Critical Proliferation-independent Window for Basic Fibroblast Growth Factor Repression of Myogenesis via the p42/p44 MAPK Signaling Pathway*

Received for publication, January 4, 2001, and in revised form, January 24, 2001
Published, JBC Papers in Press, January 25, 2001, DOI 10.1074/jbc.M100091200

Lori L. Tortorella‡, Debra J. Milasincic, and Paul F. Pilch§

From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

In many cell types including myoblasts, growth factors control proliferation and differentiation, in part, via the mitogen-activated kinase (MAPK) pathway (also known as the extracellular regulated kinase (Erk) pathway). In C2C12 myoblast cells, insulin-like growth factor-1 and basic fibroblast growth factor (bFGF) activate MAPK/Erk, and both growth factors promote myoblast proliferation. However, these factors have opposing roles with respect to differentiation; insulin-like growth factor-1 enhances muscle cell differentiation, whereas bFGF inhibits the expression of the muscle-specific transcription factors MyoD and myogenin. Cells treated with bFGF and PD98059, a specific inhibitor of the MAPK pathway, show enhanced expression of the muscle-specific transcription factors MyoD and myogenin as compared with cells not exposed to this inhibitor. Inhibiting MAPK activity also enhances myoblast fusion and the expression of the late differentiation marker myosin heavy chain. Basic FGF mediated repression of muscle-specific genes does not result from continued cell proliferation, since bFGF-treated cells progress through only one round of cell division. We have identified a critical boundary 16 to 20 h after plating during which bFGF induced MAPK activity is able to repress myogenic gene expression and differentiation. Thus, the targets of MAPK that regulate myogenesis are functional at this time and their identification is in progress.

The mitogen-activated protein kinase (MAPK)\(^1\) signaling cascade has been implicated in the regulation of numerous cellular processes including cell growth and differentiation. The activation of the MAPK pathway occurs through ligand-induced activation of receptor tyrosine kinases that results in receptor dimerization and autophosphorylation (1). The resulting phosphotyrosine residues of the activated receptors associate with various downstream targets. For MAPK or Erk activation, in general, receptor phosphorylation leads to the binding of Grb2/SOS and activation of Ras, which in turn activates the kinase cascade consisting of Raf, its target MEK1/2 (MAPK/Erk kinase), followed by MAPK activation. Activated MEK1/2 phosphorylates Thr\(^{183}\) and Tyr\(^{185}\) of p42/p44 MAPK rendering the kinase active such that it phosphorylates cytoplasmic and nuclear substrates (reviewed in Refs. 2–4). MAPK is strongly activated by many mitogens, suggesting these kinases may play an important role in cell proliferation, and by inference, a negative role in differentiation for appropriate cell types. Somewhat paradoxically, however, the sustained activation of MAPK’s appears to be necessary for the differentiation and neurite outgrowth process in the PC12 neuronal cell line (5–9). Microinjection of constitutively active MAPK into PC12 cells results in their differentiation (8). Also, co-injection of MAPK with the dual-specificity phosphatase MAP kinase phosphatase-1, or the expression of dominant negative MAPK inhibits neuronal cell differentiation (8). On the other hand, MAPK activity antagonizes the ability of 3T3-L1 preadipocytes to differentiate. Treatment of these cells with a MEK1 inhibitor (PD98059) enhances their differentiation, whereas overexpression of MAPK inhibits this process (10). Moreover, tumor necrosis factor \(\alpha\), an inhibitor of adipogenesis, activates MAPK and combined treatment of 3T3-L1 cells with tumor necrosis factor \(\alpha\) and PD98059 restores their differentiation (10).

Members of the MRF (muscle regulatory factor) family of proteins, including Myf-5, MyoD, myogenin, and MRF4, regulate the differentiation of pluripotent stem cells into multinucleated myotubes (reviewed in Refs. 11–13). These proteins are transcription factors of the basic helix-loop-helix type that can transactivate the promoters of many muscle-specific genes when associated with ubiquitously expressed E box proteins (12). Although the temporal pattern of MRF gene expression that controls the progress of muscle cell differentiation has been characterized, the signaling events that dictate whether myoblasts either proliferate or exit the cell cycle and express muscle-specific genes have not been fully described. The positive regulation of myogenic progression may be controlled by several signal transduction cascades, including phosphatidylinositol 3-kinase (PI 3-kinase) (14–16) and the stress-activated protein kinase p38 (17, 18). Insulin-like growth factor-1 promotes myogenesis in L6E9 cells by way of downstream signaling through PI 3-kinase since ectopic expression of a dominant negative p85 subunit of PI 3-kinase inhibits cell differentiation (16). The addition of the PI 3-kinase inhibitors, wortmannin or LY294002, to muscle cells inhibits their differentiation as marked by lack of myogenin expression and creatine kinase

* This work was supported by National Institute of Health Grants DK-30425 (to P. F. P.) and DK-49147 (to N. B. Ruderman). 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.

† Supported by National Institutes of Health Training Grant AG00115 from the NIA (to P. Polgar).
‡ To whom correspondence should be addressed: Dept. of Biochemistry, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118. Tel.: 617-638-4044; Fax: 617-638-5339; E-mail: pilch@biochem.bumc.bu.edu.
§ The abbreviations used are: MAPK, mitogen-activated protein kinase; Erk, extracellular regulated kinase; MEK, MAPK/Erk kinase; SOS, son of sevenless; Grb2, growth factor receptor-bound protein; MRF, muscle regulatory factor; PI 3-kinase, phosphatidylinositol 3-kinase; IGF-1, insulin-like growth factor-1; bFGF, basic fibroblast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MHC, myosin heavy chain; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline.
activity (14, 15). The stress-activated protein kinase, p38, plays a positive role in the differentiation of muscle cells, and the expression of muscle-specific genes and fusion to myotubes is inhibited by the p38 inhibitor, SB203580 (17, 18). Both insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) utilize the MAPK pathway, in part, to promote C2C12 myoblast cell growth (19) but still it is not clear what role p42/p44 MAPK plays in controlling the differentiation of skeletal muscle cells. Thus the focus of this study is to examine the growth factor activated MAPK signaling cascade in the regulation of muscle cell differentiation.

The differentiation of C2C12 myoblasts seems to be a default pathway for cells which are deprived of normal growth conditions (serum deprivation). In vivo, the differentiation process is tightly regulated by serum growth factors and by autocrine and paracrine factors. The C2C12 cell line is sensitive to bFGF, a growth factor known to regulate satellite cell function (20, 21). We show that bFGF is a more potent activator of p42/p44 MAPK compared with IGF-1 and this activation represses myogenesis. We use the specific inhibitor of MEK1 (PD98059) to show that p42/p44 MAPK activity is important for repression of muscle-specific gene expression and myoblast fusion. In addition, we determined that bFGF does not repress differentiation by maintaining the cells in a proliferative state even though it promotes one round of cell division and maintains the expression of cyclin D1. MAPK activity is critically important for the suppression of myogenesis during the 16–20 h after plating. At this time the cells are in late G1 phase and the early targets of p42/p44 MAPK appear to be expressed during this period.

Experimental Procedures

Cell Culture

Mouse myoblast cells (C2C12) (22, 23) were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose and 1-glutamine supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were rendered quiescent by suspension in methylcellulose-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM dibasic sodium phosphate, 1.8 mM monobasic potassium phosphate, pH 7.4), followed by centrifugation at 2000 g for 10 min on ice and then samples were collected in 0.3M sodium hydroxide and counted using a scintillation counter containing 25 mM Tris, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, 0.1 μM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, and 1 mM sodium orthovanadate. Cell lysates were cleared of insoluble material by centrifugation at 12,000 × g for 10 min at 4 °C. Equal amounts of protein were loaded on a 10% or 6–12% continuous gradient SDS-polyacrylamide gel (26). Separated proteins were transferred electrophoretically to a 0.2-μm nitrocellulose membrane (Bio-Rad) using Towbin's transfer buffer (390 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol) (27). Membranes were incubated in 10% nonfat milk in PBS-T (PBS plus 0.1% Tween 20) and then with the following primary antibodies in PBS-T and 2% bovine serum albumin for 1 h at room temperature; F5D monoclonal antisem (Developmental Studies Hybridoma Bank, University of Iowa) specific for myogenin, MF20 monoclonal antisem (Developmental Studies Hybridoma Bank) specific for myosin heavy chain, anti-ACTIVE MAPK antibody (Promega Corp., Madison, WI), that recognizes only the activated p42/p44 MAPK species, pan-ERK antibody (Transduction Laboratories, Lexington, KY), PC10 (PCNA) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p27kip1 antibody (Transduction Laboratories, CA), cyclin D1 antibody (PharMingen, San Diego, CA). Horseradish peroxidase-conjugated secondary antibodies (Sigma) were diluted 1:2000 in PBS-T and incubated for 1 h at room temperature. Proteins were detected using Enhanced Chemiluminescence (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and quantitated using a Molecular Dynamics Densitometer (Amersham Pharmacia Biotech, Inc.) with Molecular ImageQuant Software version 3.2.

Northern Blotting

RNA was obtained as described by Chomczynski and Sacchi (28). Cells were rinsed three times with PBS, lysed in Solution D (4 mM guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 mM β-mercaptoethanol), followed by acid phenol (pH 4.2): chloroform extraction. Total RNA was precipitated in isopropyl alcohol at −20 °C, followed by centrifugation at 10,000 g for 20 min. Ethanol precipitates (20 μg) of RNA were separated by electrophoresis in 6% formaldehyde gel and transferred to GeneScreen nylon membrane (PerkinElmer Life Sciences) by capillary action using 10 × SSC (1.5 mM NaCl, 0.15 μM sodium citrate, pH 7.0) as the liquid phase. The RNA was cross-linked to the membrane using the UV Stratalinker (Stratagene, La Jolla, CA). RNA was checked for equal loading and transfer by UV visualization of ethidium bromide RNA staining. Prehybridization of nylon membranes was for 4–6 h at 42 °C in 50% formamide, 4 × SSC, 5 × Denhardt’s (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin), 0.05 M sodium phosphate, pH 7.0, 0.5 mg/ml sodium pyrophosphate, 1% SDS, 0.1 mg/ml tRNA carrier, and the labeled probe. The cDNA inserts used for probes were MyoD (29) and myogenin (30). The cDNA probes were labeled with [32P]dATP by random priming using the method of Feinberg and Vogelstein (31) and purified with a NucTrap column (Stratagene). The specific activity of the probes were at least 10⁶ cpm. Blots were washed in a stepwise gradient at moderate stringency (0.2 × SSC, 0.1% SDS at 42 °C) and exposed to film at −80 °C.

Quantitative Analysis

Determination of Cell Number—Cells were detached from the plate with 0.05% trypsin-EDTA and then resuspended in PBS with 0.4% trypsin blue. Viable cells as determined by trypsin blue exclusion were counted using a hemocytometer.

Determination of Protein Concentration—The amount of protein was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Bovine serum albumin was used as a protein standard.
**RESULTS**

Activation of the p42 and p44 MAPK Isoforms Represses Differentiation in C2C12 Cells—Various signal transduction pathways, such as the MAPK cascade, have been implicated in controlling proliferation, growth arrest, and differentiation. The data concerning the role of the p42/p44 MAPK pathway in myogenesis is conflicting as both positive and negative regulatory roles have been suggested (32–35). The present study addresses the role of bFGF induced p42/p44 MAPK activation in C2C12 differentiation. A cell suspension method was used (24, 25) that permits growth arrest of C2C12 cells in G1, without subsequent induction of the myogenic program. This technique allows us to characterize early signaling events induced by growth factors in a synchronous way and to determine the effect of this signaling on muscle cell differentiation.

Quiescent C2C12 cells were stimulated with the indicated concentrations of bFGF or IGF-1 and MAPK phosphorylation was measured after 5 min by Western blotting with a MAPK antibody that recognizes the activated form of MAPK (Fig. 1). Activation of MAPK by bFGF or IGF-1 requires nanomolar concentrations of growth factor, with bFGF being a significantly more potent upstream activator of MAPK phosphorylation. Compared with IGF-1-treated cells, the addition of bFGF induced p42/p44 MAPK activation in C2C12 differentiation. A cell suspension method was used (24, 25) that permits growth arrest of C2C12 cells in G1, without subsequent induction of the myogenic program. This technique allows us to characterize early signaling events induced by growth factors in a synchronous way and to determine the effect of this signaling on muscle cell differentiation.

The results in Fig. 1 indicate a correlation between the level of PD98059-induced MAPK activity and myogenin expression. The MEK1 inhibitor, PD98059, was used to block MAPK activation in response to growth factor stimulation. Quiescent C2C12 cells were incubated with the indicated concentrations of PD98059 and then stimulated with bFGF or IGF-1. The dose dependence of the inhibitor on MAPK activity and myogenin protein expression is shown in Fig. 2. In agreement with the data presented in Fig. 1, IGF-1 and bFGF activate the p42 and p44 isoforms of MAPK, although to different extents. Cells treated with 3 nM IGF-1 and 20 μM PD98059 have no detectable MAPK activity, while cells treated with 1 nM bFGF and increasing concentrations of PD98059 still retain some of their kinase activity. Even at the highest concentration (100 μM) of PD98059 tested, the bFGF induced activation of MAPK is inhibited by ∼70%. Most likely, the inability of PD98059 to completely inhibit MAPK phosphorylation is due to the limited solubility of the inhibitor (19, 37). Alternatively, since PD98059 is specific for MEK1, it may not block signaling that is MEK2 dependent. The addition of PD98059 to cells correlates with a loss of MAPK activity, but not to a loss of MAPK protein expression as determined by probing the blot with pan-ERK which recognizes the 42- and 44-kDa isoforms of MAPK (data not shown).

Myogenin protein expression was analyzed under these same conditions to determine whether the level of MAPK activity correlates with the amount of muscle-specific marker expression. C2C12 cells stimulated with IGF-1 express myogenin at 48 h after addition of growth factor, in a manner similar to cells deprived of serum (Fig. 2 compare control and IGF-1-treated cells). Similar results were observed with control and IGF-1-treated cells since C2C12 cells express endogenous IGFs as they differentiate (38–42), and the addition of 3 nM IGF-1 does not have dramatic effect on differentiation. Meanwhile, the addition of bFGF inhibits the expression of myogenin (Fig. 2). Interestingly, cells treated with PD98059 in addition to either IGF-1 or bFGF exhibit enhanced expression of myogenin protein 48 h after exposure to growth factor (Fig. 2, lanes labeled FGF+PD and IGF+PD). At the higher end of the concentration range for PD98059, a 4.5-fold increase in myogenin expression is observed for cells incubated with bFGF and PD98059, while a 1.5-fold increase occurs in cells treated with IGF-1 and PD98059, compared with their counterparts that saw growth factor alone. These data suggest that inactivation of MAPK relieves a repression of myogenin expression and that MAPK activity may play a role in suppressing differentiation in C2C12 cells.

p42/p44 MAPK Activity Represses the Transcription of Muscle-specific Genes and Blocking Kinase Activity Leads to Enhanced Myotube Formation—To determine whether the MAPK-dependent reduction of myogenin protein is a result of decreased mRNA expression, Northern blot analysis was per-
formed. Total mRNA was isolated at the indicated time during the differentiation time course and the expression of myoD and myogenin were examined (Fig. 3A). Up-regulation of both myoD and myogenin mRNA is observed in control (no growth factor stimulation) and IGF-1-treated cells by 24 h post-stimulation. Cells treated with the PD98059 inhibitor, in addition to IGF-1, undergo a more rapid induction of these mRNAs (Fig. 3A, compare IGF to IGF+PD at 24 h). Alternatively, C2C12 cells stimulated with bFGF exhibit minimal expression of myoD and myogenin. Cells treated with both bFGF and PD98059 express both myoD and myogenin although to a lesser extent and in a delayed time course compared with control cells. Thus, MRF expression appears to be transcriptionally regulated by bFGF. To confirm that the lack of muscle-specific gene expression is reflective of changes in C2C12 phenotype, the expression of muscle-specific proteins and myoblast fusion was examined. Quiescent C2C12 cells were treated as in Fig. 3A and then at the indicated times the cells were harvested and analyzed by Western blotting for myosin heavy chain (MHC) and myogenin (Fig. 3B). The expression of muscle marker proteins is induced in control (no growth factor stimulation) and IGF-1-treated cells. Myogenin protein expression is evident 24–48 h after stimulation, along with subsequent induction of MHC protein by 48–72 h. On the other hand, cells treated with bFGF lack expression of myogenin and MHC, and fail to differentiate into myotubes (Fig. 3C). Cells treated with the MEK1 inhibitor exhibit enhanced expression of differentiation markers (Fig. 3B, lanes labeled FGF+PD and IGF+PD). The combined treatment of IGF-1 and PD98059 results in an earlier induction of both myogenin and MHC proteins (Fig. 3B, compare IGF to IGF+PD at 24 h). Moreover, the treatment of cells with bFGF and PD98059 also leads to fusion of cells into multinucleated myotubes (Fig. 3C) and the induction of myogenin and MHC proteins (Fig. 3B, compare FGF to FGF+PD). Cells treated with both bFGF and the inhibitor demonstrate a 6–8-fold increase in myogenin over cells treated with bFGF alone. The differentiation of bFGF-treated C2C12 cells cannot be completely restored by the addition of PD98059, perhaps because of the limited solubility of the inhibitor or because of its specificity for MEK1 as mentioned above.

Basic FGF Promotes One Round of Cell Division, but Cells Fail to Growth Arrest in G1—The inhibition of differentiation by MAPK activity may result from the ability of bFGF to maintain the cells in a proliferative state. The possibility that bFGF is causing the cells to proliferate throughout the "differentiation" time course was tested by assaying the incorporation of tritiated thymidine into DNA as a marker of cell cycle progression (S phase). Quiescent cells treated with fetal bovine serum were used as an indicator of continuous cell cycling and the amount of radiolabeled thymidine detected in these cells steadily increases over the 96-h time course (Fig. 4A). Otherwise, quiescent cells were exposed to bFGF or IGF-1 in the presence or absence of PD98059 and these cells incorporate radiolabeled thymidine primarily during the 24–48-h time period after plating (Fig. 4A). Although cells treated with both bFGF and PD98059 undergo DNA synthesis, they have a profile of thymidine incorporation that is more similar to control cells than to bFGF-treated cells. The bFGF-treated cells incorporate 20% of the labeled thymidine compared with proliferating cells at times after 48 h, but they incorporate 150% of the radiolabel compared with differentiated (control) cells, indicating that a small percentage of the cells may be maintained in the cell cycle. To confirm that the synthesis of DNA and therefore progression through S phase correlates with cell doubling, cells treated under the same conditions were counted at each 24 h period to determine changes in cell number. As shown in Fig. 4B, bFGF- and IGF-1-treated cells (> PD) undergo only one round of cell doubling, while cells treated with fetal bovine serum go through at least three doublings over the 72 h time course. These data indicate that both bFGF and IGF-1 initiate C2C12 cell cycle progression, but after one round of division, the cells growth arrest.

The proliferative state of the cells over the 72 h time course was analyzed by examining several markers of cell cycle in quiescent C2C12 cells treated with or without PD98059 and bFGF or IGF-1 (Fig. 5). Proliferating cell nuclear antigen (PCNA) is a protein that associates with DNA polymerase subunits whose expression is a marker of cycling cells. The expression of PCNA is increased in growth factor-treated cells during the first 48 h (Fig. 5, compare control with IGF-1 and bFGF lanes) and after this time drops to below basal (0 h) levels. These data are consistent with the thymidine incorporation results (see Fig. 4), and suggests that the cells are not proliferating at later times (72 h). The expression of PCNA in bFGF + PD-treated cells mirrors the expression in cells treated with bFGF alone, which is not surprising as their time course for DNA synthesis is similar.

Cyclin D1, which is expressed during G1 and S phase, was examined to determine whether the expression of cell cycle regulatory proteins may be altered in cells exposed to bFGF.

**Fig. 2.** The inhibition of MAPK activity by the MEK1 inhibitor, PD98059, is dose dependent and allows myogenin expression. Suspended C2C12 cells were replated for 1 h with the indicated concentration of PD98059, stimulated with 1 nM bFGF or 3 nM IGF-1 and then harvested 5 min (activated MAPK***) or 48 h (myogenin expression) after growth factor addition. A total protein (100 µg) was separated by 10% SDS-polyacrylamide gel electrophoresis, blotted to a nitrocellulose membrane, and examined for activated MAPK isoforms (MAPK***) or myogenin by Western blotting techniques. B, densitometry was performed with blots from part A using a Molecular Dynamics densitometer with MD ImageQuant Software version 3.2: open bar, IGF; striped bar, FGF; dotted bar, control. Moesus (-) indicates control cells that were maintained in DMEM + 0.4% calf serum. The results are representative of three independent experiments with similar results.
Cyclin D1 expression is barely detectable in cells not exposed to growth factor (Fig. 5, lanes labeled control cells). On the other hand, bFGF (± PD)-treated cells express cyclin D1 through 30 h during which time the cells are proliferating (see Fig. 4) and then its expression drops to basal levels at 48 h. Cyclin D1 is also expressed in IGF-1 (± PD)-treated cells, although to a lesser extent and for a shorter time than cells exposed to FGF. One striking difference in cyclin D1 expression is apparent at 72 h when bFGF-treated cells regain expression of cyclin D1 compared with their counterparts that were exposed to IGF-1 or to both bFGF and PD98059. Thus, the maintained expression of cell cycle regulatory proteins in bFGF-treated cells may prevent the progress of differentiation.

Cells exposed to bFGF are no longer cycling after 48 h, therefore, we determined whether or not the cells were growth arrested in G0 by examining the expression of the cyclin-dependent kinase inhibitor p27KIP1. At time 0, the p27KIP1 expression is high since the cells were made quiescent by suspension (Fig. 5, lane labeled 0 h). As the cells differentiate, they maintain their level of p27KIP1 expression, which decreases only when a small population of the cells enter the cell cycle between 24 and 30 h (Fig. 5, lanes labeled control). Also, expression of p27KIP1 is diminished in IGF (± PD)-treated cells as they proliferate, but it is regained at later times as the cells differentiate. Similarly, bFGF + PD-treated cells expressed p27KIP1, although to a lesser extent than control and IGF-1-treated cells. On the other hand, the low level of p27KIP1 expression in bFGF-treated cells leads us to conclude that these cells are not growth arrested in G0. These results give rise to an alternative hypothesis; bFGF-treated cells undergo one round of cell division and then are “stuck” at some point in the cell cycle.

To determine at what phase of the cell cycle C2C12 cells are in as a function of the differentiation time course, we performed flow cytometric analysis on differentiating, proliferating, and bFGF-treated cells (Fig. 6). Quiescent C2C12 cells were cultured in only DMEM + 0.4% calf serum. The data presented is representative of three independent experiments.
cells at each condition. At each time point at least four individual cells were detached from the cell culture plate with trypsin/EDTA, resuspended in PBS containing trypsin blue, and counted using a hemocytometer. The percentage of cell death ($<5\%$) as determined by trypsin blue exclusion was similar for cells at each condition. At each time point at least four individual plates/condition were counted. The data presented is representative of two separate experiments with similar results.

exposed to bFGF alone appear to accumulate in G$_{0}$/M phase at later times. These results suggest that after 16–20 h, the cells either proliferate or remain in G$_{0}$/G$_{1}$ and later differentiate, and this decision is dependent on the activation of MAPK (see Fig. 7).

p42/p44 MAPK Activity Is Required between 16 and 20 h After Plating to Inhibit Myogenesis—The fact that differentiating (control) cells and bFGF-treated cells exhibit similar profiles for their phases of the cell cycle up to 20 h, but differ thereafter, suggests that bFGF may be regulating events that are occurring in the 20-h period after cell plating that block the pathway to differentiation. To determine whether the continuous presence of the growth factor during this period was necessary to inhibit myogenin expression, hence differentiation, bFGF was added at time 0 and then withdrawn at the various times indicated (Fig. 7A). Progress toward differentiation was monitored by myogenin expression 48 h after plating. PD98059 was added upon withdrawal to prevent any MAPK activation that may be occurring due to the presence of internalized bFGF receptor (45) that might escape the washing procedure and addition of PD98059 did not have a significant effect on myogenin expression (not shown). Exposure of cells to bFGF for 12 h and then withdrawal resulted in no inhibition of myogenin expression, but after 16 h, a 40% decrease in this MRF was observed. Interestingly, bFGF-dependent MAPK activity was somewhat diminished at early time points, possibly due to the induction of MAP kinase phosphatase-1 upon growth factor exposure (see Ref. 44, and reviewed in Refs. 45 and 46). Next, cells were plated in DMEM and bFGF was added at various times thereafter (Fig. 7B). Myogenin expression at 48 h and MAPK activation immediately following bFGF addition were monitored as in the previous figure. Addition of bFGF up to 20 h after plating effectively inhibited myogenin expression and MHC expression (data not shown), but this treatment was ineffective when added 24 h after plating. However, bFGF added at 24 h activated MAPK to the same extent as it does when added at any other time, indicating that the ability of bFGF to activate MAPK is not lost between 20 and 24 h of incubation and therefore is not the reason for the ineffectiveness of bFGF at this time. Taken together, the data indicate that the ability of bFGF to inhibit myogenesis is mediated by MAPK activity at a time between 16 and 20 h after cell plating.

**DISCUSSION**

The growth and regeneration of postnatal and adult skeletal muscle is mediated by satellite cells that reside between the basal membrane and plasma membrane of myofibers. Satellite cells are usually mitotically quiescent in mature muscle, but the cells become active following injury, exercise, denervation, or in diseased states. Various extracellular stimuli result in the proliferation, differentiation, and fusion of satellite cells with nearby muscle fibers. Growth factors, such as bFGF, IGF-1, and transforming growth factor-β have been implicated in the regulation of satellite cell proliferation and differentiation (reviewed in Refs. 20 and 21). Basic FGF is mitogenic, but can also act as a negative regulator of myogenesis by inhibiting the expression and/or activity of the MRFs (47–50). The signaling events that mediate repression of myogenesis by bFGF have not been clearly identified. The regulation of myogenesis has mainly been studied using conditions where differentiation is achieved following withdrawal of serum components. In the present study, a cell suspension system was used that arrests the cells in G$_{0}$ without the subsequent induction of myogenic markers (25), allowing us to examine the growth factor effects on a synchronized system and enabling us to detect changes in signaling that may be crucial for the control of differentiation. Using this system, p42/p44 MAPKs were identified as potential regulators of bFGF-mediated myogenic inhibition. A robust activation of p42/p44 MAPK occurs upon bFGF stimulation of G$_{0}$ arrested C2C12 cells. A chemical inhibitor of p42/p44 MAPK signaling, PD98059, was used to show that MAPK activity represses the expression of several muscle-specific transcription factors, including myogenin and MyoD, and the subsequent expression of differentiation markers, such as myosin heavy chain. These results are consistent with reports that overexpression of MAP kinase phosphatase-1 could inhibit mitogen-induced p42 MAPK activity, resulting in the expression of MyoD, myogenin, and MHC (33). A potent bFGF induced signal is sufficient for complete inhibition of muscle-specific marker expression (our results and Ref. 36). In addition, bFGF is capable of overriding positive regulatory effects normally imposed by IGF-1 in C2C12 cells (36).

Our results also show that bFGF and IGF-1 are mitogenic in C2C12 cells and cause one round of cell proliferation, even though these growth factors differ in their level of MAPK activation and consequently, their role in differentiation. Interestingly, IGF-1, which is a positive mediator of muscle differentiation, activates MAPK to a slight extent in G$_{0}$ arrested...
C2C12 cells compared with bFGF. Obstruction of the IGF-1 induced MAPK signal by PD98059 results in an earlier induction of myogenesis. The slight activation of MAPK by IGF-1 is substantial enough to slightly delay the progression of differentiation. We suggest that in growth factor-stimulated C2C12 cells, the ability of cells to differentiate is limited by the level of activated MAPK. Since bFGF stimulates p42/p44 MAPK activity to a much greater extent than IGF-1, only the former is able to inhibit the myogenic program. In similar studies, PD98059 treatment of IGF-1-stimulated L6A1 myoblasts results in markedly enhanced myogenin expression and creatine kinase activity.3 These data suggest that the activation of MAPK is a key regulator of the myogenic process. Cells may have a sensing mechanism which distinguishes between the intensity of cellular signals that allows for fine tuned regulation of differentiation.

One striking feature of bFGF-mediated repression is the limited requirement for MAPK activity following stimulation. In fact, a MAPK signal from the time of replating is not necessary, since bFGF can be added to the cells 16–20 h after plating and the cells remain undifferentiated. The addition of bFGF 24 h after replating is not effective in repressing differentiation, even though MAPK is fully activated under these conditions. These results indicate that there is a critical time frame during which MAPK can inhibit differentiation, probably because the MAPK substrates and/or their molecular interactors are available during this window. After 24 h, the cells enter a commitment phase where they are no longer affected by bFGF. Along these lines, we have observed in serum-deprived C2C12 cells that p42/p44 MAPK activity decreases by 24 h after withdrawal.3 It is not surprising that the MAPK activity decreases as differentiation progresses, considering its strong "anti-myogenic" effects. Previous studies (32, 34) suggest that p42/p44 MAPK activity increases during myoblast differentiation. It is possible that distinctions in the cell culture methods may account for these differences from our results, and indeed the cell suspension system used in our studies are preferred to examine the regulation by signaling pathways. In any case, all of our data suggests that p42/p44 MAPK activity inhibits differentiation. Interestingly, it has been suggested that the sustained p42 MAPK activity observed in the cells is due to an IGFII autocrine loop (32). It is possible that early in differentiation an insulin/IGF stimuli coordinates differentiation via activation of PI 3-kinase, as demonstrated in various myoblast cell lines, including L6A1, L6E9 (14, 15), and in chicken embryo myoblasts (51) and that MAPK activity may be needed at later times.

Recent studies which examine the role of MAPK in adipocyte differentiation have suggested that one possible target of MAPK in preadipocytes is peroxisome proliferator-activated receptor-γ, a key regulator of adipogenesis that gets phospho-

---

3 L. Tortorella and P. Pilch, unpublished results.
rylated and this modification represses this transcription factors activity and thus suppresses differentiation (52). As peroxisome proliferator-activated receptor-γ is a key modulator of adipogenesis, one could hypothesize that the primary transcriptional regulators of myogenesis may also be regulated in this fashion. Of the four MRF family members, only MyoD and myf5 contain at least one potential p42/p44 MAPK phosphorylation site with the consensus Pro-X-(Ser/Thr)-Pro, while myogenin and MRF4 contain the minimal phosphorylation site(s) (Ser/Thr)-Pro. In the quiescent C2C12 culture system used in these studies, it is unlikely that bFGF induced MAPK activity is altering the activity of the MRFs, in particular MyoD and myogenin, since these genes are not expressed significantly in bFGF-treated cells. Basic FGF has been implicated in controlling the mRNA expression of the muscle-specific transcription factors, MyoD and myogenin (Refs. 47 and 48, and our results). On the other hand, in myoblasts which express these transcription factors post-translational modification, such as phosphorylation of the MRFs, may be the underlying regulatory mechanism for altering their transcriptional activity and/or their association with important transcriptional co-factors. For instance, PKC-dependent phosphorylation of myogenin has been shown to inhibit its transcriptional activity (49). The possibility that bFGF alters the interaction of MRFs with necessary co-factors has also been addressed (50).

It is known that bFGF acts as a mitogen and could be altering the cell cycle proteins. Studies indicate that D-type cyclins are both positively and negatively regulated by bFGF. We have shown that cyclin D1 expression is up-regulated in bFGF-treated C2C12 cells and is dependent on MAPK activation since cells treated with bFGF and PD98059 do not maintain cyclin D1 expression at later times. These results are consistent with reports that in C2C12 myoblasts, bFGF induces cyclin D1 and inhibits cyclin D3 expression whereas in the absence of mitogens cyclin D1 levels decrease (53). In addition, overexpression of cyclin D1 in C2C12 (53) and 10T1/2 (54) cells inhibits differentiation, while overexpression of other cyclin D isoforms did not have any effect on differentiation. In the latter study, it was shown that cyclin D1 acts by inhibiting MyoD from transactivating muscle-specific promoters and this inhibition was due to the phosphorylation of MyoD. The MRFs are not expressed in bFGF-treated C2C12 cells and therefore cyclin D1/cyclin-dependent kinase complexes are not regulating myogenesis by altering their transactivation function. Since the effect of bFGF on cyclin D1 expression is not apparent until 72 h, this response may or may not be directly mediated by MAPK.

Finding the targets of signaling molecules that regulate the differentiation process have been difficult since the signaling induced by extracellular mediators usually occurs within minutes, but the resulting effect on differentiation is not apparent for many hours or days. We have identified the 16–20-h period that bFGF induced MAPK activity is essential to inhibit myoblast differentiation. During this time the cells are probably in late G1 phase considering that they do not incorporate thymidine until 24 h and they have the DNA content of cells in G1 (Figs. 4 and 6). These data is consistent with the earlier results that bFGF inhibits MM14 myoblast differentiation at the G1 phase (55). Therefore, the MAPK substrates at early times in the differentiation time course may be proteins expressed during G0/G1 phase, and our efforts will now focus on finding the targets of bFGF induced MAPK that are responsible for inhibiting C2C12 differentiation.

Acknowledgments—We thank Dr. Andrew Lassar, Harvard Medical School, for the MyoD1 plasmid, Suzan Lazro, Dana Farber Cancer Institute, for assistance with the flow cytometer, Dr. Stephen Farmer, Boston University School of Medicine, for helpful discussions, reagents, and critical reading of the manuscript, and Drs. Ron Morrison and Matthew Nugent, Boston University School of Medicine, for helpful discussions and reagents.
