Insulin-like Growth Factor Binding Protein-5 Modulates Muscle Differentiation through an Insulin-like Growth Factor–dependent Mechanism

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Abstract. The insulin-like growth factor binding proteins (IGFBPs) are a family of six secreted proteins which bind to and modulate the actions of insulin-like growth factors-I and -II (IGF-I and -II). IGFBP-5 is more conserved than other IGFBPs characterized to date, and is expressed in adult rodent muscle and in the developing myotome. We have shown previously that C2 myoblasts secrete IGFBP-5 as their sole IGFBP. Here we use these cells to study the function of IGFBP-5 during myogenesis, a process stimulated by IGFs. We stably transfected C2 cells with IGFBP-5 cDNAs under control of a constitutively active promoter. Compared with vector-transfected control cells, C2 myoblasts expressing the IGFBP-5 transgene in the sense orientation exhibit increased IGFBP-5 levels in the extracellular matrix during proliferation, and subsequently fail to differentiate normally, as assessed by both morphological and biochemical criteria. Compared to controls, IGFBP-5 sense myoblasts show enhanced survival in low serum medium, remaining viable for at least four weeks in culture. By contrast, myoblasts expressing the IGFBP-5 antisense transcript differentiate prematurely and more extensively than control cells. The inhibition of myogenic differentiation by high level expression of IGFBP-5 could be overcome by exogenous IGFs, with des (1-3) IGF-I, an analogue with decreased affinity for IGFBP-5 but normal affinity for the IGF-I receptor, showing the highest potency. These results are consistent with a model in which IGFBP-5 blocks IGF-stimulated myogenesis, and indicate that sequestration of IGFs in the extracellular matrix could be a possible mechanism of action. Our observations also suggest that IGFBP-5 normally inhibits muscle differentiation, and imply a role for IGFBP-5 in regulating IGF action during myogenic development in vivo.

THE involvement of growth factors in the regulation of myoblast proliferation and differentiation is well established (for review see references 16, 32). Unlike most growth factors, the insulin-like growth factors (IGFs)[1] stimulate both mitogenesis and myogenesis in vitro (14), an apparent paradox since terminal differentiation requires withdrawal from the cell cycle. The mechanisms by which IGFs stimulate myogenesis are unclear, but may involve regulation of the helix-loop-helix transcription factors myf-5 and myogenin (17, 28), whose forced expression is sufficient for conversion of fibroblasts into myoblasts (4, 10), and which regulate many muscle-specific genes (26). Recently, the importance of IGF action in muscle formation and differentiation has been documented in vivo. Mice lacking the IGF-I receptor have impaired muscle formation and die shortly after birth (27). Therefore, regulation of IGF activity during development may be critical for proper muscle formation.

One mechanism by which IGF activity is modified is through high affinity interactions with insulin-like growth factor–binding proteins (IGFBPs). The IGFBPs are comprised of a family of six secreted proteins which share ~50% amino acid sequence identity (for review see references 3, 22). Postulated mechanisms by which IGFBPs modulate growth factor activity include (*1*) protecting IGFs from proteolytic degradation, (*2*) targeting IGFs in serum to specific tissues, and (*3*) regulating local IGF availability to receptors by sequestration in extracellular storage pools. IGFBPs have been shown to both enhance and inhibit IGF-mediated cellular proliferation (22), but their effects on IGF-stimulated cellular differentiation remain largely unknown.

Recently, we cloned the cDNA encoding murine IGFBP-5

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1. *Abbreviations used in this paper*: CK, creatine kinase; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; MHC, myosin heavy chain.

from myoblast cell lines and showed that it is the most conserved IGF-binding protein identified to date (21). In rodents, IGFBP-5 is expressed in the developing myotome (19) and in adult smooth and skeletal muscle (21). In addition, we have identified multiple skeletal muscle cell lines which secrete IGFBP-5 in a differentiation-dependent manner (21, 29, 37, 39, 41). Therefore, IGFBP-5 is a likely candidate to regulate IGF activity in muscle development and differentiation.

To investigate directly the role of IGFBP-5 during myogenic differentiation, we transfected C2 myoblasts with expression plasmids containing a murine IGFBP-5 coding region cDNA in either the sense or antisense orientation. Unlike some myoblast cell lines (29, 39), C2 cells produce no other IGFBPs (21, 41), rendering them a useful model in which to study IGFBP-5 function without potential interference from other IGFBPs. Constitutive expression of the IGFBP-5 cDNA in the sense orientation resulted in increased IGFBP-5 protein expression during myoblast proliferation, a diminished capacity to differentiate, and enhanced survival in reduced serum medium. These phenotypic changes could be reversed by addition of exogenous IGFs. By contrast, constitutive expression of the IGFBP-5 cDNA in the antisense orientation led to precocious biochemical and morphological differentiation. Our results show that IGFBP-5 can contribute to the regulation of muscle differentiation through a mechanism which involves modulation of IGF activity.

Materials and Methods

Materials

Enzymes, including restriction enzymes, ligases and polymerases were from Life Technologies (Gaithersburg, MD), Perkin-Elmer/Cetus (Norwalk, CT), New England Biolabs (Beverly, MA), and United States Biochemical (Cleveland, OH). DNA linkers were also purchased from New England Biolabs. Ribonucleotide triphosphates and deoxyribonucleotide triphosphates were purchased from Pharmacia-Biotechnology (Piscataway, NJ). Radionuclides were from Dupont-New England Nuclear (Boston, MA). Plasmid bluescript was from Stratagene (La Jolla, CA) and pcDNA1neo was from Invitrogen (San Diego, CA). The pBAT vector was obtained from Dr. Arnd Annweiler (Heidelberg, Germany) (1) and pEMSVscribeα2 was from the late Dr. Harold Weintraub (Seattle, WA). Tissue culture media, sera and geneticin were purchased from Life Technologies. Creatine kinase enzymatic reagents and secondary antibodies were obtained from Sigma Chem. Co. (St. Louis, MO). BCA protein quantitation reagents were purchased from Pierce Chemical (Rockford, IL). The enhanced chemiluminescence (ECL) Western blot detection kit was obtained from Amersham Corp. (Arlington Heights, IL). Recombinant IGF-II and des (1-3) IGF-I were purchased from GroPep (Adelaide, Australia). Recombinant IGF-I was obtained from Dr. Chris Morrison at Ciba-Geigy (St. Aubin, Switzerland).

Construction and Purification of Expression Plasmids

pBAT/mIGFBP-5, a plasmid containing 55 bp of the β-globin leader sequence and ~1.2 kb of mIGFBP-5 cDNA in the HindIII/PstI sites, was linearized with KpnI, blunt ended with T_4 DNA polymerase, ligated with EcoRI linkers, and digested with EcoRI to liberate the insert and unmask the restriction site. The insert was then ligated into pEMSVscribeα2 in the sense and antisense orientations relative to the MSV LTR (see Fig. 1). Plasmids used in transfections were purified by centrifugation through $CsCl_2$ density gradients (38).

Stable Transfection and Isolation of Colonies

C2 cells were plated at 150,000 cells/100-mm-gelatin-coated tissue culture plate (41). On the following day, the cells were washed and transfected with 5 μg of DNA at a 10:1 molar ratio (pEMSV/mIGFBP-5 :pcDNA1neo) by a modified calcium phosphate precipitation procedure (6). On day 3, cells were washed and split onto three 150-mm plates in growth media containing 400 μg/ml of active geneticin. Selection proceeded for 16 d; media was changed every 4 d. Individual colonies were isolated by trypsinization and expanded in selection medium.

Cell Culture

Transfected cells were routinely plated at 10^4 cells/ml on gelatin-coated plates in Dulbecco's modified Eagle medium supplemented with 10% FCS, 10% newborn calf serum, and 200 μg/ml of active geneticin. Differentiation was induced when the cells were ~80% confluent. Growth medium then was removed, the cells washed with EBSS, and incubated in Dulbecco's modified Eagle's medium plus 2% horse serum. Photomicrographs were taken with a 10× objective.

RNA Isolation and Analysis

Total RNA was isolated using guanidinium thiocyanate (7, 8). RNA quantity and quality were assessed by agarose-gel electrophoresis followed by ethidium bromide staining. Ribonuclease protection assays were performed as described (36). IGFBP-5 transgene expression was detected using a ^{32}P-labeled antisense riboprobe derived from the KpnI–SacI fragment of pBAT/mIGFBP-5 (including the β-globin leader sequence) transcribed using either T7 or T3 RNA polymerase (30) to detect coding or anticoding mRNAs, respectively (see Fig. 3). Endogenous IGFBP-5 mRNA was detected using a 268-nt antisense strand riboprobe derived from the 3′ untranslated region of the cDNA (21) which is not included in the transgene. Myogenin mRNA expression was detected using an antisense probe derived by subcloning the 168-nt EcoRI–SacI fragment of pEMSV/myogenin (10) into pBluescript and transcribing using T7 RNA polymerase.

Analysis of Secreted Proteins by Western or Ligand Blotting

Cell-conditioned media were harvested from differentiated cells and treated with EDTA to a final concentration of 2 mM. Aliquots were electrophoresed through SDS polyacrylamide gels in the absence of reducing agents, transferred to 0.2 μm nitrocellulose filters, and blocked with 3% BSA in tris-buffered saline (TBS) for at least 1 h. For Western blot analysis, filters were incubated with a 1:1,000 dilution of antiserum raised against human IGFBP-5, as described previously (37) and detected using ECL. For ligand blot analysis, filters were incubated overnight at 25°C with 4×10^6 cpm of ^{125}I-IGF-II in TBS containing 1% BSA and 0.1% Tween-20 (20), washed with TBS plus 0.1% Tween-20, and exposed to x-ray film at −80°C with two intensifying screens.

Analysis of Extracellular Matrix-associated IGFBPs

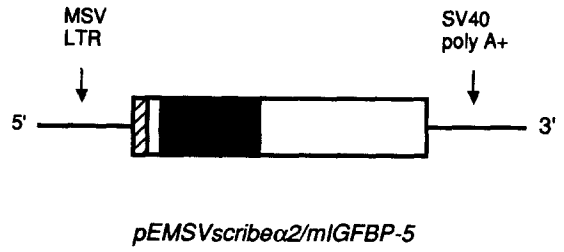
Cells were plated on 24-well cluster plates and proteins associated with the extracellular matrix (ECM) were isolated at ~80% confluence and at 1, 2, 4, 8, and 20 h after initiation of differentiation. Cells were washed with PBS and then lysed with PBS containing 1% Triton X-100 followed by incubation with 25 mM ammonium acetate (pH 9) to remove cellular membranes (24, 25). ECM proteins in each well were scraped into 50 μl of Laemmli sample buffer (24). IGFBP expression was assessed by ligand or Western blot as described above using one-half of a matrix preparation.

Analysis of Myosin Heavy Chain Expression

30 μg of total cytoplasmic proteins were reduced with β-mercaptoethanol, electrophoresed through 7.5% SDS-polyacrylamide gels and transferred to 0.2 μm nitrocellulose filters. Filters were blocked overnight at 4°C with 3% BSA in TBS and then incubated with a 1:200 dilution of anti-chicken MHC monoclonal antibody MF20 (18). Proteins were detected using ECL following incubation with HRP-conjugated anti-mouse IgG.

Cytoplasmic Lysates and Analysis of Creatine Kinase Activity

Cells were grown on 60-mm gelatin-coated dishes, and harvested while undifferentiated, and at 12, 24, 48, 72, and 96 h after incubation in differentiation medium. Cells were washed with phosphate-buffered saline



pEMSVscribeα2/mIGFBP-5

pEMSVscribeα2/mIGFBP-5 Antisense

Figure 1. Schematic representation of the pEMSVscribeα2/mIGFBP-5 expression plasmids. The pEMSVscribeα2/IGFBP-5 sense (*top panel*) and antisense (*bottom panel*) expression plasmids were constructed as described in Materials and Methods. The white boxes represent cDNAs corresponding to IGFBP-5 mRNA untranslated sequences. The black boxes indicate the IGFBP-5 coding sequences, while the hatched boxes represent the 55-bp rabbit β-globin 5′ untranslated sequence from the pBAT vector. The Moloney sarcoma virus promoter (MSV LTR) and SV40 polyadenylation sequences are marked by arrows.

(PBS) and lysed by incubation with 0.5 ml of 50 mM Tris-MES, pH 7.8, 1% Triton X-100 (TMT buffer) for 10 min at 25°C. Samples were stored at −80°C and assayed for enzymatic activity within 1 wk, as per manufacturer's instructions (Sigma procedure 47-UV). Creatine kinase activity was normalized to total protein content as determined by the BCA assay, performed in microtiter plates as per manufacturer's instructions. All data points represent the mean of duplicate determinations of representative experiments; each experiment was performed 2–4 times.

IGF Dose-response Assay

Transfected cells were plated in six-well cluster dishes and grown as above, but induced to differentiate in Dulbecco's modified Eagle's medium containing 2% horse serum and graded concentrations of either IGF-II, IGF-I, or des (1-3) IGF-I. Cells were allowed to incubate for 72 h before harvesting cytoplasmic lysates in 400 μl TMT buffer, followed by assays for creatine kinase activity and total protein content.

Results

Stable Transfection of C2 Myoblasts with An IGFBP-5 cDNA

The pEMSVscribeα2 vector, which contains the Moloney sarcoma virus LTR, was chosen to express IGFBP-5 transgenes since it has been used previously to direct expression of a variety of cDNAs in myoblasts (9, 10). The transgene contains 55 bp of the β-globin 5′ untranslated sequence,

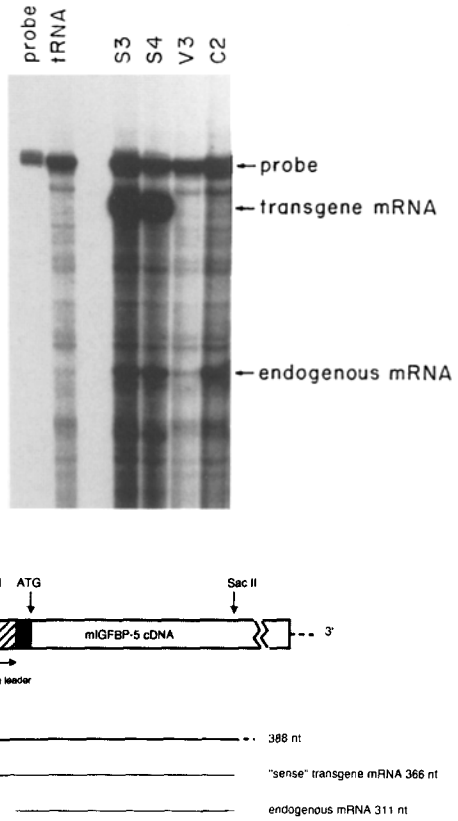


Figure 2. Two independent colonies express IGFBP-5 transgene mRNA. The top panel is an autoradiograph of a ribonuclease protection experiment using 10 μg of total cellular RNA isolated from cells stably transfected with the IGFBP-5 transgene (cell lines S3 and S4) and grown to ~80% confluence in DME containing 20% serum. Total RNA from parental C2 cells (C2), or cells transfected with the empty expression vector (V3), were included as positive controls for the endogenous IGFBP-5 mRNA. Migration of the bands protected by the transgene, the endogenous gene, or the undigested probe are indicated by the arrows on the right. Autoradiographic exposure was for 18 h at −80°C with two intensifying screens. The bottom panel is a schematic representation of the riboprobe derived from the transgene which was used in this experiment to discriminate between mRNAs which contain the β-globin sequence and those which do not.

23 bp of IGFBP-5 5′ UTR, the entire coding sequence, and ~350 bp of 3′ UTR, followed by the SV40 polyadenylation signal (Fig. 1). Cotransfection of sense or antisense IGFBP-5 expression plasmids with pcDNA1neo, which encodes the neomycin resistance gene, each resulted in several colonies which were resistant to geneticin. Two sublines expressing the IGFBP-5 sense transgene, and four expressing the antisense transcript, were characterized in detail. Cell lines transfected with the empty expression vector and selectable marker also were characterized. All cells proliferated normally in serum-containing media, with doubling times of ~15 h (data not shown).

Colonies S3 and S4 Express the IGFBP-5 Sense Transgene

RNA was harvested from sense cells that had grown to ~80% confluency and analyzed by ribonuclease protection assay. Fig. 2 shows that RNA from colonies S3 and S4

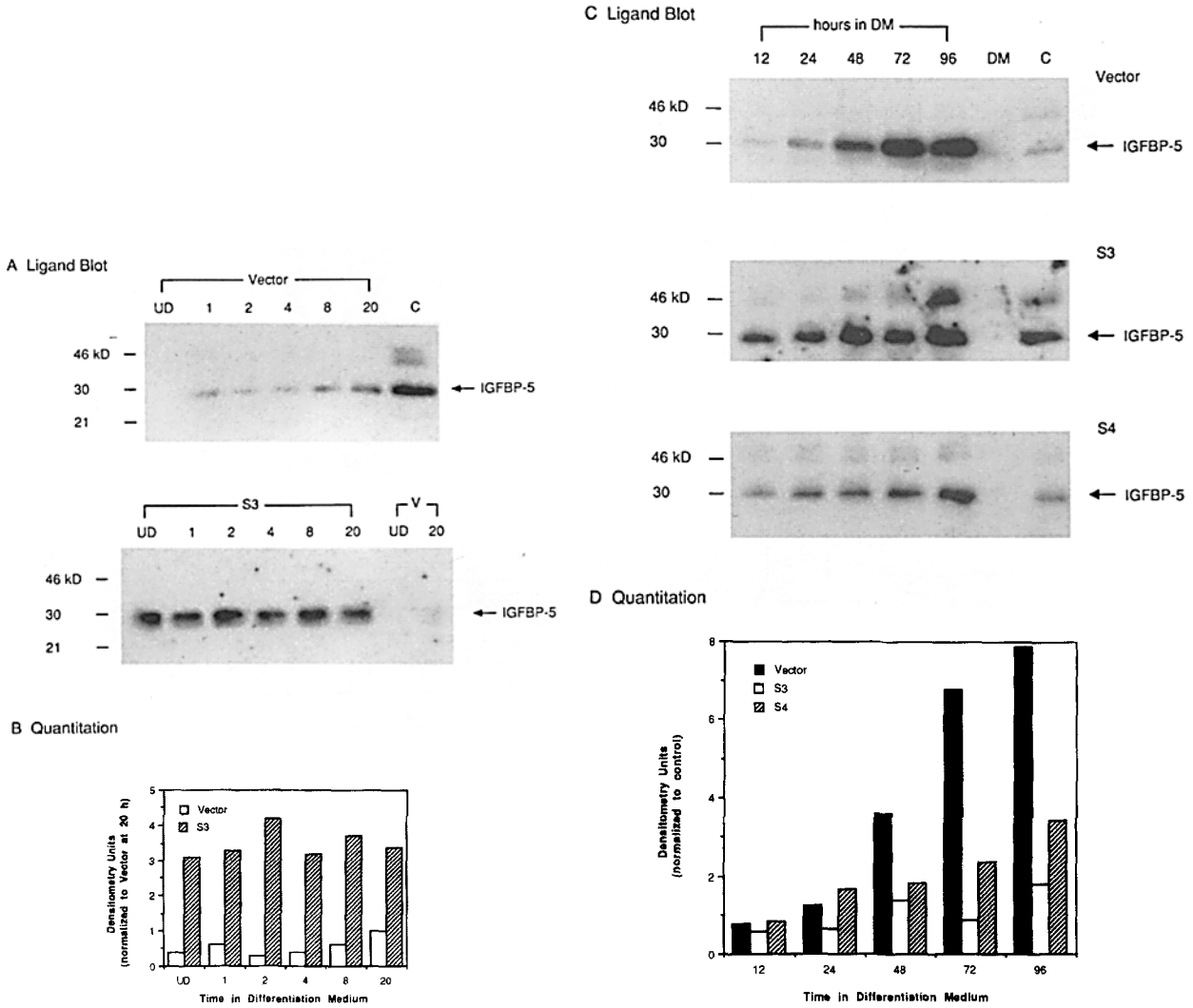


Figure 3. Analysis of IGFBPs produced by S3 and S4 myoblasts. (*Left panel*) IGFBP-5 expression in the ECM. (*A*) Extracellular matrix proteins from vector-transfected (*V*) cells (*top*) or S3 cells (*bottom*) were isolated from undifferentiated cells (*UD*) and at 1, 2, 4, 8, and 20 h after initiation of incubation in differentiation medium. Proteins from one-half of each sample were separated by electrophoresis on 12.5% SDS-PAGE, transferred to a nitrocellulose filter, and incubated with ^{125}I-IGF-II. The V-UD and 20-h samples were repeated in the bottom panel to allow for direct comparison of IGFBP expression. Autoradiographic exposure was for 4 d at −80°C with two intensifying screens. (*B*) Autoradiographs shown in *A* were scanned by densitometry to determine the intensity of IGFBP-5. Values were normalized to the vector sample at 20 h (V-20), which was arbitrarily set at 1. Open boxes represent values from vector-transfected cells, while hatched boxes indicate results from S3 cells. (*Right panel*) Accumulation of IGFBP-5 in cell-conditioned media. (*C*) Autoradiographs of ligand blots of media (50 μl/lane) conditioned by vector (*top*), S3 (*middle*), or S4 (*bottom*) cells. Media were collected at 12, 24, 48, 72, and 96 h after initiation of incubation in differentiation medium, and analyzed as in *A*. Autoradiographic exposure was for 48 h at −80°C with two intensifying screens. (*D*) Autoradiographs shown in *C* were scanned by densitometry to determine the intensity of IGFBP-5. Values were normalized to the external control, which was arbitrarily set at 1. Black boxes represent values from vector-transfected cells, while open boxes and hatched boxes indicate media conditioned by S3 and S4 cells, respectively. *DM*, nonconditioned differentiation medium; *C*, external control medium from parental C2 cells included for quantititation. The migration of IGFBP-5 (confirmed by Western blot) is marked by arrows on the right; molecular mass standards are on the left.

protected a 366-nt band derived from the transgene. This band was not seen in vector-transfected or parental C2 cells. Levels of accumulation of the transgene were severalfold higher than endogenous IGFBP-5 mRNA, indicated by the 311-nt band.

IGFBP-5 Is Detected in the Extracellular Matrix of Myoblasts

IGFBP-5 has been shown to accumulate in the ECM deposited by fibroblasts in culture (24). To determine whether IGFBP-5 was found in the matrix of myoblasts during proliferation or differentiation, ECM preparations from vector-transfected control cells and S3 myoblasts were examined by ligand blot for the presence of IGF-binding activity (Fig. 3, *A* and *B*), and by Western blot for anti–IGFBP-5 immunoreactive proteins (data not shown). In contrast to control cells, where IGFBP-5 is minimally detected in the matrix during the initial 20 h after the switch to low serum medium, IGFBP-5 levels in the ECM

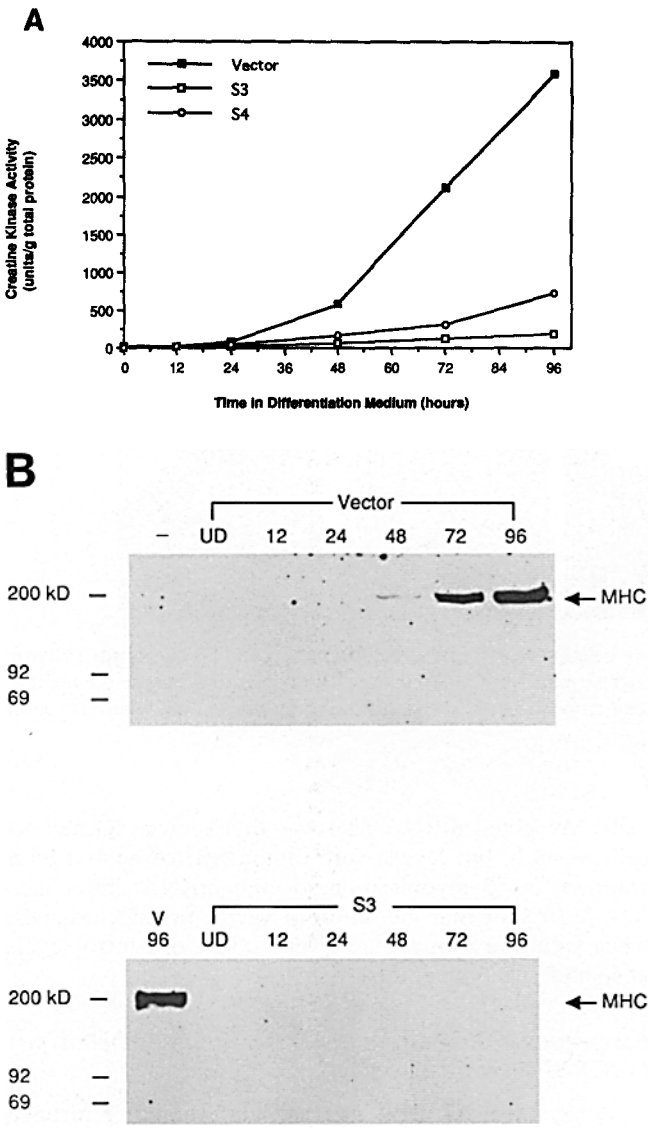


Figure 4. Minimal biochemical differentiation in myoblasts expressing the IGFBP-5 sense transgene. (*A*) Reduced creatine kinase activity in transfected cells. Lysates from cell lines which express the transgene (S3 and S4) were analyzed for expression of the muscle-specific isoform of creatine kinase, and specific activity was determined relative to total protein concentration. Cells were harvested at 80% confluence, and at 12, 24, 48, 72, and 96 h after incubation in differentiation medium. IGFBP-5 S3 cells (*open squares*) and S4 cells (*open circles*) show markedly reduced CK activity compared to vector-transfected control cells (*filled squares*). (*B*) Lack of expression of myosin heavy chain (MHC) by transfected IGFBP-5 sense myoblasts. Cytoplasmic proteins (30 μg) from vector-transfected cells (*top panel*) and S3 cells (*bottom panel*) were separated by SDS-PAGE, transferred to nitrocellulose filters, and analyzed for MHC expression by Western blot using anti-chicken MHC monoclonal antibody MF-20. Proteins were detected using ECL and quantitated by densitometry. Vector lysates harvested at 96 h (V96) were included in the bottom panel to allow direct comparison between the two experiments. MHC expression is not detected in the cytoplasm of S3 myoblasts during the 96-h time course.

of S3 cells were high before the onset of differentiation and remained constant throughout the same 20-h time course. By contrast, when compared to an external control, similar levels of IGFBP-5 were seen in conditioned medium from both control and sense myoblasts during the initial 24 h in differentiation medium (Fig. 3, *C* and *D*). As shown previously for C2 cells (21), IGFBP-5 levels in conditioned differentiation medium from vector-transfected myoblasts increased markedly during the subsequent 72 h (Fig. 3, *C* and *D*).

Diminished Myogenic Differentiation of Transfected IGFBP-5 Sense Myoblasts

Transfected myoblasts were incubated in standard differentiation medium containing 2% horse serum and examined for biochemical and morphological changes associated with muscle differentiation. Fig. 4 *A* shows results of analysis of the muscle-specific isoform of creatine kinase (CK). Vector-transfected cells showed a time-dependent rise in CK activity. Enzymatic activity was first detected by 48 h, and increased throughout the ensuing 48 h, to a level of ~4,000 U/g total protein at 96 h. By contrast, a minimal rise in CK was seen in S4 and S3 cells, to 600 and 200 U/g, respectively, at 96 h. The same cytoplasmic lysates were analyzed for myosin heavy chain (MHC) expression by Western blot (Fig. 4 *B*). In vector-transfected control cells, MHC was detectable at 48 h and increased in a differentiation-dependent manner. In contrast, no MHC was expressed in S3 cells.

Morphological differentiation also was impaired in IGFBP-5 sense myoblasts. As seen in Fig. 5, vector-transfected control cells formed myotubes within 48–72 h after onset of incubation in differentiation medium, a time course that was slightly delayed compared with parental C2 cells (21). In contrast, after 96 h in low serum medium, S3 cells appeared healthy but undifferentiated. The majority of cells were mononucleate myoblasts; multinucleated myotubes were rarely seen. At 96 h, the S4 cell line also showed substantially less myotube formation than control cells (data not shown).

The failure of S3 cells to differentiate also correlated with prolonged survival in low serum–containing medium. As noted previously, at 96 h postmedium change, the S3 cells remained alive and healthy in contrast to vector cells which were beginning to detach from the plate. We investigated the effects of prolonged incubation in medium containing 2% horse serum on cell viability and on biochemical differentiation, as measured by creatine kinase assay. As shown in Fig. 6, S3 cells maintained low levels of CK expression for 3 wk in low serum medium. In other experiments, myoblasts survival persisted for up to 4 wk (data not shown).

Diminished Biochemical and Morphological Differentiation of Sense Myoblasts Correlates with Subnormal Levels of Myogenin Gene Expression

Myogenin is a muscle-specific transcription factor that is expressed early during myoblast differentiation and which contributes to the regulation of muscle-specific genes (for review see reference 11). Myogenin has also been implicated as a target of IGF action during myogenesis (13, 15).

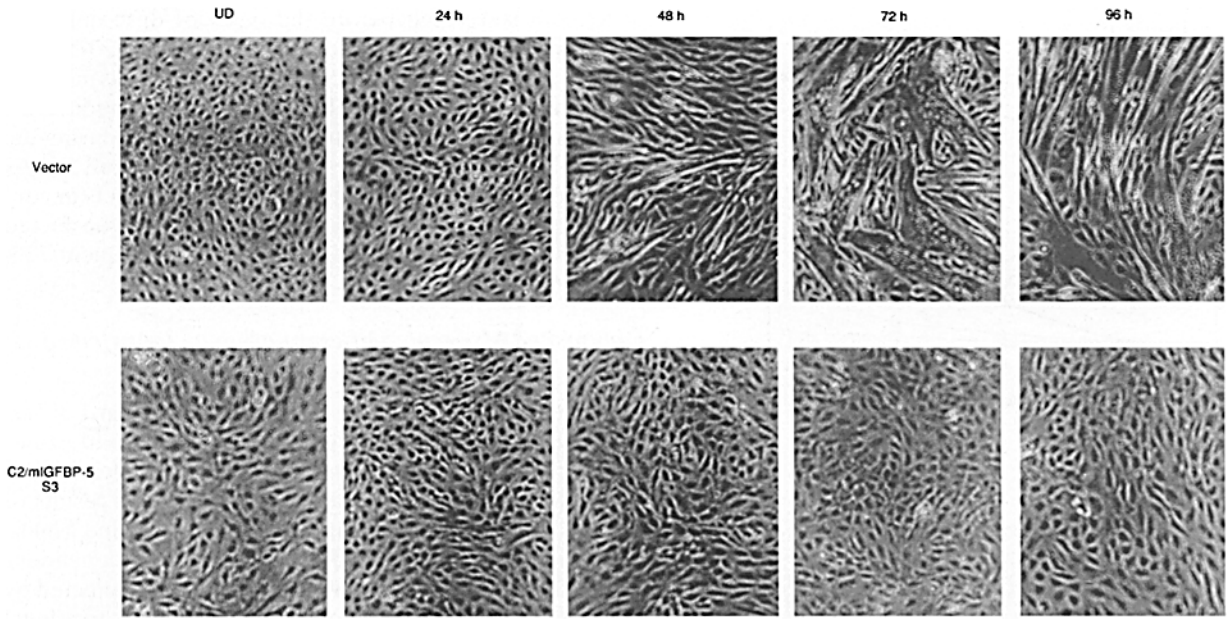


Figure 5. Lack of morphological differentiation of S3 myoblasts. Control cells (vector-transfected, *top panel*) and S3 myoblasts (*bottom panel*) were induced to differentiate for 24–96 h. Phase-contrast photomicrographs were taken using a 10 × objective, before the incubation in differentiation medium, and at 24, 48, 72, and 96 h. At 96 h, the majority of S3 cells are viable and mononucleated, whereas vector cells have formed myotubes by 48–72 h.

We examined expression of myogenin mRNA by ribonuclease protection assay in control and IGFBP-5 sense cells as a function of time in differentiation medium. As seen in Fig. 7, induction of myogenin mRNA occured by 48 h in vector-transfected cells, at which time transcript levels were more than 15-fold higher than in undifferentiated cells. Myogenin mRNA also was first seen in S3 and S4 cells at 48 h, but levels were diminished compared with controls. In S3 myoblasts, myogenin mRNA abundance was < 10% of that measured in vector-transfected cells, while values in S4 myoblasts were ~50% of control levels at 48 and 72 h time points.

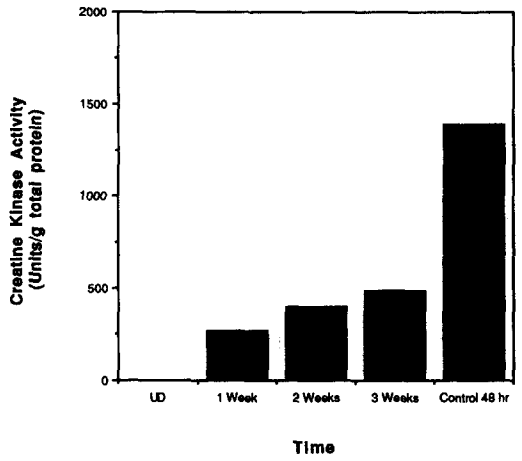


Figure 6. Long-term viability of S3 myoblasts in medium containing 2% horse serum. Parallel cultures of C2/mIGFBP-5 S3 cells were incubated in DME supplemented with 2% horse serum for up to 3 wk. Culture medium was changed twice each week and cells were harvested 1, 2, or 3 wk later. Creatine kinase activity was determined and normalized to total protein content. The majority of cells remained mononucleated and viable throughout the time course, although some cell death was evident at 3 wk. The bar labeled "Control 48 h" shows CK activity of vector-transfected control cells after a 2-d incubation in differentiation medium.

Exogenous IGFs Restore the Differentiated Phenotype to IGFBP-5 Sense Myoblasts

To determine if IGFBP-5 repressed differentiation through a mechanism dependent upon IGFs, confluent S3 cells were incubated with graded concentrations of recombinant IGF-II, IGF-I, or des (1-3) IGF-I in low serum media for 72 h and analyzed for creatine kinase activity (Fig. 8) and myotube formation (Fig. 9). Each peptide tested could restore CK activity to levels that were ~50% of those in vector cells at 72 h (compare Figs. 4 and 8). As indicated by the dose-response curves, des (1-3) IGF-I was the most effective agent. Half-maximal activity was observed with 0.37 nM des (1-3) IGF-I, ~10 nM IGF-I, and ~45 nM IGF-II. Similar dose-response curves were observed with myotube formation (Fig. 9).

Enhanced Muscle Differentiation after Forced Expression of An IGFBP-5 Antisense Transcript

Since overexpression of IGFBP-5 in sense myoblasts led to diminished differentiation, we postulated that inhibition of IGFBP-5 expression would cause accelerated differentiation. To test this hypothesis, we prepared stable cell lines with an IGFBP-5 cDNA in the antisense orientation relative to the MSV LTR (see Fig. 1). To assess expression of the transgene, RNA was harvested from four antisense lines, AS1, 3, 5, and 12, after cultures had reached ~80%

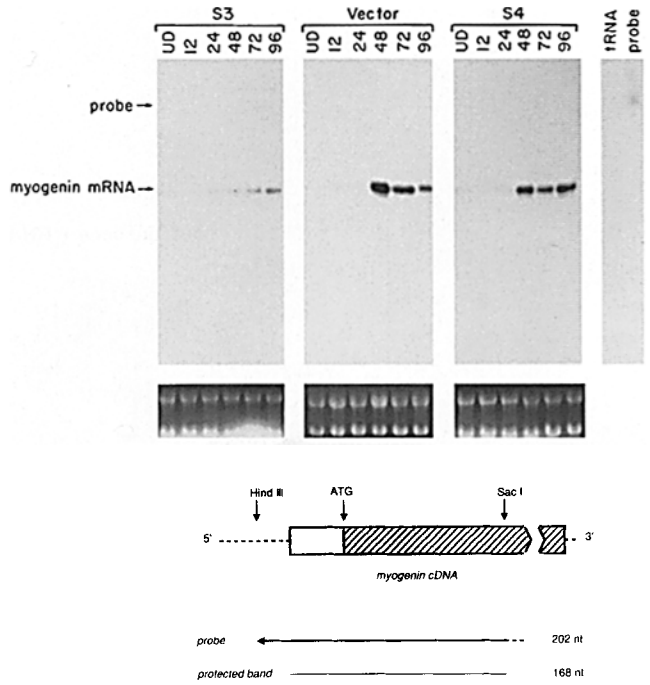


Figure 7. Expression of myogenin mRNA in cells transfected with the IGFBP-5 sense transgene. The top panel shows an autoradiograph of a ribonuclease protection experiment using 10 μg of total cellular RNA isolated from cells stably transfected with the vector (*center*) or the IGFBP-5 sense expression plasmid (*left and right*), while undifferentiated, (*UD*) and after 12, 24, 48, 72, or 96 h of incubation in differentiation medium. Two independent sense colonies were analyzed, S3 (*left*) and S4 (*right*). A sample of yeast tRNA was included as a negative control, and an aliquot of undigested probe was included on the gel as a molecular weight marker. The migration of the undigested probe and the myogenin mRNA are marked by arrows on the left. The gel was exposed to x-ray film for 24 h at −80°C with two intensifying screens. The middle panel shows ethidium-bromide stained agarose gels of the RNA samples used in this experiment. The bottom panel shows a schematic diagram of the riboprobe used in this experiment, which was derived from the 5′ untranslated and coding regions of a mouse myogenin cDNA.

confluency, and analyzed for the IGFBP-5 antisense mRNA by ribonuclease protection assay. Fig. 10 shows that RNA from each colony protected a 366-nt band derived from the transgene whereas vector-transfected and parental C2 cells did not. Levels of accumulation of transgene mRNA varied among the four stable cell lines.

Antisense cells and controls were induced to differentiate by incubation in medium containing 2% horse serum and analyzed for CK activity. As seen in Fig. 11, all four AS lines showed accelerated differentiation. In two lines, AS3 and AS12, CK activity was measurable by 24 h in differentiation medium. By 48 h, levels were 5 to 25 times higher than in vector-transfected control myoblasts.

Enhanced expression of CK in IGFBP-5 antisense cells was accompanied by elevated levels of MHC (data not shown) and by accelerated myotube formation. As shown in Fig. 12, control cells exhibited a typical fibroblast-like shape at confluence while in growth medium and for the initial 24 h in differentiation medium. Myotubes were first visible by 48 h and increased in size and abundance by 72 h.

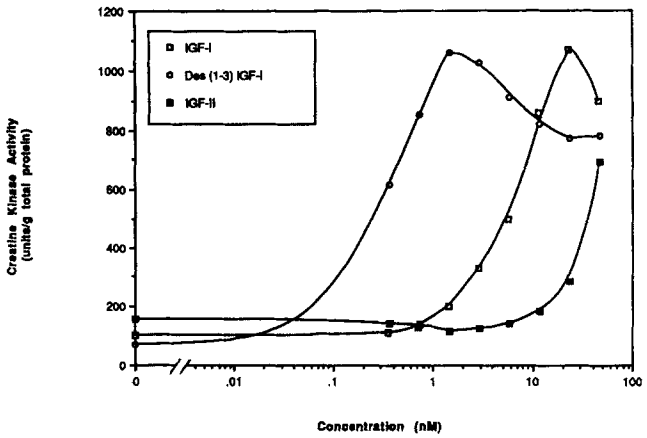


Figure 8. Restoration of creatine kinase activity in S3 myoblasts treated with exogenous IGFs. Dose-response curve for creatine kinase activity in lysates harvested after a 72-h incubation in differentiation medium (DME plus 2% horse serum) which had been supplemented with graded concentrations of IGFs. IGF-II, *filled squares*; IGF-I, *open squares*; des (1-3) IGF-I, *open circles*. Des (1-3) IGF-I is the most potent stimulus of CK activity.

By contrast, myoblast alignment was apparent after 12 h in differentiation medium for AS3 cells, myotubes were seen at 24 h, and extensive myotube formation had occurred by 48 h. Similar results were observed for the other antisense cell lines (data not shown).

Precocious Myogenin Expression Accompanies Enhanced Differentiation of IGFBP-5 Antisense Myoblasts

As noted, myogenin gene expression is induced early during muscle differentiation in vitro, and the protein contributes to the activation of many muscle-specific genes which are expressed later in differentiation (11). In vector-transfected C2 myoblasts, myogenin mRNA increased in abundance by 48 h after incubation in ifferentiation medium (Fig. 13). By contrast, in AS3 and AS12 myoblasts, myogenin transcripts were present as cells approached confluence in growth medium, and mRNA levels remained elevated throughout the time course (Fig. 13, and data not shown). The premature expression of myogenin in IGFBP-5 antisense myoblasts potentially causes the precocious CK activity and MHC expression observed in these cells, and supports the hypothesis that IGFs may regulate muscle differentiation through myogenin.

Discussion

The role of the IGF system in muscle differentiation has been under active investigation for many years (for review see reference 14). Proper functioning of all components of this system is essential for normal muscle development in vivo, since mice lacking the IGF-I receptor exhibit muscle hypoplasia (27). The functions of the IGFs in muscle seem almost contradictory: they stimulate cell cycle progression and induce differentiation, which requires withdrawal from the cell cycle (for review see reference 14). Recent studies using L6E9 myoblasts indicate that in muscle cells which have been removed from serum, IGFs initially are

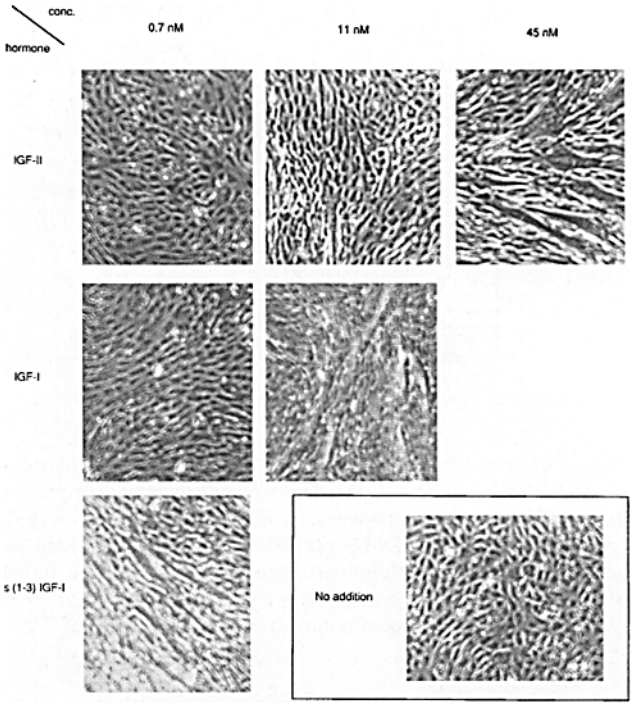


Figure 9. Myotube formation in S3 myoblasts is induced by exogenous IGFs. Phase-contrast photomicrographs of myotube formation after a 72-h incubation with differentiation medium supplemented with the specified concentration of growth factor. "No addition" indicates a 72-h incubation in differentiation medium without exogenous IGFs. The relative potency of the hormones for stimulating CK activity or myotube formation was the same: des (1-3) IGF-I > IGF-I > IGF-II.

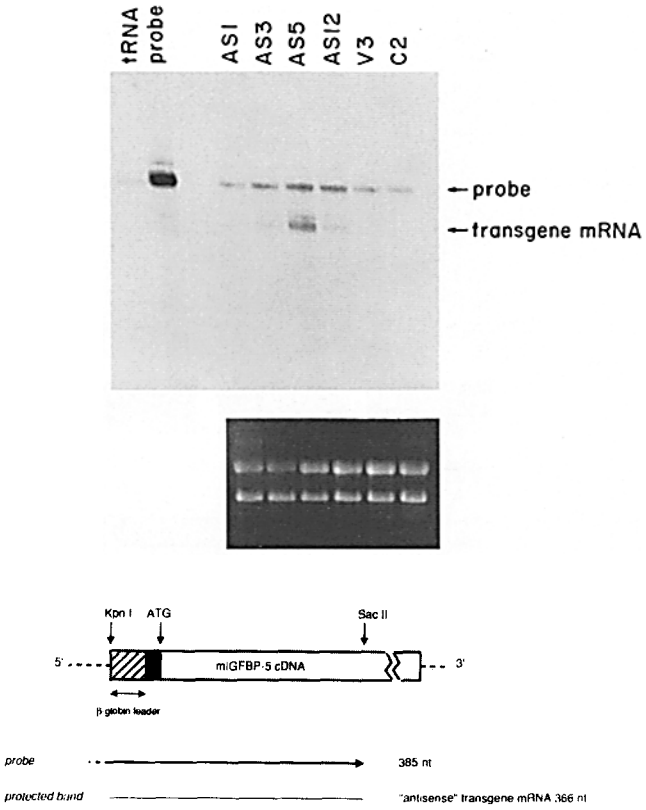


Figure 10. Four independent colonies express the IGFBP-5 antisense (*AS*) transgene mRNA. The top panel is an autoradiograph of a ribonuclease protection experiment using 10 μg of total cellular RNA isolated from cells stably transfected with the IGFBP-5 antisense transgene (*AS1*, *AS3*, *AS5*, and *AS12*) and grown to ~80% confluence in DME containing 20% serum. Total RNA from parental C2 cells (*C2*) or cells transfected with the empty expression vector (*V3*) were included as negative controls. Migration of the protected band and the undigested probe are indicated by the arrows on the right. Autoradiographic exposure was for 24 h at −80°C with two intensifying screens. The middle panel shows an ethidium-bromide stained gel of the samples used in the top panel. The bottom panel is a schematic representation of the riboprobe derived from the transgene which was used in this experiment.

mitogenic, and inhibit myogenic differentiation through a mechanism independent of proliferation; subsequent stimulation of differentiation only occurs during more prolonged (>24 h) incubation (35). It is not known how IGFs mediate these distinct actions, but one mechanism may be through use of alternative signaling pathways which are induced by different levels or different durations of receptor activation. Thus, regulation of IGF concentration near the cell surface could be an important factor in controlling differential effects on myoblast proliferation or differentiation.

One mechanism by which IGF availability can be modulated is through binding to IGFBPs (for review see reference 22). IGFBPs are expressed by several muscle cell lines (12, 21, 29, 37) and it has been reported that exogenous IGFBP-6 can block IGF-stimulated differentiation of L6A1 myoblasts (2). Recently, we demonstrated that IGFBP-5 also can inhibit muscle differentiation, since an IGF-I analogue which cannot bind to IGFBP-5 but binds normally to the IGF-I receptor was more effective than native IGF-I in stimulating creatine kinase activity and promoting myotube formation in C2I myoblasts (37), a cell line which secretes no other IGFBPs and is dependent upon exogenous IGFs for terminal differentiation (33). The involvement of IGFBP-5 in myogenesis in vivo is further suggested by its localization to the myotome and to the muscle of the limb bud during rodent embryogenesis (19). In other myoblast cell lines, where IGFBP-4, -5, and -6 are produced (12), analogous studies have concluded that these proteins generally inhibit IGF-stimulated muscle differentiation (12, 39).

Defining the functions of individual IGFBPs is complicated by several factors. First, many cell lines secrete multiple IGFBPs, which may have either redundant or antagonistic effects. Second, different IGFBPs have been found to positively and negatively modulate IGF-stimulated processes depending on cell type and experimental design (for review see references 3, 22). Third, several IGFBPs have been shown to have effects independent of their IGF ligands (for review see references 3, 22). Fourth, actions of IGFBPs are influenced by their extracellular localization. IGFBP-5 in particular has been identified on the cell surface of fibroblasts, as well as in the extracellular matrix and in cell-conditioned media (5, 24), and exhibits different IGF binding characteristics depending on its location (24). Finally, at least one IGFBP, IGFBP-1, when modi-

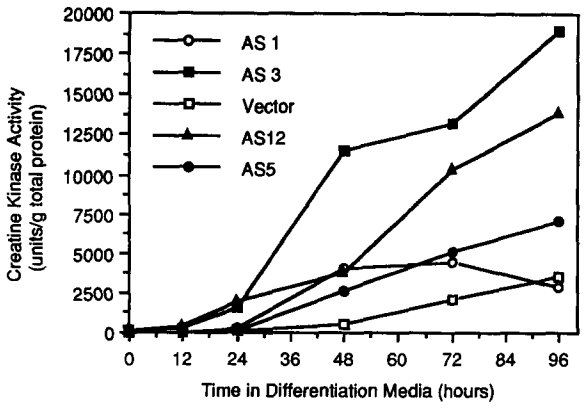


Figure 11. Premature biochemical differentiation by myoblasts expressing the IGFBP-5 antisense transgene. Lysates from cell lines which express the transgene were analyzed for expression of the muscle-specific isoform of creatine kinase, and specific activity was determined relative to total protein concentration. Cells were harvested at 80% confluence, and at 12, 24, 48, 72, and 96 h after incubation with differentiation medium containing 2% horse serum. A time course for vector-transfected cells (*open squares*) was determined as a control. IGFBP-5 AS cells (*filled squares*, *circles*, and *triangles*; *open circles*) show enhanced and early initiation of CK activity compared with control cells.

fied by reversible phosphorylation, undergoes a reduction in its affinity for IGF-I (23). In addition, many studies undertaken to determine the function of different IGFBPs have used recombinant proteins, which do not necessarily undergo normal modification or localization. Taken together, these issues stress the need to identify model systems in which a single IGFBP can be analyzed in the context of its natural environment, in order to determine its functions.

Previously, we have shown that C2 myoblasts only secrete IGFBP-5 during terminal differentiation (21, 41), and thus are a potentially excellent model in which to study the function of this protein. Through stable transfection of an IGFBP-5 cDNA under control of a constitutively active promoter, we now have generated C2 cell lines overexpressing IGFBP-5 while proliferating. These myoblasts replicate normally in growth medium, secrete IGFBP-5 and partition it into the extracellular matrix and culture medium, but fail to differentiate appropriately. Diminished differentiation may be secondary to reduced myogenin expression, with subsequent lack of activation of muscle-specific enzymes and structural proteins. By contrast, C2 cell lines stably transfected with an IGFBP-5 cDNA in the antisense orientation underwent accelerated differentiation, as evidenced by enhanced myogenin gene expression, precocious induction of CK activity, and early formation of myotubes. Based on these complementary results, we conclude that IGFBP-5 blocks differentiation of C2 myoblasts.

How might IGFBP-5 inhibit differentiation? This is clearly an IGF-dependent process, since normal differentiation could be restored by addition of IGFs to IGFBP-5 "sense" cells. The induction of differentiation by IGFs in these myoblasts appears to require saturation of growth factor–binding sites on IGFBP-5, since des (1-3) IGF-I, which does not bind to IGFBP-5 but has normal affinity for the IGF-I receptor, was more effective than IGF-I on a molar basis. It thus appears that one mechanism for controlling IGF availability is through growth factor sequestration, possibly in the extracellular matrix. The steps by which IGFBP-5 is released from the matrix to the medium

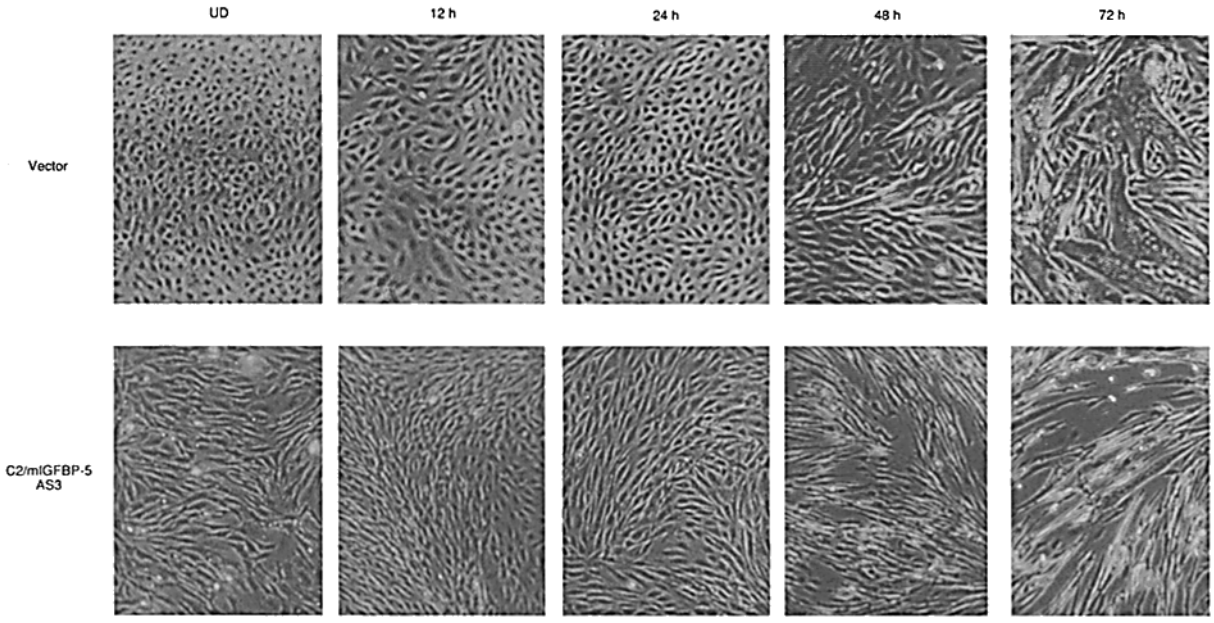


Figure 12. Enhanced morphological differentiation of C2/mIGFBP-5 AS cells. Control cells (vector-transfected, *top panel*) and AS3 myoblasts (*bottom panel*) were incubated in differentiation medium for 72 h. Phase-contrast photomicrographs were taken using a 10× objective, before the incubation in differentiation medium, and at 12, 24, 48, and 72 h during the time course. At 24 h, long, thin myotubes are beginning to appear in the AS3 cultures and by 72 h these myotubes have begun to detach from the plate. Vector cells begin to form myotubes at 48 h and remain viable throughout the time course.

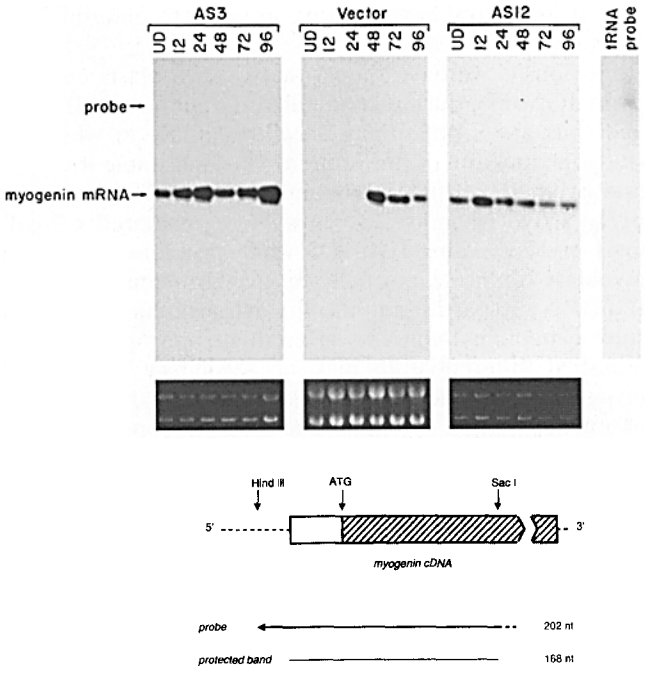


Figure 13. Precocious myogenin mRNA expression in differentiating AS cells. The top panel shows an autoradiograph of a ribonuclease protection experiment using 10 μg of total cellular RNA isolated from myoblasts stably transfected with the empty expression vector (*center*) or the IGFBP-5 antisense plasmid (*left and right*) while undifferentiated (*UD*) and after 12, 24, 48, 72, or 96 h of incubation in differentiation medium. Two independent AS colonies were analyzed (*AS3* and *AS12*). A sample (10 μg) of yeast tRNA was included as a negative control, and an aliquot of undigested probe was added to the gel as a molecular weight marker. The migration of the undigested probe and myogenin mRNA are marked by arrows on the left. All samples were processed in one assay, and were exposed to x-ray film for 14 h at −80°C with two intensifying screens. The middle panel shows ethidium-bromide stained agarose gels of the RNA samples (2 μg, AS3 and AS12; 10 μg, vector) used in this experiment. The bottom panel shows a schematic diagram of the riboprobe, which was derived from 5′ untranslated and coding regions of a mouse myogenin cDNA.

are not known, nor are the mechanisms by which the IGFs dissociate from binding proteins. An IGFBP-5 protease has been identified in fibroblasts (31) and in myoblasts (data not shown), and may be important in these processes. Since we show that C2 cells which express the IGFBP-5 sense transgene exhibit prolonged survival without differentiation in low serum medium, IGFBP-5 in the extracellular matrix may function as a source of sustained low level growth factor release. In other experiments, we have found that endogenously produced IGF-II prevents apoptotic death of C2 cells during the transition from proliferating to differentiating myoblasts (40). It is thus possible that low level secretion of IGFs from a storage pool is responsible for the prolonged survival of C2 cells expressing the IGFBP-5 sense transgene.

In summary, we developed myogenic cell lines that demonstrate key roles for the IGF system in muscle differentiation. C2 cells that prematurely express IGFBP-5 do not differentiate effectively, and have enhanced viability in low serum. By contrast, myoblasts expressing an IGFBP-5 transgene in the antisense orientation differentiate more rapidly than controls. These results are consistent with a model in which IGFBP-5 normally inhibits IGF-stimulated myogenesis, and indicate that sequestration of IGFs in the extracellular matrix could be a possible mechanism of action. Our observations complement results seen with SV40 transformed fibroblasts, in which reduced expression of IGFBP-5 on the cell surface correlates with enhanced IGF-stimulated proliferation (34), and lead to the general conclusion that IGFBP-5 normally blocks IGF action.

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References

1. Annweiler, A., R.A. Hipskind, and T. Wirth. 1991. A strategy for efficient in vitro translation of cDNAs using the rabbit β-globin leader sequence. *Nucleic Acids Res.* 19:3750.
2. Bach, L.A., and M.M. Rechler. 1995. Insulin-like growth factor binding proteins. *Diabetes Rev.* 3:38–60.
3. Bach, L.A., S. Hsieh, A.L. Brown, and M.M. Rechler. 1994. Recombinant human insulin-like growth factor (IGF)-binding protein-6 inhibits IGF-II-induced differentiation of L6A1 myoblasts. *Endocrinology.* 135:2168–2176.
4. Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H.H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:701–709.
5. Camacho-Hubner, C., W.H. Busby, Jr., R.H. McCusker, G. Wright, and D.R. Clemmons. 1992. Identification of the forms of insulin-like growth factor-binding proteins produced by human fibroblasts and the mechanisms that regulate their secretion. *J. Biol. Chem.* 267:11949–11956.
6. Chen, C.A., and H. Okayama. 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques.* 6:632–638.
7. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 24:5294–5299.
8. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
9. Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 51:987–1000.
10. Edmondson, D.G., and E.N. Olson. 1990. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* 4: 1450.
11. Edmondson, D.G., and E.N. Olson. 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. *J. Biol. Chem.* 268:755–758.
12. Ewton, D.Z., and J.R. Florini. 1995. IGF binding proteins-4, -5 and -6 may play specialized roles during L6 myoblast proliferation and differentiation. *J. Endocrinol.* 144:539–553.
13. Florini, J.R., and K.A. Magri. 1989. Effects of growth factors on myogenic differentiation. *Am. J. Physiol.* 256:C701–C711.
14. Florini, J.R., and D.Z. Ewton. 1990. Highly specific inhibition of IGF-I-stimulated differentiation by an antisense oligodeoxyribonucleotide to myogenin mRNA. No effects on other actions of IGF-T. *J. Biol. Chem.* 265:13435–13437.
15. Florini, J.R., D.Z. Ewton, and S.L. Roof. 1991. Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression. *Mol. Endocrinol.* 5:718–724.
16. Florini, J.R., K.A. Magri, D.Z. Ewton, P.L. James, K. Grindstaff, and P.S. Rotwein. 1991. "Spontaneous" differentiation of skeletal myoblasts is de-

pendent upon autocrine secretion of insulin-like growth factor-II. *J. Biol. Chem.* 266:15917–15923.
17. Florini, J.R., D.Z. Ewton, and F.J. McWade. 1995. IGFs, muscle growth, and myogenesis. *Diabetes Rev.* 3:73–92.
18. Gonzalez-Sanchez, A., and D. Bader. 1984. Immunochemical analysis of myosin heavy chains in the developing chicken heart. *Dev. Biol.* 103:151–158.
19. Green, B.N., S.B. Jones, R.D. Streck, T.L. Wood, P. Rotwein, and J.E. Pintar. 1994. Distinct expression patterns of insulin-like growth factor binding proteins 2 and 5 during fetal and postnatal development. *Endocrinology.* 134:954–962.
20. Hossenlopp, P., D. Seurin, B. Segovia Quinson, and M. Binoux. 1986. Identification of an insulin-like growth factor-binding protein in human cerebrospinal fluid with a selective affinity for IGF-II. *FEBS Lett.* 208:439–444.
21. James, P.L., S.B. Jones, W.H. Busby, Jr., D.R. Clemmons, and P. Rotwein. 1993. A highly conserved insulin-like growth factor-binding protein (IGFBP-5) is expressed during myoblast differentiation. *J. Biol. Chem.* 268:22305–22312.
22. Jones, J.I., and D.R. Clemmons. 1995. Insulin-like growth factors and their binding proteins - biological actions. *Endocrine Rev.* 16:3–34.
23. Jones, J.I., A.J. D'Ercole, C. Camacho Hubner, and D.R. Clemmons. 1991. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I. *Proc. Natl. Acad. Sci. USA.* 88:7481–7485.
24. Jones, J.I., A. Gockerman, W.H. Busby, Jr., C. Camacho-Hubner, and D.R. Clemmons. 1993. Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. *J. Cell Biol.* 121: 679–687.
25. Knudsen, B.S., P.C. Harpel, and R.L. Nachman. 1987. Plasminogen activator inhibitor is associated with the extracellular matrix of cultured bovine smooth muscle cells. *J. Clin. Invest.* 80:1082–1089.
26. Lassar, A.B., S.X. Skapek, and B. Novitch. 1994. Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr. Opin. Cell Biol.* 6:788–794.
27. Liu, J.-P., J. Baker, A.S. Perkins, E.J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (*Igf-1*) and type 1 IGF receptor (*Igf1r*). *Cell.* 75:59–72.
28. Mangiacapra, F.J., S.L. Roof, D.Z. Ewton, and J.R. Florini. 1992. Paradoxical decrease in myf-5 messenger RNA levels during induction of myogenic differentiation by insulin-like growth factors. *Mol. Endocrinol.* 6: 2038–2044.
29. McCusker, R.H., and D.R. Clemmons. 1988. Insulin-like growth factor binding protein secretion by muscle cells: effect of cellular differentiation and proliferation. *J. Cell. Physiol.* 137:505–512.
30. Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.
31. Nam, T.J., W.H. Busby, Jr., and D.R. Clemmons. 1994. Human fibroblasts secrete a serine protease that cleaves insulin-like growth factor-binding protein-5. *Endocrinology.* 135:1385–1391.
32. Olson, E.N. 1993. Signal transduction pathways that regulate skeletal muscle gene expression. *Mol. Endocrinol.* 7:1369–1378.
33. Pinset, C., D. Montarras, J. Chenevert, A. Minty, P. Barton, C. Laurent, and F. Gros. 1988. Control of myogenesis in the mouse myogenic C2 cell line by medium composition and by insulin: characterization of permissive and inducible C2 myoblasts. *Differentiation.* 38:28–34.
34. Reeve, J.G., A. Guadano, J. Xiong, J. Morgan, and N.M. Bleehen. 1995. Diminished expression of insulin-like growth factor (IGF) binding protein-5 and activation of IGF-I-mediated autocrine growth in simian virus 40-transformed human fibroblasts. *J. Biol. Chem.* 270:135–142.
35. Rosenthal, S.M., and Z.-Q. Cheng. 1995. Opposing early and late effects of insulin-like growth factor I on differentiation and the cell cycle regulatory retinoblastoma protein in skeletal myoblasts. *Proc. Natl. Acad. Sci. USA.* 92:10307–10311.
36. Rotwein, P., S.K. Burgess, J.D. Milbrandt, and J.E. Krause. 1988. Differential expression of insulin-like growth factor genes in rat central nervous system. *Proc. Natl. Acad. Sci. USA.* 85:265–269.
37. Rotwein, P., P.L. James, and K. Kou. 1995. Rapid activation of insulin-like growth factor binding protein-5 gene transcription during myoblast differentiation. *Mol. Endocrinol.* 9:913–923.
38. Sambrook, J., E.F. Fritsch, and T. Maniatis, editors. 1989. Plasmid vectors. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1.3–1.110.
39. Silverman, L.A., Z.Q. Cheng, D. Hsiao, and S.M. Rosenthal. 1995. Skeletal muscle cell-derived insulin-like growth factor (IGF) binding proteins inhibit IGF-I-induced myogenesis in rat L6E9 cells. *Endocrinology.* 136: 720–726.
40. Stewart, C.E.H., and P. Rotwein. 1996. Insulin-like growth factor-II is an autocrine survival factor for differentiating myoblasts. *J. Biol. Chem.* In press.
41. Tollefsen, S.E., R. Lajara, R.H. McCusker, D.R. Clemmons, and P. Rotwein. 1989. Insulin-like growth factors (IGF) in muscle development. Expression of IGF-I, the IGF-I receptor, and an IGF binding protein during myoblast differentiation. *J. Biol. Chem.* 264:13810–13817.