were either quiescent (verapamil or KCl treated) or were spontaneously contracting. This analysis showed that the amount of phosphorylated USF1, expressed as a ratio of the nonphosphorylated USF1 protein in each nuclear extract, was twofold higher in the contractile-arrested myocytes compared with the contracting cell cultures in five of the six cell culture preparations. These data suggest that site-specific phosphorylation of USF1 by intracellular kinase cascades may play a key role in determining DNA binding activity of USF1 and in vivo activation of USF1-mediated transcription of the α-MHC gene.

DISCUSSION

Hypertrophic growth of the cardiac myocyte in response to increased hemodynamic load is characterized by activation of intracellular signaling pathways, by induction of immediate early and secondary response genes including the α-MHC genes, and by an accumulation of myocyte-specific proteins (1, 12, 23, 25, 27). A role for cell surface integrins and focal adhesions has been suggested in transmitting the mechanical hypertrophic signal to intracellular signaling cascades that include PKC isoenzymes, c-Jun NH2-terminal kinases, and ERKs, which in turn regulate nuclear transcription factors (4, 6, 9, 19, 31). Despite recent studies (28, 33) of these intracellular signaling pathways, understanding the mechanisms by which extracellular signals result in altered transcription of relevant cardiomyocyte genes such as the myosin heavy chains remains unclear.

We previously identified that USF1 bound to an E-box HME within the proximal region of the α-MHC promoter that was necessary and sufficient to confer contractile responsiveness to the promoter in ventricular myocytes (24, 38). We also showed that overexpression of rat USF1 in contracting ventricular myocytes increased the activity of a minimum α-MHC promoter construct containing the E-box HME; however, in contractile-arrested myocyte cultures, USF1 overexpression did not stimulate the α-MHC promoter, suggesting that USF1 was modified differently under the two conditions resulting in distinct transcriptional activities (38). We therefore, hypothesized that myocyte contractile activity signaling through intracellular kinase cascades alters phosphorylation of nuclear USF1, thus acting as a potential mechanism by which mechanocellular stimuli could be transmitted to alterations in transcriptional activity of cardiac genes.

Our initial observation showing that the DNA binding activity (as measured by EMSA) of bacterially expressed rat USF1 was enhanced when incubated in the presence of rabbit reticulocyte lysate but not when the lysate was heat inactivated suggested that USF1 was covalently modified. We subsequently showed that phosphorylation of purified USF1 with exogenous classic PKC isoenzymes (mixture of PKCα, β, and γ isoforms from rat brain) not only enhanced its binding to the E-box HME but also promoted the formation of multimers. This is analogous to the basic leucine zipper protein, cAMP response element binding protein, in which PKC phosphorylation induces dimer formation, whereas PKA does not, and that the degree of phosphorylation of cAMP response element binding protein complexes modulates its transcriptional activity (17). Three consensus PKC phosphorylation sites of rat USF1 (serine-257, -262, and -309) are located within the leucine zipper domain, which has been shown for other basic leucine zipper proteins to enhance binding to DNA, whereas three potential phosphoserine residues within the proximal domain of USF1 would tend to disrupt DNA binding (26, 34). Two of these potential PKC sites (serine-257 and -262) are also consensus sites for PKA phosphorylation and may provide a mechanism by which diverse signaling pathways can converge on a single DNA element. Alternatively, the preferential formation of USF multimers by PKC phosphorylation may provide a mechanism for distinct transcriptional effects of these signaling path-
ways (37). We have shown here that activation of PKC signaling pathways by treatment of cultured cardiomyocytes with phorbol esters increased the activity of the α-MHC promoter containing the wild-type E-box enhancer element HME but not when the HME was mutated. Therefore, together these data support a potential role for distinct PKC signaling pathways in regulating USF1-mediated α-MHC transcription (25). Electrical pacing and stretch-induced cardiomyocyte hypertrophy have been shown to activate one of several kinase cascades including p38 kinase, PKCα, and PKCe, ERKs, or c-Jun NH2-terminal kinases (18, 30, 31, 35).

In the present study, we determined that phosphorylation of USF1 occurred in vivo in neonatal rat ventricular myocytes grown in culture. Two-dimensional electrophoretic analysis showed that a significant fraction (~40–50%) of the total nuclear USF1 was phosphorylated, thus providing a mechanism by which a specific kinase or phosphatase pathway could determine the transcriptional potential of USF1. We identified two distinct molecular species of phosphorylated USF1 as well as nonphosphorylated USF1 in both contracting and contractile-arrested myocyte cultures. When we compared total phosphorylated to nonphosphorylated USF1 in contracting myocytes to that in quiescent cultures, we found a higher phosphorylation ratio in the noncontracting myocytes. Because site-specific phosphorylation rather than total phosphorylation per se determines transcriptional activity, we must conclude that our observation of increased DNA binding activity of PKC-phosphorylated USF1 as well as increased α-MHC promoter activity in response to PMA and attributable to the HME enhancer reflects distinct site-specific phosphorylation of USF1. Therefore, increased phosphorylation of USF1 in contractile-arrested myocytes may occur at amino acid residues that result in disruption of DNA binding. Mutagenesis studies of each of the six potential PKC and PKA phosphorylation sites within USF1 may determine which intracellular signaling pathways are important in mediating the transcriptional activity of USF1 on target promoters.

Protein serine-threonine phosphatases have been associated with the nuclear envelope of cardiomyocytes (11), and the nuclear trafficking of variants of protein-tyrosine phosphatases has been shown in response to cellular stress (14), suggesting that dephosphorylation of nuclear proteins as well as their phosphorylation may play a role in the present model. Alternatively, induction of intracellular kinase cascades that may ultimately lead to phosphorylation of nuclear USF1 have been reported in other models of hypertrophy, namely, stretch-activated and electrically stimulated cardiomyocyte hypertrophy (6, 9, 30). Thus the signaling paradigms in these models may be distinct from the spontaneously contractile cardiomyocytes used in the present study. USF1 must also function in combination with numerous other transcription factors necessary for both inducible and tissue-specific expression of the α-MHC gene (10, 21, 32). Therefore, phosphorylation of USF1 as well as other nuclear factors, including those of the MEF2 and GATA families, may provide a mechanism by which multiple diverse stimuli can modulate transcriptional activities of specific genes that would determine cardiomyocyte phenotype (1, 28, 33).

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