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Insulin-like Growth Factors (IGF) in Muscle Development

EXPRESSION OF IGF-I, THE IGF-I RECEPTOR, AND AN IGF BINDING PROTEIN DURING MYOBLAST DIFFERENTIATION*

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The insulin-like growth factors (IGFs) I and II exert pleiotropic effects on diverse cell types through interaction with specific high affinity cell surface receptors and with locally produced binding proteins. In skeletal muscle and in myoblast cell lines, the functions of IGF-I and -II are complex. Both growth factors appear capable of stimulating cellular proliferation and differentiation, as well as exerting insulin-like effects on intermediary metabolism. We have demonstrated recently that the expression of IGF-II and its receptor is induced during the terminal differentiation of the myoblast cell line, C2, and have suggested that IGF-II may be an autocrine growth factor in these cells (Tollefsen, S. E., Sadow, J. L., and Rotwein, P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 1543–1547). We now have examined this cell line for expression of other components involved in IGF signaling. The synthesis of IGF-I is low during myoblast proliferation; IGF-I mRNA can be detected only through use of a sensitive solution hybridization assay. Typical IGF-I receptors can be measured in myoblasts, whereas IGF binding proteins cannot be detected in proliferating cells or in conditioned culture medium. During myogenic differentiation, IGF-I mRNA levels increase transiently by 6–10-fold within 48–72 h. The expression of IGF-I mRNA is accompanied by a 2.5-fold accumulation of IGF-I in the culture medium. IGF-I receptors also increase transiently, doubling by 48 h after the onset of differentiation. By contrast, secretion of a M_r 29,000 IGF binding protein is induced 30-fold to 100 ng/ml within 16 h and continues to increase throughout differentiation. These studies demonstrate that several components critical to IGF action are produced in a fusing skeletal muscle cell line in a differentiation-dependent manner and suggest that both IGF-I and IGF-II may be autocrine factors for muscle.

The insulin-like growth factors (IGFs)[1] I and II are structurally similar polypeptides with diverse biological properties (for review, see Refs. 1–3). Like other multifunctional peptide growth factors (4), the IGFs stimulate cellular replication and exert other effects not directly related to cell growth (2, 5–11). In myoblasts, IGF-I and IGF-II have been shown to enhance both proliferation and differentiation (12, 13), to promote nutrient uptake, and to inhibit protein breakdown (14–16). Many of the actions of IGFs on muscle appear to be mediated by the IGF-I receptor (16, 17), a ligand-activated tyrosine-specific protein kinase structurally related to the insulin receptor (18). In contrast, the role of the IGF-II receptor in IGF signaling is unclear, despite the apparent identification of this glycoprotein as the cation-independent mannose 6-phosphate receptor involved in targeting lysosomal enzymes (19–22). An additional complexity in determining the actions of IGFs in many tissues is the presence of binding proteins. Several cell types including myoblasts have been shown to secrete IGF binding proteins (23), and at least one binding protein appears capable of enhancing the mitogenic actions of IGF-I on fibroblasts and smooth muscle cells (24).

We have demonstrated recently the endogenous expression of both IGF-II and its receptor in a myogenic cell line, C2, in a differentiation-dependent manner (25). Because several studies have suggested that IGF-I plays a key role in muscle growth (12, 13, 26, 27), we now have examined C2 cells for other components of IGF signaling. We report here that during myoblast differentiation IGF-I is produced by C2 cells, that the IGF-I receptor is expressed coordinately, and that a single IGF binding protein of M_r 29,000 is rapidly secreted. These observations suggest that both IGF-I and IGF-II may be autocrine growth factors for skeletal muscle and that muscle differentiation may provide a useful model for elucidating the mechanisms by which the actions of these growth factors are integrated within the cell.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, DNA and RNA polymerases, DNA ligase, and other enzymes were purchased from commercial suppliers (Promega Biotec, New England Biolabs, Bethesda Research Laboratories, United States Biochemical Corp., and Stratagene Cloning Systems). All deoxynucleotide, dideoxynucleotide, and ribonucleotide triphosphates were obtained from Pharmacia LKB Biotechnology Inc. Nitrocellulose filters and sheets were from Schleicher &

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[1] The abbreviations used are: IGF, insulin-like growth factor; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Schuell. Radionuclides ($Na^{125}I$, [α-^{32}P]dATP, [α-^{32}P]dCTP, [α-^{35}S]dATP, [α-^{32}P]CTP) were purchased from Amersham Corp. and Du Pont-New England Nuclear. Plasmid Bluescript was obtained from Stratagene. Recombinant [Thr^{59}]IGF-I was purchased from Amgen Biologicals, and recombinant IGF-II was generously provided by Dr. M. M. Smith (Eli Lilly and Co., Indianapolis, IN). Disuccinimidyl suberate was obtained from Pierce Chemical Co. Sera for tissue culture (fetal and neonatal calf serum and horse serum) were purchased from Bethesda Research Laboratories-Gibco.

Cell Cultures—The mouse C2 cell line (28) was plated at 3×10^3 cells/cm^2 on gelatin-coated tissue culture plates and grown in DMEM supplemented with 10% fetal bovine serum and 10% newborn calf serum at 37 °C in humidified 5% CO_2, 95% air atmosphere. The cells reached 90% of confluent density after 48 h and were harvested as undifferentiated cells. To induce differentiation, cells were washed with DMEM, and the medium was changed to DMEM plus 2% horse serum. In initial experiments, DMEM with 10% horse serum gave equivalent results. Cells and/or media were harvested at varying times thereafter. Conditioned media were immediately clarified by low speed centrifugation and were stored in aliquots at −80 °C until assayed.

Isolation of Recombinant Bacteriophage Containing the Mouse IGF-I Gene—Approximately 5×10^5 bacteriophage plaques from a genomic library in λ Charon 28 (29) were screened by standard methods (30), using a ^{32}P-labeled (31) rat IGF-I cDNA (32) as a hybridization probe. DNA was isolated (33) from plaque-purified positive bacteriophage and was mapped by digestion with *Bam*HI, *Eco*RI, and *Hin*dIII, singly or in combination, followed by hybridization (34) to ^{32}P-labeled rat IGF-I cDNA (34).

Nucleotide Sequence Analysis—Subclones containing mouse IGF-I exons were prepared in plasmid Bluescript for DNA sequencing using dideoxy chain-terminating inhibitors (35). All sequence information was verified on both DNA strands.

RNA Isolation—Total cellular RNA was extracted from proliferating or differentiating C2 cells using a modified protocol employing guanidinium thiocyanate (36). The integrity of each RNA sample was verified by gel electrophoresis, and the quantity was determined spectrophotometrically.

RNA Analysis—Northern blots were performed following standard procedures (37). Total RNA (10 μg) was separated by size in a 1% agarose gel containing 2.2 M formaldehyde in 20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA. Following capillary transfer to nitrocellulose and baking for 3 h at 80 °C in a vacuum oven, the filters were prehybridized overnight at 42 °C in buffer containing 50% formamide (37). Hybridization to a ^{32}P-labeled canine creatine kinase cDNA (38) proceeded for 18 h at 42 °C. Post-hybridization washes followed standard protocols (37). The filter was exposed to Kodak XAR5 film using intensifying screens for 20 h.

A solution hybridization-nuclease protection assay using 8 μg of total RNA was performed as described previously (25). The assay included 1.5×10^6 dpm of a single-stranded mIGF-I exon 3 probe synthesized as an "antisense" transcript (25) from a linearized plasmid using T_7 polymerase, [α-^{32}P]CTP (800 Ci/mmol), and unlabeled ATP, GTP, and UTP (see Fig. 1). In several experiments, a mouse IGF-II exon 3 probe was similarly employed as described previously (25). In all experiments, adult or neonatal mouse liver RNA and yeast tRNA were included as positive and negative controls, respectively. The latter did not hybridize to either mouse IGF probe. Relative RNA abundance was calculated with a scanning laser densitometer (Pharmacia LKB Biotechnology Inc.) from autoradiographs of dried gels.

IGF-I Binding Studies—IGF-I was radioiodinated using lactoperoxidase (39) and was stored in 0.1 N acetic acid containing 10 mg/ml BSA at −70 °C. The specific activity ranged from 183.8 to 237.6 μCi/μg. Binding studies were performed as described previously (25, 40). Cell monolayers were washed three times in serum-free DMEM separated by 20-min incubations at 37 °C. Washed cells were then scraped from the plates with a rubber policeman, centrifuged, and resuspended in binding buffer (20 mM imidazole-HCl, pH 7.4, 250 mM NaCl, 5% glycerol, and 2.5 mg/ml BSA). Cells were added to 1.5-ml microcentrifuge tubes containing ^{125}I-labeled IGF-I and increasing amounts of unlabeled IGF-I (final volume 300 μl). After an overnight incubation at 4 °C, the cells were centrifuged for 5 min, and the supernatant fluid was removed by aspiration. The cells were washed once with 1 ml of binding buffer, and the pellet was counted. Binding data were analyzed by LIGAND (41). An aliquot of cells equal to that used in the binding assays was solubilized with 0.5 ml of 0.1% SDS for protein determination (42).

Affinity Cross-linking Analysis—Cell monolayers were washed three times with 20 ml of HEPES buffer (20 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4) containing 10 mg/ml BSA. ^{125}I-Labeled IGF-I (final concentration 0.2 nM) with or without unlabeled ligand was added to the cells in 12.5 ml of HEPES buffer containing 10 mg/ml BSA. The cells were incubated for 4 h at 15 °C and then were washed with 20 ml of ice-cold phosphate-buffered saline. Disuccinimidyl suberate (final concentration 0.1 mM) in 12.5 ml of HEPES buffer was added for 20 min at 15 °C to cross-link the bound ligand. The reaction was quenched by the addition of three volumes of 10 mM Tris-HCl, pH 7.4, with 1 mM EDTA for 20 min. After aspiration of buffer, the cells were scraped from the plates with a rubber policeman and centrifuged. The cell pellets were solubilized in 200 μl of electrophoresis sample buffer containing 2% SDS with or without 5% 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (43). Autoradiographs were obtained by exposure of the dried gels to Kodak XAR5 x-ray film in the presence of a Du Pont Cronex Lightning Plus intensifying screen at −70 °C. Molecular weight standards include myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase *b* (M_r 97,400), and BSA (M_r 66,200).

Binding Protein Assay—The binding protein content of conditioned media was determined by measuring the ability of aliquots to bind ^{125}I-labeled IGF-I (23). Media were incubated at 20 °C for 1 h with 20,000 cpm of ^{125}I-labeled IGF-I in 250 μl of buffer (100 mM HEPES, pH 6.0, 44 mM NaH_2PO_4, 0.01% Triton X-100, 0.1 mg/ml BSA, 0.02% NaN_3). Complexes of binding protein and IGF-I then were precipitated by the addition of 250 μl of 1% human immune globulin and 500 μl of 25% polyethylene glycol, followed by centrifugation at $1,200 \times g$ for 15 min. The pellet was washed once with 6.25% polyethylene glycol and counted. Results are expressed as nanograms of binding protein/milliliter of conditioned media. Pure human amniotic fluid binding protein was used as a standard. The assay has a lower limit of detection of 50 pg of pure standard (23).

Ligand Blotting—A 21-μl aliquot of conditioned media was mixed with 7 μl of 4 × concentrated Laemmli (43) sample buffer without 2-mercaptoethanol and heated to 60 °C for 10 min before being applied to a 12.5% discontinuous SDS-polyacrylamide gel. Samples were electrophoresed at 20 mA until the dye front reached the bottom of the gel (1.5 h). Proteins were transferred to a nitrocellulose filter using a semidry electroblotter at 70 mA for 1 h as described by Hossenlopp *et al.* (44). The filters were probed for IGF binding proteins with 400,000 cpm of ^{125}I-labeled IGF-I. Binding proteins then were visualized by autoradiography. Molecular weights were estimated by comparison to prestained protein standards electrophoresed in adjacent lanes of the gel.

Radioimmunoassay—Media were acidified by adding an equal volume of 0.5 N HCl and were passed through a C_{18} cartridge (Sep-Pak, Waters Associates). The cartridge was washed with 10 ml of 4% acetic acid, and IGFs were eluted using 7 ml of 50% acetonitrile in distilled H_2O (45). Aliquots were lyophilized and reconstituted in immunoassay buffer (46). Under these conditions binding proteins do not elute from the column and recovery of added ^{125}I IGF-I is ~80%.[2] Rabbit anti-human somatomedin C/IGF-I antibody (47), lot UBK487, from the National Hormone and Pituitary Program (kindly provided by Drs. J. J. Van Wyk and L. E. Underwood, University of North Carolina, Chapel Hill, N.C.) was used in the radioimmunoassay. With human recombinant [Thr^{59}]IGF-I as standard and tracer, cross-reactivity with IGF-II is ~0.5%.

RESULTS

Identification of the Mouse IGF-I Gene—Two recombinant bacteriophage were isolated and purified from the mouse genomic library by hybridization to a rat IGF-I cDNA. The two clones contained overlapping inserts of murine DNA. The mouse equivalents of rat and human IGF-I exons 3 and 4 (48, 49) were identified by blot hybridization (34), as indicated in Fig. 1*A*. Fig. 1*B* illustrates a higher resolution restriction map of exon 3 and depicts the 870-base pair probe used in subsequent studies. The sequence of exon 3 is shown in Fig. 1*C*. This exon encodes the carboxyl part of the B domain of

[2] S. E. Tollefsen, R. Lajara, R. H. McCusker, D. R. Clemmons, and P. Rotwein, unpublished observations.

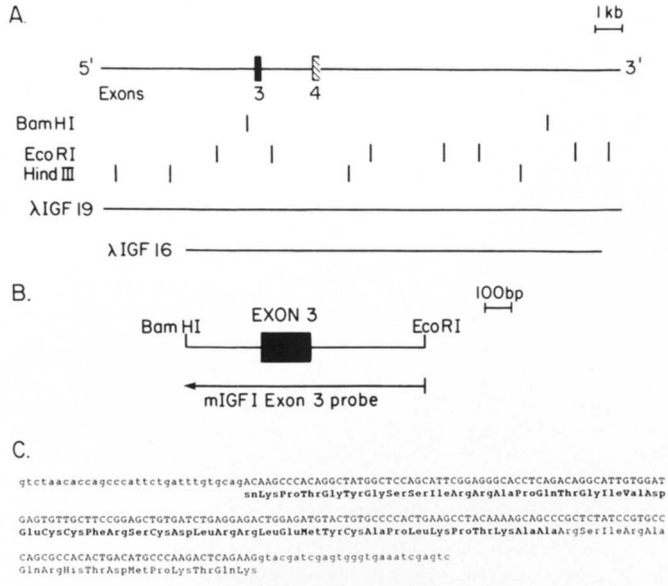

FIG. 1. **Partial characterization of the mouse IGF-I gene.** *A*, part of the 3′ end of the gene is illustrated. Exons 3 and 4 are depicted by *boxes*. The *cross-hatched* portion within exon 4 has not been characterized fully. Sites for restriction endonucleases *Bam*HI, *Eco*RI, and *Hind*III are indicated *below* the gene, as are two λ clones. *B*, a detailed map of exon 3 is shown. The 870-base pair (*bp*) probe used in gene expression studies extends from a 5′ *Bam*HI site to a 3′ *Eco*RI site. *C*, the DNA sequence of exon 3 is illustrated. Introns are in *lowercase* and the exon in *uppercase* letters. The amino acid sequence encoded by exon 3 appears *below* the corresponding DNA. *Bold type* highlights the part of the mature IGF-I peptide encoded by this exon. *kb*, kilobase pair.

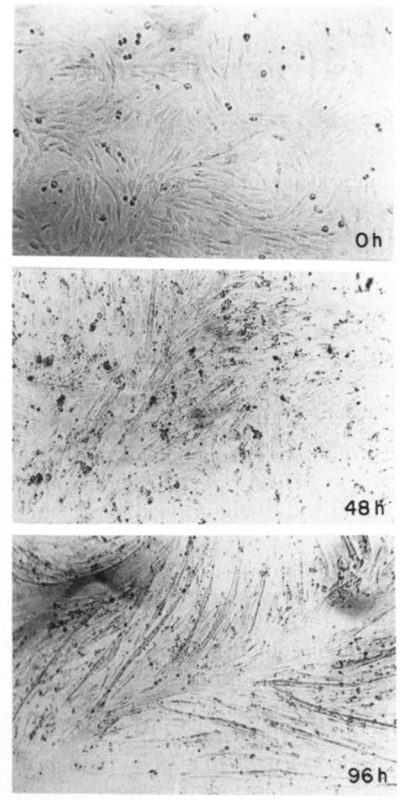

FIG. 2. **Differentiation of C2 cells into myotubes.** The *top panel* shows C2 myoblasts at the end of their proliferative phase. The cells are at approximately 90% of confluent density. The *middle panel* illustrates the extent of myogenesis after 48 h in differentiation medium as described under "Experimental Procedures." Cell fusion and the formation of myotubes has begun. The *bottom panel* shows the extent of differentiation by 96 h. Giant myotubes have developed. All phase contrast photomicrographs were obtained at × 90 magnification and depict the same field of cells.

mature IGF-I, as well as the entire C, A, and D regions, as indicated by the *boldface type*. The 16 amino-terminal residues of the E domain of the IGF-I precursor comprise the 3′ end of the exon, as depicted by *regular type*. The nucleotide and derived amino acid sequence of exon 3 presented here match data previously obtained by others (50, 51).

Analysis of IGF Gene Expression in Differentiating Muscle Cells—To determine whether muscle cells produce IGF-I, we analyzed the myogenic cell line C2 for IGF-I mRNA. This cell line provides a convenient model for studying gene expression during both myoblast proliferation and differentiation. Contracting myotubes form within 96–120 h after triggering the differentiation program by changing the cell culture medium to DMEM with 2 or 10% horse serum (see Fig. 2). Total cellular RNA, obtained at various times during C2 differentiation, was examined by a sensitive solution hybridization-nuclease protection assay using the probe depicted in Fig. 1*B*. As illustrated in Fig. 3*A*, a minimal amount of IGF-I mRNA was detected in proliferating myoblasts and during the initial 24 h of terminal differentiation into myotubes. By 48 h, levels of IGF-I mRNA increased dramatically (15-fold in this experiment) but then declined over the ensuing 72 h. By 120 h when cells are fully differentiated, the IGF-I mRNA level was only three times higher than that found in proliferating myoblasts. As shown in the *right panel* of Fig. 3, data obtained from eight similar experiments (two studies on each of four sets of differentiating cells) support the results illustrated in Fig. 3*A*. IGF-I mRNA peaked transiently between 48 and 72 h after the onset of differentiation at 6–10 times the level found in undifferentiated cells. In contrast to the transitory expression of IGF-I mRNA, mRNA for muscle-specific genes such as creatine kinase accumulated during myoblast differentiation (Fig. 3*B*).

We have shown previously that IGF-II mRNA also is induced in C2 cells with differentiation-dependent kinetics and that IGF-II is secreted into the culture medium of differentiating myoblasts (25). In order to compare the abundance of each growth factor during muscle differentiation, we examined the relative levels of both IGF mRNAs and measured both peptides in conditioned culture medium. The mRNAs were analyzed by employing IGF-I and IGF-II probes simultaneously in the solution hybridization-nuclease protection assay. As depicted in Fig. 4, RNA from adult and neonatal liver and from C2 cells protected a 182-nucleotide fragment of the mouse IGF-I exon 3 probe. Steady-state levels of IGF-I mRNA are higher in adult than in neonatal liver, consistent with the known postnatal increase in IGF-I gene expression in mice (51, 52). There is also an increase in IGF-I mRNA during C2 differentiation similar to what was illustrated in Fig. 3*A*. RNA from neonatal, but not adult liver, protected a 151–152-nucleotide fragment of the IGF-II exon 3 probe as described previously (25). In C2 cells, there is a dramatic increase in IGF-II mRNA during differentiation (>25-fold in this experiment), consistent with our previous results (25). The relative abundance of IGF-II mRNA at 72 h after induction of differentiation is at least 15 times higher than the level of IGF-I mRNA based on densitometric scanning of the autoradiograph presented in Fig. 4. Results from radioimmunoassay of conditioned culture medium corroborate the mRNA measurements. As shown in Fig. 5, radioimmunoassayable IGF-I increases by 250% during the initial 72 h of differentiation and then declines slightly. The apparent rise at 120 h may reflect slight cross-reactivity in the assay with

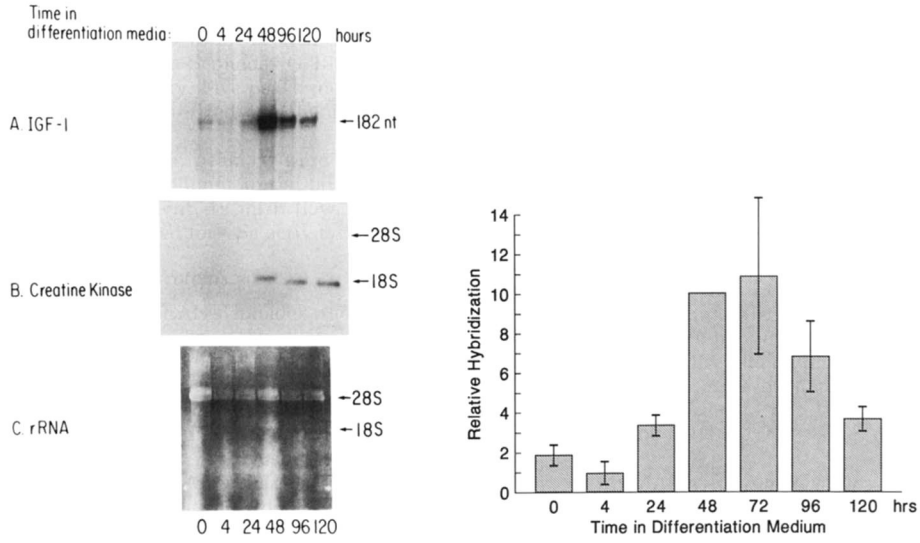

FIG. 3. **Detection of IGF-I mRNA in differentiating C2 cells.** Total cellular RNA was isolated at various intervals after exposure of cells to differentiation medium as described under "Experimental Procedures." *Left panel*: A, RNA (8 μg) was subjected to a solution hybridization assay using the IGF-I exon 3 probe (see Fig. 1*B*). Autoradiographic exposure time was for 36 h. The "protected" 182 nucleotide (*nt*) fragment is indicated. *B*, an autoradiograph of an RNA blot hybridization experiment is illustrated using 5 μg of the RNA used in *A* and a canine creatine kinase probe. Autoradiographic exposure time was for 16 h. The appearance of creatine kinase mRNA is an indicator of myogenic differentiation in these cells. *C*, the RNA gel used in the experiment pictured in *B* is shown (photographed under ultraviolet light after staining the gel with ethidium bromide). Positions of 28 S and 18 S rRNA are indicated for *B* and *C*. *Right panel:* the bar graphs depict the relative levels (mean ± S.E.) of IGF-I mRNA observed during C2 differentiation (n = 8 experiments, two for each of four sets of differentiating cells). Data were obtained using a scanning laser densitometer and have been normalized to the values obtained at 48 h after the start of differentiation, which has been set arbitrarily to 10.

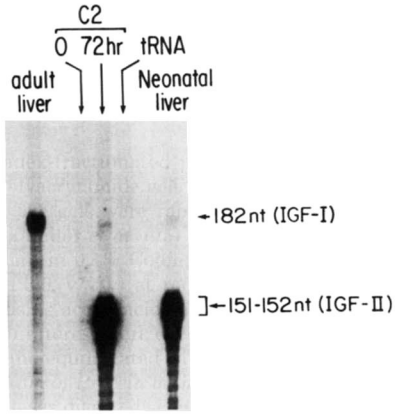

FIG. 4. **Detection of IGF-I and IGF-II mRNA in differentiating C2 cells.** Total cellular RNA was isolated at varying intervals after exposure of cells to differentiation medium as described under "Experimental Procedures." RNA (8 μg) was subjected to a solution hybridization assay simultaneously using equal amounts of IGF-I and IGF-II probes labeled to the same specific activity. Autoradiographic exposure time was for 12 h. Protected fragments of 182 nucleotides (*nt*) (IGF-I) and 151–152 nucleotides (IGF-II) are indicated.

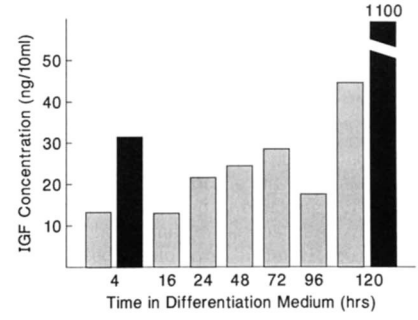

FIG. 5. **Secretion of IGF-I and IGF-II during C2 differentiation.** Conditioned culture medium was collected at varying intervals after exposure of cells to differentiation medium (DMEM plus 2% horse serum), and radioimmunoassays for IGF-I and IGF-II were performed as described under "Experimental Procedures." The mean IGF-I levels (measured in triplicate) from one of three representative experiments are depicted at the indicated time points by the *cross-hatched bars*. IGF-II levels (25) are indicated by the *solid bars*. The levels of IGF-I and IGF-II in nonconditioned 2% differentiation medium ranged from 8 to 14 ng/10 ml, and from 30 to 55 ng/10 ml, respectively.

IGF-II because IGF-II accumulates in the media to more than 1000 ng/10 ml at this time. Thus, although both IGF-I and IGF-II mRNA and protein secretion increase during C2 differentiation, the expression of IGF-II is at least an order of magnitude greater than IGF-I.

Analysis of the IGF-I Receptor in Differentiating C2 Cells—To determine if C2 cells express IGF-I receptors on their surface, IGF-I binding to undifferentiated and differentiating cells was examined in competition binding studies. Fig. 6*A* shows Scatchard plots of data from a representative study. The Scatchard plots of IGF-I binding to undifferentiated cells and to cells harvested 24–96 h after induction of differentiation are linear. In this experiment undifferentiated C2 cells bound 0.178 pmol of IGF-I/mg protein, and cells harvested 72 h after induction of differentiation bound 0.464 pmol of IGF-I/mg protein. Analysis of data obtained from seven independently performed experiments indicates that the expression of the IGF-I receptor on the cell surface of C2 cells increases transiently, but significantly, during differentiation, as illustrated in Fig. 6*B*. The number of IGF-I receptor binding sites in cells harvested 48–72 h after induction of differentiation is about two times higher than in undifferentiated cells, although there is some variability among different experiments. Correction of the data for protein content, as

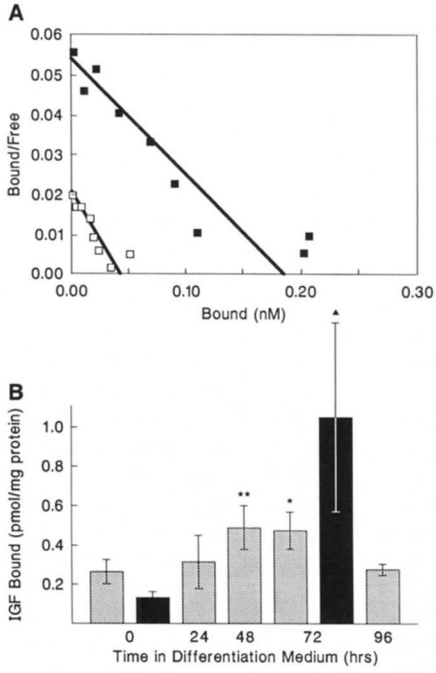

FIG. 6. **IGF-I binding to undifferentiated and differentiating C2 cells.** IGF-I binding studies were performed as described under "Experimental Procedures." *A*, Scatchard plots of IGF-I binding to cells harvested 48 h after initiation of growth (□) or 72 h after induction of differentiation (■). Binding assays contained 45,000 cpm (0.062 nM) of ^{125}I-labeled IGF-I and 0–1 μg/ml (0–130.8 nM) of unlabeled IGF-I in a final volume of 300 μl. Each point represents the mean of duplicate assays. Binding data were analyzed by LIGAND (41). *N*, the ratio of nonspecifically bound ligand to free ligand, was 0.0029 and 0.0095 for undifferentiated and differentiating C2 cells, respectively, and nonspecific binding, $N \times F$, has been subtracted. The solid lines are computer-generated best fits for a one-site binding model. The binding capacities and dissociation constants for IGF-I binding to undifferentiated and differentiating cells in this experiment are 0.042 and 2.3 nM and 0.189 and 3.51 nM, respectively. Data have not been adjusted for protein content in this study (71.2 μg/assay for undifferentiated cells and 122.0 μg/assay for differentiating cells). *B*, IGF-I receptor expression during muscle differentiation. C2 cells were harvested 48 h after initiation of growth (0 h) or 24–96 h after induction of differentiation as indicated. Data have been corrected for protein content of cells in each assay. The IGF-I binding capacity (mean ± S.E.) obtained at each time point (n = 3–6) in seven independently performed experiments and the IGF-II binding capacity (mean ± S.E.) at 0 and 72 h (25) are depicted by *cross-hatched* and *closed bars*, respectively. **, $p < 0.02$; *, $p < 0.01$ *versus* time 0 h for IGF-I binding capacity; ▲, $p < 0.001$ *versus* time 0 h for IGF-II binding capacity (Student's *t* test).

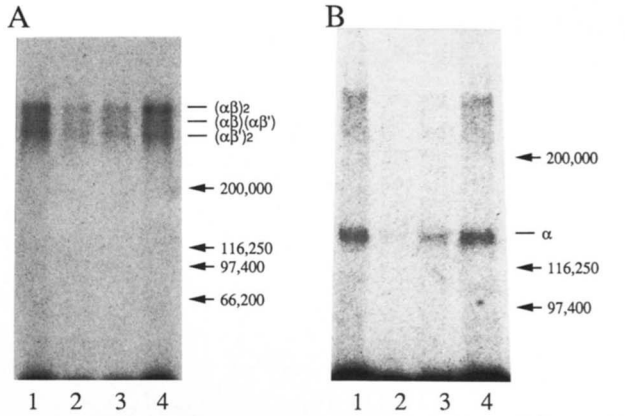

FIG. 7. **Affinity cross-linking of IGF-I to C2 cells.** C2 cells were incubated for 4 h at 15 °C with ^{125}I-labeled IGF-I (0.2 nM) in the absence (*lanes 1*) or presence of 100 ng/ml of either unlabeled IGF-I (*lanes 2*), unlabeled IGF-II (*lanes 3*), or unlabeled insulin (*lanes 4*). Affinity cross-linking was performed with 0.1 mM disuccinimidyl suberate as described under "Experimental Procedures." Shown are autoradiographs of the radioligand-receptor complexes, analyzed by SDS-polyacrylamide gel electrophoresis. *A*, 3–10% acrylamide gradient resolving gel under nonreducing conditions; *B*, 5% acrylamide resolving gel under reducing conditions. The migration of molecular weight standards is indicated.

presented in Fig. 6*B*, may underestimate the magnitude of the increase in receptor number which occurs during differentiation because total protein content of C2 cells also increases.[2] The dissociation constants (means ± S. D.) of IGF-I binding to undifferentiated and to differentiating C2 cells are 3.29 ± 1.26 nM (n = 7) and 3.71 ± 1.35 nM (n = 15), respectively, indicating that the affinity of the receptor for IGF-I does not change during differentiation. As reported previously (25), the number of IGF-II receptor binding sites is increased 8.7-fold in C2 cells harvested 72 h after induction of differentiation. In contrast, insulin binding to undifferentiated C2 cells and to cells studied 48 h after the onset of differentiation is negligible.[2]

To confirm that the receptor to which IGF-I binds in these cells is the authentic IGF-I receptor, affinity cross-linking studies were performed (Fig. 7). ^{125}I-Labeled IGF-I was cross-linked predominantly to proteins which migrated under non-reducing conditions as complexes with apparent $M_r > 300,000$ (Fig. 7*A*, *lane 1*). These proteins correspond to the $(\alpha\beta)_2$, the $(\alpha\beta)$ $(\alpha\beta')$, and the $(\alpha\beta')_2$ forms of the IGF-I receptor (53). After reduction with 2-mercaptoethanol, these complexes migrated with an apparent M_r 135,000 (Fig. 7*B*, *lanes 1*). Cross-linking of ^{125}I-labeled IGF-I was markedly inhibited in the presence of unlabeled IGF-I (*lanes 2*), was weakly inhibited by unlabeled IGF-II (*lanes 3*), and was not inhibited by unlabeled insulin (*lanes 4*). These results agree with previous IGF-I receptor cross-linking analyses (53) and demonstrate that IGF-I interacts specifically with the IGF-I receptor on the surface of C2 cells. In C2 cells, the IGF-II receptor is detected only after cross-linking with ^{125}I-labeled IGF-II (25).

Analysis of IGF Binding Proteins in Differentiating C2 Cells—To determine whether muscle cells secrete specific IGF binding proteins, ligand blots and IGF binding assays were performed using conditioned medium from undifferentiated and differentiating C2 cells. As illustrated in the ligand blot shown in Fig. 8*A*, medium collected from undifferentiated cells contains little IGF binding activity. During C2 differentiation a binding protein of M_r 29,000 appears. This binding protein is not seen in nonconditioned culture medium with 2% horse serum.[2] By densitometric analysis of the autoradiograph pictured in Fig. 8*A*, binding protein increases 4-fold within 4 h, 30-fold by 16 h, and 100-fold by 120 h after the onset of C2 differentiation (Fig. 8*A*, *bottom panel*). An increase in binding protein accumulation also is seen in detergent extracts of cell pellets.[2]

The binding capacity of conditioned culture medium for ^{125}I-labeled IGF-I rises rapidly during C2 differentiation and reaches 100 ng/ml by 16 h (Fig. 8*B*). By 48 h, binding capacity has decreased and remains low throughout the remainder of the time course. Although these observations appear to contradict the results observed with ligand blotting, in which a continual rise in IGF binding protein is seen, the decline in binding capacity coincident with an increase in total binding protein content can be explained by occupancy of binding protein with endogenously produced and secreted ligand. The kinetics of secretion of IGF-II by differentiating C2 cells appear to match the decline in binding activity observed here (see Fig. 5 and Ref. 25). Thus, the binding assay corroborates

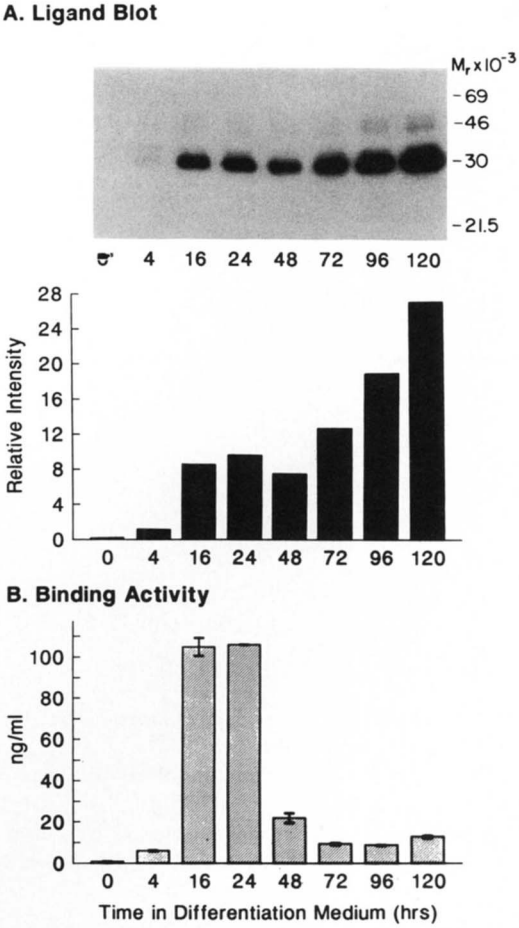

FIG. 8. **Detection of IGF binding proteins in differentiating C2 cells.** *A*, ligand blot showing binding of ^{125}I-labeled IGF-I to proteins found in conditioned culture medium. Medium (21 μl) from C2 cells was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose filter using a semidry electroblotter, and filters were probed for IGF binding proteins as described under "Experimental Procedures." The autoradiograph shown here depicts a predominant protein of M_r 29,000, which, as illustrated in the accompanying bar graph, increases >100-fold in intensity during C2 differentiation. *B*, IGF binding activity of conditioned culture medium was assessed by measuring the ability of aliquots to bind ^{125}I-labeled IGF-I. Binding content increases to 100 ng/ml within 16 h after the start of differentiation but declines by 48 h, probably because endogenously synthesized and secreted IGF-II also accumulates in the medium and occupies binding sites (see Fig. 5 and Ref. 25).

data obtained by radioimmunoassay on the differentiation-dependent secretion of IGF-II by C2 cells (25).

DISCUSSION

This study demonstrates that several components involved in modulating IGF activity are regulated in a fusing skeletal muscle cell line in a differentiation-dependent manner. IGF-I mRNA transiently increases during myogenesis, and IGF-I accumulates in conditioned culture medium, although to less than 5% of the level of IGF-II. IGF-I receptor affinity is similar in undifferentiated myoblasts and fully differentiated myocytes but receptor number increases transiently by about 2-fold during differentiation. In contrast, IGF-II receptor expression, which is less than 50% of IGF-I receptor expression in undifferentiated myoblasts, increases markedly during myogenesis and remains high. An IGF binding protein of M_r 29,000 accumulates rapidly in conditioned culture media of differentiating C2 cells. Binding protein levels measured by a ligand blotting technique increase by 100-fold during myogenic differentiation. The inducible nature of IGF ligand, receptor, and binding protein expression during C2 differentiation suggests that both IGF-I and IGF-II may function as autocrine factors in muscle.

The signals that modulate IGF-I gene expression during C2 differentiation are unknown. The best characterized regulator of IGF-I is growth hormone, which rapidly increases IGF-I transcription in whole animals and in certain cell lines (51, 54–57). Growth hormone probably does not play a role in IGF-I gene expression in C2 cells because its content in differentiation medium is negligible (<0.01 nM), and addition of growth hormone to a final concentration of 5 nM does not alter the abundance of IGF-I or IGF-II mRNA during differentiation.[2] In animals, additional signals modify IGF-I mRNA abundance. In early rodent development, IGF-I mRNA increases nearly 10-fold between embryonic days 11 and 13 (58), several days prior to the ontogeny of growth hormone synthesis and secretion by the pituitary and before the appearance of cell surface receptors for growth hormone (59, 60). In addition, in adult rats, nutritional factors modify levels of IGF-I mRNA in liver independent of growth hormone (61). Whatever the mechanisms responsible for the induction of IGF-I gene expression during muscle development in C2 cells, the signal that is generated is transitory, and the secretion of IGF-I is modest, being markedly less than that of IGF-II.

The signals that alter expression of the IGF-I receptor and the IGF binding protein during differentiation are similarly unknown. In the human IM-9 lymphoid cell line, in human fibroblasts, and in the mouse BC3H-1 myoblast cell line, preincubation with IGF-I, IGF-II, or insulin leads to down-regulation of the IGF-I receptor, resulting from a decrease in receptor number (62). In addition, in at least one subclone of the rat L6 myoblast cell line, binding of IGF-I declines by about 70% 18 days after the onset of differentiation (14). In contrast, secretion of IGF binding proteins by rat L6 myoblasts and myotubes is enhanced by exposure of cells to pharmacological concentrations of insulin (1 μg/ml). However, following differentiation there is a significant loss in sensitivity to insulin stimulation (23). In both BC3H-1 and L6 cells, IGF-I enhances binding protein secretion, with half-maximal stimulation between 12 and 24 ng/ml. These cells respond to IGF-I with increased binding protein secretion regardless of their state of differentiation (63). In C2 cells, it is unlikely that insulin, IGF-I, or IGF-II modify cell surface levels of the IGF-I receptor or trigger secretion of the IGF binding protein that is seen shortly after the cells are placed in differentiation medium because concentrations of these ligands in growth and in differentiation medium are low (30–50 pg/ml for insulin, 1–3 ng/ml for IGF-I, and 3–5 ng/ml for IGF-II). However, by 72 h after the onset of differentiation when there is substantial induction of IGF-II (25), this peptide could stimulate binding protein secretion or down-regulate the IGF-I receptor. These findings suggest that the dramatic induction in IGF binding protein that precedes the increase in IGF-I or IGF-II may be linked to early events in muscle differentiation.

What are the potential functions of IGF-I and IGF-II in muscle that support an autocrine action for these growth factors during myogenesis? Florini *et al.* (12) have suggested that relatively low concentrations of IGF-I (10 ng/ml) or moderate concentrations of IGF-II (10–300 ng/ml) promote differentiation of L6 myoblasts. Similar observations have been made by Schmid *et al.* (13) using chick embryo fibroblasts and myoblasts, although in these studies subnanomolar concentrations of either IGF enhanced myogenesis. It has

been proposed that both growth factors interact with the IGF-I receptor which then mediates the differentiative response (16, 17). In a similar context, Smith *et al.* (64) have shown that murine NIH 3T3-L1 preadipocytes differentiate into fat cells in response to 10–20 nM IGF-I (85–170 ng/ml) and have suggested that activation of the IGF-I receptor occupies a central step in the differentiation process. Other studies in L6 myoblasts and in intact rat soleus muscle provide strong evidence that IGF-I and IGF-II enhance nutrient uptake and that these actions appear to be mediated through the IGF-I receptor (16, 17, 65). In contrast, in human myoblasts, IGF-II appears to be equipotent with IGF-I in stimulating uptake of the nonmetabolizable amino acid analogue, α-aminoisobutyric acid and antibodies to the IGF-I receptor only inhibit about half of the IGF-II effect (15). Thus, the interactions of the IGFs with their receptors may produce pleiotropic effects in muscle.

The role of the M_r 29,000 IGF binding protein during muscle cell differentiation is not well-defined. Other IGF binding proteins, most notably that derived from human amniotic fluid (24), appear to potentiate the mitogenic actions of IGF-I on mouse and chicken embryo fibroblasts, human fibroblasts, and porcine aortic smooth muscle cells at concentrations (100 ng/ml) similar to those that C2 cells produce during differentiation. Because the binding protein derived from amniotic fluid adheres to the cell surface, possibly by an Arg-Gly-Asp sequence near its carboxyl terminus (66–68), it is conceivable that interaction with extracellular matrix protein receptors, or integrins (69), modulates binding protein action during myoblast differentiation. In this context, because antibodies to an integrin have been shown to reversibly inhibit muscle differentiation (70), the IGF binding protein described here could be a critical factor in induction of myogenesis. Additional study of this protein will be required to clarify its role in muscle development.

In summary, we have described the differentiation-dependent expression and/or modulation of five components in the IGF-I and IGF-II signaling pathways in a fusing skeletal muscle cell line. Characterization of the mechanisms of regulation and the modes of action of IGF-I and IGF-II, their receptors, and their binding proteins in this model system should provide insight into the processes of cellular differentiation.

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