Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10307–10311, October 1995
Cell Biology

Opposing early and late effects of insulin-like growth factor I on differentiation and the cell cycle regulatory retinoblastoma protein in skeletal myoblasts

(cyclin D1/cyclin-dependent kinase 4/myogenin)

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Communicated by William H. Daughaday, Balboa Island, CA, July 31, 1995

ABSTRACT **The mechanisms by which insulin-like growth factors (IGFs) can be both mitogenic and differentiation-promoting in skeletal myoblasts are unclear because these two processes are believed to be mutually exclusive in this tissue. The phosphorylation state of the ubiquitous nuclear retinoblastoma protein (Rb) plays an important role in determining whether myoblasts proliferate or differentiate: Phosphorylated Rb promotes mitogenesis, whereas un- (or hypo-) phosphorylated Rb promotes cell cycle exit and differentiation. We hypothesized that IGFs might affect the fate of myoblasts by regulating the phosphorylation of Rb. Although long-term IGF treatment is known to stimulate differentiation, we find that IGFs act initially to inhibit differentiation and are exclusively mitogenic. These early effects of IGFs are associated with maintenance of Rb phosphorylation typical of proliferating cells; upregulation of the gene expression of cyclin-dependent kinase 4 and cyclin D1, components of a holoenzyme that plays a principal role in mediating Rb phosphorylation; and marked inhibition of the gene expression of myogenin, a member of the MyoD family of skeletal muscle-specific transcription factors that is essential in muscle differentiation. We also find that IGF-induced inhibition of differentiation occurs through a process that is independent of its mitogenic effects. We demonstrate, thus, that IGFs regulate Rb phosphorylation and cyclin D1 and cyclin-dependent kinase 4 gene expression; together with their biphasic effects on myogenin expression, these results suggest a mechanism by which IGFs are initially mitogenic and subsequently differentiation-promoting in skeletal muscle.**

Cell proliferation and differentiation are believed to be mutually exclusive events in skeletal muscle and in a wide variety of cell types (1). Although growth factors, in general, inhibit muscle differentiation, the insulin-like growth factors (IGFs) are distinctive in that they are the only known mitogens that, when free of other serum components, stimulate both proliferation and differentiation of skeletal muscle cells (2, 3). The mechanisms by which the IGFs can be both mitogenic and differentiation-promoting in muscle, however, are unclear.

The decision of a cell to proliferate or differentiate is linked to mechanisms that regulate the cell cycle (1). Recent studies have demonstrated that the retinoblastoma gene product (Rb) is an important regulator of cell cycle progression (4). Rb is a ubiquitous nuclear factor, the activity of which is regulated by phosphorylation (5, 6). When un-(or hypo-)phosphorylated in the early G_1 phase of the cell cycle, Rb is thought to act as a growth suppressor by interacting with and inactivating E2 promoter binding factor (E2F)/differentiation-regulated transcription factor 1 protein (DP) complexes that play a role in the transcription of genes necessary for DNA synthesis (7). However, in mid-to-late G_1 phase, Rb becomes phosphorylated, E2F/DP complexes are released, and Rb is no longer active as a growth suppressor (8).

Recent studies have demonstrated that the Rb protein, which is present in phosphorylated and un-(or hypo-) phosphorylated forms in proliferating myoblasts but is present virtually only in the unphosphorylated form in differentiated myocytes (9), is required for terminal myogenic differentiation. Inactivation of unphosphorylated Rb by simian virus 40 T antigen results in reinitiation of DNA synthesis and mitosis in previously differentiated myocytes (9). Furthermore, unlike myotubes from normal mice, cultured myotubes from mice genetically deficient in Rb retain the ability to synthesize DNA, demonstrating that Rb is required to maintain the postmitotic state of differentiated myocytes (10). In addition, the unphosphorylated but not the phosphorylated form of Rb can interact directly with members of the MyoD family of skeletal muscle-specific transcription factors, including myogenin (9), which plays an essential role in muscle differentiation (11, 12). This interaction may be required for myoblast differentiation because Saos-2 osteosarcoma cells, which express a nonfunctional Rb protein, can be converted to skeletal muscle cells by cotransfection with Rb and myogenin expression constructs but cannot be so converted with a myogenin construct alone (9).

In view of the critical role of Rb phosphorylation in determining whether myoblasts proliferate or differentiate, we hypothesized that IGFs might affect the fate of myoblasts by regulating Rb phosphorylation. While long-term IGF treatment is known to stimulate differentiation (3), we find that IGFs act initially to inhibit differentiation and are exclusively mitogenic. These early effects of IGFs are associated with marked inhibition of the gene expression of myogenin, maintenance of Rb phosphorylation typical of proliferating cells, and upregulation of the gene expression of cyclin-dependent kinase 4 (cdk4) and cyclin D1, components of a holoenzyme that plays a principal role in mediating Rb phosphorylation (13). We also find that IGF-induced inhibition of muscle differentiation occurs through a process that is independent of its mitogenic effects. We thus demonstrate that IGFs regulate Rb phosphorylation and cyclin D1 and cdk4 gene expression; together with their biphasic effects on myogenin expression, these results suggest a mechanism by which IGFs are initially mitogenic and subsequently differentiation-promoting in skeletal muscle.

MATERIALS AND METHODS

Materials. The following chemicals were purchased: [α-^{32}P]deoxy-CTP (3000 Ci/mmol; 1 Ci = 37 GBq) from DuPont

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Abbreviations: IGF, insulin-like growth factor; Rb, retinoblastoma gene product; cdk4, cyclin-dependent kinase 4; araC, cytosine β-D-arabinofuranoside; BrdUrd, 5-bromo-2′-deoxyuridine.

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New England Nuclear; cytosine β-D-arabinofuranoside (araC) from Sigma; a monoclonal antibody (G3-245) to the human Rb gene product (which cross-reacts with rat Rb protein) from PharMingen; and tissue culture medium components and fetal bovine serum from the Cell Culture Facility (University of California, San Francisco). IGF-I was a gift from CIBA–Geigy and des(Gly^1–Glu^3)-IGF-I was a gift from Genentech. A rat myogenin cDNA was provided by W. E. Wright (University of Texas Southwestern Medical Center, Dallas), a mouse cyclin D1 cDNA was provided by C. J. Sherr (St. Jude Children's Research Hospital, Memphis, TN), and a rat cdk4 cDNA was provided by T. E. Weaver (Children's Hospital Medical Center, Cincinnati).

Cell Culture. Rat L6E9 skeletal muscle cells [from B. Nadal-Ginard (Harvard Medical School, Boston)], which have been used as a model system for studying the events that occur during muscle cell differentiation (14), were grown in Dulbecco's modified Eagle's medium (DMEM)/20% fetal bovine serum/1% glutamine and antibiotics. Subconfluent cells maintained an appearance typical of myoblasts and were studied 3 days after plating (4×10^3 cells/cm^2) in 16-mm 24-well plates, 35-mm 6-well plates, or 150 × 25-mm tissue culture dishes. For IGF-treatment studies, cells were placed in serum-free medium/1% bovine serum albumin with vehicle (0.1 M acetic acid) or the indicated peptide.

mRNA Analysis. Total RNA was isolated by extraction in guanidinium isothiocyanate (15). RNA was quantitated by spectrophotometric determination at 260 nM. Twenty-five micrograms of RNA per sample was denatured in formaldehyde, subjected to electrophoresis in 1% agarose gels, and transferred to nitrocellulose. Myogenin, cyclin D1, and cdk4 cDNAs were labeled by using random primers to 10^9 cpm/μg. Nitrocellulose membranes were prehybridized, hybridized, and washed as described (16). Autoradiography was done, and mRNA abundance was determined by laser densitometry.

Immunoblotting Analysis. For Rb protein phosphorylation studies, cells were lysed in sample buffer [100 mM Tris·HCl, pH 6.8/4% SDS/0.2% bromophenol blue/20% (vol/vol) glycerol] that was brought to a boil for 5 min just before addition to the cells. Lysates were immediately boiled for 5 min and frozen at −70°C. Dithiothreitol was subsequently added to 30 μl of cell lysate (final concentration, 0.2 M). Samples were boiled and analyzed by SDS/7.5% PAGE. After electrotransfer, nitrocellulose blots were incubated with an anti-Rb protein antibody that recognizes both a larger phosphorylated form and a smaller, hypophosphorylated form. Blots were treated with alkaline phosphatase-conjugated rabbit anti-mouse IgG and subsequently incubated with 5-bromo-4-chloro-3-indolyl phosphate at 150 μg/ml and nitro blue tetrazolium at 300 μg/ml dissolved in 100 mM NaCl/5 mM $MgCl_2$, pH 9.5 until blue color appeared on the membrane. After soaking in 20 mM Tris/1 mM EDTA, pH 2.9, membranes were rinsed with water and air-dried (17). Images of the blots were video-digitized by a charge-coupled device camera (NEC America, Inc., T1-24A, Irving, TX) and image software (Image Version 1.54, National Institutes of Health, Bethesda, MD). The images of the blots were standardized by capturing them at the same focal length and exposure, and the intensity and area of the bands were quantified.

5-Bromo-2′-Deoxyuridine (BrdUrd) Incorporation into DNA. BrdUrd incorporation into DNA as an indication of DNA synthesis was measured using the BrdUrd labeling and detection kit II (no. 1299 964) from Boehringer Mannheim. Briefly, L6E9 myoblasts were grown on 16-mm coverslips in DMEM/20% fetal bovine serum/1% glutamine and antibiotics. After incubation in serum-free medium/1% bovine serum albumin in the absence or presence of IGF peptides and/or araC, cells were labeled with BrdUrd (final concentration, 10 μmol/liter) at 37°C and 5% CO_2/95% air for 30 min. Cells were washed, fixed in 70% (vol/vol) ethanol (in 50 mM glycine buffer, pH 2.0), and treated with an anti-BrdUrd monoclonal antibody (clone BMC 9318, IgG1). Cells were then treated with alkaline phosphatase-conjugated anti-mouse IgG and subsequently incubated in a phosphate/nitro blue tetrazolium solution for 30 min at room temperature. Cells were examined with a light microscope; nuclei that had incorporated BrdUrd appeared blue.

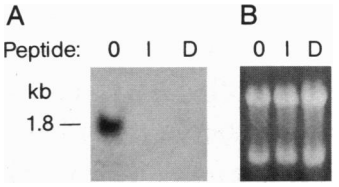

FIG. 1. (*A*) Northern blot of myogenin mRNA in control (lane 0) and IGF-I (lane I) or des(Gly^1–Glu^3)-IGF-I (lane D)-treated (20 ng/ml) cells after 6 hr. (*B*) Ethidium bromide staining of the gel. A representative of three independent experiments is shown.

RESULTS

Early IGF Treatment Decreases Myogenin mRNA. Myogenin mRNA is either low or nondetectable in proliferating myoblasts and increases spontaneously upon serum withdrawal (18–20). Although skeletal myoblasts differentiate merely upon serum withdrawal, IGFs have been shown to augment this process in association with increased levels of myogenin mRNA (19, 20). Studies demonstrating IGF-induced increases in myogenin mRNA, however, involved IGF treatment periods of >24 hr (19, 20). In contrast, when cells were treated for 6 hr with an equivalent concentration of IGF-I (20 ng/ml), myogenin mRNA markedly decreased (Fig. 1). A similar degree of inhibition of myogenin gene expression was seen when myoblasts were treated with des(Gly^1–Glu^3)-IGF-I,

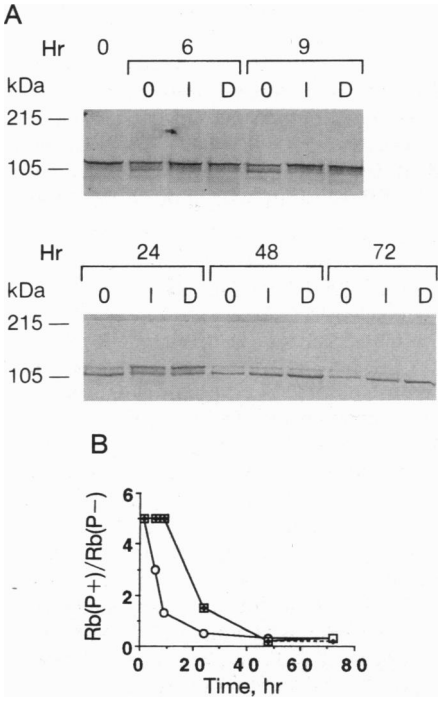

FIG. 2. (*A*) Immunoblots of Rb protein phosphorylation in control (lanes 0) and IGF-I (lanes I) or des(Gly^1–Glu^3)-IGF-I (lanes D)-treated (20 ng/ml) cells over time. The hypophosphorylated Rb is the 105-kDa form. (*B*) Ratio of phosphorylated [Rb (P+)]/un-(or hypo-) phosphorylated Rb [Rb (P−)]. ○, Control; □, treated with IGF-I; ◆, treated with des(Gly^1–Glu^3)-IGF-I. A representative of four independent experiments is shown.

a naturally occurring IGF-I analog with markedly reduced affinity for IGF-binding proteins (21) but with an affinity for the IGF-I receptor comparable to that for native IGF-I (22) (Fig. 1). Thus, short- and long-term treatment with IGFs have opposing effects on muscle cell differentiation.

IGFs Regulate Rb Protein Phosphorylation and Cyclin D1 and cdk4 Gene Expression. Because phosphorylation of Rb protein promotes proliferation and inhibits differentiation, we investigated whether the early inhibitory effects of IGFs on muscle differentiation were associated with inhibition of Rb dephosphorylation. When analyzed by SDS/7.5% PAGE, phosphorylated Rb migrates above a hypophosphorylated form of ≈105 kDa (9). Rb in proliferating myoblasts (time 0) was present predominantly in the phosphorylated form (Fig. 2). Cells switched into serum-free medium demonstrated a progressive decrease in phosphorylated Rb and an increase in hypophosphorylated Rb, such that by 24 hr, the ratio of phosphorylated to hypophosphorylated Rb decreased from 5.0 to 0.5 (Fig. 2). In contrast, myoblasts treated with either IGF-I or des(Gly^1–Glu^3)-IGF-I demonstrated persistence of Rb phosphorylation typical of proliferating myoblasts for at least 9 hr, with a prolonged increase in the ratio of phosphorylated to hypophosphorylated Rb for at least 24 hr (Fig. 2).

In view of the ability of IGFs to regulate Rb phosphorylation, we examined the ability of IGFs to regulate the gene expression of cyclin D1 and cdk4, the regulatory and catalytic components, respectively, of a holoenzyme that plays a principal role in mediating the phosphorylation of Rb (13). For these studies, the des(Gly^1–Glu^3)-IGF-I analog was used to minimize any differentiation-inhibitory effects of endogenous IGF-binding proteins seen in these myoblasts with more prolonged IGF treatment (20). On Northern analysis, proliferating myoblasts (time 0) expressed a cyclin D1 transcript of ≈4.0 kb and a cdk4 transcript of 1.4 kb; myogenin mRNA (1.8 kb) was virtually nondetectable (Fig. 3). When control cells were placed in serum-free medium, cyclin D1 mRNA decreased within 6 hr and cdk4 mRNA decreased between 12 and 24 hr. Conversely, myogenin mRNA increased in control cells within 6 hr with a peak increase at 24 hr, indicating spontaneous differentiation upon serum withdrawal. In contrast, cells treated with des(Gly^1–Glu^3)-IGF-I for up to 24 hr expressed cyclin D1 and cdk4 mRNA at levels that were greater than or equal to those in proliferating myoblasts; concurrently, des(Gly^1–Glu^3)-IGF-I markedly inhibited myogenin gene expression. An inverse relationship between the effects of des(Gly^1–Glu^3)-IGF-I on cyclin D1/cdk4 and on myogenin mRNA could be seen at each time point studied between 6 and 24 hr (Fig. 3).

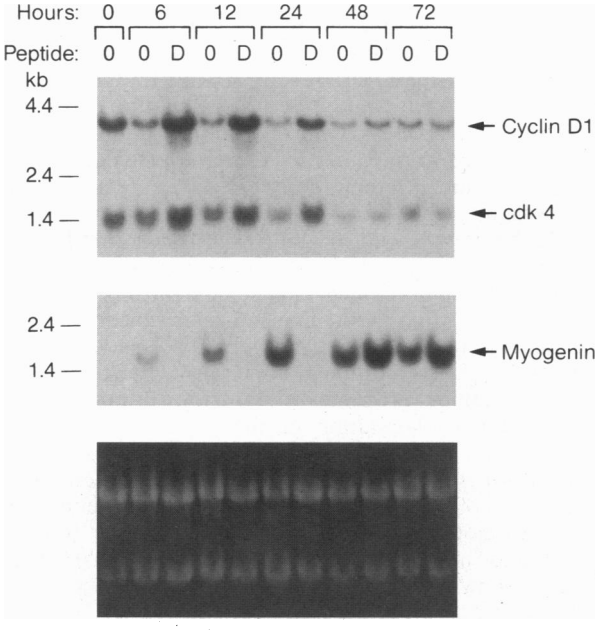

FIG. 3. Northern blots of cyclin D1, cdk4, and myogenin mRNA in control (lanes 0) and des(Gly^1–Glu^3)-IGF-I (lanes D)-treated (20 ng/ml) cells over time. Ethidium bromide staining of the gel is shown below. A representative of four independent experiments is shown.

In contrast to the marked inhibitory effect of IGFs on myogenin gene expression at early time points (up to 24 hr), more prolonged des(Gly^1–Glu^3)-IGF-I treatment (48–72 hr) resulted in an increase in myogenin mRNA similar to that previously reported in this cell line (20) (Fig. 3). Concurrent with this switch of des(Gly^1–Glu^3)-IGF-I from an inhibitor to a stimulator of myogenin gene expression, IGF treatment no longer upregulated Rb phosphorylation (Fig. 2) or cyclin D1 and cdk4 gene expression (Fig. 3).

Because IGFs were added to cells only at time 0, it is possible that the IGF-induced upregulation of Rb phosphorylation and inhibition of myogenin gene expression at 24 but not at 48 hr could have been due to depletion of the added IGF peptide after 24 hr. To address this possibility, myoblasts were studied at 48 hr of IGF treatment, with the peptide added at time 0 only or at both time 0 and 24 hr. Despite readdition of the IGF analog at 24 hr, Rb phosphorylation was not upregulated at 48 hr (Fig. 4*A*), and myogenin gene expression was not inhibited but rather was increased at 48 hr (Fig. 4*B*). Thus, IGF signaling in myoblasts results in a time-dependent altered response that is probably not a consequence of IGF peptide depletion.

IGF-Induced Inhibition of Muscle Differentiation Occurs Through a Process That Is Independent of Its Mitogenic Effects. Because IGFs inhibit muscle differentiation (decreased myogenin mRNA) concurrent with upregulation of Rb phosphorylation and of cyclin D1 and cdk4 gene expression, we examined whether the differentiation-inhibitory effects of IGFs were a consequence of their mitogenic effects. DNA synthesis as measured by BrdUrd incorporation decreased in

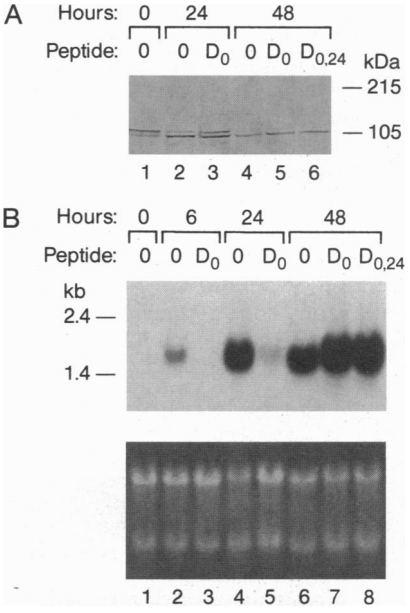

FIG. 4. (*A*) Immunoblot of Rb protein phosphorylation in control (lanes 0) and des(Gly^1–Glu^3)-IGF-I (lanes D)-treated (20 ng/ml) cells: effect of repeated des(Gly^1–Glu^3)-IGF-I treatment. In lanes 3 and 5, des(Gly^1–Glu^3)-IGF-I was added at time 0 only; in lane 6, it was added at time 0 and 24 hr. (*B*) (*Upper*) Northern blot of myogenin mRNA in control (lanes 0) and des(Gly^1–Glu^3)-IGF-I (lanes D)-treated (20 ng/ml) cells: effect of repeated des(Gly^1–Glu^3)-IGF-I treatment. In lanes 3, 5, and 7, des(Gly^1–Glu^3)-IGF-I was added at time 0 only; in lane 8, des(Gly^1–Glu^3)-IGF-I was added at time 0 and 24 hr. (*Lower*) Ethidium bromide staining of the gel. A representative of four independent experiments is shown.

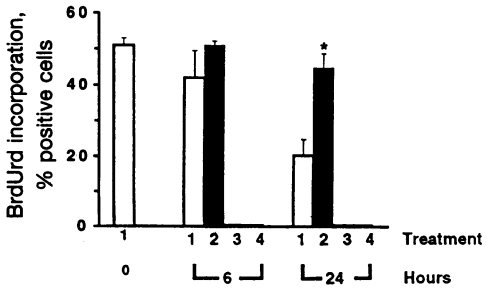

FIG. 5. BrdUrd incorporation into DNA: effect of des(Gly^1–Glu^3)-IGF-I (20 ng/ml) and araC (2 μg/ml). Lanes: 1, control; 2, des(Gly^1–Glu^3)-IGF-I; 3, araC; 4, des(Gly^1–Glu^3)-IGF-I plus araC. Data represent the mean ± SD of six replicate values. A representative of four independent experiments is shown. ⋆, $P < 0.001$ vs. control cells at 24 hr.

control cells after 24 hr in serum-free medium (Fig. 5). In contrast, treatment with des(Gly^1–Glu^3)-IGF-I for 6–24 hr maintained BrdUrd incorporation at a level comparable to that in proliferating myoblasts (time 0) (Fig. 5). Of note, myoblasts were 50–60% confluent at time 0. No change in cell density was seen in control or IGF-treated cells at 6 hr or after 24 hr in control cells; however, consistent with the known mitogenic response of myoblasts to IGFs (2), cells treated with des(Gly^1–Glu^3)-IGF-I had divided and were 90–95% confluent at 24 hr (data not shown).

To examine the relationship between IGF-induced inhibition of differentiation and stimulation of mitogenesis, we inhibited DNA synthesis with araC; araC at 2.0 μg/ml has been previously shown to completely inhibit proliferation of L6E9 skeletal myoblasts without inducing cell lysis or cell death (14). This dose of araC completely inhibited DNA synthesis not only in serum-free but also in des(Gly^1–Glu^3)-IGF-I-treated cells at 6 and 24 hr (Fig. 5).

We therefore used araC at 2 μg/ml for up to 24 hr, during which DNA synthesis was completely suppressed in basal and des(Gly^1–Glu^3)-IGF-I-treated cells, and reexamined the effects of des(Gly^1–Glu^3)-IGF-I on differentiation. As seen in Fig. 6, myogenin mRNA was barely detectable in proliferating myoblasts (time 0) but increased spontaneously in serum-free medium in the absence or presence of araC at 9 and 24 hr. When cells were treated with the IGF analog alone, myogenin mRNA accumulation was markedly inhibited at both 9 and 24 hr, as expected (see Fig. 3). However, when myoblasts were treated concurrently with des(Gly^1–Glu^3)-IGF-I and araC, myogenin gene expression continued to be markedly suppressed (Fig. 6), demonstrating that IGF-induced inhibition of

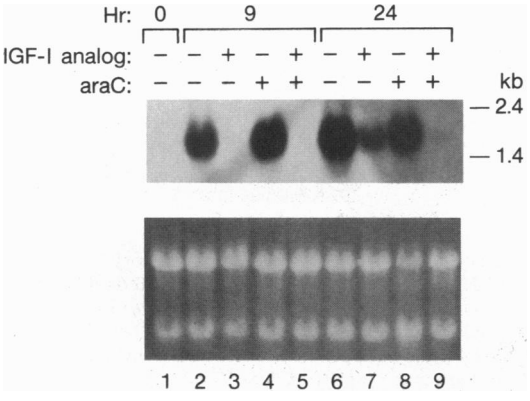

FIG. 6. Effect of araC (2 μg/ml) on des(Gly^1–Glu^3)-IGF-I (20 ng/ml; IGF-I analog)-induced inhibition of myogenin mRNA accumulation. (*Upper*) Northern blot. (*Lower*) Ethidium bromide staining of the gel. A representative of four independent experiments is shown.

differentiation can occur through a process that is independent of its mitogenic effects.

DISCUSSION

Skeletal muscle cell differentiation is a process whereby mesodermal stem cell-derived myoblasts cease proliferation and acquire the biochemical and morphologic phenotype of myocytes and fused myotubes (1). The decision of myoblasts to proliferate or differentiate is strongly linked to the cellular environment, influenced by cell–extracellular matrix interactions (23), direct cell–cell interactions (24), and by soluble growth factors (25). Although growth factors, in general, inhibit myogenic differentiation (25), IGFs are distinctive in that they are the only known mitogens that, when free of other serum components, stimulate both the proliferation and differentiation of skeletal muscle cells (2, 3, 19, 20, 25). This dual effect of IGFs is of particular interest because these two processes are believed to be mutually exclusive in these cells (1).

In contrast to the previously reported stimulation of myogenic differentiation by IGFs, requiring treatment periods of >24 hr (3, 19, 20), we find that IGFs act initially to inhibit differentiation associated with a marked reduction in myogenin mRNA. To explore a potential mechanism by which IGFs initially inhibit and subsequently stimulate myogenic differentiation, we examined the effects of IGFs on phosphorylation of the cell cycle regulatory Rb protein. The inactive, phosphorylated form of Rb promotes proliferation, whereas the un-(or hypo-)phosphorylated form promotes the terminally differentiated state of myocytes (9). Concurrent with IGF-induced inhibition of myogenin gene expression, we find that IGFs inhibit Rb dephosphorylation and upregulate gene expression of cyclin D1 and cdk4, components of a holoenzyme that plays a principal role in mediating Rb phosphorylation (13). Although IGF-I has also been shown to increase cyclin D1 expression in MG63 human osteosarcoma cells (26), our results are, to our knowledge, the only demonstration that IGFs regulate Rb phosphorylation and cdk4 gene expression.

Inhibition of Rb dephosphorylation and upregulation of cyclin D1/cdk4 gene expression by IGFs can potentially inhibit myogenic differentiation through a variety of mechanisms. Recently a direct interaction between myogenin and Rb has been shown to occur and may be required for skeletal muscle differentiation (9); however, this interaction involves only the un-(or hypo-)phosphorylated form of Rb (9). Thus, maintenance of Rb phosphorylation by IGFs would be expected to inhibit interaction of Rb with myogenin. In addition, IGFs upregulate cyclin D1 mRNA, and overexpression of cyclin D1 inhibits myogenic differentiation by blocking the activation of muscle-specific genes by MyoD (27, 28).

While the present studies with BrdUrd show that IGFs are mitogenic at the same time that they inhibit differentiation, studies with araC, in which DNA synthesis was completely prevented, show that IGF-I-induced inhibition of myogenic differentiation occurs through a process that is independent of its mitogenic effects. Although basic fibroblast growth factor is thought to inhibit myogenic differentiation by protein kinase C-mediated phosphorylation of the basic helix–loop–helix muscle-specific transcription factors (29), and transforming growth factor β is thought to inhibit myogenic differentiation by repression of the transcriptional activity of these myogenic basic helix–loop–helix proteins (30), both basic fibroblast growth factor and transforming growth factor β, like IGF-I, have been shown to negatively regulate muscle differentiation without stimulating proliferation (31, 32).

While other growth factors such as basic fibroblast growth factor, transforming growth factor β, and platelet-derived growth factor are exclusively inhibitory of myogenic differentiation under serum-free conditions (31–33), our work dem-

onstrates that IGFs can initially inhibit and subsequently stimulate differentiation of the same tissue type. Just as IGF-induced inhibition of myogenic differentiation occurs through a process independent of its mitogenic effects, IGF-induced stimulation of muscle differentiation is not simply a consequence of increased cell number: IGFs for 48 hr stimulated myogenic differentiation in subconfluent L6 myoblasts, even when DNA synthesis was completely inhibited by araC (34). The initial inhibition and subsequent stimulation of skeletal muscle differentiation by IGFs may reflect a divergence in IGF-I receptor signaling pathways or may reflect quantitative differences in components of the same signaling pathway. With respect to the latter possibility, the response of PC-12 chromaffin cells to receptor tyrosine kinase activation is thought to be determined by duration of extracellular signal-regulated kinase (ERK) activation (35). Transient extracellular signal-regulated kinase activation is associated with a proliferative response in PC-12 cells, whereas more sustained extracellular signal-regulated kinase activation is associated with differentiation (35).

While exogenous IGFs have a time-dependent biphasic effect on myogenic differentiation, endogenous IGF production by skeletal muscle cells is required for differentiation in tissue culture (36, 37). After serum withdrawal, IGF peptide production by muscle cells increases spontaneously (38–40), and IGF antisense oligodeoxynucleotides or cRNAs inhibit the differentiation process (36, 37). Taken together, these data suggest that IGFs function as "maintenance factors" in skeletal muscle cells: When subconfluent, proliferating myoblasts are exposed to exogenous IGFs, these IGFs initially maintain the cycling state of these cells. However, once myoblasts leave the cell cycle, autocrine production of IGFs is induced (38–40), which promotes and maintains the differentiated state of these cells. These studies suggest that in developing skeletal muscle *in vivo*, endocrine and/or paracrine sources of IGFs contribute initially to a proliferative response in myoblasts. Subsequently, when environmental cues favor differentiation (e.g., reaching a critical threshold of cell density), an autocrine source of IGFs is induced that potentiates the differentiated state. A proliferative role for IGFs during early muscle development is supported by the marked skeletal muscle hypoplasia observed in transgenic mice homozygous for a targeted mutation of the IGF-I receptor (41).

In summary, IGF-I can no longer be viewed only as a positive regulator of myogenic differentiation. While both long-term IGF treatment as well as autocrine/paracrine production of IGFs by muscle cells promote the differentiated state, proliferating myoblasts respond to IGFs initially with a marked inhibition of differentiation and a parallel mitogenic response. The proliferative and differentiation-inhibiting responses to IGF-I can be separated, however, because blocking the mitogenic effects of IGF-I did not prevent inhibition of differentiation by IGF-I. These early effects of IGFs on myogenesis may be mediated, at least in part, by a marked decrease in myogenin mRNA, inhibition of Rb dephosphorylation, and upregulation of the gene expression of cyclin D1 and cdk4.

We thank Dr. David O. Morgan and Dr. Ellen H. Filvaroff for helpful discussions and Carol Dahlstrom for typing the manuscript. This work was supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (1R29 DK-44181) and by Basic Research Grant 1-FY92-0700 from the March of Dimes Birth Defects Foundation.

1. Olson, E. N. (1992) *Dev. Biol.* **154,** 261–272.
2. Ewton, D. Z. & Florini, J. R. (1980) *Endocrinology* **106,** 577–583.
3. Ewton, D. Z. & Florini, J. R. (1981) *Dev. Biol.* **86,** 31–39.
4. Goodrich, D. W., Wang, N. P., Qian, Y.-W., Lee, E. Y.-H. P. & Lee, W.-H. (1991) *Cell* **67,** 293–302.
5. Buchkovich, K., Duffy, L. A. & Harlow, E. (1989) *Cell* **58,** 1097–1105.
6. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J. & Lee, W.-H. (1989) *Cell* **58,** 1193–1198.
7. La Thangue, N. B. (1994) *Curr. Opin. Cell Biol.* **6,** 443–450.
8. Sherr, C. J. (1994) *Cell* **79,** 551–555.
9. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V. & Nadal-Ginard, B. (1993) *Cell* **72,** 309–324.
10. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V. & Nadal-Ginard, B. (1994) *Science* **264,** 1467–1471.
11. Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J., Olson, E. N. & Klein, W. H. (1993) *Nature (London)* **364,** 501–506.
12. Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. & Nabeshima, Y. (1993) *Nature (London)* **364,** 532–535.
13. Kato, J.-Y., Matsushime, H., Hiebert, S. W., Ewen, M. E. & Sherr, C. J. (1993) *Genes Dev.* **7,** 331–342.
14. Nadal-Ginard, B. (1978) *Cell* **15,** 855–864.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), Vol. 1, pp. 7.19–7.22.
16. Hartmann, K. K. P., Papa, V., Brown, E. J., Ullrich, D., Rosenthal, S. M. & Goldfine, I. D. (1990) *Endocrinology* **127,** 2038–2040.
17. Liu, X.-J., Malkowski, M., Guo, Y., Erickson, G. F., Shimasaki, S. & Ling, N. (1993) *Endocrinology* **132,** 1176–1183.
18. Edmondson, D. G. & Olson, E. N. (1989) *Genes Dev.* **3,** 628–640.
19. Florini, J. R., Ewton, D. Z. & Roof, S. L. (1991) *Mol. Endocrinol.* **5,** 618–724.
20. Silverman, L. A., Cheng, Z.-Q., Hsiao, D. & Rosenthal, S. M. (1995) *Endocrinology* **136,** 720–726.
21. Bagley, C. J., May, B. L., Szabo, L., McNamara, P. J., Ross, M., Francis, G. L., Ballard, F. J. & Wallace, J. C. (1989) *Biochem. J.* **259,** 665–671.
22. Ballard, F. J., Francis, G. L., Ross, M., Bagley, C. J., May, B. & Wallace, J. C. (1987) *Biochem. Biophys. Res. Commun.* **149,** 398–404.
23. Heino, J. & Massague, J. (1990) *J. Biol. Chem.* **265,** 10181–10184.
24. Rosen, G. D., Sanes, J. R., LaChance, R., Cunningham, J. M., Roman, J. & Dean, D. C. (1992) *Cell* **69,** 1107–1119.
25. Florini, J. R., Ewton, D. Z. & Magri, K. A. (1991) *Annu. Rev. Physiol.* **53,** 201–216.
26. Furlanetto, R. W., Harwell, S. E. & Frick, K. K. (1994) *Mol. Endocrinol.* **8,** 510–517.
27. Rao, S. S., Chu, C. & Kohtz, D. S. (1994) *Mol. Cell. Biol.* **14,** 5259–5267.
28. Skapek, S. X., Rhe, J., Spicer, D. B. & Lassar, A. B. (1995) *Science* **267,** 1022–1024.
29. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P. & Olson, E. N. (1992) *Cell* **71,** 1181–1194.
30. Brennan, T. J., Edmondson, D. G., Li, L. & Olson, E. N. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3822–3826.
31. Olson, E. N., Sternberg, W., Hu, J. S., Spizz, G. & Wilcox, C. (1986) *J. Cell Biol.* **103,** 1799–1805.
32. Spizz, G., Roman, D., Strauss, A. & Olson, E. N. (1986) *J. Biol. Chem.* **261,** 9483–9488.
33. Jin, P., Sejersen, T. & Ringertz, N. R. (1991) *J. Biol. Chem.* **266,** 1245–1249.
34. Turo, K. A. & Florini, J. R. (1982) *Am. J. Physiol.* **243,** C278–C284.
35. Marshall, C. J. (1995) *Cell* **80,** 179–185.
36. Florini, J. R., Magri, K. A., Ewton, D. Z., James, P. L., Grindstaff, K. & Rotwein, P. S. (1991) *J. Biol. Chem.* **266,** 15917–15923.
37. Montarras, D., Pinset, C., Perez, M.-C., Ilan, J. & Gros, F. (1993) *C.R. Acad. Sci.* **316,** 1029–1031.
38. Tollefsen, S. E., Lajara, R., McCusker, R. H., Clemmons, D. R. & Rotwein, P. (1989) *J. Biol. Chem.* **264,** 13810–13817.
39. Tollefsen, S. E., Sadow, J. L. & Rotwein, P. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1543–1547.
40. Rosenthal, S. M., Brunetti, A., Brown, E. J., Mamula, P. W. & Goldfine, I. D. (1991) *J. Clin. Invest.* **87,** 1212–1219.
41. Liu, J.-P., Baker, J., Perkins, A. S., Robertson, E. J. & Efstratiadis, A. (1993) *Cell* **75,** 59–72.