

# ERK1/2 Is Required for Myoblast Proliferation but Is Dispensable for Muscle Gene Expression and Cell Fusion

NATHAN C. JONES,<sup>1</sup> YURI V. FEDOROV,<sup>1</sup> R. SCOTT ROSENTHAL,<sup>1,2</sup>  
AND BRADLEY B. OLWIN<sup>1\*</sup>

<sup>1</sup>Department of Molecular, Cellular, and Developmental Biology,  
University of Colorado, Boulder, Colorado

<sup>2</sup>Bayer Corporation, Research Triangle Park, North Carolina

Skeletal muscle satellite cells, which are found between the muscle fiber and the basal lamina, remain quiescent and undifferentiated unless stimulated to remodel skeletal muscle or repair injured skeletal muscle tissue. Quiescent satellite cells express c-met and fibroblast growth factor receptors (FGFR) 1 and 4, suggesting these receptors are involved in maintaining the undifferentiated quiescent state or involved in satellite cell activation. Although the signaling pathways involved are poorly understood, the mitogen activated protein kinase (MAPK) cascade has been implicated in the regulation of skeletal muscle growth and differentiation by FGFs. In this study, we investigated if activation of the Raf–MKK1/2–ERK1/2 signaling cascade plays a role in FGF-dependent repression of differentiation and proliferation of MM14 cells, a skeletal muscle satellite cell line. Inactivation of the Raf–MKK1/2–ERK1/2 pathway in myoblasts through the overexpression of dominant negative mutants of Raf-1 blocks ERK1/2 activity and prevents myoblast proliferation. Additionally, inhibition of MKK1/2 by treatment with pharmacological inhibitors also blocks FGF-mediated stimulation of ERK1/2 and blocks the G1 to S phase transition of myoblasts. Unexpectedly, we found that inactivation of the Raf–ERK pathway does not activate a muscle reporter, nor does inactivation of this pathway promote myogenic differentiation. We conclude that FGF-stimulated ERK1/2 signaling is required during the G1 phase of the cell cycle for commitment of myoblasts to DNA synthesis but is not required for mitosis once cells have entered the S-phase. Moreover, ERK1/2 signaling is not required either to repress differentiation, to promote skeletal muscle gene expression, or to promote myoblast fusion. *J. Cell. Physiol.* 186:104–115, 2001. © 2001 Wiley-Liss, Inc.

Skeletal muscle satellite cells are the primary source of cells for skeletal muscle growth following exercise and injury and are maintained in a quiescent undifferentiated state (Mauro, 1961; Moss and Leblond, 1970). The mechanisms and factors involving satellite cell activation as well as control of their proliferation and differentiation are not well understood (Schultz and McCormick, 1994). Two polypeptide growth factors have been implicated in activation and proliferation of satellite cells, these include hepatocyte growth factor (HGF) and the fibroblast growth factors (FGFs). Recent data has demonstrated that both FGF (FGFR-1 and FGFR-4) and HGF (c-met) receptors are present in quiescent satellite cells and thus, are implicated in activation and proliferation of satellite cells (Cornelison and Wold, 1997). The MM14 cell line, derived from an adult mouse hindlimb most closely resembles primary satellite cells reflected by a common pattern of expressed genes (Cornelison, 1998) and the capacity to maintain

*Abbreviations:* caMKK1, constitutively active MKK1; caRAF-1, constitutively active Raf-1; CMV, cytomegalovirus; dnMKK1, dominant negative MKK1; dnRaf-1, dominant negative Raf-1; ERK, extracellular signal regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; MAPK, mitogen activated protein kinase; MHC, myosin heavy-chain; MKK, mitogen activated protein kinase kinase; MKP3, MAPK phosphatase 3; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PLC- $\gamma$ , phospholipase C- $\gamma$ ; SH2, Src homology 2; TBS, Tris-buffered saline; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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\*Correspondence to: Bradley B. Olwin, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309. E-mail: bradley.olwin@colorado.edu

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MM14 cells in a quiescent undifferentiated state similar to satellite cells. MM14 cells exhibit a unique dependence on FGFs, which are required to repress terminal differentiation (Linkhart et al., 1981; Olwin and Hauschka, 1986). Interestingly, activation of the FGFR-1 tyrosine kinase is sufficient to repress terminal differentiation but is not sufficient to promote proliferation (Kudla et al., 1998). Thus, use of chimeric receptors to analyze FGFR-1 signaling in MM14 cells has revealed a previously unknown requirement for FGFs in promoting cell proliferation as well as a requirement for repression of differentiation (Kudla et al., 1998). In the present study, we have focused our efforts on the involvement of the Raf/ERK signaling pathway and its role in regulation of FGF-dependent satellite cell growth and differentiation.

Previous studies examining repression of myogenesis by fibroblast growth factors (FGFs) have demonstrated that activation of a MAPK cascade is likely to play an important role (Robinson and Cobb, 1997). Although FGFs weakly activate ERK1/2 in skeletal muscle myoblasts (Campbell et al., 1995; Kudla et al., 1995), MKK1/2 is reported to be strongly activated (Campbell et al., 1995). Other recently published studies implicate ERK1/2 in regulation of myogenesis; however, the results conflict. Both the 23A2 and L6 myoblast cell lines are reported to commit to terminal differentiation upon treatment with the MKK1/2 inhibitor, PD098059, leading to the conclusion that MKK1/2 and probably ERK1/2 activation is necessary for inhibition of differentiation (Coolican et al., 1997; Weyman and Wolfman, 1998). In contrast, Gredinger et al. (1998) report increased ERK1/2 activity in differentiating C2C12 cells, as well as in C3H10T1/2-MyoD cells (C3H10T1/2 fibroblast cells converted to a skeletal muscle phenotype by expression of MyoD). PD098059 treatment of C3H10T1/2-MyoD cells inhibits muscle-specific gene expression, suggesting that ERK1/2 activation positively regulates myogenic differentiation, specifically, expression and activation of MyoD (Gredinger et al., 1998). Discrepancies within the cited results can be attributed to differences in cell-specific signaling, to the use of serum as opposed to the use of a single growth factor in experimental conditions, or to the correlation of the steady state activities of signaling intermediates with biological responses at time points far beyond the critical signaling events that regulate commitment to myogenic differentiation. Unlike satellite cells *in vivo*, which are maintained in a quiescent, undifferentiated state, the skeletal muscle cell lines used in the studies above differentiate upon cessation of proliferation and thus, inhibition of their proliferation is likely to indirectly induce myogenesis.

The mechanisms involved in coupling FGF signaling to intracellular responses have not been elucidated. Recent data from our laboratory and others suggest that signals emanating from the activated FGFR-1 may activate intracellular signaling cascades through non-conventional mechanisms. Seven autophosphorylated tyrosine residues have been identified on the intracellular domain of activated FGFR-1 (Mohammadi et al., 1996). The only substrate that has been shown to bind a phosphotyrosine on FGFR-1 is phospholipase C- $\gamma$  (PLC- $\gamma$ ) (Mohammadi et al., 1991). However, mutation of five

of those autophosphorylated tyrosines to phenylalanine, including the site recognized by the PLC- $\gamma$  SH2 domain, had no detectable effect on FGFR-1 stimulation of cellular proliferation (Mohammadi et al., 1992, 1996; Peters et al., 1992). Additionally, activation of FGFR-1 in a variety of cell types induces phosphorylation of a 90 kDa product (Klint et al., 1995; Vainikka et al., 1996; Wang et al., 1996; Kouhara et al., 1997) for which two isoforms have been identified (SNT1 and SNT2) that bind weakly to FGFR-1 (Xu et al., 1998). Consistent with the lack of a phosphotyrosine requirement to initiate intracellular signal transduction, the SNTs bind to FGFR-1 via a PTB domain but do not require phosphotyrosines to bind (Xu et al., 1998). These data and recent data from our group demonstrating that a G<sub>i/o</sub> protein may be involved in transducing FGF signals in myoblasts (Fedorov et al., 1998) suggest that FGFR-1 activation of intracellular signaling pathways is unusual and complex.

In this study, we use a combination of pharmacological inhibitors, dominant negative mutants, and constitutively active mutants in the Raf/ERK signaling pathway to demonstrate that activation of this pathway is necessary for FGF-mediated stimulation of cellular proliferation, but is not involved in regulation of myogenic differentiation. In contrast to recent reports (Bennett and Tonks, 1997; Gredinger et al., 1998), this pathway appears dispensable for differentiation and fusion of myoblasts into multinucleated myotubes. We demonstrate that the activity is critical for commitment of myoblasts to DNA synthesis but is not required for mitosis once cells have entered the S-phase.

## MATERIALS AND METHODS

### Cell culture

MM14 cells were grown on gelatin coated plates in growth media consisting of Ham's F-10C media supplemented with 15% horse serum as previously described (Clegg et al., 1987). FGF-2 was added in increasing concentrations (0.3–2.5 nM) every 12 h, depending on cell density. Growth media were replaced every 24 h. Differentiation media consisted of Ham's F-10C media supplemented with 1.5% horse serum.

### DNA constructs

The  $\alpha$ -cardiac actin/luciferase and CMV-*LacZ* expression constructs were provided by Dr. Stephen Konieczny, Purdue University, West Lafayette, IN. The dnMKK1 [MKK1 (K97M)] and caMKK1 [MKK1 ( $\Delta$ N3/S218F/S222D)] were provided by Dr. Natalie Ahn, University of Colorado, Boulder, CO. The dnRaf-1 (Raf C4) and caRaf-1 (Raf BXB) expression constructs (Bruder et al., 1992) were provided by Dr. Ulf Rapp, National Cancer Institute, Bethesda, MD.

### Western analysis

MM14 cells, at a density of  $5 \times 10^5$  cells per 100 mm plate, were washed three times with 5 ml of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) and then grown for 4 h in growth media containing 1.5% horse serum in the absence of added FGF-2. PD098059

(50  $\mu$ M) or U0126 (25  $\mu$ M) was added 2 h prior to stimulation. Cells were stimulated with either 0.1 nM FGF-2 or 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 10 min.

Cell lysates were prepared by washing the plates in 4°C PBS followed by incubation on ice for 20 min in 300  $\mu$ l of RIPA (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM *p*-nitrophenyl phosphate, 1 mM sodium *o*-vanadate, 10 mM tetrasodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin). Lysates were collected and particulates were removed by centrifugation at 14,000  $\times g$  for 15 min at 4°C. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce Chemical Company, Rockford, IL). Extracted proteins (15  $\mu$ g) resolved by SDS-PAGE were electrophoretically transferred to Immobilon-P (Millipore Corp., Bedford, MA) in 25 mM ethanolamine, 25 mM glycine, 20% methanol, pH 9.5. Nonspecific binding sites were blocked with 5% nonfat dried milk, 0.05% Tween-20 in PBS. Active MAPK was detected using a polyclonal antibody specific to dual-phosphorylated ERK1 (Promega, Madison, WI). Phosphorylated MEK1/2 was detected using Phospho-Plus MEK1/2 (Ser217/221) (New England Biolabs, Inc., Beverly, MA). Total ERK1/2 and MKK1/2 were detected with either a polyclonal antibody that recognizes ERK1 (K-23) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or an anti-MKK1 antibody (Transduction Laboratories, Lexington, KY), respectively. Bound polyclonal antibodies were detected with either anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase (Promega, Madison, WI). Bound antibody complexes were visualized by Renaissance Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA). Blots were stripped by incubating in stripping buffer (100 mM  $\beta$ -mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.7) at 50°C for 30 min, followed by six washes in PBST (0.05% Tween-20 in PBS).

#### Clonal cell proliferation assay

MM14 cells were synchronized by mitotic shake-off and plated in 6-well plates at clonal density (200 cells per well) and grown in the presence of 0.3 nM FGF. The cells were left untreated or treated with either 0.2% DMSO or 25  $\mu$ M U0126 at the indicated time points post plating. Eighteen hours after plating, cells were washed with PBS, fixed for 10 min in 70% EtOH, and the number of cells per clone was determined. Data shown are mean values from one representative experiment performed in triplicate. Error bars represent confidence limits for  $P = 0.05$ . No less than 30 colonies were counted per point per experiment. The experiment was repeated two times with similar results.

#### DNA synthesis assay

DNA synthesis was assayed by [<sup>3</sup>H] thymidine incorporation. Briefly, MM14 cells were synchronized by mitotic shake-off and plated in 24-well plates at 2,000 cells per well. The cells were grown with the addition of 2.5  $\mu$ l of DMSO or increasing concentrations of PD098059 or U0126 for 8 h and then given 2  $\mu$ Ci of [<sup>3</sup>H] thymidine (DuPont-NEN, Boston, MA) and incubated

for an additional 6 h. The amount of [<sup>3</sup>H] thymidine incorporated into DNA was determined by liquid scintillation counting as described previously (Olwin and Hauschka, 1986).

#### Transient transfections

**Muscle-specific reporter gene.** MM14 cells were plated out on 6-well tissue culture plates at 12,500 cells per well and allowed to attach for 4–5 h. A calcium phosphate–DNA precipitate was made containing 0.3  $\mu$ g of a muscle-specific reporter construct containing four E-boxes from the muscle creatine kinase enhancer (4RTK) and a basal thymidine kinase promoter driving the expression of luciferase, 0.15  $\mu$ g of a reporter construct containing the cytomegalovirus promoter driving the expression of  $\beta$ -galactosidase (CMV-*LacZ*), and 6  $\mu$ g of an expression construct encoding either dnRaf or dnMKK1. Equivalent DNA concentrations were maintained by the addition of pBluescript (pBSSK+) (Stratagene, La Jolla, CA). DNA was resuspended in *N*-[2-hydroxyethyl]piperazine-*N'*[2-ethanesulfonic acid] (HEPES)-buffered saline (23 mM HEPES, pH 7.05, 140 mM NaCl, 5 mM KCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM dextrose) and 2 M CaCl<sub>2</sub> was added dropwise while vortexing, to a final concentration of 110 mM. DNA was allowed to precipitate for 20 min at room temperature. Growth media were removed from the tissue culture plates and 150  $\mu$ l of the precipitated DNA was added to the plates and incubated at room temperature for 20 min. Growth media were then added back to the plates. Following a 4 h incubation, the growth media were again removed and the cells were incubated in 0.5 ml of 15% glycerol in HEPES-buffered saline for 2.5 min. The plates were then washed once with HEPES-buffered saline and growth medium was re-added containing FGF-2 or anti-FGF-2 antibodies ( $\alpha$ -FGF-2) as indicated. Additional FGF-2 was added every 12 h as described above. For MKK1/2 inhibitor treatments, either PD098059 or U0126 was added immediately following the glycerol treatment to a final concentration of 50 and 25  $\mu$ M, respectively. For all transfections, cells were maintained in growth media for 36 h after glycerol incubation, and the cells were then washed with PBS and harvested by scraping into 100  $\mu$ l of lysis buffer (2 mM Tris, pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1  $\mu$ g/ml leupeptin, 2 mM DL-dithiothreitol, 2 mM phenylmethylsulfonyl fluoride). Lysates were incubated on ice for 20 min, then centrifuged (14,000  $\times g$ ) at 4°C to remove particulates, and assayed for luciferase and  $\beta$ -galactosidase activity using the Dual-Light Assay kit (Tropix, New Bedford, MA). Luciferase activity values (RLU) were normalized to  $\beta$ -galactosidase activity values (RLU) to correct for differences in transfection efficiency.

**MAPK activation reporter gene.** MAPK activity was determined using the PathDetect Elk-1 reporting system (Stratagene, La Jolla, CA) (Xu et al., 1997). MM14 cells were plated on 6-well plates at a density of 40,000 cells/well or on 24-well plates at a density of 8,000 cells/well and cotransfected with 2.5  $\mu$ g (500 ng for a 24-well plate) of pFR-Luc reporter vector, 200 ng (40 ng for a 24-well plate) of pFA-Elk-1, 1  $\mu$ g (200 ng for 24-well plate) of CMV-*LacZ*, and 1.5  $\mu$ g (500 ng for a 24-well plate) of either an expression vector encoding dnRaf-1, dnMKK1, caRaf-1, caMKK1, or pBSSK+ per well. Twelve to sixteen hours after the transfection cells were washed twice with PBS

and incubated in 2.5% horse serum without FGF for 6 h. The cells were then either kept in the same media or stimulated with 0.1 nM FGF-2. Cells were harvested 6 h following FGF stimulation and luciferase and  $\beta$ -galactosidase activities were determined and normalized as described above.

**Myotube formation assay.** MM14 cells were plated and transfected as described for the muscle-specific reporter assays. DNA precipitates were made using CMV-*LacZ* (0.5  $\mu$ g) and either pBSSK+ (10 or 20  $\mu$ g), dnRaf-1 (20  $\mu$ g), or caMKK1 (10  $\mu$ g) maintaining constant DNA concentrations using pBSSK+. Following glycerol incubation, cells were cultured in growth media with or without added FGF-2 as indicated. For caMKK1 transfections, cells were grown in either growth media with added FGF-2 or in differentiation media in the absence of FGF-2 as indicated. Additional FGF-2 was added every 12 h as described above. Cells were washed with PBS 72 h after glycerol incubation and fixed for 10 min in 2% formaldehyde, 0.05% glutaraldehyde in PBS. Plates were then rinsed three times with PBS and incubated in X-gal stain (4 nM ferricyanide, 4 mM ferrocyanide, and 1 mg/ml X-Gal in PBS). Stained cells were washed three times with PBS. A minimum of 100 transfected cells per plate was scored for the presence of two or more nuclei. Nontransfected cells (at least 1,000 per plate) were also scored.

**Clonal cell proliferation assay.** MM14 cells were plated on 100-mm tissue culture plates at a density of 3,000 cells per plate and transfected as described above. DNA precipitates were made using 1  $\mu$ g of CMV-*LacZ* and 20  $\mu$ g of either pBSSK+, dnRaf-1, or dnMKK1. Following glycerol incubation, cells were cultured in growth media with or without added FGF-2 as indicated. Cells were fixed and stained in X-gal stain 36 h post glycerol shock as described above. MM14 cells to be transfected with caMKK1 or caRaf-1 were plated on 6-well tissue culture plates at a density of  $1 \times 10^4$  per well and transfected as described above. DNA precipitates were made with 5  $\mu$ g of CMV-*LacZ* and 2  $\mu$ g of control DNA (pcDNA3) or expression vectors encoding caRaf-1 or caMKK1. The cells were washed once with PBS, 1 ml of trypsin added, and the cells were transferred onto 100-mm tissue culture plates at a density of 100 cells/plate 1 h post glycerol shock. Trypsinization was utilized in order to remove all FGF from the cell surface to eliminate mitogenic effects of residual growth factors, thereby ensuring that all biological activities observed are solely due to expression of the constitutively active signaling intermediates. After passage onto 100 mm plates, transfectants were grown for 36 h, fixed, and stained in X-gal stain as described above.

#### Analysis of myosin heavy-chain expression

MM14 cells were plated onto 6-well plates at a cell density of 8,000 cells per well in growth medium. After 6 h, either 2.5  $\mu$ l of DMSO, PD098059 (50  $\mu$ M), or U0126 (25  $\mu$ M) was added to the wells and cells were grown in the presence or absence of FGF-2 as described above. 24 h post-treatment, cells were washed with Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.5, 100 mM NaCl), fixed in AFA (60% EtOH, 3% formaldehyde, 4% glacial acetic acid) for 2 min, and rinsed three times in TBS. Myosin heavy-chain (MHC) was visualized as described previously (Kudla et al., 1995). MHC positive cells were scored as a percentage of entire cell

population. A minimum of 1,000 total cells were scored per plate.

## RESULTS

In accord with the behavior of skeletal muscle satellite cells *in vivo*, we have shown previously that distinct signaling pathways are required to repress differentiation and promote proliferation of MM14 cells (Clegg et al., 1987; Kudla et al., 1998). However, the signal transducers that function subsequent to FGFR activation are largely unknown. Since activation of satellite cells is known to activate ERK1/2 (Yablonka-Reuveni et al., 1999), we initiated a series of studies to determine the role(s) of Raf/ERK pathway in regulation of growth and differentiation of MM14 cells.

#### Muscle cell proliferation is blocked by addition of PD098059 or U0126

In agreement with two previously published reports (Campbell et al., 1995; Kudla et al., 1995), FGF stimulation of MM14 cells activates both MKK1/2 and ERK1/2. The relatively weak FGF-dependent activation of ERK1/2 compared to the positive control (TPA treatment) has been attributed to both regulatory feedback control of ERK1/2 activity by MKP3, an ERK1/2-specific phosphatase (Mourey et al., 1996), or to the low number of FGFR1 on the cell surface (Kudla et al., 1998). Increasing the number of FGFR1 by ectopic overexpression also increases FGF-dependent ERK1/2 activation, but does not alter the biological responses regulated by FGFs in MM14 cells (Kudla et al., 1998). Treatment of MM14 cells with inhibitors of MKK1/2 activity, either PD098059 (Dudley et al., 1995) or U0126 (Favata et al., 1998), blocks the stimulation of both MKK1/2 and ERK1/2 activity (Fig. 1A, B).

The role of FGF-dependent activation of the Raf-MKK1/2-ERK1/2 pathway in regulating specific biological functions in MM14 skeletal muscle cells has not yet been addressed. We thus asked if treatment with PD098059 or U0126 affected either proliferation or differentiation of skeletal muscle myoblasts. MM14 cells synchronized at the M/G1 boundary by mitotic shake-off exhibit an immediate and absolute requirement for FGF to prevent terminal differentiation. Withdrawal of FGF from synchronized cells for 3 h prevents S-phase entry, stimulates expression of muscle-specific genes within 6 h, and cell fusion by 12 h (Clegg et al., 1987). Addition of increasing concentrations of either PD098059 or U0126 to synchronized MM14 cells blocks FGF-dependent S-phase entry (Fig. 2A). Western analysis of lysates prepared from MM14 cells treated with increasing concentrations of either drug indicate a strong correlation between ERK1/2 activity and exit of the cells from the cell cycle (Fig. 2B). These data clearly demonstrate that activation of the Raf/ERK pathway is essential for FGF-2-dependent S-phase entry.

#### MKK1/2 activity is required for the G1 to S-phase transition but not for mitosis

Although MM14 cells exhibit an absolute requirement for FGFs to promote a G1 to S-phase transition (Clegg et al., 1987) and prevent terminal differentiation, FGF activity appears dispensable throughout the remainder of the cell cycle. We investigated if FGF-2-dependent

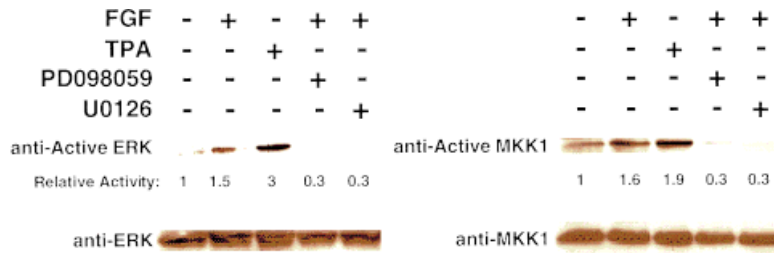


Fig. 1. PD098059 and U0126 inhibit FGF-2-dependent activation of MKK1/2 and ERK1/2 in proliferating myoblasts. MM14 cells were incubated in 2% horse serum for 4 h and then stimulated with FGF-2 or TPA for 10 min as indicated. PD098059 or U0126 were added at the time of serum starvation. Cell extracts were then subjected to western analysis using anti-active ERK polyclonal antibody (A, upper panel) or

anti-active MKK1 polyclonal antibodies (B, upper panel). Blots were stripped and probed with anti-ERK1 (K-23) polyclonal antibody (A, lower panel) or anti-MKK1 polyclonal antibody (B, lower panel). Relative activities were determined from blots using Molecular Analyst Software (Bio-Rad Laboratories).

activation of MKK1/2 was required beyond the G1/S phase transition by plating synchronized MM14 cells at clonal density and treating at different times with U0126 (25  $\mu$ M). The cells were allowed to proliferate in full growth media containing FGF-2 for 18 h, then fixed and the number of cells per clone was determined. Untreated cells would be expected to undergo an average of one cell doubling in that time. Only cells treated within

5 h of plating were significantly affected by addition of the MKK1/2 inhibitors (Fig. 3). Addition at 7 h or later time points had no detectable effect on DNA synthesis or cell division. Cells grown in the presence of FGF from  $t = 0$  averaged  $3 \pm 0.4$  cells per clone and cells grown in the absence of FGF from  $t = 0$  averaged  $1.5 \pm 0.3$  cells per clone. The majority of cells isolated from mitotic shake-off did not undergo cytokinesis and thus yielded an

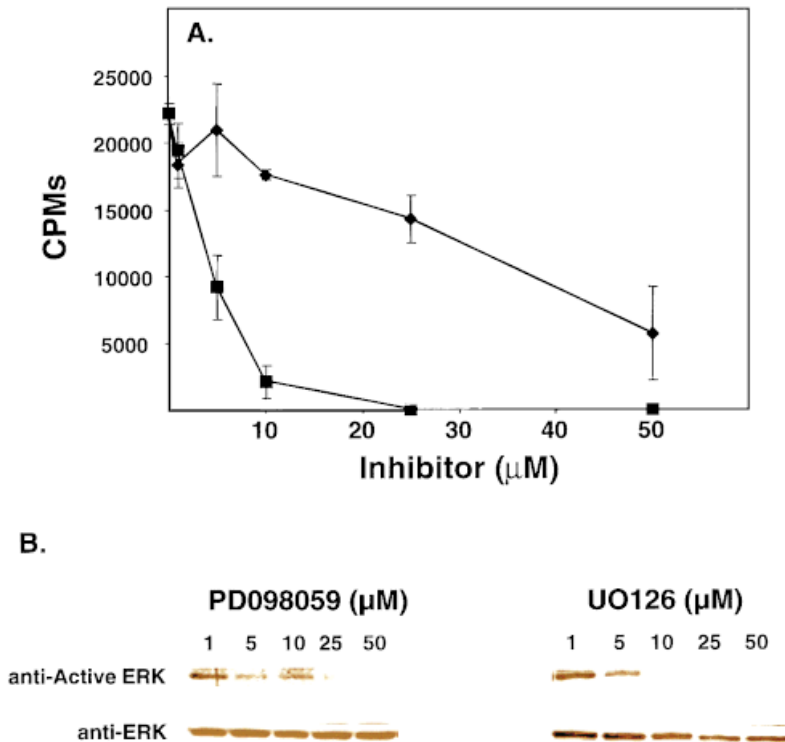


Fig. 2. PD098059 and U0126 block FGF-dependent proliferation of MM14 cells. (A) MM14 cells were synchronized by mitotic shake-off and were plated at 2,000 cells per well and incubated for 12 h with either PD098059 ( $\blacklozenge$ ) or U0126 ( $\blacksquare$ ), then given 2  $\mu$ Ci of [ $^3$ H]thymidine, and incubated for an additional 6 h. Cells were harvested and [ $^3$ H]thymidine incorporation was quantified as described in Materials and Methods. Data represent one of three similar experiments. Error bars represent SD of three independent samples from one experiment. NOTE: The low [ $^3$ H]thymidine incorporation at high MEK inhibitor concentrations is not the result of cell death; the cell number at high

MKK1/2 inhibitor concentrations equals the cell number of MM14 cells in differentiation conditions (data not shown). Data represent one of three similar experiments. (B) MM14 cells incubated in 1.5% serum without FGF-2 for 2 h. DMSO or increasing concentrations of either PD098059 or U0126 was added and cells were incubated for an additional 2 h. Cells were then stimulated with 0.1 nM FGF-2 for 10 min and harvested as described in Materials and Methods. Cell extracts were then subjected to western analysis using anti-active ERK (upper panel) or anti-ERK1 (K-23) polyclonal antibody (lower panel).

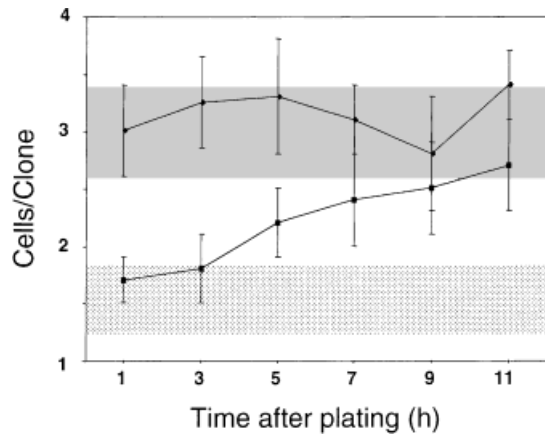


Fig. 3. Activation of MKK/ERK is required only for the G1 to S-phase transition. The MKK1 inhibitor (U0126) negatively affects growth of MM14 cells only when added early in the G1 phase of the cell cycle. MM14 cells were synchronized by mitotic shake-off and plated in 6-well plates at a density of 200 cells per well. The cells were left untreated or treated with either 0.2% DMSO (◆) or 25  $\mu$ M U0126 (■) at the indicated time points. As a control, cells were grown in either the presence (grey box) or absence (stippled box) of FGF-2 for the entire time course of the assay (18 h). Cells were fixed and the number of cells per clone was determined 18 h after plating. Data shown are mean values from one representative experiment performed in triplicate. Error bars represent confidence limits for  $P = 0.05$ . No less than 30 colonies were counted per point per experiment. The experiment was repeated two times with similar results.

average of two cells per clone following plating. Thus, activation of the Raf/ERK pathway by FGF appeared critical for commitment of skeletal muscle myoblasts to DNA synthesis but did not appear to be required for maintenance of S-phase or for mitosis. However, stimulation of ERK1/2 activity by TPA treatment is incapable of inducing DNA synthesis in MM14 cells (data not shown). Together these results suggest that activation of MKK1/2-ERK1/2 is necessary, but not sufficient, to promote MM14 cell proliferation.

#### Inhibition of Raf/ERK pathway blocks proliferation of skeletal muscle cells

To confirm that ERK1/2 activation is indeed critical for cellular proliferation, ectopic expression of a dominant negative MKK1 mutant (dnMKK1) should prevent skeletal muscle myoblast cell proliferation. Similarly, if activation of MKK1/2 is via Raf, then ectopic expression of a dominant negative Raf-1 mutant (dnRaf-1) would also be expected to block proliferation. MM14 cells plated at clonal density were transiently cotransfected with a CMV-*lacZ* construct (to identify transfected cells) and expression vectors encoding either dnMKK1 or dnRaf-1. Cell clones were scored for proliferation 36 h following transfection. Typically, 10–20% of the total number of clones stained positive with X-gal, consistent with our previously reported transfection efficiency (Hannon et al., 1996). Ectopic expression of either dnRaf-1 or dnMKK1 blocked proliferation of MM14 cells (Fig. 4A), and thus, we conclude that inactivation of the Raf/ERK pathway blocks myoblast proliferation. The slightly greater cell number in the presence of the dominant negative mutants as compared to the control cell population grown in the absence of FGF (Fig. 4A)

reflects the time needed for the transfected cDNAs to be expressed (approximately 12 h) and would be expected to allow approximately one additional cell cycle prior to inhibition of proliferation.

To demonstrate that dnRaf-1 and dnMKK1 mutants block FGF-dependent activation of ERK1/2, we used the PathDetect Elk-1 reporting system (Stratagene, La Jolla, CA). Control experiments with cells transiently transfected either with pFA-Elk-1 or pFR-Luc alone, or

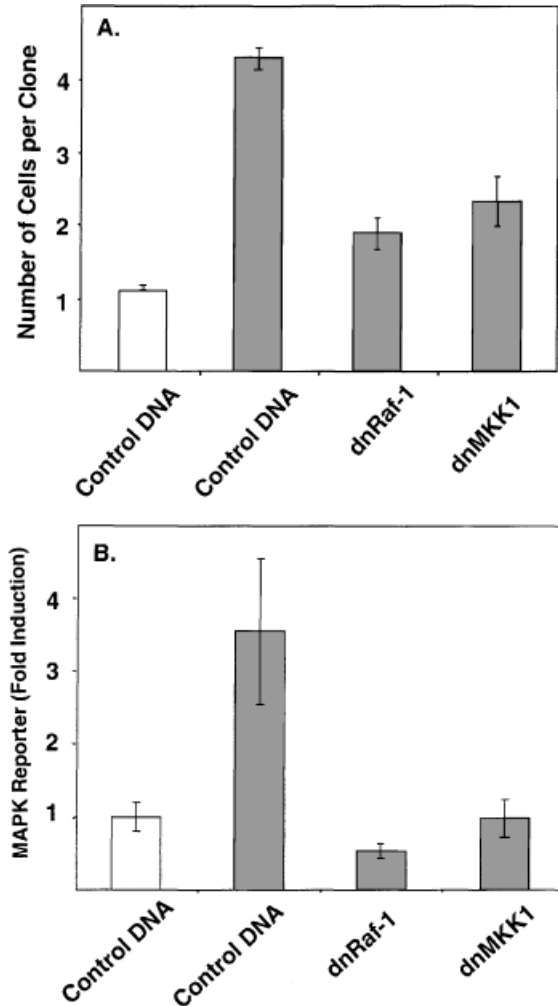


Fig. 4. Expression of dnRaf-1 or dnMKK1 blocks FGF-dependent proliferation of MM14 myoblasts. (A) MM14 cells were plated at clonal density (3000 cells per 100 mm plate) and transfected with control DNA (pBSSK+), dnRaf-1, dnMKK1, and CMV-*lacZ* as described in Materials and Methods. Transfected cells were grown for 36 h in the presence (■) or absence (□) of added FGF as indicated, fixed, and stained for  $\beta$ -galactosidase expression as described in Materials and Methods. The average number of cells in per transfected clone was determined (at least 150 clones per data point). Data represent one of three similar experiments. Error bars represent SD of total population of cells quantified. (B) MM14 cells were transiently co-transfected with pFR-Luc, pFA-Elk-1, and CMV-*LacZ* expression vectors and either dnRaf-1 or dnMKK1 as indicated. Transfected cells were grown for 36 h in the presence (■) or absence (□) of added FGF, harvested, and assayed as described in Materials and Methods. Data represent one of three similar experiments. Error bars represent SD of three independent transfections from one experiment.

with a combination of pFR-Luc and a mutant transactivating vector lacking the Elk-1 domain displayed no luciferase activity (data not shown). Cotransfection of MM14 cells with either dnRaf-1 or dnMKK1 and the two PathDetect vectors (pFA Elk-1 and pFR-Luc) show that expression of either dominant negative mutant is capable of blocking MAPK reporter activity (Fig. 4B).

#### Inhibitors of Raf/ERK pathway do not activate a muscle-specific reporter gene construct

For most myoblast cell lines, inhibition of proliferation will induce terminal differentiation. In contrast, MM14 cells can be maintained in a quiescent, undifferentiated state (Clegg et al., 1987; Fedorov et al., 1998) similar to satellite cells in vivo. Removal of FGF from MM14 cells induces irreversible cell-cycle withdrawal followed by terminal myogenic differentiation of >95% of the entire population. To determine the role of the Raf–MKK1/2–ERK1/2 signaling cascade in regulating the expression of muscle-specific genes, the activity of a skeletal muscle reporter construct containing four E-boxes from the creatine kinase enhancer element was determined upon inhibition of ERK1/2 with MKK1/2 inhibitors or upon ectopic expression of dnRaf-1. Elimination of FGF signaling by ectopic expression of a dominant negative FGFR-1 mutant in the presence of FGF-2 yields a 5- to 6-fold activation of the 4RTKLuc reporter (Fig. 5). In contrast to other reports (Coolican et al., 1997; Weyman and Wolfman, 1998), we observed that treatment with PD098059 or U0126 in the presence of FGF-2 did not activate the muscle-specific reporter. These data unexpectedly suggest that inhibition of ERK1/2 activity does not result in activation of MyoD family members. Since activation of skeletal muscle reporter constructs may not accurately predict the

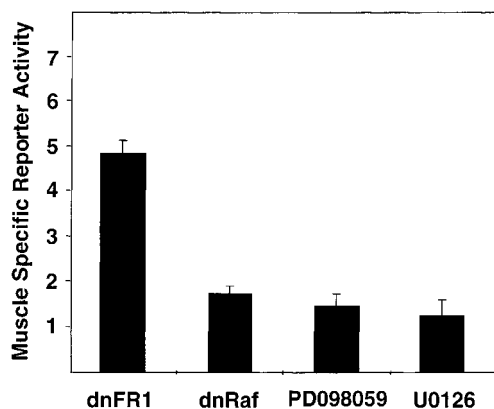


Fig. 5. Muscle gene expression is not induced upon treatment with either PD098059 or U0126. MM14 cells were transiently cotransfected with expression vectors encoding CMV-*lacZ* 4RTKLuc, a muscle-specific reporter gene, and treated with DMSO, or PD098059, or U0126, as indicated. Cells were then harvested and assayed 36 h later as described in Materials and Methods. The fold-induction of luciferase activity of cells maintained in FGF-2 is shown for each treatment. Induction of  $\leq 2$ -fold for muscle-reporters is not considered a reliable indicator of muscle-specific gene induction. Data represent one of three similar experiments. Error bars represent SD of the mean for three independent transfections from one experiment.

extent of myogenic differentiation, we also determined directly the capacity of dnRaf-1 and the MKK1/2 inhibitors to induce myogenic differentiation at the cellular level.

#### MKK1/2 inhibitors PD098059 and U0126 do not induce myogenic differentiation

Since treatment of MM14 cells with MKK1/2 inhibitors did not induce muscle gene expression, the Raf/ERK pathway may reflect FGF-dependent signaling that impinges only on cellular proliferation. To further test this hypothesis, cells treated with either MKK1/2 inhibitor were analyzed for expression of myosin heavy chain (MHC), a skeletal muscle-specific protein exclusively expressed in terminally differentiated muscle. MM14 cells maintained in FGF-2 and serum proliferate and exhibit a round phase-bright morphology and do not stain positively for MHC (Fig. 6A), while removal of FGF-2 induces differentiation resulting in MHC expression and cell fusion (Fig. 6B). Treatment of cells with PD098059 (50  $\mu$ M) or U0126 (25  $\mu$ M) in the presence of FGF-2 does not significantly induce fusion or myosin expression (Fig. 6C, D, respectively). Quantitative analysis of these data demonstrates that concentrations of the MKK1/2 inhibitors, which block proliferation, do not significantly induce MHC expression (Fig. 6E). In agreement with the muscle-specific reporter data, these data show that inhibition of ERK1/2 activity via MKK1/2 inhibitors does not induce skeletal muscle differentiation. Although the extent of differentiation in the presence of U0126 is greater than that observed for PD098059, a low level of MHC expression (8–20%) is also seen when MM14 cells are maintained in a quiescent state (i.e., in the absence of serum with FGF present) for 24–48 h (Clegg et al., 1987; Fedorov et al.,

Fig. 6. Treatment on MM14 cells with PD098059 or U0126 does not induce myosin heavy chain expression. MM14 cells were grown for 60 h in the presence (A) and absence (B) of nM FGF-2 or in the presence of 1 nM FGF-2 and either 50  $\mu$ M PD098059 (C) or 25  $\mu$ M U0126 (D). The cells were fixed and stained for myosin heavy chain as described in Materials and Methods. Identical results were obtained when PD098059 was replenished 12 h after the initial treatment (data not shown). Quantitative analysis of myosin staining is shown for each treatment (E). Myosin heavy-chain positive cells were scored as a percentage of entire population (3,000 total cells were scored for each data point). Data presented represent one of three independent experiments. Error bars represent SD of three independent samples from one experiment.

Fig. 7. Inhibition of Raf in MM14 cells does not induce myoblast fusion. MM14 cells were transiently cotransfected with expression vectors encoding CMV-*lacZ* and either control DNA (pBSSK+) in the presence (A) or absence (B) of 1 nM FGF-2, or dnRaf-1 in the presence of FGF-2 (C) and scored for the number of myonuclei present. Solid head arrows indicate transfected cells, while the open-head arrows indicate nontransfected cells. (D) Quantitative analysis of the data are indicated for both transfected (◊) (at least 300 cells per data point) and non-transfected cells (■) cells (1,000 cells per data point). Cells were grown for 36 h in the presence or absence of added FGF-2 as indicated, harvested, and assayed as described in Materials and Methods. All cells were fixed and stained for  $\beta$ -galactosidase activity 72 h post-transfection to identify transfected cells. The plates were then scored for the number of nuclei per cell. Data presented represent one of three similar experiments. Error bars represent SD of the mean of all cells counted from three independent transfections in one experiment.

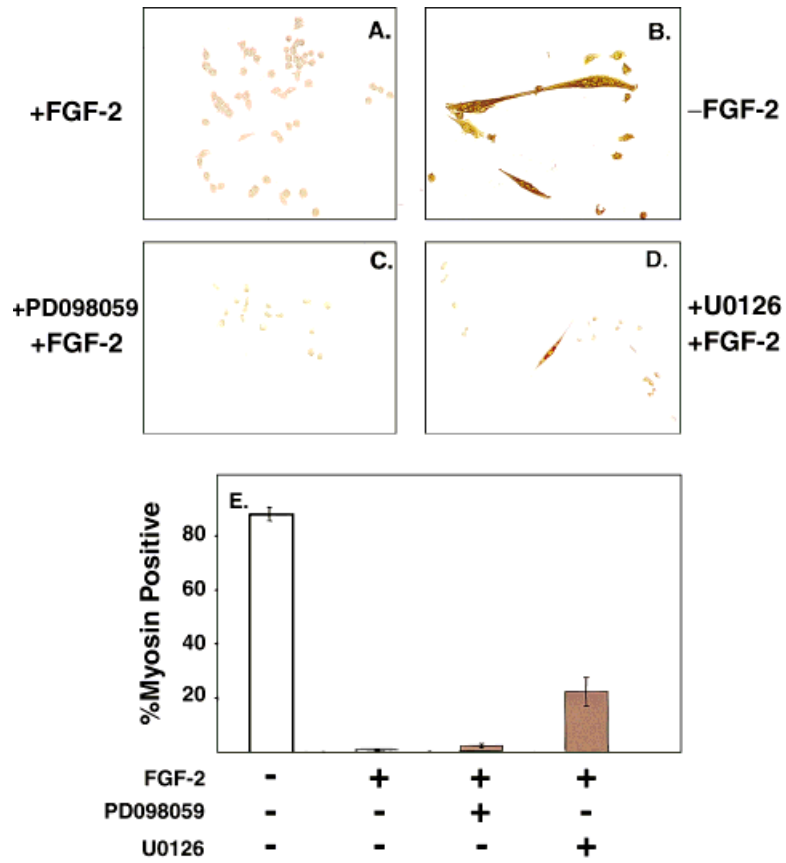


Fig. 6.

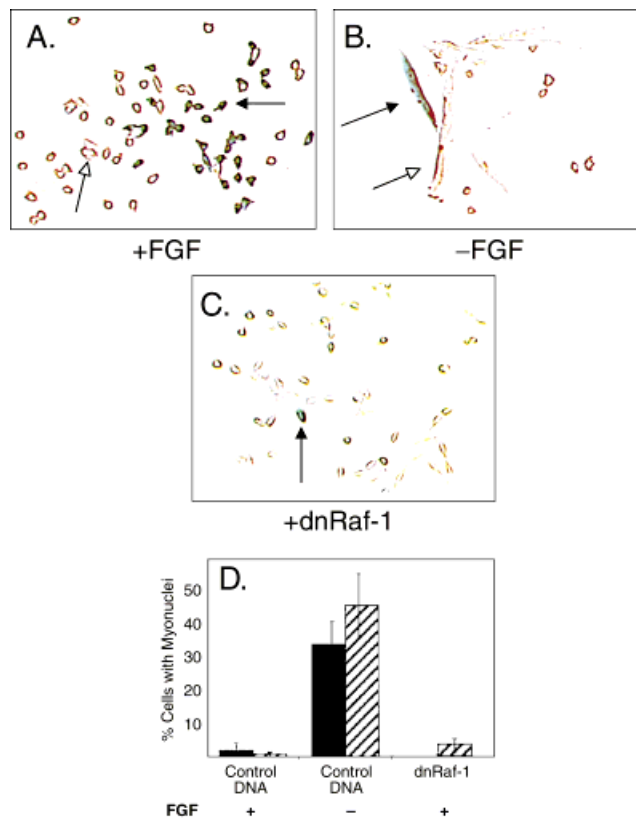


Fig. 7.

1998). Thus, the level of differentiation observed in the presence of either MKK1/2 inhibitor is consistent with the spontaneous differentiation rate previously established for noncycling MM14 cells (Clegg et al., 1987; Fedorov et al., 1998).

#### Effect of ectopic expression of dnRaf-1 on myoblast fusion

Since treatment of MM14 cells with the MKK1/2 inhibitors prevents S-phase entry but does not promote differentiation, we predicted that inhibition of the Raf/ERK pathway at the level of Raf would yield similar results. Since pharmacological agents that inhibit Raf function are not available, MM14 cells were transiently co-transfected with a dnRaf1 expression vector and cell fusion was determined. Transfected MM14 cells were readily distinguished by staining for  $\beta$ -galactosidase (Fig. 7A). Removal of FGF-2 from the medium for 72 h induces cell fusion for both untransfected and transfected cell populations (Fig. 7B). Ectopic expression of dnRaf-1 does not induce fusion of myoblasts (Fig. 7C). Single cell transfected clones are prevalent due to the inhibition of cell proliferation by ectopically expressed dnRaf-1. Quantitative analysis of these data demonstrates that ectopic dnRaf-1 does not induce cell fusion (Fig. 7D). Thus, inhibition of the Raf/ERK pathway at the level of Raf or MKK1/2 does not induce myogenesis, suggesting that independent FGFR-1-mediated signals are required for proliferation and repression of terminal differentiation.

#### Activation of Raf-MKK1/2-ERK1/2 pathway is not sufficient to promote cell proliferation or repress myogenic differentiation

Activation of the Raf/ERK signaling cascade is sufficient to promote DNA synthesis and cell division in many cell types (Greulich and Erikson, 1998; Lewis et al., 1998). Our data demonstrate that activation of the Raf/ERK pathway by FGFs is necessary for proliferation of skeletal muscle cells. To address whether or not activation of this pathway is sufficient to stimulate cell proliferation, MM14 cells were transiently cotransfected with constitutively active Raf-1 and MKK1 mutants and a CMV-*LacZ* expression vector. The extent of cell proliferation was determined by plating cells at clonal density and counting the number of cells per clone. Ectopic expression of neither caRaf-1 nor caMKK1 promoted cell proliferation in the absence of FGF-2 (Fig. 8A). Although unlikely, it is possible that the constitutively active mutants are not functional in MM14 cells and thus cannot promote proliferation. Therefore, we examined the ability of both constitutively active mutants to activate the MAPK reporter system in the absence of FGF-2. Both caRaf-1 and caMKK1 robustly stimulated MAPK reporter activity (Fig. 8B). As a further control to show that activation of the MKK1/2-ERK1/2 pathway does not replace FGF-dependent signaling, we determined the ability of caMKK1 to repress differentiation in MM14 cells grown under differentiation conditions. Ectopic expression of caMKK1 is not sufficient to repress terminal myogenic differentiation (Fig. 9). Thus, while MKK1/2 activity is necessary for proliferation, caMKK1 is sufficient for neither promoting proliferation nor repressing myogenic differen-

tiation, indicating that additional FGF-dependent signals are required for both cellular processes.

## DISCUSSION

To understand the molecular mechanisms involved in regulating proliferation and differentiation of skeletal muscle satellite cells, we have focused our efforts on elucidating the FGF-dependent intracellular signaling pathways that repress myogenesis and promote proliferation in MM14 cells, a model skeletal muscle satellite cell line. MM14 cells express only FGFR-1 (Kudla et al.,

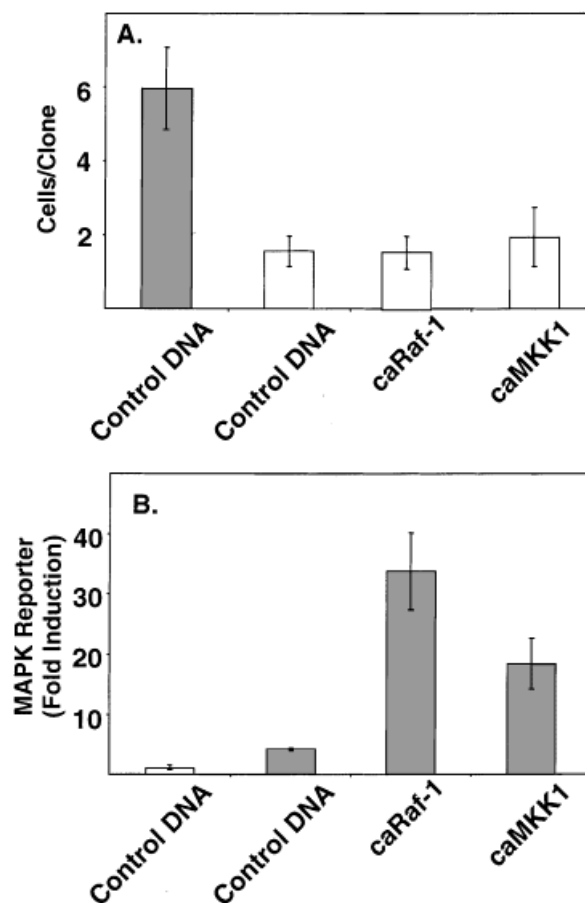


Fig. 8. Constitutively active MKK1 is not sufficient to stimulate myoblast proliferation. (A) Ectopic expression of constitutively active mutants of MKK1 or Raf-1 cannot support MM14 cell growth in the absence of exogenous FGF. MM14 cells plated in 6-well plates were co-transfected with CMV-*LacZ* together with control DNA (pcDNA3) or vectors encoding caRaf-1 or caMKK1. Cells were passaged and replated at low density (1,000 cells/100 mm plate) 1 h after transfection and grown in the presence (■) or absence (□) of added FGF-2. Thirty-six hours after replating, the cells were fixed and stained for  $\beta$ -galactosidase expression. The average number of cells in per transfected clone was determined. Mean values represent three independent experiments performed in triplicate. Error bars represent credibility limits for  $P \leq 95\%$ . No less than 30 colonies were counted per point per experiment. (B) MM14 cells plated in 6-well plates were cotransfected with the CMV-*LacZ* reporter, the Elk-1 reporter plasmids, and either control DNA or caRaf-1 or caMKK1 expression vectors as described in (A). Transfected cells were grown for 36 h in the presence (■) or absence (□) of added FGF-2, harvested, and assayed as described in Materials and Methods. Data presented represent one of three similar experiments. Error bars represent SD of the mean for three independent transfections from one experiment.

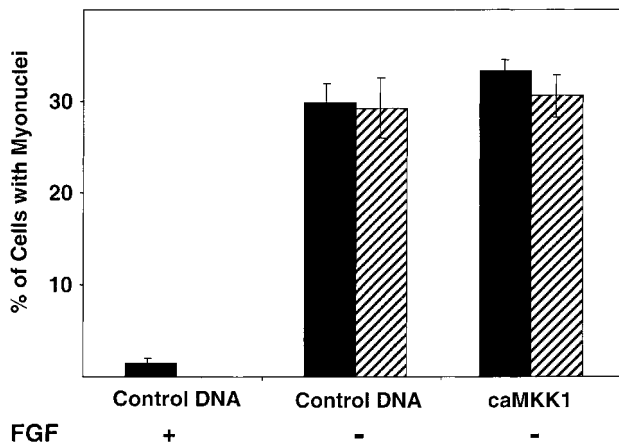


Fig. 9. Constitutively active MKK-1 is not sufficient to repress terminal myogenic differentiation of MM14 cells. MM14 cells were transiently cotransfected with expression vectors encoding CMV-*lacZ* and either control DNA (pBSSK+) or caMKK1 in growth media with 1 nM FGF-2 or in differentiation media without FGF-2, as indicated. All cells were fixed and stained for  $\beta$ -galactosidase activity 72 h post transfection to identify transfected cells as described in Materials and Methods. The plates were then scored for the number of nuclei per cell. Quantitative analysis of the data are presented for both transfected (▨) (at least 150 cells per data point) and nontransfected (■) cells (600 cells per data point). Data presented represent one of three similar experiments. Error bars represent SD of the mean of all cells counted from three independent transfections in one experiment.

1998) and the intracellular signals transduced by FGFR-1 cannot be replaced by activation of other growth factor receptors either already present (Lim and Hauschka, 1984) or ectopically expressed (Kudla et al., 1995). Work from several laboratories suggests that the Ras/Raf/ERK pathway is activated in skeletal muscle cells and may play a role in the regulation of myogenesis (Campbell et al., 1995; Mourey et al., 1996; Bennett and Tonks, 1997; Kudla et al., 1998). Using both mutants of Raf and MKK1, as well as specific pharmacological inhibitors, we have examined the role of the Raf/ERK signaling in regulating FGF-mediated repression of myogenic differentiation and stimulation of myoblast proliferation. Our group and others have demonstrated activation of ERK1/2 in skeletal muscle myoblasts by growth factors (Campbell et al., 1995; Kudla et al., 1995; Milasincic et al., 1996; Mourey et al., 1996). Interestingly, FGFR-1 stimulation does not activate ERK1/2 as strongly as MKK1/2, an apparent paradox that has been attributed to the action of MKP3 (also known as rVH6), a dual specificity phosphatase (Mourey et al., 1996). Ectopic overexpression of FGFR-1 leads to a stronger activation of ERK1/2 (Kudla et al., 1998), suggesting that ERK1/2 activity may be tightly regulated in the cell. Thus, we sought to determine if inhibition of ERK1/2 and the Raf/ERK pathway would affect skeletal muscle satellite cell growth and differentiation.

Conflicting reports from several groups using a skeletal muscle cell line derived from 5-AzaC treatment of 10T1/2 cells (Weyman and Wolfman, 1998), L6 myoblasts (Coolican et al., 1997), and C2C12 cells (Bennett and Tonks, 1997) suggest that ERK1/2 activity is required to inhibit differentiation, or that ERK1/2 acts as a positive mediator of differentiation in C2C12 or C3H10T1/2-MyoD cells (Gredinger et al., 1998).

Although these reports conflict, several factors can account for the discrepancies between these previously reported data and ours. Unlike MM14 cells and satellite cells found in vivo, these cell lines will differentiate when proliferation is inhibited. Thus, they do not represent the state of the majority of quiescent satellite cells in vivo. Inhibition of proliferation in most myoblast cell lines would be expected to indirectly induce myogenesis, leading to the erroneous conclusion that ERK1/2 is directly involved in myogenic differentiation. A second important issue is the requirement for removal of serum to initiate myogenesis in most skeletal muscle cell lines. Removal or reduction of serum is expected to alter concentrations of a large number of growth factors and affect several intracellular signaling pathways. If the number of signaling pathways converging on myogenesis is large, delineation of individual signals critical for inhibiting myogenesis are likely to be difficult to identify. Whereas some of the studies cited above have looked at the role of Raf/ERK1/2 activation in the late stages of myogenesis, we have addressed the role of Raf/ERK pathway activation during the initial FGF-dependent signaling events. Exposure of synchronized MM14 cells to FGFs during G1(3h) is sufficient to prevent terminal differentiation. Thus, inhibition of FGFR1 signals that repress differentiation during G1 would lead to cell cycle withdrawal, muscle-specific gene expression and fusion, while inhibition of signals required only for cell proliferation would maintain a quiescent, undifferentiated state. FGFs are required for the G1 to S-phase transition in myoblasts but appear to be dispensable for the remainder of the cell cycle; withdrawal of FGFs prior to the G1-phase leads to terminal differentiation, while withdrawal after the G1-phase does not affect the subsequent mitosis or cytokinesis (Clegg et al., 1987).

We have previously demonstrated that repression of differentiation and stimulation of cell division are separable processes and that FGFR-1 signals appear to be required for both biological activities (Lim and Hauschka, 1984; Kudla et al., 1998). Finally, we (Flanagan-Steet et al., 2000) and others (Seed and Hauschka, 1988; Rando and Blau, 1994; Floss et al., 1997; Cornelison, 1998) demonstrated that myoblasts and satellite cells in vivo behave similarly to MM14 cells. These properties make MM14 cells an ideal model system for delineating the signals from identified growth factors that are responsible for repressing myogenesis or stimulating proliferation.

The requirement for ERK1/2 activity in mediating proliferation and repression of differentiation was examined in synchronized MM14 cells. All inhibitors of the Raf/ERK pathway that we tested eliminated ERK1/2 activity when measured directly or by an Elk reporter assay. We found that ERK1/2 activity was required in the G1-phase for transit of cells from the G1 to S-phase, consistent with an FGF requirement at this transition. Loss of ERK1/2 activity thus prevented myoblasts from entering S-phase, causing the cells to be maintained in a quiescent, undifferentiated state. A similar state is seen for satellite cells in vivo, for serum-deprived MM14 cells maintained in the presence of FGF-2 (Clegg et al., 1987), and for MM14 cells expressing FGFR-1 chimeras (Kudla et al., 1998). Neither Raf, MKK1/2, nor-ERK1/2 activity

appeared to be required for continuation of DNA synthesis or mitosis. In contrast to our results, some cell types may exhibit a requirement for MKK1/2 and ERK 1/2 in mitosis (Shapiro et al., 1998; Zecevic et al., 1998), suggesting that mitosis in skeletal muscle myoblasts and other cell types may differ.

Previously, we have demonstrated that activation of the FGFR1 tyrosine kinase is sufficient to inhibit differentiation but not sufficient to promote DNA synthesis (Kudla et al., 1998). Our prior studies utilizing chimeric receptors suggested that stimulation of proliferation in MM14 cells required signals in addition to activation of FGFR1 (Kudla et al., 1998). Here we show that FGFs participate in at least two independent signaling pathways emanating from FGFR1, one is required for proliferation and a second is required to repress myogenic differentiation. We propose that a minimum of two FGF-dependent pathways are involved in stimulating proliferation and repressing terminal differentiation (Fig. 9). Although overexpression of constitutively active mutants of Raf-1 and MKK1 function as oncogenes in a variety of cell types (Stanton et al., 1989; Mansour et al., 1994; Kerkhoff and Rapp, 1997), MM14 clones expressing caRaf-1 and caMKK1 were indistinguishable from wild type cells and did not proliferate. Thus, it appears that stimulation of the Raf/ERK pathway is necessary but not sufficient for myoblast proliferation.

We found that inhibition of the Raf/ERK pathway blocked myoblast proliferation and inhibited MKK1/2 and ERK1/2 activity. Of the independent Raf/ERK pathway inhibitors employed, all blocked proliferation and only U0126 was capable of promoting limited (10%) skeletal muscle differentiation. While this treatment results in a measurable level of differentiation, we see similar levels of differentiation in quiescent MM14 cells maintained in the absence of serum for 36–48 h (Clegg et al., 1987; Fedorov et al., 1998). Recent data from our laboratory and others suggests that specific MAPKs are involved in regulation of MM14 cell myogenesis since inhibition of p38 $\alpha$  prevents differentiation (manuscript in preparation) and different MAPK phosphatases are capable of either blocking FGF-mediated proliferation or blocking FGF-mediated repression of myogenesis (manuscript in preparation). These data serve to underscore the importance of using multiple inhibitors to determine physiological roles for the Raf/ERK pathway in myoblasts. While we conclude that activation of ERK1/2 is necessary for commitment of MM14 cells to DNA synthesis, neither Raf1, MKK1/2 nor ERK1/2 activity appears to be required for withdrawal of myoblasts from the cell cycle, for induction of muscle gene expression or for cell fusion.

MM14 cells provide a good model for the behavior of skeletal muscle satellite cells. Both the primary satellite cells and MM14 cells exhibit three distinct phenotypes that depend on the growth conditions: (a) active proliferation, (b) quiescent and undifferentiated, and (c) quiescent and terminally differentiated. Quiescent satellite cells express both FGFR4 and FGFR1 (Cornelison, et al., 2000). Consistent with our results and the presence of FGFRs on satellite cells, a requirement for FGF-6 has been demonstrated for regeneration of skeletal muscle (Floss et al., 1997). Elucidating MM14 signals

necessary to support cell growth may be relevant to satellite cells during skeletal muscle growth and regeneration. We are currently attempting to determine if primary satellite cells possess signaling pathways that exhibit requirements similar to those of MM14 cells and determining if additional MAPKs present in MM14 cells play critical roles in repressing terminal differentiation or promoting proliferation of skeletal muscle cells.

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