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### Research Article

Muscle is an important target tissue for insulin-like growth factor (IGF) action. The presence of specific, high affinity IGF receptors, as well as the expression of IGF peptides and binding proteins by muscle suggest that a significant component of IGF action in this tissue is mediated through autocrine and/or paracrine mechanisms. To explore autocrine/paracrine action of IGFs in muscle, we studied the regulation of the IGF-I receptor and the expression of IGF peptides during differentiation of the mouse BC3H-1 muscle cell line. Differentiation from myoblasts to myocytes was associated with a 60% decrease in IGF-I receptor sites determined by Scatchard analysis. Analysis of mRNA abundance and protein labeling studies indicated that the decrease in IGF-I receptor sites was associated with similar reductions in IGF-I receptor gene expression and receptor biosynthesis. IGF-II peptide gene expression was detected in myoblasts and increased 15-fold with differentiation; the increase in IGF-II gene expression preceded the decrease in IGF-I receptor gene expression. In contrast, IGF-I peptide gene expression was low in myoblasts and decreased slightly with differentiation. To explore the potential role of endogenous IGF-II in the differentiation-associated decrease in IGF-I receptor expression, we investigated the effects of IGF-II treatment in myoblasts. The addition of IGF-II to undifferentiated myoblasts resulted in downregulation of the IGF-I receptor which was associated with decreased IGF-I receptor biosynthesis and [...]

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# Regulation of Insulin-like Growth Factor (IGF) I Receptor Expression during Muscle Cell Differentiation

## Potential Autocrine Role of IGF-II

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### Abstract

Muscle is an important target tissue for insulin-like growth factor (IGF) action. The presence of specific, high affinity IGF receptors, as well as the expression of IGF peptides and binding proteins by muscle suggest that a significant component of IGF action in this tissue is mediated through autocrine and/or paracrine mechanisms. To explore autocrine/paracrine action of IGFs in muscle, we studied the regulation of the IGF-I receptor and the expression of IGF peptides during differentiation of the mouse BC3H-1 muscle cell line. Differentiation from myoblasts to myocytes was associated with a 60% decrease in IGF-I receptor sites determined by Scatchard analysis. Analysis of mRNA abundance and protein labeling studies indicated that the decrease in IGF-I receptor sites was associated with similar reductions in IGF-I receptor gene expression and receptor biosynthesis. IGF-II peptide gene expression was detected in myoblasts and increased 15-fold with differentiation; the increase in IGF-II gene expression preceded the decrease in IGF-I receptor gene expression. In contrast, IGF-I peptide gene expression was low in myoblasts and decreased slightly with differentiation. To explore the potential role of endogenous IGF-II in the differentiation-associated decrease in IGF-I receptor expression, we investigated the effects of IGF-II treatment in myoblasts. The addition of IGF-II to undifferentiated myoblasts resulted in downregulation of the IGF-I receptor which was associated with decreased IGF-I receptor biosynthesis and decreased IGF-I receptor mRNA abundance. These studies suggest, therefore, that IGF-I receptor expression during muscle cell differentiation may be regulated, at least in part, through autocrine production of IGF-II. (*J. Clin. Invest.* 1991. 87:1212-1219.) Key words: insulin-like growth factor I receptor • insulin-like growth factor II • muscle cell differentiation • downregulation • gene expression

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### Introduction

Insulin-like growth factor (IGF)-I,<sup>1</sup> IGF-II, and insulin are distinct members of a family of peptide hormones with a broad range of metabolic and mitogenic actions (1, 2). While it is well established that muscle is a principal target tissue for insulin, it has become apparent in the last several years that muscle is also an important site of action for the IGFs (3-16). Specific high affinity receptors for IGF-I and II have been identified in various muscle cell lines as well as in primary monolayers of normal muscle (3-10), and both IGF peptides are capable of stimulating growth and differentiation of muscle cells (11-14).

In distinction to insulin, however, there is evidence that a significant component of IGF action in muscle is mediated through autocrine or paracrine mechanisms. In addition to high affinity receptors specific for IGF-I and II, mRNA and peptide for both IGFs have been detected in cloned muscle cell lines, normal fetal and adult muscle tissues, in postinjury regenerating muscle, and in muscle during growth hormone-stimulated hypertrophy (9, 10, 17-22). Furthermore, IGF binding proteins, which may modify the biological activity of the IGFs, are also secreted by muscle cells (23).

Whether locally secreted or derived from the circulation, the biological effects of the IGFs in muscle and other tissues are mediated through interaction with specific cell-surface receptors. The IGF-I receptor is a tetrameric glycoprotein composed of two extracellular  $\alpha$ -subunits (135 kD) which bind the ligand and the two transmembrane  $\beta$ -subunits (90 kD) which have intrinsic tyrosine kinase activity (24-26). The primary structure of the human and partial structure of the rat IGF-I receptors have been recently determined by cDNA cloning (26, 27), and thus the regulation of the IGF-I receptor can be studied at the level of gene expression.

Regulation of IGF-I receptor expression in muscle cells has been controversial. IGF-I receptors have been reported to either decrease or increase during muscle cell differentiation (4, 10), though the mechanisms responsible for these changes are not well understood. In order to clarify how the IGF-I receptor is regulated in muscle, we characterized the IGF-I receptor during differentiation of the BC3H-1 mouse muscle cell line. Furthermore, we determined whether this cell line expressed IGF

1. *Abbreviations used in this paper:* DMEH, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor.

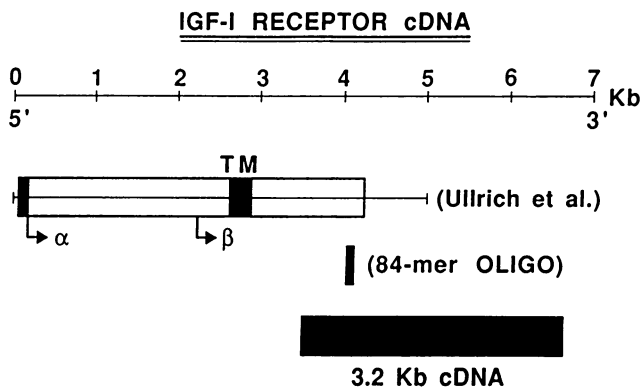
peptides and explored the potential autocrine role of these peptides in the regulation of IGF-I receptor expression.

## Methods

**Materials.** The following products were purchased: [<sup>35</sup>S]methionine (1,129 Ci/mmol) and [<sup>125</sup>I]-insulin (2,200 Ci/mmol) from New England Nuclear, Boston, MA; [<sup>125</sup>I]-IGF-I, [Thr<sup>39</sup>] (2,000 Ci/mmol) from Amersham Corp., Arlington Heights, IL; and porcine insulin from Elanco Products, Indianapolis, IN. IGF-I was a gift from CIBA-GEIGY Corp., Summit, NJ; IGF-II was a gift from Eli Lilly and Co., Indianapolis, IN. Anti-insulin receptor antiserum Bd was a gift from Dr. G. Boden (Temple University, Philadelphia, PA). Anti-receptor antiserum L1-10, which recognizes both insulin and IGF-I receptors, was prepared as described (28). Rat IGF-I (29) and IGF-II (30) cDNAs were kindly provided by Dr. S. Casella (Johns Hopkins University, Baltimore, MD) and Dr. M. Rechler (National Institute of Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD), respectively. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was kindly provided by Dr. G. Chazenbalk (Veterans Administration Medical Center, University of California, San Francisco, CA).

**IGF-I receptor cDNA.** A λgt11 human placental cDNA library (Clontech, Palo Alto, CA) was screened with an 84-base oligonucleotide probe (provided by Dr. M. S. Urdea, Chiron, Emeryville, CA) complementary to bases 3976–4059 of the human IGF-I receptor cDNA according to the numbering of Ullrich, et al. (26). From ~ 5 × 10<sup>5</sup> plaques, 4 positives were identified; the one containing the longest insert (3.2 kb) (Fig. 1) was plaque-purified and subcloned into pUC18. Its identity was confirmed by sequencing the ends by the dideoxy chain termination technique (31). This 3' cDNA was used in studies of IGF-I receptor mRNA abundance, as it contained the region of least homology with the closely related insulin receptor which is also expressed in this cell line (32).

**Cell culture.** BC3H-1 cells, a nonfusing mouse cell line with characteristics of both smooth and skeletal muscle (33), were grown in Dulbecco's modified Eagle's medium H-21 (DMEH-21) supplemented with 20% fetal bovine serum (FBS), 1% glutamine, and antibiotics. Subconfluent cells maintained an appearance typical of myoblasts. Cells were plated at 4 × 10<sup>3</sup> cells/cm<sup>2</sup> in either 75-cm<sup>2</sup> tissue culture flasks or 35-mm 6-well plates. After 3 d, differentiation was initiated by



**Figure 1.** IGF-I receptor cDNA. A λgt11 human placental cDNA library was screened with an 84-base oligonucleotide probe complementary to bases 3976–4059 of the human IGF-I receptor cDNA according to the numbering of Ullrich et al. (26). From ~ 5 × 10<sup>5</sup> plaques, 4 positives were identified; the one containing the longest insert (3.2 kb) was plaque-purified and subcloned into pUC18. Its identity was confirmed by sequencing using the dideoxy chain termination technique. The α- and β-subunits and the transmembrane (TM) domain of the cDNA reported by Ullrich are indicated.

replacing medium containing 20% FBS with medium containing 1% FBS. Myocytes were studied for up to 7 d in differentiation medium. Undifferentiated cells were studied 3 d after plating in medium containing 20% FBS.

**Hormone binding assays.** Cell monolayers in 6-well plates were rinsed with 2 ml of 120 mM NaCl, 1.2 mM MgSO<sub>4</sub>, 15 mM NaOAc, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, 1% bovine serum albumin, and 100 mM Hepes, pH 7.6, and incubated in 2 ml of the same buffer containing 4.5 pM [<sup>125</sup>I]-IGF-I and varying concentrations of unlabeled ligand for 18 h at 4°C (32). The incubation medium was aspirated, the monolayers were washed with 10 mM Tris HCl (pH 7.4) and 154 mM NaCl, and the cells from each well were lysed in 1 ml 0.03% NaDodSO<sub>4</sub> (SDS) for measurement of cell-associated radioactivity. Binding was corrected for nonspecific [<sup>125</sup>I]-IGF-I binding as determined in the presence of 3 × 10<sup>-8</sup> M unlabeled IGF-I. Binding determinations were carried out in triplicate for each concentration of unlabeled ligand. Binding in each well was normalized to total protein (34).

**IGF-I receptor biosynthesis studies.** BC3H-1 cells were plated in 75-cm<sup>2</sup> flasks at 4 × 10<sup>3</sup> cells/cm<sup>2</sup> in DMEH-21 containing 20% FBS. Differentiation was induced as described above. Cells were washed twice in PBS and labeled by adding 1.5 mCi [<sup>35</sup>S]methionine to 5 ml of medium lacking methionine. Cells were incubated for 4 h at 37°C. Under these conditions, [<sup>35</sup>S]methionine incorporation into IGF-I receptor increased linearly for up to 4 h, indicating that receptor degradation was not a significant variable during this time period. The cells were then washed with PBS, scraped off, and solubilized for 60 min with Triton X-100 plus 0.1 mM phenylmethylsulfonyl fluoride and 2 mg/ml bacitracin (28). Suspensions were then centrifuged at 100,000 g for 60 min and the solubilized IGF-I and insulin receptors were partially purified by wheat germ agglutinin chromatography (32). Labeled receptors were immunoprecipitated using polyclonal antibodies, denatured in Laemmli sample buffer containing 50 mM dithiothreitol, and analyzed by electrophoresis in 7.5% SDS polyacrylamide gels and fluorography. Since an antiserum that recognizes only the murine IGF-I receptor was not available, IGF-I receptors were precipitated by a two-step method. First, insulin receptors were removed by double precipitation with antiserum Bd that immunoprecipitates the insulin receptor (32). Then, IGF-I receptors were immunoprecipitated with antiserum L1-10 which recognizes both receptors.

**RNA analysis.** Poly(A)<sup>+</sup> RNA was isolated by lysing cells in 500 mM NaCl, 1% SDS, 10 mM EDTA, and 10 mM Tris (pH 7.2) in the presence of fungal Proteinase K (Bethesda Research Laboratories, Gaithersburg, MD) (35). Lysates were then pushed through a 25-gauge needle to shear DNA and incubated at 37°C for 3 h to digest proteins. Oligo (dT) cellulose (Collaborative Research, Bedford, MA) was added to a final concentration of 2 mg/ml lysate and rotated overnight at room temperature. Oligo (dT)/poly(A)<sup>+</sup> RNA hybrids were then washed in high salt buffer (500 mM NaCl, 0.2% SDS, 0.1 mM EDTA, 10 mM Tris, pH 7.2). Poly(A)<sup>+</sup> RNA was then eluted, ethanol precipitated, and resuspended in H<sub>2</sub>O (36). Poly(A)<sup>+</sup> RNA concentrations were determined by optical density and normalized by hybridization with oligo (dT) on a nitrocellulose slot blot (35, 37). For RNA transfer blots, 10 μg of poly(A)<sup>+</sup> RNA were denatured in formaldehyde, subjected to electrophoresis in 1% agarose gel, and transferred to nitrocellulose (38). cDNA probes for the IGF-I receptor as well as for IGF-I, IGF-II, and GAPDH were labeled using random primers (39) to a specific activity of 10<sup>9</sup> cpm/μg. Nitrocellulose membranes were prehybridized, hybridized and washed as previously described (38), and autoradiography was carried out.

**Radioimmunoassay.** IGF-I and II were measured in conditioned media of BC3H-1 cells after 10-fold lyophilization. For IGF-I determination, binding proteins were removed by G-50 chromatography in 0.25 M formic acid as described by Hintz et al. (40). For IGF-II, binding proteins were removed by extraction with 0.8 M formic acid, 0.05% Tween-20, and 70% acetone according to the method of Bowsher et al. (41). Antibody UBK 487 was used in the IGF-I RIA, as it recognizes murine IGF-I (42). Assay sensitivity was 12 ng/ml. For IGF-II determi-

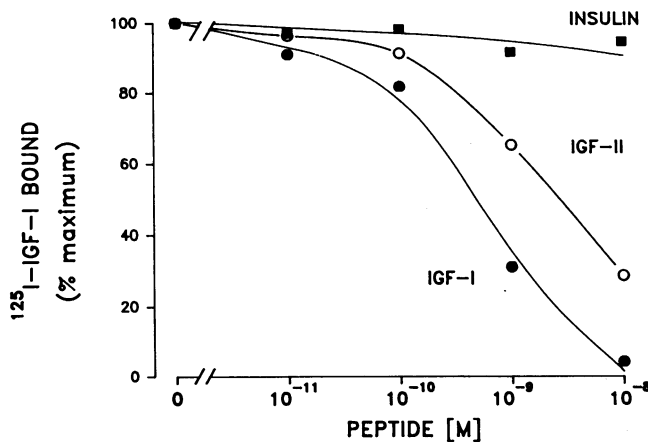
nations, a monoclonal antibody against human IGF-II (Amano Pharmaceutical Co., Nagoya, Japan) having 100% cross-reactivity with rat IGF-II was used. Assay sensitivity was 10 ng/ml.

**Downregulation studies.** The effect of IGF-II treatment on IGF-I binding, IGF-I receptor biosynthesis, and IGF-I receptor mRNA abundance was studied in BC3H-1 myoblasts. (a) For binding studies, subconfluent myoblasts were studied 3 d after plating, as described above. Cells were preincubated in serum-free DMEH-21 containing 1% BSA with vehicle (0.1 N acetic acid) or the indicated peptide for 18 h at 37°C. Cells were then washed twice at 4°C with buffer containing 0.5 M sodium acetate, 150 mM NaCl (pH 4.5) for 6 min to remove preincubated peptide from the cell surface (43). The cells were then washed three times with binding buffer at 4°C. Binding with [<sup>125</sup>I]-IGF-I was subsequently carried out as described above. Data were analyzed by analysis of variance with subsequent comparisons to control values made by unpaired *t* test. (b) For IGF-I receptor biosynthesis studies, myoblasts were preincubated with IGF-II or vehicle, as described above, and determination of IGF-I receptor biosynthesis by [<sup>35</sup>S]methionine labeling and immunoprecipitation was carried out. (c) For IGF-I receptor mRNA abundance studies, myoblasts were preincubated with IGF-II or vehicle as described for binding and receptor biosynthesis studies, after which poly(A)<sup>+</sup> RNA was isolated and analyzed as described above.

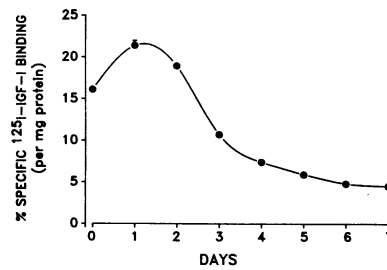
## Results

**IGF-I binding studies.** To investigate the specificity of <sup>125</sup>I-IGF-I binding in BC3H-1 myoblasts, competition-inhibition studies were carried out (Fig. 2). Specific <sup>125</sup>I-IGF-I binding was inhibited by unlabeled IGF-I one-half maximally at 0.14 nM. IGF-II also interacted with the IGF-I receptor, though one-half-maximal inhibition of IGF-I binding occurred at 1.3 nM IGF-II. At concentrations up to 10 nM, insulin had little effect in inhibiting IGF-I binding.

To investigate the effect of differentiation on IGF-I binding in BC3H-1 cells, we compared binding in myoblasts with that in myocytes during 7 d of differentiation (Fig. 3). Specific <sup>125</sup>I-IGF-I binding per mg protein was 16.1±0.3% (mean±SE) in myoblasts, increased transiently to a peak of 21.4±0.6% after 1

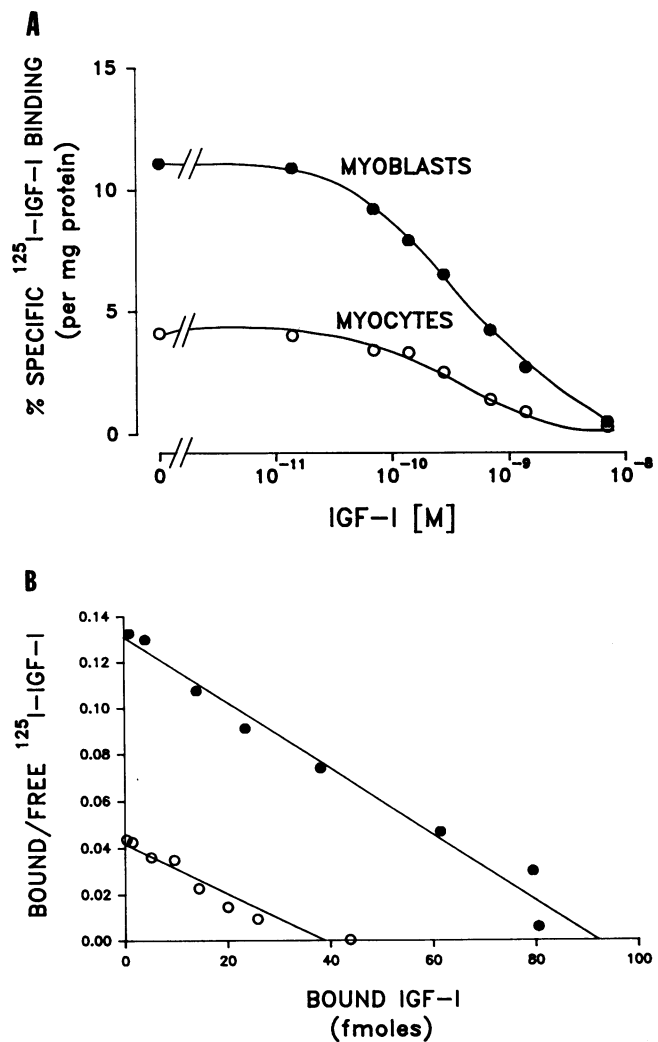


**Figure 2.** Specificity of <sup>125</sup>I-IGF-I binding in BC3H-1 myoblasts. Cells were incubated with 4.5 pM <sup>125</sup>I-IGF-I and varying concentrations of unlabeled IGF-I, IGF-II, or insulin for 18 h at 4°C. Bound radioligand was determined after aspiration of incubation medium and cell washing. Binding is expressed as a percentage of maximal specific <sup>125</sup>I-IGF-I binding per mg of protein. A representative of three separate experiments is shown. Each point represents the mean of triplicate values.



**Figure 3.** Effect of differentiation on <sup>125</sup>I-IGF-I binding in BC3H-1 cells: time course. Cells were plated in 35-mm wells and specific <sup>125</sup>I-IGF-I binding per mg of protein was measured. On day 0, when myoblasts were 80–90% confluent, differentiation was initiated by replacing medium containing 20% FBS with medium containing 1% FBS. Each point is the mean (±SE) of three separate experiments performed in triplicate.

d, and decreased by 7 d to a plateau of 4.5±0.2%. Concurrently, <sup>125</sup>I-insulin binding increased during differentiation, as previously reported (32) (data not shown).

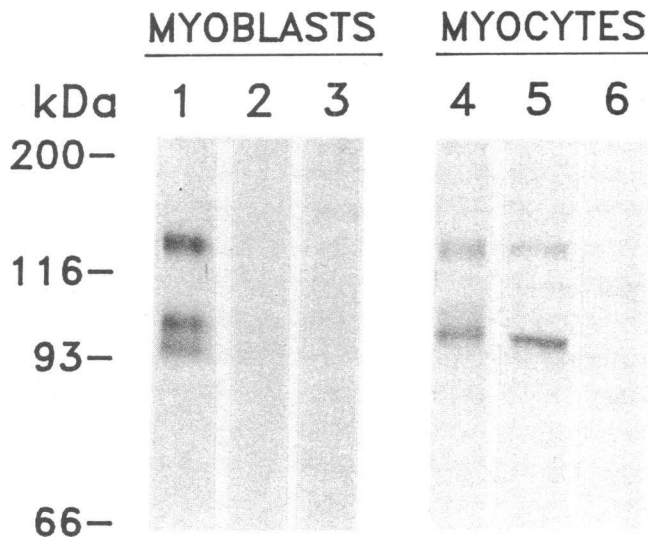


**Figure 4.** IGF-I binding in BC3H-1 cells. (A) Specific <sup>125</sup>I-IGF-I binding per mg protein in myoblasts (●) and in myocytes (○) after 7 d of differentiation was measured in the presence of increasing concentrations of IGF-I. (B) Scatchard plot of the same data. A representative of three separate experiments is shown. Each point represents the mean of triplicate values.

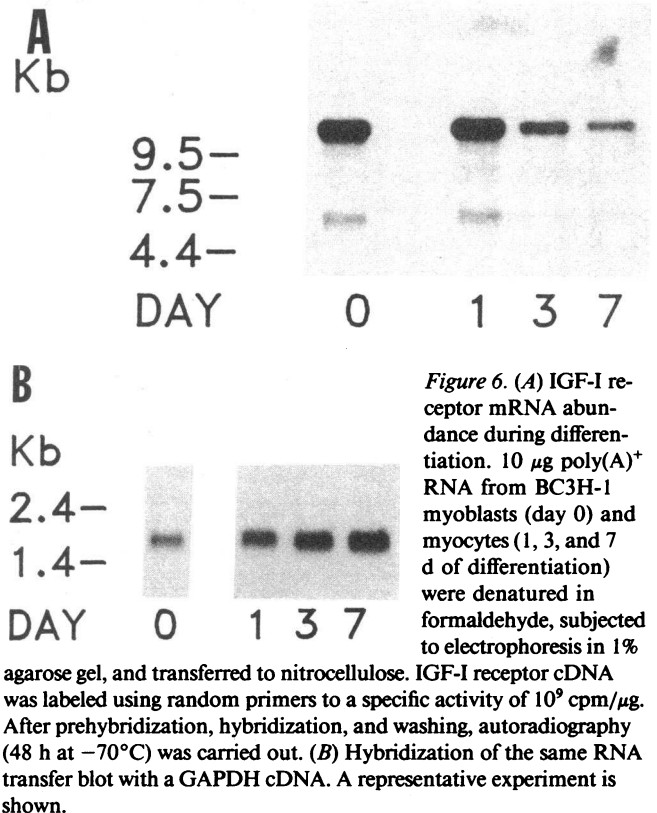
To determine whether the differentiation-associated decrease in IGF-I binding reflected a change in the affinity or number of IGF-I receptors, Scatchard analyses were carried out (Fig. 4). Binding of IGF-I to both myoblasts and myocytes was best fit by a one-site model. With differentiation, IGF-I receptors decreased from  $\sim 27,000 \pm 1,000$  (mean  $\pm$  SE) binding sites per cell in myoblasts to  $11,000 \pm 1,000$  binding sites per cell in myocytes. In contrast, IGF-I receptor affinity was not significantly different between myoblasts and myocytes ( $0.34 \pm 0.02$  nM, mean  $\pm$  SE,  $n = 3$  vs.  $0.43 \pm 0.05$  nM, respectively).

**IGF-I receptor biosynthesis.** Cell labeling studies with [ $^{35}$ S]methionine followed by immunoprecipitation with antireceptor antisera showed that the differentiation-associated decrease in IGF-I binding reflected a decrease in receptor biosynthesis. In both myoblasts and myocytes, a single band of  $M_r$  135,000 corresponding to the  $\alpha$ -subunit of the IGF-I receptor was apparent. In addition, two bands of  $\sim M_r$  95,000 and 97,000 corresponding to IGF-I receptor  $\beta$ -subunits were seen in myoblasts with a slightly larger  $\beta$ -subunit doublet seen in myocytes (Fig. 5). With differentiation, there was a 50–60% decrease in the biosynthesis of the IGF-I receptor  $\alpha$ -subunit. In addition, myocytes demonstrated a 60% decrease in the biosynthesis of the larger IGF-I receptor  $\beta$ -subunit but no apparent change in the smaller  $\beta$ -subunit. In contrast, there was a marked differentiation-associated increase in the biosynthesis of the insulin receptor  $\alpha$ - and  $\beta$ -subunits as previously reported (32).

**IGF-I receptor mRNA abundance during differentiation.** To determine whether the differentiation-associated decrease in IGF-I receptor biosynthesis was associated with a decrease in IGF-I receptor mRNA abundance, RNA transfer blot analyses



**Figure 5.** IGF-I receptor biosynthesis in BC3H-1 cells. Cells were labeled by adding 1.5 mCi [ $^{35}$ S]methionine to medium lacking methionine. Cells were incubated for 4 h at 37°C, washed, and solubilized. IGF-I receptors were partially purified by wheat germ agglutinin chromatography, immunoprecipitated with polyclonal antibodies (lanes 1 and 4) or normal rabbit serum (lanes 3 and 6), reduced, and analyzed by 7.5% SDS-PAGE and fluorography. For comparison, insulin receptor biosynthesis (lanes 2 and 5) is also shown. All lanes were run on the same gel. The positions of molecular weight markers (in thousands) are indicated.



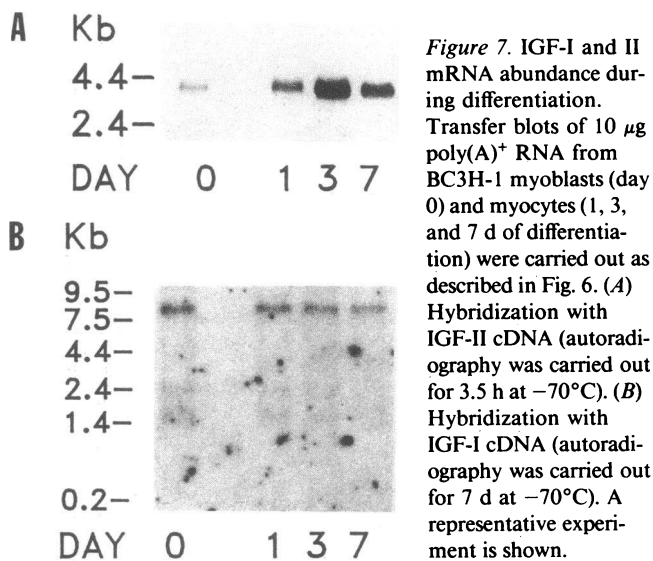
**Figure 6.** (A) IGF-I receptor mRNA abundance during differentiation. 10  $\mu$ g poly(A) $^{+}$  RNA from BC3H-1 myoblasts (day 0) and myocytes (1, 3, and 7 d of differentiation) were denatured in formaldehyde, subjected to electrophoresis in 1% agarose gel, and transferred to nitrocellulose. IGF-I receptor cDNA was labeled using random primers to a specific activity of  $10^9$  cpm/ $\mu$ g. After prehybridization, hybridization, and washing, autoradiography (48 h at  $-70^{\circ}$ C) was carried out. (B) Hybridization of the same RNA transfer blot with a GAPDH cDNA. A representative experiment is shown.

were carried out (Fig. 6). Autoradiographs of mRNA from myoblasts revealed a major band at 11.0 kb and a minor band at 5.5 kb. After 1 d of differentiation, there was no change in IGF-I receptor mRNA abundance. However, at 3 d there was a 75% decrease, and by 7 d of differentiation, an 80–90% decrease in IGF-I receptor mRNA abundance was observed.

**IGF-II expression and secretion during differentiation.** In order to determine whether differentiation was associated with an increase in IGF-II expression and secretion, transfer blot analyses of mRNA and radioimmunoassay of peptide in conditioned media were carried out. In myoblasts, RNA transfer blots revealed a single mRNA species of  $\sim 4.0$  kb, identical in size to the principal IGF-II transcript reported to be present in extracts from murine muscle (18, 19) (Fig. 7 A). After 1 d of differentiation, there was a 2- to 3-fold increase in IGF-II mRNA abundance. After 3 d there was a 15-fold increase in IGF-II mRNA abundance, which then decreased to a level that was 5-fold greater than that seen in myoblasts. RIA of IGF-II in conditioned media from myoblasts demonstrated levels that were  $\sim 10$  ng/ml greater than those in control 20% serum-supplemented media (90 ng/ml). However, with differentiation, concentrations of IGF-II in conditioned media reached a peak of 50 ng/ml on day 5 in comparison to levels of  $\sim 15$  ng/ml in unconditioned 1% serum-supplemented media (Fig. 8).

In contrast to IGF-II, RNA transfer blots revealed low IGF-I peptide mRNA abundance in myoblasts (three species of 7.8, 2.0, and 1.0 kb) which decreased slightly during differentiation (Fig. 7 B). IGF-I was not detected in conditioned media from either myoblasts or myocytes.

**IGF-I receptor downregulation by IGF-II.** To investigate the potential role of IGF-II in the differentiation-associated



decrease in IGF-I receptor expression, we studied the effects of the addition of IGF-II to myoblasts. As noted above, these cells have a relatively high level of IGF-I receptor expression and a relatively low level of IGF-II peptide expression. Preincubation of myoblasts for 18 h with IGF-II demonstrated a decrease in specific IGF-I binding (Fig. 9). At a concentration of 100 ng/ml, IGF-II induced a 60% decrease in IGF-I binding, while comparable concentrations of IGF-I and insulin induced 80 and 40% decreases in IGF-I binding, respectively.

Next, we determined whether IGF-II treatment of myoblasts influenced IGF-I receptor biosynthesis. After preincubation of myoblasts for 18 h with IGF-II (100 ng/ml), a 35% decrease in the IGF-I receptor  $\alpha$ -subunit and a 50% decrease in the larger  $\beta$ -subunit (but not the smaller  $\beta$ -subunit) were seen (Fig. 10). This decrease in IGF-I receptor biosynthesis in myoblasts treated with IGF-II was similar to the decrease in IGF-I receptor biosynthesis that was observed when myoblasts differentiated into myocytes.

In addition, we examined whether IGF-II treatment of myoblasts influenced IGF-I receptor mRNA abundance. Myoblasts were preincubated with IGF-II (100 ng/ml) or vehicle for 18 h and mRNA was isolated. RNA transfer blots demonstrated that IGF-II treatment resulted in a 60% decrease in IGF-I receptor mRNA abundance (Fig. 11).

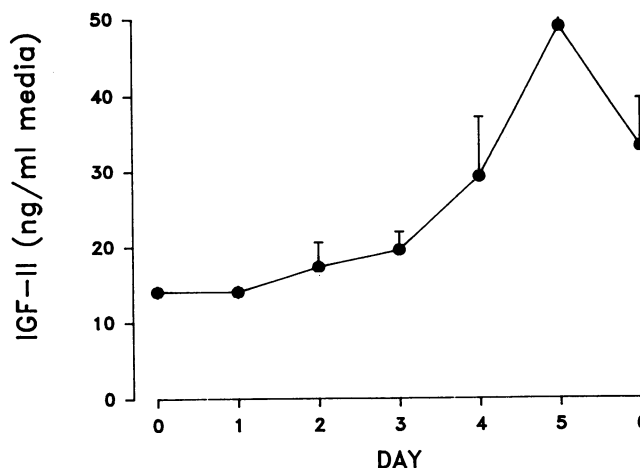
## Discussion

In view of the ability of the IGFs to promote the growth and differentiation of muscle cells (11–14), we investigated the regulation in this tissue of the IGF-I receptor, which mediates the mitogenic effects of IGF-I and possibly those of IGF-II. While some studies indicate that IGF-II can signal through its own receptor (44–46), other reports have demonstrated that both IGF peptides can induce mitogenic effects through the IGF-I receptor (14–16). A muscle cell line in culture provides an opportunity to study changes in IGF-I receptor expression during the differentiation process, and to investigate potential mechanisms responsible for these changes. For this purpose, we employed BC3H-1 cells, a nonfusing mouse cell line with characteristics of both smooth and skeletal muscle (33). These cells

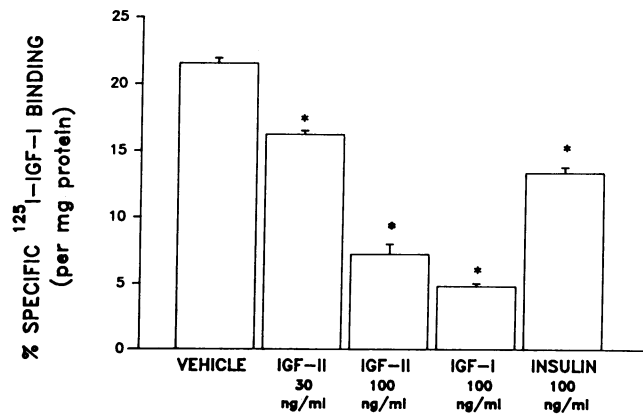
have been extensively used as a model system for studying the events that occur when myoblasts differentiate into myocytes (47, 48).

In this study, we found, as previously reported (4, 32), that IGF-I binding is decreased during differentiation of myoblasts to myocytes. This decrease in IGF-I receptor binding reflected a decrease in IGF-I receptor gene expression and receptor biosynthesis. To explain the mechanisms of IGF-I receptor downregulation during differentiation, we investigated the principal ligands that interact with the IGF-I receptor, IGF-I and II. Concomitant with differentiation into myocytes there was a marked increase in IGF-II gene expression and secretion by BC3H-1 cells. This increase in IGF-II gene expression preceded the decrease in IGF-I receptor gene expression. In contrast to IGF-II, the gene expression of IGF-I, which was low in myoblasts, did not increase with differentiation. To determine whether the increase in IGF-II expression mediated the differentiation-associated decrease in IGF-I receptor expression, we added IGF-II directly to undifferentiated myoblasts. Exogenous IGF-II reduced IGF-I receptor binding, biosynthesis, and receptor gene expression. These observations indicate, therefore, that the decrease in IGF-I receptor gene expression during differentiation of myoblasts to myocytes may be accounted for through autocrine mechanisms involving expression of IGF-II.

It is noteworthy that the concentration of IGF-II in conditioned media from BC3H-1 muscle cells undergoing differentiation ranged from  $\sim 20$ –50 ng/ml, while 30–100 ng/ml of exogenous IGF-II were required to induce IGF-I receptor downregulation in myoblasts. It is conceivable that during differentiation, the IGF-I receptor is downregulated by endogenously produced IGF-II principally through intracellular mechanisms. This type of receptor regulation has been recently described for platelet-derived growth factor (PDGF) receptors in *v-sis*-transformed normal rat kidney cells, where concomitant expression of a PDGF analogue led to downregulation of the PDGF receptor in intracellular compartments (49). Thus, secreted IGF-II may not accurately reflect intracellular IGF-II



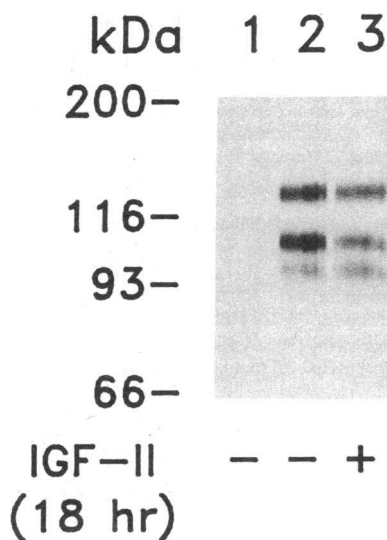
**Figure 8.** IGF-II secretion during differentiation. IGF-II (mean  $\pm$  SE) was determined by RIA of conditioned media from BC3H-1 cells for up to 6 d of differentiation. The levels of IGF-II in control 1% serum-supplemented media were  $\sim 15$  ng/ml. Each point represents the mean of four samples. A representative experiment is shown.



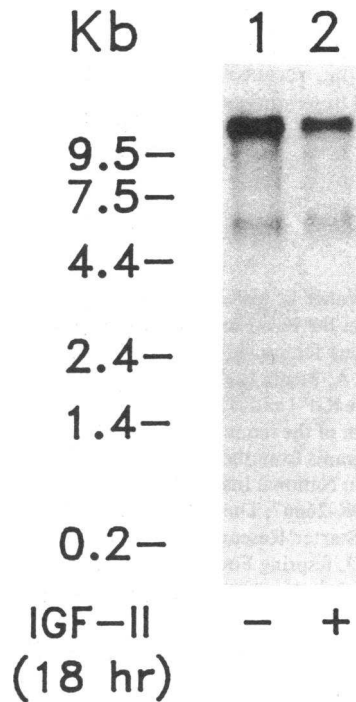
**Figure 9.** IGF-I receptor downregulation by IGF-II. BC3H-1 myoblasts were preincubated in serum-free DMEH-21 containing 1% BSA with vehicle (0.1 N acetic acid) or the indicated peptide for 18 h at 37°C. Specific <sup>125</sup>I-IGF-I binding was then determined. Each bar represents the mean of six separate experiments carried out in triplicate. \**P* < 0.0001 vs. vehicle.

concentrations, which could be higher. In addition, IGF-I receptor studies during differentiation and during downregulation by exogenous IGF-II are not directly comparable, as their experimental designs were not equivalent. In the downregulation studies, a relatively high concentration of IGF-II (100 ng/ml) was used in undifferentiated cells, but for a relatively brief period (18 h). In contrast, cells undergoing differentiation, while exposed to lower IGF-II concentrations in media (up to 50 ng/ml), were exposed to endogenously produced IGF-II for 3–7 d.

Previous studies of IGF-I receptor expression during muscle cell differentiation have yielded conflicting observations. Similar to BC3H-1 cells, IGF-I receptor content as determined by Scatchard analysis also decreased during differentiation of the rat L6 skeletal muscle cell line (4). When similar analyses were carried out in mouse C2 muscle cells, it was reported that IGF-I receptor content increased transiently during differentiation (10). However, comparison of IGF-I binding in these cell lines is limited by differing experimental designs. L6 cells were



**Figure 10.** Effect of IGF-II preincubation on IGF-I receptor biosynthesis in myoblasts. BC3H-1 myoblasts were preincubated in serum-free DMEH-21 containing 1% BSA with vehicle (0.1 N acetic acid; lanes 1 and 2) or IGF-II (100 ng/ml; lane 3) for 18 h at 37°C. Cell labeling with [<sup>35</sup>S]methionine, solubilization, immunoprecipitation with anti-receptor antisera, lanes 2 and 3 (or normal rabbit serum, lane 1), SDS-PAGE and fluorography were performed.



**Figure 11.** Effect of IGF-II preincubation on IGF-I receptor mRNA abundance in myoblasts. BC3H-1 myoblasts were preincubated in serum-free DMEH-21 containing 1% BSA with vehicle (0.1 N acetic acid; lane 1) or IGF-II (100 ng/ml; lane 2) for 18 h at 37°C. Transfer blots of 10 μg poly(A)<sup>+</sup> RNA were carried out and hybridized with the IGF-I receptor cDNA. A representative experiment is shown.

studied only before and after 18 d of differentiation (4), while C2 (10) and BC3H-1 cells were studied daily for 4 and 7 d, respectively.

Concomitant expression of the IGF-I receptor and the IGFs has been reported in a number of cells and tissues (9, 10, 50). This observation suggests that IGF-I receptor regulation may occur via autocrine/paracrine mechanisms. In addition to signaling cellular functions via the IGF-I receptor, the IGF ligands also regulate the IGF-I receptor (3, 51, 52). These studies, however, are the first to demonstrate an increase in IGF-II expression during differentiation that is associated with a concomitant decrease in IGF-I receptor biosynthesis and gene expression. It is of interest that Tollefsen et al. found that IGF-II and, to a lesser extent, IGF-I expression increased during differentiation of the C2 mouse muscle cell line (9, 10). However, in these studies, they observed an increase in IGF-I binding after 2–3 d of differentiation followed by a decrease after 4 d to levels equivalent to that in myoblasts (10). In this study, we also noted a transient increase in IGF-I binding in BC3H-1 cells during the first 2 d of differentiation. However, after 3 d in differentiation media, when IGF-II gene expression in BC3H-1 cells was markedly increased, IGF-I receptor expression was suppressed below levels seen in myoblasts. Since serum contains IGF peptides that can interact with the IGF-I receptor, it is possible that, when first placing the cells into a low serum-containing medium (in order to induce differentiation), such peptides are decreased, allowing transient receptor upregulation. The observation that IGF-I receptor mRNA abundance is similar in BC3H-1 myoblasts and in cells after 1 d of differentiation (Fig. 6 A), suggests that the transient increase in IGF-I binding (on days 1–2) may be accounted for by translational or posttranslational mechanisms.

In summary, differentiation of BC3H-1 mouse muscle cells is associated with decreased IGF-I receptor content, biosynthesis and gene expression, and a marked increase in IGF-II pep-

tide gene expression. Furthermore, treatment of undifferentiated myoblasts with IGF-II results in IGF-I receptor downregulation at the levels of binding, receptor biosynthesis, and receptor mRNA abundance. These studies suggest, therefore, that IGF-I receptor expression during muscle cell differentiation may be regulated, at least in part, through autocrine production of IGF-II.

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