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Cloning and Sequence Determination of Bovine Insulin-Like Growth Factor Binding Protein-2 (IGFBP-2): Comparison of Its Structural and Functional Properties With IGFBP-1

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Abstract Insulin-like growth factor binding proteins (IGFBPs) are secreted by several cell types and can modify IGF actions. Mandin-Darby Bovine Kidney (MDBK) cells have been shown to secrete a 34,000 Da form of IGF binding protein whose N-terminal sequence is similar to a form of IGFBP purified from rat BRL-3A cells that has recently been named IGFBP-2. These studies report the complete amino acid sequence of bovine IGFBP-2 and compare its functional properties with human IGFBP-1. The protein is 81% identical to rat IGFBP-2. When compared with both rat IGFBP-2 and human IGFBP-1, the positions of all 18 cysteine residues are conserved. Similarly an RGD sequence is present near the carboxyl terminus in both proteins. IGFBP-2 has a higher affinity for IGF-II than for IGF-I and its affinity for both forms of IGF is greater than for human IGFBP-1. Like IGFBP-1 the protein can enhance the DNA synthesis response of porcine aortic smooth muscle cells to IGF-I; however, IGFBP-2 was much less potent. The maximum potentiation of the IGF-mediated mitogenic response that could be achieved was approximately 42% that of IGFBP-1. This potentiation is dependent upon a factor contained in platelet poor plasma and if this factor is omitted from the incubation medium, IGFBP-2 inhibits DNA synthesis. The purification of IGFBP-2 will allow more detailed comparisons to be made between it and other forms of IGFBPs in physiologic test systems.

Key words: cell growth, somatomedins, growth regulation, binding protein, smooth muscle

Insulin-like growth factors I and II (IGF-I and II) are peptides that are present in several types of physiologic fluids and stimulate mitogenesis or potentiate differentiated cellular functions in a wide variety of cell types (Van Wyk, 1985). The IGFs are present in plasma and extracellular fluids bound to specific binding proteins (IGFBPs) (Ooi and Herington, 1988; Binoux et al., 1986). Recently, six forms of IGFBPs have been isolated and their structures determined. IGFBP-1 (Brewer et al., 1988; Julkunen et al., 1988) and IGFBP-2 (Szabo et al., 1988; Binkert et al., 1989; Brown et al., 1989) are distinct proteins whose plasma concentrations are inversely related to growth hormone (Binoux et al., 1986). IGFBP-3 is a glycoprotein whose plasma concentrations are directly related to GH secretory status (Baxter and Martin, 1986). The structures of IGFBP-4 (Shimasaki et al., 1990) and IGFBP-5 and 6 (Kiefer et al., 1991) have been determined, but their regulation by GH and IGF-I has not been determined.

While one source of circulating IGFBPs appears to be the liver (Schwander et al., 1983),

Abbreviations used: IGF-I and IGF-II, insulin-like growth factors I and II; IGFBP, IGF-binding proteins; TEMED, N,N,N′,N′-tetramethylethylenediamine; BSA, bovine serum albumin; PEG, polyethylene glycol; TFA, trifluoroacetic acid; DMEM, Dulbecco's modified Eagle's Minimal Essential Medium; NC, nitrocellulose; TBS, Tris buffered saline; RT, room temperature; RP-HPLC, reverse phase-high pressure liquid chromatography; FBS, fetal bovine serum; PPP, platelet poor plasma; SDS, sodium dodecyl sulfate; MDBK, Madin Darby Bovine Kidney; Tris, Tris (hydroxymethyl) amino methane; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; RIA, radioimmunoassay; HBSS, Hanks Basic Salt Solution.

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IGFBPs have been detected in amniotic fluid (Busby et al., 1988a; Drop et al., 1984; Povoa et al., 1984), cerebrospinal fluid (Hossenlopp et al., 1986a), and additionally are secreted by various cell types, including skeletal and smooth muscle (McCusker and Clemmons, 1988; Hill et al., 1985), fibroblasts (Clemmons et al., 1987; Adams et al., 1984; Atkinson et al., 1980), and hepatocytes (Mottola et al., 1986; Romanus et al., 1987). Some of these cell types also secrete IGF-I and II or IGF-like peptides. The interaction of the IGFs with several different IGFBPs in the local cellular microenvironment compounds the difficulty in understanding the mechanism by which the IGFs interact with specific cell surface receptors to regulate cell growth. Many diverse functions have been proposed for the IGFBPs, including transport of IGFs in blood, storage of IGF, inhibition of IGF action by preventing IGF access to IGF receptors, potentiation of the mitogenic response by providing continuously available hormone and providing a mechanism of tissue specific IGF actions (Hintz, 1984; Hall et al., 1988; Elgin et al., 1987; Rutanen et al., 1988). In addition to an incomplete understanding of these IGFBP functions, it is not clear what the net effect of more than one type of IGFBP would be on IGF action. In order to address these questions, our laboratory and others have purified individual forms of the IGFBPs. This paper describes the complete primary structural determination of bovine IGFBP-2 and compares its growth promoting, IGF binding, and cell surface associating properties with human IGFBP-1.

MATERIALS AND METHODS

Materials

Phenyl-Sepharose (CL-4B) was obtained from Pharmacia-LKB, Uppsala, Sweden. Tris, SDS, TEMED, and polyacrylamide were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Urea, HPLC grade acetonitrile, EDTA, and Tween 20 were obtained from Fisher Scientific, Fair Town, NJ. Ammonium persulfate, BSA, sodium phosphate, and PEG (8,000) were obtained from Sigma Chemical Co., St. Louis, MO. TFA was purchased from Pierce Chemical Co., Rockford, IL, and glycine, bromophenol blue, and glycerol were obtained from Serva, Heidelberg, Germany. Prestained high molecular weight standards (Amersham Corp., Arlington Heights, IL) were used for gel electrophoresis utilizing a Mighty Small II apparatus (Hoeffer Scientific Instruments, San Francisco, CA). Transfer of proteins to nitrocellulose filters (Schleicher and Schull, Inc., Keene, NH) was performed with the semidry electroblotter (Janssen Life Sciences Products, Piscataway, NJ). X-omat RP film was purchased from Eastman Kodak Co., Rochester, NY.

An MDBK cDNA library that was prepared in lambda ZAP II and a picoBlue immunoscreening kit (goat anti-rabbit) were purchased from Stratagene, LaJolla, CA. Nitrocellulose was obtained from Schleicher and Schuell, Keene, NH. The reagents for DNA sequencing were supplied by U.S. Biochemical, Cleveland, OH. ^{35}S radionucleotide was ordered from New England Nuclear, Wilmington, DE. DNA restriction endonucleases and modifying enzymes were purchased from New England Biolabs, Beverly, MA.

Methods

Protein purification. *Preparation of conditioned media.* MDBK cells (ATCC CCL22) were grown to confluence in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum in Nunc Cell Factories (Roskilde, Denmark). Confluent cultures were then maintained in serum-free Dulbecco's modified Eagle's Minimal Essential Medium without phenol-red. Conditioned media was collected every 72 hours and stored at −20°C.

IGF-I affinity column. Approximately 3 g of Affi-gel 10 (Bio-Rad, Richmond, CA) was exposed to 1.1 mg of recombinant IGF-I (Monsanto, St. Louis, MO) in 0.1 M Hepes, pH 7.5 for 4 hours. The unreacted sites were blocked with 1.5 ml of 1.5 M Tris-HCl, pH 8.0. The total binding capacity of the gel was approximately 11–12 nmoles of IGFBP-2 at pH 7.0.

Chromatographic procedures. Twenty liters of MDBK conditioned medium (MDBK cm) were centrifuged to remove cellular debris. The pH of the supernatant was adjusted to 7.2 and the salt content of the medium was increased to 0.5 M NaCl. Between 2 to 2.6 L were loaded onto a phenyl-Sepharose column (3.1 × 6.5 cm) previously equilibrated with 0.05 M Tris, 0.5 M NaCl, pH 7.2. After sample loading, the column was washed with equilibrating buffer, and subsequently eluted in three steps with: 0.02 M Tris, pH 9.0, H_2O, and 4 M urea. The activity of each fraction was measured by the IGF-I binding assay (see following).

The activity eluted at pH 9.0 and with H_2O. These fractions were pooled separately and the pH of each adjusted to 7.0. Each pool was then applied to the IGF-I affinity column (pre-equilibrated with 0.05 M Tris, pH 7.0), at 0.24 ml/min over periods of 5–18 hours. Following washing with 0.05 Tris, pH 7.0 and 0.002 M Tris, pH 7.0, [^{125}I]-IGF-I binding activity was eluted with 0.5 M acetic acid. Since extraction of the IGF binding activity was incomplete (average 40% after a single pass over the affinity column), samples were re-run until the binding activity measured in the "flow through" was 15% or less of the starting sample.

The IGF-affinity purified IGFBP fractions were pooled and 1–2 ml aliquots further purified using a Vydac C-4 HPLC. The major protein peak, containing IGF-I binding activity was lyophilized and reconstituted with H_2O.

Analytical methods. *^{125}I-IGF-I binding assay.* The presence of IGF-I binding activity in column fractions was determined using a previously described PEG precipitation method (McCusker and Clemmons, 1988). A 5–20 μl aliquot of each fraction was incubated with 20,000 cpm [^{125}I]-IGF-I (340 μCi/μg) and binding activity determined. The iodinated IGF-I was a gift from Dr. Louis Underwood. Nonspecific binding was determined by measuring the amount of ^{125}I-IGF-I that could be precipitated in the presence of 1.0 μg/ml unlabeled IGF-I. This value was consistently < 10% and was subtracted from the total radioactivity that was precipitated. To determine the capacity of the pure IGFBP-2 to bind IGF-I (recombinant human; Bachem, Inc., Torrance, CA) and II (recombinant bovine; Monsanto, Inc., Chesterfield, MO) between 1.0 and 75 ng/ml of each growth factor was incubated with 4.0 ng/ml IGFBP-2 and 20,000 cpm [^{125}I]-IGF-I (0.1 ng/ml) in the buffer described previously at 4°C. After 18 hours bound and free [^{125}I]-IGF-I were separated (McCusker et al., 1988).

IGFBP-2 iodination and radioimmunoassay. Following purification, pools of active fractions that had been saved at each step were quantified using a specific IGFBP-2 radioimmunoassay to determine recovery. Pure bovine IGFBP-2 was iodinated using a procedure that was nearly identical to that previously described (Busby et al., 1988a). Briefly 1.0 mCi [^{125}I]-NaI (Amersham, Arlington Heights, IL) was added to 75 μl of 0.5 M NaH_2PO_4, pH 7.5, containing 10 μg pure protein, followed by the addition of chloramine-T (0.20 mM final conc.) for 3.5 minutes. Iodinated protein was purified and stored as previously described (Busby et al., 1988b). Specific activity was approximately 20 μCi/μg. The RIA was performed also as previously described (Busby et al., 1988b) using a 1:200,000 final dilution of antibody (see below). The lower limit of detection was 40 pg/ml with 50% binding occurring at approximately 250 pg/ml.

SDS gel electrophoresis. The purity of the 34,000 Da IGFBP was determined by SDS-polyacrylamide gel electrophoresis with silver staining. The running gel was 12.5% acrylamide containing 0.375 M Tris, 0.1% SDS, pH 8.8, while the stacking gel was 4% acrylamide in 0.125 M Tris, 0.1% SDS, pH 6.8. Aliquots of samples were lyophilized and reconstituted in 27 μl of 0.1 M Tris, pH 6.8, 10% glycerol, 2% SDS, and 0.002% bromophenol blue. Samples to be analyzed following reduction of disulfide bonds were reconstituted in this same buffer containing 0.135 M dithiothreitol. Samples were then heated to 60°C for 10 minutes, clarified by centrifugation, and 25 μl loaded per lane. Samples were electrophoresed at 25 mA per gel until the dye front reached the bottom of the gel. Following electrophoresis, gels were either silver stained according to Oakley et al. (1980) or samples were transferred to nitrocellulose and ligand blotted (see below).

Ligand blotting. Following SDS gel electrophoresis, proteins were transferred to nitrocellulose using a semidry electroblotter for 75 minutes at 70 mA. Transfer, probing, and washing buffers were as described by Hossenlopp et al. (1986b). To probe for IGFBPs, 100,000 CPM of [^{125}I]-IGF-I/ml was added. Following sequential washing steps, the IGFBPs were visualized by autoradiography. Molecular weight estimates were determined from prestained standards.

Preparation of antisera. One hundred twenty micrograms of pure bovine IGFBP-2 was reconstituted in 800 μl of H_2O and added to an equal volume of complete Freunds adjuvant (Sigma, St. Louis, MO). After thoroughly mixing, 25 μl aliquots were injected intradermally, at multiple sites, in a single New Zealand white rabbit (Franklin Rabbitry, Wake Forest, NC). After 4 weeks, the rabbit was reinjected subcutaneously with 60 μg IGFBP-2 in 1 ml 50% incomplete Freund's adjuvant. Thereafter, the rabbit was bled at 2 week intervals (10–15 ml/bleed) and reimmunized with 20 μg IGFBP-2 every 4 weeks.

Immunoblotting. Following SDS gel electrophoresis and transfer of proteins to nitrocellulose (NC), the filters were washed for 5 minutes in 50 mM Tris HCl, 200 mM NaCl, pH 7.4 (TBS) and then blocked for 15 minutes at RT with TBS containing 3% BSA. The filters were incubated for 90 minutes at RT in TBS containing 1% BSA and a 1:2000 dilution of IGFBP-2 antisera. Using this dilution, 0.1 ng of pure IGFBP-2 could be detected. Following 3 10-minute washes in TBS containing 0.1% NP-40 and 0.03% Triton X-100, and a final wash in TBS, the filters were incubated for 2 hours at RT with anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) diluted 1:500 in TBS containing 1% BSA. The NC was again washed sequentially with TBS containing 0.1% NP 40 and 0.03% Triton X-100 (3 × 10 minutes), followed by TBS without detergent. The filters were then washed for 2 minutes in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.2. Color was developed using the ProtoBlot System (Promega Biotec, Madison, WI) by transferring the NC paper to 5 ml of 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.2, containing 33 μl Nitro Blue tetrazolium chloride substrate and 16.5 μl 5-Bromo-4-chloro-3-indolylphosphate substrate. Color was allowed to develop for up to 45 minutes at 22°C and was stopped by the addition of 10 ml of 20 mM Tris-HCl, 5 mM EDTA, pH 8.0. Molecular weight estimates were determined from prestained standards.

Amino acid composition and sequencing. HPLC-purified MDBK cell 34,000 Da IGFBP-2 was lyophilized and reconstituted into HPLC-grade water (Burdick and Jackson, Muskegon, MI). Approximately 60 pmoles were sequenced directly using an Applied Biosystems, Inc., model 470A sequencer equipped with on-line analysis using an Applied Biosystems, Inc., model 120A PTH Analyzer (Hunkapiller et al., 1983). An additional aliquot of 180 pmoles was reduced and S-pyridylethylated in solution according to the method of Hawke and Yuan (1987) in order to verify the presence of cysteinyl residues.

A subsequent aliquot of 1,800 pmoles of HPLC purified binding protein was reconstituted after lyophilization with 200 μl of 100 mM Tris-HCl, pH 8.1. An aliquot (45 pmoles) was subjected to acid hydrolysis (6N HCl, 1% phenol, evacuated sealed tube) at 115°C for 24 hours. Compositional amino acid analysis was performed on the hydrolysate using post-column ninhydrin derivatization and a Beckman model 6300 Auto-analyzer. In addition, approximately 1.3 nmoles of the sample was digested with V8 protease (Endoproteinase Glu-C, Boehringer-Mannheim, Indianapolis, IN) in order to release internal fragments, specifically following glutamic acid residues. The proteolysis was carried out using an enzyme to substrate ratio of 1:45 in 100 mM Tris-HCl, pH 8.1 and a time course of 1 hour at 37°C followed by 16 hours at RT. After acidification with 10% trifluoroacetic acid (TFA), 90% of the digest was subjected to reverse-phase HPLC on an Aquapore (RP-300) 2.1 × 30 mm column (Brownlee Laboratories, Santa Clara, CA) in 0.1% TFA with acetonitrile as the mobile phase using an Applied Biosystems, Inc., Foster City, CA, model 130A Separation System. The peptides were separated and eluted using a gradient of 0 to 70% B over 50 minutes followed by ramping to 100% over an additional 10-minute period (where B = 70% acetonitrile in 0.085% TFA). A flow-rate of 100 μl/min was used. Column fractions were hand-collected directly onto TFA-etched, GF/C filter discs (Applied Biosystems, Inc.). After air-drying and storage at −20°C, fragments were sequenced as per above albeit after topical application of 30 μl of Polybrene solution containing 3% polybrene, 0.1 M NaCl.

c-DNA clone isolation and sequencing. To isolate the bovine IGFBP-2 cDNA, an MDBK cell cDNA library in the lambda ZAP II vector was used to express the IGFBP-2 protein. 1.0×10^6 recombinant plaques from this library were screened in *E. coli* XL-1 Blue cells with a 1:2000 dilution of a rabbit polyclonal antibody, prepared using purified MDBK IGFBP-2 as described (above). Positive signals were detected using an alkaline phosphatase conjugated goat anti-rabbit second antibody as described by the manufacturer (Stratagene). The resulting positive clones were in vivo excised using the helper phage R408. The rescued phagemid clones were sequenced using dideoxy chain termination sequence analysis with modified T7 DNA polymerase (Sequenase TM) (Sanger et al., 1977). Both strands of DNA were completely sequenced through the coding region. The non-coding regions were determined by sequencing only a single strand of the DNA.

DNA synthesis determinations. Porcine aortic smooth muscle cells were grown in 10 cm dishes (Falcon 3001) in DMEM supplemented with 10% fetal bovine serum (FBS). The culture media were changed every three days. The cells

were passaged weekly, as previously described (Elgin et al., 1987). To test for ^{3}H-thymidine incorporation, the cells were seeded at 5,000 cells/well in 96-well microtest plates in DMEM with 10% FBS. After 5 days the wells were washed once with serum-free DMEM, then exposed to DMEM containing 0.1% human platelet poor plasma (PPP) (McCusker and Clemmons, 1988) and 0.5 μCi ^{3}H-thymidine. Test wells received IGF-I (10 ng/ml) and various concentrations of IGFBP-2 (0.5–10 ng/ml). Following a 36-hour incubation, ^{3}H-thymidine incorporation into DNA was determined as previously described (Elgin et al., 1987).

Cell adherence. MDA-231 cells (gift from Dr. K. Osborne, University of Texas, San Antonio), a human breast carcinoma cell line, were grown to confluency in EMEM supplemental with 10% bovine serum. The confluent cultures were washed twice with serum-free DMEM and exposed to pure IGFBP-1 or 2 for 6 hours at 8°C. After that time the plates were rinsed in HBSS, [^{125}I]-IGF-I (80,000 cpm/well) was added and IGF-I binding determined as previously described (Clemmons et al., 1987). Non-specific binding was determined in an excess (100 ng/ml) of unlabeled IGF-I and was subtracted from all values.

RESULTS

Elution of IGF binding activity from the phenyl-Sepharose column demonstrated that approximately 46% (as determined by RIA) of the starting activity was recovered. An approximately 100-fold purification was achieved at this step (Table I). The pool of active fractions was further purified by IGF-I affinity chromatography. Approximately 85 to 90% of the total activity was eluted with 0.5 M acetic acid. Final purification was achieved using reverse phase HPLC. The 34,000 Da form of IGFBP (IGFBP-2) eluted as a single peak at 29% acetonitrile (Fig. 1, fraction 9). Recovery of binding protein was determined at each step using the IGFBP-2 RIA (Table I). The final yield was estimated to be 6.5 mg. This represented 38% overall recovery. The IGF affinity column provided the most effective purification step, removing greater than 95% of the contaminating proteins while recovering 85–90% of the total binding activity.

Purity of the final product was determined by SDS PAGE analysis with silver staining. The purified material showed two bands—a major one of 34,000 Da and a minor one of 72,000 Da

TABLE I. Recovery of IGFBP-2 During Purification

Step	Total protein mg	IGFBP-2 mg	Recovery[a] % Step	Recovery[a] % Overall
1. MDBK cm (19 L)	—	17.2	—	—
2. Initial supernatant	4,106.0	17.2	100	100
3. Phenyl-Sepharose	40.6	7.95	46	46
4. IGF affinity	8.0	7.6	96	44
5. C-4 HPLC	6.5	6.5	86	38

[a]Recoveries based on known quantities of pure IGFBP-2 used as standard in the RIA (see Methods). Total protein estimates based upon bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL) using BSA as standard.

(Fig. 2, panel A, lane 1). Ligand and immunoblotting of the pure material showed that both bands were immunoreactive and that both bound IGF-I (Fig. 2, panel B). Two findings suggest that the minor band represents a dimer of the 34,000 Da binding protein: 1) Ligand blotting analysis showed that the band at 72,000 Da has the capacity to bind IGF-I (Fig. 2, panel B, lane 1); 2) Reduction of the purified protein with dithiothreitol resulted in the disappearance of the 72,000 Da band with only a single band of 34,000 Da remaining (Fig. 2, lane 2). This 34,000 Da band could be detected with anti-IGFBP-2 antiserum, but reduction destroyed its capacity to bind IGF-I (data not shown). The antibody had a high degree of specificity for IGFBP-2. There was $< 0.2\%$ crossreactivity for hIGFBP-1 and bIGFBP-3, $< 0.1\%$ for hIGFBP-4, and no crossreactivity for hIGFBP-5.

The N-terminal sequence and several internal sequences derived from V8 protease digestion were obtained (Fig. 3). To complete the sequence analysis cDNA sequence analysis was performed; 1.0×10^{6} recombinant plaques were screened with the anti-IGFBP-2 antisera. Seven plaques that gave positive hybridization signals were plaque purified in vivo, excised, and recircularized with the lambda vector to form the phagemid pBluescript containing the cloned insert. The clones had inserts ranging in size from approximately 1.0 Kb to 1.7 Kb. Restriction analysis with EcoRI and BamHI showed that all the clones were related. The longest cDNA clone (1,772 bp) was sequenced. The nucleotide sequence and the predicted amino-acid sequence are shown in Figure 3. This cDNA contains 388

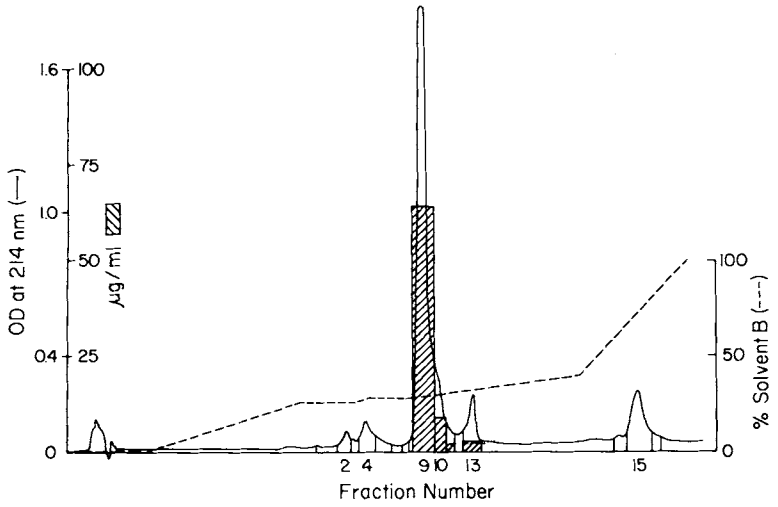

Fig. 1. HPLC purification of IGF binding protein. The IGF-affinity purified binding protein was reconstituted in H_2O and injected onto a Vydac C-4 reverse phase column (4.6 mm × 25 cm). The sample was eluted isocratically for 5 minutes with 100% solvent A (0.04% trifluoroacetic acid in dH_2O), followed by the indicated (----) gradient to 100% solvent B (0.04% trifluoroacetic acid in acetonitrile) over the next 60 minutes. The flow rate was 1.5 ml/min and the absorbance was monitored at 214 nM (----). The 34,000 Da IGF binding protein (detected by RIA) eluted as the major absorbance peak (□) at 29% B (fraction 9).

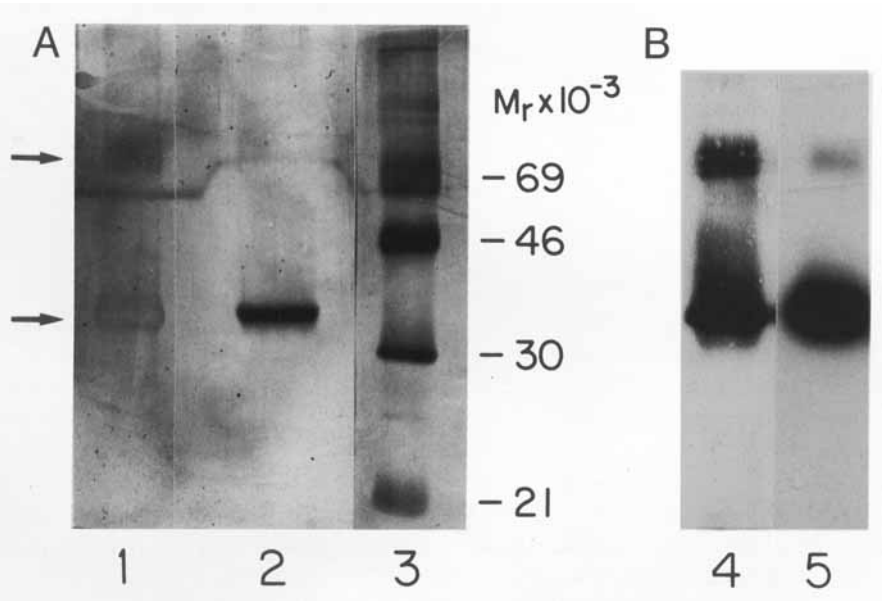

Fig. 2. Panel A: Protein purity. One to two micrograms of protein from fraction 9 was analyzed by 12.5% SDS polyacrylamide gels using the buffer system and silver staining procedures as described in Methods. **Lane 1:** HPLC purified IGFBP-2. This sample contained the 34,000 Da IGFBP-2 and its dimeric 72,000 Da form (arrows). It was used for amino acid sequence determination and biological studies. **Lane 2:** same as lane 1, but following reduction with 0.13 M dithiothreitol. **Lane 3:** molecular weight standards. **Panel B:** Ligand and immunoblotting of pure IGFBP-2. Proteins from fraction 9 of the HPLC column in Figure 1 were separated by SDS PAGE (12.5% gel) and following transfer to nitrocellulose, were blotted with [^{125}I]-IGF-I (**lane 5**) or anti-IGFBP-2 antisera (**lane 4**) as described in Methods. The arrows indicate the 72,000 and 34,000 Da IGFBP-2 bands.

nucleotides of 5′ untranslated sequence followed by an open reading frame encoding 317 amino acids, which is followed by 381 nucleotides of 3′ untranslated sequence and includes a poly A tail. The predicted amino acid sequence from this cDNA clone contains peptide sequences that exactly match the sequences of all six peptides that had been determined from the native protein (Fig. 3). The signal peptide of IGFBP-2 is 33 amino acids in length based on

Fig. 3. Nucleotide and predicted amino acid sequences of IGFBP-2. The underlined regions indicate peptides that were sequenced from the native protein. Asterisks show the first amino acid of the mature protein. The RGD sequence is indicated in a box.

the position of the first amino acid, determined by protein sequence analysis and confirmed by the position of the first amino acid in both the human protein and rat protein sequence. Therefore, the mature IGFBP-2 protein is most likely 284 amino acids in length. The predicted amino acid sequence contains one RGD sequence and no N-linked glycosylation sites (Bahl and Shah, 1977). There are a total of 18 cysteine residues in the mature protein analogous to those in rat IGFBP-2, hIGFBP-1, and hIGFBP-3. During the preparation of this manuscript the complete sequence of bovine IGFBP-2 was reported by Upton et al. (1990). Our sequence differs from that reported at positions 27, 28, 81, 95, 101, and 280. Comparison of our sequence results with the human IGFBP-2 sequence reveals that the amino acids reported at positions 28, 95, 101, and 280 in our sequence are identical to those in the human. Bovine IGFBP-2 has a high degree of amino acid homology to IGFBP-2 sequences reported from other species. Specifically, there is 81% identity to the rat IGFBP-2 and 87% identity to the human IGFBP-2 sequence (Fig. 4).

The IGF binding specificity of the pure protein was quantitated. Figure 5 shows competition for binding between ^{125}I-IGF-I and unlabeled IGF-I or II. Fifty percent competition occurred at approximately 8 ng/ml IGF-I and 2 ng/ml IGF-II (Fig. 5a). To determine the affinity of the protein for IGF-I and II, Scatchard analysis was performed. The results show binding affinities of 3.6×10^9 M^{-1} for IGF-II and 2.1×10^9 M^{-1} for IGF-I (Fig. 5b).

To compare the biologic activity of bovine IGFBP-2 to human IGFBP-1, 10 ng/ml of IGF-I and increasing concentrations of IGFBP-1 or -2 were added to quiescent smooth muscle cell cultures and their effects on DNA synthesis were determined. As shown in Figure 6A, IGFBP-2 was not as active in stimulating DNA synthesis compared with IGFBP-1. Specifically, this form of IGFBP induced an approximately

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                                                                  *        50
Bovine  MQPRLGGPAL .LLLPP.... ..LLLLLLLG AGGGDCGARA EVLFRCPPCT
 Human  MlPRvGcPAL .pLpPPpllp llpLLLLLLG AsGGggGARA EVLFRCPPCT
   Rat  MlPRLGGPAL pLLLPs.... ..LLLLLLLG AGGcgpGvRA EVLFRCPPCT

                                                                  100
Bovine  PESLAACKP. ...PPGAAAG PAGDARVPC. ELVREPGCGC CSVCARLEGE
 Human  PErLAACgPp pvaPPaAvAa vAGgARmPCa ELVREPGCGC CSVCARLEGE
   Rat  PErLArCgP. ...PPdApca ..........  ELVREPGCGC CSVCARqEGE

                                                                  150
Bovine  RCGVYTPRCG QGLRCYPNPG SELPLRALVH GEGTCEKHGD AEYSASPEQV
 Human  aCGVYTPRCG QGLRCYPhPG SELPLqALVm GEGTCEKrrD AEYgASPEQV
   Rat  aCGVYiPRCa QtLRCYPNPG SELPLkALVt GaGTCEKr.. .rvgAtPqQV

                                                                  200
Bovine  ADNGEEHAEG GLVENHVDGN VNLMGGGGGA GRKPLKSGMK ELAVFREKVT
 Human  ADNGddHsEG GLVENHVDst mNmlGGGGsA GRKPLKSGMK ELAVFREKVT
   Rat  ADseddHsEG GLVENHVDGt mNmlGGssag rkpP.KSGMK ELAVFREKVn

                                                                  250
Bovine  EQHRQMGKGG KHHLGLELPK KLRPPPARTP CQQELDQVLE RISTMRLPDE
 Human  EQHRQMGKGG KHHLGLEEPK KLRPPPARTP CQQELDQVLE RISTMRLPDE
   Rat  EQHRQMGKGa K.HLsLEEPK KLRPPPARTP CQQELDQVLE RISTMRLPDd

                                                                  300
Bovine  RGPLEHLYSL HIPNCDKHGL YNLKQCKMSL NGERGECWCV NPNTGKLIQG
 Human  RGPLEHLYSL HIPNCDKHGL YNLKQCKMSL NGqRGECWCV NPNTGKLIQG
   Rat  RGPLEHLYSL HIPNCDKHGL YNLKQCKMSL NGqRGECWCV NPNTGKpIQG

                                                          331
Bovine  APTIRGDPEC HLFYNEQQCA RGVHTQRMQ
 Human  APTIRGDPEC HLFYNEQQeA cGVHTQRMQ
   Rat  APTIRGDPEC HLFYNEQQen dGVHaQRvQ
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Fig. 4. Homology between rat, human, and bovine IGFBP-2. Sequences are aligned and gaps introduced to maximize homologies. Amino acids which are conserved between bovine and either rat or human IGFBP-2 are shown by upper case letters, whereas lower case letters denote differences. The asterisk indicates the first amino acid of the mature proteins.

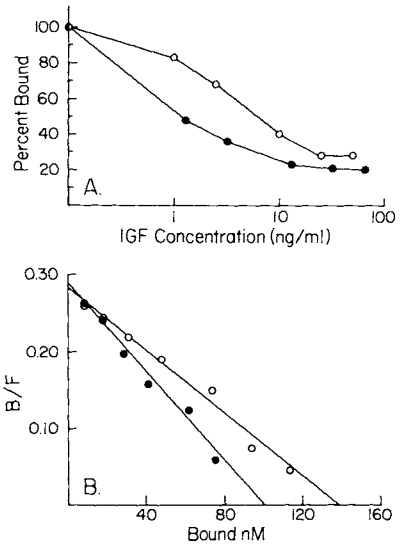

Fig. 5. IGF binding properties of pure IGFBP-2. Increasing concentrations (1–75 ng/ml) of IGF-I (○—○) or IGF-II (●—●) were added with a constant amount (4 ng/ml) of IGFBP-2 **(panel A)** and [^{125}I]-IGF-I (20,000 cpm). To determine the binding affinity, the assay was repeated using concentrations of IGF-I (○—○) and IGF-II (●—●) between 0.4 and 100 ng/ml and a constant amount (2 ng/ml) of IGFBP-2 **(panel B).** Specific binding was determined in each IGF binding capacity assay as described in Methods. The data were analyzed by the method of Scatchard.

2.2-fold increase in smooth muscle cell ^{3}H-thymidine incorporation compared with a 4.8-fold increase induced by IGFBP-1. To exclude the possibility that IGFBP-2 was potentiating the cellular replication response by simply inhibiting degradation of IGF-I, radiolabelled IGF-I was incubated with IGFBP-2 and the smooth muscle cell cultures with and without PPP. The rate of degradation was slow (e.g., a 15% loss of TCA precipitable activity in 24 hours) and was not altered by the presence of IGFBP-2. If platelet poor plasma was omitted from the incubation medium, IGFBP-2 inhibits the IGF-I stimulated increase in ^{3}H-thymidine incorporation. The maximum inhibitory response that was induced with IGFBP-2 was 3.3-fold greater than IGFBP-1 (Fig. 6B). Inhibition appears to require IGF-I binding to IGFBP-2, since we have shown that IGF analogues that do not bind IGFBP-2 are more potent than native IGF-I in stimulating smooth muscle cell DNA synthesis in the absence of PPP (Clemmons et al., 1990). Since the smooth muscle cells secrete IGFBP-2 (McCusker et al., 1989), the analogues appear to be more effective because they have reduced affinity for IGFBP-2.

To determine if IGFBP-2 adhered to cell surfaces, MDA-231 cells were transiently exposed to IGFBP-1 or 2 and [^{125}I]-IGF-I binding assessed. Both proteins increased [^{125}I]-IGF-I binding to cell surfaces. However, there were significant differences between the two proteins, since IGFBP-1 enhanced [^{125}I]-IGF-I binding by 48%, whereas IGFBP-2 was less potent, increasing binding by 11% (Fig. 7).

DISCUSSION

The analysis of the primary structure of IGFBP-2 reveals several similarities to IGFBP-1 that may be related to important functional properties of these proteins. Comparison of the bovine IGFBP-2 sequence to human IGFBP-1 reveals identical alignment of the 18 cysteine residues (Brewer et al., 1988). These cysteines also align with those that are present in IGFBP-3, IGFBP-5, and IGFBP-6, suggesting that the cysteine positioning is a property of IGFBPs that is necessary for a critical function, such as IGF binding. Unlike IGFBP-3, -4, and -5, IGFBP-1 and -2 contain no glycosylation sites, and treatment of either protein with N or O glycanase does not result in a change in esti-

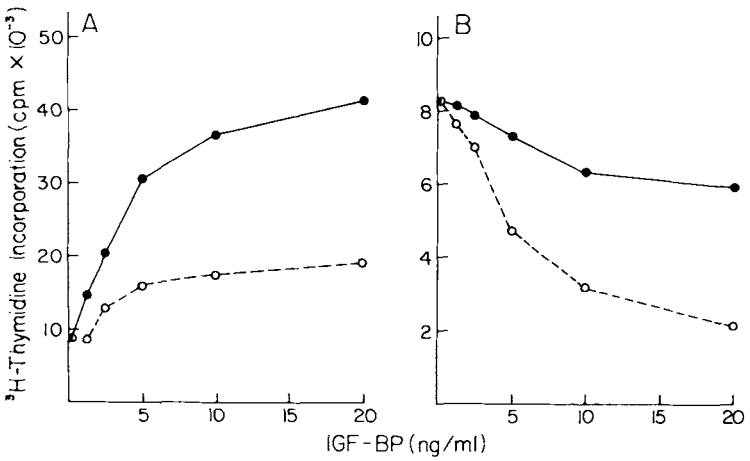

Fig. 6. Cellular replication response to IGFBP-2. Quiescent smooth muscle cell cultures were exposed to increasing concentrations of IGFBP-2 (O—O) or IGFBP-1 (●—●) in the presence of IGF-I (10 ng/ml) and 0.2% PPP in 0.2 ml DMEM **(Panel A).** After 36 hours ^{3}H-thymidine incorporation was determined as described in Methods. Each point represents the mean of triplicate determinations. Cultures in **Panel B** were exposed to increasing concentrations of IGFBP-1 (●—●) or IGFBP-2 (O—O) plus IGF-I (10 ng/ml) in the absence of PPP.

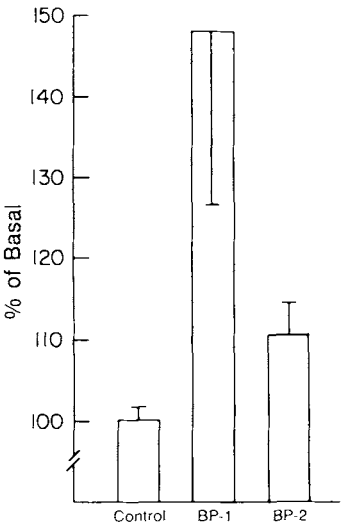

Fig. 7. Adherence of IGFBP-1 and 2 to cell cultures. Pure hIGFBP-1 or bIGFBP-2 (50 ng/ml) was incubated with confluent MDA-231 cell monolayers for 6 hours at 8°C as indicated. At that time the monolayers were washed and [^{125}I]-IGF-I binding determined as in Methods. The results are expressed as the percentage increase in binding above the basal level. Basal specific binding varied between 1,600 and 2,500 cpm. The results are the mean of duplicate determinations from 3 separate experiments.

mated molecular size. An arg-gly-asp sequence near the carboxyl terminus is present in both IGFBP-1 and 2. This sequence has been shown to mediate cellular attachment to matrix proteins and it has been proposed that it may be required for IGFBP-1 binding to cell surfaces (Busby et al., 1988a). The role of the RGD sequence in mediating IGFBP-2 bioactivity has not been determined. These structural similarities suggest possible functional similarities between IGFBP-1 and -2.

The 72,000 M_r band that was detected (Fig. 2) appears to represent a disulfide linked dimeric form of IGFBP-2, since the band is lost upon exposure of IGFBP-2 to DTT and a single 34 kDa immunoreactive band is detected. We have reported previously that IGFBP-1 forms multimeric units that are detected during the purification procedure and that these multimers result from intermolecular disulfide bond formation (Busby et al., 1989). These results suggest that either the individual cysteine residues that are involved in oligomer formation in IGFBP-1 and -2 may be similar or that other factors that control oligomer assembly of the two proteins are similar.

IGFBP-1 and -2 differ in their degree of sequence conservation across species. The N-terminal amino acid sequence of the bovine IGFBP-2 is similar to rat IGFBP-2 purified from Buffalo rat liver conditioned medium (Brown et al., 1989; Mottola et al., 1986; Romanus et al., 1987). Major homology with the rat and human forms of IGFBP-2 is shown by sequence identities of 81% and 87% (Brown et al., 1989; Margot et al., 1989), respectively. This homology indicates a high degree of conservation of the IGFBP-2 structure across species and contrasts sharply with IGFBP-1, wherein the sequence of the rat protein compared with human has many fewer identical amino acids (e.g., 50%) (Murphy et al.,

1990). The reason for this high degree of conservation compared with IGFBP-1 is not clear.

To analyze the role of IGFBP-2 in modulating IGF action, three types of studies were performed. Binding specificity studies showed that IGF-II had approximately 1.8-fold greater affinity for this protein compared with IGF-I. This result is in agreement with the results of Ballard and coworkers (Szabo et al., 1988). Scatchard analysis of the binding of IGF-I to IGFBP-2 revealed approximately 1:1 stoichiometry (Fig. 5), and the plot was linear. In contrast, when the binding of IGF-I to IGFBP-1 is analyzed, curvelinear Scatchard plots are obtained with the ligand (Busby et al., 1988a). We have recently shown that the curvelinear plot obtained with IGFBP-1 purified from amniotic fluid is due to the presence of both phosphorylated and nonphosphorylated forms of this protein. When the binding characteristics of pure phosphorylated or dephosphorylated IGFBP-1 are analyzed, the Scatchard plots are linear, and the phosphorylated form has a 6-fold higher affinity for IGF-I (Jones et al., 1991). This suggests that IGFBP-2 purified from MDBK cell conditioned medium is composed entirely of either a phosphorylated or dephosphorylated form. Compared with IGFBP-1 purified from amniotic fluid, IGFBP-2 has an approximately 3-fold higher affinity for IGF-I at pH 7.4. This finding suggests that in solution when equimolar concentrations of IGFBP-2 and IGFBP-1 are present, the IGF's will preferentially associate with IGFBP-2. However, it should be noted that this depends upon the phosphorylation state of IGFBP-1, since pure phosphorylated IGFBP-1 has a higher affinity than IGFBP-2.

Biologic activity studies showed that like IGFBP-1, IGFBP-2 had the capacity to potentiate the smooth muscle cell DNA synthesis response to IGF-I. However, IGFBP-2 was less potent than IGFBP-1, having only about 42% of its bioactivity. Since IGFBP-2 has a higher affinity for IGF-I at neutral pH compared with amniotic fluid-derived IGFBP-1, it might be expected to be a better inhibitor of IGF-I binding to cell surface receptors. This enhanced affinity may partially account for its decreased capacity to potentiate IGF-I actions. Since the phosphorylation state of IGFBP-1 is associated with significant changes in its affinity, our findings suggest that this post-translational modification of this protein may account for differences in its biologic activity. This might also account for the difference between our results and those of Upton et al., since the exact phosphorylation state of the IGFBP-2 that we purified and that purified by Upton et al. is unknown. Whether a particular form of IGFBP inhibits or potentiates IGF activity is not only dependent on its affinity for the ligand, but also on its capacity to bind cell surfaces (Busby et al., 1988a). When IGF-I binding to MDA-231 cells (a cell type that has no detectable IGFBPs associated with its cell surface) was assessed, IGFBP-1 induced a greater increase in IGF-I binding, compared with IGFBP-2. Since IGFBP-1 has a lower affinity for IGF-I, our binding data in Figure 7 suggests that IGFBP-1 binds to cell surfaces more avidly. The findings support the conclusion that both the ability to bind to cell surfaces and ligand affinity are physiologically important characteristics of IGFBPs and that differences in these properties may account for the observed differences in cell growth responsiveness to IGF-I.

We have previously shown that a factor in platelet poor plasma is required for IGFBP-1 to potentiate the cellular DNA synthesis response to IGF-I (Clemmons and Gardner, 1990). When PPP was omitted from the incubation medium both IGFBP-1 and -2 inhibited replication and IGFBP-2 was 3-fold more potent than IGFBP-1. The mechanism that accounts for this difference is not defined; however, it may relate to the observed differences in the affinity of each binding protein for IGF-I.

IGFBP-2 has been shown to be present in multiple body fluids, suggesting that it may have a major physiologic regulatory role. Not only has the protein been noted in blood and cell culture supernatants, but it also appears to be produced by hepatocytes, decidual cells, ovarian granulosa cells, aortic smooth muscle cells, cardiac muscle cells, and kidney (MBDK) cells. These findings suggest that the protein may be present in the local microenvironment of several cell types and available to modulate IGF action. Insulin appears to be a major regulatory variable that inhibits the synthesis of this protein (Ooi et al., 1990). Likewise, increased levels of this protein have been noted in hypophysectomized animals compared with normal animals (Ooi et al., 1990), and IGFBP-2 mRNA has been shown to be developmentally regulated in the rat fetus (Brown et al., 1989). These findings suggest that the protein may have a systemic

function in modulating the target cell actions of IGF-I and II.

In summary, we have purified and sequenced bovine IGFBP-2 and compared its functional properties with human IGFBP-1. The homogeneous product has very high sequence similarity to the rat and human forms of IGFBP-2. Like IGFBP-1, the protein can multimerize and can act as an inhibitor or a potentiator of IGF-I mediated DNA synthesis. These findings suggest that this protein will have an important role in mediating IGF-I actions at the target cell level and point to the need for future studies to address the role of how this protein functions in combination with other IGF binding proteins in extracellular fluids to modulate IGF actions.

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