Mitogen-activated Protein Kinase Pathway Is Involved in the Differentiation of Muscle Cells*

(Received for publication, December 10, 1997, and in revised form, February 12, 1998)

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The differentiation of muscle cells is controlled by the MyoD family of transcription factors. This family is regulated by extracellular growth factors that transmit largely unknown signals into the cells. Here we show that the activity of extracellular signal-regulated protein kinase (ERK), a kinase that is part of the mitogen-activated protein kinase (MAPK) cascade, is low in myoblasts and is induced with the onset of terminal differentiation of C2 cells. ERK activity is also induced in fibroblasts that were modified to express MyoD, but not in the parental fibroblast cells. Thus, ERK induction is an intrinsic property of muscle cells. A specific MAPK kinase inhibitor (PD98059) that was added to C2 cells partially inhibited the fusion of myoblasts to multinucleated myotubes without affecting the expression of muscle-specific markers. This inhibitor blocked the induction of MyoD expression that normally takes place during terminal differentiation. Two lines of evidence suggest that the MAPK cascade induces the activity of MyoD: 1) the expression of constitutively activated forms of MEK1 or Raf1 enhanced the transcriptional activity of MyoD in 10T1/2 fibroblasts; and 2) the addition of PD98059 to fibroblast cells expressing a conditional MyoD-estrogen fusion protein significantly inhibited the expression of MyoD-responsive genes. Our results indicate that the MAPK pathway is activated in differentiating muscle cells and that it positively regulates the expression and activity of MyoD protein.

The development of skeletal muscle is a multistep process in which pluripotent mesodermal cells give rise to myoblasts that subsequently withdraw from the cell cycle and differentiate into myotubes. Each of these steps is subjected to positive and negative regulatory mechanisms. The first stage, commitment of mesodermal cells to the myogenic lineage, is induced by positive cues of extracellular molecules like Sonic Hedgehog and Wnt family members secreted from neighboring tissues (1, 2). These positive cues induce the expression of two members of the MyoD family, Myf5 and MyoD. Cells that express either MyoD or Myf5 are dividing myoblasts that are committed to the myogenic lineage. However, these proteins are not functional in myoblasts, and their activities are induced only upon withdrawal from the cell cycle and during terminal differentiation. Once activated, MyoD and Myf5 contribute to the arrest of the cell cycle, transcription of muscle-specific genes, and terminal differentiation (3). Induction of the activity of MyoD and Myf5 proteins may be a result of the appearance of positive regulator(s) or, alternatively, the disappearance of negative regulator(s). Extracellular growth factors and intracellular proteins that inactivate MyoD proteins have been studied extensively (3–5). Less is known at present about the mechanisms that activate these proteins. Insulin and insulin-like growth factors are the only extracellular growth factors known to promote terminal differentiation of myoblasts (6, 7). Insulin and insulin-like growth factors are involved in activation of phosphatidylinositol 3-kinases and mitogen-activated protein kinases (MAPKs)† via tyrosine kinase receptors within many cells. In muscle cells, insulin activates different MAPK pathways as well as the phosphatidylinositol 3-kinase (8, 9). However, the involvement of these kinase cascades in muscle differentiation is largely unknown. A recent work has demonstrated that phosphatidylinositol 3-kinase inhibitors suppressed morphological and biochemical changes associated with L6E9 myoblast terminal differentiation (10). Therefore, it is possible that these kinase cascades participate in promoting terminal muscle differentiation.

A potential signal transduction pathway that might be involved in myoblast differentiation is the MAPK pathway, which is known to play a complex pleiotropic role in cell growth and differentiation of many lineages. In fibroblasts, this pathway transmits mitogenic signals and interferes with normal differentiation of hepatocytes. However, in other lineages, such as neuronal cells, adipocytes, oocytes, T cells, and photoreceptor cells of the fruit fly Drosophila, activation of this pathway promotes cell differentiation (11). The simplest explanation for the different manifestations of the MAPK pathway is that it affects the regulation of diverse target genes in different cell lineages. However, other explanations are also possible; in the rat pheochromocytoma PC12 cells that serve as a model for neuronal differentiation, temporal parameters of MAPK activation are critical for the definition of growth versus differentiation signals (12). Sustained activation of ERK leads to differentiation, whereas short-lived activation leads to proliferation. The effect of MAPK on muscle cells is controversial; some suggest that it is mitogenic and prevents differenti-

* This work was supported by a United States-Israel Binational Science Foundation (BSF) (to E. B. and S. J. T.), by an Israel Cancer Association grant (to E. B.), by the Rappaport Foundation for Medical Research, and by the Foundation for Promotion of Research in the Technion. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated protein kinase; MBP, myelin basic protein; ER, estrogen receptor; PBS, phosphate-buffered saline; MHC, myosin heavy chain; MLC, myosin light chain; MCK, muscle creatine kinase; GST, glutathione S-transferase; JNK, Jun N-terminal kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.
tion, others believe that it is not involved in differentiation, while still others consider that it may promote differentiation (see “Discussion”).

In view of the involvement of MAPK in the differentiation of various cell lineages and its largely disputed role in myogenesis, we investigated the involvement of MAPK in muscle cell differentiation. In this study, we show that the MAPK pathway plays a positive role in myogenesis. We report that ERK activity is substantially induced during terminal muscle differentiation of C2 cells. The level of c-Fos mRNA, a target of the MAPK pathway, was found to be elevated during differentiation. The induction of ERK activity was specific to differentiating muscle cells and did not take place in fibroblast progenitors of these muscle cells. Activation of the MAPK pathway in transfected cells or its inhibition by the specific inhibitor PD098059 suggests that the MAPK pathway is involved in the induction of MyoD expression and activity. Therefore, we conclude that MAPK activity is induced during muscle differentiation and cooperates with MyoD to activate muscle-specific transcription.

EXPERIMENTAL PROCEDURES

Materials

PD098059 was a product of New England Biolabs Inc. Rabbit polyclonal antibody raised against the C-terminal epitope corresponding to amino acids 245-258 of rat ERK2 was obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibody against a synthetic phosphotyrosine (residue 204) peptide that corresponded to residues 196-209 of human p44 MAPK was purchased from New England Biolabs Inc. Protein A-Sepharose, estradiol, and myelin basic protein (MBP) were supplied by Sigma.

Plasmids

pEMSV-MyoD has been described by Tapscott et al. (13). The 4R-thick CAT reporter gene was described by Weintraub et al. (14). Plasmids pGEX-Jun, CMV-act.MEK1, and Raf1-CAAX were gifts from Drs. Ami Aronheim and Michael Karin. The activated allele of MEK1 was originally described by Mansour et al. (15), and Raf-CAAX by Leevers et al. (16). The constitutively activated MEK1 used in this work was S218E/S222D. The dominant-negative form of MEK1 used was K97M (15).

Cell Culture

C2 cells were a gift of Dr. David Yaffe (17). 10T1/2 cells were obtained from American Type Culture Collection. 10T1/2-MyoD cells were prepared by transfecting pEMSV-MyoD together with pBABE-puro into 10T1/2 cells. Cells were selected in the presence of 2 μg/ml puromycin (Sigma), and myogenic clones were isolated. The preparation of 10T1/2 cells that express MyoD-ER fusion protein was described previously (18). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 15% calf serum (HyClone Laboratories), penicillin, and streptomycin (growth medium). To induce differentiation, we used Dulbecco's modified Eagle's medium supplemented with 10 μg/ml insulin and 10 μg/ml transferrin (differentiation medium). 10T1/2-MyoD-ER cells were induced in differentiation medium supplemented with 10^{-3} M β-estradiol.

Growth of Cells in the Presence of PD098059

PD098059 was dissolved in dimethyl sulfoxide to a concentration of 20 mM and was added directly to differentiation medium to a final concentration of 20–100 μM. Control cells were incubated with the same volumes of dimethyl sulfoxide without PD098059. The medium was replaced every 12 h with medium containing fresh PD098059.

Transfections

Transfections were performed by calcium phosphate precipitation as described (19). Cells in 6-cm TC dishes (Corning) were transfected for 12 h with a total amount of 10 μg of the following plasmid DNAs: 1 μg of pCMV-LucZ, 3 μg of chloramphenical acetyltransferase reporter gene, 3 μg of myogenic expression plasmid, and 3 μg of expression vector of activated MEK1 or Raf-CAAX. Following transfection, the medium was replaced with either growth medium or differentiation medium for another 24–48 h. Transfection efficiency was tested in soluble 5-bromo-4-chloro-3-indolyl β-D-galactopuranoside assays as described (20), and the amount of extracts used for the chloramphenicol acetyltransferase assays was adjusted accordingly (21).

Immunohistochemical Staining

Cells were washed three times in phosphate-buffered saline (PBS), fixed in 2% parafomaldehyde for 7 min, washed three times in PBS, and permeabilized in 0.25% Triton X-100 for 10 min. Subsequently, the cells were incubated for 90 min with a primary antibody. The primary antibodies used were monoclonal anti-MyoD (5.8A), polyclonal anti-MHC (Sigma), and polyclonal anti-MCK. After three washings in PBS, the cells were incubated for 90 min with a secondary antibody conjugated to fluorescein isothiocyanate or to rhodamine (Jackson ImmunoResearch Laboratories, Inc.). The cells were washed three times in PBS, and the final wash contained 1 ng/ml 4',6-diamidino-2-phenylindole. The immunohistochemically stained cells were viewed at a magnification ×200 in a fluorescence microscope (Olympus Model BX50).

In Vitro Kinase Assays

Expression of GST Fusion Proteins—Using the bacterial expression vector pGEX2T-Jun-1 (22), the Jun protein was expressed in E. coli strain BL21 and purified from extracts on glutathione beads as described (22).

Preparation of Cell Extracts—To prepare cell extracts, cells were washed three times in PBS and then extracted in lysis buffer (25 mM HEPES (pH 7.7), 0.5 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 0.1 mM NaVO₃, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 20 mM p-nitrophenol phosphate). The suspension was rotated at 4 °C for 30 min, and the extract was cleared by centrifugation at 10,000 × g for 10 min. Protein concentration was determined by the Bio-Rad protein assay.

Solid-phase Kinase Assay (SNK Assay)—Equal amounts of cell extracts were collected at different times after the initiation of cell differentiation and were mixed with 15 μl of GSH-agarose beads to which GST or GST-Jun proteins were bound. The mixtures were rotated at 4 °C for 3 h, pelleted at 10,000 × g for 10 s, and washed extensively four times in 1 ml of HEPES binding buffer (20 mM HEPES (pH 7.5), 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, and 0.5% Triton X-100). The GST-agarose beads were resuspended in 30 μl of kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl₂, 20 mM p-nitrophenol phosphate, 0.1 mM NaVO₃, and 2 mM dithiothreitol) containing 20 μCi ATP and 5 μCi of [γ-32P]ATP (3000 μCi/mmole). After 20 min at 30 °C, the kinase reaction was terminated by washing the beads with HEPES binding buffer, and phosphorylated proteins were eluted in 30 μl of Laemmli sample buffer and resolved on SDS-polyacrylamide gel.

In Vitro Kinase Assay for MAPK (ERKs)—Cells were extracted in lysis buffer. Equal amounts of protein from each time point of differentiation were mixed with 10 μl of anti-ERK2 antibody (Santa Cruz Biotechnology) for 2.5 h at 4 °C with rotation. Protein A-Sepharose was added, and the mixtures were rotated for an additional hour. Following removal of the supernatant, protein A beads were washed in 3 × 1 ml of 0.5 mM NaCl and 0.1% Triton X-100 and in 1 × 1 ml of 10 mM HEPES and 10 mM MgCl₂. Complexes were then resuspended in 10 μl of substrate (2 mM mg/ml MBP) and 10 μl of a solution containing 5 μCi of [γ-32P]ATP, 50 μM ATP, 10 mM MgCl₂, and 10 mM HEPES (pH 7.6) and incubated for 30 min. The phosphorylation reaction was terminated by the addition of Laemmli sample buffer. The mixture was heated to 90 °C for 5 min and then separated on 12.5% SDS-polyacrylamide gel to directly visualize MBP phosphorylation.

RNA Analysis

Cells were harvested, and RNA was extracted with Tri Reagent solution (Molecular Research Center, Inc.) according to the manufacturer's directions. Total RNA (5 μg) was used for Northern blot analysis on 1.5% agarose gels containing 6.7% formaldehyde. Gels were transferred to Magna nylon membrane by Micron Separations Inc. and were UV-cross-linked. Blots were hybridized with probes for myoD (pEMSV-MyoD), myogenin (pEMSV-myogenin), mtc2 (pVZLC2), p21 (pDNA-Waf1), and GAPDH (pMGAP) that were prepared with random hexamers and the Klenow reaction. The order of hybridization was determined by Bio-Rad Biomax light-sensitive x-ray film. Autoradiographs were quantitated using a laser densitometer (Molecular Dynamics). Northern blots were hybridized with probes for v-myb (pEMSV-vmyb), myoD (pEMSV-myogenin), pdx2 (pMVZLC2), p21 (pDNA-Waf1), and GAPDH (pMGAP) that were prepared with random hexamers and the Klenow reaction. The order of hybridization was determined by Bio-Rad Biomax light-sensitive x-ray film. Autoradiographs were quantitated using a laser densitometer (Molecular Dynamics).
acrylamide gel electrophoresis, and transferred to polyvinylidene difluoride filters. Immunoblotting was conducted with the anti-ERK2 (1:100 dilution) and anti-phospho-MAPK (1:100 dilution; New England Biolabs Inc.) antibodies. Proteins were visualized using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Reverse Transcription-PCR Analysis

Total RNA (200 and 500 ng treated with RQ DNase I (Promega)) was incubated with Moloney murine leukemia virus reverse transcriptase for 30 min at 55 °C as described (23). Reverse transcription reactions were used for PCR amplification of specific cDNAs. One-fifth of the reverse transcription sample was added to the PCR buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM each dNTP, 1 μCi of [α-32P]dCTP, 25 pmol of each primer, and 1 unit of Taq polymerase (Promega) in a total volume of 50 μl. DNA amplification was performed under the following conditions: denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, and primer extension at 72 °C for 1 min. One-fifth of the PCR sample was electrophoresed on 6% polyacrylamide gel, and PCR fragments were viewed by autoradiography.

To enable quantification, a cycle number was chosen for each primer pair that maintained approximately exponential amplification with the most enriched sample. Thus, for c-fos and the L7 ribosomal gene primers, 30 and 22 cycles were applied, respectively (18). The following pairs of primers were used: c-fos, 5'-ACG TGT AAG TAG TGC AGC CC-3' and 5'-CAG ACP CTT AGG AGG CCT TC-3' (315 nucleotides); and L7, 5'-GAA GCT CAT CTA TGA GAA GGC-3' and 5'-AAG ACG AGG GAG CTG CAG AAC-3' (202 nucleotides).

RESULTS

ERK Activity Is Induced During Muscle Differentiation—To study the in vitro activity of the ERK pathway in differentiating C2 muscle cells, extracts were prepared from cells that were grown in growth medium (15% bovine calf serum) and from cells grown in differentiation medium, as described under “Experimental Procedures,” and collected at different stages of differentiation. ERK proteins were immunoprecipitated from these extracts using polyclonal anti-ERK1/2 antibodies, and the kinase activity of the immunoprecipitates was measured by phosphorylation of MBP. The activity of ERKs was induced concomitantly with the onset of muscle differentiation (Fig. 1, compare the activity of ERK with the expression of MLC2). To test the specificity of the ERK pattern of induction, the activity of JNK, which belongs to a separate MAPK pathway, was determined in the same extracts. As shown in Fig. 1, in contrast to ERK, the activity of JNK was reduced during muscle differentiation.

To further verify that ERK activity is induced during muscle differentiation, a phospho-specific ERK antibody was used to detect the active forms of ERK proteins. Two phosphorylated forms of ERK, p42 and p44, were detected, and their levels were found to increase during differentiation (Fig. 2A). The increase in the phosphorylated form of ERK was not due to an increase in the total level of ERK proteins, as demonstrated by the similar amounts of ERK proteins at different stages of differentiation that were detected by antibodies to all forms of ERK (Fig. 2B).

Transcription of the c-fos gene is directly induced by the MAPK pathway (24, 25). ERK participates in c-fos induction via phosphorylation of p62, which is part of the serum response complex activating the c-fos promoter (24). To test whether induction of ERK activity in muscle cells is followed by expression of the c-fos gene, c-fos transcripts were detected at different stages of differentiation. Transcripts of c-fos were barely detected by standard Northern analysis in C2 cells (data not shown); therefore, a quantitative reverse transcription-PCR analysis of c-Fos was performed (Fig. 3). Levels of c-Fos mRNA were low in myoblasts and increased during the process of differentiation (Fig. 3A). Induction of c-Fos occurred concomitantly with induction of ERK activity in the same cells (Fig. 3B). It therefore appears that the induction of ERK activity takes place simultaneously with the expression of the c-fos gene. The similar pattern of expression suggests that the transcription of c-fos may be induced in myotubes as a result of ERK activation.

ERK Activity Is Induced Only in Muscle Cells That Undergo Differentiation, and Not in Parental Fibroblast Cells That Do Not Differentiate to Muscle—Subsequently, we asked whether
the induction of ERK activity is an intrinsic property of differentiating muscle cells or is a result of the transfer of cells to insulin-containing differentiation medium. For this purpose, 10T1/2 fibroblasts were transfected with an expression vector of MyoD, and clones were isolated that stably expressed MyoD and became myogenic (10T1/2-MyoD). These cells, as well as progenitor 10T1/2 fibroblasts, were grown in high serum-containing medium (growth medium) and then transferred to medium that contained insulin (differentiation medium). Protein extracts were prepared from myogenic 10T1/2-MyoD cells and from 10T1/2 fibroblast cells that were grown in growth medium and in differentiation medium for 48 h, and ERK activity was assayed. As shown in Fig. 4A, ERK activity was high in proliferating 10T1/2 fibroblasts and was reduced after their growth in differentiation medium for 48 h. By contrast, ERK activity in 10T1/2-MyoD myoblasts was low in proliferating cells and was markedly induced in myotubes that were grown in differentiation medium for 48 h. Levels of ERK activity in myoblasts of 10T1/2-MyoD clones and C2 cells were always lower than in 10T1/2 fibroblast cells grown in high serum-containing medium (Figs. 1 and 4). The possibility that ERK activity is suppressed in the presence of MyoD in proliferating myoblasts was tested. For this purpose, ERK activity was studied in another cell line that originated from 10T1/2 fibroblasts. This cell line expresses a MyoD-estrogen receptor chimeric protein whose activity is induced by the addition of estradiol to the medium (18). The conditional MyoD protein is inactive in the cytoplasm of cells that have not been treated with estradiol. Proliferating 10T1/2-MyoD-ER cells expressed high levels of ERK activity under high serum conditions (Fig. 4B, lanes 1 and 4). Levels of ERK activity in this cell line were similar to levels of proliferating 10T1/2 fibroblasts and distinctly higher than levels of proliferating myoblasts (Fig. 4). However, when differentiation was induced by the addition of estradiol and differentiation medium, ERK activity remained high at the onset of differentiation (48 h in differentiation medium) (Fig. 4B, lanes 5 and 6). In the absence of estradiol and in the presence of differentiation medium, ERK activity declined in differentiation medium as seen in fibroblasts (Fig. 4B, lanes 1 and 2). Hence, we conclude that (a) ERK activity is significantly lower in dividing myoblasts than in dividing fibroblasts and (b) the induction of ERK is an inherent property of muscle cells, and not of the parental fibroblast cells.

Inhibition of the MAPK Pathway by PD98059 Prevents the Induction of MyoD Expression and Reduces Fusion of C2 Cells—The pattern of ERK activity during myogenesis sug-
gests a positive role for this pathway in myogenesis. To test this possibility, we investigated whether the inhibition of the MAPK pathway affects myogenesis. The specific noncompetitive inhibitor of MAPK kinase (PD098059) blocks its activation by Raf1 (26, 27). This inhibitor was shown to prevent the in vivo activation of MAPK by growth factors and to reverse the transformed phenotype induced by Ras overexpression or by nerve growth factor-induced differentiation (27, 28). First, PD098059 levels that inhibit ERK activity in C2 cells were determined. Differentiation medium was added with PD098059 to C2 cells; 72 h later, the cells were lysed, and the activity of MAPK in the extracts was determined (Fig. 5A). Treatment of cells with 50 μM PD098059 inhibited >80% of ERK activity (Fig. 5A). Under these conditions, PD098059 did not affect the activity of another MAPK, JNK (data not shown).

Whereas untreated C2 cells differentiated and fused to form multinucleated myotubes, PD098059-treated cells differentiated to form less developed myotubes that contained fewer nuclei (Fig. 5C). Immunohistochemical staining of MyoD (nuclear) and MCK (cytoplasmic) indicated that both muscle-specific markers were expressed in drug-treated and untreated cells. The reason why PD098059-treated cells did not fuse properly was not due to a reduced density of these cells, as can be seen by the nuclear staining of all cells in the microscopic field (Fig. 5C, lower right panel). The inhibitory effect of PD098059 was reversible; removal of the drug allowed the cells to fuse and form multinucleated myoblasts (data not shown).

Multinucleated myotube formation normally entails induction of the expression of muscle-specific marker proteins. The effect of the ERK pathway on the expression of several muscle-specific marker proteins was investigated. None of the examined muscle-specific markers was inhibited in PD098059-treated cells (Fig. 5B, MLC2; and C, MCK). Also, induction of p21 (Waf1), causing the exit of myoblasts from the cell cycle, was not inhibited in PD098059-treated cells. The expression of myogenin, a member of the MyoD family, was induced to a similar extent in the drug-treated and untreated cells (Fig. 5B). Myf5 expression was not detected in C2 cells using Northern analysis (data not shown). However, MyoD, whose expression was induced during differentiation, remained at low basal levels in cells that were treated with the drug (Fig. 5B). Therefore, from the differentiation markers that were examined, only MyoD expression was affected. We conclude that treatment of C2 cells with PD098059 had a limited effect on differentiation; MyoD expression was not induced, and cells did not fuse as efficiently as untreated cells. We suspected that the decrease in cell fusion could be a result of the reduced expression of MyoD in these cells. To test this possibility, another myogenic cell line that does not express detectable levels of MyoD was treated with the drug. Like C2 cells, this cell line (L-8) differentiated to form multinucleated myotubes when grown in differentiation medium. However, unlike C2 cells, the fusion of L-8 cells was not affected by the drug (data not shown).

**Differentiation of a Cell Line That Expresses a Conditional MyoD Protein Is Inhibited by PD098059**—MyoD is known to activate its own transcription during myogenesis (29). Treatment of C2 cells with PD098059 prevented the elevation in MyoD mRNA (Fig. 5B), an effect that could be due to the inactivation of the MyoD protein itself.

To find out whether MyoD activity was affected by the MAPK pathway, the above-described cell line that expresses a conditional MyoD protein (10T1/2-MyoD-ER) was used (18). In this cell line, unlike in C2 cells, only MyoD protein initiates myogenesis (18). Therefore, inhibition of MyoD in these cells is expected to abolish myogenesis. The activity of the chimeric protein was induced by the addition of estradiol to cells that
were grown in the presence or absence of PD098059. In these cells, as in C2 cells, 80% of ERK activity was inhibited by 50 μM PD098059 (data not shown). Cells were grown in the presence of estradiol for 36 h, at which time myogenin mRNA levels were induced to detectable levels (Fig. 6, lane 5) (30). If cells were treated with PD098059 during that period, the induction of myogenin expression was severely affected (Fig. 6, lanes 2–4). In this cellular system, myogenin expression is directly induced by the MyoD-ER chimeric protein (18). Also, we found that in contrast to C2 cells, mRNA levels of another muscle-specific marker, MLC2, were inhibited in these cells when treated with the drug (Fig. 6). The seemingly general inhibition of the myogenic program in this experimental system indicates that the existing MyoD-ER protein was inactivated in cells treated with PD098059. These results suggest that inhibition of the ERK pathway blocks the activity of the existing MyoD protein and consequently the expression of its target genes such as myogenin and mlc2.

Activity of MyoD Protein Is Up-regulated by the ERK Pathway—The effect of MAPK on MyoD activity was further studied by transiently transfecting 10T1/2 fibroblasts with expression vectors of MyoD and active forms of kinases that are members of the MAPK pathway. The active form of MEK1 was described by Mansour et al. (15) and Alessi et al. (31), and the active form of Raf1 was described by Leevens et al. (16). Most 10T1/2 fibroblasts transfected with the MyoD expression vector also expressed the differentiation marker MHC when grown for 24 h in differentiation medium (Fig. 7B, lane 1). However, if the transfected cells were continuously grown in high-serum-containing medium (15% bovine calf serum), only ~20–30% of the cells that expressed MyoD also expressed MHC (Fig. 7B, lane 2). Under these serum conditions, cells that were cotransfected with the activated allele of MEK1 or, alternatively, the activated allele of Raf1 exhibited a higher proportion of MHC staining in the cytoplasm of MyoD-expressing cells (Fig. 7B, lanes 3 and 4). Thus, activation of the ERK pathway appears to contradict the inhibitory effects of serum on the function of MyoD as judged by the activation of endogenous MHC expression.

The transcriptional activity of MyoD was also studied by cotransfection of the same expression vectors with a reporter gene whose expression was driven by a minimal promoter that contained MyoD-binding sites. As shown in Fig. 8, the transfected reporter gene behaved similarly to the endogenous mhc gene, i.e. was specifically activated by MyoD in the differentiation medium (lane 8). MyoD activation of the reporter gene was lower in medium containing high serum (growth medium) (Fig. 8, lane 2), and under these conditions, activated MEK1 or activated Raf1 induced the reporter gene activity to levels similar to those observed in differentiation medium (lanes 3 and 4). Therefore, under conditions of high serum, in which MyoD was only partially active, activated forms of kinases from the MAPK pathway could rescue its activity. On the other hand, under media conditions in which MyoD was fully active (differentiation medium), the transfection of a dominant-negative allele of MEK1 inhibited MyoD activity as indicated by the expression of the reporter gene (Fig. 8, lanes 8 and 9).

**DISCUSSION**

Several growth factors and hormones have been associated with the growth and differentiation of muscle cells, but so far, little evidence has accumulated on the participation of intracellular signaling pathways in these processes. The results of this study strongly indicate that the MAPK pathway is involved in the differentiation of muscle cells. We have shown for the first time that ERK1/2 kinase activity is induced concomitantly with the onset of muscle differentiation (Figs. 1 and 2). Induction of ERK activity was found in C2 cells and in muscle cells derived from 10T1/2 fibroblasts (Figs. 1 and 4). ERK activity was not induced in 10T1/2 fibroblasts that were grown under the same medium conditions as muscle cells (Fig. 4), suggesting that the process of ERK induction is directly linked to the differentiation process. To study the effects of ERK activity on the differentiation of muscle cells, we used two approaches: activation of or interference with ERK activity. To activate ERK in muscle cells, constitutively activated forms of MEK1 or Raf1 were expressed in 10T1/2 fibroblasts. Expression of MyoD in 10T1/2 fibroblasts growing in a medium containing a high serum concentration induced the expression of endogenous MHC only in a subset of the transfected cells (Fig. 7B). Coexpression of the activated form of MEK1 or Raf1 increased significantly the number of transfected cells that expressed MHC (Fig. 7B). Coexpression of each of these kinases augmented MyoD activity as measured by the activation of a reporter gene (Fig. 8). To inhibit ERK activity in muscle cells,
a specific noncompetitive inhibitor of MAPK kinase (PD098059) was used (Fig. 5). The treatment of muscle cells with this drug repressed MyoD expression and activity (Figs. 5 and 6). Based on these observations, we suggest that the MAPK pathway modulates the expression and activity of MyoD.

Can ERK Induction Be Involved in Promoting Muscle Differentiation?—Previous studies showed that overexpression of an activated form of Ras (Ha-Ras61), Src, or c-Fos, all of which are effectors and effectors of the MAPK cascade, prevented the differentiation of myoblasts (32). On the basis of these observations, how can one explain the distinct induction of MAPK activity at the onset of muscle differentiation? A possible answer to this question can be derived from two recent works that studied Ras-transformed muscle cells (33, 34). In these studies, PD098059 used to inhibit MAPK activity did not reverse the Ras phenotype. Therefore, it was concluded that inhibition of muscle differentiation by oncogenic Ras occurred by unknown pathways other than the MAPK pathway. However, these works did not identify that the MAPK pathway promoted myogenesis. Ramocki et al. (34) transfected activated forms of MEK1 or Raf1 into 10T1/2 fibroblasts and found that Raf1 inhibited the function of MyoD and that MEK1 did not affect its activity, whereas we found that the same kinases augmented MyoD activity in the same cells (Figs. 7 and 8). This controversy may be explained by the different experimental conditions used in the two works. Whereas Ramocki et al. studied Raf1 and MEK1 under conditions that promoted differentiation (differentiation medium), we studied their effects in high serum-containing medium. We chose these conditions due to the low endogenous activity of ERK in dividing myoblasts (Fig. 1). Ramocki et al. measured the effects in cells that were grown for 48 h in differentiation medium. Under these conditions, the
endogenous ERK activity was high and could mask the effects of the transfected alleles (see Fig. 1).

Opposing effects of the MAPK pathway on growth and differentiation were suggested in analogous systems such as PC12 neuronal cells. These cells either differentiate or proliferate in response to growth factor stimulation. The “decision” to enter each of these pathways is determined by the strength, duration, or both the strength and duration of the stimulus (12, 35, 36). The mechanism of this phenomenon is unknown, but it is instructive to consider how small quantitative differences in signal duration or amplitude are converted into remarkable qualitative differences in gene expression. By analogy, an accurate temporal and quantitative induction of ERK activity might explain its role in differentiation and not in proliferation. Inducing the ERK pathway at different times and intensities could result in either cell growth or differentiation. In fact, a recent work suggested that the MAPK cascade functions at two stages of muscle differentiation (37). At an early stage, MAPK activity inhibits the exit of myoblasts from the cell cycle, whereas at a later stage of differentiation, the same activity is needed for myotube formation. In this respect, it is also interesting to note that the extracellular growth factor transforming growth factor-β, whose signaling pathway is only partially understood, plays both a positive and a negative role in the development of muscle cells (38, 39). Transforming growth factor-β inhibits differentiation of cells that are triggered to differentiate in differentiation medium (38), but augments differentiation in an environment rich in mitogens (39). Also, insulin-like growth factors were shown to have complex effects on the growth and differentiation of muscle cells (40). At first, insulin-like growth factors activate proliferation, and subsequently, they stimulate events leading to the expression of muscle-specific genes.

Is c-Fos Directly Involved in the Differentiation Process?—We have found that c-fos expression is induced during muscle differentiation (Fig. 3). Presently, we do not know whether the induction of c-fos expression is a consequence of ERK induction, nor do we know whether c-fos participates in the differentiation of myoblasts. However, a recent work suggested that the serum response factor is indispensable for myogenic differentiation of C2 cells (41). The serum response factor mediates the induction of c-fos expression in response to serum and growth factors (42, 43), and it is therefore possible that like the serum response factor, c-fos is required for myogenic differentiation.

Activation of ERK Is Inherent to Muscle Differentiation—We suggest that the induction of ERK activity is an inherent property of differentiating myoblasts. This suggestion is based on the observation that ERK activity is induced in 10T1/2 cells that ectopically express MyoD, but not in 10T1/2 cells (Fig. 4). The peak of ERK activity usually appears 24–48 h after differentiation medium containing insulin is added to myoblasts. It also appears concomitantly with the expression of muscle-specific genes such as mck and mlc. We therefore suggest that the transmission of the MAPK signal is not a direct consequence of the exogenous insulin, but rather that an autocrine loop that activates this signaling pathway is being generated in differentiating muscle cells.

Another observation made in this study is that ERK activity in proliferating myoblasts is very low compared with its high levels in proliferating fibroblasts (Fig. 4). Very low levels of ERK activity were observed in C2 cells and in 10T1/2-MyoD myoblasts. Unlike 10T1/2-MyoD cells, 10T1/2-MyoD-ER cells, which express the conditional MyoD protein, maintain high levels of ERK activity (Fig. 4). Other results of this work demonstrate that coexpression of MyoD and activated MEK1 or activated Raf1 promotes muscle differentiation in high serum-containing medium (Fig. 7). A selection against myoblasts that maintain high ERK activity because of precocious differentiation could be the cause for the low levels of ERK activity in myoblasts. Alternatively, MyoD protein may suppress ERK activity in myoblasts. Campbell et al. (44) studied the signals transmitted by basic fibroblast growth factor in MM14 muscle cells. In agreement with our observations, they demonstrated that MAPK activity was not induced in proliferating myoblasts. Basic fibroblast growth factor stimulated the activity of MAPK only in committed myotubes. This work further showed that the signal generated by basic fibroblast growth factor did not progress downstream to MAPK in proliferating myoblasts (44). Later, Campbell and co-workers (45) identified a novel dual specificity protein-tyrosine phosphatase (DUSP6) shown to dephosphorylate activated MAPK. The phosphatase was highly expressed in proliferating MM14 cells and declined rapidly during differentiation. Bennett and Tonks (37) found that the expression of MAPK phosphatase 1, another phosphatase of MAPK, declined significantly during differentiation of C2 cells. Therefore, the expression of known MAPK phosphatases is in inverse correlation to the activity of MAPK as found in this work.

The MAPK Pathway Augments MyoD Activity—Two lines of evidence presented in this work show that the ERK pathway affects MyoD activity. First, treatment of C2 cells with the specific inhibitor of MEK1 (PD098059) repressed the induction of MyoD expression that usually occurs during terminal differentiation (Fig. 5). In addition to its effect on MyoD expression, the inhibitor also repressed MyoD activity in cells expressing a conditional MyoD protein (Fig. 6). In these cells, treatment with PD098059 inhibited the expression of muscle-specific markers like myogenin and MLC. In a second set of results, we noted that the expression of an activated form of MEK1 or Raf1 increased significantly the activity of MyoD in transfected 10T1/2 fibroblast cells (Fig. 7). Whether the effect of MAPK is restricted to MyoD only or other members of the myogenic family are also affected is still unclear. However, the pattern of muscle-specific gene expression and the phenotype of C2 cells that were treated with PD098059 suggest that its effect is probably restricted to MyoD. Similarly to untreated cells, these cells expressed all the muscle cell markers that were examined: MLC, MHC, MCK, and p21. Moreover, the expression of the MyoD family member myogenin was normally induced in the drug-treated cells. The normal expression of myogenin in these cells suggests that it is responsible for inducing the expression of muscle marker genes.

Fusion of C2 Cells Is Decreased by Treatment with PD098059—Surprisingly, C2 cells that were treated with PD098059 fused rather poorly under differentiation conditions. This is a distinct phenotype, especially because these cells express normal levels of several muscle-specific genes. An identical phenotype was recently described by Bennett and Tonks (37) using a different approach to inhibit ERK activity in C2 cells. In their work, overexpression of MAPK phosphatase 1 during differentiation prevented the fusion of cells despite the appropriate expression of myosin heavy chain. We found one distinct difference between Bennett and Tonks’ results and our own: in their work, Bennett and Tonks saw no effect of MAPK phosphatase 1 on the expression of MyoD, whereas we found that PD098059 inhibited the expression and activity of MyoD. The differences in expression of MyoD could have been a result of a substantial difference between the systems used; in their work, MAPK phosphatase 1 inhibited both ERK and JNK, whereas in our work, PD098059 inhibited only ERK. Another possible way to explain the difference is that we measured the steady-state mRNA levels of MyoD, whereas Bennett and
Tonks measured the protein levels of MyoD.

The observed linkage between the specific inhibition of MyoD and the appearance of the distinct phenotype indicates a possible involvement of MyoD in the activation of muscle cell fusion. This idea is strongly supported by a study of Brennan et al. (46) on the BC2H1 muscle cell line. This cell line expresses myogenin and other muscle-specific genes, but not MyoD during differentiation (47). Also, the lack of MyoD expression in this cell line is correlated with the cells’ inability to form multinucleated myotubes during differentiation. Expression of exogenous myoD cDNA in these cells was sufficient, however, to induce a phenotype that forms multinucleated myotubes during differentiation. These results, together with those of the present work, suggest that the effects of MyoD and myogenin are not redundant and that MyoD may be required in these cell lines to allow the fusion of muscle cells. These studies and others suggest an independent segregation of cell fusion and activation of muscle structural genes (47, 48).

Several recent works that studied the effect of PD098059 on muscle differentiation arrived at different conclusions (33, 49, 50). Two studies (33, 50) suggested that the drug had no effect on myoblast differentiation. Another work (49) suggested that the drug caused dramatic enhancement of differentiation. While our work suggests no effect on the expression of differentiation markers, it does indicate a certain inhibitory effect on cell fusion. The controversy may be explained by the use of different cell lines in these studies. Coolican et al. (49) studied L-6 cells, whereas we studied C2 and L-8 cells. In C2 cells, we found that the effects of PD098059 were probably directed to the expression and activity of MyoD protein. MyoD protein is not detectable in L-6 and L-8 cell lines. This may explain why differentiation of L-8 cells was not affected by PD098059. The unexpected stimulation of L-6 differentiation by PD098059 (49) could have been a consequence of the antimitotic effect of the drug that induced earlier withdrawal from the cell cycle and consequently faster differentiation (37). Therefore, different results could also reflect the dual role of MAPK in muscle differentiation: mitogenic at early stages and differentiation-promoting at later stages (37).

ERK1 and ERK2 may not be the only members of MAPK to activate myogenesis. Recently, a newly isolated member of the family (ERK6) was found to be highly expressed in human skeletal muscle cells and appeared to function as a signal transducer during the differentiation to myotubes (51). A comparison between ERK6 and ERK1/2 demonstrated that they represent two separate pathways that do not share the same activating signaling cascades or regulatory systems (51). Additional studies are needed to determine whether or not ERK6 operates in addition to ERK1 and ERK2 in mouse muscle cells.

In conclusion, it appears that like the multiple inhibitory pathways of muscle differentiation, positive regulation of muscle development and differentiation involves multiple pathways that regulate this multistep process.

Acknowledgments—We thank Drs. Ami Aronheim and Michael Karfin for providing the pGEX-Jun plasmid and the expression vectors of different kinases. We thank Drs. Uri Nudel and David Yaffe for the C2 and L-8 cells. We thank Drs. Michael Fry and Bianca Raikhan-Eisenkraft for critical reading of the manuscript.