Coordinate expression of insulin-like growth factor II and its receptor during muscle differentiation
(gene regulation/RNA hybridization/trophic factors)

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ABSTRACT The role of polypeptide growth factors in promoting muscle differentiation is uncharacterized. We have used a fusing skeletal muscle cell line, C2, to examine the endogenous expression of one peptide, insulin-like growth factor II (IGF-II), and its receptor during differentiation. The synthesis of IGF-II is low during proliferation of myoblasts; IGF-II mRNA can be detected only through use of a highly sensitive solution-hybridization assay. Competition binding studies reveal that the IGF-II receptor is similarly nonabundant in myoblasts. During differentiation IGF-II mRNA rises rapidly. A nearly 4-fold increase is seen within 16 hr of onset of the differentiation process, and levels are 25 times higher than those in myoblasts by 96 hr, when myoblasts have formed and muscle-specific α-actin mRNAs are synthesized. IGF-II accumulates in conditioned culture medium with similar kinetics. The expression of IGF-II receptors on the cell surface increases almost 6-fold 24 hr after the onset of differentiation and remains high. These studies suggest that IGF-II and its receptor are coordinately regulated during myogenic differentiation in C2 cells and that IGF-II may be an autocrine factor for skeletal muscle.

The formation of skeletal muscle involves proliferation of a committed stem cell, the myoblast, and subsequent differentiation and fusion into myotubes. Recent studies have suggested (1, 2) that commitment to the myoblast lineage is a consequence of induction of a limited number of genes whose expression is required for the later ordered activation of the differentiation program. Although the phenomenology of myoblast differentiation is well described, the mechanisms involved at the onset of myogenesis in withdrawal from the cell cycle or terminally in expression of muscle-specific proteins (3–6) are not understood. In this context, it has been shown that specific mitogens and activated oncogenes inhibit myogenesis (7–13). Whether other growth factors are capable of promoting muscle differentiation or whether specific differentiation agents are produced by skeletal muscle cells has not been established.

The insulin-like growth factors (IGFs) I and II are structurally related polypeptides with several actions on muscle. IGFs appear able to stimulate both proliferation and differentiation of myoblasts at subnanomolar concentrations and are able to promote nutrient uptake and inhibit proteolysis in muscle cell lines (14–17). In some of these actions, IGF-I is nearly an order of magnitude more potent than IGF-II. It has been suggested that both growth factors exert their effects on myoblasts through the IGF-I receptor (15, 18), an IGF-sensitive tyrosine-specific protein kinase structurally similar to the insulin receptor (19). In contrast, the role of the IGF-II receptor in IGF action is less clear, although this protein appears to be identical to the cation-independent mannose-6-phosphate receptor involved in lysosomal enzyme targeting (20–24).

Several studies have suggested that IGFs might be produced by skeletal muscle (25–27) and thus might be autocrine or paracrine stimulatory agents. The demonstration of induction of IGF mRNAs in muscle in response to a growth stimulus (28) prompted examination of IGF gene expression during muscle differentiation in a model cell line. The mouse C2 cell line undergoes terminal myogenic differentiation upon serum withdrawal (4, 5) and expresses many muscle-specific proteins (4, 5, 8, 29). In this report we demonstrate that one growth factor, IGF-II, is produced by C2 cells in a differentiation-dependent manner and is accompanied by coordinate induction of its cognate receptor.

MATERIALS AND METHODS Cell Culture. The mouse C2 cell line (30) was plated at a density of 3 × 10⁵ cells per cm⁴ on gelatin-coated tissue culture plates 150 mm in diameter and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) newborn calf serum and 10% (vol/vol) fetal bovine serum (BRL–GIBCO) at 37°C in humidified 5% CO₂/95% air atmosphere. After 48 hr the cells reached 85–90% of confluent density and were harvested as undifferentiated cells. To induce differentiation, plates were washed with DMEM, and the medium was changed to DMEM supplemented with either 2% or 10% (vol/vol) horse serum (BRL–GIBCO) with equivalent results. Cells were harvested at various times thereafter.

Molecular Cloning. The mouse IGF-II gene was isolated by screening a DBA/2J cosmid library with a 32P-labeled human IGF-II cDNA probe. Potentially positive colonies were purified by sequential rounds of hybridization. DNA was isolated, and the identity of the gene was verified by DNA sequence analysis (31, 32). A recombinant plasmid containing most of exon 3 and its adjacent 3′ intron was prepared in Bluescript (Stratagene) for use as a hybridization probe (Fig. 1).

RNA Isolation and Analysis. Total cellular RNA was extracted from cell pellets by homogenization in guanidinium thiocyanate as described (33). The integrity of each RNA sample was analyzed by gel electrophoresis, and the quantity was determined spectrophotometrically.

Gel-blot hybridization of electrophoretically fractionated RNA was performed (34) after capillary transfer of RNA from a 1% agarose, 2.2 M formaldehyde gel (35). A single-stranded IGF-II probe (see Fig. 1) was synthesized as an “antisense” transcript from a linearized plasmid, using T7 polymerase (Stratagene), [α-32P]CTP (800 Ci/mmol, DuPont/NEN; 1 Ci = 37 GBq), and unlabeled ATP, GTP, and UTP (36). A rat β-actin cDNA was labeled (37) to a specific activity of 10⁹

Abbreviation: IGF, insulin-like growth factor.

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extends by The portion 1544 Cell specific and derived DNA were calculated IGF-II (23, 40). DuPont/NEN). Ci/mmol, Kilobases; dpm/ug, [alpha-32P]dATP and [alpha-32P]dCTP (800 Ci/mmoll, DuPont/NEN). A solution-hybridization nuclelease-protection assay using 8 µg of total cellular RNA was performed by following previously described methods (38) and using 1.0–1.5 × 10^6 dpm of labeled probe (36). In all experiments serial dilutions of neonatal mouse liver RNA and yeast tRNA were included as positive and negative controls, respectively. The latter did not hybridize to the IGF-II probe. Relative RNA abundance was calculated with a scanning laser densitometer (LKB).

Receptor Binding Studies. Recombinant IGF-II (Mr = 7471; provided by M. Smith, Lilly), and recombinant [Th35]IGF-I (Mr = 7649; purchased from Agenm Biologicals) were radioiodinated using lactoperoxidase and NaI219 (39). The specific activity ranged from 133 to 198 µCi/µg.

IGF-II binding studies to C2 cells were performed as described (23, 40). Cell monolayers were washed three times in serum-free DMEM separated by 20-min incubations at 37°C. Cells were scraped from the plates with a rubber policeman, centrifuged, and resuspended in binding buffer [20 mM imidazole-HCl (pH 7.4) with 250 mM NaCl, 5% (vol/vol) glycerol, and bovine serum albumin at 2.5 mg/ml]. Cells were added to 1.5-ml polypropylene microcentrifuge tubes containing 121-labeled IGF-II and various amounts of unlabeled IGF-II. The final volume was 300 µl. After overnight incubation at 4°C, cells were centrifuged for 5 min, and supernatant fluid was removed. Cells were washed once with 1 ml of binding buffer, and radioactivity in the pellet was measured. Binding data were analyzed by the computer program LIGAND (41). Protein was measured (42) in an aliquot of cells solubilized with 0.5 ml of 0.1% NaDodSO4, using bovine serum albumin as the standard.

Affinity cross-linking of IGF receptors was performed as described (16). Cell monolayers were washed three times with 20 ml of Heps buffer (20 mM Heps, pH 7.4/120 mM NaCl/5 mM KCl/1.2 mM MgSO4/10 mM NaHCO3/1.3 mM CaCl2/1.2 mM KH2PO4 containing bovine serum albumin at 10 mg/ml. 125I-labeled IGF-II (final concentration, 0.2 nM) was added with or without unlabeled ligand to cells in 12.5 ml of Hepes buffer plus bovine serum albumin at 20 mg/ml. The cells were incubated for 4 hr at 15°C and washed with 20 ml of ice-cold phosphate-buffered saline. Disuccinimidyl suberate (final concentration, 0.1 mM) in 12.5 ml of Heps buffer was added for 20 min at 15°C to cross-link bound ligand. After quenching the reaction with three vol of 10 mM Tris-HCl/1 mM EDTA, pH 7.4, and aspirating the buffer, cells were scraped from the plates with a rubber policeman and centrifuged. Cell pellets were solubilized in 200 µl of electrophoresis sample buffer with 2% (wt/vol) NaDodSO4. Gel electrophoresis was performed (43) with 5% polyacrylamide resolving gels, and autoradiographs of the dried gels were obtained.

Radioimmunoassay. Conditioned media were collected, clarified by low-speed centrifugation, and stored at −80°C until use. Radioimmunoassays for insulin and growth hormone followed standard techniques. For the IGF-II immunoassay (44) media were acidified by adding an equal volume of 0.5 M HCl and were passed through a C18 cartridge (Sep-Pak, Waters). The cartridge was washed with 10 ml of 4% (vol/vol) acetic acid, and IGFs were eluted using 7 ml of 50% (vol/vol) acetonitrile in distilled H2O. Aliquots were lyophilized and reconstituted in immunoassay buffer (44). Under these conditions binding proteins do not elute from the column. The antibody is an anti-rat IGF-II monoclonal antibody (Amano Pharmaceutical, Nagoya, Japan). Using bovine IGF-II as standard and tracer, the B/B0 is ~200 pg/ml, and cross-reactivity with recombinant or highly purified human IGF-I is <3%.

RESULTS

Identification of the Mouse IGF-II Gene. Five overlapping clones containing IGF-II sequences were isolated. The two cosmids pictured in Fig. 1A span 54 kilobases of mouse genomic DNA. By mapping and DNA sequence analysis five IGF-II exons were identified (Fig. 1A). The initial two exons are noncoding and are expressed as distinct mRNA species through use of different promoters and by alternative RNA splicing as in rat IGF-II (45). Exons 3 and 4 are composed entirely of coding information, while exon 5 contains both coding and 3′ nontranslated sequences. A higher resolution map of the first coding exon (exon 3) appears in Fig. 1B. The DNA sequence is illustrated in Fig. 1C. Within the 163 nucleotides there are three substitutions between mouse and rat IGF-II (45). The protein sequence of the NH2-terminal 28 residues of mature mouse IGF-II (in boldface script) differs by 2 amino acids from the rat peptide, and by 3 residues from human IGF-II (46). The detailed structure and sequence of the gene will be presented elsewhere (P.R., unpublished results).

Analysis of IGF-II Gene Expression in Differentiating Muscle Cells. To determine whether muscle cells produce IGF-II, we analyzed the myogenic cell line, C2, for IGF-II mRNA. Total cellular RNA, from two groups of proliferating myoblasts and from two sets of differentiated myotubes, was fractionated on agarose/formaldehyde gel and was transferred to a nitrocellulose filter for hybridization to the mouse IGF-II probe depicted in Fig. 1B. As seen in Fig. 2, a predominant RNA of ~4.2 kilobases was detected in differentiated cells but not in proliferating myoblasts. This RNA species appears identical in size to the IGF-II mRNA found in fetal mouse liver. These results, which confirm other observations in mouse placenta (47), contrast with data from other mammalian species, where a complex pattern of IGF-II mRNAs exists (27).

We next sought to define the kinetics of accumulation of IGF-II mRNA during differentiation of C2 cells to determine whether IGF-II is secreted by this cell line. Using a sensitive
rRNA are the myogenic in RNA shown in doublet of 151 solution-hybridization of Total cellular RNA 1B). Autoradiographic substantial increase detected from ribonuclease digestion, corresponding a experiments detected in proliferating assay, sure or differentiated hr from autoradiograph of exon 3 after initiation mRNAs is indicated. HR1 mRNA in medium. Detection changing mRNA of IGF-II mRNA in hr. The RNA isolation and D2 were taken 152 48 hr after induction of differentiation is linear. Undifferentiated C2 cells bind small amounts of IGF-II (0.066 pmol/mg of protein in this experiment). Larger amounts of IGF-II (0.576 pmol/mg of protein in this experiment) are bound by C2 cells 72 hr after induction of differentiation.

In six independently performed studies the IGF-II binding capacity per mg of protein of differentiating C2 cells was 3.1–17.9 times higher than that of undifferentiated cells. The dissociation constants (mean ± SD) of IGF-II binding to undifferentiated and to differentiating cells were 2.90 ± 1.46 nM (n = 7) and 3.17 ± 1.50 nM (n = 10), respectively, indicating that the affinity of the receptor does not change during differentiation. To determine total IGF-II receptor content, we solubilized C2 cells harvested before and 48 hr after induction of differentiation with 1.0% Triton X-100. At both times 60–70% of receptors were located intracellularly, as reported (48, 49). Total IGF-II binding capacity was increased 4.4 times in differentiating C2 cells, suggesting that the increase in cell surface IGF-II binding results from increased receptor synthesis.

To confirm that IGF-II binds to its receptor in C2 cells and not to the IGF-I receptor and/or to one or more IGF binding proteins, affinity cross-linking studies were performed (Fig. 5). 125I-labeled IGF-II was cross-linked predominantly to a protein that migrated under nonreducing conditions as a complex with an apparent Mr 220,000 in both undifferentiated and differentiating C2 cells (lanes 1). After reduction

![Fig. 2](image-url) Detection of IGF-II mRNA in differentiated C2 cells. An autoradiograph of a blot hybridization experiment using 5 µg of total RNA from late fetal mouse liver from two sets of undifferentiated (U) or differentiated (D) C2 cells is shown. The U1 and U2 time samples were taken 48 hr after initiation of cell growth. D1 and D2 were taken 168 hr after changing to differentiation medium. Mobility of size markers (28S and 18S rRNA) is indicated. Autoradiographic exposure time was for 16 hr.

![Fig. 3](image-url) Detection of IGF-II mRNA in differentiating C2 cells. Total cellular RNA was isolated at various intervals after exposure of cells to differentiation medium. (A) RNA (8 µg) was subjected to a solution-hybridization assay using the IGF-II exon 3 probe (see Fig. 1B). Autoradiographic exposure time was for 10 hr. The ‘‘protected’’ doublet of 151 or 152 nucleotides is indicated. (B) An autoradiograph of a gel-blot hybridization experiment is pictured using 5 µg of the RNA shown in A and a rat β-actin probe. Autoradiographic exposure time was for 12 hr. The appearance of α-actin mRNAs and the decline in β- and γ-actin mRNA levels are typically observed indicators of myogenic differentiation in these cells (29). (C) The RNA gel used in the experiment illustrated in B is shown. Positions of 28S and 18S rRNA are indicated for B and C.

![Fig. 4](image-url) Scatchard plots of IGF-II binding to C2 cells. IGF-II binding studies were performed using cells harvested 48 hr after initiation of growth (u) or 72 hr after induction of differentiation (m). Binding assay mixtures contained 46,000 cpm (0.07 nM) of 125I-labeled IGF-II and unlabeled IGF-II at 0–1 µg/ml (0–133.9 nM) in a final volume of 300 µl. Each point represents the mean of duplicate assays. Binding data were analyzed by the program LIGAND (41). Nonspecific binding has been subtracted. Solid lines are computer-generated best fits for a one-site binding model. Dissociation constants for binding to undifferentiated and differentiating cells are 1.34 and 1.86 nM, respectively.
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with 2-mercaptoethanol, this complex migrated at M, 250,000 (data not shown). Cross-linking of 125I-labeled IGF-II was markedly inhibited by unlabeled IGF-II (lanes 3) but not by unlabeled IGF-I (lanes 2) or insulin (lanes 4). These results agree with previous 125I-labeled IGF-II-receptor cross-linking analyses (16). C2 cells also bind IGF-I with high affinity, although there is a <2-fold increase in IGF-I receptor number during differentiation (S.E.T. and P.R., unpublished results). In these cells, 125I-labeled IGF-II was not cross-linked substantially to the IGF-I receptor or to proteins of lower molecular weight (not shown). These results demonstrate that IGF-II interacts primarily with the IGF-II receptor on the surface of C2 cells.

Coordinate Expression of IGF-II and Its Receptor During Myogenic Differentiation. Fig. 6 summarizes data on IGF-II mRNA, IGF-II secretion, and IGF-II receptor levels during differentiation of C2 cells. As shown here, IGF-II mRNA increases markedly during myogenesis. A measurable (3.9-fold) increment occurs within 16 hr of induction of differentiation. Peak levels of ≈25-fold over baseline are reached by 96 hr, at which time myotubes have formed. IGF-II, measured by immunoassay, is secreted into the medium and accumulates over the period of differentiation. By 120 hr IGF-II levels are ≈20-fold above baseline (55 vs. 1178 ng/10 ml). The expression of the IGF-II receptor on the surface of C2 cells also increases after induction of differentiation. After 24 hr, IGF-II bound per mg of protein in differentiating cells is 3.7 times higher than in underdifferentiated cells, and IGF-II receptor expression remains high during the next 72 hr. Thus these studies suggest that IGF-II and its receptor are coordinately regulated during myogenic differentiation of C2 cells.

DISCUSSION

This study demonstrates that IGF-II and its receptor are produced in a fusing skeletal muscle cell line in a differentiation-dependent manner. The increase in the IGF-II receptor parallels the increase in IGF-II mRNA and the accumulation of IGF-II in culture medium. The inducible nature of IGF-II and IGF-II receptor expression during C2 differentiation suggests that IGF-II may be an autocrine factor for muscle.

The signals that modulate IGF-II gene expression during cellular growth or differentiation are not known. In rodents IGF-II is primarily a fetal growth factor, whose synthesis is attenuated shortly after birth (27). Whether this decline reflects decreased gene transcription, increased mRNA degradation, or other events, is unknown. Unlike IGF-I, whose transcription is enhanced by growth hormone (50), no trophic agents have been shown to specifically alter IGF-II gene expression in vivo. The structure of the IGF-II gene is complicated. In rats and in mice (Fig. 1), the gene contains at least two promoters. The proximal promoter appears to consist of minimal control elements found in many other genes (51). Interaction of these cis-acting sequences with ubiquitous transcription factors SPI and the TATA-box binding factor (51) is insufficient to explain the pattern of developmental regulation of IGF-II found in vivo or the results described here. The functional anatomy of the more distal promoter has not been defined. We only may speculate
that the massive induction seen in C2 cells is a consequence of transcriptional activation of the IGF-II gene.

The signal(s) that modulate the IGF-II receptor during cellular growth or differentiation are similarly unknown. Insulin at concentrations of >0.5 nM has been shown to cause a translocation of the receptor from an intracellular site to the plasma membrane (48, 49), resulting in a 3- to 10-fold increase in receptor number. In our studies insulin levels in growth and differentiation medium were 2 micromol/ml (0.013 nM) and 1 micromol/ml (0.007 nM), respectively, much less than the amount reported to stimulate translocation. In addition, cell surface and total IGF-II receptor levels increase proportionately during C2 cell differentiation, indicating that the increase in cell-surface IGF-II binding results from enhanced receptor synthesis rather than a subcellular redistribution of preformed receptors. The IGF-II receptor appears to constitutively recycle in the cell (52); neither IGF-II nor IGF-I has been shown to regulate IGF-II receptor number or its subcellular distribution. It is thus possible that the IGF signal activates transcription of both the IGF-II and IGF-II receptor genes during muscle differentiation.

The functions of IGF-II and its receptor in muscle differentiation remain to be defined. Beguinot et al. (16) have suggested that IGF-II acts through the IGF-II receptor to stimulate uptake of 2-deoxyglucose and α-aminoisobutyric acid in rat L6 muscle cells. In contrast, using a similar experimental approach, Yu and Czech (53) have proposed that IGF-II acts through the IGF-I receptor to enhance nutrient uptake in intact rat soleus muscle. Their conclusion is supported by observations of Kiiess et al. (18), who found that dose–response curves for stimulation of nutrient uptake and leucine incorporation into protein by IGF-II in L6 myoblasts were not altered by an anti-IGF-II receptor antibody at concentrations that blocked IGF-II binding to the receptor. The effects of antibodies to the IGF-II receptor on muscle cell proliferation and differentiation have not been studied, although the IGF-II receptor does not appear to mediate IGF-II action on DNA synthesis in the H-35 hepatoma cell line (54). The remarkable finding that the IGF-II receptor and the orphan-independent mannose-6-phosphate receptor are the same protein (20–24) defines a function for the receptor in lysosomal enzyme targeting but not in IGF-II action. Examination of the interaction of IGF-II with its receptor during muscle differentiation may suggest mechanism(s) by which these two processes are integrated within the cell.

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