The differentiation and maturation of skeletal muscle require interactions between signaling pathways activated by hormones and growth factors and an intrinsic regulatory network controlled by myogenic transcription factors. Insulin-like growth factors (IGFs) play key roles in muscle development in the embryo and in regeneration in the adult. To study mechanisms of IGF action in muscle, we developed a myogenic cell line that overexpresses IGF-binding protein-5. C2BP5 cells remain quiescent in low serum differentiation medium until the addition of IGF-I. Here we use this cell line to identify signaling pathways controlling IGF-mediated differentiation. Induction of myogenin by IGF-I and myotube formation were prevented by the phosphatidylinositol (PI) 3-kinase inhibitor, LY294002, even when included 2 days after growth factor addition, whereas expression of active PI 3-kinase could promote differentiation in the absence of IGF-I. Differentiation also was induced by myogenin but was blocked by LY294002. The differentiation-promoting effects of IGF-I were mimicked by a modified membrane-targeted inducible Akt-1 (iAkt), and iAkt was able to stimulate differentiation of C2 myoblasts and primary mouse myoblasts incubated with otherwise inhibitory concentrations of LY294002. These results show that an IGF-regulated PI 3-kinase-Akt pathway controls muscle differentiation by mechanisms both upstream and downstream of myogenin.

Skeletal muscle development is a multi-step process that begins with the determination of myogenic precursors from pluripotent mesodermal stem cells and concludes with the terminal differentiation of committed myoblasts, which is characterized by cell cycle withdrawal, expression of muscle-specific proteins, and formation and maturation of myofibers (1–3). The muscle-restricted basic helix-loop-helix transcription factors MyoD, MRF4, myogenin, and myf5 were identified initially based on their ability to confer a myogenic fate on non-muscle cells (4, 5). These proteins activate genes that are required for muscle determination or differentiation through formation of heterodimers with other ubiquitous basic helix-loop-helix factors and binding to DNA control elements termed E boxes that are found in the promoter regions of muscle-restricted genes (3–5). Several genes regulated by myogenic basic helix-loop-helix proteins also contain binding sites for members of the MEF2 family (6), and MEF2 proteins have been shown to function as accessory transcription factors to enhance muscle gene expression and to facilitate muscle differentiation (7).

Environmental cues also modulate muscle differentiation (6, 8–10). Many peptide growth factors are able to stimulate myoblast proliferation and also prevent differentiation through signal transduction pathways induced upon growth factor binding to specific high affinity cell surface receptors (9, 10). The Ras-Raf-Mek-Erk pathway has been implicated in growth factor-stimulated muscle cell proliferation and the coordinate inhibition of differentiation (11–15). Unlike most growth factors, the insulin-like growth factors, IGF-I1 and IGF-II, are also capable of promoting muscle differentiation in cell culture (8, 16–20), and their actions through the IGF-I receptor have been linked to the formation, maintenance, and regeneration of skeletal muscle in vivo (21–26). In cultured muscle cells, activation of the IGF-I receptor through endogenously produced IGF-II promotes terminal differentiation through signal transduction pathways that have not been elucidated completely, although current evidence suggests a role for PI 3-kinase in this process (11, 27–32). Autocrine IGF-II also plays a central role in maintaining cell viability during the transition from proliferating to differentiating myoblasts, and recent studies from our laboratory identify a signaling pathway requiring PI 3-kinase, Akt, and the cyclin-dependent kinase inhibitor, p21/waf1/cip1 in IGF-mediated myoblast survival (33–35).

The current experiments were initiated to identify mechanisms of IGF-regulated myoblast differentiation. We previously established a myogenic cell line derived from C2 myoblasts by stable transfection of the IGF-binding protein, IGFBP-5 (36). C2BP5 myoblasts exhibit minimal differentiation under typical low serum conditions but can be induced to express muscle-specific mRNAs and proteins and form multinucleated myotubes after incubation with IGF analogues that activated the IGF-I receptor (36). Using these cells as well as parental C2 myoblasts and neonatal mouse myoblasts in primary culture, we show that IGF-stimulated activation of PI 3-kinase and Akt are sufficient to promote full myogenic differentiation.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture supplies, fetal calf serum, newborn calf serum, horse serum, Ham's F-10 medium, phosphate-buffered saline (PBS), G418, and platelet-derived growth factor-bb were purchased from Life Technologies, Inc. Dulbecco's modified Eagle's medium

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(DME) was obtained from Mediatech-Cellogrow, Herndon, VA. RIGF-1 was purchased from Gropep, Adelaide, Australia, and human fibroblast growth factor-2 was purchased from Promega, Madison, WI. LY294002 was from Biomol Research Laboratories, Plymouth Meeting, PA, and 4-hydroxymatoxin was from Sigma-Aldrich. Collagenase type II was obtained from Worthington Biochemical Corp., Lakewood, NJ, and penicillin was from Sigma. 4-hydroxytamoxifen was from Sigma and p38 mitogen-activated protein kinase inhibitor (SB203580) was from Calbiochem. PBS, and fresh growth medium was added. When the cells reached >90% confluent density, growth medium was replaced with DM with or without supplements as indicated, incubated at 37 °C for various times, and fixed for immunocytochemistry with 4% paraformaldehyde.

Construction and Use of an Inducible Akt Adenovirus—The previously described iAkt plasmid (33) was used to create a recombinant adenovirus with the AdEasy Vector kit (Quantum Biotechnologies). First, the iAkt cDNA was isolated from its plasmid by restriction digestion with SalI and NheI endonucleases. After both sites were blunt with the Klenow fragment of DNA polymerase I, the DNA was ligated into the EcoRV site of a modified pShuttle plasmid (AdEasy kit) from Clontech. The recombinant plasmid was linearized and recombined with the pAdEasy plasmid in E. coli strain BW31583 as described by the manufacturer. Five micrograms of purified pAdEasy:TetR/iAkt plasmid was then digested with Pael and transfection and transfected into low passage HEK293 cells using Fugene-6 at a ratio of 1 μg of plasmid DNA and 5 μg of pAdEasy plasmid (37). A pShuttle/TetR/iAkt recombinant plasmid was linearized and recombined with the pAdEasy plasmid in E. coli strain BW31583 as described by the manufacturer. After microinjection into newborn male C57BL/6 mice, the left eye was injected with 5 × 10^6 pfu of virus and the right eye was injected with 1 × 10^6 pfu of virus. Treatment was given at various times after birth. After 4 weeks of age, all mice were prepared for immunohistochemical and immunofluorescent analyses as described.
resuspension in 40 μl of kinase assay buffer containing the Akt substrate GSKit-3α and 200 μM ATP, the reaction was allowed to proceed at 30 °C for 30 min. After stopping the reaction by the addition of concentrated electrophoresis loading buffer, samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, as described above. Immunoblotting was performed using rabbit polyclonal antibody to phospho-GSK-3α/β, and immunoreactive proteins were visualized by enhanced chemiluminescence and detected using the Molecular Imager FX and Quantity One software, as above.

Statistical Analysis—Results are presented as the means ± S.E. Statistical significance was determined using independent Student's t test for paired samples. Results were considered statistically significant when p < 0.05.

RESULTS

IGF-I Stimulates Differentiation of C2BP5 Myoblasts—We previously established muscle cell lines derived from C2 myoblasts stably transfected with a mouse IGFBP-5 cDNA (C2BP5 cells). These cells did not undergo significant morphological or biochemical differentiation unless IGF-I, IGF-II, or analogues that activated the IGF-I receptor were added to low serum DM (36). Fig. 1 shows that C2BP5 cells expressed little MHC or myogenin after up to 4 days in DM and formed few multinucleated myotubes unless the analogue R3IGF-I, which binds to the IGF-I receptor but not IGFBP-5 (39), was included in the medium. After 4 days, nearly 30% of IGF-treated cells produced myogenin compared with <2% of myoblasts incubated in DM alone (Fig. 1B), and large myotubes were seen in IGF-treated cultures versus almost none in DM (Fig. 1A). As seen by immunoblotting, in IGF-I-treated cells myogenin was readily induced within 2 days, and expression was increased for up to 4 days, whereas little myogenin was detected in myoblasts incubated in DM alone (Fig. 1C). A similar pattern of differential induction of differentiation was observed in myoblasts cultured for up to 7 days in DM without or with R3IGF-I (data not shown). Thus, the addition of IGF-I promotes differentiation of C2BP5 muscle cells.

Sustained PI 3-Kinase Activity Is Required for IGF-stimulated Muscle Differentiation—Previous studies with several muscle cell lines indicate that inhibition of PI 3-kinase activity could interfere with myoblast differentiation (11, 27, 29–32, 40). To determine the role of the PI 3-kinase pathway in IGF-mediated muscle differentiation, C2BP5 cells were incubated with R3IGF-I in the presence of the drug LY294002, a specific PI 3-kinase inhibitor (41). As seen in Fig. 2, A and B, LY294002 added at day 0 prevented IGF-stimulated production of myogenin, blocked expression of MHC, and abrogated production of multinucleated myofibers for up to 4 days but did not alter cell viability. Similar results were observed in parental C2 cells, where LY294002 diminished expression of myogenin (Fig. 2C), and in mouse limb myoblasts in primary culture, where LY294002 blocked myogenin and MHC expression and blunted myotube formation (Fig. 2D). In the latter case, LY294002 diminished survival by ~20%. Thus, LY294002 inhibits differentiation in C2BP5 myoblasts and in other cultured muscle cells.

We next asked if the blockade of PI 3-kinase activity interfered with muscle differentiation even after it was initiated. C2BP5 cells were incubated in DM plus R3IGF-I for 2 days, after which LY294002 or vehicle (MeSO₄) was added in the presence of additional R3IGF-I for a further 1 or 2 days. As shown in Fig. 3, LY294002 prevented the further increase in myogenin protein expression that normally occurred during this period and also attenuated production of myotubes. These results indicate that continued signaling through the IGF-I receptor and PI 3-kinase are needed for full differentiation of C2BP5 myoblasts.

As a further test of the requirement for PI 3-kinase activity in muscle differentiation, C2BP5 cells were transfected with expression plasmids encoding either a constitutively active or kinase-deficient form of PI 3-kinase (p110α or p110Δkin, respectively). After incubation of cells in DM without IGF-I for 2 days, only 5 ± 0.4% of myoblasts transfected with p110Δkin expressed MHC, a value not different from results obtained using an enhanced green fluorescent protein expression vector (2.5 ± 0.5%, Fig. 4). By contrast, 25.7 ± 2% of cells transfected with p110α were also positive for MHC (p < 0.008), results equivalent to those observed with a myogenin expression plasmid (27 ± 1.2%). Surprisingly, the ability of myogenin to stimulate myoblast differentiation was significantly inhibited when cells were incubated with LY294002 (Fig. 4, 7.5 ± 1.1%, p < 0.009). Taken together, the results in Figs. 3 and 4 suggest that sustained IGF-stimulated PI 3-kinase activity is needed to maintain muscle differentiation. These observations additionally indicate that IGF-mediated PI 3-kinase activation is part of a signaling pathway involved both in induction of myogenin expression and in potentiation of myogenin action on differentiation. Thus, PI 3-kinase appears to function both upstream
and downstream of myogenin during muscle differentiation in C2BP5 cells.

**IGF-I Causes Sustained Stimulation of Akt Activity in C2BP5 Cells**—Akt is one of the major downstream effector molecules for PI 3-kinase-regulated signaling pathways (42) and has been linked to a variety of biological effects in skeletal muscle cells, including control of early and late events in differentiation (15, 43). To explore the role of Akt in IGF-I-mediated myoblast differentiation, we first evaluated the ability of the growth factor to activate this protein kinase. As seen in Figs. 5, A and B, treatment of C2BP5 cells with R3IGF-I led to readily increased and sustained phosphorylation of Akt on serine 473 that was prevented in cells co-incubated with LY294002. IGF-I also promoted prolonged stimulation of Akt kinase activity. As shown in Fig. 5C, growth factor treatment caused a prompt induction of enzymatic activity (nearly 3-fold within 5 min) that peaked by 2 h but remained at nearly peak levels (5 times that of control cells) for at least 12 h. Phosphorylation of Akt on serine 473 also was maintained for up to 24 h after incubation of cells with IGF-I (data not shown). Thus, the addition of IGF-I leads to sustained Akt activity in C2BP5 myoblasts.

**Forced Expression of Akt Stimulates Muscle Differentiation**—We next evaluated the ability of Akt to promote differentiation of C2BP5 myoblasts. Cells were co-infected with two recombinant adenoviruses, one encoding a tTA and the other encoding a tTA-regulated gene expressing HT-inducible HA-tagged membrane-targeted Akt (iAkt). Upon infection in the absence of tetracycline or derivatives, Akt protein is expressed, but its full enzymatic activity requires induction by HT (44). As shown in Fig. 6, when virus-infected cells were incubated in DM without HT for up to 4 days, fewer than 10% of myoblasts expressed myogenin (Fig. 6A) and very few MHC-positive cells were seen despite significant expression of iAkt (Fig. 6B). In contrast, in the presence of HT, nearly 34% of cells were positive for myogenin by day 4 (p < 0.04), and extensive MHC expression and myotube formation was observed. In the presence of the last-lasting tetracycline analogue, doxycycline, little Akt expression was observed (data not shown).

To extend these results further, C2 myoblasts and mouse limb myoblasts in primary culture were co-infected with the

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**Fig. 2. Inhibition of muscle differentiation by LY294002.** A, immunoblots for myogenin and cdk4 using whole cell protein extracts of C2BP5 cells incubated in DM plus R3IGF-I (2 nM) without or with LY294002 (LY, 30 μM) for up to 4 days. B, results of immunocytochemistry of C2BP5 cells for myogenin (green) and MHC (red) after incubation for 4 days in DM plus R3IGF-I (IGF-I, 2 nM) without or with LY294002 (LY, 30 μM). Below are nuclei stained with Hoechst dye. C, immunoblots for myogenin and cdk4 using whole cell protein extracts of C2 cells incubated in DM without or with LY294002 (LY, 30 μM) for the times indicated. D, results of immunocytochemistry of primary mouse myoblasts for myogenin (green) and MHC (red) after incubation for 4 days in DM without or with LY294002 (LY, 30 μM). Below, nuclei are stained with Hoechst dye.

**Fig. 3. Sustained IGF-I action is required for myogenin expression.** A, immunoblots for myogenin and cdk4 using whole cell protein extracts of C2BP5 cells incubated in DM plus R3IGF-I (IGF-I, 2 nM) for up to 4 days. After 2 days in DM, LY294002 (30 μM) or vehicle (Me2SO4) was added to cultures. The graph shows results (mean ± S.E.) of three independent experiments. Values obtained after 4 days of IGF-I treatment have been arbitrarily set to 100. Myogenin expression after treatment with IGF-I for 3 or 4 days is significantly greater than after incubation with IGF-I plus LY294002 (p < 0.003). B, immunocytochemistry of C2BP5 cells using antibody against MHC (red) and myogenin (green) after incubation for 4 days in DM with R3IGF-I without or with LY294002 (LY) added for the last 2 days.
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DISCUSSION

Muscle differentiation is a multi-stage process involving cell cycle withdrawal, expression, and activation of muscle-specific genes and proteins and fusion of mononucleated myocytes into multinucleated myofibers (1–3, 7). In this study we have focused on mechanisms of IGF-mediated induction of differentiation. In previous papers we have established that IGF-I-activated signaling pathways involving PI 3-kinase and Akt promote myoblast survival during the earliest phases of differentiation by stimulating expression of the cyclin-dependent kinase inhibitor, p21/waf1/cip1 (34, 35). We now show that the same molecules play key roles in later stages of muscle differentiation and, when ectopically expressed, bypass the need for IGF signaling.

We previously characterized C2-derived myoblast cell lines overexpressing the IGF-binding protein, IGFBP-5 (36). C2BP5 cells failed to differentiate normally when incubated in low serum differentiation medium and showed enhanced viability under these conditions. Differentiation could be restored by exogenous IGF-I or by analogues that activated the IGF-I receptor (31) demonstrate that forced expression of active PI 3-kinase could positively regulate myogenic differentiation. We now have extended their studies to show that ectopic expression of PI 3-kinase by itself was sufficient to stimulate differentiation and, in C2BP5 cells, was as potent as myogenin. In addition we find that active PI 3-kinase is required to maintain differentiation, since the addition of LY294002 after 2 days of incubation of C2BP5 cells with IGF-I blocked further increases in myogenin expression and diminished myotube formation.

Although as expected, exogenous myogenin was able to stimulate differentiation of C2BP5 myoblasts, its effects, surprisingly, were blocked by LY294002, indicating that IGF-regulated PI 3-kinase activity was involved not only in induction of myogenin gene and protein expression but also in potentiation of myogenin action. In agreement with these results, Xu and Wu (45) recently showed that active PI 3-kinase could enhance the effects of ectopically expressed MyoD or MEF2C on co-transfected reporter genes, suggesting a collaborative role for signaling pathways regulated by PI 3-kinase in myogenic tran-
scription factor function. Similar observations are reported by Tamir and Bengal (40), who were able to blunt the actions of MEF2C on differentiation of MyoD-transfected 10T1/2 fibroblasts with the PI 3-kinase inhibitor, LY294002. It is thus reasonable to postulate that at least part of the inhibition of myogenin-stimulated differentiation by LY294002 that we observe in C2BP5 cells is secondary to interference with the activity of MEF2C, thus minimizing its ability to collaborate with myogenin (7).

The serine-threonine kinase Akt has been implicated as a central downstream target of PI 3-kinase action (42). Akt has been found to play a key role in the survival of many cell types (46–50), including skeletal myoblasts (34, 35, 43), where we have demonstrated its importance in induction of p21 gene and protein expression (34, 35). As shown here, IGF-I treatment of C2BP5 cells led to a rapid increase in phosphorylation of Akt and to a rapid and sustained induction of Akt enzymatic activity. These findings are in general agreement with published results demonstrating persistently enhanced phosphorylation of Akt in L8 and C2C12 myoblasts (40, 51). More importantly, we find that ectopic expression of iAkt could promote extensive differentiation of C2BP5 cells in the absence of IGF-I and could reverse the inhibitory effects of LY294002 on differentiation of C2 cells and primary mouse myoblasts. These results extend observations by Jiang et al. (52), who demonstrated a role for Akt in modulating differentiation of chick embryonic myoblasts, and by Rommel et al. (25), who found that forced expression of Akt mimicked the actions of IGF-I in promoting myofiber hypertrophy of C2C12 myoblasts. Thus, we now show that Akt by itself is sufficient to promote differentiation in model muscle cell systems.

It has been suggested recently that Akt-2 (or protein kinase Bβ) plays a more fundamental and essential role than Akt-1 (protein kinase Bα) in muscle differentiation in culture (53). Expression of Akt-2 but not Akt-1 was induced during differentiation (53, 54), and Akt-2 but not Akt-1 was found in nuclei of differentiated C2.7 myoblasts (53). In addition, Akt-2 appeared to be more potent than Akt-1 in transactivating a muscle-specific reporter gene, and an Akt-2-specific inactivating antibody blocked differentiation, whereas an Akt-1-specific antibody had little effect (53). Our results, although not addressing the potency of different Akt isoforms, indicate that nuclear expression of Akt is not obligatory for its stimulation of differentiation. As seen in Fig. 6, iAkt was not found in the nucleus of infected muscle cells, yet upon activation with HT, was able to promote extensive myofiber formation. Based on published

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**FIG. 5.** IGF-I stimulates Akt activity in C2BP5 myoblasts. A, immunoblots of phosphorylated Akt (p-Akt) and total Akt using whole cell protein extracts of C2BP5 cells incubated in DM without or with R1 IGF-I (IGF-I, 2 nm) for the times indicated. B, immunoblots of phosphorylated Akt and total Akt using whole cell protein extracts of C2BP5 cells treated with R1 IGF-I (IGF-I, 2 nm) plus vehicle (MeSO4) or LY294002 (LY, 30 μM) for the times indicated. C, results of in vitro Akt kinase assays performed with whole cell protein extracts of C2BP5 myoblasts incubated in DM without or with R1 IGF-I (IGF-I, 2 nm) for up to 12 h. The graph summarizes results (mean ± S.E.) of three independent experiments. Akt activity measured after treatment of cells with IGF-I for 2 h has been assigned a value of 100%. The asterisks indicate significant increases in kinase activity (*, p < 0.02, **, p < 0.002).

**FIG. 6.** Akt promotes differentiation of C2BP5 myoblasts. A, quantitation of myogenin expression in C2BP5 myoblasts infected with a recombinant adenovirus expressing iAkt. One day after infection, cells were incubated for the times indicated in DM plus either vehicle (ethanol; gray bars) or HT (1 μM, black bars) and immunostained using antibodies to the HA epitope (for Akt) or to myogenin. Results are presented as the percentage of HA-expressing cells that co-express myogenin (mean ± S.E. of 3 independent experiments, each representing at least 200 cells counted). There was a significant increase in myogenin expression after activation of Akt with HT (p < 0.04). B, immunocytochemistry of adenovirus-infected C2BP5 myoblasts using antibodies to the HA epitope (for Akt) or MHC after cells were incubated for 4 days in DM supplemented with either vehicle (ethanol (EtOH)) or HT (1 μM).
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studies showing that active Akt could transactivate musclespecific promoters in transient transfection assays (45, 53), our results suggest the existence of an additional uncharacterized nuclear component in the IGF-I-PI 3-kinase-Akt-muscle differentiation pathway.

Although the physiological relevance of the IGF-stimulated signaling pathways defined here have not been investigated, previous studies have demonstrated that IGF-I action plays key roles in the formation and maintenance of skeletal muscle. Mice engineered to lack the IGF-I receptor or deficient in IGF-I exhibited marked muscle hypoplasia during embryonic development and died shortly after birth because of inadequate muscle mass to inflate their lungs (22, 23). Conversely, forced expression of IGF-I in skeletal muscle caused myofiber hypertrophy (24, 26) and was responsible for sustaining muscle mass during aging (25, 26). Further dissection of the mechanisms of regulation and actions of the IGF-I-PI 3-kinase-Akt pathway should lead to new insights with therapeutic implications for diseases affecting skeletal muscle.

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