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Identification of the Forms of Insulin-like Growth Factor-binding Proteins Produced by Human Fibroblasts and the Mechanisms That Regulate Their Secretion*

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Human fibroblasts secrete insulin-like growth factor-binding proteins (IGFBPs) that can modify insulin-like growth factor (IGF) I action. We have determined the molecular identities of three forms of IGFBPs that are secreted by human fibroblasts *in vitro*. Ligand blot analysis of fibroblast conditioned media revealed that the M_r 43,000 and 39,000 forms were the most abundant, but that M_r 31,000 and 24,000 forms were also present. An antiserum that was specific for IGFBP-5 reacted with the M_r 31,000 form, and an IGFBP-4-specific antiserum recognized only the M_r 24,000 form. The M_r 39,000 and 43,000 forms were detected by IGFBP-3 antiserum. Further proof that fibroblasts synthesized these forms of IGFBPs was obtained by Northern blotting. A cDNA probe for IGFBP-3 hybridized with a 2.4-kilobase (kb) transcript, whereas a cDNA probe for IGFBP-5 recognized a single 6.0-kb transcript, and an IGFBP-4 cDNA probe recognized 2.2- and 2.0-kb transcripts. IGF-I and -II caused a minimal (<43%) increase in IGFBP-5 mRNA abundance and had no effect on IGFBP-4 mRNA abundance. IGF-I and -II (100 ng/ml) stimulated 6–8-fold increases in IGFBP-5 levels, whereas IGFBP-4 was inhibited. Insulin failed to elicit any change in IGFBP-5, suggesting that binding of the IGFs to IGFBPs was required to detect the increase. Immunoblotting for IGFBP-5 revealed an M_r 23,000 (non-IGF-I-binding) fragment. To determine if the IGFs were influencing proteolytic degradation of IGFBP-5, pure IGFBP-5 was added to fibroblast cultures and incubated for 4 h at 37 °C. The amount of fragment formation was attenuated by the presence of IGF-I and -II, but not insulin, suggesting that this is a mechanism by which the IGFs act to modulate IGFBP-5 concentration. In contrast to the IGFs, forskolin, which increased IGFBP-4 and -5 mRNA abundance and secretion, had no effect on fragment formation. The results show that human fibroblasts synthesize and secrete IGFBP-3, -4, and -5 and that changes in intracellular cAMP regulate synthesis, whereas the IGFs regulate IGFBP-4 and -5 levels by post-transcriptional mechanisms.

Several cell types have been shown to produce insulin-like growth factors (IGFs)[1] I and II and their specific binding proteins (IGFBPs) (1). Human dermal fibroblasts have been used as a model system to determine the factors that regulate the secretion of IGF-I and -II (2, 3) and to study their effects on fibroblast replication (4). Purified IGF-I-like peptides that are secreted by fibroblasts have been shown to bind to IGF-I receptors on the fibroblast surface (5); however, the fate of the secreted IGF-like peptides prior to receptor binding has not been determined. In addition to IGF-like peptides, human fibroblasts secrete several forms of IGFBPs (6). The exact molecular identities of each of the forms of IGFBPs that are secreted by human fibroblasts have not yet been determined; however, two of these forms (M_r 43,000 and 39,000) can be specifically immunoprecipitated with an IGFBP-3 antiserum (7). These two immunoreactive forms of IGFBP-3 are believed to be glycosylation variants of the same protein. Fibroblasts also secrete an M_r 31,000 form of IGFBP that could not be immunoprecipitated with an antisera to IGFBP-1 (7). However, a different antiserum prepared against pure IGFBP-1 did react with this M_r 31,000 form in concentrated conditioned medium (6). This M_r 31,000 form was shown to be increased 6–8-fold following IGF-I treatment (6). An additional M_r 24,000 form is present in fibroblast conditioned media, and its identity has not been determined. Because IGFBPs can modify the growth response of human fibroblasts to IGF-I (8, 9), it is important to determine the molecular identities of the M_r 31,000 and 24,000 forms and to examine the variables that regulate secretion of these IGFBPs. This information will be necessary to determine if these proteins also have a role in regulating the fibroblast growth response to IGF-I. Therefore, the purpose of this study was to determine the molecular identities of the M_r 31,000 and 24,000 IGFBP forms and to analyze the mechanisms that regulate their secretion.

EXPERIMENTAL PROCEDURES

Materials—EMEM was purchased from Hazelton (Denver, PA). Calf serum was purchased from Colorado Laboratories, Inc. (Logan, VT). Cycloheximide, Cohn fraction V, BSA, polyethylene glycol 8000, dexamethasone, hydrocortisone, progesterone, 17β-estradiol, ammonium persulfate, sodium phosphate, isopropyl-1-thio-β-D-galactopyranoside, and 5-bromo-4-chloro-3-indolyl β-D-galactoside were obtained from Sigma. Human γ-globulins were purchased as an 18% solution (Cutter Biological, Berkeley, CA). Recombinant human insulin was purchased from Lilly. ^{125}I-IGF-I (150–250 μCi/μg) was prepared by a previously described method (10). Recombinant human

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[1] The abbreviations used are: IGFs, insulin-like growth factors; IGFBPs, insulin-like growth factor-binding proteins; BSA, bovine serum albumin; EMEM, Eagle's minimum essential medium; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); PCR, polymerase chain reaction; TGF-β, transforming growth factor-β; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

IGF-I and -II were obtained from Bachem, Inc. (Torrance, CA). Human platelet-derived growth factor was purchased from PDGF, Inc. (Boston, MA); and recombinant human basic fibroblast growth factor was provided by Dr. A. Sommer (Synergen, Boulder, CO). TGF-β from porcine platelets was obtained from R & D Systems, Inc. (Minneapolis, MN); phenyl-Sepharose CL-4B and oligo(dT)-cellulose were obtained from Pharmacia LKB Biotechnology Inc. Tris, sodium dodecyl sulfate (SDS), Temed, RNA and DNA ladders, and polyacrylamide were purchased from Bethesda Research Laboratories. Urea, acetonitrile, EDTA, isoamyl alcohol, and Tween 20 were obtained from Fisher. Trifluoroacetic acid was purchased from Pierce Chemical Co. Glycine, bromphenol blue, and glycerol were from Serva (Heidelberg, Federal Republic of Germany). Agarose (Sea Kem, Seaplaque, and NuSieve) was obtained from FMC Bioproducts (Rockland, ME). A Mighty Small 2 gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) was used for protein electrophoresis. Transfer of proteins to nitrocellulose filters (Schleicher and Schuell) was performed using a semidry electroblotter (Janssen Life Sciences, Piscataway, NJ). Autoradiograph X-Omat RP film was purchased from Eastman Kodak Co. The reagents used in cDNA sequencing were bought from United States Biochemical Corp., and the reagents used in PCR were obtained from Perkin-Elmer Cetus Instruments. PCR was performed in a Perkin-Elmer Cetus thermocycler. ^{35}S-dATP, [α-^{32}P]dCTP, and Rainbow high molecular weight standards were purchased from Amersham Corp. The pBS vector was obtained from Stratagene (San Diego, CA). Phenol, ethidium bromide, and trypsin were purchased from Boehringer Mannheim. DNA restriction endonuclease and modifying enzymes were purchased from Promega Biotec. Tissue culture plates were obtained from Falcon Labware (Becton, Dickinson & Co., Oxnard, CA), and calf serum was obtained from Colorado Serum Co. (Denver, CO).

Cell Cultures—Human fetal dermal fibroblasts (GM10, Human Mutant Genetic Cell Repository, Camden NJ) were cultured in 10-cm dishes using EMEM supplemented with serine (21 μg/ml), pyruvate (110 μg/ml), asparagine (30 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% calf serum. Cells were passaged weekly using phosphate-buffered saline plus 0.1% trypsin and 0.02 M EDTA and were replated at a density of 1×10^4 cells/cm^2 in either 24- or 6-well tissue culture plates. The medium was replaced on the 3rd day after plating, and cells were used on day 6 or 7. Cells were used between passages 8 and 14. For the experiments, cells were washed twice with serum-free EMEM and then incubated in 0.5 ml of EMEM supplemented with 0.01% BSA with or without other additives for 24 h. Conditioned media were collected and stored at −20 °C for further analysis. SV40-transformed human fetal lung fibroblast (AG2804) cultures were obtained from the Aging Cell Repository (Camden, NJ) and were maintained as described previously (11).

Preparation of Conditioned Medium for IGFBP Purification—A human glioblastoma tumor cell line (T98G; American Type Culture Collection, Rockville, MD) was grown to confluence in EMEM supplemented with 10% calf serum in Nunc cell factories (Roskilde, Denmark). Confluent cultures were then maintained in serum-free minimum essential medium without phenol red for 48 h, and conditioned medium was harvested and stored at −20 °C. Approximately 19 liters of conditioned medium were collected in this manner.

Preparation of Cellular Extracts—Cellular proteins were extracted using the procedure of Knudsen *et al.* (12). Briefly, cells were plated in 6-well plates and grown to confluence. Conditioned media samples were collected in siliconized tubes, and the cells were washed twice with phosphate-buffered saline and then solubilized with phosphate-buffered saline containing 0.5% Triton X-100 for 20 min at room temperature. Cellular extracts were transferred to siliconized tubes, lyophilized to dryness, and then redissolved in H_2O and stored at −20 °C.

IGFBP Purification—Nineteen liters of T98G cell-conditioned medium were centrifuged at 16,000 × *g* for 30 min to remove cellular debris; the pH was adjusted to 7.2; and the salt concentration was increased to 0.5 M NaCl. Aliquots (1.5 liters) were loaded onto a phenyl-Sepharose column (4.4 × 5.7 cm) previously equilibrated with 0.05 M NaH_2PO_4, 0.15 M NaCl, 0.002 M EDTA, pH 6.6. After sample loading, the column was washed with the same buffer until the absorbance (280 nm) returned to base line. Proteins were eluted using a linear gradient of 0–100% elution buffer (0.04 M NaH_2PO_4, 1.7 mM EDTA, 15% acetonitrile, pH 6.6) over 2 h. Twelve-milliliter fractions were collected, and 20 μl of a 1:50 dilution of each fraction were assayed for IGF binding activity. Pools of active fractions were subjected to ligand blotting to determine the molecular weight estimates of each form of IGFBP present. The binding protein activity eluted as a single broad peak between 10 and 15% acetonitrile. Recovery ranged between 60 and 90%. The fractions containing IGF binding activity were pooled; the acetonitrile was removed by partial lyophilization; and the remaining material was pumped directly onto a reverse-phase HPLC C_4 column (0.46 × 25 cm; Vydac, Hespevia, CA) previously equilibrated with 0.04% trifluoroacetic acid in H_2O. The column was washed with 0.04% trifluoroacetic acid until absorbance (214 nm) returned to base line and was then eluted using a linear gradient of 0–35% acetonitrile for 30 min. This was followed by isocratic elution for 20 min and then a linear gradient to 100% acetonitrile over 30 min. Fractions containing IGF binding activity eluted between 30 and 35% acetonitrile. The IGFBPs were incompletely separated, but did elute in the following sequence: IGFBP-5, -4, and -3, followed by IGFBP-2. Recovery of total IGFBP activity was 85%. Fractions containing predominantly either IGFBP-4 or -5 (100–200 μg of total protein/column) were pooled and diluted with H_2O to a final concentration of 0.04% trifluoroacetic acid, 10% acetonitrile and were then reapplied to a Vydac C_4 column (0.46 × 2.5 cm). The column was eluted with a linear gradient (0–20% acetonitrile) over 10 min, followed by a 40-min linear gradient of 20–40% acetonitrile. The IGFBPs eluted between 23 and 28% acetonitrile. The purity of the IGFBP-5 and -4 fractions was determined by SDS-PAGE with silver staining. Fractions that contained a mixture of intact IGFBP-4 and -5 were pooled and rerun. The fractions that were eluted at 22–23% acetonitrile when analyzed by SDS-PAGE and silver staining were noted to contain two bands (M_r 23,000 and 18,00). Ligand blotting using ^{125}I-IGF-I showed no detectable binding activity; however, when ^{125}I-IGF-II was used, both fragments were detected. These fractions were further purified and submitted for N-terminal amino acid sequence analysis. The concentrations of pure intact IGFBP-4 and -5 were low, *e.g.* 1–5 μg/ml. Since attempts to concentrate the samples by lyophilization resulted in a 50% loss of each protein, the fractions containing each protein were pooled separately, and then each pool was reapplied to the C_4 column and eluted using a linear gradient of 0–100% acetonitrile over 40 min. Single fractions containing an ~20–25 μg/ml concentration of each protein were obtained. Final purity was assessed by SDS-PAGE followed by silver staining, ligand blotting, or amino acid sequence analysis.

N-terminal Sequence Determination—Pure preparations of intact IGFBP-4 or -5 or the M_r 23,000 and 18,000 fragments were lyophilized. Approximately 148 pmol of IGFBP-5, 125 pmol of IGFBP-4, and 420 pmol of the fragments were reconstituted separately in HPLC-grade water (Burdick & Jackson Laboratories Inc., Muskegon, MI). Each was sequenced directly using an Applied Biosystems Model 470A Sequencer equipped with on-line analysis using an Applied Biosystems Model 120A phenylthiohydantoin analyzer. To verify the presence of cysteine residues, 180 pmol of each of the intact proteins were reduced and *S*-pyridylethylated in solution according to the method of Hawke and Yuan (13) prior to sequencing.

IGF Binding Activity in Chromatographic Fractions—IGF binding activity was determined using a polyethylene glycol precipitation method (14). In brief, conditioned media aliquots were incubated with 20,000 cpm of ^{125}I-IGF-I for 1 h at 22 °C and pH 6.0. Bound ^{125}I-IGF-I was separated from unbound by precipitation with 0.25% human globulin and 12.5% polyethylene glycol.

Generation of Antisera to IGFBP-4 and -5—Intact pure IGFBP-4 was concentrated, and ~80 μg were reconstituted in 800 μl of water and added to an equal volume of Freund's complete adjuvant (Sigma). The inoculum was thoroughly mixed to reach an emulsion, and 25 μl were injected intradermally at multiple sites in a single New Zealand White rabbit (Franklin Rabbitry, Wake Forest, NC). After 4 weeks, the injection was repeated using 30 μg of IGFBP-4 in 1 ml of 50% Freund's incomplete adjuvant. Thereafter, the rabbit was bled at 3-week intervals and was reimmunized with 20 μg of protein five times. The antisera used in these studies were obtained at the fourth and fifth bleeds. They were specific for IGFBP-4 as proven by their inability to react with 50 ng of pure IGFBP-1, -3, or -5 by immunoblotting. In contrast, 0.5 ng of the pure IGFBP-4 could be detected by this method. Antisera against IGFBP-5 were prepared in a similar manner, with the exception that intact IGFBP-5 was not immunogenic in rabbits. Therefore, two fragments of IGFBP-5 with molecular weight estimates of 23,000 and 18,000 were pooled, and the pool was used as an immunogen. These fragments were sequenced, and it was shown that the first nine amino acid assignments were identical to the N terminus of intact IGFBP-5. A guinea pig was immunized with 90 μg of the fragment mixture intradermally, followed by a repeat injection of 40 μg subcutaneously after 4 weeks. The animal was

reimmunized with 25 μg at 4-week intervals. The guinea pig was bled every 2 weeks. These studies used antiserum from the third bleed. The antiserum had no cross-reactivity with 50 ng of IGFBP-1, -2, -3, or -4, but could detect 0.2 ng of intact IGFBP-5.

Ligand Blotting—To study the regulation of distinct forms of IGFBP, ligand blot analyses were performed. Aliquots of conditioned media or of cellular extracts were electrophoresed through 12.5% SDS-polyacrylamide gel (15). The separated proteins were then transferred to nitrocellulose filters. The transfer and probing buffers were as described by Hossenlopp *et al.* (16). The membranes were probed for IGF binding activity by incubation with ~500,000 cpm of ^{125}I-IGF-I overnight at 4 °C. The filters were then washed as described (16). IGFBP bands were visualized by autoradiography and were quantified by scanning densitometry. To correctly quantify these changes, the band intensities of increasing concentrations of pure IGFBP-5 that had been ligand-blotted were analyzed. It was determined that the increases in band intensity were linear between 1000 and 25,000 scanning units. Molecular weights were estimated by comparing the electrophoretic mobility of the IGFBPs to prestained molecular weight markers.

Immunoblotting—Conditioned media samples were electrophoresed and transferred to nitrocellulose filters as described above for ligand blotting analysis. The polyclonal antiserum against human IGFBP-1 that was used had been prepared as described previously (17). The polyclonal antiserum against bovine IGFBP-2 was prepared as previously described (18) and was used at a final dilution of 1:1000. IGFBP-3 antiserum was prepared as described for IGFBP-1 with the following modifications. Seventy micrograms of recombinant human IGFBP-3 (a gift from Biogrowth, Inc.) were used for the primary immunization, and 20 μg were used for reimmunization. The antiserum obtained could detect <1.0 ng of IGFBP-3 at a 1:1000 dilution; and when tested at a final dilution of 1:100, it had no cross-reactivity with IGFBP-1, -4, or -5 and <1% with IGFBP-2 (data not shown). For immunoblot analysis, nitrocellulose filters were incubated with the appropriate primary immune serum in Tris-buffered saline containing 1% BSA, pH 7.0, for 90 min at room temperature unless stated otherwise. The filters were then rinsed three times with Tris-buffered saline and incubated for 2 h with anti-rabbit IgG-alkaline phosphatase conjugate at a final dilution of 1:500 in Tris-buffered saline plus BSA, followed by two rinses with Tris-buffered saline containing 0.01% Tween 20. Bands were visualized using the ProtoBlot system immunoblotting reagents following the technique recommended by the manufacturer (Promega Biotec). The lower limit of sensitivity for this technique is ~0.2 ng of human IGFBP-2, 1 ng of IGFBP-1, and 1 ng of IGFBP-3. Neither IGFBP-1 nor -2 antiserum cross-react with IGFBP-3 at the dilution used in these studies. The antisera against IGFBP-5 and -4 were used at final dilutions of 1:200 and 1:1000, respectively.

RNA Isolation—RNA was isolated from cell cultures by the guanidinium thiocyanate method (19) and was quantified by spectrophotometry. Poly(A)$^+$ RNA was prepared from 1.0 mg of total cellular RNA (T98G cells) using an oligo(dT)-cellulose column as per the manufacturer's instructions (Pharmacia). Two cycles of purification were used.

Preparation of DNA Probes for IGFBP-4 and -5—PCR was used to prepare specific probes for human IGFBP-4 and -5. To prepare a human IGFBP-4 cDNA probe, two sets of primers were designed. A set of two 27-mers in the 5′ to 3′ orientation (5′-GATACTGAATTCGGCTGCGGCTG(C/T)TGC-3′) and a set of eight 27-mers in the 3′ to 5′ orientation (5′-GTAGAGGAATTCGTC(A/G)CAGTT (G/T)GG(A/G)AT-3′) were synthesized. The primers were designed to amplify a 462-base pair segment of DNA that was located in the center of the protein-coding region (20). Both sets of primers contained *Eco*RI sites at the 5′-end. Two micrograms of poly(A)$^+$ RNA from T98G cells were reverse-transcribed in a 20-μl reaction containing 0.1 M Tris, 0.15 M KCl, 0.02 M dithiothreitol, 0.006 M $MgCl_2$, pH 8.3, 2.5 μg of BSA, 80 units of RNase inhibitor, 0.5 μg of random hexamers, 0.5 mM deoxynucleotide triphosphates, and 200 units of Moloney murine leukemia virus reverse transcriptase. After 30 min at 37 °C, 200 units of the enzyme were added again, and incubation was repeated. The reaction mixture was adjusted to contain 17 mM Tris, pH 8.3, 50 mM KCl, 2 mM dithiothreitol, 2 mM $MgCl_2$, 198 μM deoxynucleotide triphosphates, and 0.12 μg/ml BSA in 100 μl. To allow amplification by PCR, the 5′- and 3′-primer sets were added at 1 and 4 μM, respectively. The reaction mixture was covered with mineral oil and heated for 3–5 min at 95 °C. After the addition of 5 units of DNA polymerase from *Thermus aquaticus* (Taq polymerase), the cycling reaction was initiated. Two initial cycles with an annealing temperature of 45 °C were followed by 40 cycles with an annealing temperature of 55 °C. Melting and extension temperatures were 95 and 72 °C in all cycles, respectively. Ten microliters of the reaction mixture were electrophoresed through a 1.5% agarose gel (Sea Kem) to show that the 462-base pair product was amplified. Sizes were compared to the relative migration of the bands in the DNA ladder. The remaining material was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and was then extracted with chloroform/isoamyl alcohol (24:1) and precipitated with 0.3 M sodium acetate, pH 5.0, 67% ethanol. The dried DNA pellet was resuspended, digested with *Eco*RI, and separated by electrophoresis on a 1.5% low melting temperature agarose gel (NuSieve). The DNA of interest was excised from the gel and then extracted with phenol only followed by chloroform/isoamyl alcohol (24:1) and was precipitated as described above. The DNA was ligated into the *Eco*RI site of the pBS vector that had been *Eco*RI-digested and whose 5′-ends had been dephosphorylated using calf intestinal alkaline phosphatase. The ligation proceeded for 4 h at 22 °C using 1.5 units of T_4 DNA ligase for 200 ng of DNA (vector:insert molar ratio of 1:4) in a total reaction volume of 5 μl. The reaction mixture was then diluted to 25 μl with H_2O and was used to transfect *Escherichia coli* JM109 cells (21). Colonies were grown on Luria broth agar plates containing ampicillin (100 μg/ml). Isopropyl-1-thio-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside were added upon plating to perform a white and blue colony assay. White colonies were collected and grown in 5 ml of Luria broth containing ampicillin for 6 h. Plasmid DNA was prepared from a 1.5-ml culture using the rapid boiling DNA preparation (22). Positive clones were identified by *Eco*RI digest of the DNA followed by electrophoresis on a 1.5% agarose gel (Sea Kem). An alkaline DNA preparation (21) was performed for some of the positive clones to sequence double-stranded DNA using the dideoxy sequencing method (23). The DNA sequence of the amplified 462-base pair product was identical to the sequence published for human IGFBP-4 (20, 24).

The cDNA fragment of IGFBP-5 was prepared in the same manner as IGFBP-4 with a few modifications. The sequence used for the 5′ to 3′ primer was the same as that used for IGFBP-4, and the sequence for the 3′ to 5′ primer was a set of four 18-mers (5′-GTCCCCATC-CAC(G/A)TA (C/T)TC-3′). The primers were designed to cover a region containing 627 base pairs within the protein-coding region of IGFBP-5 (25). An *Eco*RI site was contained only in the 5′-end of one set of primers. PCR was performed using 37 °C as the annealing temperature in the initial two cycles. Otherwise, temperatures were as described for amplifying IGFBP-4 DNA. The cloning vector was prepared by an *Sma*I digest followed by an *Eco*RI digest and was gel-purified as described above on an agarose gel (Seaplaque). The vector:insert molar ratio used in the ligation reaction was 1:3. The ligation reaction was allowed to proceed for 2 h at room temperature and then overnight at 16 °C. Positive clones were identified by *Eco*RI/*Hin*dIII restriction digest.

The IGFBP-3 cDNA probe was a full-length cDNA (2585 base pairs) that had been cloned into pUC119 and was prepared as previously described (26).

Northern Blot Analysis—RNA samples were denatured by heating to 55 °C for 1 h with 1 M glyoxal and 50% dimethyl sulfoxide in 10 mM sodium phosphate, pH 6.8. Samples (15 μg in 12 μl) were combined with 3 μl of dye solution (0.1% bromphenol blue, 0.1% xylene cyanol, and 50% sucrose) and were subjected to electrophoresis on a 1% agarose gel with recirculating buffer (10 mM sodium phosphate, pH 6.8). To confirm that similar amounts of RNA were loaded, the gels were stained with ethidium bromide, and ribosomal RNAs (28 S and 18 S) were visualized by UV transillumination. RNA was transferred onto nylon membrane filters (Biotrans, ICN Biomedical, Inc., Irvine, CA) using 20 × SSC (1 × SSC = 15 mM sodium citrate, 0.15 M NaCl, pH 7.0), and the filters were baked under vacuum for 2 h at 80 °C. For hybridization, gel-purified DNA fragments of cDNAs corresponding to IGFBP-4, -3, or -5 were labeled with [^{32}P]dCTP by the random priming method (27). Prehybridization and hybridization were performed in a solution of 1 mM EDTA, 0.25 M $NaHPO_4$, pH 7.2, 7% SDS (28), and 0.2 mg/ml denatured salmon sperm DNA at 55 °C for 12 and 24 h, respectively. Filters were washed in 2 × SSC, 0.1% SDS for 15 min at 22 °C followed by 15 min at 50 °C and were then washed in 0.1 × SSC, 0.1% SDS for 1 h at 65 °C. Autoradiography was performed at −80 °C. Molecular weight estimation of the hybridizing band was obtained by comparison with the relative migration rate of a commercial RNA ladder containing 9.5-, 7.5-, 4.4-, 2.4-, 1.4-, and 0.24-kb RNA standards. Changes in band intensity were analyzed by scanning densitometry. To determine linearity,

between 5.0 and 20 μg of total RNA from T98G cells were processed by Northern blotting, and the scanning densitometry units were determined. The results were linear between 8000 and 24,000 scanning units.

RESULTS

Conditioned medium from the GM10 fibroblast cell line was analyzed by ligand blotting. Under basal conditions, this cell type secreted predominantly an IGFBP that migrated as a doublet between M_r 43,000 and 39,000 (Fig. 1). A previous study had reported that these two bands represented different glycosylation forms of IGFBP-3 (7). Bands with molecular weight estimates of 31,000 and 24,000 were also detected in the conditioned medium. IGFBP-3 antiserum detected only the M_r 39,000 and 43,000 bands and did not react with the M_r 31,000 or 24,000 band (Fig. 1). Because we had previously shown that the abundance of the M_r 31,000 band was markedly stimulated by IGF-I (6), we wished to determine its identity. To determine if the M_r 31,000 and 24,000 bands were related to other forms of IGFBPs that had been previously characterized, these ligand blots were immunoblotted with antisera that are specific for human IGFBP-1 (17) and bovine IGFBP-2 (18). Neither antisera reacted with these bands. Therefore, the M_r 31,000 and 24,000 bands do not appear to be proteolytic degradation products or deglycosylated forms of IGFBP-3 or intact IGFBP-1 or -2.

To determine the molecular identity of the M_r 31,000 and 24,000 forms of IGFBP, conditioned medium from T98G cells, which had been shown to secrete two IGFBPs of identical electrophoretic mobility, was used to purify these proteins. Approximately 900 μg of the M_r 31,000 protein and 1.2 mg of the M_r 24,000 protein were recovered during purification to homogeneity. Proof of purity was obtained by amino acid sequence determination of each peak. N-terminal amino acid sequence determination of the M_r 24,000 protein revealed that the first 32 amino acids had the following sequence: DEAIAHCPPCSEEKLARCRPBVGCEELVREPG. This sequence is identical to that published for human IGFBP-4 (24). Likewise, the M_r 31,000 protein revealed the following N-terminal sequence: LGSFVHCEPCDEKALSMCPPSPLGCELVKE, corresponding to the first 30 residues of human IGFBP-5 (25). Purified IGFBP-4 and -5 were then analyzed by silver staining and immunoblotting. SDS-PAGE with silver staining revealed a single band for IGFBP-5 with a molecular weight estimate of 31,000 (Fig. 2). Antiserum that was prepared from IGFBP-5 fragments reacted intensely with this band (Fig. 2) and detected concentrations as low as 0.2 ng (data not shown). IGFBP-4 migrated as a single band at M_r 24,000 and antisera prepared using this protein reacted with the M_r 24,000 band.

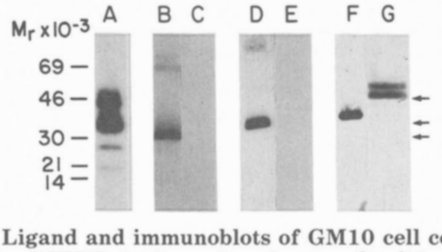

FIG. 1. **Ligand and immunoblots of GM10 cell conditioned media using IGFBP-1, -2, or -3 antiserum.** Conditioned medium (24 h) from IGF-I (50 ng/ml)-treated fibroblast cultures was ligand-blotted (*lane A*) or immunoblotted with IGFBP-1 (*lane C*), IGFBP-2 (*lane E*), or IGFBP-3 (*lane G*) antiserum as detailed under "Experimental Procedures." *Lanes B, D,* and *F* represent pure human IGFBP-1 (25 ng), bovine IGFBP-2 (10 ng), and recombinant human deglycosylated IGFBP-3 (25 ng), respectively. Shown are the relative positions of the molecular weight standards. The *arrows* (*top* to *bottom*) indicate IGFBP-3, -2, and -1.

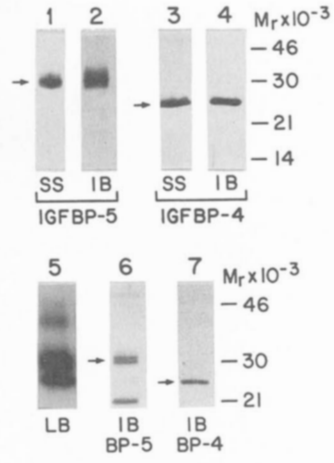

FIG. 2. **Silver staining and immunoblot analysis of IGFBP-4 and -5.** Twenty nanograms of IGFBP-5 and -4 that were purified from the T98G cell conditioned media were analyzed by silver staining (*SS*) (*lanes 1* and *3*, respectively). The pure proteins were also electrophoresed and transferred onto nitrocellulose and then incubated overnight with antiserum that was specific for either IGFBP-5 or -4 (*lanes 2* and *4*, respectively). Shown are the relative positions of the molecular weight standards. To confirm that the antisera were specific, 20 μl of conditioned medium from T98G cells were electrophoresed and analyzed by ligand blotting (*lane 5*) or by immunoblotting (*IB*) with IGFBP-5 antiserum (*lane 6*) and IGFBP-4 antiserum (*lane 7*). The *arrows* show the positions of intact immunoreactive IGFBP-5 and -4. A non-IGF-binding immunoreactive band is noted in *lane 6*. This band has identical electrophoretic mobility to a fragment of IGFBP-5 that was purified from T98G cell conditioned medium.

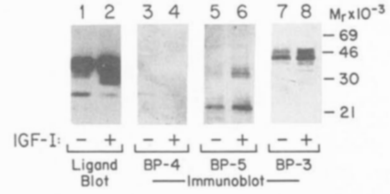

FIG. 3. **Immunoblot of fibroblast conditioned medium with IGFBP-3, -4, or -5 antiserum.** Confluent cultures of GM10 cells were exposed to EMEM (−) or EMEM plus 100 ng/ml IGF-I (+). The conditioned media samples were analyzed by ligand blotting (*lanes 1* and *2*) or by immunoblotting (*lanes 3–8*). *Lanes 3* and *4, 5* and *6,* and *7* and *8* were exposed to IGFBP-4, -5, and -3 antisera, respectively. The conditioned media used for the immunoblots were concentrated 10-fold. Shown are the molecular weight standards. The IGFBP-5 antiserum was prepared using IGFBP-5 fragments and appears to have a higher affinity for fragments compared to intact protein. Therefore, the use of this antiserum may overestimate fragment abundance relative to changes in intact IGFBP-5.

When conditioned medium obtained from the human glioblastoma cells (a cell line that secretes IGFBP-2, -3, -4, and -5) was immunoblotted with IGFBP-5 antiserum, only the M_r 31,000 band was reactive (Fig. 2). Similarly, the IGFBP-4 antiserum did not cross-react with IGFBP-3, -5, and -2, but did recognize the M_r 24,000 band (Fig. 3). To further test the specificity of these antisera, conditioned medium from AG2804 cells was analyzed by immunoblotting. This cell line has been shown to secrete an M_r 32,000 form of IGFBP (11) with an N-terminal sequence identical to IGFBP-6 (11, 25). Neither IGFBP-4 nor -5 antiserum bound to this protein (data not shown). These data indicate that IGFBP-4 and -5 were pure and that the antibodies are specific for the appropriate forms of IGFBPs.

To determine whether the M_r 31,000 and 24,000 proteins in fibroblast conditioned medium corresponded to the pro-

teins purified from T98G cell conditioned medium, GM10 cell conditioned medium was analyzed. Ligand blotting showed the M_r 31,000 and 24,000 bands and that the intensity of the M_r 31,000 band was stimulated 6-fold by IGF-I exposure (Fig. 3). Immunoblotting this ligand blot with IGFBP-5 antiserum showed that the M_r 31,000 band could be detected and that the intensity of the immunoreactive band was increased following IGF-I exposure. An intensely staining M_r 23,000 band was also detected. This band did not bind IGF-I, as shown on the ligand blot. These findings suggest that the polyclonal IGFBP-5 antiserum recognizes a protein that is identical or nearly identical to the purified IGFBP-5 and that its regulation by IGF-I is similar to the M_r 31,000 band previously described (6, 7). The M_r 24,000 IGFBP secreted by the GM10 fibroblasts diminished in intensity following IGF-I exposure. Immunoblotting showed that the M_r 24,000 band was detected by IGFBP-4 antiserum and that its intensity decreased following IGF-I exposure. This suggests that the M_r 24,000 protein secreted by GM10 fibroblasts is very similar or identical to IGFBP-4 and that its levels in conditioned medium are diminished by IGF-I treatment. The ligand blot bands at M_r 39,000 and 43,000 were recognized by antiserum against IGFBP-3, and both ligand and immunoblot activities in GM10 conditioned medium were increased by IGF-I treatment.

To further strengthen the conclusion that IGFBP-4 and -5 are synthesized by fibroblasts, specific cDNA probes were prepared using the polymerase chain reaction. cDNA sequence analysis revealed that the probes contained the correct sequences predicted for IGFBP-4 and -5. As shown in Fig. 4, a single 6.0-kb messenger RNA band corresponding to the previously reported size of the human IGFBP-5 transcript (20) was detected in RNA isolated from fibroblast cultures. IGFBP-5 message was slightly increased (~40 and 29%, respectively) following 6 and 12 h of exposure to IGF-I (Fig. 4). No greater increase in band intensity could be detected in four separate experiments. There was no detectable change in mRNA abundance in cultures that had been exposed to IGF-I for 24 h compared to controls. Two transcripts of 2.2 and 2.0 kb were detected with the IGFBP-4 cDNA probe (Fig. 5). Although scanning densitometry showed minimal changes in band intensity induced by IGF-I at 12 and 24 h when corrected for the amount of RNA loaded per lane, exposure to IGF-I did not result in a change in mRNA abundance at any time point. These findings show that IGF-I-induced changes in the levels of IGFBP-5 and -4 are not primarily due to changes in either mRNA transcription or altered mRNA degradation rates. In contrast, when changes in IGFBP-3 mRNA were analyzed, a 2-fold increase (as determined by scanning densitometry) in the 2.4-kb transcript was noted following IGF-I exposure (Fig. 6). This corresponds to the relative change in protein abundance that occurs following IGF-I exposure.

To determine whether other factors known to stimulate IGFBP secretion were also mediating their effects at a post-transcriptional level, fibroblast cultures were exposed to forskolin (10 μM), and total RNA as well as conditioned medium were collected after 24 h. Northern blotting revealed that forskolin increased IGFBP-4 and -5 mRNA abundance (Fig. 7). Scanning densitometry showed that the 2.2- and 2.0-kb forms of IGFBP-4 mRNA were increased 2.6- and 2.8-fold, respectively, and that IGFBP-5 was increased 2.5-fold. This suggests that IGFBP-4 and -5 synthesis is regulated by

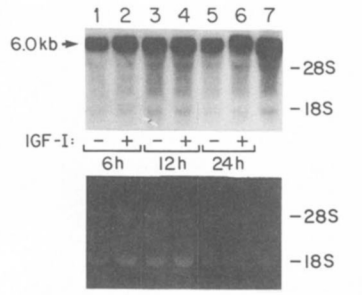

FIG. 4. **IGFBP-5 mRNA abundance in cultured human fibroblasts.** Total cellular RNA was prepared as described under "Experimental Procedures" from GM10 cultures, and 15-μg aliquots were electrophoresed and transferred to a nylon membrane and then hybridized with a ^{32}P-labeled human IGFBP-5 cDNA probe (*upper*). The *arrow* denotes the 6-kb message that is present in these cultures. The RNA samples loaded in *lanes 1, 3,* and *5* are from unstimulated human fetal fibroblast cultures, whereas the samples in *lanes 2, 4,* and *6* are from cultures that were exposed to IGF-I (50 ng/ml) for the times indicated. *Lane 7* is 15 μg of total RNA isolated from the T98G cells. Also shown is the ethidium bromide staining of total RNA loaded in each lane (*lower*). Ribosomal RNA electrophoretic migration positions are denoted.

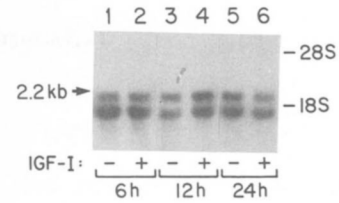

FIG. 5. **IGFBP-4 mRNA abundance in cultured human fibroblasts.** Human fibroblast RNA was prepared as described for Fig. 4, electrophoresed, and transferred to nylon filters. The filters were probed with a ^{32}P-labeled human IGFBP-4 cDNA probe. Two transcripts with molecular size estimates of 2.2 and 2.0 kb were detected. If higher stringency washes were used, the lower band could be removed; however, there was a marked parallel reduction in the intensity of the upper band. RNA samples loaded in *lanes 1, 3,* and *5* are from unstimulated cultures, whereas samples in *lanes 2, 4,* and *6* are from cultures that were exposed to IGF-I (50 ng/ml) for the times indicated prior to harvesting the RNA.

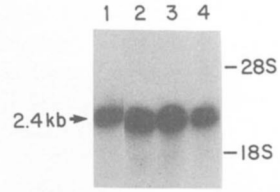

FIG. 6. **Change in IGFBP-3 mRNA abundance following IGF-I exposure.** Total RNA (15 μg) from quiescent GM10 fibroblast cultures that had been exposed to EMEM alone (*lane 1*), IGF-I (50 ng/ml) (*lane 2*), IGF-II (50 ng/ml) (*lane 3*), or forskolin (10 μM) (*lane 4*) for 24 h was electrophoresed and transferred to nylon membranes. The RNA was hybridized with the ^{32}P-labeled human IGFBP-3 cDNA probe. A single 2.4-kb transcript was detected after autoradiography.

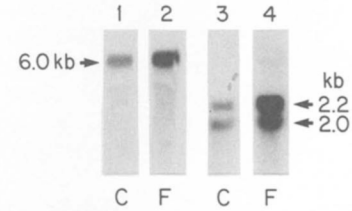

FIG. 7. **Effect of forskolin on IGFBP-4 and -5 mRNA expression.** Confluent fibroblast monolayers were incubated for 12 h in EMEM alone (*C*) or in EMEM plus forskolin (10 μM) (*F*). Fifteen micrograms of total RNA were electrophoresed, transferred to nylon membranes, and hybridized with ^{32}P-labeled IGFBP-5 and -4 cDNA probes. The *arrows* indicate the 6.0-kb transcript of IGFBP-5 and the 2.2- and 2.0-kb transcripts of IGFBP-4.

changes in intracellular cAMP at the level of changes in mRNA abundance. In contrast, the mechanism(s) by which IGF-I regulates IGFBP-4 and -5 levels may be post-transcriptional.

Immunoblot analysis of GM10 conditioned medium with an IGFBP-5 antiserum revealed a prominant band at M_r 23,000. This band had a molecular weight estimate that was identical to one of the proteolytic fragments of IGFBP-5 that was purified from T98G cell conditioned medium. N-terminal sequence analysis of the T98G cell-derived fragment showed that its 9 N-terminal residues were identical to the N-terminal sequence of IGFBP-5, proving that it was a non-IGF-I-binding fragment of that protein. To determine if the IGF-I-stimulated increase in IGFBP-5 ligand blot activity in conditioned medium was associated with a decrease in the amount of M_r 23,000 fragment generated, GM10 cells were incubated with pure IGFBP-5 (1.0 μg/ml) in the presence or absence of IGF-I or -II or insulin for 4 h. Exposure to either IGF-I or -II (200 ng/ml), but not insulin (5 μg/ml), inhibited the formation of the M_r 23,000 fragment (Fig. 8). To determine the specificity of this protease, pure IGFBP-1 was added to the cultures, and its abundance was analyzed after 24 h. No proteolytic degradation fragments were noted after immunoblotting with IGFBP-1 antisera.

To determine the specificity of the fibroblast response to IGF-I, confluent cultures were incubated with concentrations of insulin and IGF-II that were sufficient to stimulate IGF-I receptor activation. In contrast to IGF-I and -II, insulin stimulated secretion of IGFBP-4 but had no effect on IGFBP-3 and -5 (Fig. 9). As noted previously, IGFBP-3 and -5 were preferentially induced by both IGF-I and -II. These increases were dependent upon the concentrations of IGF-I and -II that were used. Scanning densitometry showed that IGF-I (100 ng/ml) invoked a 6.5-fold increase in IGFBP-5, whereas the IGFBP-3 band intensity increased only 2.1-fold (Table I). Similarly, IGF-II induced only a 1.4-fold increase in IGFBP-3 band intensity, but increased IGFBP-5 by 8-fold. Secretion of IGFBP-4 was inhibited by IGF-I and -II and stimulated 3.2-fold by insulin.

The regulation of secretion of IGFBPs by other hormones and growth factors was also analyzed. Scanning densitometry showed that forskolin increased conditioned medium levels of IGFBP-3 and -5 1.3- and 3.5-fold, respectively (Fig. 10*A*). In contrast, TGF-β and dexamethasone appeared to decrease the levels of both of these forms of IGFBP. Cycloheximide completely inhibited the secretion of all forms of IGFBP. Platelet-derived growth factor, fibroblast growth factor, estradiol, and progesterone had no effect (data not shown).

IGFBPs on fibroblast surfaces have been shown to modu-

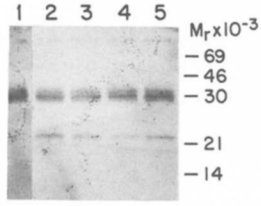

FIG. 8. **Effect of IGF-I and -II and insulin on IGFBP-5 fragment formation.** Pure IGFBP-5 was incubated with quiescent fibroblast cultures in EMEM for 4 h at 37 °C. At that time, the conditioned media samples were removed, and 25 μl of each sample were analyzed by SDS-PAGE and immunoblotting. *Lane 1* contains 25 ng of pure IGFBP-5 and shows no fragment. *Lane 2* shows an identical amount of IGFBP-5 that had been exposed to cultures that contained no additives during the 4-h incubation at 37 °C. It shows the appearance of an M_r 23,000 fragment. *Lanes 3* and *4* contain conditioned medium from cultures that had been incubated with 1.0 μg/ml IGFBP-5 and 200 ng/ml IGF-I or -II, respectively. *Lane 5* shows conditioned medium from a culture that was exposed to IGFBP-5 plus 5 μg/ml human insulin.

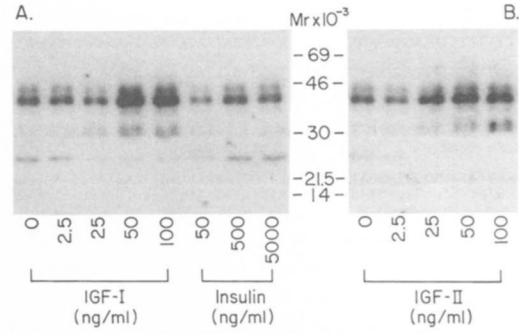

FIG. 9. **Regulation of IGFBP abundance by IGF-I and -II and insulin.** Confluent cultures of human fetal fibroblasts (GM10 cells) were incubated with or without IGF-I and insulin (*A*) or IGF-II (*B*) for 24 h. Conditioned media samples were analyzed by ligand blotting as described for Fig. 1. The molecular weights of protein standards run in parallel lanes are indicated.

TABLE I

Effect of IGF-I and -II and insulin on IGFBP abundance in GM10 cell conditioned media

Treatment	IGFBP		
	M_r 24,000	M_r 31,000	M_r 39,000–43,000
IGF-I (ng/ml)			
0	587[a]	346	5106
2.5	481	0	4131
25	186	1078	4841
50	270	2100	8976
100	144	1986	10,991
Insulin (ng/ml)			
50	211	173	2422
500	819	291	5015
5000	1007	337	4302
IGF-II (ng/ml)			
0	292	333	5140
2.5	241	373	3514
25	264	817	5860
50	257	2051	9367
100	206	2591	7122

[a] Areas represent densitometry units from Fig. 9. The M_r 39,000–43,000 IGFBP appears as a doublet, and due to difficulty in distinguishing between the peaks by scanning densitometry analysis, the total area was determined and presented.

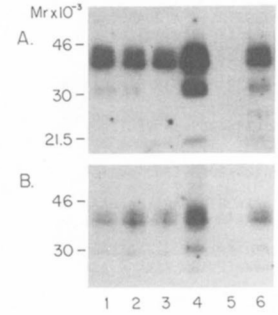

FIG. 10. **Regulation of abundance of IGFBPs in GM10 cells and their levels in conditioned medium.** Confluent monolayers were treated with TGF-β (1 ng/ml) (*lane 2*), dexamethasone (10^{-6} M) (*lane 3*), IGF-I (50 ng/ml) (*lane 4*), cycloheximide (25 μg/ml) (*lane 5*), or forskolin (10 μM) (*lane 6*) and were compared to control cultures (*lane 1*). After 24 h, conditioned medium was collected, and the cells were solubilized with Triton X-100. *A*, IGFBPs in fibroblast conditioned medium; *B*, IGFBPs found in Triton X-100-solubilized cells. Shown are the relative positions of molecular weight standards.

late IGF-I binding to IGF-I receptors (29). Therefore, the amount of the various forms of cell-associated IGFBPs was determined under basal conditions and after exposure to modulators of IGFBP secretion. Fig. 10*B* shows the abundance of the various forms of IGFBPs that were associated with cellular extracts. The cell-associated IGFBPs showed relative changes in their abundance that paralleled the changes found in the culture media. IGF-I induced significant increases in cell-associated IGFBP-3 and -5.

DISCUSSION

This study demonstrates that cultured human fibroblasts secrete three structurally distinct forms of IGFBPs. Four bands are detected by SDS-PAGE; however, the two bands with molecular weight estimates of 39,000 and 43,000 appear to represent different glycosylation variants of IGFBP-3 since they are both immunoprecipitable with IGFBP-3 antiserum (7) and both react with IGFBP-3 antiserum by immunoblotting. Scanning densitometry showed that these two forms constituted ~70–80% of the IGF-I binding activity in the basal state. Several other published studies have also shown that these two forms are the predominant species of IGFBP secreted by this cell type (6, 7).

Whereas IGFBP-3 is the predominant form released by human fibroblast cultures, these cells also release detectable quantities of M_r 31,000 and 24,000 forms. The molecular identities of these forms were determined to be IGFBP-5 and -4, respectively. This was proven by purifying these proteins to homogeneity from a glioblastoma cell line, establishing their identities with amino acid sequencing, and preparing specific antisera. The IGFBP-4 antiserum bound the M_r 24,000 form and the IGFBP-5 antiserum bound the M_r 31,000 form of IGFBP that were present in the fibroblast conditioned medium and did not recognize other forms of IGFBPs detected by ligand blotting. Further proof was provided by the observation that the cDNA probes for these two transcripts hybridized specifically to mRNAs that had molecular weight estimates that were identical to those reported for IGFBP-4 and -5 (20, 24). Martin *et al.* (11) have reported that transformed human fibroblast cultures secrete a form of IGFBP that has an electrophoretic mobility similar to that of IGFBP-5. However, that protein has a different N-terminal sequence, and our IGFBP-5 antiserum did not recognize that protein in transformed fibroblast conditioned medium. Based on these findings, we conclude that the M_r 31,000 IGFBP in the conditioned medium is IGFBP-5 and that the M_r 24,000 form is IGFBP-4.

Although IGFBP-5 was a minor component of the total IGFBP activity in fibroblast conditioned medium obtained from unstimulated cultures, its abundance in conditioned medium was enhanced 6–8-fold following exposure to IGF-I or -II. This confirms our previous finding (6) and that of Martin and Baxter (7) that showed an 8.4-fold induction of an M_r 31,000 protein in conditioned medium from a neonatal fibroblast cell line. Clearly, this 6–8-fold induction in IGFBP-5 by IGF-I and -II has the potential to be a major modulator of the cellular response to the IGFs. We have noted that an M_r 31,000 IGFBP is associated with fibroblast surfaces and is released from fibroblast surfaces with IGFBP-3 during binding assays (4) and that the released IGFBP-5 alters IGF association with cell-surface binding sites. In addition, Andress and Birbaum (30) reported that IGFBP-5 released by a human osteoblast cell line can potentiate the mitogenic effect of IGF-I by 2-fold on this same cell type. Therfore, like IGFBP-3, IGFBP-5 has the potential to be an important modulator of IGF-I binding to fibroblast surfaces and could modify the fibroblast growth response to IGF-I. Its regulation by exposure to IGF-I suggests a model in which these growth factors may be able to autoregulate their interaction with cell-surface binding sites.

The change that occurred in IGFBP-5 binding activity in response to IGF-I and -II did not appear to be totally explained by changes in mRNA abundance. In 6-h stimulated cultures, IGF-I induced a 43% increase in IGFBP-5 mRNA abundance; but after 24 h, no change was detectable. Therefore, this small change in IGFBP-5 mRNA expression appears to be an early effect that is not quantitatively similar to the 6–8-fold increase in binding protein abundance that was detected in the conditioned medium. In contrast, IGFBP-3 mRNA was increased ~2-fold in response to IGF-I, which is similar to the change in binding protein abundance in media. Likewise, when quiescent cultures were exposed to forskolin, a 2.5-fold increase in IGFBP-5 mRNA abundance and a 3.5-fold increase in protein levels were noted, suggesting that the forskolin-mediated change in binding protein abundance was mediated by increased protein synthesis and was distinct from the mechanism by which the effects of IGF-I and -II were mediated.

Since insulin can bind to the IGF-I receptor, it would have been predicted that insulin exposure would result in an increase in the abundance of IGFBP-5 in conditioned medium. However, insulin had no effect, suggesting that the difference may be related to the fact that insulin cannot bind to the IGFBPs. We have previously reported that IGF analogs that have reduced binding affinity for IGFBPs did not alter the level of the M_r 31,000 IGFBP that was detected (31). More important, an analog that did not bind to the IGF-I receptor, but did bind the binding proteins, was equipotent in stimulating an increase in the M_r 31,000 form of IGFBP. These results suggest that IGF-I and -II must associate with IGFBP-5 to stimulate the increase in detectable IGFBP-5 in conditioned medium and that receptor binding is not required. It would be difficult to invoke a mechanism to explain this change that was based solely on increased mRNA abundance. Therefore, it seemed reasonable that this response might be related to the disappearance of IGFBP-5 fragments that were detected by immunoblotting in conditioned medium from IGF-treated cultures. The addition of IGF-I or -II to cultures containing intact pure IGFBP-5 resulted in diminution of fragment band intensity, suggesting that when the IGFs bind to IGFBP-5 in the media, proteolytic cleavage of this protein is inhibited. Although we have not definitively demonstrated the presence of a protease that degrades IGFBP-5, our results suggest that IGF-I is indirectly increasing IGFBP-5 abundance by inhibiting its proteolysis. Since insulin does not bind to IGFBP-5 and does not inhibit the formation of its M_r 23,000 fragment, this supports the conclusion that IGF-I and -II are inhibiting formation of the M_r 23,000 fragment by binding to the intact binding protein. We propose that this change accounts for part of the 6–8-fold increase in IGFBP-5 that is induced by IGF-I and -II. However, since the increase in intact IGFBP-5 was not always accompanied by a concomitant decrease in fragments (Fig. 3), we cannot exclude the possibility that IGF-I and -II may have a direct effect on IGFBP-5 secretion. The importance of IGF-induced inhibition of IGFBP-5 proteolysis is that it could provide a distinct means for modulating the effect of the IGFs on this cell type and for regulating the amount of IGFs that are available to bind to receptors, extracellular matrix, or other cell surface-associated binding sites. Because IGFBP-5 has been shown to potentiate the effects of IGF-I on cultured osteoblasts (30), it appears that this may be a positive feedback system wherein

the effect of IGF-I is augmented by modulating IGFBP proteolytic activity. This interesting potential mechanism deserves further study.

This response appears to be specific for IGFBP-5 since no fragments of IGFBP-3 or -4 were detected. Furthermore, the band intensity of IGFBP-4 decreased after exposure to IGF-I, and this change did not appear to be due to acceleration of proteolysis. However, no change in IGFBP-4 mRNA abundance was noted, suggesting that the effect was mediated by inhibiting its release into medium. Because IGFBP-4 has been shown to be a potent inhibitor of IGF-mediated cell growth (32), coordinate stimulation of IGFBP-5 with concomitant inhibition of IGFBP-4 by IGF-I would provide a mechanism for regulating the effects of these binding proteins to allow for induction of a mitogenic signal by IGF-I. Likewise, agents such as forskolin that increase IGFBP-4 might inhibit IGF-mediated fibroblast growth through this mechanism. Whether or not such coordinate signaling occurs in other cell types deserves further analysis.

Control of IGFBP-3 secretion by cultured fibroblasts has been analyzed by several investigators. Martin and Baxter (33) have reported that TGF-β induces a 4-fold increase in IGFBP-3 secretion. We detected no effect of TGF-β, but our cultures were grown in calf serum, whereas theirs were grown in fetal bovine serum. Because culturing fibroblasts in fetal bovine serum results in a decrease in the basal rate of IGFBP-3 secretion,[2] the difference in our results could be due to the presence of lower amounts of TGF-β in calf serum or some other factor that is necessary for TGF-β to stimulate IGFBP-3 secretion. Our study also shows that forskolin caused an increase in IGFBP-3. In addition, in this study and in that reported by Martin and Baxter (7), IGF-I and -II increase the secretion of IGFBP-3, and this change appears to be due to enhanced synthesis. We have shown that IGFBP-3 that is released into culture medium by fibroblasts can directly alter IGF-I interaction with cell-surface binding sites (34) since its affinity for IGF-I is significantly greater than that of the IGF-I receptor. Both Blum *et al.* (35) and DeMellow and Baxter (8) have shown that exposure of fibroblast cultures to IGFBP-3 potentiates the cellular growth response to IGF-I. Therefore, factors that control the secretion of IGFBP-3 in fibroblasts, such as IGF-I or cAMP, may modulate IGF interaction with the cell-surface binding sites and cellular growth responses. Likewise, because unsaturated IGFBP-3 in medium can inhibit IGF binding (34) and cell growth (8), coordinate regulation of IGFBP-3 secretion may be a mechanism for determining whether IGFBP-3 potentiates or inhibits IGF target cell actions.

These results provide several potential mechanisms by which the IGFs may regulate IGFBP abundance in fibroblast conditioned medium. Because the IGFBPs are modulators of cellular responsiveness to the IGFs, delineation of these mechanisms may provide new insights into how this family of growth factors functions to stimulate cell growth.

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[2] C. Camacho-Hubner, W. H. Busby, Jr., R. H. McCusker, G. Wright, and D. R. Clemmons, unpublished data.

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