MOLECULAR AND CELLULAR BIOLOGY, Nov. 1996, p. 5964–5973 Vol. 16, No. 11
0270-7306/96/$04.00+0

Stimulation of C2C12 Myoblast Growth by Basic Fibroblast Growth Factor and Insulin-Like Growth Factor 1 Can Occur via Mitogen-Activated Protein Kinase-Dependent and -Independent Pathways

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Received 11 December 1995/Returned for modification 20 February 1996/Accepted 15 August 1996

It is now well-recognized that the mitogen-activated protein (MAP) kinase cascade facilitates signaling from an activated tyrosine kinase receptor to the nucleus. In fact, an increasing number of extracellular effectors have been reported to activate the MAP kinase cascade, with a significant number of cellular responses attributed to this activation. We set out to explore how two extracellular effectors, basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1), which have both been reported to activate MAP kinase, generate quite distinct cellular responses in C2C12 myoblasts. We demonstrate here that bFGF, which is both a potent mitogen and inhibitor of myogenic differentiation, is a strong MAP kinase agonist. By contrast, IGF-1, which is equally mitogenic for C2C12 cells but ultimately enhances the differentiated phenotype, is a weak activator of the MAP kinase cascade. We further demonstrate that IGF-1 is a potent activator of both insulin receptor substrate IRS-1 tyrosyl phosphorylation and association of IRS-1 with activated phosphatidylinositol 3-kinase (PI 3-kinase). Finally, use of the specific MAP kinase kinase inhibitor, PD098059, and wortmannin, a PI 3-kinase inhibitor, suggests the existence of an IGF-1-induced, MAP kinase-independent signaling event which contributes to the mitogenic response of this factor, whereas bFGF-induced mitogenesis appears to strongly correlate with activation of the MAP kinase cascade.

The binding of hormones or growth factors to their cognate cell surface receptor/tyrosine kinases initiates an intricate array of phosphorylation events that have been implicated in the control of cellular growth, differentiation, and metabolism. The mitogen-activated protein (MAP) kinase phosphorylation cascade is one mechanism by which a signal generated by ligand binding at the cell membrane is transduced to the nucleus. The MAP kinase signaling cascade is triggered by hormone or growth factor binding to a receptor/tyrosine kinase followed by receptor autophosphorylation and recruitment of the Src homology 2 (SH2)-domain-containing proteins to phosphorylated tyrosine residues on the receptor (7, 48). Subsequent to these receptor-proximal events, a protein phosphorylation cascade that involves association of GTP-bound Ras with Raf followed by activation of MAP kinase kinase (MEK) and MAP kinase (23, 43) is stimulated. In turn, activated MAP kinases translocate to the nucleus, where they phosphorylate appropriate transcription factors and ultimately exert their effects on cellular growth and differentiation (9, 13, 37). A paradox exists, however, because activation of the MAP kinase signaling pathway by various extracellular effectors appears to be initiated by a common cascade of phosphorylation events, whereas the downstream cellular responses which have been attributed to activation of this pathway can vary dramatically. For instance, in neuronal cells, MAP kinase activity results from stimulation with nerve growth factor, basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), or epidermal growth factor (EGF). Interestingly, IGF-1 and EGF are mitogenic for these cells, whereas bFGF and nerve growth factor induce neuronal differentiation (17, 32). Mitogenicity correlates with a transient peak of MAP kinase activity, whereas differentiation results from prolonged activation of the MAP kinase signal. The role of the MAP kinases during adipogenesis has also been the focus of much recent research (for a review, see reference 11). While there is evidence favoring a role for the MAP kinases in the growth-promoting effects of EGF and insulin in adipocytes, activation of the MAP kinase cascade does not appear to be sufficient to stimulate insulin-regulated metabolic effects such as glucose transport and increases in glycogen synthase activity (9).

In comparison with our understanding of the role of MAP kinase signaling during adipogenesis and neuronal differentiation, the role of the MAP kinases in myogenic growth and differentiation remains unclear. In vivo, it has been demonstrated that intravenous injection of insulin into rats causes a two- to threefold increase in MAP kinase activity detected in muscle cell extracts (18, 53). In myogenic cell lines, a role for the MAP kinases in processes ranging from the differentiation of myoblasts into postmitotic myotubes to the bFGF-induced inhibition of myogenic differentiation and the insulin- or IGF-1-induced inhibition of glycogen synthase kinase-3 activity has been suggested (6, 10, 16, 25). We questioned how bFGF, IGF-1, and insulin, all reportedly acting through the MAP kinase pathway, ultimately could elicit such disparate effects on the myogenic phenotype. Specifically, we sought to elucidate the potential role of the MAP kinases in myoblast growth. To address this question, we have employed a cell culture system in which C2C12 myoblasts are growth arrested in G_0, without a concomitant induction of muscle-specific genes, by being cultured in methylcellulose-containing suspension medium (28). Upon reattachment to a solid substratum, these quiescent, synchronized myoblasts can be activated either to enter the cell cycle or to differentiate by varying the growth factor composition of the media in which the cells are plated. We had previously demonstrated that bFGF or IGF-1 caused quiescent myoblasts to progress synchronously through one round of the cell cycle (28). After this initial round of cell division, however, bFGF inhibited induction of the muscle-specific phenotype,

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whereas IGF-1 enhanced the myogenic response. Quiescent myoblasts plated in bFGF and IGF-1 in combination progressed through multiple rounds of the cell cycle and remained undifferentiated.

We demonstrate here that bFGF, IGF-1, and insulin all elicit a mitogenic response in C2C12 myoblasts cultured as described previously (28). bFGF-stimulated mitogenesis is accompanied by a robust peak of MAP kinase activity between 2 and 10 min following stimulation, whereas myoblasts treated with either IGF-1 or insulin exhibit only minimal MAP kinase activity. The combination of either IGF-1 or insulin with bFGF results in the highest levels of DNA synthesis; however, this increased mitogenicity could not be accounted for by an increase in MAP kinase activity. When the signaling events following stimulation with IGF-1 or insulin were characterized, we found that both IGF-1 and insulin stimulate tyrosyl phosphorylation of the physiologic insulin receptor substrate, IRS-1 (49, 50), and phosphatidylinositol 3-kinase (PI 3-kinase) activity. IGF-1 and insulin stimulation also resulted in a significant increase in the PI 3-kinase activity associated with IRS-1. Both bFGF- and IGF-1-induced activation of DNA synthesis was sensitive to the MEK inhibitor, PD098059, whereas a wortmannin-insensitive signaling event involving association of PI 3-kinase with IRS-1 is believed to contribute to IGF-1-induced mitogenesis. Therefore, the data presented here represent at least a partial explanation for the divergent effects resulting from signaling via the bFGF and IGF-1 receptor tyrosine kinases during the mitogenic response of C2C12 myoblasts to these soluble factors.

MATERIALS AND METHODS

Cell culture. C2C12 mouse myoblasts (4) were passaged and maintained as subconfluent monolayers in high-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum. To growth arrest myoblasts, subconfluent cells were trypsinized and suspended at a final density of 3×10^5/ml in DMEM containing 1.5% Methocel A4M (Dow Chemical Company, Midland, Mich.) supplemented with 20% fetal bovine serum. After 48 to 72 h, the suspended cells were recovered for replating by dilution of methylcellulose-containing medium with 4 volumes of sterile phosphate-buffered saline (PBS) followed by centrifugation at $2000 \times g$ for 10 min at 15°C. All culture media contained 100 U of penicillin per ml and 100 μg of streptomycin per ml. Quiescent cells (10×10^6/150-mm-diameter dish) were replated and, unless otherwise indicated, allowed to adhere for 1 h prior to stimulation by the following soluble growth factors: recombinant human bFGF and IGF-1 (PeproTech, Rocky Hill, N.J.) or insulin from bovine pancreas (Sigma Chemical Co., St. Louis, Mo.). Where indicated, PD098059 (a kind gift from Alan R. Saltiel, Parke-Davis Pharmaceutical Research, Ann Arbor, Mich.) or wortmannin (Sigma) was added to preadhered cells, which were then incubated for 30 min prior to growth factor stimulation.

MAP kinase activity assays. Cells were washed with ice-cold PBS and then scraped into lysis buffer, consisting of 70 mM β-glycerophosphate (pH 7.2), 100 μM sodium orthovanadate, 2 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5% Triton X-100, 5 μg of leupeptin per ml, 2 μg of aprotinin per ml, and 1 mM dithiothreitol, on ice. The lysates were then cleared by centrifugation at 10,000 rpm at 4°C for 10 min followed by filtration through Spin-X 0.22-μm-pore-size cellulose acetate centrifuge filter units (Costar, Cambridge, Mass.) at 10,000 rpm (Eppendorf model 5414; Brinkmann Instruments, Westbury, N.Y.) at 4°C for 10 min. Soluble extracts (0.5 to 1 mg of protein) were fractionated by anion-exchange chromatography on a 1-ml Resource Q column by the Pharmacia FPLC system (Pharmacia Biotech, Uppsala, Sweden). Chromatography was performed and fractions were assayed exactly as described previously (17) with 0.33 mg of myelin basic protein (MBP) per ml used as a substrate.

PI3-kinase assays. Cells were washed with PBS–1 mM $CaCl_2$–1 mM $MgCl_2$–100 μM sodium orthovanadate and then with buffer A (20 mM Tris [pH 7.4], 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM sodium orthovanadate). The cells were scraped into buffer A containing 1% Nonidet P-40 (NP-40) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and were incubated on ice for 30 min with occasional vortexing. Samples were microcentrifuged for 10 min at 12,000 rpm (Eppendorf model 5414) at 4°C to remove insoluble material, and 500 μg of supernatant was incubated for 90 min with 2 μl of anti-PI3-kinase antibody (Upstate Biotechnology Inc., Lake Placid, N.Y.) at 4°C. A 40-μl volume of protein A-TrisAcryl (Pierce, Rockford, Ill.) was added, and the samples were rotated for 1 h at 4°C. The immunocomplexed beads were washed three times with PBS containing 1% NP-40, three times with 100 mM Tris (pH 7.4)–5 mM LiCl, and twice with TNE buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA). All washes included 100 μM sodium orthovanadate. After the last wash, the beads were incubated with 50 μl of TNE buffer, 20 μg of phosphatidylinositol (Avanti Polar Lipids, Alabaster, Ala.), 10 μl of 100 mM $MgCl_2$, and 5 μl of ATP solution (0.88 mM ATP, 20 mM $MgCl_2$, 30 μCi of [γ-^{32}P]ATP) for 10 min with constant agitation. Reactions were stopped with 20 μl of 6 N HCl. Lipids were extracted with $CHCl_3$-methanol ($CHCl_3$-MeOH) (1:1), and the lower phase was spotted on thin-layer chromatography plates treated with oxalate. Lipids were resolved by chromatography in $CHCl_3$-MeOH-H_2O-NH_4OH (60:47:11.3:2). The plates were dried and autoradiographed, and, where quantitation is indicated, spots were scraped from the thin-layer chromatography plates and radioactivity was measured in liquid scintillation fluid (Liquiscint; National Diagnostics, Atlanta, Ga.).

Western blot (immunoblot) analysis. (i) MAP kinase. Cells were washed with ice-cold PBS and then scraped into Western lysis buffer (25 mM Tris [pH 7.4], 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2% sodium dodecyl sulfate [SDS], 1 mM PMSF, 1 μM aprotinin, 1 μM leupeptin). After 20 min on ice with occasional vortexing, the lysates were cleared by centrifugation at 10,000 rpm (Eppendorf model 5414) at 4°C for 10 min. Soluble proteins were quantitated by using the bicinchoninic acid assay (Pierce). Alternatively, Resource Q fractions (9 volumes) were precipitated with 72% trichloroacetic acid (TCA)–0.15% sodium deoxycholate (1 volume) in a Microfuge tube for 2 h at 4°C and then centrifuged at 15,000 rpm at 4°C for 10 min. Samples were separated on SDS–12% polyacrylamide gels (26) and then electrophoretically transferred to nitrocellulose at 4°C for 16 to 18 h (the transfer buffer contains 39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol). The membranes were incubated in PBST (PBS plus 0.1% Tween 20) containing 4% nonfat dry milk for 30 min at 25°C to block nonspecific binding, then incubated with monoclonal anti-pan-Erk antibody (Transduction Laboratories, Lexington, Ky.) diluted 1:500 in PBST–1% bovine serum albumin–0.02% NaN_3 for 1 h at 25°C, and finally incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (1:1,000; Sigma) in PBST for 1 h. The blots were then developed with a Renaissance chemiluminescence kit (DuPont, Boston, Mass.) and exposed to X-ray film for approximately 1 min to visualize specific proteins.

(ii) Activated MAP kinase. Western blot analysis was performed as described above except that the membranes were incubated with a polyclonal antiserum which recognizes only the activated 42- and 44-kDa forms of MAP kinase (obtained from Quality Controlled Biochemicals, Inc., Hopkinton, Mass.) at a 1:2,300 dilution. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1,000; Sigma) was used as the secondary antibody.

(iii) IRS-1. Adherent cells were washed twice in ice-cold PBS and then scraped into lysis buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM PMSF, 0.3 μM aprotinin, 1 mM sodium orthovanadate). Western blot analysis was performed as described above for MAP kinase except that the samples were fractionated on 6% gels and the membranes were incubated with affinity-purified polyclonal anti-IRS-1 antibody (2 μg/ml; a kind gift from Michael Gibbs, Pfizer, Inc., Groton, Conn.). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1,000; Sigma) was used as the secondary antibody.

(iv) Phosphotyrosine. IRS-1 was immunoprecipitated as described below and then fractionated on 6% polyacrylamide gels. The primary antibody was mouse monoclonal PY20 antibody (1:1,000; Transduction Laboratories). The secondary antibody was the same as that used for detecting MAP kinase above.

IRS-1 immunoprecipitation. Adherent cells were washed twice with ice-cold PBS and then scraped into 1 ml of lysis buffer (50 mM Tris-base [pH 8.0], 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg of aprotinin per ml). The lysates were incubated on ice for 20 min with occasional vortexing and then cleared by centrifugation at 10,000 rpm (Eppendorf model 5414) at 4°C for 10 min. The lysates were then incubated with 10 μl of rabbit polyclonal anti-IRS-1 antiserum R2656 (also a gift from Michael Gibbs) on ice for 1 h, after which 40 μl of protein A-TrisAcryl (Pierce) was added. After rotation for 1 h at 4°C, immunocomplexed beads were washed briefly three times with lysis buffer. Lastly, the beads were boiled in 1× Laemmli sample buffer for 5 min to elute immunocomplexes, and the supernatants were processed for further Western blot analysis as described above. Where indicated, IRS-1 immunocomplexes were substituted for PI 3-kinase immunoprecipitates and assayed for PI 3-kinase activity.

DNA synthesis assay. Cells were labeled for 19 to 23 or 24 to 28 h with 2 μCi of [*methyl*-^{3}H]thymidine per ml (1 to 10 Ci/mmol; Amersham, Arlington Heights, Ill.). The cells were rinsed thoroughly with PBS and then fixed with 100% MeOH. The fixed cells were coated with a thin layer of autoradiography emulsion, type NTB2 (Eastman Kodak Company, Rochester, N.Y.), and labeled nuclei were detected 3 days later by developing the emulsion with D-19 developer followed by Rapid Fix (Kodak).

RESULTS

MAP kinase activity in C2C12 myoblasts stimulated with bFGF and IGF-1. Quiescent myoblasts were recovered from suspension culture and plated on tissue culture dishes for 1 h

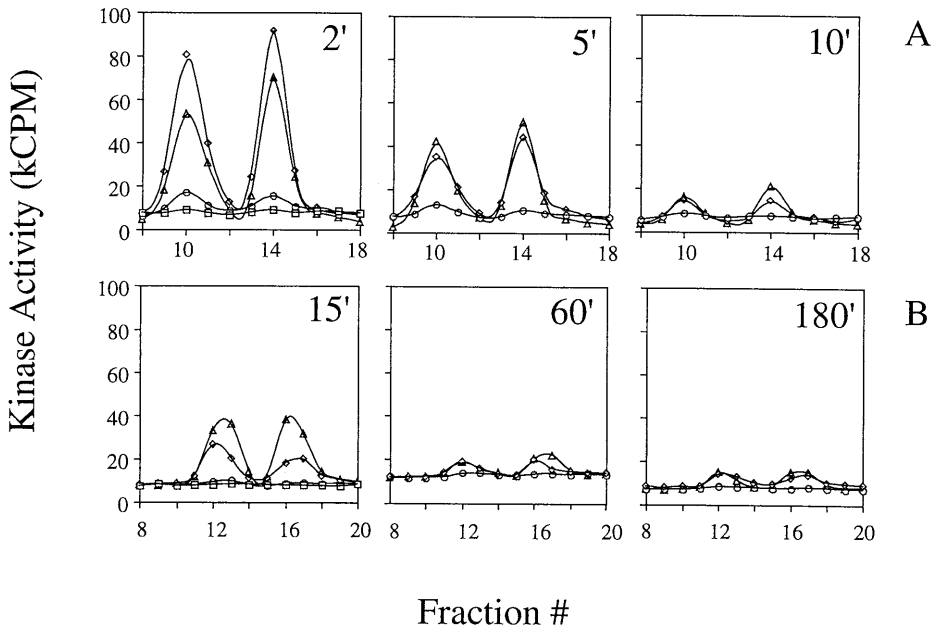

FIG. 1. bFGF, but not IGF-1, markedly stimulates MAP kinase activity in C2C12 myoblasts. C2C12 cells were cultured in suspension for 48 h to arrest growth and then plated for 1 h in the absence of growth factors. The cells (10×10^6) were then stimulated with bFGF (1 nM) (◇), IGF-1 (3 nM) (○), or bFGF and IGF-1 in combination (△) or were left untreated (□) and harvested as described in the text. Cytosolic proteins (1 mg per condition) were fractionated by Resource Q chromatography, and fractions (20-μl aliquots) were assayed for the ability to phosphorylate MBP. Panels A and B represent separate experiments.

in DMEM alone to facilitate attachment and spreading. Adhered myoblasts were then stimulated with either 1 nM bFGF, 3 nM IGF-1, or bFGF and IGF-1 in combination for the times indicated, and cell lysates were fractionated by Resource Q anion-exchange chromatography as described in Materials and Methods. As Fig. 1A demonstrates, stimulation of quiescent myoblasts with 1 nM bFGF resulted in a transient activation of two peaks of MAP kinase activity between 2 and 10 min when fractions were assayed for their ability to phosphorylate MBP. When cell extracts were assayed between 15 and 180 min (Fig. 1B, from a separate experiment), it is clear that MAP kinase activity has decreased to near baseline levels by 180 min following stimulation. When myoblasts were exposed to 3 nM IGF-1, kinase activity was barely detectable between 2 and 10 min and undetectable when assayed 15 to 180 min following stimulation. Activation of quiescent myoblasts with 3 nM IGF-1 in combination with 1 nM bFGF results in slightly enhanced MAP kinase activity between 5 and 15 min following stimulation; however, this bFGF–IGF-1-induced activity also returns to near baseline levels by 180 min poststimulation. Finally, extracts from adhered myoblasts to which no exogenous growth factor had been added (2 or 15 min) exhibit no detectable MAP kinase activity when assayed in vitro for the ability to phosphorylate MBP.

Identification of specific protein kinases in supernatants from bFGF- and IGF-1-stimulated myoblasts. In order to identify the specific proteins responsible for the two peaks of kinase activity demonstrated above, cells were stimulated for 15 min with the various effectors and fractions from Resource Q were TCA precipitated, electrophoresed, and immunoblotted with anti-pan-Erk, a monoclonal antibody which recognizes various members of the MAP kinase family, including extracellular signal-regulated kinases 1 and 2 (Erk1 and Erk2). When the MAP kinase profiles from unstimulated cells are compared with those of myoblasts stimulated with bFGF, alone or in combination with IGF-1, two distinctions become apparent (Fig. 2). First, a portion of the 42-kDa MAP kinase, Erk2, migrates with a slightly slower electrophoretic mobility attributable to a change in phosphorylation state of this species (1) (fractions 11 and 12, appearance of middle species). Second, in both myoblasts treated with bFGF alone and those treated with bFGF and IGF-1 together, a new species of MAP kinase appears in fractions 15 and 16 at approximately 44 kDa,

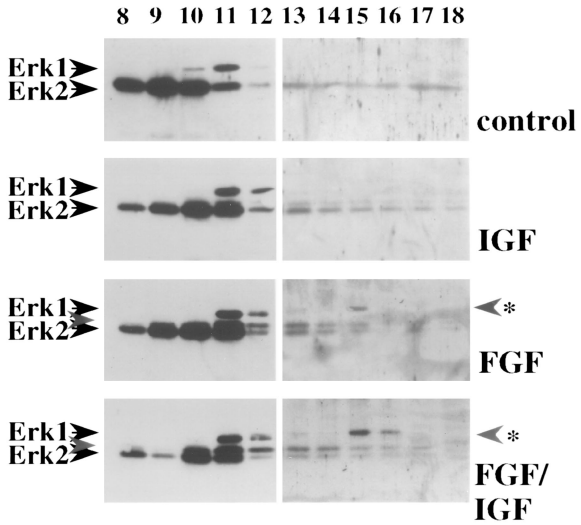

FIG. 2. Immunodetection of various MAP kinase isoforms in fractionated extracts from quiescent C2C12 myoblasts stimulated with bFGF, IGF-1, or bFGF and IGF-1 together for 15 min. After being assayed for protein kinase activity, fractions eluted from a Resource Q column (fractions 8 to 18) were TCA precipitated, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with anti-pan-Erk antibody, which recognizes both the 42- and the 44-kDa MAP kinase family members. ➤ and *➤, more slowly migrating, phosphorylated Erk2 and Erk1 species, respectively.

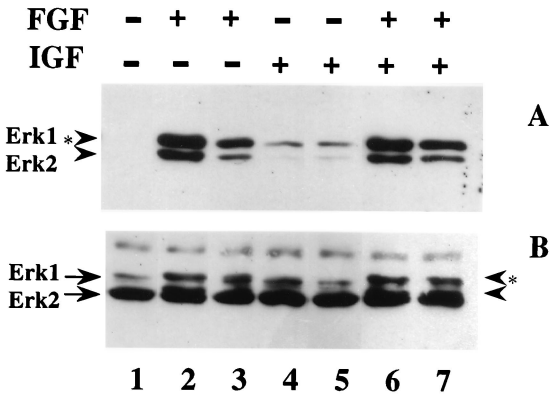

FIG. 3. MAP kinase activation by growth factors can be demonstrated by an antibody specific for the activated species. C2C12 myoblasts were growth arrested by being cultured in suspension for 48 h and then preplated for 1 h prior to stimulation with growth factors. Cytosolic proteins (50 μg) were separated by SDS-PAGE and then immunoblotted with either polyclonal anti-activated MAP kinase (A) or monoclonal anti-pan-Erk antibody (B). Lanes 1, untreated myoblasts; lanes 2 and 3, cells treated with 1 nM bFGF for 5 or 10 min, respectively; lanes 4 and 5, cells treated with 3 nM IGF-1 for 5 or 10 min, respectively; and lanes 6 and 7, cells treated with bFGF and IGF-1 together for 5 or 10 min, respectively. ➤ and *➤, more slowly migrating, phosphorylated Erk2 and Erk1 species, respectively.

again with a slightly slower electrophoretic mobility than its unmodified 44-kDa counterpart eluting earlier in the NaCl gradient (fractions 11 and 12, highest-molecular-mass species). These data indicate that the MAP kinase activity in peak 1 is due to phosphorylated Erk2 and that peak 2 activity results from phosphorylated Erk1, and these results are in agreement with those of detailed studies performed elsewhere to identify the MAP kinase activity peaks from nerve growth factor-treated PC12 cells and EGF-stimulated NIH 3T3 fibroblasts (1, 5). Finally, when quiescent myoblasts are treated with IGF-1, phosphorylated Erk1 and Erk2 are virtually undetectable with this antibody. The abundance of the slower-mobility phosphorylated species correlates well with levels of kinase activity assayed as in Fig. 1.

It is clear from Fig. 1 and 2 that while there are appreciable levels of MAP kinase activity in extracts from cells stimulated for 15 min with either bFGF alone or bFGF and IGF-1 in combination, only a fraction of the total MAP kinases are activated following stimulation for this time. The same was true for extracts from cells stimulated for 5 or 10 min. Immunoblotting of unfractionated extracts with anti-pan-Erk antibody demonstrated that <50% of the detectable Erk2 protein migrated as the slower-mobility, phosphorylated species following 5-min stimulation with bFGF alone or bFGF and IGF-1 (Fig. 3B, lanes 2 and 6). However, immunoblotting of extracts with an antibody that recognizes only the phosphorylated forms of Erk1 and Erk2 demonstrates that the abundance of these species is dramatically increased relative to that in unstimulated cells (Fig. 3A). Immunoblotting with an activated MAP kinase-specific antibody also confirms the observation made from Fig. 1 that IGF-1 stimulates only very low levels of MAP kinase activation (Fig. 3A, lanes 4 and 5).

Signaling through the IGF-1 receptor in C2C12 myoblasts results in tyrosine phosphorylation of IRS-1. The low levels of MAP kinase activity in IGF-1-treated myoblasts interested us, especially in light of recent reports that implicate the Ras signaling pathway in both IGF- and insulin-induced mitogenicity (3, 15, 42, 45, 51). In order to demonstrate that these growth factors were in fact transmitting a functional signal via their respective receptors, we first measured ligand-dependent tyrosine phosphorylation of the physiologic insulin receptor substrate, IRS-1 (35, 36). Figure 4A shows the relative abundance of IRS-1 protein in cell extracts isolated from cells treated with 1 nM bFGF, 3 nM IGF-1, or the two factors in combination. Cells were harvested between 15 and 180 min poststimulation, and 100 μg of total cell protein per condition was analyzed by immunoblotting with anti-IRS-1 antibody. IRS-1 was readily detectable in suspended, quiescent myoblasts, and the level of protein was not influenced by attachment to a solid substratum. Furthermore, protein levels remained roughly constant throughout the time course studied, independent of growth factor exposure. However, when the corresponding phosphotyrosine levels in IRS-1 are measured, the changes due to IGF-1 exposure are quite evident. In the experiment whose results are shown in Fig. 4B, cells were treated as for Fig. 4A and then harvested between 15 min and 24 h following stimulation. IRS-1 was first immunoprecipitated from lysates as described in Materials and Methods and then subjected to Western blot analysis using a monoclonal antiphosphotyrosine antibody. As expected, suspended myoblasts, preplated control cells, and myoblasts treated with bFGF alone exhibit virtually undetectable levels of phosphotyrosine. By contrast, cells treated with either IGF-1 alone or IGF-1 in concert with bFGF exhibit abundant phosphotyrosine in IRS-1 immunocomplexes.

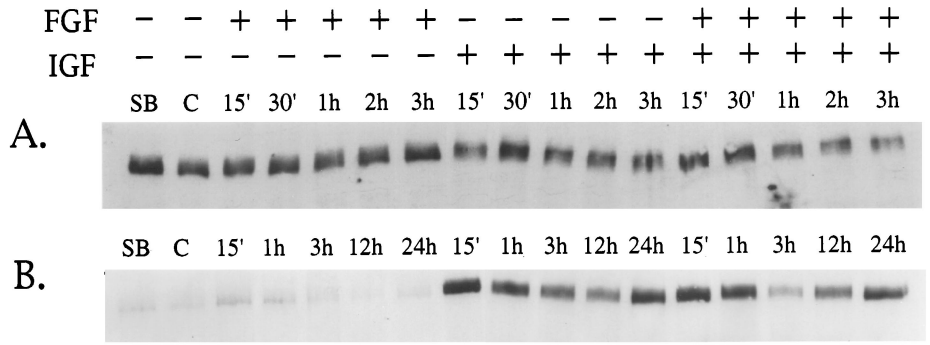

FIG. 4. IGF-1, but not bFGF, can stimulate tyrosine phosphorylation of IRS-1 in C2C12 myoblasts. C2C12 cells were growth arrested and plated as described in the text. The cells were then stimulated with either bFGF (1 nM) or IGF-1 (3 nM) or the two soluble factors in combination for the indicated times prior to harvesting. Cytosolic extracts were separated by SDS-PAGE and immunoblotted with polyclonal anti-IRS-1 antibody (A) or, conversely, IRS-1 was immunoprecipitated with anti-IRS-1 antiserum and then immunoblotted with monoclonal antiphosphotyrosine antibody (B). SB, suspended myoblasts harvested prior to preplating; C, preadhered myoblasts not subjected to growth factor stimulation.

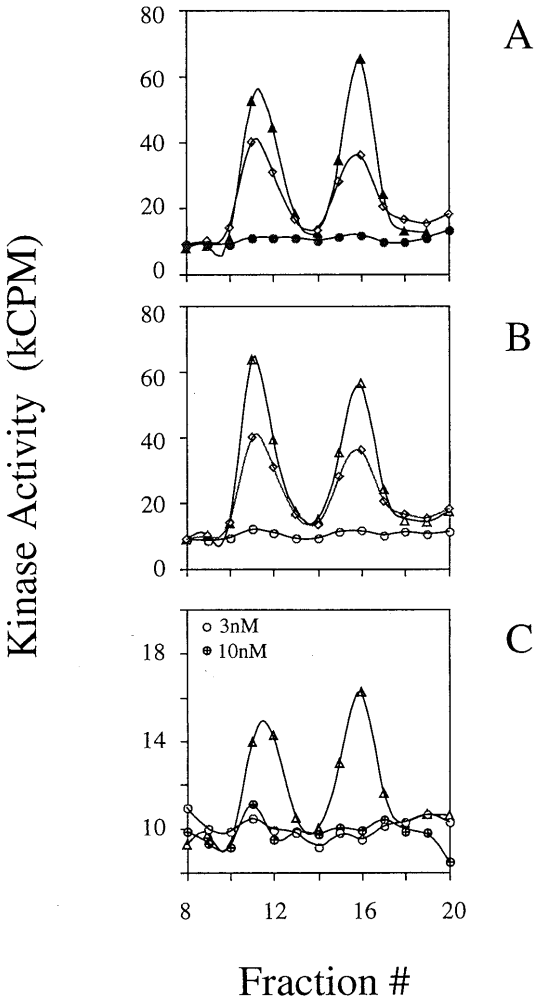

FIG. 5. Insulin can mimic the effects of IGF-1 on MAP kinase activation. Growth-arrested C2C12 myoblasts were harvested from suspension culture and preplated for 1 h in DMEM alone. After growth factor stimulation, cytosolic proteins were harvested and fractionated (1 mg per condition) by Resource Q chromatography. Fractions (20-μl aliquots) were assayed for the ability to phosphorylate MBP. (A) Myoblasts stimulated with insulin (10 nM) (●), bFGF (1 nM) (◇), or bFGF and insulin together (▲). (B) Cells stimulated with IGF-1 (3 nM) (○), bFGF (1 nM) (◇), or bFGF and IGF-1 together (△). (C) Cells stimulated with IGF-1 (3 nM) (○), IGF-1 (10 nM) (⊕), or 3 nM IGF-1 plus 1 nM bFGF (△).

The effects of IGF-1 on MAP kinase activity and IRS-1 tyrosyl phosphorylation can be mimicked by insulin. The issue of whether the MAP kinases are important for signaling via the insulin or IGF-1 receptors is currently unresolved (for a review, see reference 11). Indeed, the MAP kinases were first identified in insulin-stimulated 3T3L1 adipocytes (39, 41), and a number of more recent studies have utilized these cells in an attempt to understand the role of the MAP kinase signaling pathway in insulin action (12, 36, 40). Insulin has also been shown to activate MAP kinase in the L6 myoblast cell line (10), in the myeloid progenitor $32D^{IR}$ cell line (insulin receptors expressed at levels 30-fold higher than those of parental 32D cells) (30), in rat 1 fibroblasts overexpressing insulin receptors (12×10^6 receptors per cell, a 750-fold increase over the parental line) (5), and in HIR cells (NIH 3T3 cells expressing 6×10^6 human insulin receptors per cell) (32).

We therefore assayed for the ability of insulin to stimulate MAP kinase activity in G_0 growth-arrested C2C12 myoblasts. Preadhered quiescent myoblasts were stimulated for 15 min with either 10 nM insulin or 3 nM IGF-1, alone or in combination with 1 nM bFGF. Figure 5A demonstrates that insulin-stimulated MAP kinase activity was virtually undetectable when cells were stimulated with insulin alone, as is the case with IGF-1 (Fig. 5B). Furthermore, increasing the IGF-1 concentration from 3 to 10 nM does not result in enhanced MAP kinase activity, indicating that the absence of activity is not merely due to a suboptimal concentration of available growth factor (Fig. 5C). Interestingly, insulin stimulation resulted in an apparently synergistic effect on MAP kinase activity when insulin was added in combination with 1 nM bFGF (Fig. 5A), as was the case at 15 min poststimulation with IGF-1 and bFGF (Fig. 5B and 1B). This synergy has been observed in several separate experiments comparing the effects of bFGF versus bFGF in combination with IGF-1 but is detectable only between 15 and 30 min poststimulation, when the peak of MAP kinase activity has passed. Thus, while this result is highly reproducible, the significance of this synergy is obscure at this time.

Figure 6 shows the relative abundance of tyrosyl-phosphorylated IRS-1 detected in insulin- versus IGF-1-stimulated myoblasts. Consistent with the data presented in Fig. 4, phosphotyrosine is virtually undetectable in IRS-1 immunoprecipitated from either control or bFGF-treated cells (Fig. 6, lanes 2 and 3). By contrast, treatment with either IGF-1 alone, IGF-1 and bFGF in combination, or insulin alone stimulated rapid phosphorylation on tyrosine in IRS-1 immunoprecipitates (Fig. 6, lanes 4 to 6, respectively).

Correlation between growth factor-induced MAP kinase activity in adhered myoblasts and progression through the cell cycle. Previously, it was demonstrated that quiescent suspended myoblasts adhered in the presence of either bFGF or IGF-1 progress through S phase between 18 and 28 h after attachment, with the peak of DNA synthesis of cells adhered in the presence of IGF-1 lagging slightly behind that of cells adhered in bFGF (28). We therefore questioned whether activation of MAP kinase by bFGF, IGF-1, or insulin at early times correlated with mitogenicity as evidenced by progression through S phase. Quiescent myoblasts were plated in the presence of the indicated concentrations of soluble factors, and [^{3}H]thymidine was added to the media, which were allowed to incubate for 4 h, either 19 to 23 or 24 to 28 h following stimulation. As is demonstrated in Fig. 7, 39% of cells adhered in the presence of bFGF incorporated [^{3}H]thymidine during the first window of labeling and 42% incorporated label 24 to 28 h poststimulation. This correlates well with the fact that bFGF potently induced MAP kinase activity at the earlier

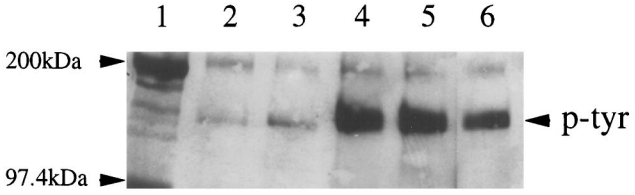

FIG. 6. Insulin stimulation results in tyrosine phosphorylation of IRS-1 in C2C12 myoblasts. C2C12 cells were cultured as described in the text prior to stimulation with 10 nM insulin (lane 6), 3 nM IGF-1 (lane 4), 1 nM bFGF (lane 3), bFGF and IGF-1 together (lane 5), or no growth factors (lane 2). IRS-1 was first immunoprecipitated from cytosolic extracts (1 mg) with anti-IRS-1 antiserum and then subjected to SDS-PAGE and immunoblotting with monoclonal antiphosphotyrosine (p-tyr) antibody. Lane 1 contains electrophoretic molecular mass markers that are nonspecifically detected with immunoblotting reagents.

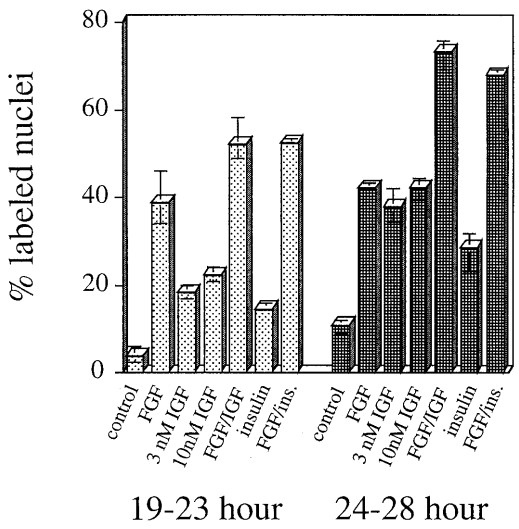

FIG. 7. Levels of DNA synthesis in suspension-arrested C2C12 myoblasts adhered in the presence of defined growth factors for 19 to 28 h. G_0 myoblasts were recovered after 48 h from suspension culture and plated in the presence of either 1 nM bFGF, 3 or 10 nM IGF-1, bFGF plus 3 nM IGF-1, 10 nM insulin, or bFGF plus 10 nM insulin. [^{3}H]thymidine was added and the cells were incubated for 4 h prior to fixation of the cells at 23 or 28 h. A total of 300,000 cells were plated per 2-cm^2 well, and each condition was analyzed in triplicate. At least 500 cells were counted per well.

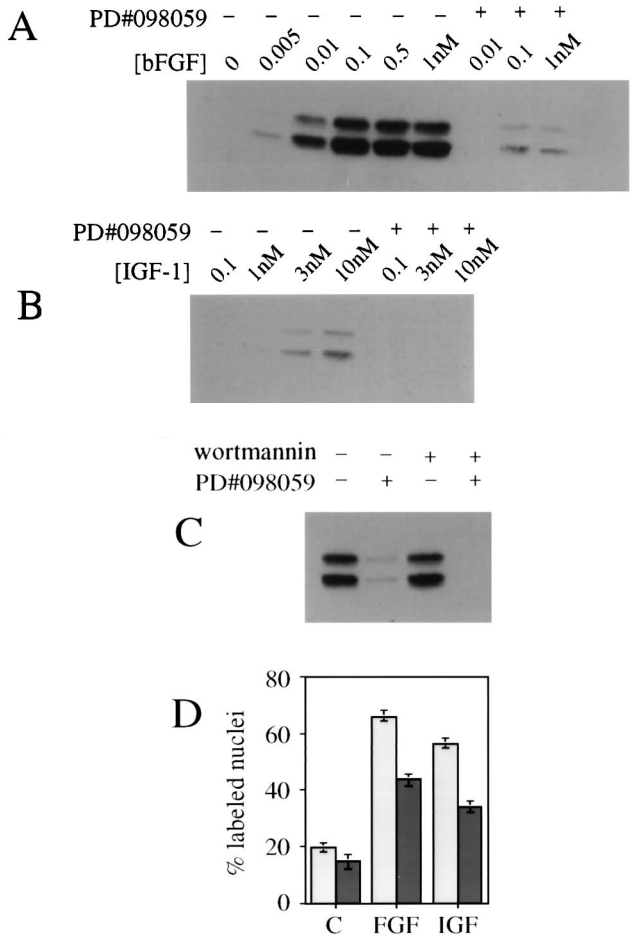

FIG. 8. The MEK inhibitor, PD098059, inhibits both growth factor-induced MAP kinase activation and DNA synthesis. (A) Preadhered quiescent myoblasts were treated for 30 min with either DMSO or 20 μM PD098059 prior to stimulation with increasing concentrations of bFGF. The cells were lysed after 5 min, and proteins were harvested for Western blot analysis with anti-activated MAP kinase antibody. (B) Myoblasts were pretreated with either DMSO or PD098059 prior to stimulation with increasing concentrations of IGF-1. (C) Cells were pretreated with either DMSO alone, 20 μM PD098059, 100 nM wortmannin, or PD098059 and wortmannin together. (D) Quiescent myoblasts were treated with either DMSO or 20 μM PD098059 for 30 min prior to the addition of 1 nM bFGF or 3 nM IGF-1. The cells were labeled with [^{3}H]thymidine from 22 to 26 h and then fixed and processed as described in the text. ▨, DMSO-treated myoblasts; ■, cells pretreated with PD098059.

times assayed. Interestingly, stimulation with 3 or 10 nM IGF-1 or with 10 nM insulin alone, which only poorly activated the MAP kinases, facilitated progression of a significant number of cells through S phase 24 to 28 h poststimulation, as indicated by 38% of nuclei being labeled in 3 nM IGF-1, 42% being labeled in 10 nM IGF-1, and 28% being labeled in 10 nM insulin. Furthermore, the combination bFGF–IGF-1 or bFGF-insulin was the most mitogenic, with 73% of nuclei being labeled in cells stimulated with bFGF–IGF-1 and 68% being labeled in bFGF-insulin (24 to 28 h poststimulation).

One possible interpretation of the data presented in Fig. 7 is that low-level signaling of the MAP kinase cascade via IGF-1 or the insulin receptor is sufficient to drive a significant percentage of myoblasts through the cell cycle. An alternative explanation is that MAP kinase activation plays only a minor role in IGF-1- or insulin-mediated proliferation and that other MAP kinase-independent signaling pathways contribute to the mitogenic response. In order to explore these possibilities, we made use of the MEK inhibitor, PD098059 (35), to inhibit signaling via the MAP kinase cascade. In the experiment whose results are shown in Fig. 8A, growth-arrested myoblasts were adhered to tissue culture dishes for 60 min and then incubated with 20 μM PD098059 or dimethyl sulfoxide (DMSO) for an additional 30 min. The cells were then stimulated with increasing concentrations of bFGF for 5 min and harvested. Alternatively, myoblasts were stimulated with increasing concentrations of IGF-1, with or without PD098059 pretreatment (Fig. 8B). In the case of bFGF, PD098059 inhibited the activated MAP kinase species by >90% and IGF-1-induced MAP kinase activity was completely inhibited. Furthermore, the inhibitory effect on activated MAP kinase was specific to the MEK inhibitor, since the fungal metabolite wortmannin, which inhibits PI 3-kinase by irreversibly binding to the 110-kDa catalytic subunit, had no effect (Fig. 8C).

When identically treated cells were assayed for progression through S phase 24 h poststimulation, PD098059 pretreatment of both bFGF- and IGF-1-stimulated cells resulted in an approximately 30 to 40% decrease in the percentage of cells undergoing DNA synthesis (Fig. 8D). While it is clear that PD098059 inhibition of MAP kinase activity correlates with a decrease in S-phase progression when cells were stimulated with either bFGF or IGF-1, it is important that in neither case was DNA synthesis inhibited to control levels. Of particular interest is the fact that a significant percentage of the cells pretreated with PD098059 prior to IGF-1 stimulation were capable of S-phase progression even in the complete absence of detectable activated MAP kinase (see Discussion).

In an attempt to identify a potential MAP kinase-independent pathway that may contribute to the mitogenic effect of IGF-1 on C2C12 myoblasts, we measured the activity of PI 3-kinase, an SH2-domain-containing protein that interacts with IRS-1 in an IGF-1- or insulin-dependent manner (14, 29, 46, 52). The stimulation of PI 3-kinase activity has been implicated in the metabolic effects of both IGF-1 and insulin in

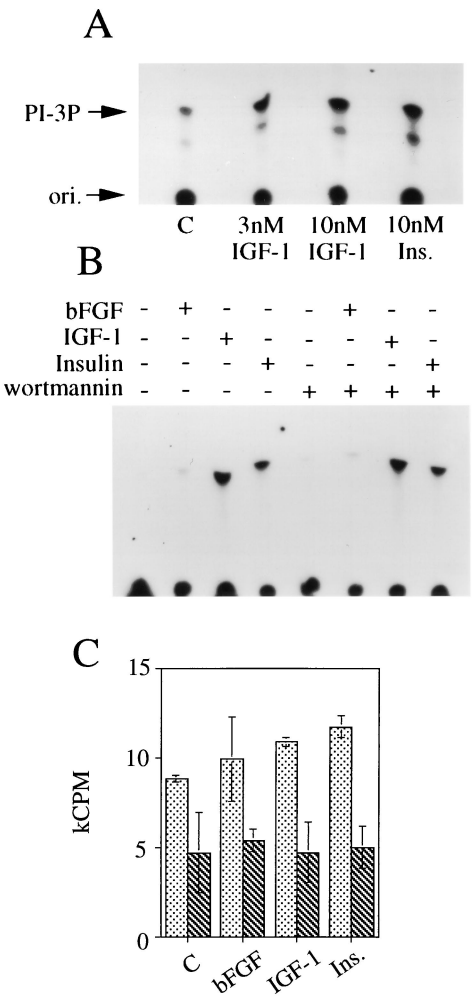

FIG. 9. IGF-1 and insulin activate PI 3-kinase activity in both p85 and IRS-1 immunoprecipitates. (A) Quiescent myoblasts were stimulated with IGF-1 (3 or 10 nM) or insulin (Ins.) (10 nM) for 5 min. The cells were lysed in PI 3-kinase lysis buffer (see Materials and Methods), and anti-p85 antibody was added to cleared lysates. PI 3-kinase assays were then performed on immunoprecipitates, with phosphatidylinositol used as a substrate. ori. (origin), material insoluble in the organic phase. (B) Myoblasts were first treated with either DMSO or wortmannin (100 nM) prior to growth factor stimulation. The cells were then stimulated for 5 min with either bFGF (1 nM), IGF-1 (3 nM), or insulin (10 nM). Cleared lysates were subjected to immunoprecipitation with anti-IRS-1 antibody, and PI 3-kinase assays were performed on the immunoprecipitated material. (C) Cells were treated as for panel B except the lysates were immunoprecipitated with anti-p85 antibody prior to the assays for PI 3-kinase activity. Error bars indicate standard errors from three separate experiments. ▨, DMSO-treated myoblasts; ▩, cells pretreated with wortmannin. C, control cells.

adipogenic and myogenic cell systems (for reviews, see references 21 and 31). Furthermore, activation of PI 3-kinase has been correlated with mitogenicity in PC12 cells (8, 33, 38). Figure 9A shows the levels of PI 3-kinase activity in cells treated with either IGF-1 (3 and 10 nM) or 10 nM insulin. A 500-μg sample of total cell proteins was immunoprecipitated with a monoclonal antibody to the 85-kDa subunit of PI 3-kinase. The immunoprecipitates were then assayed for the ability to phosphorylate the 3′ hydroxyl group of the inositol ring of phosphoinositide. Both IGF-1 and insulin are capable of stimulating PI 3-kinase activity to a level above that measured in control cells. However, the basal level of total PI 3-kinase activity was significant. Data from three separate experiments indicated that 3 nM IGF-1 and 10 nM insulin increased PI 3-kinase activity an average of 1.6- and 2.1-fold, respectively, whereas bFGF-stimulated increases in PI 3-kinase activity were not consistently observed (Fig. 8C and data not shown). While there was a high level of basal PI 3-kinase activity in p85 immunoprecipitates from control cells, the PI 3-kinase activity in IRS-1 immunoprecipitates was virtually undetectable in control and bFGF-treated cells. Furthermore, both IGF-1 and insulin treatment of myoblasts resulted in a dramatic increase of PI 3-kinase activity in IRS-1 immunoprecipitates (Fig. 9B). It is interesting that in vivo addition of wortmannin had no effect on the IGF-1- or insulin-induced PI 3-kinase activity in IRS-1 immunoprecipitates (Fig. 9B), whereas it inhibited the activity in p85 immunoprecipitates approximately 40 to 50% (Fig. 9C). In addition, wortmannin had no significant effect on IGF-1-induced DNA synthesis (data not shown). Collectively, these data suggest the possibility that a wortmannin-insensitive signaling event involving complex formation between IRS-1 and active PI 3-kinase may contribute to the IGF-1-induced mitogenic effect in myoblasts.

DISCUSSION

What role do the MAP kinases play in bFGF-induced regulation of growth and differentiation in myogenic cells? In myogenic cells, bFGF is both a mitogen and a potent inhibitor of differentiation. We demonstrate here that G_0 growth-arrested C2C12 myoblasts undergo a robust peak of MAP kinase activity within 2 to 10 min after stimulation with bFGF. This bFGF-induced activation of the MAP kinases correlates with progression of virtually the entire population of cells through S phase within 24 h following bFGF treatment (Fig. 7) (28). In further support of the fact that signaling via the MAP kinase pathway is important for the mitogenic response of myoblasts to bFGF, Lim and coworkers reported a bFGF-stimulated increase in MAP kinase activity in asynchronously proliferating C2C12 myoblasts which had been serum starved for 4 h prior to stimulation with bFGF (27). This fibroblast growth factor (FGF)-induced MAP kinase activity correlated with increased expression of the immediate-early response gene, *TIS8* (also known as *egr1*, *NGFIA*, and *zif268*), although the study left open the question of whether these bFGF-induced responses were playing a role in myogenic growth or differentiation processes (27). In addition, Shaoul and coworkers reported that expression of FGF receptor 1 (FGFR1) in L6E9 myogenic cells (which normally do not express FGFRs) conferred on them FGF-dependent growth as well as MAP kinase responsiveness (44). When we treated G_0 myoblasts with the MEK inhibitor, PD098059, bFGF-stimulated DNA synthesis was inhibited 30 to 40%, demonstrating that S-phase progression is at least partially MEK dependent. It was not possible to completely inhibit either bFGF-induced DNA synthesis or bFGF-stimulated MAP kinase activity by using this compound, presumably because of limited solubility at higher concentrations (unpublished data and reference 2). As has been previously suggested, use of this inhibitor is limited when a strong MAP kinase agonist such as EGF, platelet-derived growth factor, or bFGF is being studied (reference 2 and this study), especially in light of the potential of amplification by molecules further downstream in the cascade of any signal that is not inhibited. While inhibition of MAP kinase clearly has an effect on DNA synthesis, we suggest that the inability to completely block DNA synthesis may be due to the fact that the bFGF-activated MAP kinase cannot be fully inhibited. Alternatively, bFGF may stimulate some MAP kinase-independent processes that contribute to the mitogenic phenotype. For instance, it has previously

been suggested that PI 3-kinase is involved in the mitogenic response of PC12 cells to bFGF (38). In our system, however, wortmannin pretreatment of C2C12 myoblasts had no effect on bFGF-induced DNA synthesis (data not shown).

It is interesting that two recent reports have suggested that bFGF-dependent inhibition of differentiation does not involve activation of the MAP kinase pathway. Campbell and coworkers suggested that the bFGF-induced repression of differentiation in the MM14 myogenic cell line was not dependent on MAP kinase signaling because they were able to detect MAP kinase activity in cells serum starved for 10 h, at a time when a majority of cells were no longer responsive to bFGF's differentiation-inhibiting effects (6). Additional studies by Kudla et al. confirmed this result with MM14 cells serum starved for 4.5 h prior to bFGF stimulation (25). Although these data suggest that bFGF-induced inhibition of differentiation occurs in a MAP kinase-independent manner, a possible role for the MAP kinases in inhibition of myogenic differentiation cannot conclusively be ruled out. For instance, Kudla and coworkers reported that MAP kinase activity was detectable in MM14 cells overexpressing FGFR1 at a time when myoblasts were still responsive to the differentiation-inhibiting effects of bFGF. It is possible that the relatively low number of FGFRs on MM14 cells (700 compared with ~5,000 on the C2C12 surface [34]) or the asynchronous nature of the MM14 cell culture system utilized in the aforementioned studies could obscure a potentially important role of bFGF-stimulated MAP kinase activity in the inhibition of myogenic differentiation. We are in the process of addressing the possible role of MAP kinase in myogenic differentiation. Preliminary data suggest that treatment of bFGF-stimulated G_0 myoblasts with PD098059 enhances the expression of at least two markers of the differentiated phenotype, myogenin and myosin heavy chain (data not shown). However, it is not yet clear whether this is a direct effect of the drug on the myogenic program per se or a secondary effect resulting from the inhibition of growth. Studies are under way to more fully address this question.

Does insulin and IGF-1 signaling in myogenic cells utilize the MAP kinase pathway? In a recent review by Denton and Tavaré, the cases for and against a role of MAP kinase in insulin action were presented (11). In light of the studies reviewed, the authors suggested that the MAP kinases may play a role in insulin-stimulated growth events such as c-Fos induction and DNA synthesis, whereas the metabolic effects of insulin occur in a MAP kinase-independent manner. In the study presented here, we were able to detect only very low levels of MAP kinase activity when quiescent, undifferentiated C2C12 myoblasts were challenged with either 3 nM IGF-1 or 10 nM insulin (Fig. 1 to 4 and 8A). In addition, both IGF-1 and insulin stimulated rapid tyrosyl phosphorylation of IRS-1, PI 3-kinase activity in p85 immunoprecipitates, PI 3-kinase activity in IRS-1 immunoprecipitates, and subsequent activation of DNA synthesis 20 to 30 h after stimulation. Three pieces of evidence suggest that the mitogenic effects of IGF-1 are due to both MAP kinase-dependent and -independent signaling pathways.

First, as was the case with bFGF, IGF-1-stimulated DNA synthesis was inhibited ~40% when cells were treated with PD098059 prior to addition of growth factor, indicating the probable existence of a MAP kinase-dependent component of the mitogenic response. In contrast to the case with bFGF, however, complete inhibition of IGF-1-induced MAP kinase activity was obtained with the MEK inhibitor, PD098059 (even after overnight exposure of the autoradiographic film shown, no activated MAP kinase was detectable in either control or IGF-1-treated cells following PD098059 exposure). This is in good agreement with the complete inhibition of MAP kinase activity observed with this compound when other relatively weak agonists, including insulin, were tested (2). The fact that a significant portion of the cell population was still capable of undergoing S-phase progression even in the complete absence of activated MAP kinase indicates that a MAP kinase-independent event may also be contributing to the IGF-1-induced mitogenic effect.

Second, there is clearly a signaling pathway involving IRS-1 and PI 3-kinase which is unique to IGF-1 signaling in myoblasts. Both IGF-1 and insulin stimulation resulted in a dramatic increase in IRS-1 phosphorylation, and increased PI 3-kinase activity was detected in p85 immunoprecipitates. Interestingly, whereas PI 3-kinase activity in p85 immunoprecipitates was sensitive to in vivo addition of wortmannin, the fraction of total cellular PI 3-kinase that associated with IRS-1 upon stimulation with either IGF-1 or insulin did so in a wortmannin-insensitive manner. This correlated with the fact that DNA synthesis in these cells was wortmannin insensitive. This finding is in good agreement with data presented previously by researchers studying the potential role of PI 3-kinase in the insulin-sensitive metabolic functions of L6 myotubes (47). In these studies, wortmannin was able to inhibit PI 3-kinase activity in IRS-1 immunoprecipitates only when added to the in vitro kinase assay (47). PI 3-kinase activity has been implicated in a wide array of cellular processes, including inhibition of apoptosis, regulation of glucose transport, and mitogenesis (21). The diversity of this signaling molecule may be regulated by both its subcellular localization and its association with other key signaling molecules (19, 20, 22, 24). Detection of both wortmannin-sensitive and -insensitive signaling events in myogenic cells upon which IGF-1 and insulin have pluripotent effects lends support to this theory. Studies are under way to address these questions and, in particular, the question of whether the activation of a specific signaling event results in distinct cellular functions depending on the cell context in which that signaling pathway is activated.

A third and last point should be made concerning the data obtained when myoblasts are treated with both bFGF and IGF-1 or bFGF and insulin in combination. Clearly, treatment of myoblasts with either bFGF–IGF-1 or bFGF-insulin causes them to reach their full mitogenic potential (Fig. 7). However, the levels of MAP kinase activity in cells treated with bFGF and IGF-1 in combination were remarkably similar to those detected when myoblasts were treated with bFGF alone, as analyzed by both the in vitro ability of MAP kinase to phosphorylate MBP and Western blotting with an antibody specific for only activated Erk1 and Erk2 (Fig. 1 and 3). Since IGF-1 and bFGF are synergistic in their overall mitogenic response but are not obviously so in their ability to stimulate MAP kinase, this also supports the theory that a MAP kinase-independent mitogenic pathway is stimulated by IGF-1.

Conclusion. The data presented here indicate a strong role for the MAP kinase signaling cascade in bFGF-induced mitogenesis in muscle, whereas IGF-1 or insulin appears to be capable of initiating a growth response in both a MAP kinase-dependent and a MAP kinase-independent manner. Studies are under way to clarify the potential role of the MAP kinases in myogenic differentiation. Clearly, processes as complex as cellular growth and differentiation involve activation of multiple signaling cascades and cross-talk between these pathways. Furthermore, the pluripotent effects of factors such as bFGF, IGF-1, and insulin may depend on the cell context in which each receptor is activated. We feel that the data presented here represent at least a partial explanation for the divergent effects resulting from signaling via the bFGF and IGF-1 receptor tyrosine kinase receptors during myogenesis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DK-44269 to P.F.P. and by NIH grant DK45048 to S.R.F.

REFERENCES

1. **Ahn, N. G., R. Seger, R. L. Bratlien, C. D. Diltz, N. K. Tonks, and E. G. Krebs.** 1991. Multiple components in an epidermal growth factor-stimulated protein kinase cascade. J. Biol. Chem. **266:**4220–4227.
2. **Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel.** 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem. **270:**27489–27494.
3. **Baltensperger, K., L. M. Kozma, A. D. Cherniak, J. K. Klarlund, A. Chawla, U. Banerjee, and M. P. Czech.** 1993. Binding of the Ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science (Washington, D.C.) **260:**1950–1952.
4. **Blau, H. M.** 1985. Plasticity of the differentiated state. Science (Washington, D.C.) **230:**758–766.
5. **Boulton, T. G., S. H. Nye, D. J. Robbins, N. Y. Ip, E. Radziejewska, S. D. Morgenbesser, R. A. DePinho, N. Panayotatos, M. H. Cobb, and G. D. Yancopoulos.** 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell **65:**663–675.
6. **Campbell, J. S., M. P. Wenderoth, S. D. Hauschka, and E. G. Krebs.** 1995. Differential activation of mitogen-activated protein kinase in response to basic fibroblast growth factor in skeletal muscle cells. Proc. Natl. Acad. Sci. USA **92:**870–874.
7. **Carpenter, G.** 1992. Receptor tyrosine kinase substrates: *src* homology domains and signal transduction. FASEB J. **6:**3283–3289.
8. **Carter, A. N., and C. P. Downes.** 1992. Phosphatidylinositol 3-kinase is activated by nerve growth factor and epidermal growth factor in PC12 cells. J. Biol. Chem. **267:**14563–14567.
9. **Chen, R.-H., C. Sarnecki, and J. Blenis.** 1992. Nuclear localization and regulation of *erk*- and *rsk*-encoded protein kinases. Mol. Cell. Biol. **12:**915–927.
10. **Cross, D. A. E., D. R. Alessi, J. R. Vandenheede, H. E. McDowell, H. S. Hundal, and P. Cohen.** 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem. J. **303:**21–26.
11. **Denton, R. M., and J. M. Tavaré.** 1995. Does mitogen-activated-protein kinase have a role in insulin action? The cases for and against. Eur. J. Biochem. **227:**597–611.
12. **Fingar, D. C., and M. J. Birnbaum.** 1994. Characterization of the mitogen-activated protein kinase/90-kilodalton ribosomal protein S6 kinase signaling pathway in 3T3-L1 adipocytes and its role in insulin-stimulated glucose transport. Endocrinology **134:**728–735.
13. **Gille, H., A. D. Sharrocks, and P. E. Shaw.** 1992. Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. Nature (London) **358:**414–417.
14. **Giorgetti, S., R. Ballotti, A. Kowalski-Chauvel, M. Cormont, and E. Van Obberghen.** 1992. Insulin stimulates phosphatidylinositol-3-kinase activity in rat adipocytes. Eur. J. Biochem. **207:**599–606.
15. **Giorgetti, S., P. G. Pelicci, G. Pelicci, and E. Van Obberghen.** 1994. Involvement of Src-homology/collagen (SHC) protein in signaling through the insulin receptor and the insulin-like-growth-factor-I-receptor. Eur. J. Biochem. **223:**195–202.
16. **Hashimoto, N., M. Ogashiwa, and S. Iwashita.** 1995. Role of tyrosine kinase in the regulation of myogenin expression. Eur. J. Biochem. **227:**379–387.
17. **Heasley, L. E., and G. L. Johnson.** 1992. The β-PDGF receptor induces neuronal differentiation of PC12 cells. Mol. Biol. Cell **3:**545–553.
18. **Hei, Y.-J., J. H. McNeill, J. S. Sanghera, J. Diamond, M. Bryer-Ash, and S. L. Pelech.** 1993. Characterization of insulin-stimulated seryl/threonyl protein kinases in rat skeletal muscle. J. Biol. Chem. **268:**13203–13213.
19. **Heller-Harrison, R. A., M. Morin, and M. P. Czech.** 1995. Insulin regulation of membrane-associated insulin receptor substrate 1. J. Biol. Chem. **270:**24442–24450.
20. **Joly, M., A. Kazlauskas, F. S. Fay, and S. Corvera.** 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. Science (Washington, D.C.) **263:**684–687.
21. **Kapeller, R., and L. C. Cantley.** 1994. Phosphatidylinositol 3-kinase. Bioessays **16:**565–576.
22. **Kelly, K. L., and N. B. Ruderman.** 1993. Insulin-stimulated phosphatidylinositol 3-kinase. Association with a 185-kDa tyrosine-phosphorylated protein (IRS-1) and localization in a low density membrane vesicle. J. Biol. Chem. **268:**4391–4398.
23. **Krebs, E. G., N. G. Ahn, J. S. Campbell, L. M. Graves, R. Seger, and J. E. Weiel.** 1993. Regulation of protein serine/threonine kinases by tyrosine kinases. Adv. Second Messenger Phosphoprotein Res. **28:**227–244.
24. **Kublaoui, B., J. Lee, and P. F. Pilch.** 1995. Dynamics of signaling during insulin-stimulated endocytosis of its receptor in adipocytes. J. Biol. Chem. **270:**59–65.
25. **Kudla, A. J., M. L. John, D. F. Bowen-Pope, B. Rainish, and B. B. Olwin.** 1995. A requirement for fibroblast growth factor in regulation of skeletal muscle growth and differentiation cannot be replaced by activation of platelet-derived growth factor signaling pathways. Mol. Cell. Biol. **15:**3238–3246.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lim, R. W., C. Y. Zhu, and B. Stringer.** 1995. Differential regulation of primary response gene expression in skeletal muscle cells through multiple signal transduction pathways. Biochim. Biophys. Acta **1266:**91–100.
28. **Milasincic, D. J., J. Dhawan, and S. R. Farmer.** 1996. Anchorage-dependent control of muscle-specific gene expression in C2C12 mouse myoblasts. In Vitro Cell. Dev. Biol. Anim. **32:**90–99.
29. **Myers, M. G., J. M. Backer, X. J. Sun, S. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen, and M. White.** 1992. IRS-1 activates phosphatidylinositol 3′-kinase by associating with src homology 2 domains of p85. Proc. Natl. Acad. Sci. USA **89:**10350–10354.
30. **Myers, M. G., Jr., T. C. Grammer, L.-M. Wang, X. J. Sun, J. H. Pierce, J. Blenis, and M. F. White.** 1994. Insulin receptor substrate-1 mediates phosphatidylinositol 3′-kinase and $p70^{S6k}$ signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. J. Biol. Chem. **269:**28783–28789.
31. **Nakanishi, S., H. Yano, and Y. Matsuda.** 1995. Novel functions of phosphatidylinositol 3-kinase in terminally differentiated cells. Cell. Signalling **7:**545–557.
32. **Nguyen, T. T., J.-C. Scimeca, C. Filloux, P. Peraldi, J.-L. Carpentier, and E. Van Obberghen.** 1993. Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. J. Biol. Chem. **268:**9803–9810.
33. **Ohmichi, M., L. Pang, V. Ribon, A. Gazit, A. Levitzki, and A. Saltiel.** 1993. The tyrosine kinase inhibitor tyrphostin blocks the cellular actions of nerve growth factor. Biochemistry **32:**4650–4658.
34. **Olwin, B. B., and S. D. Hauschka.** 1989. Cell type and tissue distribution of the fibroblast growth factor receptor. J. Cell. Biochem. **39:**443–454.
35. **Pang, L., T. Sawada, S. J. Decker, and A. R. Saltiel.** 1995. Inhibition of MAP kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. **270:**13585–13588.
36. **Porras, A., A. R. Nebreda, M. Benito, and E. Santos.** 1992. Activation of Ras by insulin in 3T3 L1 cells does not involve GTPase-activating protein phosphorylation. J. Biol. Chem. **267:**21124–21131.
37. **Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki, and J. R. Woodgett.** 1991. Phosphorylation of c-jun mediated by MAP kinase. Nature (London) **353:**670–674.
38. **Raffioni, S., and R. A. Bradshaw.** 1992. Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. Proc. Natl. Acad. Sci. USA **89:**9121–9125.
39. **Ray, L. B., and T. W. Sturgill.** 1988. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine *in vivo*. Proc. Natl. Acad. Sci. USA **85:**3753–3757.
40. **Robinson, L. J., Z. F. Razzack, J. C. Lawrence, Jr., and D. E. James.** 1993. Mitogen-activated protein kinase activation is not sufficient for stimulation of glucose transport or glycogen synthase synthase in 3T3-L1 adipocytes. J. Biol. Chem. **268:**26422–26427.
41. **Rossomando, A. J., D. M. Payne, M. J. Weber, and T. W. Sturgill.** 1989. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc. Natl. Acad. Sci. USA **86:**6940–6943.
42. **Sasaoka, T., D. W. Rose, B. H. Jhun, A. R. Saltiel, B. Draznin, and J. M. Olefsky.** 1994. Evidence for a functional role of Shc proteins in mitogenic signaling by insulin, insulin-like growth factor-1, and epidermal growth factor. J. Biol. Chem. **269:**13689–13694.
43. **Seger, R., and E. G. Krebs.** 1995. The MAPK signaling cascade. FASEB J. **9:**726–735.
44. **Shaoul, E., R. Reich-Slotky, B. Berman, and D. Ron.** 1995. Fibroblast growth factor receptors display both common and distinct signaling pathways. Oncogene **10:**1553–1561.
45. **Skolnik, E. Y., A. Batzer, N. Li, C.-H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis, and J. Schlessinger.** 1993. The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science (Washington, D.C.) **260:**1953–1955.
46. **Sun, X. J., P. Rothenberg, C. R. Kahn, J. M. Backer, E. Araki, P. A. Wilden, D. A. Cahill, B. J. Goldstein, and M. F. White.** 1991. Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (London) **352:**73–77.
47. **Tsakiridis, T., H. E. McDowell, T. Walker, C. P. Downes, H. S. Hundal, M. Vranic, and A. Klip.** 1995. Multiple roles of phosphatidylinositol 3-kinase in regulation of glucose transport, amino acid transport, and glucose transport-

ers in L6 skeletal muscle cells. Endocrinology **136:**4315–4322.
48. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212.
49. **White, M. F.** 1995. The IRS-1 signaling system. Curr. Opin. Genet. Dev. **4:**47–54.
50. **White, M. F., and C. R. Kahn.** 1994. The insulin signaling system. J. Biol. Chem. **269:**1–4.
51. **Yamauchi, K., K. Holt, and J. E. Pessin.** 1993. Phosphatidylinositol 3-kinase functions upstream of Ras and Raf in mediating insulin stimulation of c-*fos* transcription. J. Biol. Chem. **268:**14597–14600.
52. **Yonezawa, K., H. Ueda, K. Hara, K. Nishida, A. Ando, A. Chavanieu, H. Matsuba, K. Shii, K. Yokono, and Y. Fukui.** 1992. Insulin-dependent formation of a complex containing an 85-kDa subunit of phosphatidylinositol 3-kinase and tyrosine-phosphorylated insulin receptor. J. Biol. Chem. **267:** 25958–25965.
53. **Zhou, G. X., K. E. Meier, and M. G. Buse.** 1993. Sequential activation of two mitogen activated protein (MAP) kinase isoforms in rat skeletal muscle following insulin injection. Biochem. Biophys. Res. Commun. **197:**578–584.