The Mitogenic and Myogenic Actions of Insulin-like Growth Factors Utilize Distinct Signaling Pathways*

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It is well established that mitogens inhibit differentiation of skeletal muscle cells, but the insulin-like growth factors (IGFs), acting through a single receptor, stimulate both proliferation and differentiation of myoblasts. Although the IGF-I mitogenic signaling pathway has been extensively studied in other cell types, little is known about the signaling pathway leading to differentiation in skeletal muscle. By using specific inhibitors of the IGF signal transduction pathway, we have begun to define the signaling intermediates mediating the two responses to IGFs. We found that PD098059, an inhibitor of mitogen-activated protein (MAP) kinase activation, inhibited IGF-stimulated proliferation of L6A1 myoblasts and the events associated with it, such as phosphorylation of the MAP kinases and elevation of c-fos mRNA and cyclin D protein. Surprisingly, PD098059 caused a dramatic enhancement of differentiation, evident both at a morphological (fusion of myoblasts into myotubes) and biochemical level (elevation of myogenin and p21 cyclin-dependent kinase inhibitor expression, as well as creatine kinase activity). In sharp contrast, LY294002, an inhibitor of phosphatidylinositol 3-kinase, and rapamycin, an inhibitor of the activation of p70S6 kinase (p70S6k), completely abolished IGF stimulation of L6A1 differentiation. We found that p70S6k activity increased substantially during differentiation, and this increase was further enhanced by PD098059. Our results demonstrate that the MAP kinase pathway plays a primary role in the mitogenic response and is inhibitory to the myogenic response in L6A1 myoblasts, while activation of the phosphatidylinositol 3-kinase/p70S6k pathway is essential for IGF-stimulated differentiation. Thus, it appears that signaling from the IGF-I receptor utilizes two distinct pathways leading either to proliferation or differentiation.

It is widely believed that most mitogens stimulate skeletal muscle cell proliferation but inhibit differentiation. However, the insulin-like growth factors (IGF-I and IGF-II) are unique among growth factors in that they stimulate both proliferation and differentiation of muscle cells in culture. Recently, it has been shown that IGF actions occur through two phases (reviewed in Ref. 1). This involves an initial mitogenic response in which intracellular mediators of proliferation such as cyclin D mRNA and c-fos mRNA are up-regulated and mediators of the myogenic response such as elevation of myogenin mRNA are suppressed (2), followed by a strong myogenic response characterized by expression of the muscle-specific transcription factor myogenin and fusion of myoblasts into myotubes.

Although IGF binding has been shown for both IGF receptors, the type I IGF-I receptor and the type II IGF-II receptor, most biological actions of IGF-I and IGF-II on L6A1 muscle cells appear to be mediated by the type I IGF-I receptor (3). It is not obvious how stimulation of two mutually exclusive processes such as proliferation and differentiation can be mediated by the same receptor. A likely possibility is that there is a step at which signal transduction pathways leading to proliferation or differentiation diverge. Although many of the signaling intermediates for the mitogenic actions of IGFs have been described for other cell types, little is known about signal transduction pathways leading to muscle differentiation.

The results of studies on signaling by the insulin and IGF-I receptors in other cell types have revealed two primary pathways by which these signals might be transmitted. Binding of IGF-I to the type I receptor induces a conformational change resulting in receptor autophosphorylation, followed by tyrosine phosphorylation of several cellular substrates. Two primary substrates of the activated IGF receptor are IRS-1 and Shc (4, 5). Phosphorylated tyrosines on IRS-1 serve as docking sites for multiple proteins containing SH-2 domains. Among those proteins shown to bind to IRS-1 are the p85 regulatory subunit of PI 3-kinase (6), SH-PTP2 (Syp) (7), the adaptor protein Nck (8), and Grb-2 (9). This latter protein associates with mSos, which activates Ras in turn activating the Raf-1/MAP kinase pathway (10). Phosphorylated Shc also associates with Grb-2 and activates this pathway (5, 11). Although both IRS-1 and Shc can link the activated IGF-I receptor with Grb-2/Sos, Sasaoka et al. (12) have recently shown that the Shc/Grb2/Sos complex is more important for Ras activation.

A second pathway involving PI 3-kinases is also observed in insulin/IGF-I receptor signal transduction (4, 13). Association of IRS-1 with p85/p110 PI 3-kinase results in its activation, leading to downstream phosphorylation and activation of the serine/threonine kinase p70S6k (14). This in turn leads to phos-kinase inhibitor; p70S6k, p70 S6 kinase; PD, PD098059, an inhibitor of the activation of MEK by Raf-1; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; R3 IGF-I, the R3 analog of IGF-I in which an arginine has replaced glutamate in the third residue to suppress binding to IGF-binding proteins; Rapa, rapamycin, an inhibitor of the activation of p70S6k; CK, creatine kinase; PBS, phosphate-buffered saline; IGFBP, IGF-binding protein; EGF, epidermal growth factor.

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§ The abbreviations used are: IGF, insulin-like growth factor; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular regulated kinase; PGF, fibroblast growth factor; Grb-2, growth factor receptor binding protein-2; IRS, insulin receptor substrate; LY, LY294002, an inhibitor of the activation of PI 3-kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase kinase; p21, a cyclin-dependent kinase inhibitor; p70S6k, p70 S6 kinase; PD, PD098059, an inhibitor of the activation of MEK by Raf-1; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; R3 IGF-I, the R3 analog of IGF-I in which an arginine has replaced glutamate in the third residue to suppress binding to IGF-binding proteins; Rapa, rapamycin, an inhibitor of the activation of p70S6k; CK, creatine kinase; PBS, phosphate-buffered saline; IGFBP, IGF-binding protein; EGF, epidermal growth factor.

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phorylation of the ribosomal S6 protein and PHAS-1, resulting in an increased translation of mRNAs containing polypyrimidine tracts (15). In addition, Weng et al. (16) have shown that constitutively active PI 3-kinase indirectly activates p70\(^{S6k}\), possibly through the kinase Akt (17). However, p70\(^{S6k}\) may also be activated through a PI 3-kinase-independent pathway, since mutation of the PDGF receptor has been shown to abolish PI 3-kinase activation in transfected 293 cells, while p70\(^{S6k}\) activation remains unaffected (18). In addition, Lenormand et al. (19) have recently shown that expression of an estradiol-regulation remains unaffected (18). In addition, Lenormand conditional inhibition of the cell cycle at the G1 phase. Rapamycin blocks acti-

## Mitogenic and Myogenic Signals of IGFs in Myoblasts

Northern Blot Analyses—Total RNA was isolated from cultures using the RNeasy total RNA kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Ten-μg samples were analyzed on Northern blots. Consistency of RNA loading was monitored by visualization of ethidium bromide-stained ribosomal RNA bands. 32P-Labeled probe for c-fos cDNA probe was obtained from ATCC (Rock-

## EXPERIMENTAL PROCEDURES

### Materials—The IGF-I analog, Ris IGF-I, was a gift from Paul Walton and John Ballard (GroPep Pty Ltd, Adelaide, Australia). Rapamycin was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA), and LY294002 was purchased from Calbiochem. The MEK inhibitor, PD098059, was a gift from Alan Saltiel (Parke-Davis Pharmaceuticals, Ann Arbor, MI). The c-fos cDNA probe was obtained from ATCC (Rock-

### Electrophoresis and Immunoblotting—Total cell lysates were prepared by washing the cell monolayers (100-mm dish) twice with ice-cold PBS followed by lysis in 0.5 ml of boiling 1 x Laemmli sample buffer containing 100 mM dithiothreitol. Proteins (10 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis following the procedures of Laemmli (30) and transferred onto Immobilon-nitrocellulose (Milli-

### RESULTS

**Effects of R3 IGF-I on L6A1 Myoblast Proliferation and Differentiation**—The actions of IGF-I are modified by six IGF-binding proteins (IGFBPs), three of which are secreted by L6A1 muscle cells and have both inhibitory and stimulatory effects on proliferation and differentiation (Refs. 31–33). In order to minimize the actions of these IGFBPs in this study, we used an analog of IGF-I with a low affinity for IGFBPs, R3 IGF-I. We have previously reported that several similar IGF-I analogs with reduced affinity for IGFBPs were not only more potent

### Quantitation of Proliferation and Differentiation—To quantitate cell proliferation, cells were trypsinized and counted in a model ZBI Coulter Counter 1 day after the addition of growth factors and inhibitors. Differentiation was quantitated 3 days after the addition of growth factors and inhibitors by measuring the elevation in CK activity using the NAD-coupled microtiter assay (27). CK levels were normalized to DNA content as described previously (28).

**Northern Blot Analyses**—Total RNA was isolated from cultures using the RNeasy total RNA kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Ten-μg samples were analyzed on Northern blots. Consistency of RNA loading was monitored by visualization of ethidium bromide-stained ribosomal RNA bands. 32P-Labeled probe for c-fos cDNA probe was obtained from ATCC (Rockville, MD), and the myogenin cDNA probe (E26) was a gift from Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, TX). The anti-cyclin D antibody, p70S6k assay kit, and recombinant protein complex was captured with 50 μl of protein-A-agarose (50% slurry) for 1 h at 4°C. The beads were washed twice with an equal volume of cold PBS followed by one wash with assay dilution buffer. The beads were resuspended in 20 μl of assay dilution buffer, 10 μl of substrate, 10 μl of inhibitor mixture, and 10 μl of [γ-32P]ATP mixture (75 mM MgCl2, 500 μM ATP, 10 μCi of [γ-32P]ATP). The reaction mixtures were incubated for 10 min at 30°C and then centrifuged for 1 min. Aliquots (20 μl) of the supernatants were spotted onto P81 phosphocellulose paper squares and washed three times in 0.75% phosphoric acid, followed by one wash in acetone. The squares were transferred to scintillation vials and counted in a Beckman LS 7500 scintillation counter.

### RESULTS

**Effects of R3 IGF-I on L6A1 Myoblast Proliferation and Differentiation**—The actions of IGF-I are modified by six IGF-binding proteins (IGFBPs), three of which are secreted by L6A1 muscle cells and have both inhibitory and stimulatory effects on proliferation and differentiation (Refs. 31–33). In order to minimize the actions of these IGFBPs in this study, we used an analog of IGF-I with a low affinity for IGFBPs, R3 IGF-I. We have previously reported that several similar IGF-I analogs with reduced affinity for IGFBPs were not only more potent
than IGF-I in stimulating L6A1 myoblast proliferation and differentiation, but caused much more extensive myotube formation (31). The effects of adding increasing concentrations of R3 IGF-I on L6A1 cell proliferation and differentiation are shown in Fig. 1. Stimulation of proliferation after 1 day reached a maximum at approximately 30–100 ng/ml R3 IGF-I. Maximum stimulation of differentiation measured after 3 days was attained with 1–3 ng/ml of R3 IGF-I, while exposure to higher concentrations resulted in progressively lower CK levels accompanied by decreased fusion of myoblasts into myotubes as well as increased cell division; this biphasic response was also seen at higher concentrations of unmodified IGF-I (34). Therefore, in subsequent studies, R3 IGF-I was used at low concentrations of 1–3 ng/ml in experiments monitoring inhibitor effects on differentiation and at higher concentrations of 10–30 ng/ml when we studied effects on proliferation.

Effects of Specific Inhibitors of the IGF-I Signal Transduction Pathway on L6A1 Myoblast Proliferation and Differentiation—In order to determine which signaling pathways play a role in mediating R3 IGF-I-stimulated proliferation and differentiation, we used three compounds that have been reported to inhibit specific signaling molecules in other cells. PD098059 specifically inhibits the activation of MEK by Raf-1, thus suppressing MAP kinase activation (20). LY294002 is a specific inhibitor of PI 3-kinase (23), and rapamycin treatment inhibits p70S6k (25). When these inhibitors were added in the presence of R3 IGF-I, we observed striking differences in the response of L6A1 myoblast cultures. Fig. 2 summarizes the concentration dependencies of the effects of each of the three inhibitors on cell proliferation and differentiation when added in the presence of 3 ng/ml R3 IGF-I. As shown in Fig. 2, increasing concentrations of PD098059 gave progressive decreases in the mitogenic response to R3-IGF-I, inhibiting proliferation approximately 90% at concentrations of 30 μM, while the same levels of inhibitor showed a dramatic 3-fold enhancement of R3 IGF-I-stimulated differentiation quantitated as CK in units/mg of DNA. In sharp contrast, incubation with increasing concentrations of either LY294002 or rapamycin progressively inhibited the myogenic response to R3 IGF-I but had much less effect on the mitogenic response. At concentrations of 10 μM and 1 ng/ml, LY294002 and rapamycin, respectively, completely abolished R3 IGF-I-stimulated differentiation but inhibited cell proliferation only 30–40%.

There were equally striking changes in cell morphology following treatment with these inhibitors. Upon the removal of growth medium, cells maintained in serum-free DMEM remained relatively quiescent, with little, if any cell division or fusion into myotubes (Fig. 3A). After the addition of R3 IGF-I to L6A1 cultures, the myoblasts first underwent a round of cell division before fusing into myotubes; after 2 days the cultures consisted of both unfused myoblasts and differentiated myotubes (Fig. 3B). However, when PD098059 was added in the presence of R3 IGF-I, there was less cell division, and the myoblasts began to differentiate sooner, forming an extensive network of myotubes; after 2 days in culture, few if any unfused myoblasts could be detected (Fig. 3C). In sharp contrast, differentiation was completely inhibited in cultures treated with either LY294002 or rapamycin in the presence of R3 IGF-I (Fig. 3, D and E). Myoblasts treated with these two inhibitors continued to proliferate, as demonstrated by the increased cell density when compared with those in DMEM, but they did not fuse into myotubes. These results (Figs. 2 and 3) suggest that signaling by the MAP kinase pathway is required for R3 IGF-I-stimulated L6A1 proliferation, but not for differentiation, and that inhibition of this pathway by PD098059 permits, even enhances, R3 IGF-I-stimulated L6A1 myoblast differentiation.

In contrast, a functional PI 3-kinase/p70S6k pathway is an absolute requirement for IGF-stimulated differentiation.

Effects of Combinations of Inhibitors—As shown in Figs. 2 and 3, the inhibitors used here have dramatically opposite effects on responses of myoblasts to R3 IGF-I. This raises the question of which effect predominates when cells are incubated with combinations of these inhibitors. As illustrated in Fig. 4B, the results are unequivocal; the inhibitory effects of LY294002 and rapamycin override both the stimulation of myogenesis by R3 IGF-I and its enhancement by PD098059. Differentiation of myoblasts in response to R3 IGF-I is completely blocked by these inhibitors, both in the absence and presence of PD098059; morphological differentiation (i.e. fusion) of the cultures closely parallels the quantitation by CK levels presented in Fig. 4B. Thus, it appears that the PI 3-kinase/p70S6k pathway is essential for myogenesis even when the mitogenic response through MAP kinase is blocked.

The results presented in Fig. 4A indicate a major effect of the MAP kinase pathway and a smaller contribution of the PI 3-kinase/p70S6k pathway to the mitogenic response; there was much greater inhibition of proliferation by PD098059 than by either of the other inhibitors, but when added in combination with PD098059, there was an even lower level of cell proliferation; indeed, there was some loss of cells below the level found in DMEM controls.

Effect of Inhibitors on Intracellular Mediators of Proliferation—We usually measure proliferative responses of L6A1 myoblasts to mitogenic stimuli by counting the actual number of cells in the cultures 1 day after the specific treatment. In addition, we also determined the effects of these inhibitors on several intracellular mediators closely associated with early aspects of the proliferative response in other cell systems. Of these, c-fos mRNA is an established early marker for cell proliferation and has been shown to be induced during IGF-I-stimulated mitogenesis in L6 myoblasts (35, 36). We investigated the effects of PD098059, LY294002, and rapamycin on c-fos mRNA levels after 30 min as shown in Fig. 5A. In the presence of R3 IGF-I, c-fos mRNA levels were enhanced about 5-fold above control levels. As expected, this increase in c-fos mRNA was significantly reduced in the presence of the MEK inhibitor, PD098059. In contrast, LY294002 and rapamycin, at concentrations that abolished myogenic differentiation, had little or no effect on c-fos mRNA levels compared with R3 IGF-I treatment alone. Both combinations of inhibitors resulted in...
decreased c-fos mRNA levels similar to those seen for PD098059 plus R3 IGF-I alone.

The primary substrates of MEK are the p42/p44MAPK isoforms, ERK2 and ERK1, respectively (37). PD098059 prevents the activation of MEK, thereby inhibiting phosphorylation and activation of MAP kinases. It has been postulated that the MAP kinases are required for activation of the G1 cyclin-dependent complexes (38). Therefore, levels of phosphorylated MAP kinase were measured in cells incubated with PD098059, LY294002, and rapamycin, both individually and in combination. As shown in Fig. 5B, PD098059 had the expected effect of inhibiting R3 IGF-I-induced phosphorylation of both ERK1 and 2 within 1 h. LY294002 and rapamycin, on the other hand, had little or no effect. When LY294002 and rapamycin were added in combination with PD098059, the inhibition was equivalent to PD098059 on its own. This confirms that the action of PD098059 is upstream of MAP kinase and that LY294002 and rapamycin have no effect on the MAP kinase pathway or, therefore, on c-fos transcription.

The inhibitory effects of PD098059 on the levels of cyclin D1, a protein essential to cell cycle progression, were similar to, although not as dramatic as those seen for phosphorylated MAP kinase. However, PD098059 in the presence of either LY294002 or rapamycin was almost completely inhibitory (Fig. 5C). This suggests that although proliferation occurs primarily through the MAP kinase pathway, inhibiting this pathway does not block all aspects of the proliferative response, suggesting that the PI 3-kinase/p70^S6k^ pathway may also be involved. However, since R3 IGF-I and LY294002 or rapamycin in the absence of PD098059 had no effect on cyclin D1 levels, the proliferative role of the PI 3-kinase/p70^S6k^ pathway is most probably minor.

Effect of Inhibitors on Intracellular Mediators of Myogenesis—In a similar study on early mediators of differentiation, we measured levels of myogenin and p21 mRNA 18 and 42 h after the R3 IGF-I addition in the absence or presence of the inhibitors (Fig. 6). Myogenin is a basic-helix-loop-helix transcription factor of the MyoD family that is required for terminal differentiation and myotube formation (reviewed in Ref. 1).

Induction of the mRNA for the cyclin-dependent kinase inhibitor, p21, is an early event in myogenic differentiation marking the exit of myoblasts from the cell cycle (39). At early times of incubation (18 h), R3 IGF-I-treated cells exhibited very little increase in myogenin mRNA compared with DMEM control cells (Fig. 6A). In the presence of R3 IGF-I and PD098059, myogenin mRNA levels were elevated more than 3-fold compared with R3 IGF-I treatment alone, indicating an early enhancement of the myogenic response. LY294002 and rapamycin-treated cells showed a significant reduction in myogenin mRNA at that time. The combination of PD098059 with rapamycin and LY294002 reduced the enhancing effect of PD098059 on myogenin mRNA levels, although they were still slightly elevated above control and R3 IGF-I levels.

By 42 h, myogenin mRNA in R3 IGF-I-treated cells was elevated almost 4-fold above DMEM-treated control cells. This is consistent with observations that myogenin mRNA levels rise rather late after the IGF-I addition (2, 29). Cells treated with R3 IGF-I and PD098059 had myogenin levels similar to those in R3 IGF-I-treated cells; i.e. by this late time, myogenin levels in R3 IGF-I-treated cells reached those levels seen in cells treated with both R3 IGF-I and PD098059. LY294002 and rapamycin with R3 IGF-I alone or in combination with PD098059 showed patterns similar to those seen at 18 h.

At 18 h, myogenin protein levels correlated well with mRNA values showing enhanced myogenin protein in the presence of R3 IGF-I and PD098059 (Fig. 6B). The effect of the inhibitors on myogenin protein levels was less pronounced by 48 h, although R3 IGF-I-treated cells showed enhanced levels of myogenin compared with DMEM-treated cells. This suggests that translation of myogenin mRNA may be affected differently than transcription of the myogenin gene in the presence of these inhibitors.

Levels of p21 mRNA were also elevated in the presence of R3 IGF-I and R3 IGF-I plus PD098059 at 18 and 42 h (Fig. 6C). However, the inhibitors LY294002 and rapamycin, alone or in combination with PD098059, completely blocked p21 mRNA expression compared with DMEM-treated cells, again suggesting the importance of the PI 3-kinase/p70^S6k^ pathway in...
differentiation. These results reflect the well established coordination of cell cycle exit with the onset of myogenic differentiation.

Effects of Inhibitors on p70S6 Activity—Results with the inhibitors LY294002 and rapamycin strongly indicated a major role for the PI 3-kinase/p70S6k pathway in the stimulation of myogenesis by the IGFs, suggesting that p70S6k activity should be elevated during differentiation. To test this possibility, we measured the enzymatic activity of p70S6k in lysates from cells treated with R3 IGF-I as shown in Fig. 7. R3 IGF-I increased p70S6k activity above control levels over an extended period of time (48 h), showing an initial increase over control values (Fig. 7, inset), followed by a much larger increase at 48 h. The later activity was substantially elevated in the presence of PD098059, just as was differentiation of the cells. Treatment with rapamycin and LY294002 reduced p70S6k activity to the level found in DMEM controls, demonstrating that inhibition of p70S6k activity parallels the effects of these inhibitors on myogenesis. These results suggest that the early increase in p70S6k activity may contribute to the relatively rapid mitogenic response, while the larger subsequent increase is necessary for the stimulation of L6A1 myoblast differentiation.

FIG. 3. Morphological effects of R3 IGF-I and inhibitors on L6A1 myoblasts. L6A1 myoblast cultures were prepared as described in the legend to Fig. 1. R3 IGF-I (3 ng/ml) was added in the presence of 30 μM PD098059, 10 μM LY294002, or 1 ng/ml rapamycin. After 2 days, the cultures were washed with PBS, fixed with MeOH, and stained with Wright’s and Giemsa stains.
The results presented here represent the first steps toward understanding the signaling pathways involved in two major responses of myoblasts to the IGFs. When we (40) first reported that the known mitogens, IGFs, stimulate differentiation as well as proliferation of skeletal muscle cells in culture, there was some reluctance to accept this observation because of the long held (41) and well supported (42–45) view that mitogens block myogenesis by forcing myoblasts in the G_1 stage of the cell cycle to reenter S phase rather than to fuse to form postmitotic myotubes. However, the stimulation of myogenic differentiation by the IGFs has been demonstrated independently in a number of laboratories (reviewed in Ref. 1), and the effect is now well established although not fully explained.

It has been difficult to understand how the same agent, IGF-I (or IGF-II or insulin at high concentrations), acting through a single receptor (3), can stimulate these two responses, which are generally considered to be mutually antagonistic. One suggested explanation for our observations was that IGFs acted only to maintain the cells in a viable state, thus allowing differentiation rather than actively stimulating the process. In earlier studies, we demonstrated that IGFs did not stimulate differentiation solely by preventing cell death by showing that the IGFs caused significant differentiation in medium containing horse serum at levels as high as 5% (34). Another possibility, that the greater fusion rate might result from the higher cell density in cultures stimulated to proliferate by IGF-I, was eliminated by experiments (46) that showed that the stimulation of myogenesis by IGFs was readily detectable in cells incubated under conditions in which DNA synthesis was blocked or proliferating cells were killed by cytosine arabinoside. However, it has recently been shown that IGF-II does, in fact, act as an autocrine survival factor for differentiating myoblasts (47). Although our earlier results show that this does not account for the stimulation of differentiation, we had previously shown that IGF-II was produced by differentiating muscle cells and that the rate of differentiation of different muscle cell lines correlated with the amount of IGF-II secreted by the cell (48). Additional direct evidence implicating IGF-II as an autocrine myogenic factor was provided by Stewart et al. (49) and Quinn et al. (50), who showed that overexpression of IGF-II or the type I IGF-I receptor in C2 cells resulted in greatly accelerated differentiation. Thus, it appears that although the IGFs may act as survival factors and stimulate proliferation, the role of IGFs as positive regulators of myogenesis is firmly established.

A beginning of an explanation of the two responses (proliferation and differentiation) to IGFs was provided by the finding that there is a temporal separation in the responses of myoblasts to IGFs; cells first proliferate and then differentiate (2, 28). But this does not explain how myoblasts can respond in the two different ways or what signals mediate those responses.

The results presented here suggest that the signaling pathways leading to proliferation or differentiation diverge at a very early step, possibly as early as the interaction of the IGF-1 receptor or of IRS-1 with intracellular signaling pathways. As in many other cell types, the stimulation of proliferation in L6A1 skeletal muscle cells by IGF appears to be mediated by the Ras/Raf-1/MAP kinase pathway. Several years ago it was shown that overexpression of ras oncogenes in myoblasts inhibited muscle differentiation (51, 52). Since Ras activation is a critical component of the signaling pathway leading to MAP kinase activation and proliferation, it is reasonable to predict that the stimulation of the MAP kinase pathway would inhibit differentiation and that inhibition of the MAP kinase pathway would stimulate differentiation. We have now shown that the addition of PD098059, the specific inhibitor of MEK activation by Raf-1, not only blocked cell proliferation, but resulted in a dramatic increase in myogenesis above that obtained with R3 IGF-I alone (Figs. 2 and 3). This was evident not only by morphologic changes, but also by changes in specific mediators of proliferation and differentiation. Levels of mRNA for the transcription factor, c-fos, an early marker of proliferation, and of cyclin D protein were suppressed by PD098059 (Fig. 5, A and C). In contrast, the increase in myogenin mRNA and protein was significantly enhanced in the presence of PD098059, particularly during early stages of differentiation (18 h) (Fig. 6, A and B) when myogenin levels are not normally elevated in IGF-I-treated myoblasts (2, 29). This suggests the possibility that proliferation is the primary response of muscle cells to IGFs and that differentiation occurs when there is an interruption of the MAP kinase pathway. What external stimuli bring about this inhibition in vivo is unclear. Our results are similar to those of Lazar et al. (22) who, using PD098059 to block MEK activation by insulin, demonstrated that the MAP kinase pathway is not required for many of the metabolic actions of insulin, such as glucose uptake, lipogenesis, and glycogen synthesis. However, in their studies, PD098059 treatment did not result in additional stimulation of any of the metabolic actions as we...
have observed in the case of IGF-stimulated L6A1 myogenesis.

In apparent disagreement with our finding that activation of the MAP kinase pathway is not required for L6A1 myogenesis, Hashimoto et al. (53) concluded that activation of MAP kinase played a positive role in the expression of myogenin and subsequent differentiation of C2C12 myoblasts. Their conclusion was based on their observations that genistein, a tyrosine kinase inhibitor, inhibited phosphorylation of MAP kinase, myogenin expression, and fusion of myoblasts into myotubes. Since genistein is a general inhibitor that can affect the phosphorylation of many signaling intermediates, it is possible that the inhibitory effects on myogenesis could result from effects on mediators other than MAP kinase. When we added genistein to L6A1 myoblasts at concentrations lower than the 50 μM used by Hashimoto et al., we found that it inhibited proliferation as well as differentiation (data not shown), and concluded that this was not a useful inhibitor for our studies on the separate responses.

The importance of the MAP kinase pathway in proliferation has been shown in several cell types for many different mitogens (37). Lavoie et al. (38) have reported that activation of the p42/p44MAPK isoforms, which leads to activation of various transcription factors, also results in positive regulation of cyclin D1 expression. Growth factor-dependent accumulation of cyclin D1 has been shown to be necessary in order for cells to pass the G1 restriction point of the cell cycle (54).

As shown in Fig. 5, A and B, neither LY294002 nor rapamycin was inhibitory to ERK 1 and 2 phosphorylation or c-fos expression, and neither further enhanced the inhibition of ERK 1 and ERK 2 phosphorylation by PD098059. However, the decrease in cyclin D1 levels at 4 h by PD098059 (Fig. 5C), although significant, was further enhanced by either LY294002 or rapamycin, although neither had any effect individually. Cyclin D1 has been postulated to be the “nuclear sensor” of extracellular signals (38), and our results indicate that cyclin D1 levels may be regulated by both the MAP kinase and the PI 3-kinase/p70S6k pathways. This suggests that although the MAP kinase pathway is essential for proliferation, the p70S6k pathway also plays a role. This is further substantiated by our finding (Fig. 7) that there was an initial increase of p70S6k activity 30 min after R3 IGF-I stimulation, a time when proliferative signals such as c-fos mRNA are up-regulated. IGF-I, at 80 ng/ml, which gives a greater mitogenic response than R3 IGF-I, caused an even greater early increase in p70S6k activity (data not shown), supporting the role of p70S6k in proliferation. At the high levels required for complete suppression of myogenesis, both LY294002 and rapamycin exhibit some (40%) inhibition of the proliferative response to R3 IGF-I.

However, it appears that the PI 3-kinase/p70S6k pathway plays a much more critical role in the myogenic response to IGFs. Inhibition of PI 3-kinase or p70S6k by LY294002 or rapamycin, respectively, resulted in little or no myotube formation (Fig. 3) accompanied by sharply decreased levels of myogenin analyzed on Northern blots (middle part), and c-fos mRNA abundance was quantitated by densitometry (upper part); equal loading was verified with ethidium bromide-stained ribosomal 18 S RNA (lower part). B, phospho-MAPK. Cell lysates were prepared 1 h after the addition of R3 IGF-I with or without inhibitors, and 10 μg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-phospho-MAPK. The Western blot for phospho-MAPK is shown in the lower part, and the densitometric quantitation of ERK 1 (bars on left) and ERK 2 (bars on right) is shown in the upper part. C, cyclin D. Cell lysates were prepared 4 h after the addition of R3 IGF-I with or without inhibitors, and 10 μg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with an antibody to cyclin D. The Western blot is shown in the lower part, and the densitometric quantitation is shown in the upper part.
genin and p21 mRNA (Fig. 6). In addition, p70S6k activity increased with time as R3 IGF-I-stimulated differentiation proceeded (Fig. 7). The elevated activity was further enhanced by PD098059, suggesting that inhibition of the MAP kinase pathway stimulates differentiation. The increase in p70S6k activity was completely abolished in the presence of LY294002 and rapamycin (Fig. 7), supporting our interpretation that myogenic signaling proceeds from PI 3-kinase to p70S6k. Our results are in direct contrast with those reported by Jayaraman and Marks (55), who concluded that rapamycin treatment of BC3H1 myoblasts induced differentiation. BC3H1 myoblasts are not typical of most skeletal muscle cells, since they do not fuse to form myotubes, and biochemical differentiation can be reversed. In the cited study, much higher levels of rapamycin were used, and elevation of α-actin expression was the only myogenic response that was reported.

Our results also suggest that changes in the expression of biochemical markers alone do not indicate differentiation. Myogenin mRNA and protein levels were elevated at 42 h in cells treated with R3 IGF-I and the combination of PD98059 and LY294002 or rapamycin (Fig. 6). However, under these conditions, no differentiation was seen to occur (Fig. 4B). Therefore, elevated myogenin levels alone are not sufficient to induce differentiation; LY294002 and rapamycin may be inhibiting an additional component of the differentiation pathway, possibly the myogenic factor MEF-2 (56).

As this manuscript was being completed, Pilch's group (57) published the results of a study of effects of FGF and IGF on the proliferative response of C2C12 myoblasts. They found complete inhibition of MAP kinase activation but only partial

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**FIG. 6.** Effects of inhibitors on R3 IGF-I stimulation of myogenin and the cyclin-dependent kinase inhibitor, p21. Cultures were prepared and treated as described under Fig. 5, except that R3 IGF-I was added at 3 ng/ml. The following analyses were made in parallel cultures. A, myogenin mRNA. Total RNA was prepared 18 and 42 h after the addition of R3 IGF-I with or without inhibitors, 10 µg was analyzed on Northern blots (middle part), and myogenin mRNA abundance was quantitated by densitometry (upper part); equal loading was verified with ethidium bromide-stained ribosomal 18 S RNA (lower part). B, myogenin protein. Total cell lysates were prepared, and 10 µg of total protein was separated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-myogenin. The Western blot is shown in the lower part, and densitometric quantitation is shown in the upper part. C, p21 mRNA. RNA preparation and Northern analyses were performed as described in A for myogenin.

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**FIG. 7.** Assay of p70S6k after the addition of R3 IGF-I and inhibitors. L6A1 myoblast cultures were established overnight in growth medium at 1.2 × 10^6 cells/100-mm dish, washed once with DMEM, and then treated with R3 IGF-I (1 ng/ml) with or without PD098059 (10 µM), LY294002 (10 µM), or rapamycin (1 ng/ml). At the indicated times, p70S6k immunoprecipitates were prepared and assayed for p70S6k activity as described under “Experimental Procedures.” The inset provides a clearer indication of the effect of treatments after 30 min. The data obtained with R3 IGF-I plus LY294002 or R3 IGF-I plus rapamycin were essentially identical to those obtained with the DMEM controls, so separate points are not shown for them.
inhibition of [3H]thymidine incorporation by PD098059 and concluded that a MAP kinase-independent pathway makes a substantial contribution to the mitogenic response to IGF-I. To the extent that they overlap, our results are in good overall agreement with theirs; we too find that PI 3-kinase activation plays some role in the mitogenic response, and the myogenic response is suppressed by LY294002, which was not used by Milasinic et al. (57).

Our findings are consistent with the recent report of Kali- man et al. (24), who also observed that LY294002 and wortmannin inhibited the capacity of L6E9 myoblasts to form myotubes when the cultures were placed in low serum differentiation medium and concluded that PI 3-kinase activation was required for differentiation. The effects of IGFs on their system were not considered, although our previous observations of autocrine expression of IGFs by myoblasts in low serum medium (48) indicate that IGFs may have been acting in those cells as well.

Although other mitogens have been shown to stimulate PI 3-kinase activity in non-muscle cells, we have found no reports of stimulation of PI 3-kinase in muscle by FGF, PDGF, or EGF. Milasinic et al. (57) have recently reported that in C2C12 cells, IGF-I and insulin increase PI 3-kinase activity by approximately 2-fold, whereas basic FGF, a potent inhibitor of myogenesis, was inconsistent in stimulating PI 3-kinase activity. Also, Kudla et al. (58) have shown that PDGF-BB stimulation of MM14 cells overexpressing the PDGF-B receptor resulted in autophosphorylation of the PDGF-β receptor, activation of MAP kinase, and increased jun B and c-fos mRNA. However, no effect on proliferation or differentiation was observed. In other muscle cell types, PDGF has been shown to repress myogenesis and promote proliferation (59–61). However, the signaling mechanisms involved were not investigated.

We cannot rule out the possibility that these other mitogens may also act through PI-3-kinase in skeletal muscle. It has already been shown that different mitogens acting through the same signaling pathway can generate separate responses. In PC12 cells, FGF or nerve growth factor stimulation results in differentiation, whereas EGF stimulation leads to increased cell proliferation (reviewed in Ref. 62). All of these mitogens stimulate MAP kinase activity; however, the duration of the MAP kinase signal is sustained in FGF/nerve growth factor stimulation but is transient in EGF stimulation. In addition to signal duration, other factors may potentially play a role in modulating signaling pathways for different mitogenic stimuli.

The role of Raf-1 and its importance in linking Ras to the MAP kinase pathway is well established. However, some studies suggest that Raf-1 may have actions other than activation of the Ras/MAP kinase pathway. Alessi et al. (21) have reported that inhibition of MEK by PD098059 in PDGF- and insulin-stimulated Swiss 3T3 cells or IGF-I-stimulated L6 cells resulted in an increase in Raf-1 activity, although the consequence of this elevated Raf-1 activity were not investigated. They suggest that downstream components of the MAP kinase pathway may act to suppress Raf-1 activity, possibly through hyperphosphorylation. However, in hippocampal neuronal cells, elevated levels of Raf-1 activity, but not MEK or ERK, were sufficient for differentiation (63). Transfection with a Raf-1:estrogen receptor fusion gene, ΔRaf-1:ER, resulted in increased Raf-1 activity and extended differentiation following stimulation with estradiol. Prolonged activation of MAP kinases was not sufficient for differentiation, suggesting an alternative pathway where Raf-1, but not MAP kinases, functions in differentiation of neuronal cells. Yen et al. (64) have reported that expression of an activated form of Raf-1 accelerates terminal differentiation of promyelocytic leukemia cells, and this is accompanied by down-regulation of retinoblastoma gene product phosphorylation. However, Ming et al. (18) have reported that neither Ras nor Raf-1 plays a role in activation of p70S6K. Dominant negative mutants of Ras or Raf-1 both failed to inhibit p70S6K activation by EGF in human 293 cells but did inhibit MAP kinase activation. We have shown that p70S6K activity is critical for differentiation in L6A1 skeletal muscle cells, and we are currently exploring the specific role that Raf-1 plays in this process.

It is striking that two such different responses of myoblasts to the IGFs are mediated by quite distinct signaling pathways rather than resulting from subtle differences in timing or concentrations of signaling intermediates. Although many recent studies have examined IGF signaling pathways, none have directly addressed the issue of how IGF-I can stimulate both proliferation and differentiation in a single cell type such as skeletal muscle. In this study we have now shown that different signaling pathways are specifically associated with each response; cell proliferation is mediated primarily by the Ras/Raf-1/MAP kinase pathway, whereas stimulation of differentiation utilizes the PI 3-kinase/p70S6K pathway.

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