CAMP-dependent Protein Kinase Represses Myogenic Differentiation and the Activity of the Muscle-specific Helix-Loop-Helix Transcription Factors Myf-5 and MyoD*

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Myf-5 and MyoD are members of a family of muscle-specific basic helix-loop-helix (bHLH) proteins that are fundamental for myogenic cell differentiation and transcriptional activation of muscle-specific genes. Here we report that elevated levels of the intracellular signaling molecule CAMP and overexpression of CAMP-dependent protein kinase (PKA) inhibit myogenic differentiation. PKA represses the transcriptional activation of muscle-specific genes by the myogenic regulators Myf-5 and MyoD. The repression is directed at the basic HLH domain and is mediated through the E-box DNA consensus motif to which these proteins bind. However, phosphorylation of Myf-5 and MyoD by PKA in vitro does not affect their ability to bind to DNA. PKA specifically inhibits the activity of myogenic bHLH proteins, but not of other HLH proteins, such as the ubiquitously expressed E2A gene products E12 and E47 (E2-5). Our results demonstrate that PKA mediates the CAMP-induced inhibition of muscle cell differentiation by repressing the activity of Myf-5 and MyoD. The inhibition by PKA occurs post-translationally and presumably affects the transactivation process at a step following DNA-binding. The regulation of Myf-5 and MyoD function by a CAMP-dependent pathway may partly explain how external signals generated by serum and certain peptide growth factors can be transduced to the nucleus and inhibit dominant-acting factors that are responsible for myoblast differentiation.

Differentiation of skeletal muscle cells is characterized by the coordinate activation of an array of genetically unlinked muscle-specific genes and the arrest of cell proliferation. Serum components and peptide growth factors, such as basic fibroblast growth factor and transforming growth factor-β, specifically inhibit the differentiation of muscle cells and the transcriptional activation of muscle-specific genes (for review, see Florini et al. (1991)). These and other observations have led to the concept that differentiation and growth are mutually exclusive events in myogenic cell lines. Little, however, is known about the mechanisms underlying the antagonism between molecules that promote growth and those that regulate cell differentiation. Therefore, the elucidation of networks that link signals at the cell surface to transcriptional events in the nucleus requires the identification of intracellular signaling molecules as well as the characterization of trans-acting factors that serve as nuclear targets for the regulatory pathways.

Myoblasts offer an excellent model system for studying mechanisms whereby differentiation may be controlled, because a family of muscle-specific nuclear factors that can activate the complete differentiation program has recently been identified (for reviews see, Emerson (1990), Olson (1990), and Weintraub et al. (1991a)). This family of myogenic control proteins which includes MyoD (Davis et al., 1987), myogenin (Wright et al., 1989; Edmondson and Olson, 1989), Myf-5 (Braun et al., 1989a), and MRF4 (Rhodes and Konieczny, 1989), also called herculin (Miner and Wold, 1990) or Myf-6 (Braun et al., 1990a), shares extensive sequence homology within a basic region and an adjacent putative helix-loop-helix (HLH) domain which mediate sequence-specific DNA binding and dimerization, respectively (Murre et al., 1989; Davis et al., 1990; Brennan et al., 1991; Winter et al., 1992). High affinity binding to the DNA consensus sequence, CANNTG, referred to as E-box, requires heterodimerization of the myogenic bHLH proteins with the more widely expressed HLH proteins E12 or E47 (E2-5). Transactivator domains have been identified outside of the bHLH region in the NH2 terminus of MyoD (Weintraub et al., 1991b) and the NH2 and COOH termini of myogenin (Schwartz et al., 1992) and Myf-5 (Braun et al., 1990b). Winter et al. (1992). Numerous muscle-specific genes containing one or more E-box motifs within their control regions can be transactivated through the interaction with the myogenic HLH proteins (for review, see Olson (1990), Emerson (1990), and Weintraub et al. (1991a)). Thus, these proteins constitute cell type-specific transcription factors and may be potential targets for signals generated by serum components and peptide growth factors.

An approach taken to identify signaling pathways through which growth factors may inhibit myogenesis has been to transfect myoblasts with activated oncogenes known to be involved in signal transduction and growth control. These investigations have revealed that activated ras proteins (Lasar et al., 1989; Olson et al., 1987; Payne et al., 1987), membrane-associated tyrosine protein kinases, such as v-src (Falcone et al., 1985), and nuclear protooncogene products, such

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†The abbreviation used are: HLH, helix-loop-helix; bHLH, basic HLH; PKC, protein kinase C; PKA, protein kinase A; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; MHC, myosin heavy chain; kb, kilobase pair(s); MLC, myosin light chain; EMSA, Electrophoretic mobility shift assay; DM, differentiation medium.
as c-myc (Miner and Wold, 1991; Schneider et al., 1987), c-fos, and jun (Rahm et al., 1988; Li et al., 1992a; Bengal et al., 1992), and adenovirus E1A protein (Webster et al., 1988; Enkemann et al., 1990; Braun et al., 1992) can disrupt myogenesis and inhibit muscle-specific gene expression. Some of these oncogene products act in pathways regulated by protein kinase C (PKC), which by itself can inhibit myogenesis.

Phosphorylation of myogenin by PKC in vitro prevents DNA binding and constitutively expressed PKC inhibits transactivation by myogenin.2 Other than PKC, no pathways that may link components acting close to the cell membrane with those that function in the nucleus have yet been established. The availability of cloned regulatory proteins that induce muscle differentiation and directly activate transcription of muscle-specific genes offers the unique opportunity to explore mechanisms whereby negative regulators of myogenesis prevent muscle-specific transcription.

cAMP is one of the important intracellular messengers that may be involved in the control of cellular events in response to external signals. It has been reported previously that dibutyryl cAMP and other agents that increase intracellular cAMP levels inhibit muscle cell differentiation and the expression of muscle-specific genes (Hu and Olson, 1988; Salmen et al., 1991). Regulation of gene transcription by cAMP is frequently mediated through cAMP-dependent protein kinase (PKA) which phosphorylates nuclear targets (Kemp and Pearson, 1990) when cAMP is bound to the regulatory subunits of the holoenzyme, thereby releasing and activating the catalytic subunits (Clegg et al., 1987).

To begin to dissect mechanisms whereby cAMP-dependent pathways may inhibit the muscle differentiation program, we investigated the role of PKA in the activation of muscle-specific gene expression. In the present report, we demonstrate that transactivation of endogenous and exogenous muscle-specific genes by MyoD and Myf-5 are substrates for PKC, which by itself can inhibit myogenesis. Phosphorylation of myogenin by PKC in vitro prevents DNA binding and constitutively expressed PKC inhibits transactivation by myogenin.2 Other than PKC, no pathways that may link components acting close to the cell membrane with those that function in the nucleus have yet been established. The availability of cloned regulatory proteins that induce muscle differentiation and directly activate transcription of muscle-specific genes offers the unique opportunity to explore mechanisms whereby negative regulators of myogenesis prevent muscle-specific transcription.

MATERIALS AND METHODS

Cell Cultures, Transfections, and Plasmid Constructs—C3H 10T1/2 fibroblasts and mouse C2 and rat L6 myoblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. To induce differentiation cells were shifted to differentiation medium containing DMEM supplemented with 10% horse serum and 5 μg/ml insulin. Dibutyryl cAMP was used at 3 μmol/ml final concentration. Transfections were performed using calcium phosphate precipitation as described previously (Braun et al., 1988b). In transient assays cells were shifted to differentiation medium 24 h after transfection and cultured for additional 48 h. Chloramphenicol acetyltransferase (CAT) and β-galactosidase activities were determined in cell extracts according to standard procedures (Gorman, 1985). All CAT activities were standardized to β-galactosidase obtained from 5 μg of cotransfected plasmid RSV-βgal.

Immunohistochemical Staining of Cells—Immunohistochemical staining of cells expressing sarcomeric myosin heavy chain (MHC) was performed with the monoclonal antibody MF-20 (Bader et al., 1992) and the Vectastain ABC Kit as described previously (Braun et al., 1989a).

For transactivation assays, 5 or 10 ng of the following reporter genes were used; MLC-CAT plasmid containing the muscle-specific enhancer of the rat myosin light chain 1/3 (MLC) gene and the proximal promoter of the MLC1 gene linked to the CAT gene (Rosenthal et al., 1990); 4R-tk CAT plasmid containing a 1.1 kb 5′ upstream fragment of the human myogenin (Myf-4) promoter linked to CAT (Weintraub et al., 1990); Myf-CAT plasmid containing a 1.1 kb 5′ upstream fragment of the human myogenin (Myf-4) promoter linked to CAT (Salmen et al., 1991); plasmid (E2+5) TATA-CAT containing six tandem copies of E2 and E5 sites fused to the alkaline phosphatase TATA box (Henthorn et al., 1987) and plasmid (E2+5) VP16 expressing chimeric proteins between the VP16 transactivator domain (Cress and Triezenberg, 1991) and the Myf-5 open reading frame of amino acids 1-149 and Myf-5 amino acids 75-135, respectively (Braun et al., 1992; Winter et al., 1992); GALmyf5 (amino acids 155-253) expressing a fusion protein between the GAL4 DNA binding domain (amino acids 1-147) and the carboxyl terminus of Myf-5 (amino acids 135-255) (Braun et al., 1990b; Winter et al., 1992); plasmid pSIV2AE2-5 expressing the E2-5 (E47) cDNA (Henthorn et al., 1990). The expression vectors for the catalytic and regulatory subunits of PKA were MT-CEVA myoD and MT-REVA-neo, respectively (Lohse and Arnold, 1988; Lohse et al., 1992). The control plasmids δ-actin CAT and GAL-VP16 have been described previously (Lohse and Arnold, 1988; Martin et al., 1990).

Isolation of RNA and Northern Blot Analysis—RNA was isolated from tissue culture cells by the method described by Chomczynski and Sacchi (1987). Purified RNA was separated on formaldehyde gels, transferred to a nylon membrane, and hybridized as described previously (Braun et al., 1989b). The following cDNA probes were used for specific hybridization: MyoD represented by the 0.8-kb HpaII/EcoRI fragment of the 3′-coding region of the mouse cDNA (Davis et al., 1987); myogenin encoded by a 0.7-kb fragment of the rat cDNA as described previously (Sassoon et al., 1988); Myf-5 represented by a 0.3-kb ApaLI/BamH1 genomic fragment encompassing part of the first exon of the mouse Myf-5 gene (Ott et al., 1991); Bober et al., 1991); glyceraldehyde phosphate dehydrogenase containing 1.1 kb of the mouse glyceraldehyde phosphate dehydrogenase cDNA.

Expression of Bacterial-Expressed pGEX-Myf5 and pGEX-MyoD Fusion Proteins—The construction of the vectors pGEX-Myf5 and pGEX-MyoD fusion proteins between glutathione S-transferase (GST) and MyoD or Myf-5 has been described previously (Braun et al., 1990a). The expression and purification were performed as reported by Smith and Johnson (1988) and Lasseur et al. (1989). Purified protein preparations were stored in 20% glycerol at −20°C.

DNA Binding Assays and In vitro Phosphorylation—DNA binding of GST-MyoD fusion proteins was measured by gel retardation assays as described (Braun et al., 1989a, 1991). Typical band shift experiments were performed with 500 ng of purified fusion proteins and 100 fmol of poly(dI-dC). End-labeled oligonucleotide corresponding to the high affinity binding site of the MLC1/3 enhancer (GTACAAACCAAGAGCCGCAAATAAGT) was used as target DNA. The specificity of binding was assessed by competition with wild-type and mutant oligonucleotides (GTACAACTAGTATCTTGGAGAAATG) and 10 μM of the corresponding mutant oligonucleotide. Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels in 0.25× TBE running buffer at 160 V for 5 h at 4°C. In vitro phosphorylation of bacterially produced fusion proteins was done in 1 μl of kinase reaction buffer containing 17.5 mM Tris-HCl, pH 7.4, 2.5 mM magnesium chloride, 40 μM ATP plus 10 μCi of γ-[32P]ATP (specific activity, 3000 Ci/mmol), and 500 ng of purified fusion protein. The reaction was started by adding 10 units of PKA, catalytic subunit (Sigma Inc.) and incubated at 37°C for 15 min.

Preparation of Nuclear Extracts from Tissue Culture Cells—Nuclear extracts from undifferentiated and differentiating C2C12 cells were prepared from 20 dishes containing approximately 3 × 106 cells each and processed as described previously (Braun et al., 1992). Briefly, cells were grown for 3 days in DMEM plus 10% fetal calf serum and 3 μM dibutyryl cAMP (Sigma) or in differentiation medium alone containing 10 μM forskolin, 40 g/liter glucose, 100 μM 1,2-phenylenediamine dihydrochloride (DMEM, 10% horse serum), or DMEM, harvested, pelleted at 1400 rpm, and resuspended in 8 ml of buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, and 1 mM MgCl2 in the mix of protease inhibitors containing 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 40 μg/ml benzamidine. Cells were lysed with 0.8 ml of ice-cold buffer containing 0.8 ml of ice-cold buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diamidithio-reitol, and protease inhibitor mix. Nuclei were extracted

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2 E. Olson, personal communication.
Inhibition of Myogenesis by cAMP and PKA

Fig. 1. cAMP inhibits differentiation of mouse C2 and rat L6 myoblasts. Cells were cultured in differentiation medium with or without Bt2cAMP (3 μmol/ml) for 3 days, fixed, and immunostained with anti-MHC monoclonal antibody MF-20. Essentially all myotubes stained positive. The upper two panels show C2 cells, and the lower panels show L6 cells.

Table I

<table>
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<tr>
<th>Plasmids</th>
<th>Myosin positive cells</th>
<th>Inhibition by PKA</th>
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<tr>
<td>pEMSV-a ccribe</td>
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<tr>
<td>pEMSV-MyoD</td>
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</table>
| +PKA (10 μg) | 36 | 74  
| +PKA (15 μg) | 23 | 83  
| pEMSV-Myf5 | 52 |  
| +PKA (10 μg) | 16 | 70  
| +PKA (15 μg) | 8 | 85  

*Transfections were performed with 10 μg of activator plasmid and 10 or 15 μg of MT-CEVneo plasmids on 5 × 10⁶ cells/plate.

RESULTS

cAMP Attenuates Myotube Formation of Mouse C2 and Rat L6 Myogenic Cell Lines—It has been reported previously (Hu and Olson, 1988) that cAMP analogs and compounds that increase intracellular cAMP levels inhibit the activation of muscle gene expression in the fusion-defective cell line BC3H1. To analyze the effect of elevated cAMP on the differentiation program of other myogenic cell lines, mouse C2 and rat L6 myoblasts were cultured in medium which

TABLE I

Myogenic conversion of 10T1/2 fibroblasts by MyoD and Myf-5 is inhibited by PKA

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Numbers represent the mean of 30 fields counted for each transfection.
Inhibition of Myogenesis by cAMP and PKA

FIG. 3. The catalytic subunit of PKA inhibits transactivation of the muscle-specific reporter genes Myf41-CAT and MLC-CAT by Myf-5 and MyoD. A. 10T1/2 fibroblasts were cotransfected with 10 μg of Myf41-CAT reporter plasmid, 7.5 μg of pEMSV-Myf5 transactivator plasmid, and varying amounts of MT-CEV<sub>α</sub> plasmid expressing the catalytic subunit of PKA or MT-REV<sub>β</sub>-neo plasmid expressing the regulatory subunit of PKA as specified. pRSV-E1a plasmid was used as a control for a known suppressor of Myf-5 transactivation (Braun et al., 1992). Myf41-CAT plasmid alone is inactive in 10T1/2 fibroblasts as shown previously (Salminen et al., 1991). Cells were cultured in differentiation medium for 48 h, and CAT activity was determined according to standard procedures. 5 μg of pRSV-βgal plasmid was included in each transfection, and CAT values were standardized according to β-galactosidase activity. B. cotransfections of 15 μg of MLC-CAT or β-actin CAT reporter plasmids with 10 μg of pEMSV-Myf5 or pEMSV-MyoD transactivator plasmids were performed as described in A. The specified amounts of MT-CEV<sub>α</sub> inhibitor plasmid were included where indicated. pEMSVα-2 plasmid containing no insert cDNA was used as control vector. CAT activities are given relative to the activity obtained in the absence of inhibitor plasmid. A representative result of three independent experiments is shown.

FIG. 4. The inhibition by PKA is directed at the E-box DNA binding motif but does not affect the nonmyogenic bHLH protein E2-5 (E47). 10T1/2 fibroblasts were transfected with 5 μg of 4R-tkCAT (A) or (E2+5)-tkCAT (B) reporter plasmids and 10 μg of the indicated activator plasmids pEMSV-Myf5, pEMSV-MyoD, and pSV2βE2-5. The inhibitor plasmid MT-CEV<sub>α</sub> was included as specified. pEMSVα-2 was used as the vector control. The efficiency of each transfection was controlled by cotransfection of 5 μg of RSV-βgal, and all CAT values were calibrated accordingly. Columns indicate CAT activities relative to control transactivation (without inhibitor). The shown result is representative for three independently performed transfections.
Inhibition of Myogenesis by cAMP and PKA

supports differentiation in the absence and presence of dibutyryl cAMP (Bt2cAMP). Although both cell lines in medium differentiation (DM) readily fused into multinucleated myotubes, a marked reduction of morphological differentiation was observed in the presence of Bt2cAMP (Fig. 1). The expression of several muscle-specific marker genes, such as MHC measured by immunostaining with the monoclonal antibody MF20, muscle creatine kinase, myosin light chains, and sarcomeric actin was also suppressed (data not shown). At the applied concentration of Bt2cAMP, the cells remained firmly attached to the substratum and appeared morphologically normal with no signs for general toxicity. We therefore conclude that the differentiation of skeletal muscle cells is subject to specific negative regulation by the cAMP signal transduction pathway.

Myoblasts in the Presence of Bt2cAMP Express Normal Levels of Myf-5 and MyoD but No Myogenin mRNA—Since cAMP inhibits not only the expression of single muscle-specific genes but seems to affect the complete myogenic differentiation program, we examined the expression of the myogenic regulatory genes of the bHLH family of proteins which are believed to be essential for establishing the muscle phenotype. C2 and L6 myoblasts are known to constitutively express MyoD and Myf-5, respectively, and accumulate myogenin at the onset of differentiation (Braun et al., 1989b). Therefore, RNA from both cell lines grown in DM medium in the absence or presence of Bt2cAMP was analyzed on Northern blots using cDNA hybridization probes specific for Myf-5, MyoD, and myogenin. As shown in Fig. 2, expression of Myf-5 and MyoD mRNA was essentially unaltered by Bt2cAMP, whereas the expression of myogenin mRNA was almost completely inhibited in both cell lines. These results suggest that the expression of the various myogenic bHLH genes is differentially sensitive to cAMP-dependent regulation. Although transcription of the MyoD and Myf-5 genes was not affected by cAMP, expression of the myogenin gene was nearly totally suppressed.

PKA Can Substitute for cAMP in the Inhibition of Myogenesis and Represses Transactivation by Myf-5 and MyoD—Many effects of cAMP on gene regulation are mediated through cAMP-dependent PKA. To investigate whether PKA would be involved in the mechanism by which cAMP inhibits muscle differentiation, we overexpressed the catalytic subunit of PKA in 10T1/2 fibroblasts and examined its effect on myogenic conversion by MyoD or Myf-5. Transient transfection of the expression plasmids pEMSV-MyoD and pEMSV-Myf5 converted a substantial number of 10T1/2 cells into myosin-positive myoblasts, but cotransfection of the vector MT-CEVneo expressing the catalytic subunit of PKA resulted in a significant reduction of myosin containing cells as measured by immunostaining with the monoclonal antibody MF-20 which recognizes sarcomeric MHC (Table I). Comparable inhibition was also observed with 10 independently derived C2 clones which had been stably transfected with the PKA expressing vector MT-CEVneo (data not shown). These observations indicate that PKA can substitute for cAMP in the repression of muscle cell differentiation, suggesting that PKA may mediate the inhibitory effect of cAMP.

It has been shown that some of the oncogene products and external mitogenic stimuli inhibit the activity of constitutively expressed myogenic factors (from viral LTR promoters) by a post-translational mechanism without affecting their synthesis (Vaidya et al., 1989; Lassar et al., 1989; Li et al., 1992a; Bengal et al., 1992; Braun et al., 1992). In order to examine whether PKA would also interfere with the trans-
The glutathione S-transferase (pGEX) alone is not phosphorylated. The weaker bands are primarily due to residual bacterial proteins. Fused pGEX fusion proteins were phosphorylated, aliquots were separated on 10% SDS-polyacrylamide gel and exposed.

The activation function of constitutively expressed Myf-5 or MyoD was also strongly suppressed by PKA. Other transactivating factors and enhance their transactivating function.

**PKA Inhibition of Transactivation by Myf-5**

- **Fig. 6. In vitro phosphorylation of bacterially produced pGEX-MyoD and pGEX-Myf5 by PKA.** 500 ng of affinity-purified pGEX fusion proteins were phosphorylated in vitro with [32P]ATP and PKA as described under “Materials and Methods.” 10 μg aliquots were separated on 10% SDS-polyacrylamide gel and exposed on x-ray film overnight. The positions of the chimeric proteins were determined by Coomassie Blue staining and are indicated by arrowheads. pGEX-Myf5 contains a faster migrating degradation product. The weaker bands are primarily due to residual bacterial proteins. The glutathione S-transferase (pGEX) alone is not phosphorylated.

- **Inhibition of Myogenesis by cAMP and PKA**

  - MyoD
  - Myf-5
  - pGEX
  - pGEX-MyoD
  - pGEX-Myf5
  - + PKA

  - pGEX-MyoD
  - pGEX-Myf5
  - pGEX

  **Fig. 6.** In vitro phosphorylation of bacterially produced pGEX-MyoD and pGEX-Myf5 by PKA. 500 ng of affinity-purified pGEX fusion proteins were phosphorylated in vitro with [32P]ATP and PKA as described under “Materials and Methods.” 10 μg aliquots were separated on 10% SDS-polyacrylamide gel and exposed on x-ray film overnight. The positions of the chimeric proteins were determined by Coomassie Blue staining and are indicated by arrowheads. pGEX-Myf5 contains a faster migrating degradation product. The weaker bands are primarily due to residual bacterial proteins. The glutathione S-transferase (pGEX) alone is not phosphorylated.
Inhibition of Myogenesis by cAMP and PKA

A

<table>
<thead>
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<th>Protein (Phosph.)</th>
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<td>pGEX-MyoD</td>
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<tr>
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<td>Phosphorylated</td>
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retic. lysate

Fig. 7. Sequence-specific DNA binding of in vitro phosphorylated pGEX-MyoD and pGEX-Myf5. A, Gel mobility shift assays (EMSA) were performed with 500 ng of unphosphorylated pGEX-Myf5 protein and pGEX-Myf5 protein phosphorylated in vitro by PKA. Synthetic oligonucleotide encompassing the high affinity E-box of the MLC1/3 enhancer was used as the DNA binding site (for sequence, see "Materials and Methods"). Complexes formed with unphosphorylated control protein in binding buffer (+) or in phosphorylation buffer (buffer) and with phosphorylated protein (phosph.) are shown. The same experiment performed with pGEX-MyoD is shown in lanes 4-6. B, EMSA performed with ³²P-labeled proteins and unlabeled oligonucleotide. Only phosphorylated complexes can be detected in this assay. The positions of the pGEX-Myf5 and pGEX-MyoD complexes were determined in parallel with labeled oligonucleotides as indicated. Unbound proteins migrated faster on the gel (data not shown). C, EMSA performed with E12/pGEX-MyoD and E12/pGEX-Myf5 heterodimeric complexes on the E-box oligonucleotide. 50 ng of unphosphorylated (-) or PKA phosphorylated (+) fusion proteins were mixed with E12 protein produced in reticulocyte lysate (10 μl) as described previously (Braun and Arnold, 1991). Only parts of the gels containing the relevant complexes are shown. The faster moving Myod/E12 complex was seen inconsistently and may be a degradation product during the phosphorylation reaction.

by the VP16 activator region as described previously (Braun et al., 1992). Plasmid Myf5N10-VP16 was derived by deletion of the NH₂-terminal transactivator region (amino acids 14-74) from Myf5-VP16 (Winter et al., 1992). Both of these Myf5-VP16 chimeric proteins retained the bHLH region of Myf-5 and proved to be strong transcriptional activators of the endogenous MHC gene (Braun et al., 1992) and the muscle-specific reporter construct MLC-CAT in 10T1/2 fibroblasts. Coexpression of increasing concentrations of MT-CEVsneo plasmid expressing the catalytic subunit of PKA resulted in a severe repression of MLC-CAT activation (Fig. 5A). In a control experiment, we showed that the GAL4-VP16 transactivator tested on the appropriate reporter plasmid G5E1B-CAT was not affected by PKA, indicating that the VP16 transactivator domain itself is not inhibited by PKA (data not shown). These results then suggested that within the Myf-5 molecule the bHLH domain was sufficient to mediate the inhibitory effect of PKA.

To control whether the bHLH region of Myf-5 was also required, we replaced this motif by the DNA binding domain of the yeast transcription factor GAL4 (GALmyf5). Transfection of the plasmid expressing the GALmyf5 fusion protein together with G5E1B-CAT reporter plasmid into 10T1/2 fibroblasts showed strong activation, which was not inhibited by coexpression of the catalytic or regulatory subunits of PKA. From these results we conclude that the transactivator domain of Myf-5 in conjunction with a heterologous DNA binding domain constitutes no target for the repression by PKA, whereas the Myf-5 bHLH region can confer this regulation onto a heterologous transactivator, such as VP16. Therefore, the bHLH domain represents the primary target for the inhibitory action of PKA.

MyoD and Myf-5 Can Be Phosphorylated by PKA in Vitro but This Does Not Affect Their Ability to Bind to DNA—It has been reported previously that the myogenic bHLH factors are nuclear phosphoproteins (Tapscott et al., 1988; Brennan and Olson, 1990). To examine whether MyoD and Myf-5 might serve as substrates for phosphorylation by PKA in vitro, we incubated bacterially produced fusion proteins between glutathione S-transferase and human MyoD (pGEX-MyoD) or Myf-5 (pGEX-Myf-5) with PKA enzyme and [³²P] ATP and separated the reaction products on SDS-polyacrylamide gels. As shown in Fig. 6, both chimeric proteins pGEX-MyoD and pGEX-MyoD were highly phosphorylated, whereas
the glutathione S-transferase protein alone (pGEX) was not. We observed frequently a degradation product of pGEX-Myf5 which was also phosphorylated. Because the inhibition of MyoD and Myf-5 activity by PKA was directed at the bHLH region and was mediated through the E-box DNA sequence, we next investigated whether protein phosphorylation might alter the DNA binding properties. Electrophoretic mobility shift assays (EMSA) using control and in vitro phosphorylated pGEX-MyoD and pGEX-Myf5 proteins on a synthetic oligonucleotide containing the muscle creatine kinase E-box revealed similar DNA binding between control proteins and their phosphorylated counterparts (Fig. 7A). The same result was obtained at protein concentrations ranging from 200 to 800 ng (data not shown). Although we estimated from the amount of radioactive phosphate residues incorporated into the proteins that the majority of molecules had been phosphorylated and therefore the observed DNA binding should not be due to remaining unphosphorylated protein, we wanted to confirm this in EMSAs using 32P-labeled protein and unlabeled oligonucleotides. In this experiment, a band shift can only be observed when the phosphorylated protein species actually binds to the DNA. As shown in Fig. 7B, both labeled pGEX-MyoD and pGEX-Myf5 proteins formed DNA complexes that migrated to the same position on the gel as the control complexes of unlabelled fusion proteins and radioactively labeled oligonucleotide but slower than free proteins. These results suggest that phosphorylation of MyoD and Myf-5 by PKA in vitro does not prevent sequence-specific DNA binding.

Similar experiments performed in the presence of in vitro synthesized E12 which favors the binding of heterodimers also revealed no alterations in the DNA binding capacity of phosphorylated MyoD and Myf-5 (Fig. 7C). Nuclear extracts prepared from differentiated control and Bt2cAMP-treated C2 cells were tested for binding at an oligonucleotide encompassing the high affinity E-box of the myosin light chain enhancer, including the adjacent MEF-2 binding site (Roth et al., 1990). As shown in Fig. 8, nuclear extracts from control and Bt2cAMP-treated cells formed similar complexes in EMSAs, suggesting that DNA binding of the cognate transcription factors was not affected by cAMP. Because the shifted complexes were successfully competed by excess of wild-type oligonucleotide, but not by oligonucleotide carrying a mutated E-box, we believe that they correspond to bHLH factors. This result provides preliminary evidence that elevated intracellular levels of cAMP, which presumably activate phosphorylation by PKA, probably do not interfere with DNA binding of HLH proteins in muscle cell lines.

**DISCUSSION**

cAMP-dependent Protein Kinase Conveys Inhibitory Signals to the Myogenic Regulators Myf-5 and MyoD—Serum and peptide growth factors have long been known to suppress differentiation of muscle cells in culture and prevent the activation of muscle-specific genes (reviewed by Florini et al. (1991)). It was also shown that MyoD, as well as myogenin and Myf-5, are phosphorylated in proliferating myoblasts (Tapscott et al., 1988; Brennan and Olson, 1990). In an attempt to identify intracellular pathways that may transduce signals generated at the cell surface to the nucleus, we examined the role of cAMP and PKA during myogenesis. Our results demonstrate that stimulation of the PKA pathway inhibits myoblast differentiation and prevents transcriptional activation of muscle-specific genes by the myogenic activators Myf-5 and MyoD. The repression of Myf-5 and MyoD function by PKA is mediated through a post-transcriptional mechanism that is directed at the bHLH domain of the myogenic factors. Other bHLH proteins such as the E2A gene products, E12 and E2-5 (E47), are not inhibited by PKA, indicating that the effect is specific for myogenic bHLH proteins. Although the repression is mediated through the E-box DNA binding site, phosphorylation of Myf-5 or MyoD by PKA in vitro does not affect sequence-specific DNA binding. We therefore conclude that some other events necessary for transactivation are inhibited. Whether the repression of muscle differentiation is a direct result of Myf-5 or MyoD phos-
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phorylation remains to be determined. Taken together, our observations provide evidence that members of the MyoD family of regulatory proteins constitute effective nuclear targets for the control by which cAMP-dependent pathways attenuate muscle differentiation. Although we do not know how the levels of cAMP and PKA might change during differentiation and how this may affect the phosphorylation of the myogenic factors in vivo, PKA conceivably participates in regulatory networks that convey inhibitory signals in myoblasts to the important myogenic control factors present in the nucleus. Whether high PKA activity may also partly account for the poor responsiveness of certain cell types to the myogenic conversion by the muscle regulatory bHLH proteins (Schäfer et al., 1990; Weintraub et al., 1989) is currently under investigation.

Possible Mechanisms for the Repression of Myogenesis by PKA—What types of mechanisms may account for the inhibition of Myf-5 and MyoD function by PKA? In most cases in which phosphorylation by PKA affects transactivation, the exerted control is positive. The only example to date in which transactivation is inhibited by phosphorylation relates to the yeast transcription factor ADRI which enhances the expression of the ADH2 gene via an upstream activating sequence (UAS) (Cherry et al., 1989). When ADRI is phosphorylated by PKA, its ability to interact with the general transcriptional machinery appears to be abolished, whereas it still binds to DNA normally (Taylor and Young, 1990). Similar to ADRI, PKA also suppresses transactivation by Myf-5 and MyoD without affecting their ability to bind to DNA. Significantly, however, the inhibition of Myf-5 activity does not require the transactivator domains which would be expected to be the candidate regions for interactions with the basal transcriptional machinery. Although we have shown that Myf-5 and MyoD can be phosphorylated by PKA in vitro, we have no evidence that direct phosphorylation of the bHLH proteins causes the block in transactivation. It is equally possible that proteins which interact with Myf-5 or MyoD for transactivation constitute the critical targets for PKA. In fact, Li et al. (1992b) have recently demonstrated by mutagenesis of one PKA site in the basic region of myogenin that phosphorylation of this site is not important for the inhibition of myogenin activity by PKA. It is unlikely, however, that the known dimerization partners E12 or E47 mediate the inhibitory effect as their transactivating capacity was not affected by PKA. Inspection of the primary amino acid sequence within the basic HLH domain of Myf-5 that is required and sufficient for the repression by PKA revealed two regions that resemble the consensus sequence for phosphorylation by PKA. These putative PKA sites are located outside of the basic clusters that presumably mediate DNA contacts but are contained within a sequence that is highly conserved among the myogenic bHLH proteins and therefore may serve some fundamental function. Mutational analysis to assess the importance of the putative phosphorylation sites in Myf-5 is in progress. It will be interesting to see whether the loss of Myf-5 activity might be caused by phosphorylation-induced conformational changes or by interference with additional factors that may be necessary as coactivators of Myf-5.

An alternative scenario for the repression of myogenesis by PKA might be that PKA induces the expression of one or several negative regulators of muscle-specific transcription. Indeed, it has been shown that cAMP activates the expression of the immediate early response genes c-fos and c-jun which both inhibit myogenesis and the function of myogenic bHLH proteins (Lassar et al., 1989; Bengal et al., 1992; Li et al., 1992a). Although the exact mechanism whereby these factors interfere with the activity of the myogenic regulators has not been elucidated, it is clear that the repression requires the bHLH domain and is directed at the E-box (Li et al., 1992a). Therefore, Fos and Jun or both proteins conceivably are candidates for effectors of PKA-induced inhibition of myogenesis. Whether the cAMP response element-binding protein (CREB) known to be activated by PKA-dependent phosphorylation may be involved in the induction of negative regulators remains a possibility. However, CREB cannot play a direct role in the repression of muscle-specific gene activation, because it does not bind to the E-box sequence that is sufficient to mediate the inhibition by PKA.

The results of this study identify one of numerous signal transduction pathways that regulate the activity of the important myogenic HLH transcription factors whereby the differentiated phenotype of muscle cells can be modulated. Although we have not yet defined the precise events by which PKA inhibits myogenesis, we have provided evidence for a cellular control component that can regulate the transactivation capacity of members of the MyoD family of proteins at the post-translational level. We suggest a mechanism which probably acts at a step downstream of DNA binding.

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