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A Highly Conserved Insulin-like Growth Factor-binding Protein (IGFBP-5) Is Expressed during Myoblast Differentiation*

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Insulin-like growth factor-binding proteins (IGFBPs) are a family of secreted proteins that bind insulin-like growth factors I and II (IGFs I and II) and are capable of modulating IGF actions on target cells. We have shown previously that C2 myoblasts secrete a single ~29-kDa IGFBP during their terminal differentiation (Tollefsen, S. E., Lajara, R., McCusker, R. H., Clemmons, D. R., and Rotwein, P. (1989) *J. Biol. Chem.* 264, 13810–13817). In this study, we have purified the protein from C2 cell-conditioned media by conventional and IGF-affinity chromatography, cloned its cDNA by PCR-based and traditional library screening, and identified it as mouse IGFBP-5. The resultant 5561 nucleotide cDNA encodes a 252-amino acid mature protein (predicted M_r ~28,400) that is 97% identical to rat and human IGFBP-5. In differentiating C2 myoblasts and in F3 azamyoblasts the >6-kilobase IGFBP-5 mRNA accumulates concomitantly with induction of myogenin mRNA, an early marker of muscle differentiation. Ligand blot analysis shows that IGFBP-5 protein is secreted within 12 h of the onset of differentiation in these cells and that it is the only IGFBP produced in several fusing skeletal muscle cell lines. *In vivo*, IGFBP-5 transcripts are expressed in a variety of mouse tissues including striated muscle, but, unlike other IGFBPs, it is barely detectable in liver. IGFBP-5 is more conserved than other IGFBPs in mammals; its conserved structure and sequence also extends to non-mammalian vertebrates. Hybridization of a mouse BP5 coding region probe to RNA from several chicken and *Xenopus* tissues demonstrated similarly sized transcripts in these species. A partial *Xenopus* cDNA is identical in 38/45 deduced amino acids to the mammalian proteins. Identification of an IGFBP that is produced during myoblast differentiation provides a model system in which to study the potential modulatory role of IGFBPs in development.

Insulin-like growth factors I and II (IGF I and II)[1] comprise a related pair of circulating proteins with pleiotropic effects on diverse cell types (reviewed in Refs. 1 and 2). Actions of both IGFs are mediated by interaction with the IGF I receptor, a ligand-activated tyrosine protein kinase that is structurally similar to the insulin receptor (3). IGF II also binds with high affinity to the IGF II receptor. This protein, which is also known as the cation-independent mannose-6-phosphate receptor, plays a major role in lysosomal enzyme targeting, although its function in modulating actions of IGF II remains controversial (4). IGFs also can bind to a family of secreted proteins, termed insulin-like growth factor-binding proteins (IGFBPs), that are capable of enhancing or inhibiting IGF action in cultured cells (for review, see Ref. 5).

The six IGFBPs that have been identified in mammals have a conserved structure that is distinct from the two IGF receptors. IGFBPs all contain clusters of similarly placed cysteine residues that are located in the NH_2 and COOH thirds of the proteins. Different IGFBPs bind IGF I and II with differing affinities, and binding affinity can be altered by one or more post-translational modifications, including phosphorylation (6, 7) and proteolytic cleavage (8, 9). Several IGFBPs are found in cell surface-associated (5, 10) as well as secreted forms.

Previously, we have shown that expression of IGF II and its receptor increase during C2 myoblast differentiation (11–13) and have demonstrated through use of antisense oligonucleotides that IGF II functions in an autocrine or paracrine manner in promoting C2 cell differentiation (14). In the course of these studies, we identified an ~29-kDa IGFBP that also was secreted during C2 myoblast differentiation (11). Since IGFBPs have been shown to both enhance and inhibit IGF actions (5), the expression of an IGFBP during terminal differentiation could provide a mechanism for modulating IGF action in this system. In this study, we have purified this IGFBP, cloned its cDNA, and identified it as the mouse homologue of IGFBP-5. IGFBP-5 mRNA and protein are expressed in C2 cells and in F3 azamyoblasts *in vitro*, and its mRNA is synthesized in many tissues *in vivo*, including striated muscle. We also demonstrate that IGFBP-5 mRNA

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L12447.

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; UTR, untranslated region; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); BP, binding protein.

is found in tissues of non-mammalian vertebrates and show, by comparative protein analysis, that IGFBP-5 is the most conserved IGF-binding protein identified to date.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture supplies (fetal bovine serum, newborn calf serum, horse serum, Dulbecco's modified Eagle's medium) were purchased from Life Technologies, Inc. Restriction endonucleases and other enzymes (Taq polymerase, Sequenase, SP6 RNA polymerase, Moloney murine leukemia virus reverse transcriptase) were obtained from various manufacturers (American Allied Biochemical, Life Technologies, Inc., Perkin-Elmer Cetus Instruments, U. S. Biochemical Corp., New England Biolabs, Promega-Biotec). Radionuclides were purchased from Du Pont NEN or Amersham Corp.

TABLE I
Purification of a mouse IGFBP from C2 cell-conditioned media

Purification step	Estimated[a] total protein	Total activity[b]
	mg	*units*
C2 cell-conditioned media	1,470[c]	2,685
Ammonium sulfate fractionation	1,250	1,880
Phenyl-Sepharose chromatography	275	1,683
Sephadex G-150 chromatography	17.9	4,548
IGFI affinity chromatography	0.34	42,140
C-4 HPLC	0.061	36,500

[a] Protein recovery was quantitated by either direct protein assay or absorbance at 214 or 280 nM.

[b] One unit of activity is defined as the amount necessary to achieve half-maximal binding in an IGF binding assay.

[c] Estimated based on approximately 1.4 mg/ml protein in 2% horse serum.

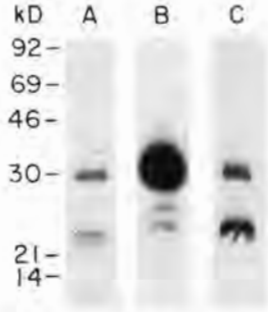

FIG. 1. **Analysis of purified C2 cell IGF-binding protein.** HPLC fractions were subjected to 12.5% PAGE and either silver stained (*A*), or transferred to a nitrocellulose filter and incubated with ^{125}I-labeled IGF I (*B*), or a 1:1000 dilution of an antiserum raised against human IGFBP-5 (*C*). The ligand and immunoblot experiments were performed on different lanes from the same gel, whereas analogous fractions from a different HPLC fractionation were silver-stained.

Cell Cultures—Mouse C2 myoblasts (15) and F3 azamyoblast clone b (16) were plated at 5×10^3 cells/ml on gelatin-coated 15-cm diameter tissue culture dishes and grown in medium containing 10% fetal bovine serum and 10% newborn calf serum. Upon attaining ~80% of confluent density, cells were washed with Earle's balanced salt solution, and the media were replaced with a differentiation medium containing Dulbecco's modified Eagle's medium plus 2% horse serum. Differentiation medium conditioned for 48–72 h (1050 ml) was collected and used for protein purification. Cells used for RNA isolation were harvested at ~80% of confluent density and after 2, 4, 12, 24, 48 or 72 h of exposure to differentiation medium. At each time point, medium conditioned by parallel cultures was collected, and the cultures refed. All medium samples were clarified by low speed centrifugation and stored at −20 °C until use.

Protein Purification—Material from the supernatant after addition of ammonium sulfate to 25% (w/v) was collected and precipitated by bringing the NH_4SO_3 concentration to 75%, followed by centrifugation. The pellet was redisolved in 50 mM Tris-HCl, pH 7.4, 10% ammonium sulfate (w/v). The sample was applied twice to a phenyl-Sepharose column (3 cm × 4.4 cm), which had been equilibrated in the same buffer. Fractions that eluted in 0.02 M Tris-HCl, pH 9.0, contained IGF binding activity when assayed by polyethylene glycol precipitation (17). These fractions were lyophilized, extracted with 0.5 M acetic acid, pooled, and applied to a G-150 Sephadex column equilibrated with 0.5 M acetic acid. Active fractions determined by IGF binding assay as above were lyophilized, extracted with 0.05 M Tris-HCl, pH 6.4, pooled, and applied to an IGF I affinity column equilibrated with the extraction buffer. Active fractions eluted in 0.5 M acetic acid were assayed by polyethylene glycol precipitation assay and chromatographed through a C-4 reverse phase HPLC column (4.6 mm × 2.5 cm) (Vydac, Hesperion, CA) using an acetonitrile gradient. Two incompletely separated peaks eluted at ~26% acetonitrile. This fraction contained two bands on a silver-stained 12.5% PAGE (see Fig. 1*A*). Samples from each band were subjected to direct amino acid sequencing and to digestion with trypsin followed by sequencing (18). Both protein bands demonstrated the same limited amino acid sequences.

Ligand and Western Blotting—HPLC fractions or conditioned medium were electrophoresed through 12.5% PAGE under nonreducing conditions. Samples were transferred to nitrocellulose using a semi-dry electroblotter as described by Hossenlopp *et al.* (19). For ligand blot, filters were hybridized with 650,000 cpm (150,000 cpm/ml) of ^{125}I-labeled IGF I and visualized by autoradiography. For Western blot, filters were probed with guinea pig antiserum raised against fragments of human IGFBP-5 as described elsewhere (18), followed by incubation with alkaline phosphatase-conjugated rabbit anti-guinea pig IgG. Bands were visualized using the Protoblot system immunoblotting reagents per manufacturer's direction (Promega Biotec).

Mouse IGFBP-5: cDNA Cloning and Sequencing—Initial cDNA clones were obtained by PCR amplification of cDNA that was reverse transcribed from differentiated C2 cell mRNA by using mixed oligonucleotide primers that were designed based on amino acid sequences obtained from the purified protein (see Fig. 3). Additional clones were isolated by 3′ rapid amplification of cDNA ends (20), using C2 cell cDNA as the template. DNA sequences obtained from these clones enabled the design of specific oligonucleotide primers, which were used to amplify a cDNA from an F3 azamyoblast library in λgt10 (a

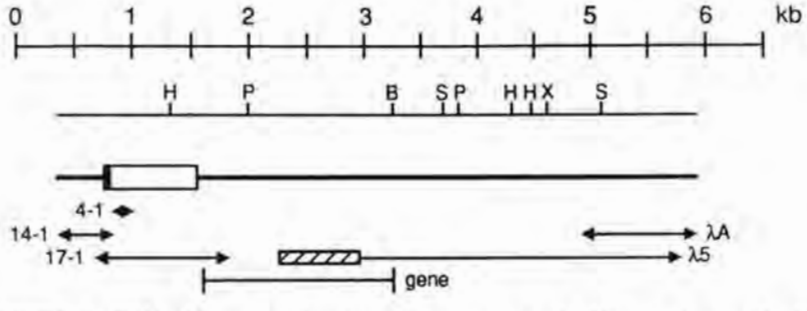

FIG. 2. **Map of mouse IGFBP-5 cDNA clones.** Overlapping clones spanning ~5.5 kb were obtained by PCR-based and conventional screening of cDNAs synthesized from F3 azamyoblast or C2 myoblast mRNA (see "Experimental Procedures"). Restriction sites for the following enzymes are indicated: *Hin*dIII (*H*), *Pst*I (*P*), *Bam*HI (*B*), and *Xho*I (*X*). The signal peptide and mature protein are denoted by the *shaded* and *open boxes*, respectively. Individual cDNAs are indicated *below* the *map*. The hatched box denotes a 739-nucleotide inversion in λ5. The region that was sequenced from a genomic clone is indicated.

FIG. 3. **cDNA sequence of mouse IGFBP-5.** cDNA and genomic clones corresponding to 5561 base pairs were sequenced on both strands. Two in-frame stop codons (*underlined*) preceding the first ATG were found in the 5′-UTR. The 5′- and 3′-UTR are shown in *lower case letters*, and the coding region is shown in *upper case letters*. The first amino acid of the mature protein is indicated by an *arrow*. The 18 cysteines conserved among most IGFBPs are shown in *italicized type*. Amino acids corresponding to NH_2-terminal and tryptic peptide sequences are *underlined*. The stop codon is indicated by *asterisks*. Direct repeats in the 3′-UTR are denoted by *double underlines*.

gift of Dr. Harold Weintraub) (21). This clone was then used to screen the F3 library by standard methods (22) but was subsequently shown to have ~3.2-kb PCR-induced deletion. The cDNA screening resulted in the identification of two sets of nonoverlapping clones, which were purified by successive rounds of hybridization. The gap between these two groups of clones could not be amplified by PCR, hence the region surrounding and containing this gap was sequenced from the mouse IGFBP-5 gene. Comparison of the nucleotide sequences of the gene and cDNAs revealed a 739-nucleotide inversion at the 5′ end of one of the cDNA clones, λ5. The correct orientation of this region was determined by ribonuclease protection assay (23), using single-stranded cRNA probes (24) derived from cDNAs and genomic fragments. The same approach was used to establish colinearity of the mRNA and the gene in the region of the gap described above. To obtain further 3′ sequence, the library was rescreened with the last 1.2 kb of λ5 (see Fig. 2). Six additional cDNA clones were purified. cDNA and genomic fragments were subcloned into plasmid vectors and were sequenced on both strands by the dideoxy chain-termination method (25) with Sequenase, using vector-specific or cDNA-specific oligonucleotide primers. DNA sequence information was compiled and analyzed using Geneworks (Intelligenetics) and DNA Strider 1.2 (Christian Marck, France).

Xenopus IGFBP-5 cDNA Cloning—cDNA was synthesized from *Xenopus* skeletal muscle mRNA using Mouse murine leukemia virus reverse transcriptase (22). Subsequently, a fragment corresponding to the NH_2 terminus of the protein was amplified by PCR using primers from the mouse IGFBP-5 cDNA.

Computer-assisted Amino Acid Analysis—Amino acid sequences of

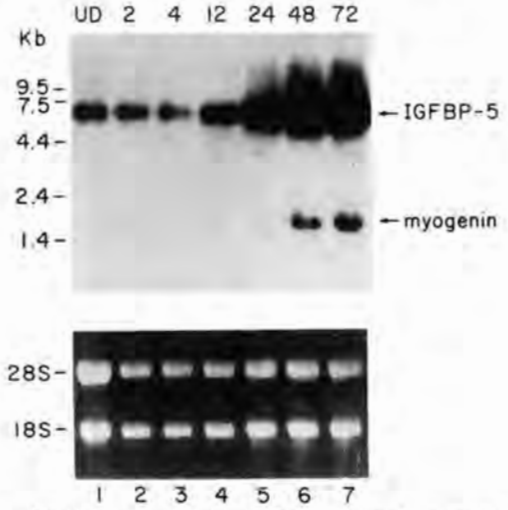

FIG. 4. **Northern analysis of RNA from C2 myoblasts.** C2 cells were grown to ~80% confluence and induced to differentiate. Total RNA was isolated from undifferentiated (*UD*) cultures or at various time points after exposure to differentiation medium. Total RNA (5 μg) was hybridized concurrently to ^{32}P-labeled DNA probes for mouse IGFBP-5 and myogenin (28). Filters were exposed to Kodak XAR5 x-ray film for 21 h at −80 °C with two intensifying screens. The *lower panel* shows an ethidium bromide-stained agarose gel of the same samples. The pictured experiment is representative of three independent studies.

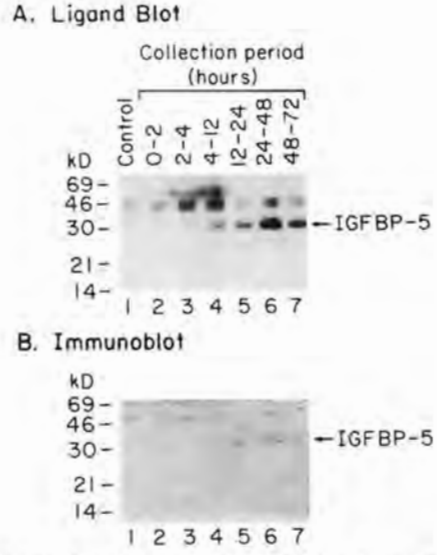

FIG. 5. **Ligand and immunoblot of C2 cell-conditioned media.** Media from cultures grown in parallel to those examined in Fig. 4 were collected at the specified intervals after initiation of differentiation. Samples were analyzed by ligand blot using ^{125}I-labeled IGF I (*A*) or by Western blot using polyclonal antiserum raised against human IGFBP-5 (*B*). The control lane represents unconditioned media (Dulbecco's modified Eagle's medium plus 2% horse serum). These experiments were performed in duplicate with essentially identical results.

rat and human IGFBP-5 and other IGFBPs were obtained from Genbank. Alignments and evolutionary relationships among different IGFBPs were established using Geneworks.

RNA Isolation and Analysis—Total RNA was extracted from cell pellets or animal tissues using a modified guanidinium thiocyanate method (26, 27) and quantitated by spectrophotometry. Samples for Northern blot analysis were electrophoresed through 1% agarose, 2.2 M formaldehyde gels and stained with ethidium bromide to determine their integrity. The RNA was UV cross-linked (Stratalinker-Stratagene) following transfer to supported nitrocellulose by capillary action. Myogenin (28) and IGFBP-5 DNA probes were labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP using the random primer method (29). Hybridization was performed at 42 °C in the presence of 50% formamide (30). cRNA probes were synthesized by *in vitro* transcription using SP6 RNA polymerase in the presence of [α-^{32}P]CTP (24). Hybridization occurred at 55 °C in solution containing 50% formamide (24). After hybridization, filters were washed in solution containing 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, and 0.1% SDS first for 15 min at 25 °C and then for 60 min at 68 °C (mouse) or 55 °C (chicken and *Xenopus*). Filters were exposed to x-ray film (Kodak XAR5) using two intensifying screens. Signal intensities on the filters were quantified using a β emission scanner (Betagen).

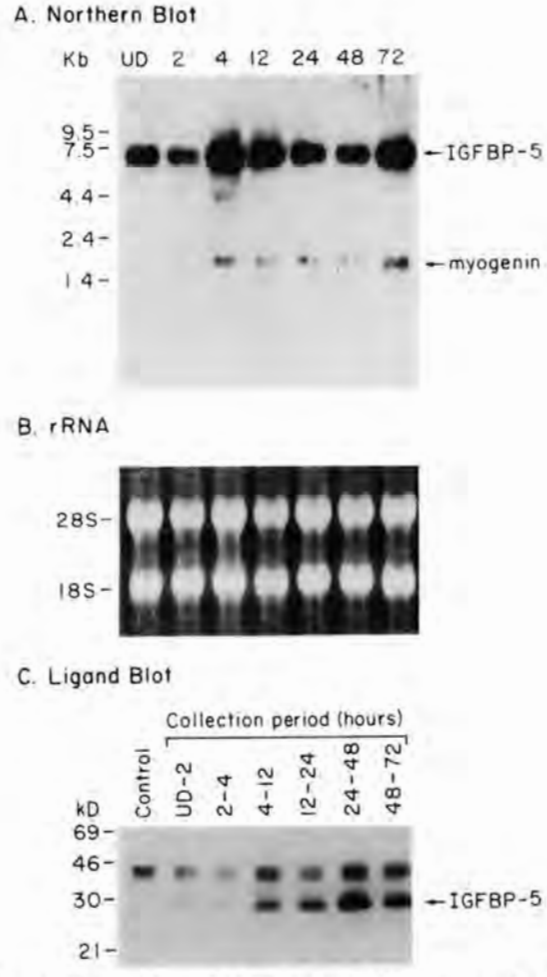

FIG. 6. **Expression of IGFBP-5 mRNA and protein during F3 azamyoblast differentiation.** F3 azamyoblasts were grown to ~80% confluence and induced to differentiate. Total RNA was isolated from undifferentiated (*UD*) cultures or at various time points after exposure to differentiation medium. Media were collected at the same time points from parallel cultures. *A*, total RNA (5 μg) was hybridized concurrently to ^{32}P-labeled DNA probes for mouse IGFBP-5 and myogenin (28). Filters were exposed to Kodak XAR5 x-ray film for 21 h at −80 °C with two intensifying screens. *B*, ethidium bromide-stained agarose gel prior to transfer. *C*, ^{125}I-labeled IGF I ligand blot of cell-conditioned media. The control lane contains unconditioned differentiation medium. These experiments were performed in duplicate with essentially identical results.

RESULTS

Purification of an IGFBP from C2 Cell-conditioned Media—Confluent cultures of C2 cells were induced to differentiate in medium containing 2% horse serum for 48–72 h. Conditioned culture medium was collected, fractionated, and chromatographed as indicated under "Experimental Procedures" and as summarized in Table I. Purification was monitored by assessing IGF binding activity and resulted in the recovery of

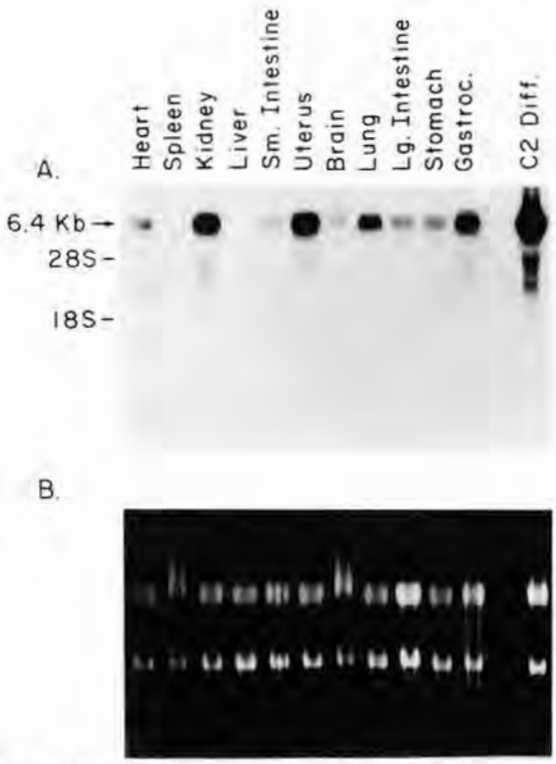

FIG. 7. **Northern analysis of IGFBP-5 mRNA in adult mouse tissues.** Total RNA (5 μg/lane) was hybridized to a ^{32}P-labeled cRNA probe from IGFBP-5 clone 4–1 (*upper panel*). The >6.0-kb IGFBP-5 transcript is indicated with an *arrow*. C2 cell mRNA was used as a positive control. The filter was exposed to Kodak XAR5 x-ray film for 20 h at −80 °C using two intensifying screens. The *lower panel* shows the ethidium bromide-stained gel prior to transfer.

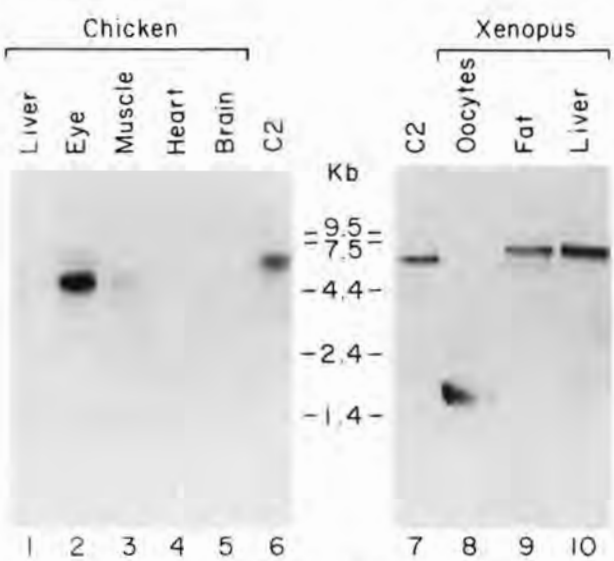

FIG. 8. **Northern analysis of IGFBP-5 mRNA in chicken and *Xenopus* tissues.** Total RNA was isolated from tissues of 7-week post-hatch chicks and from adult frogs. Chicken (10 μg) and *Xenopus* (~25 μg) samples were hybridized to a ^{32}P-labeled cRNA probe from mouse IGFBP-5 clone 4–1. C2 cell RNA (5 μg) was included as a control. Filters were exposed to Kodak XAR5 x-ray film at −80 °C using two intensifying screens for 20 h (chicken) or 2 h (*Xenopus*).

~61 μg of protein. The purified protein was visualized as two silver-stained bands of M_r = 29,000 and 23,000 after electrophoresis through a 12.5% nondenaturing polyacrylamide gel (Fig. 1*A*). The band of higher M_r could bind IGF II with higher affinity than the band of lower molecular mass as estimated by Western ligand blot (Fig. 1*B*), although both proteins were recognized equivalently by polyclonal antiserum raised against fragments of human IGFBP-5 (Fig 1*C*). The NH_2-terminal 20 amino acids obtained from each of these proteins were identical, indicating that the 23-kDa band was truncated at its COOH terminus.

cDNA Cloning—At the time that the C2 cell IGF-binding protein was purified, the sequences of only three IGFBPs were known. We therefore used protein sequence information to design mixed oligonucleotide primers and isolated a partial length cDNA by reverse transcription-PCR (31), using mRNA isolated from differentiated C2 cells. The derived protein sequence of this 190-nucleotide cDNA contained several peptides that matched those obtained from the purified protein. Additional cDNAs spanning 5.5 kb of the >6.0-kb mRNA were cloned by both PCR-based and traditional screening methods from a cDNA library made from differentiating F3 azamyoblasts (21) and from C2 cell RNA, as described under "Experimental Procedures." An uncloned gap in the 3′ UTR was bridged by isolating and characterizing a fragment of the mouse gene (Fig. 2). The genomic sequences corresponding to this region of the 3′ UTR were shown to be contiguous in the mRNA by ribonuclease protection assay.[2]

DNA Sequence Analysis—The assembled cDNA sequence is presented in Fig. 3 and consists of 5561 nucleotides, including a 5′ UTR of 387 base pairs, an 816-nucleotide coding region, and a 3′ UTR of 4358 base pairs. An open reading frame of 271 codons begins with the first ATG in the cDNA and is preceded by two in-frame stop codons. The open reading frame encodes a 19-amino acid signal peptide followed by a 252-residue mature protein with a predicted molecular mass of 28.4 kDa, in close agreement with the size of the protein as estimated by SDS-PAGE. There was perfect correlation between the amino acid sequences deduced from the cDNA and the peptides, except at Ser[179], which had been tentatively identified as a tryptophan residue. The deduced amino acid sequence of the mature protein is 97% identical to rat and human IGFBP-5. The protein contains 18 cysteines, which are conserved among five of six IGFBPs. Unlike other IGFBPs, the IGFBP-5 mRNA has a very large 3′ UTR and a long >700-nucleotide 5′ UTR.[3] The 3′ UTR contains a single direct repeat with 21/22 identities (Fig. 3), has several potential stem loops,[2] and has heterogeneous 3′ ends.[3] Of the 30 potential open reading frames encoded by both strands of this cDNA, the authentic open reading frame is the largest.

Expression of IGFBP-5 during Myogenic Differentiation—IGFBP-5 expression is induced during muscle differentiation *in vitro* (Figs. 4, 5, and 6). As seen in Fig. 4, IGFBP-5 mRNA is detectable in proliferating C2 cells, but steady-state levels increase during terminal differentiation. A rise in mRNA is first observed 12 h after exposure to differentiation medium (*lane 4*), and by 48 h (*lane 6*), a 10–15-fold accumulation has occurred. As seen in Fig. 4, the increase in IGFBP-5 mRNA paralleled the rise in myogenin, a myogenic regulatory transcription factor whose mRNA is up-regulated during muscle differentiation *in vitro* (32).

Studies of IGFBP-5 protein expression revealed a differentiation-dependent rise nearly identical to its mRNA (Fig. 5). Analysis of conditioned culture media showed the appearance of a 29-kDa band by ligand blot during the interval from 4 to 12 h after initiation of differentiation (*lane 4*). Accumulation of this protein peaked between 24 and 48 h. Western

[2] P. L. James and P. Rotwein, unpublished observations.
[3] Kou Kou, P. L. James, and P. Rotwein, unpublished observations.

A

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Mouse     LGSFVHCEPCDEKALSMCPPSPLGC ELVKEPGCGCCMTCALAEGQSCGVY TERCAQGLRCLPRQDEEKPLHALLH   75
Rat       ......................... ......................... .........................
Human     ......................... ......................... .........................
Xenopus         ..D.V.............. .......HR......H..K...... E

Mouse     GRGVCLNEKSYGEQTKIERDSREHE EPTTSEMAEETYSPKVFRPKHTRIS ELKAEAVKKDRRKKLTQSKFVGGAE   150
Rat       ......................... ......................... .........................
Human     ..........R..V.......... ..............I.......... .........................
Xenopus

Mouse     NTAHPRVIPAPEMRQESEQGPCRRH MEASLQEFKASPRMVPRAVYLPNCD RKGFYKRKQCKPSRGRKRGICWCVD   225
Rat       ................D........ ......................... .........................
Human     ......I.S.............. .......L.................. .........................
Xenopus

Mouse     KYGMKLPGMEYVDGDFQCHAFDSSN VE    252
Rat       ......................... ..
Human     ...................T..... ..
Xenopus
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B

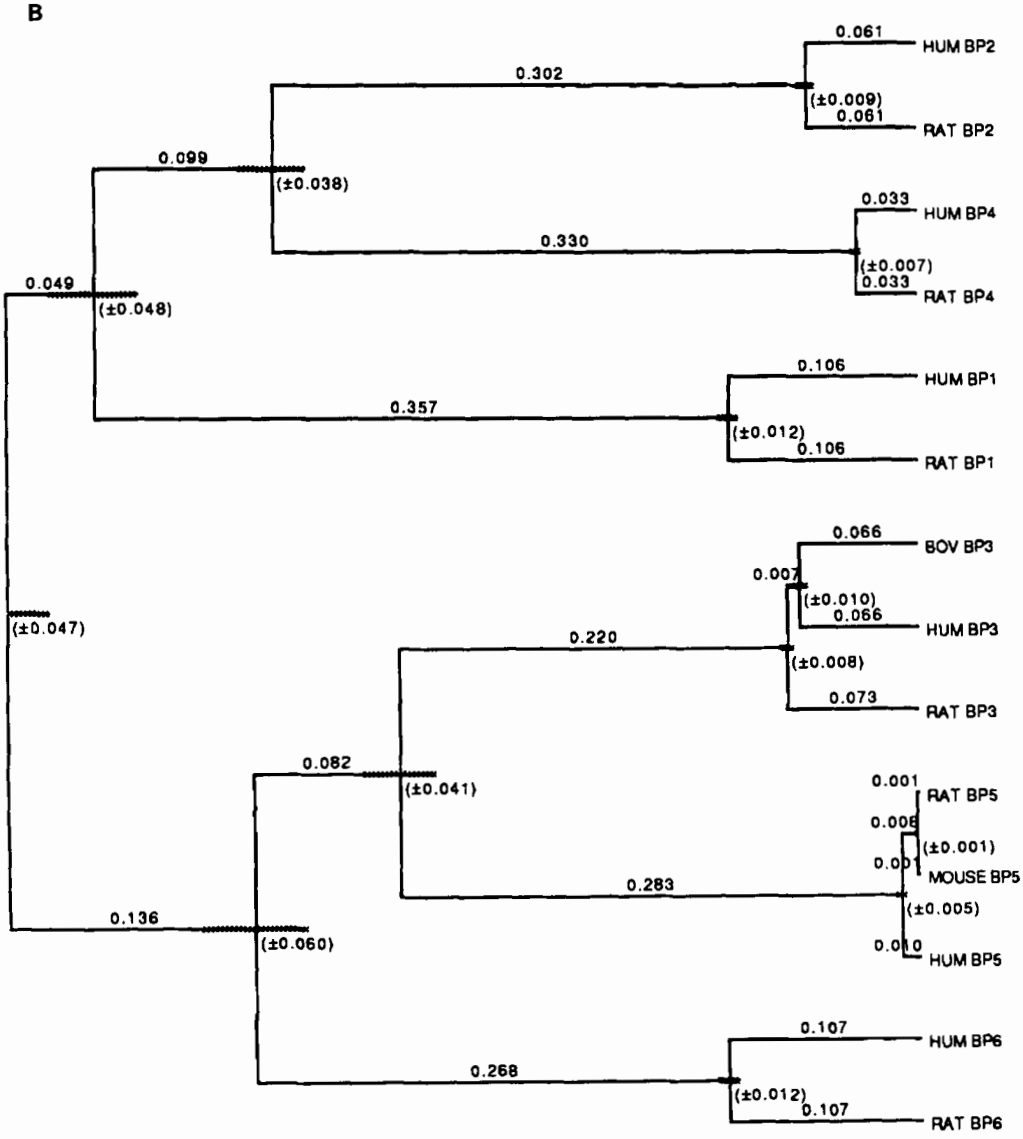

FIG. 9. **Analysis of IGFBP-5 amino acid sequences.** *A*, sequence comparison of IGFBP-5 among vertebrate species. The deduced protein sequences for mouse, rat, human, and *Xenopus* (partial) IGFBP5 were aligned using Geneworks. *B*, evolutionary relationship among IGF-binding proteins. The full-length amino acid sequences of IGFBPs were extracted from Genbank and analyzed using the tree subroutine

blot analysis of the same filter using anti-IGFBP-5 antiserum also detected a 29-kDa band with a similar time course of appearance, although this method was less sensitive than ligand blotting. As indicated by the results shown in Fig. 5*A*, no other IGFBPs accumulated in the media during C2 myoblast differentiation.

IGFBP-5 also is produced by F3 azamyoblasts (Fig. 6), a cell line derived by myogenic conversion of fetal fibroblasts (10T1/2 cell line) by 5-azacytidine treatment (16). Both BP5 and myogenin transcripts are more highly expressed in proliferating azamyoblasts than in C2 cells (compare Figs. 4*A* and 6*A*), and the subsequent rise in mRNA abundance is lower, ~3-fold. IGFBP-5 protein secretion increased rapidly during azamyoblast differentiation; ligand blot analysis demonstrates the appearance of an ~29-kDa band within 2 h of the onset of differentiation (Fig. 6*C*), with peak accumulation between 24 to 48 h. RNA analyses and protein studies also demonstrated induction of IGFBP-5 expression during the terminal differentiation of several other fusing rodent muscle cell lines.[2]

Expression of IGFBP-5 in the Mouse Tissues—To determine potential sites of synthesis of IGFBP-5 in the mouse, total RNA from various adult tissues was extracted and analyzed by Northern blot. As seen in Fig. 7, the >6.0-kb IGFBP-5 mRNA is highly expressed in the kidney, uterus, and gastrocnemius muscle and is present at lower levels in several other tissues. Very little BP5 mRNA was seen in liver, a major site of synthesis of other IGFBPs (33), or in spleen.

IGFBP-5 mRNA Is Expressed by Non-mammalian Vertebrates—Because comparative protein sequence analysis suggested that IGFBP-5 was highly conserved in mammals (see Refs. 34 and 35 and Fig. 9*B*), we asked if this BP was present in other vertebrate species. RNA studies using a probe derived from the NH_2-terminal coding region of mouse IGFBP-5 identified similarly sized transcripts in chicken and *Xenopus* tissues (Fig. 8). A partial length cDNA was then cloned from *Xenopus* skeletal muscle RNA by PCR using oligonucleotide primers derived from mouse IGFBP-5. Comparison of the deduced amino acid sequence of this cDNA with mouse, rat, and human IGFBP-5 revealed 38/45 identical residues (Fig. 9*A*). Additionally, as shown in Fig. 9*B*, IGFBP-5 proteins are more conserved than are any other IGFBPs identified to date. Mouse, rat, and human IGFBP-5 are 97% identical, whereas amino acid sequence identity of the rat and human homologues of other BPs range from only 66 to 91%. Fig. 9*B* also indicates that IGFBP-5 is more similar to IGFBP-3 and -6 than to the other binding proteins.

DISCUSSION

This study shows that the predominant IGF-binding protein secreted by differentiating C2 myoblasts is IGFBP-5. Expression of IGFBP-5 mRNA increases rapidly during C2 cell differentiation, with kinetics of accumulation similar to those of myogenin, a muscle-specific transcription factor. Secretion of BP5 occurs in parallel to the rise in mRNA expression, with increases being measurable in conditioned culture media within 12 h of the onset of terminal differentiation. By contrast, other binding proteins do not appear to be synthesized or up-regulated in C2 cells. Our experiments also demonstrate that IGFBP-5 mRNA is produced *in vivo* by a variety of mouse tissues, including skeletal muscle, and show that this binding protein is highly conserved among vertebrates, both in amino acid sequence and in the large size of its mRNA.

During the purification of ~29 kDa IGFBP-5 from C2 cell conditioned culture media, some proteolysis occurred as evidenced by the 23-kDa band identified by silver staining in Fig. 1. The apparent absence of this band in other Western blots (Fig. 5*A*) may be due to differences in sample preparation, with proteolysis occurring during one of the steps in purification procedure. A similarly sized COOH-terminally truncated protein has been identified in other cell lines (8, 10, 18). In human fibroblasts and in rat granulosa cells, this smaller protein appears to be the cleavage product of a specific protease (8, 18). Thus, it is possible that a protease became activated in the culture medium or during protein purification. A 23-kDa form of IGFBP-5 also was isolated from human osteoblasts, where it appears to have mitogenic activity even in the absence of IGF I (10). It therefore seems reasonable to postulate that the two forms of IGFBP-5 may have different actions and that a protease that recognizes BP5 also may be secreted by differentiating C2 myoblasts.

IGFs have been shown to promote the differentiation of many cell types, including muscle (2), and it has been suggested that IGF II regulates myogenesis *in vitro* (for reviews, see Refs. 36 and 37). IGFBP-5 may modulate the actions of IGF II during differentiation, since BP5 expression precedes the induction of IGF II during myogenesis. In this paper, we show that C2 cells and F3 azamyoblasts both express IGFBP-5 during differentiation. These two cell lines have different origins: C2 cells are derived from the thigh muscle of 2-month-old mice after crush injury (15), whereas azamyoblasts were cloned after treatment of 10T1/2 fibroblasts with 5-azacytidine (16). Although in C2 myoblasts the increase in IGFBP-5 mRNA during differentiation is more pronounced than in azamyoblasts (compare Figs. 4*A* and 6*A*), in both cell lines there is a prompt and sustained induction in IGFBP-5 protein secretion soon after the onset of differentiation (see Figs. 5*A* and 6*C*). Also, IGFBP-5 appears to be the only IGFBP produced by either cell line during differentiation (Figs. 5*A* and 6*C*). In this context, we cannot explain the differences between our results and those of Ernst *et al.* (38), who did not detect a 29-kDa IGFBP on ligand blots of conditioned media from C2C12 cells. One possibility may be differences between C2 cells and the C2C12 sub-line, although we have detected IGFBP-5 mRNA and protein in several other muscle cell lines.[2] Alternatively, media conditioned by C2C12 cells could have contained proteolytically cleaved IGFBP-5, which is difficult to detect by ligand blot (see Fig. 1).

The precise mechanisms by which different IGFBPs modulate IGF action are unknown. IGFBP-1 and -3 have been shown to both enhance and inhibit IGF-stimulated DNA synthesis in fibroblasts (39–41). Several IGF-binding proteins are capable of binding to the cell surface at sites distinct from either IGF receptor (18, 42). IGFBP-1 and -2 each contain Arg-Gly-Asp sequences (43), which have been shown in other proteins to bind integrins (44). IGFBP-3 and -5 also are capable of attachment to the surface of fibroblasts, although the mechanism of association has not been established. IGFBP-5 has been shown to bind IGF II preferentially (45). This binding could prevent the interaction of IGF II with either IGF receptor, or, alternatively, the ability of IGFBP-5

of the Geneworks program. The genetic distance (displayed *above* each *branch*) is estimated by comparing the number of identical residues in two proteins with the length of the shorter sequence using the Unweighted Pair Group Method with Arithmetic Mean (51). This analysis shows that BP5 is the most conserved IGF-binding protein and that it is more closely related to BP3 and BP6 than to the other IGFBPs.

to bind to the cell surface (18) could direct IGF II to one or both receptors. Binding to the cell surface may also provide a mechanism by which IGFBP-5 could transduce a signal independent of the presence of IGF II in the culture medium. Additionally, proteolytic processing, which apparently reduces the affinity of BP5 for IGFs (see Fig. 1), could make IGFs available to their receptors. Each of these possibilities can be tested by modulating IGFBP-5 expression in myoblasts.

IGFs exert multiple effects on target cells. In addition to enhancing cellular differentiation, they have been shown to stimulate proliferation, to increase nutrient uptake, and to inhibit protein breakdown (2). IGFs are highly conserved; IGF I has been identified in a variety of species, from salmon to man (for review, see Ref. 46). IGF II also has been found in mammalian and non-mammalian vertebrates, and IGF signaling pathways have been elucidated in amphibian, avian, and mammalian species (reviewed in Ref. 46). Our demonstration of the existence of at least one IGFBP in non-mammalian vertebrates potentially indicates that modulation of IGF action is also an important aspect of IGF signaling. The presence of BP5 mRNA in the chicken eye (Fig. 8) also suggests that a 28-kDa IGF-binding protein previously identified by ligand blotting in the vitreous humor of embryonic and post-hatch chicks (47) is IGFBP-5. It remains to be determined which of the other IGFBPs also are present in non-mammalian species.

The analysis presented in Fig. 9*B* shows that IGFBP-5 is the most highly conserved of all known IGFBPs. There is 97% identity among BP5 homologues in mice, rats, and humans, while amino acid sequence identity for other IGFBPs ranges from only 66 to 91% between rat and human proteins. In addition, the deduced amino acid sequence of the NH_2-terminal region of *Xenopus* IGFBP-5 indicates that IGFBP-5 also is conserved among non-mammalian vertebrates. Although the reasons for the presence of IGFBP-5 in avian and amphibian species are not known, one possibility for the strong sequence conservation between the *Xenopus* and mammalian-binding protein is structural, since it appears that more sites on IGF I are required for optimal interaction with IGFBP-5 than are needed for binding to other IGFBPs (45).

The unusual size (>6.0 kb) of the IGFBP-5 mRNA also has been conserved among *Xenopus*, chicken, mouse, rat, and human species (Fig. 8) (35, 48); all other known IGFBPs are encoded by transcripts that are smaller than 2.5 kb. Sequences in the 3′ UTR of other mammalian genes have been shown to affect RNA stability (reviewed in Ref. 49), while translational regulatory elements have been demonstrated in both 5′ and 3′ UTRs (reviewed in Refs. 49 and 50). It is presently unknown whether the untranslated regions of the IGFBP-5 mRNA regulate these processes.

In summary, we have demonstrated that the ~29-kDa IGFBP we identified previously in differentiating C2 myoblasts is the mouse homologue of IGFBP-5. Both IGFBP-5 mRNA and protein are rapidly up-regulated during myoblast differentiation, with kinetics of accumulation that parallel those of the myogenic regulatory factor myogenin. IGFBP-5 is the most conserved of all IGFBPs, and a *Xenopus* homologue has been identified. Further investigation into the function of IGFBP-5 in myoblasts and in other cell types may provide insight into the role of IGFBPs in cellular development and differentiation.

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