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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†

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The distinct effects of cytokines on cellular growth and differentiation suggest that specific signaling pathways mediate these diverse biological activities. Fibroblast growth factors (FGFs) are well-established inhibitors of skeletal muscle differentiation and may operate via activation of specific signaling pathways distinct from recently identified mitogen signaling pathways. We examined whether platelet-derived growth factor (PDGF)-activated signaling pathways are sufficient to mediate FGF-dependent repression of myogenesis by introducing the PDGF β receptor into a mouse skeletal muscle cell line. Addition of PDGF-BB to cells expressing the PDGF β receptor activated the PDGF β receptor tyrosine kinase, stimulated mitogen-activated protein (MAP) kinase, and increased the steady-state levels of *junB* and c-*fos* mRNAs. Despite the activation of these intracellular signaling molecules, PDGF β receptor activation elicited no detectable effect on cell proliferation or differentiation. In contrast to PDGF-BB, addition of FGF-2 to myoblasts activated signaling pathways that resulted in DNA synthesis and repression of differentiation. Because of the low number of endogenous FGF receptors expressed, FGF-stimulated signaling events, including tyrosine phosphorylation and activation of MAP kinase, could be detected only in cells expressing higher levels of a transfected FGF receptor cDNA. As the PDGF β receptor- and FGF receptor-stimulated signaling pathways yield different biological responses in these skeletal muscle cells, we hypothesize that FGF-mediated repression of skeletal muscle differentiation activates signaling pathways distinct from those activated by the PDGF β receptor. Activation of PDGF β receptor tyrosine kinase activity, stimulation of MAP kinase, and upregulation of immediate-early gene expression are not sufficient to repress skeletal muscle differentiation.

Intracellular signaling cascades initiated by growth factor binding to cell surface tyrosine kinase receptors transmit signals that control cell proliferation, differentiation, cell migration, and cell fate. We are examining the fibroblast growth factor (FGF)-mediated signaling pathways that control proliferation and differentiation of skeletal muscle cells. The effects of FGF on both skeletal muscle cell proliferation and differentiation have been well documented in studies using cell lines (40) and primary cell cultures (1, 46, 53).

A satellite cell line (MM14) derived from an adult mouse muscle exhibits an absolute dependence on exogenously supplied FGFs (12, 34). Removal of FGF from the culture medium results in terminal differentiation, which initiates with an irreversible withdrawal from the cell cycle, is followed by expression of muscle-specific genes and culminates with fusion into multinucleated myotubes. The biological activity of FGFs on these cells requires an interaction with heparan sulfate proteoglycans (HSPGs) (43, 48). HSPGs have been proposed to form a high-affinity FGF-binding and signaling complex with the FGF receptor tyrosine kinase 1, the only identified FGF receptor tyrosine kinase in MM14 cells (30, 57). Although a pathway involving the receptor kinase is likely to be required for generating the FGF response, signaling by other FGF receptors, including the HSPGs and a cysteine-rich FGF receptor (8), has not been excluded. Furthermore, a role for intracellular FGF in other cell types has been reported, but the mechanism of action has not been determined (5, 24, 61).

Distinct signaling pathways may be involved in modulating proliferation and differentiation of skeletal muscle cells as MM14 cell proliferation requires both FGF and serum. In the absence of serum and the presence of FGF, MM14 cells do not terminally differentiate but are reversibly quiescent (12). Only when FGF is withdrawn do the cells terminally differentiate (12). To analyze the signaling pathways activated by FGF, we determined if signaling by the platelet-derived growth factor (PDGF) β receptor could replace FGF signaling in skeletal muscle cells. In PC12 cells expressing the PDGF β receptor, both FGF and PDGF stimulate neuronal differentiation via activation of Ras and mitogen-activated protein (MAP) kinase (23). We have examined PDGF-BB-activated signaling in skeletal muscle cells overexpressing the PDGF β receptor. We report that PDGF-BB stimulation of PDGF β receptor-expressing MM14 myoblasts activated signaling pathways that result in autophosphorylation of the PDGF β receptor, activation of MAP kinase, and increases in the steady-state levels of *junB* and c-*fos* mRNAs. However, we observed no effect of

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PDGF β receptor stimulation on skeletal muscle cell growth or differentiation. These data indicate that activation of endogenous MAP kinase, immediate-early genes, and other signaling events initiated by an activated PDGF β receptor are not sufficient to regulate skeletal muscle cell growth or differentiation. Thus, FGF mediates repression of skeletal muscle differentiation through signaling pathways distinct from those activated by the PDGF β receptor.

MATERIALS AND METHODS

Cell culture and stable transfection. Mouse MM14 cells (34) were cultured on gelatin-coated plates in growth medium consisting of Ham's F-10 (Gibco) supplemented with 0.8 mM $CaCl_2$, 100 U of penicillin G per ml, 5 μg of streptomycin sulfate per ml, and 15% horse serum. The concentration of FGF-2 (purified from a yeast strain expressing human FGF-2 [47]) was increased from 0.3 to 2.5 nM with increasing cell density.

A human PDGF β receptor cDNA controlled by the simian virus 40 enhancer and an adenovirus late promoter (pDX-hPDGFR expression vector) (19) and a vector containing the neomycin resistance gene (pKO-neo) (59) were introduced into MM14 cells by calcium phosphate-mediated transfection (52). PDGF β receptor-expressing cells were isolated from neomycin-resistant cells by fluorescence-activated cell sorting, using anti-PDGF receptor monoclonal antibody PR7212 (22), biotinylated goat anti-mouse immunoglobulin G (IgG), and fluorescein isothiocyanate-avidin. The isolated cells were subcloned to derive cell clones (MM14/PR clones) that had morphology and differentiation kinetics similar to those of nontransfected MM14 cells.

An expression vector containing the mouse FGF receptor 1 cDNA under the control of the Molony murine leukemia virus long terminal repeat (63) (provided by David Ornitz, Washington University Medical School, St. Louis, Mo.) was cotransfected by the calcium phosphate method (52) with pHyg (56) to confer hygromycin resistance to transfected cells. Hygromycin-resistant cells (MM14/FR1 cells) were selected. Binding of ^{125}I-FGF-2 to nontransfected and FGF receptor 1-transfected cells indicates that transfected cells express 7.5-fold more FGF-2 high-affinity binding sites than do nontransfected cells (30).

^{125}I-PDGF binding. Specific binding of ^{125}I-PDGF was determined essentially as previously described (54). Briefly, the wells to be used for determination of nonspecific binding were preincubated for 2 h with 200 ng of PDGF-BB per ml. Wells were then incubated at 4°C for 3 h with increasing concentrations of ^{125}I-PDGF, and cell-associated radioactivity was harvested with 1% Triton X-100. Specific binding was calculated by subtracting binding not eliminated by the preincubation with unlabeled PDGF-BB. Three additional wells were used to determine cell number. Specific activities were 19,369 cpm/ng for ^{125}I-PDGF-AA and 2,104 cpm/ng for ^{125}I-PDGF-BB.

Immunoblot analysis. (i) Anti-PDGF receptor. Cell lysates were prepared by solubilizing cells in a mixture containing 50 mM NaH_2PO_4 (pH 7.4), 100 mM NaCl, 4 mM EDTA, 1% Triton X-100, 1 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Extracts were clarified by centrifugation, and the protein content was determined by the bicinchoninic acid protein assay (Pierce). Extracts containing 50 μg of protein were separated on 7.5% acrylamide gels by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions, and proteins were electrophoretically transferred to an Immobilon membrane (Millipore). Nonspecific membrane binding sites were blocked with 50 mM Tris (pH 7.4)–100 mM NaCl–0.1% Tween 20–0.25% gelatin. The membrane was incubated with 10 μg of anti-PDGF receptor antibody PR7212 (22) per ml followed by ^{125}I-labeled goat anti-mouse IgG. Bands were visualized by autoradiography.

(ii) Antiphosphotyrosine. Proliferating cells were washed and incubated in growth medium without FGF-2 for 10 h. Cells were stimulated as indicated for 10 min and then solubilized in a mixture containing 25 mM Tris (pH 7.4), 50 mM NaCl, 5 mM EDTA, 50 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate, 1% Triton X-100, 2 μg of leupeptin per ml, 2 μg of aprotinin per ml, 1 mM PMSF, and 2 mM EGTA. Solubilized cell lysates were clarified by centrifugation, and protein content was quantitated by the bicinchoninic acid protein assay. Twenty micrograms of protein from MM14 and MM14/PR cells and 10 μg of protein from MM14/FR1 cells were separated by SDS-PAGE on 7.5% acrylamide gels, and proteins were electrophoretically transferred to an Immobilon membrane. Phosphotyrosine-containing proteins were detected by using horseradish peroxidase-conjugated antiphosphotyrosine monoclonal antibody RC20 (Transduction Laboratories) and chemiluminescence as instructed by the manufacturer.

MAP kinase assays. Proliferating MM14/PR cells or MM14/FR1 cells were washed and incubated in growth medium with 2% horse serum and without FGF-2 for 4 to 5 h. Cells were stimulated with the specified factor for the indicated time. Cell lysates were prepared by washing cells with iced Tris-buffered saline (50 mM Tris [pH 7.4], 150 mM NaCl), scraping into homogenization buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 5 mM *p*-nitrophenyl phosphate, 1 mM sodium orthovanadate, 10 mM pyrophosphate decahydrate, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM PMSF), sonicating, and centrifuging to remove insoluble particulates. Lysates were adjusted to 1% Triton X-100–0.5% sodium deoxycholate–0.1% SDS. Anti-MAP kinase polyclonal antibody bound to protein A-Sepharose was incubated with each lysate for 2 to 3.5 h. The immunoprecipitates were washed with homogenization buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS and then subjected to a final wash with 20 mM Tris (pH 7.4). The washed immunoprecipitates were resuspended in reaction buffer (12.8 mM Tris [pH 7.4], 5.5 mM *p*-nitrophenyl phosphate, 51.5 mM $MgCl_2$, 2 mg of myelin basic protein per ml, 0.5 mCi of [γ-^{32}P]ATP [~3,000 Ci/mmol; Amersham] per ml) and incubated at 30°C for 30 min. The reaction mix was separated by electrophoresis on 12% polyacrylamide gels by SDS-PAGE. Phosphorylation of myelin basic protein was visualized by autoradiography and quantitated on a Molecular Dynamics PhosphoImager.

The anti-MAP kinase antibody was a gift from Curtis Ashendel (Purdue University) and was generated by immunizing rabbits with a mouse MAP kinase glutathione *S*-transferase fusion protein (13). The antibodies recognize the 42- and 44-kDa forms of MAP kinase, as determined by immunoblotting of crude soluble cell lysates with both the anti-MAP kinase and antiphosphotyrosine primary antibodies (2).

RNase protection assay. Proliferating MM14/PR cells were washed and incubated in growth medium with 2% horse serum and without FGF-2 for 10 h. Cells were stimulated for 45 min with the indicated growth factor, and total RNA was isolated (10). Analysis of immediate-early gene mRNA levels over a 4-h time course of growth factor treatment demonstrated that expression levels peaked at 45 min (27). Radiolabeled RNA probes with a specific activity of 3×10^8 cpm/μg were transcribed in vitro according to Promega's standard transcription protocol (44), using [α-^{32}P]UTP (~3,000 Ci/mmol; Amersham) and the following DNA templates. A *Hin*dIII fragment of mouse c-*myc* cDNA (55) in the *Hin*dIII site of pGEM-4 (Promega) (construct provided by Elizabeth Taparowsky, Purdue University) was linearized with *Xmn*I, and the probe was transcribed with T7 RNA polymerase; mouse *junB* cDNA (51) in the *Eco*RI site of pGEM-2 (Promega) (construct provided by Eric Olson, The University of Texas M. D. Anderson Cancer Center) was linearized with *Xho*I, and the probe was synthesized with SP6 RNA polymerase; mouse *junD* cDNA (50) in the *Eco*RI site of pBluescript (Stratagene) (construct provided by Eric Olson) was linearized with *Dra*I, and the probe was transcribed with T7 RNA polymerase; mouse c-*fos* cDNA (58) in the *Eco*RI site of pGEM-4 (Promega) (construct provided by Anthony Lanahan, Johns Hopkins University) was linearized with *Hga*I, and the probe was synthesized with T7 RNA polymerase. Following transcription, the DNA template was digested with DNase, and the entire reaction mix was separated by electrophoresis through a 6% Long Ranger acrylamide (AT Biochem) denaturing gel. The area of the gel containing the probe was excised, and the probe was eluted in 0.5 M ammonium acetate with 1 mM EDTA and 0.2% SDS. RNase protection assays were performed with an RPA II RNase protection assay kit (Ambion, Inc.) as instructed by the manufacturer. Briefly, the indicated amount of total RNA was mixed with 9×10^4 cpm of gel-purified radiolabeled RNA probe. Hybridization buffer was added, and the RNA was denatured by heating at 95°C. Two hybridizations with yeast RNA and probe were also done as controls. The samples and probes were allowed to hybridize at 43°C for approximately 24 h. Unhybridized single-stranded RNA was digested with 0.5 U of RNase A and 10 U of RNase T_1 at 37°C for 30 min. RNases were added to only one of the two yeast RNA control hybridizations. The RNase was inactivated, and the hybridized RNAs were precipitated, solubilized in gel loading buffer, denatured, and analyzed by electrophoresis through a 6% Long Ranger acrylamide denaturing gel. The gel was dried, and phosphoimages were collected and quantitated on a Bio-Rad Molecular Imager system.

Clonal growth assays. Cells were plated onto 60-mm-diameter plates at 200 cells per plate in growth medium with the indicated growth factor. Cells were cultured for 36 h, fixed (70% ethanol–37% formaldehyde–glacial acetic acid [20:2:1 volume ratio]), nuclei were stained with hematoxylin, the number of cells per colony was determined, and the average number of population doublings was calculated.

Analysis of progression into S phase of the cell cycle. Mitotic cells were collected by gently rocking plates of proliferating MM14/PR cells, removing the medium, and pelleting the suspended cells. The pelleted cells were incubated with 0.05% trypsin and 0.53 mM EDTA for 3 min at 37°C. Iced growth medium was added, and the cells were pelleted. The cells were plated in 24-well plates in growth medium with the indicated growth factor(s). At the indicated time, 2 μCi of [^{3}H]thymidine (70 to 90 Ci/mmol; DuPont) per ml was added, the cells were incubated for an additional 2 h, and [^{3}H]thymidine incorporation was measured in a Microbeta liquid scintillation plate counter (Wallac, Inc.).

Cell cycle exit assay. Cell cycle exit was assessed by incorporation of [^{3}H]thymidine into MM14/PR cells as previously described (41). Briefly, cells were plated in 24-well plates at 2,000 cells per well in growth medium with the indicated growth factor(s). Cells were incubated for 18 h and then incubated for 9 h with 2 μCi of [^{3}H]thymidine per ml. [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

Transient transfection assays. Cells were plated at 10^5 cells per 100-mm-diameter plate 6 to 8 h before transfection. A calcium phosphate-DNA precipitate containing 5 pmol of α-cardiac actin/luciferase or 10 pmol α-cardiac actin/luciferase with 25 pmol of pDX-hPDGFR (19) was prepared in 0.55 ml of *N*-2-piperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (25 mM HEPES [pH 7.05], 140 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 6 mM

dextrose) containing 0.11 M $CaCl_2$. (The α-cardiac actin/luciferase reporter construct was a gift from Stephen Konieczny, Purdue University. The *Eco*RI site of a 245-bp *Eco*RI-*Hin*dIII fragment of pHCA177CAT [36] containing the region of the human α-cardiac actin gene from −177 bp to +68 bp was filled in, and the insert was cloned into the *Sma*I-*Hin*dIII site at the 5′ end of the luciferase gene in the pGL2-basic vector from Promega Co.) The cells were incubated with 0.5 ml of the precipitate for 20 min before addition of growth medium and 0.5 nM FGF-2. After 4 h, the cells were glycerol shocked for 2.5 min with 15% glycerol in HEPES-buffered saline. Growth medium containing 1 μM insulin and FGF-2 or PDGF-BB as indicated was added. Additional FGF-2 or PDGF-BB was added every 12 h as described for FGF-2 in "Cell culture and stable transfection" above. To block the activity of any PDGF-BB that may be in the horse serum of the growth medium in experiments in which pDX-hPDGFR was transiently transfected, 10 μl of ascites fluid containing anti-human PDGF β receptor monoclonal antibody 162.6.2.6.2 (supplied by Charles Hart, ZymoGenetics, Inc., Seattle, Wash.), which neutralizes PDGF-BB activity (21), was added to the medium of cells treated with FGF-2 or with no growth factor. The cells were harvested for assay of luciferase activity and protein content 36 to 38 h following the glycerol incubation. Luciferase activity was assayed in a Berthold Lumat luminometer by using the luciferase assay system as instructed by the manufacturer (Promega) except for the addition of 2 mM PMSF and 1 μg of leupeptin per ml to the cell solubilization buffer. Protein content was determined by the method of Bradford (6). Light intensity per microgram of protein was calculated for each cell extract. Relative activity of α-cardiac actin/luciferase was calculated by dividing the light intensity per microgram of protein for each treatment by the light intensity per microgram of protein for non-growth factor-treated cells and multiplying by 100. All transfections were done in triplicate.

Analysis of DNA synthesis and myosin heavy-chain expression in single cells. Cells were plated in six-well plates at 1.5×10^4 cells per well in growth medium containing 300 pM FGF-2. After 19 h, the cells were washed and growth medium alone or with 1 nM FGF-2 or 0.7 nM PDGF-BB was added. The medium was replaced every 12 h. After 48 h, 2 μCi of [^{3}H]thymidine (70 to 90 Ci/mmol; DuPont) per ml was added and the cells were incubated for an additional 8.5 h. The cells were treated with 2% trichloroacetic acid and 0.1% sodium pyrophosphate and then fixed as described for clonal growth assays. Myosin heavy chain was immunostained with monoclonal antibody MF-20 (3) followed by biotinylated anti-mouse IgG (Vector Laboratories). Detection was by avidin/biotinylated horseradish peroxidase and diaminobenzidine as instructed by the manufacturer (Vector Laboratories). For detection of [^{3}H]thymidine, the plates were coated with NTB2 autoradiography emulsion (Kodak), exposed for 9 days, and developed according to Kodak's directions.

RESULTS

MM14 skeletal muscle cells were transfected with the PDGF β receptor cDNA to determine if signaling from this receptor can elicit the biological responses mediated by FGF-2. Similar experiments performed with PC12 cells showed that PDGF-BB elicits neurite outgrowth by using pathways common to those activated by the endogenous FGF receptor (23). PDGF β and PDGF α receptor expression are undetectable in MM14 cells (Fig. 1). Following transfection with a PDGF β receptor-expressing plasmid, cells expressing the highest levels of PDGF β receptor were isolated by fluorescence-activated cell sorting. Individual clones exhibiting morphologies and differentiation kinetics similar to those of nontransfected cells were isolated and expanded. Saturation binding analysis of one of these clones, MM14/PR 1, indicates approximately 35,000 PDGF β receptors per cell (Fig. 1A), 50-fold more than the number of endogenous FGF receptors (42). As expected, cells expressing the PDGF β receptor do not bind PDGF-AA (Fig. 1B). Immunoblot analysis of MM14/PR 1 cells with an anti-PDGF β receptor antibody confirmed expression of PDGF β receptors (Fig. 1C). The results subsequently shown are from the MM14/PR 1 clone; however, we have isolated a second PDGF β receptor-expressing clone that responds similarly to PDGF-BB.

To assess whether addition of PDGF-BB to cells expressing the PDGF β receptor activated the receptor tyrosine kinase, we removed FGF from MM14/PR cells and then added PDGF-BB. Tyrosine phosphorylation of the receptor kinase and other intracellular proteins was observed (Fig. 2A). FGF-stimulated tyrosine phosphorylation could be detected only in MM14 cells that were transfected with FGF receptor 1 (Fig. 2B), suggesting that the low number of endogenous FGF receptors expressed (only approximately 700 receptors per cell) (42) is not sufficient to generate a detectable change in tyrosine

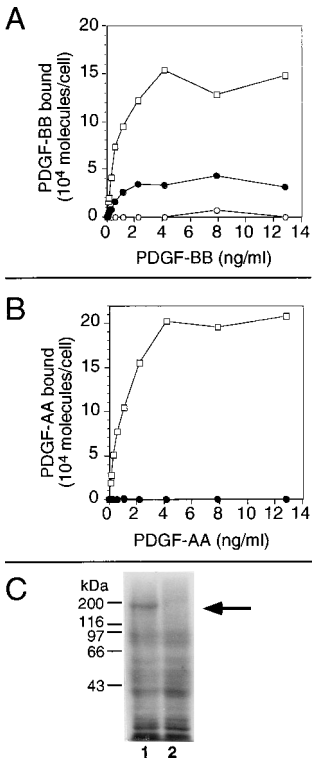

FIG. 1. PDGF receptor expression in MM14 and MM14/PR 1 cells. ^{125}I-PDGF-BB (A) and ^{125}I-PDGF-AA (B) binding to nontransfected MM14 cells (○), MM14 cells transfected with the PDGF β receptor (●), and human dermal fibroblasts (□) was determined. Data are expressed as the number of PDGF molecules specifically bound per cell and are averages of triplicate analyses. Standard deviation was always less than 10% of the average. (C) Immunoblot analysis of PDGF β receptor expression in MM14 and MM14/PR 1 cells. PDGF β receptor expression was detected by an anti-PDGF receptor monoclonal antibody and visualized with ^{125}I-labeled anti-mouse IgG and autoradiography. Lane 1, lysate from MM14/PR 1 cells; lane 2, lysate from untransfected MM14 cells. The position of the PDGF receptor is indicated by an arrow. Migration of molecular mass markers is indicated on the left.

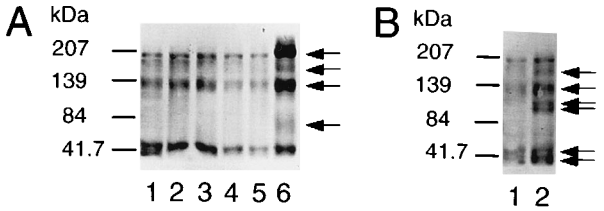

FIG. 2. Stimulation of tyrosine phosphorylation by PDGF-BB in MM14/PR cells and by FGF-2 in MM14/FR1 cells. (A) Nontransfected (lanes 1 to 3) and PDGF β receptor-transfected (lanes 4 to 6) MM14 cells were deprived of FGF for 10 h and were then left untreated (lanes 1 and 4) or stimulated for 10 min with 1 nM FGF-2 (lanes 2 and 5) or 1 nM PDGF-BB (lanes 3 and 6). Cell lysates were prepared as described in Materials and Methods and were analyzed by immunoblotting with an antiphosphotyrosine antibody conjugated to horseradish peroxidase. Bound antibody was visualized by using a chemiluminescent peroxidase substrate. (B) FGF receptor 1-transfected cells were deprived of FGF for 10 h and were then left unstimulated (lane 1) or stimulated with 1 nM FGF-2 (lane 2). Antiphosphotyrosine immunoblot analysis of cell lysates was as described for panel A. Arrows indicate proteins tyrosine phosphorylated in response to PDGF-BB (A) and FGF-2 (B). In panel A, the top arrow indicates the PDGF β receptor. Migration of molecular weight markers is indicated on the left.

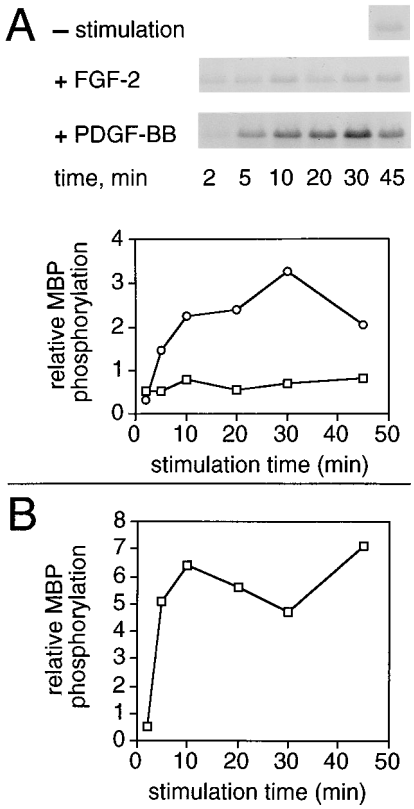

FIG. 3. Growth factor activation of MAP kinase in MM14/PR and MM14/FR1 cells. (A) Proliferating MM14/PR cells were incubated in 2% horse serum for 4.5 h. Cells were then treated for the indicated time with either 1 nM FGF-2 (□) or 1 nM PDGF-BB (○). MAP kinase was immunoprecipitated from cell lysates by using an anti-MAP kinase polyclonal antiserum. The MAP kinase substrate myelin basic protein (MBP) and [γ-^{32}P]ATP were incubated with immunoprecipitated MAP kinase, and the reaction products separated by SDS-PAGE. Phosphorylation of myelin basic protein was visualized by autoradiography and quantitated by phosphoimage analysis. (B) Proliferating MM14/FR1 cells were incubated in 2% horse serum for 5 h. Cells were then stimulated with 1 nM FGF-2, and MAP kinase activity was assayed as described for panel A.

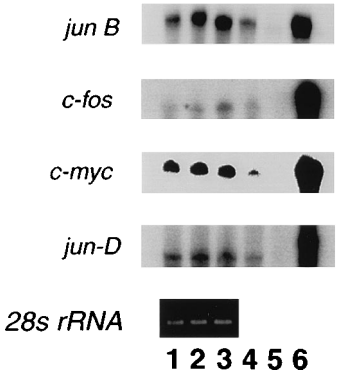

FIG. 4. FGF-2 and PDGF-BB regulation of immediate-early gene expression in MM14/PR cells. Proliferating cells were washed and incubated in 2% horse serum for 10 h and then either left unstimulated (lane 1) or stimulated for 45 min with 1 nM FGF-2 (lane 2) or 1 nM PDGF-BB (lanes 3 and 4). RNase protection analyses were done as described in Materials and Methods, using 20 μg of total RNA for the assays shown in lanes 1 to 3 and 5 μg of total RNA for the assays shown in lane 4. Lanes 5 and 6 are controls in which the probe was incubated with yeast RNA and then treated with RNase (lane 5) as were the samples in lanes 1 to 4 or mock treated without RNase added (lane 6). The amount of probe hybridized to each of the indicated RNA samples was quantitated by phosphoimage analysis. Ethidium bromide staining of 0.25 μg of RNA isolated from cells treated with each of the growth factors confirms that equal amounts of RNA were used in the RNase protection assays.

phosphorylation. FGF and PDGF stimulation yield different patterns of tyrosine phosphorylation, suggesting activation of unique intracellular signal transduction pathways. These data indicate that the expressed PDGF β receptor is capable of autophosphorylation and phosphorylation of intracellular substrates in a cell type that does not normally express either PDGF receptor isoform. Thus, the PDGF β receptor is competent to activate intracellular signaling pathways; however, these pathways may be different from those activated by the FGF receptor.

Because tyrosine kinase activity is stimulated following addition of PDGF-BB to PDGF β receptor-expressing MM14 cells, we predicted that intracellular signaling pathways dependent on receptor autophosphorylation would be activated. Therefore, we examined the ability of the activated PDGF β receptor to stimulate MAP kinase, as this signaling protein has been implicated in cellular differentiation (26). We observed activation of MAP kinase (3.3-fold) upon addition of PDGF-BB to cells expressing the PDGF β receptor (Fig. 3A). As expected, an intact PDGF signaling pathway is present from the cell membrane to the cytoplasm. In contrast, FGF addition resulted in minimal elevation of MAP kinase activity over a 45-min time course (Fig. 3A). FGF-stimulated MAP kinase activation could also not be detected in parental MM14 cells (30). It is possible that the FGF signaling pathway does not include activation of MAP kinase or that the low level of endogenous FGF receptor (42) does not activate MAP kinase sufficiently for detection in our assay. The latter is suggested by the observation that activation of MAP kinase by FGF-2 can be detected in MM14 cells overexpressing mouse FGF receptor 1 (Fig. 3B).

As our data demonstrate that PDGF binding to its receptor transduces a signal to the cytoplasm, we examined, by RNase protection analysis of the mRNA levels of four nuclear proto-oncogenes (*junB*, c-*fos*, c-*myc*, and *junD*), whether the signal was relayed into the nucleus. In serum-starved BALB/c 3T3 cells, activation of PDGF receptors by PDGF induces transcription of *junB*, c-*fos*, and c-*myc* (18, 28, 32, 51); therefore, we expect a similar transcriptional induction to occur in PDGF β receptor-expressing MM14 cells upon stimulation by PDGF-BB. Because complete mitogen withdrawal for extended times leads to terminal differentiation, we were able to withdraw mitogens for only a short time before growth factor stimulation and RNA analysis. The short withdrawal times used may be responsible for the elevated background levels of mRNA in nonstimulated cells and for the low levels of gene induction following growth factor stimulation. Addition of PDGF resulted in 1.9- and 2.4-fold increases in the steady state levels of *junB* and c-*fos*, respectively (Fig. 4). The level of c-*myc* changed only 1.2-fold, while the level of *junD* remained unchanged following PDGF-BB stimulation (Fig. 4). Similar results were observed by Northern (RNA) blot analysis (30). The changes in gene expression demonstrate that the signal generated by PDGF-BB binding to its receptor is transduced to the nucleus.

In contrast to PDGF, FGF stimulation increased proto-oncogene steady-state mRNA levels less than twofold (Fig. 4). Increases of 1.5- and 1.2-fold were observed for *junB* and c-*fos*, respectively (Fig. 4). The levels of c-*myc* and *junD* increased 1.2- and 1.1-fold, respectively (Fig. 4). Similar results were observed by Northern blot analysis (30). As was suggested in the analyses of FGF-stimulated tyrosine phosphorylation (Fig.

2) and FGF-stimulated MAP kinase activity (Fig. 3), the inability to detect significant changes in FGF-stimulated gene expression could be due to the low number of endogenous FGF receptors expressed. Alternatively, proto-oncogene expression may be cell cycle regulated, as FGF is required only during the G_1 phase of the cell cycle (12). However, despite the low level of immediate-early gene expression in FGF-treated cells, the cells respond exquisitely to FGF, suggesting that either the immediate-early genes are not intimately involved in FGF mediated repression of differentiation or a low level of gene expression is sufficient to mediate the effects of FGF.

The PDGF β receptor expressed in MM14 cells was activated by PDGF-BB and transduced intracellular signals, as demonstrated by three independent assays: (i) autophosphorylation of the receptor, a cell membrane-associated signal; (ii) activation of MAP kinase, a cytoplasmic signal; and (iii) induction of immediate early proto-oncogenes, a nuclear signal. To determine if activation of these signals is sufficient to replace all or part of the signaling pathways required for myoblast growth, the effects of PDGF-BB on differentiation and proliferation were independently examined. To analyze proliferation, a clonal growth assay was performed with MM14/PR cells. Addition of PDGF-BB did not alter the number of population doublings of clones cultured in the absence of FGF (Fig. 5A). In contrast, both nontransfected control and PDGF β receptor-expressing cells proliferated in the presence of FGF-2 (Fig. 5A). Even at PDGF-BB concentrations as high as 2 nM, no cell growth response was seen (30). Moreover, PDGF-BB does not have an additive or synergistic effect on proliferation when combined with FGF-2, nor does it decrease the number of population doublings when combined with FGF-2 (30). As PDGF-BB exerts neither a positive nor a negative influence on the response to FGF-2, the PDGF β receptor-stimulated signaling pathway is unlikely to converge with the FGF signaling pathway involved in cell growth despite the fact that PDGF β receptors are expressed at levels 50-fold greater than those of the endogenous FGF receptors.

Although PDGF-BB cannot stimulate cell growth, activation of the PDGF β receptor may promote DNA synthesis. We determined if PDGF-BB could stimulate DNA synthesis by examining [^{3}H]thymidine incorporation into mitotically synchronized MM14/PR cells. In response to FGF-2, cells isolated at the M/G_1 boundary remain synchronized through the first S phase, as evidenced by their pattern of [^{3}H]thymidine uptake (Fig. 5B). Minimal [^{3}H]thymidine uptake was seen in mitotically synchronized cells if FGF-2 was absent (Fig. 5B); indistinguishable results were observed in the presence of PDGF-BB (Fig. 5B). These data suggest that PDGF-BB cannot replace the FGF signal required for passage of MM14 cells through G_1 and into S phase.

Continued growth of MM14 cells requires FGF and 15% horse serum. FGF represses myogenesis in the absence of serum but cannot promote DNA synthesis, demonstrating a requirement of serum for maintenance of cell growth. Our data demonstrate that PDGF-BB cannot replace FGF-2 for maintenance of cell growth and DNA synthesis. However, PDGF-BB may be capable of replacing the requirement of serum for proliferation and thus promote cell growth in the presence of FGF-2. To address this possibility, the ability of PDGF-BB and FGF-2 to maintain MM14/PR cells in the cell cycle was determined in different concentrations of serum. If the requirement for serum can be replaced by PDGF-BB, then at low serum concentrations in the presence of FGF-2 and PDGF-BB, DNA synthesis should be stimulated. In addition, if PDGF-BB blocks FGF signaling, then PDGF-BB in combination with FGF-2 would lead to a reduction in DNA synthesis.

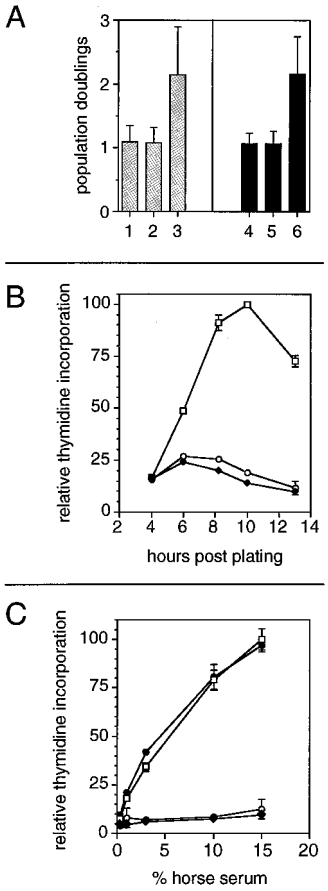

FIG. 5. PDGF receptor does not promote cell proliferation or DNA synthesis in MM14/PR cells. (A) Lack of cell proliferation in PDGF-BB-treated MM14/PR cells. Clonal growth assays of MM14 cells (▨) and MM14/PR cells (■) were performed as described in Materials and Methods. Cells were cultured at low density in 15% horse serum alone (columns 1 and 4) or in 15% horse serum supplemented with 0.2 nM PDGF-BB (columns 2 and 5) or 0.2 nM FGF-2 (columns 3 and 6). After 36 h, the number of cells in each clone was counted. A minimum of 20 clones were scored for each point. Error bars represent the standard deviation. This experiment was repeated three times with similar results. (B) PDGF-BB does not replace FGF signaling required for progression from G_1 into S phase in MM14/PR cells. Cells synchronized at the M/G_1 boundary were plated in medium containing 15% horse serum (◆), 15% horse serum plus 0.2 nM FGF-2 (□), or 15% horse serum plus 0.2 nM PDGF-BB (○). At the indicated number of hours after plating, [^{3}H]thymidine was added, and after an additional 2 h, the cells were harvested for scintillation counting. Error bars represent the standard deviation of triplicate points. This experiment was repeated twice with similar results. (C) PDGF-BB does not replace serum to maintain MM14/PR cells in the cell cycle. Cells were plated in growth medium containing the indicated concentration of horse serum supplemented with no growth factors (◆), 0.2 nM FGF-2 (□), 0.2 nM PDGF-BB (○), or 0.2 nM FGF-2 plus 0.2 nM PDGF-BB (●). After 18 h, DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation as described in Materials and Methods. Similar results were seen in two repetitions of this experiment.

In the presence of FGF-2, increasing serum concentrations resulted in a concomitant increase in DNA synthesis, reaching a maximum at 15% serum (Fig. 5C). In the absence of FGF-2 or in the presence of PDGF-BB alone, no increase in DNA synthesis was observed (Fig. 5C). When PDGF-BB and FGF-2 were combined, the results were identical to those obtained for FGF-2 alone (Fig. 5C). These data indicate that PDGF-BB cannot replace the serum requirement for MM14 cell proliferation or antagonize FGF signaling.

FGF-2 represses differentiation independently of cell proliferation, and thus the signaling pathways for these two events

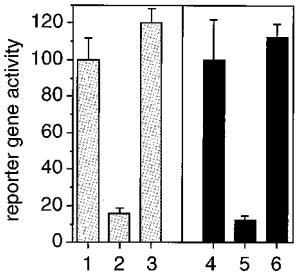

FIG. 6. Expression of a skeletal muscle-specific gene is not repressed by activation of the PDGF β receptor. Nontransfected cells (columns 1 to 3) and PDGF β receptor-transfected cells (columns 4 to 6) were transiently transfected with a luciferase reporter gene controlled by the skeletal muscle-specific α-cardiac actin promoter. Following transfection, cells were cultured in either serum alone (columns 1 and 4) or serum supplemented with FGF-2 (columns 2 and 5) or PDGF-BB (columns 3 and 6), and luciferase activity was assayed. Luciferase activity relative to activity in cells cultured in serum alone is shown. Error bars represent standard deviations.

are experimentally separable. One component of differentiation that is repressed by FGF-2 is transcription of skeletal muscle-specific genes. We examined whether the PDGF β receptor signaling pathway was capable of modulating skeletal muscle-specific gene expression by examining the ability of PDGF-BB to affect expression of a muscle-specific reporter gene (a cardiac α-actin promoter controlling expression of luciferase). Differentiation of skeletal muscle cells activates this promoter, resulting in an increase in luciferase activity. A repressor of differentiation is expected to inhibit luciferase expression, reflected in low luminescence levels. Inclusion of FGF-2 represses activation of the reporter gene in comparison with non-growth factor-treated controls (Fig. 6). These data indicate that FGF-2 functions as a repressor of skeletal muscle gene transcription. Activation of the PDGF β receptor by PDGF-BB yielded luminescence levels indistinguishable from those of 15% serum controls, demonstrating that PDGF has no detectable effect on skeletal muscle gene expression (Fig. 6).

Upon FGF withdrawal, MM14 myoblasts exit the cell cycle, express muscle-specific genes, and fuse to form multinucleated myotubes, demonstrating that FGF represses all of these phenotypes associated with terminal differentiation (12). We have shown that PDGF β receptor activation cannot maintain MM14 cells in the cell cycle and has no effect on a muscle-specific reporter gene construct. To examine whether PDGF-BB affected endogenous muscle-specific gene expression or fusion of MM14/PR cells into multinucleated myotubes, we compared the expression of skeletal muscle-specific myosin heavy chain and cellular morphology in FGF-2-treated cultures with that in PDGF-BB-treated cultures. In the absence of FGF-2, the cells do not incorporate [^{3}H]thymidine, express skeletal muscle-specific myosin heavy-chain protein, and fuse (Fig. 7A). In the presence of FGF, cells incorporate [^{3}H]thymidine but do not express myosin heavy chain and do not fuse (Fig. 7B). Myoblasts cultured in the presence of PDGF-BB (Fig. 7C) were similar to those cultured in the absence of FGF-2, indicating that activation of PDGF β receptor signaling pathways has no effect on the morphology associated with the differentiated skeletal muscle cell phenotype. In MM14 myoblasts, a low percentage of cells that have lost the capacity to differentiate even when growth factors are removed (differentiation-defective cells) arise. Additional experiments have shown that differentiation-defective cells isolated from the MM14/PR cell line respond to PDGF-BB as a mitogen (30). In the population of MM14/PR cells treated with PDGF-BB, a cell that has

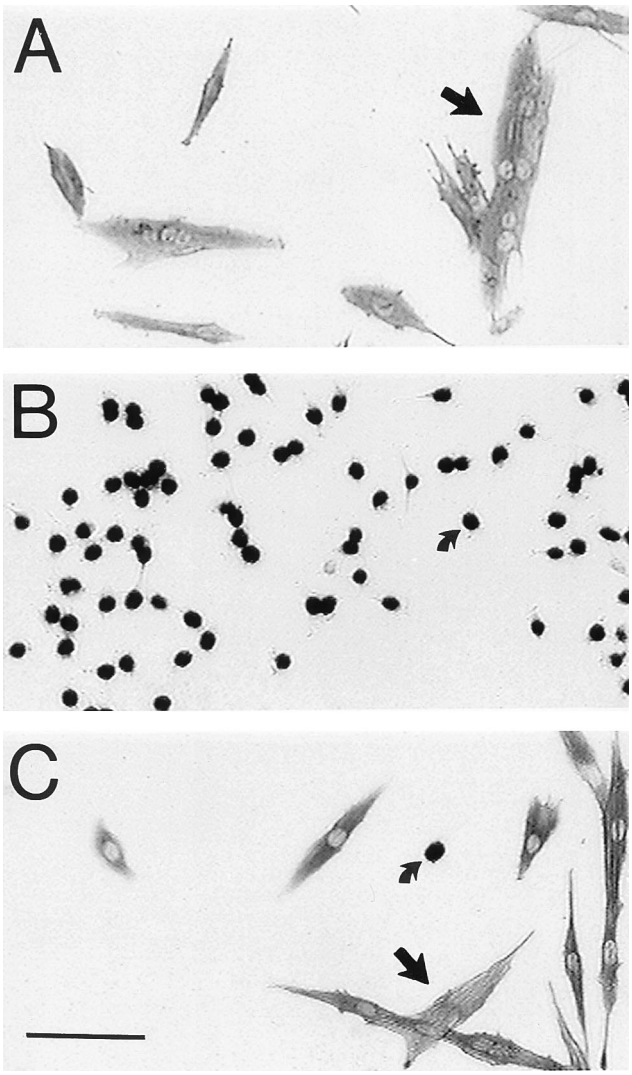

FIG. 7. PDGF receptor activation does not inhibit endogenous muscle-specific gene expression or fusion to form multinucleated cells. MM14/PR cells were cultured for 48 h in 15% horse serum alone (A) or in combination with 1 nM FGF-2 (B) or 0.7 nM PDGF-BB (C). [^{3}H]thymidine was added, and the cells were incubated for an additional 8.5 h before fixation. As described in Materials and Methods, the cells were immunostained for myosin heavy chain. [^{3}H]thymidine was detected by autoradiography. For all panels, the bar represents 0.1 mm. The light gray color represents myosin heavy chain staining in differentiated cells (large arrows), and the black dots are silver grains representing [^{3}H]thymidine incorporated by proliferating cells (small arrows).

incorporated [^{3}H]thymidine is indicated (Fig. 7C). This most likely represents a differentiation-defective cell. Because these cells arise at a very low frequency, their mitogenic response to PDGF-BB would not be detectable in our other growth assays (Fig. 5).

We have isolated two independent cell clones that express PDGF β receptor. The two clones respond similarly to PDGF-BB. To address the possibility that our cloning procedure selected against cells responsive to PDGF-BB, we transiently cotransfected MM14 cells with plasmids containing the PDGF β receptor cDNA and the cardiac α-actin/luciferase reporter gene. As expected, luciferase activity was increased in the absence of FGF-2, while inclusion of FGF repressed luciferase activity (Fig. 8). Consistent with the data for the clones of MM14 cells stably expressing the PDGF β receptor, addition

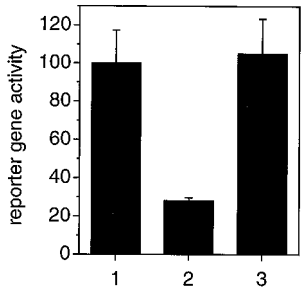

FIG. 8. PDGF-BB does not repress muscle differentiation in MM14 cells transiently transfected with the PDGF β receptor. MM14 cells were cotransfected transiently with the PDGF β receptor and the α-cardiac actin promoter/luciferase gene construct and then cultured in 15% serum alone (lane 1) or serum in addition to FGF-2 (lane 2) or PDGF-BB (lane 3). Luciferase activity was assayed and is expressed as activity relative to activity in cells cultured in serum alone. Error bars represent standard deviations.

of PDGF-BB to cells transiently transfected with the PDGF β receptor did not inhibit expression of the cardiac α-actin/luciferase reporter gene (Fig. 8).

DISCUSSION

The mechanisms involved in generating specificity in growth factor responses in different cell types are not understood. Among the diverse activities mediated by the FGF family are induction of mesoderm, limb outgrowth and patterning, stimulation of neurite outgrowth and neuronal survival, angiogenesis, and repression of myogenic differentiation (4, 16, 37, 49). Some of these specific signals in different cell types are initiated by binding of the same growth factor (FGF-2) to the cells and activation of similar or identical tyrosine kinase receptors. Specific responses elicited by the same growth factor in different cell types could be generated by (i) differences in the extent of activation of common signaling pathways, (ii) activation of distinct signaling pathways, (iii) presence of different negative regulators of a pathway, or (iv) differences in the activation of additional growth factors that act in an autocrine fashion.

Different growth factors can also elicit the same or a different effect in a single cell type. In PC12 cells, neurite outgrowth is stimulated by FGF and nerve growth factor (NGF), while epidermal growth factor (EGF) promotes mitogenesis. FGF and NGF signal the cells to withdraw from the cell cycle and differentiate, while EGF signals DNA synthesis and mitosis (9). Introduction of the PDGF β receptor into PC12 cells results in neurite outgrowth following PDGF-BB addition (23). Thus, the PDGF β receptor signals via a common pathway used by NGF and FGF. Using PDGF β receptor point mutants, critical modulators of neurite outgrowth stimulation can be identified.

We introduced the PDGF β receptor into the MM14 skeletal muscle cell line to determine if PDGF-BB could replace all or part of the FGF signaling pathway, which is required for repression of differentiation and for cellular proliferation. Addition of PDGF-BB to MM14 cells expressing PDGF β receptors activates an intact signaling pathway that can be traced from the cell surface, through the cytoplasm, and into the nucleus. However, PDGF-BB surprisingly does not replace FGF signaling, nor does PDGF-BB interfere with FGF signaling even though the PDGF β receptor is expressed at levels 50-fold in excess of the endogenous FGF receptor (i.e., 35,000 and 700 receptors per cell, respectively).

Addition of PDGF-BB to MM14 cells expressing the PDGF β receptor activated the receptor kinase, resulting in receptor autophosphorylation. Although a number of signaling pathways are activated by autophosphorylation of the PDGF β receptor (11), stimulation of these pathways is insufficient to modulate FGF-dependent growth or differentiation of skeletal muscle cells. We analyzed the activation of MAP kinase, an enzyme stimulated by activation of the PDGF β receptor and involved in growth factor signaling (35). We have shown that PDGF-BB activates MAP kinase in PDGF β receptor-transfected MM14 cells and yet has no effect on cell proliferation or differentiation. Moreover, treatment of MM14 cells with the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate also activates MAP kinase activity without an effect on MM14 cell growth or differentiation (30). Activation of MAP kinase by FGF was not detected in nontransfected or in PDGF β receptor-transfected MM14 cells. In contrast, FGF activation of MAP kinase was observed in MM14 cells expressing elevated levels of FGF receptor 1, suggesting that our inability to detect MAP kinase activation may be due to the low number of endogenous FGF receptors. Activation of MAP kinase by PDGF-BB is insufficient to replace FGF signaling, and FGF fails to detectably activate MAP kinase in MM14 myoblasts expressing only endogenous FGF receptors. Thus, we postulate that FGF receptor 1 activates a pathway distinct from the MAP kinase pathway activated by the PDGF β receptor.

A nuclear event activated by stimulation of growth factor signaling pathways is gene transcription. Transcription of several immediate-early genes is enhanced upon activation of the PDGF β receptor (18, 28, 32, 51). Among these genes are c-*myc*, c-*fos*, and *junB*, all of which are proposed to repress myogenic differentiation when expressed under nonphysiological conditions in skeletal muscle cells (39). However, increases in the steady-state levels of mRNA for c-*fos* and *junB* following PDGF-BB stimulation of MM14 cells expressing the PDGF β receptor did not affect differentiation or cell growth. The levels of immediate-early gene activation seen after stimulation by both PDGF and FGF in MM14 cells are lower than levels in studies with noncycling mouse fibroblasts (28, 29). This difference could be a consequence of the inability to serum starve MM14 cells for prolonged periods of time. Under nonphysiological conditions of extensive serum starvation, immediate-early genes can be induced 20- to 40-fold (28, 29). However, under conditions in which MM14 cells are capable of exhibiting a complete biological response to FGF, minimal induction of c-*fos*, c-*myc*, and *junB* is seen, and moreover, a low level of induction of these genes by PDGF-BB is insufficient to mediate FGF signaling.

In skeletal muscle cells, FGFs repress the permanent acquisition of a terminally differentiated state independent of promoting proliferation (12). Repression of myogenesis is ultimately achieved by inhibiting the transcriptional and posttranscriptional activation of one or more of four skeletal muscle specific transcription factors: MyoD, myogenin, MRF4, and Myf-5 (15, 31, 38, 60). Ser/Thr phosphorylation is proposed to play an important role in regulating the activity of myogenin (33). However, the activities of MRF4 and MyoD are not regulated analogously, suggesting phosphorylation of the transcription factors is not a general regulatory mechanism (20, 31). Our data suggest that PDGF-BB is not capable of regulating muscle-specific transcription factors in PDGF β receptor-transfected MM14 cells.

Two hypotheses can be generated from the data presented in this report: the FGF intracellular signal transduction pathways activated by the cell surface FGF receptor are distinct from those activated by the PDGF β receptor, and/or a com-

plete response to FGF requires intracellular transport of the ligand and perhaps the FGF receptor (45). Evidence from other laboratories supports the latter hypothesis. First, replacement of a single lysine residue of FGF-1 with glutamic acid by site-directed mutagenesis results in a protein that has decreased mitogenic activity but still binds to cell surface receptors, stimulates tyrosine kinase activity, and induces expression of c-*fos*, c-*jun*, and c-*myc* (7). Second, removal of a putative nuclear localization sequence in FGF-1 renders the FGF-1 incapable of stimulating cell proliferation while still allowing activation of tyrosine phosphorylation and upregulation of c-*fos* expression (24). Third, FGF-1 introduced into cells that lack FGF tyrosine kinase receptors stimulates DNA synthesis in the absence of any change in tyrosine phosphorylation (61). Therefore, FGF may function intracellularly in addition to activating its cell surface receptor, and this intracellular function may be critical for FGF-dependent cell proliferation or repression of differentiation.

Although PDGF does not affect MM14 cell differentiation, it has been shown to repress myogenesis and promote proliferation of other muscle cell lines (25, 62) and primary muscle cell cultures (14, 62). In these studies, the mechanisms involved were not investigated, and it is possible that PDGF was stimulating FGF activity indirectly. Consistent with this hypothesis, three recent studies demonstrate that FGFs play a direct role in the regulation of myogenesis. First, Rando and Blau have shown that optimum growth of primary mouse myoblasts requires FGF-2 (46), consistent with previous studies using partially purified FGF (34). A variety of other growth factors tested, including PDGF, could not replace the requirement for FGF-2. Second, many skeletal muscle cell lines can be grown in fetal bovine serum that contains no biologically active FGFs. One such cell line, Sol 8, has been recently shown to be ultimately dependent on FGFs. Inhibition of endogenous FGF-1 expression by antisense RNA confers dependence on exogenous FGF for repression of differentiation (17). Sol 8 cells expressing antisense FGF-1 thus exhibit an FGF dependence similar to that of MM14 cells and mouse primary cultures. It will be interesting to determine if other skeletal muscle cell lines exhibit a similar requirement for FGF that has been masked by endogenous FGF expression. Third, recent data from our laboratory have demonstrated that autocrine activation of FGF receptors in MM14 cultures by expression of a transfected FGF cDNA is sufficient to replace exogenously added FGFs. Together, these data suggest that FGFs play a direct, critical role in skeletal muscle growth and development.

In contrast to results of experiments using PC12 cells in which PDGF β receptor signaling elicits the same end result (neuronal differentiation) as does FGF initiated signaling (23), we have provided direct evidence that activation of intracellular signaling by the PDGF β receptor is insufficient to mediate FGF-dependent regulation of skeletal muscle growth and differentiation. These data suggest the FGF and PDGF signal transduction pathways are different in MM14 skeletal muscle cells and PC12 neuronal cells or that modulations of the pathways differ in the two cell types. Continued analysis of these systems will help in understanding the unique responses of different cell types to the same growth factor. We are pursuing characterization of the FGF-dependent signal transduction pathways that regulate myogenic differentiation. Further elucidation of these pathways will aid in our understanding of the mechanisms involved in regulation of myogenesis by the FGF family.

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REFERENCES

1. **Allen, R. E., and L. K. Boxhorn.** 1989. Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. J. Cell. Physiol. **138:**311–315.
2. **Ashendel, C. (Purdue University).** 1994. Personal communication.
3. **Bader, D., T. Masaki, and D. A. Fischman.** 1982. Biochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J. Cell Biol. **95:**763–770.
4. **Basilico, C., and D. Moscatelli.** 1992. The FGF family of growth factors and oncogenes. Adv. Cancer Res. **59:**115–165.
5. **Bouche, G., N. Gas, H. Prats, V. Baldin, J. P. Tauber, J. Teissie, and F. Amalric.** 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0-G1 transition. Proc. Natl. Acad. Sci. USA **84:**6770–6774.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Burgess, W. H., A. M. Shaheen, M. Ravera, M. Jaye, P. J. Donohue, and J. A. Winkles.** 1990. Possible dissociation of the heparin-binding and mitogenic activities of heparin-binding (acidic fibroblast) growth factor-1 from its receptor-binding activities by site-directed mutagenesis of a single lysine residue. J. Cell Biol. **111:**2129–2138.
8. **Burrus, L. W., M. E. Zuber, B. A. Lueddecke, and B. B. Olwin.** 1992. Identification of a cysteine-rich receptor for fibroblast growth factors. Mol. Cell. Biol. **12:**5600–5609.
9. **Chao, M. V.** 1992. Growth factor signaling: where is the specificity? Cell **68:**995–997.
10. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
11. **Claesson-Welsh, L.** 1994. Signal transduction by the PDGF receptors. Prog. Growth Factor Res. **5:**37–54.
12. **Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka.** 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell Biol. **105:**949–956.
13. **Crews, C. M., A. A. Alessandrini, and R. L. Erikson.** 1991. Mouse *Erk-1* gene product is a serine/threonine protein kinase that has the potential to phosphorylate tyrosine. Proc. Natl. Acad. Sci. USA **88:**8845–8849.
14. **Doumit, M. E., D. R. Cook, and R. A. Merkel.** 1993. Fibroblast growth factor, epidermal growth factor, insulin-like growth factors, and platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. J. Cell. Physiol. **157:**326–332.
15. **Emerson, C. P.** 1993. Skeletal myogenesis: genetics and embryology to the fore. Curr. Opin. Genet. Dev. **3:**265–274.
16. **Fallon, J. F., A. López, M. A. Ros, M. P. Savage, B. B. Olwin, and B. K. Simandl.** 1994. Apical ectodermal ridge growth signal for chick limb development. Science **264:**104–107.
17. **Fox, J. C., A. Y. Hsu, and J. Swain.** 1994. Myogenic differentiation triggered by antisense acidic fibroblast growth factor RNA. Mol. Cell. Biol. **14:**4244–4250.
18. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. Nature (London) **311:**433–438.
19. **Gronwald, R. G. K., F. J. Grant, B. A. Haldeman, C. E. Hart, P. J. O'Hara, F. S. Hagen, R. Ross, D. F. Bowen-Pope, and M. J. Murray.** 1988. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: evidence for more than one receptor class. Proc. Natl. Acad. Sci. USA **85:**3435–3439.
20. **Hardy, S., Y. Kong, and S. F. Konieczny.** 1993. Fibroblast growth factor inhibits MRF4 activity independently of the phosphorylation status of a conserved threonine residue within the DNA-binding domain. Mol. Cell. Biol. **13:**5943–5956.
21. **Hart, C. E. (ZymoGenetics, Inc., Seattle, Wash.).** 1994. Personal communication.
22. **Hart, C. E., R. A. Seifert, R. Ross, and D. F. Bowen-Pope.** 1987. Synthesis, phosphorylation, and degradation of multiple forms of the platelet-derived growth factor receptor studied using a monoclonal antibody. J. Biol. Chem. **262:**10780–10785.
23. **Heasley, L. E., and G. L. Johnson.** 1992. The β-PDGF receptor induces neuronal differentiation of PC12 cells. Mol. Biol. Cell. **3:**545–553.
24. **Imamura, T., K. Engleka, X. Zhan, Y. Tokita, R. Forough, D. Roeder, A. Jackson, J. A. M. Maier, T. Hla, and T. Maciag.** 1990. Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. Science **249:**1567–1570.

25. **Jin, P. T., T. Sejersen, and N. R. Ringertz.** 1991. Recombinant platelet-derived growth factor-BB stimulates growth and inhibits differentiation of rat L6 myoblasts. J. Biol. Chem. **266:**1245–1249.
26. **Johnson, G. L., and R. R. Vaillancourt.** 1994. Sequential protein kinase reactions controlling cell growth and differentiation. Curr. Opin. Cell Biol. **6:**230–238.
27. **Jones, N. C., A. J. Kudla, and B. B. Olwin.** Unpublished data.
28. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35:**603–610.
29. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature (London) **312:**711–716.
30. **Kudla, A. J., and B. B. Olwin.** Unpublished data.
31. **Lassar, A., and A. Münsterberg.** 1994. Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. Curr. Opin. Cell Biol. **6:**432–442.
32. **Lau, L. F., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc. Natl. Acad. Sci. USA **84:**1182–1186.
33. **Li, L., J. Zhou, G. James, R. Heller-Harrison, M. P. Czech, and E. N. Olson.** 1992. FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell **71:**1181–1194.
34. **Linkhart, T. A., C. H. Clegg, and S. D. Hauschka.** 1980. Control of mouse myoblast commitment to terminal differentiation by mitogens. J. Supramol. Struct. **14:**483–498.
35. **Lubinus, M., K. E. Meier, E. A. Smith, K. C. Gause, E. C. LeRoy, and M. Trojanowska.** 1994. Independent effects of platelet-derived growth factor isoforms on mitogen-activated protein kinase activation and mitogenesis in human dermal fibroblasts. J. Biol. Chem. **269:**9822–9825.
36. **Minty, A., and L. Kedes.** 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. **6:**2125–2136.
37. **Niswander, L., and G. R. Martin.** 1993. FGF-4 and BMP-2 have opposite effects on limb growth. Nature (London) **361:**68–71.
38. **Olson, E. N.** 1992. Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. **154:**261–272.
39. **Olson, E. N.** 1992. Proto-oncogenes in the regulatory circuit for myogenesis. Semin Cell. Biol. **3:**127–136.
40. **Olwin, B. B., K. Hannon, and A. J. Kudla.** 1994. Are fibroblast growth factors regulators of myogenesis *in vivo*? Prog. Growth Factor Res. **5:**145–158.
41. **Olwin, B. B., and S. D. Hauschka.** 1986. Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. Biochemistry **25:**3487–3492.
42. **Olwin, B. B., and S. D. Hauschka.** 1988. Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. J. Cell Biol. **107:**761–769.
43. **Olwin, B. B., and A. Rapraeger.** 1992. Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. J. Cell Biol. **118:**631–639.
44. **Promega Corporation.** 1991. Promega protocols and applications guide, 2nd ed. Promega Corporation, Madison, Wis.
45. **Prudovsky, I., N. Savion, X. Zhan, R. Friesel, J. Xu, J. Hou, W. L. McKeehan, and T. Maciag.** 1994. Intact and functional fibroblast growth factor (FGF) receptor-1 trafficks near the nucleus in response to FGF-1. J. Biol. Chem. **269:**31720–31724.
46. **Rando, T. A., and H. M. Blau.** 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J. Cell Biol. **125:**1275–1287.
47. **Rapraeger, A. C., S. Guimond, A. Krufka, and B. B. Olwin.** 1994. Regulation by heparan sulfate in fibroblast growth factor signaling. Methods Enzymol. **245:**219–240.
48. **Rapraeger, A. C., A. Krufka, and B. B. Olwin.** 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science **252:**1705–1708.
49. **Riley, B. B., M. P. Savage, B. K. Simandl, B. B. Olwin, and J. F. Fallon.** 1993. Retroviral expression of FGF-2 (bFGF) affects patterning in chick limb bud. Development **118:**95–104.
50. **Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans.** 1989. *Jun-D*: a third member of the *Jun* gene family. Proc. Natl. Acad. Sci. USA **86:**1500–1503.
51. **Ryder, K., L. F. Lau, and D. Nathans.** 1988. A gene activated by growth factors is related to the oncogene v-*jun*. Proc. Natl. Acad. Sci. USA **85:**1487–1491.
52. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
53. **Seed, J., and S. D. Hauschka.** 1988. Clonal analysis of vertebrate myogenesis. VIII. Fibroblast growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development. Dev. Biol. **128:**40–49.
54. **Seifert, R. A., C. E. Hart, P. E. Phillips, J. W. Forstrom, R. Ross, M. J. Murray, and D. F. Bowen-Pope.** 1989. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J. Biol. Chem. **264:**8771–8778.
55. **Stanton, L. W., R. Watt, and K. B. Marcu.** 1983. Translocation, breakage and truncated transcripts of c-*myc* oncogene in murine plasmacytomas. Nature (London) **303:**401–406.
56. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. **5:**410–413.
57. **Templeton, T. J., and S. D. Hauschka.** 1992. FGF-mediated aspects of skeletal muscle growth and differentiation are controlled by a high affinity receptor, FGFR1. Dev. Biol. **154:**169–181.
58. **Van Beveren, C., F. van Straaten, T. Curran, R. Müller, and I. M. Verma.** 1983. Analysis of FBJ-MuSV provirus and *c-fos* (mouse) gene reveals that viral and cellular *fos* gene products have different carboxy termini. Cell **32:**1241–1255.
59. **Van Doren, K., D. Hanahan, and Y. Gluzman.** 1984. Infection of eucaryotic cells by helper-independent recombinant adenoviruses: early region 1 is not obligatory for integration of viral DNA. J. Virol. **50:**606–614.
60. **Weintraub, H.** 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell **75:**1241–1244.
61. **Wiedlocha, A., P. Ø. Falnes, I. H. Madshus, K. Sandvig, and S. Olsnes.** 1994. Dual mode of signal transduction by externally added acidic fibroblast growth factor. Cell **76:**1039–1051.
62. **Yablonka-Reuveni, Z., and R. A. Seifert.** 1993. Proliferation of chicken myoblasts is regulated by specific isoforms of platelet-derived growth factor: evidence for differences between myoblasts from mid and late stages of embryogenesis. Dev. Biol. **156:**307–318.
63. **Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz.** 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell **64:**841–848.