Structural Determinants of Ligand and Cell Surface Binding of Insulin-like Growth Factor-binding Protein-3*

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Among the well defined insulin-like growth factor (IGF)-binding proteins (IGFBPs), IGFBP-3 is characterized by its interaction with an acid-labile glycoprotein (ALS) in the presence of IGFs. To identify the structural determinants on IGFBP-3 required for ligand binding and cell association, five recombinant human IGFBP-3 variants were expressed in Chinese hamster ovary cells: deletions of amino acids 89–264, 89–184, and 185–264, and site-specific mutations 228KGRKR → MDGEA and 253KED → RGD. The basic carboxyl-terminal region of IGFBP-3 was required for binding to heparin. The deletion variants had greatly decreased IGF binding ability as assessed by ligand blotting and solution binding assays; affinity cross-linking indicated at least a 20-fold decrease in IGF affinity. The RGD mutant had a 4–6-fold reduced affinity for both IGFs, but the MDGEA mutant bound IGF-1 with near normal affinity and IGF-II with a decrease in IGF affinity. The RGD mutant had a 4–6-fold reduced affinity for both IGFs, but the MDGEA mutant bound IGF-1 with near normal affinity and IGF-II with a 3-fold reduction in affinity. The three deletion variants were incapable of binding ALS; but of the site-specific variants, the MDGEA mutant bound ALS with 90% lower affinity (K_a = 2.5 ± 0.9 liters/nmol) than seen for rhIGFBP-3 (K_a = 24.3 ± 5.2 liters/nmol), whereas the RGD mutation had no effect on ALS affinity (K_a = 21.7 ± 4.5 liters/nmol). The ability of IGFBP-3 to associate with the cell surface was lost in variants lacking residues 185–264 and in the 228KGRKR → MDGEA variant. We conclude that residues 228–232 of IGFBP-3 are essential for cell association and are required for normal ALS binding affinity.

The insulin-like growth factor-binding proteins (IGFBPs) are a family of at least six related proteins involved in regulating the bioavailability of insulin-like growth factors, IGF-I and IGF-II. The IGFBP structure may be divided into cysteine-rich amino- and carboxyl-terminal domains, which show considerable structural conservation among IGFBP-1 to -6, and a central domain that is unique for each IGFBP. IGFBP-3, a glycoprotein of 40–45 kDa, is characterized by its ability to bind to another glycoprotein, the 85-kDa acid-labile subunit (ALS), in the presence of either IGF-I or IGF-II to form a ternary complex of 150 kDa (1). Because the majority of serum IGFs are bound in this form (2, 3), the ternary complex acts essentially as a circulating reservoir of IGFs and regulates the delivery of the IGFs to target tissues.

IGFBP-3 has been implicated at the cellular level as a modulator of IGF-I action (4, 5). Soluble IGFBP-3 can inhibit IGF-I activity by sequestering the peptide and consequently preventing interaction with its receptor. Prior to binding, potentialization of IGF-I action has been attributed to cell surface-associated IGFBP-3 (5). Several studies have also suggested that IGFBP-3 may modulate cell growth independently of IGFs (6–8). This IGF-independent growth-inhibitory effect was recently shown to be mediated by the direct induction of apoptosis by IGFBP-3 (9).

This multifaceted role of IGFBP-3 has led to extensive studies on its regulation, expression, distribution, and function in cultured cells and in animal models (3, 10–12). To date however, there have been few studies aimed at elucidating the structural determinants involved in the protein-protein and protein-cell interactions required for IGFBP-3 function. In this study we describe the generation and expression of cDNAs encoding the normal form, three deletion mutants, and two site-specific mutants of human IGFBP-3. The resulting recombinant proteins have allowed the delineation of domains involved in IGF-I and ALS binding and in cell surface association.

MATERIALS AND METHODS

Reagents—All radiolabeled proteins used were prepared as described previously (13, 14). Restriction enzymes were from Promega Corp. (Madison, WI). T7 DNA polymerase was from Pharmacia Biotech Inc. (Uppsala, Sweden). Pfu DNA polymerase was from Stratagene (La Jolla, CA). Hexahistidine bromide (Polybrene), dexamethasone, hydroxyamine, xanthine, thymidine, and mycophenolic acid were purchased from Sigma Chemical Co. (St. Louis, MO) and aminopterin from Life Technologies Inc. (Gaithersburg, MD). Nuclease-free α-modified Eagle’s medium (α-MEM) and fetal calf serum were from Cytosystems (North Ryde, NSW, Australia).

cDNA Constructs—A 1,080-base pair EcoRI-PstI fragment, excised from ipb.118 (15), containing the full coding sequence of hIGFBP-3 (provided by Dr. W. I. Wood, Genentech, South San Francisco, CA) was inserted into pSELECT (Promega). Using this recombinant plasmid as cDNA template and pairs of oligonucleotides (see below), fragments containing full or partial hIGFBP-3 coding sequences were amplified by Pfu DNA polymerase in polymerase chain reactions and cloned into the expression vector pMSG (Pharmacia). This generated expression plasmids rhIGFBP-3, rhIGFBP-3[89–264], rhIGFBP-3[185–264], and rhIGFBP-3[3a89–184] (Fig. 1A). Site-directed mutagenesis (16), employing oligonucleotides and the pSELECT-hIGFBP-3 plasmid as the mutagenesis vector, was carried out to introduce specific mutations in vitro. cDNA fragments amplified from these mutated plasmids were cloned into pMSG to generate expression plasmids rhIGFBP-3[3a253KED → RGD] and rhIGFBP-3[228KGRKR → MDGEA], each carrying the IGFBP-1 sequence analogous to the mutated region (Fig. 1A). The hIGFBP-3 coding sequences of each construct were verified by plasmid DNA sequencing (17).

Oligonucleotides were synthesized on an Oligo 1000 DNA Synthesizer (Beckman Instruments, Palo Alto, CA). Oligonucleotides I (5'-GTACGCTAGCCTGATCTGCGCCGCCATCC) and II (5'-CGGGTTCGAC-
AGGGGTCTACTTGCTGTC) introduce an NheI and SalI restriction site (indicated in italics), respectively, to aid cloning of the amplified product into pMSG. Oligonucleotides III (5'-GTATGCACTAGGGGCGCGGCGAGCCCGA) and IV (5'-CTTGTGACGACGAGCCAAGAGGAAGGCGTCTACTTGCTGTC) introduce a stop codon (indicated in bold) after amino acid residues 188 and 184, respectively, as well as a downstream SalI restriction site (indicated in italics). Oligonucleotides V (5'-ATTTAGGATCCCTTGAGGACGAGCCCGA) and VI (5'-GACTGGAATGAGAGAAAAGCAGTGTCGCCCTTCCAtgGaCgGGgAGgcGGGCTTCTGCTGG) contain BamHI sites (indicated in italics) which enabled the in-frame ligation of the amplification products using oligonucleotides I and V and oligonucleotides VI and VII. Oligonucleotides VIII (5'-CTTACAGCTCAACAGGGGGGCGGAGTCTCTGGTCGACCTCAGTACAGGG) and IX (5'-TFFTTATAGAAAAAGGTGCTGGCCCTTTCAAgCGGGAAGGGTCTGCTGGTGTTGGAGTTATGAGGGG) were used for replacing 3'X-EcoRI (AAG GAG) with RG (ggc Ggc) and 3'X-KRKK (AAA GGC AGG AAC CGG) with MDGEA (Arg GTC GGG GGA GGG), respectively. Nucleotides that differ from the IGFBP-3 sequence are indicated in lowercase letters.

Cell Culture and Transfection—CHO cells were grown in α-MEM supplemented with 10% (v/v) fetal calf serum at 37 °C. For transfection, the cells were plated out at 6 × 106 cells/75-cm² flask, incubated for 24 h, and then transfected with 20 μg of DNA of each rhIGFBP-3 expression plasmid or pMSG in the presence of 100 μg of Polybrene (18). The plasmids contain the gusA gene phosphoribosyltransferase (gpt) gene, which confers resistance to mycophenolic acid. The transfected cells were cultured in GPT selection medium for 21 days to select for stably transfected. The medium for GPT selection consists of α-MEM supplemented with 10% (v/v) fetal calf serum, 250 μg/ml xanthine, 25 μg/ml mycophenolic acid, 2 μg/ml aminopterin, 10 μg/ml thymidine, and 15 μg/ml hypoxanthine. Expression of the recombinant proteins is driven by the mouse mammary tumor virus long terminal repeat promoter on pMSG which has a glucocorticoid-responsive element, hence expression is inducible by dexamethasone. Following the selection period, the mixed population of each transfected cell line was grown to confluence and the media changed to serum-free α-MEM supplemented with 0.1% (v/v) bovine serum albumin (BSA) and 10 μM dexamethasone. After 72 h, the conditioned media were collected and stored at −15 °C before assaying for rhIGFBP-3 by a radioimmunoassay (RIA) specific for hIGFBP-3 (13).

Purification of IGFBP-3 Variants—Serum-free media conditioned for 48–72 h by each of the transfected CHO populations were collected and clarified by centrifugation at 15,300 × g for 20 min. A mixture of protease inhibitors (500 units/ml apotinin, 5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.5 mg/ml Nα-EDTA) was added to the medium. Conditioned media were applied to 1-ml heparin-Sepharose columns (HiTrap heparin, Pharmacia) at 0.4 ml/min at 4 °C. After extensive washes with 50 mM sodium phosphate (pH 6.5), rhIGFBP-3 was eluted by applying a step gradient of 0.3–1.0 M sodium chloride (made in 10 mM sodium phosphate, pH 6.5). Five fractions of 2 ml each were collected at each step of the elution gradient and assayed for rhIGFBP-3 by RIA.

One ml of polyclonal antiserum (R-100) raised against rhIGFBP-3 was purified on a protein A-Sepharose CL-4B column, essentially as recommended by the manufacturer (Pharmacia). The IgG fraction that was eluted with 0.5 M acetic acid was adjusted to pH 7 immediately and then coupled covalently to Affi-Gel 10-activated support (Bio-Rad, Hercules, CA). This antibody affinity matrix was then packed into 0.5–2.5-cm columns and washed extensively with 50 mM sodium phosphate (pH 6.5). Conditioned medium was then pumped onto the column at 0.4 ml/min at 4 °C, followed by washes as before. rhIGFBP-3 was eluted with 0.5 M acetic acid (pH 3.0) at 0.3 ml/min. Thirty fractions of 1 ml each were collected and assayed for rhIGFBP-3 by RIA.

Recombinant proteins purified by either heparin- or antibody-affinity chromatography were purified further by reverse phase high pressure liquid chromatography (HPLC) as described previously (19).

Imuno- and Ligand Blotting—Conditioned media were concentrated by centrifugation through either Centricon-3 or Centricon-10 microcentrifuges (Amicon Inc., Beverly, MA) and the IGFBP-3 concentration in each sample determined by RIA. Each protein (approximately 50 ng) was reconstituted in 50 μl of Laemmli sample buffer, heated at 95 °C for 5 min, and fractionated under nonreducing conditions on a 12% SDS-polyacrylamide gel overnight at 100 V (20). After extensive washes with 50 mM sodium phosphate buffer (pH 6.5), the conditioned media were collected and stored at −15 °C before assaying for rhIGFBP-3 by a radioimmunoassay (RIA) specific for hIGFBP-3 (13).
with 125I-IGF-I or 125I-IGF-II (0.4 × 10^4 cpm) in the presence of unla-
beled IGF-I or IGF-II, respectively, at concentrations over the range of
10 to 10^\text{12} \text{ St}. The reactions were made up to a final volume of 45 µl
with 50 mM sodium phosphate (pH 6.5) containing 0.05% (w/v) BSA.
The complexes were cross-linked with 0.25 mM disuccinimidyl suberate
(Pierce, Rockford, IL), and after 30 min incubation at 4 °C, the reactions
were terminated by the addition of 2 µl of 1.0 M Tris base. Reactions
were heated to 95 °C before electrophoresis on either 10% or 12%
PAGE. The gels were processed for autoradiography, and radiola-
beled protein bands were quantified by densitometry (Video Densitom-
eter model 620, Bio-Rad).

Binding Assays—Binary and ternary complex formation in the pres-
ence of rhIGFBP-3 or variants was measured essentially as described
previously (21). Briefly, reactions containing 125I-IGF-I or 125I-IGF-II
(10,000 cpm) and rhIGFBP-3 or analogs over the concentration range of
0–5 ng in a total volume of 0.3 ml of 50 mM sodium phosphate con-
taining 1% (w/v) BSA and 2.5 µl of goat anti-rabbit immunoglobulin in
the presence of rhIGFBP-3 antibody and 2.5 µl of goat anti-rabbit immuno
globulin in the presence of a 4% final concentration of polyethylene glycol. Ternary complex formation was mea-
sured by incubating 125I-ALS (10,000 cpm) with mixtures of IGF-I (50 ng)
and varying concentrations of rhIGFBP-3 or variants (over the range of
0–20 ng) in a total volume of 0.3 ml of 50 mM sodium phosphate contain-
ing 1% (w/v) BSA at 22 °C for 2 h. Ternary complexes were then sepa-
rated from unbound tracer by immunoprecipita-
tion as described above. The affinity of IGF binding to rhIGFBP-3 analogs
was measured essentially as described above except that the concentra-
tion of the rhIGFBP-3 analogs was held constant at 0.5 ng. Unlabeled IGF-I
was added over the concentration range of 0.0025–1 ng in a total volume of
0.3 ml. Complexes were separated from unbound tracer by immuno-
precipitation as described above. The affinity of ALS binding to the rhIGFBP-
3:IGF-I binary complex was determined as described previously (14)
except that the concentrations of rhIGFBP-3 analogs (0.5 ng) and IGF-I
(50 ng) were held constant while unlabeled ALS was added over the
concentration range of 2.5–200 ng in a total volume of 0.3 ml. Bound
tracers were separated from free tracer, as described above. Scatchard
analysis was as described previously (14).

Detection of Cell-associated IGFBP-3—Cell surface association of
rhIGFBP-3 produced endogenously by the transfected cell lines was
measured by an immunological assay described previously (22). Briefly,
cells were plated at 2 × 10^6 cells/well in 24-well plates for 48 h. Cultures
were changed to serum-free media supplemented with 0.1% (w/v) BSA
and incubated for a further 48 h. The cell monolayers were then washed
and incubated with either hIGFBP-3 antibody (R-100) or normal rabbit
serum (as control for nonspecific effects) diluted 1:5,000 in 0.5 ml of
medium. After a 1 h incubation at 22 °C, the cell monolayers were
washed again before incubation with 125I-labeled protein A (20,000 cpm
in 0.5 ml of medium) for 2 h. Unbound tracer was removed by washing,
and the cells were solubilized with 0.5% (w/v) SDS. The cell lysates
were collected, and radioactivity was determined in a γ-counter.

Statistical Analysis—Statistical analysis was carried out using Stat-
View 4.02 (Abacus Concepts Inc., Berkeley, CA). Differences between
groups were evaluated by Fisher’s Protected Least Significant Differ-
ence test after analysis of variance, and a significant difference was
defined as p < 0.05.

RESULTS

rhIGFBP-3 proteins, detectable by RIA, were secreted by each of
the transfected cell lines, and the expression of these proteins was inducible up to 7-fold by dexamethasone (Fig. 1B). The differences in levels of stimulation by dexamethasone are probably due to the heterogeneous population of transfectants in each cell line. There was no detectable hIGFBP-3 in media conditioned by CHO cells transfected with pMSG as a control. The polyclonal IGFBP-3 antibody recognized all variant pro-
tiens including the deletion variants, indicating that an anti-
genic epitope is present in the amino-terminal portion of the
protein, the only region common to all variants. In the RIA, the full-length analogs and rhIGFBP-3 yielded displacement
curves that were parallel to pure serum-derived IGFBP-3. In
contrast, the deletion variants displayed parallelism up to 1–2
ng, but the displacement curves became nonparallel at higher
concentrations (data not shown). All samples were assayed
under conditions where parallelism was observed. Under these
conditions, the RIA was considered the best method available
for quantifying low amounts of these proteins.

Immunoblots of the five variants are shown in Fig. 2A. rhIGFBP-3,
rhIGFBP-3(253KED → RGD), and rhIGFBP-3(228KGRKR → MDGEA)
produced a 40–45-kDa doublet as well as a 30-kDa form, essentially identical to serum-derived rhIGFBP-3. rhIGFBP-3(Δ185–264) migrated as a doublet of
30–35 kDa. Adding an estimated mass of 10–15 kDa of carbo-
hydrate to the calculated 19-kDa core protein, the observed
masses of rhIGFBP-3, rhIGFBP-3(Δ89–264) and rhIGFBP-3(Δ89–264)
probably represents different glycoforms of the protein consistent with the current view that the 40–45-kDa
doublet of IGFBP-3 consists of glycoforms (23); IGFBP-3 con-
tains three potential N-glycosylation sites at 58NAS, 109NAS,
and 172NFS (15). rhIGFBP-3(Δ89–264) corresponded to an
apparent size of approximately 15 kDa, whereas rhIGFBP-3(Δ89–184)
migrated as a major band at 21 kDa and a minor band at 17 kDa. Because the deduced molecular masses for the
Δ89–264 and Δ89–184 variants are 9 and 18 kDa, respectively,
it would appear that the proteins are migrating aberrantly on SDS-PAGE. Presumably, the smaller 17-kDa form of rhIGFBP-3-[Δ89–184] represents a proteolyzed form of the protein, comparable to the 30-kDa form of rhIGFBP-3, rhIGFBP-3-[228KGRKR → MDGEA], and rhIGFBP-3-[Δ89–184] (panel D, ●). The 2-ml fractions were assayed for rhIGFBP-3 in the RIA.

The ability of the rhIGFBP-3 proteins to bind IGF-I was examined by ligand blot using 125I-IGF-I. A preliminary experiment revealed that there were two forms of IGF-I-binding protein present in the media conditioned by cells transfected with the vector, pMSG (Fig. 2B). Based on the apparent sizes of these proteins (approximately 23 and 28 kDa), we assume that they are glycoforms of CHO-derived IGFBP-4. Immunoprecipitation of the sample with hIGFBP-3 antibody before electrophoresis removed these proteins (Fig. 2B). Ligand blotting of immunoprecipitated IGFBP-3 variant proteins (Fig. 2C) indicated that rhIGFBP-3-[228KGRKR → RGD] and rhIGFBP-3-[228KGRKR → MDGEA] have an IGF-I binding function. In contrast, none of the deletion variants showed detectable IGF binding by this method.

To examine their binding properties in more detail, the full-length rhIGFBP-3 analogs were purified from conditioned media by heparin-Sepharose chromatography followed by reverse phase HPLC. The wild-type protein was eluted from the heparin column as a single peak by 0.75 M NaCl (Fig. 3A). rhIGFBP-3-[228KGRKR → RGD] showed a similar elution profile (Fig. 3B). In contrast, rhIGFBP-3-[228KGRKR → MDGEA] eluted at a concentration of only 0.5 M (Fig. 3C), and rhIGFBP-3-[Δ89–184] eluted as two peaks at 0.5 and 0.75 M NaCl (Fig. 3D). Among the deletion analogs, the Δ89–184 variant showed a marked reduction in binding activity, but the other two analogs did not bind to heparin-Sepharose at all (data not shown). Endogenous IGFBP-4 from CHO cells also bound to heparin-Sepharose and eluted at 0.5 M NaCl (data not shown), consistent with previous studies (24). The CHO-derived IGFBP-4 was separated from rhIGFBP-3-[228KGRKR → MDGEA] on reverse phase HPLC.

Because of the poor binding of the rhIGFBP-3 deletion analogs to heparin-Sepharose, these analogs were purified from an antibody affinity column followed by reverse phase HPLC, as described under “Materials and Methods.” The proteins purified by either heparin or antibody affinity were analyzed by SDS-PAGE to check for protein integrity and purity. Dose-response curves for the binding of various IGFBP-3 analogs to either 125I-IGF-I (panel A) or 125I-IGF-II (panel B) to increasing amounts of rhIGFBP-3 is shown. The binding curves are representatives of at least two independent measurements for each analog. Competition for the binding of 125I-IGF-I (panel C) or 125I-IGF-II (panel D) to 0.5 ng of each rhIGFBP-3 by increasing concentrations of unlabeled IGF-I or IGF-II, respectively, is shown. BB, represents the ratio of 125I-IGF bound to rhIGFBP-3 in the presence of unlabeled IGF to that bound in the absence of unlabeled IGF. Data points shown are mean ± S.E. of at least three independent measurements.
TABLE I

| IGFBP-3 analog | Association constant ($K_a$) | liters/nmol |
|----------------|-----------------------------|-------------|
| rhIGFBP-3      | 152.2 ± 33.2                | 262.5 ± 26.5| 24.3 ± 5.2   |
| rhIGFBP-3[KED→RGD] | 39.6 ± 11.1*               | 47.8 ± 8.6*  | 21.7 ± 4.5*  |
| rhIGFBP-3[KGRKR→MDGEA] | 94.1 ± 25.7               | 90.4 ± 9.7*  | 2.6 ± 0.9*   |

*a Significant difference from value for rhIGFBP-3, $p < 0.05$.

It has been shown previously that IGFBP-3 synthesized by fibroblasts can associate with the cell surface and can be displaced by the addition of IGF-I (22). The interaction between the various endogenously produced rhIGFBP-3 forms and the cell surface was examined (Fig. 7). Cell surface-associated rhIGFBP-3 proteins were only detected in cell lines transfected with rhIGFBP-3, rhIGFBP-3[L89–184], and rhIGFBP-3-L89–264. The absence of cell-associated forms of recombinant proteins in cells expressing the L89–264 and L185–264 variants indicates that the carboxyl-terminal region of the protein is necessary for cell surface association. Furthermore, the basic residues (K228KGRKR) in the carboxyl-terminal region, shown above to be important determinants of affinity for ALS, are also integral to the cell association domain, as mutation of these residues abolished the ability of rhIGFBP-3-K228KGRKR→MDGEA to interact with the cell surface. Consistent with previous evidence indicating that IGF-I displaces cell surface-associated IGFBP-3 into the extracellular medium (22), rhIGFBP-3- and rhIGFBP-3(K253KED→RGD)-transfected cells showed a decrease (approximately 40 and 60%, respectively) in cell-associated binding proteins when incubated with IGF-I (Fig. 7). On the other hand, there was no difference in the levels of cell-associated protein when cells transfected with rhIGFBP-3[L89–184] were incubated in the presence or absence of IGF-I. This is in accord with previous observations that displacement of cell surface-associated IGFBP-3 by IGF-I requires a direct interaction between the two proteins because rhIGFBP-3[L89–184] has a greatly reduced affinity for IGF-I.

**DISCUSSION**

Comparison of the primary sequences of the six well characterized IGFBPs indicates that the strongest homology is in the amino- and carboxyl-terminal regions of the proteins, whereas the central regions are unique to each protein. The amino- and carboxyl-terminal regions contain 18 cysteine residues, at identical positions in the IGFBPs (IGFBP-6 lacks two of the residues), which probably confer similar constraints on the structure-function of these proteins (26). The IGF binding domain could therefore involve either or both of these regions. In this study we examined the role of the carboxyl-terminal conserved region in the binding of IGF-I, IGF-II, and ALS by generating three deletion variants of IGFBP-3.

The deletion of either the conserved carboxyl-terminal region (rhIGFBP-3[Δ185–264]) or the nonconserved central region (rhIGFBP-3[Δ89–184]) or the combined deletion of both regions (rhIGFBP-3[L89–264]) abolished IGF-I and IGF-II binding when analyzed by either ligand blotting or solution binding. However, all three deletion analogs showed some affinity for both IGF ligands in affinity labeling experiments, in agreement with a previous preliminary report, unsupported by data, that rhIGFBP-3[Δ89–264] and rhIGFBP-3[Δ162–264] are capable of binding to IGF-I when analyzed by a similar method (27). It was also reported that deletion of amino acid residues 92–184 or 92–223 did not abolish IGF-I binding. When competitive binding curves for IGF-I or IGF-II binding to rhIGFBP-3 were compared for the solution binding and affinity labeling methods, similar affinity estimates (as determined by half-maximal displacement of radioligand) were obtained, providing some validation of the affinity labeling technique. However, the failure of binding assays to detect IGF-I binding to the deletion mutants unless stabilized by affinity cross-linking suggests that these relatively low affinity interactions must have quite rapid off-rates.

Deletion of the central region from IGFBP-3 (L89–184) appears to have decreased both IGF-I and IGF-II binding by approximately 40-fold. Whether this nonconserved region is directly involved in the binding interactions or simply helps to...
maintain the spatial configuration of the amino- and carboxy-terminal domains is unknown. Deletion of the carboxy-terminal region (Δ185–264) alone affected IGF-II binding (~40-fold reduction) more than IGF-I binding (~20-fold reduction). This region is likely to make a specific contribution to the IGF binding site (particularly IGF-II) because carboxyl-terminal fragments of IGFBP-2 have been shown to retain considerable IGF binding abilities of the deletion analogs.

The specific mutations in rhIGFBP-3[253KED] and rhIGFBP-3[253KED → RGD] did not abolish either IGF-I or IGF-II binding as determined by solution binding assays. The relative binding affinities of rhIGFBP-3[253KED → RGD] for IGF-I and IGF-II were decreased by approximately 4- and 6-fold, respectively. In both of the site-specific IGFBP-3 variants, the altered amino acids were replaced by analogous sequences from IGFBP-1. Interestingly, it has been reported that when the RGD sequence in IGFBP-1 was replaced by KED (the corresponding IGFBP-3 sequence), the IGFBP-1 variant lost its IGF-I binding activity which was attributed to the formation of dimers by the IGFBP-1 variant. When the RGD sequence in IGFBP-1 was replaced by KGD, however, the mutation did not abolish IGF-I binding (29). The mutation in rhIGFBP-3[253KGRKR → MDGEA] decreased its affinity for IGF-I and IGF-II by 2- and 3-fold, respectively (Table I). Taken together, these results would suggest that these mutated sequences, compared with the deletion analogs, make a relatively minor contribution to the IGF-I and IGF-II binding sites. The carboxy-terminal region, where these specific mutations reside, appears to be more important for IGF-II binding than IGF-I binding, which is consistent with the relative IGF-I and IGF-II binding abilities of the deletion analogs.

In support of the participation of carboxy-terminal residues in IGFBP binding activity, it has been reported that deletions of the carboxy-terminal region of IGFBP-1 abolished IGF-I
integrin receptors. It has been shown that IGFBP-1 associates with the recognition motif on proteins that adhere to the cell surface via the carboxyl-terminal region of IGFBP-1 and IGFBP-2 serves as a bind to cells (22, 24, 34–36). The RGD sequence within the terminal domain was retained despite the deletion of one-third of the carboxyl-terminal region (5). This association of the central region deletion variant with the cell surface suggests that the structural integrity of the carboxyl-terminal domain was retained despite the deletion of one-third of the molecule.

In various studies, IGFBPs-1 to -5 have all been shown to bind to cells (22, 24, 34–36). The RGD sequence within the carboxyl-terminal region of IGFBP-1 and IGFBP-2 serves as a recognition motif on proteins that adhere to the cell surface via integrin receptors. It has been shown that IGFBP-1 associates with the α5β1 integrin and can stimulate the migration of transfected CHO cells (37). The RGD motif is not present in the sequence of native IGFBP-3. The observation that cell-associated IGFBP-3 could be displaced from human fibroblasts by heparin suggested that IGFBP-3 may interact with proteoglycans or other negatively charged molecules on the cell surface (22), possibly via the carboxyl-terminal portion of the protein that is relatively abundant in basic amino acid residues. However, recent evidence suggests that the heparin-inhibitable cell binding of IGFBP-3 may not be to heparan sulfate or chondroitin sulfate glycosaminoglycans (38). This highly basic carboxyl-terminal region is also present in IGFBP-5 and has been shown to be involved in the binding of glycosaminoglycans (39).

There are two putative heparin binding motifs in IGFBP-3, located at amino acids 148–153 and 219–226 in the central and carboxyl-terminal regions, respectively. A recent study (40) showed that synthetic peptides containing either one of these heparin binding consensus sequences bound heparin and that the peptide containing the carboxyl-terminal motif had a 4-fold higher affinity for heparin. The inability of rhIGFBP-3[Δ185–284] to bind heparin-Sepharose would suggest that the carboxyl-terminal region contributes structurally to the major heparin binding site. This is supported by the finding that rhIGFBP-3[Δ89–184] bound to heparin-Sepharose, although with less avidity than rhIGFBP-3. Furthermore, heparin binding was affected when amino acid residues adjacent to the carboxyl-terminal putative heparin binding motif were mutated in rhIGFBP-3[228KGRKR → MDGEA].

Partial replacement of the carboxyl-terminal basic region in hIGFBP-3 with the homologous acidic residues of hIGFBP-1 (rhIGFBP-3[228KGRKR → MDGEA]) abolished its ability to associate with the cell surface. On the other hand, the presence of the analogous RGD sequence in IGFBP-3 did not appear to affect the ability of the protein to bind to the cell surface. This suggests that the amino acid residues 228KGRKR form a key part of the cell surface association domain of IGFBP-3, lending further support to the study in which synthetic peptides corresponding to the basic region were shown to decrease IGFBP-3 and IGFBP-5 binding to the cell surface (24).

Previous studies from this laboratory have shown by several independent methods, gel permeation chromatography, affinity labeling, and solution binding assays with immunoprecipitation of complexes, that little or no specific binding of human ALS to hIGFBP-3 occurs in the absence of IGF-I or IGF-II. This concept has been challenged recently on the basis of studies with rat ALS and a partially proteolyzed form of rat IGFBP-3 (41) and a study using human nonglycosylated IGFBP-3 (42), both of which were interpreted to show that ALS may bind to IGFBP-3 in the absence of IGFs. Whatever the explanation for these conflicting results, our studies with natural human ALS and IGFBP-3 consistently support the notion that the binary IGF-IGFBP-3 complex, rather than IGFBP-3 itself, forms a high affinity binding site for ALS. Indeed, in the presence of IGF-I variants (for example, with substitutions in the B domain) with low affinity for IGFBP-3, the total binding of ALS is low, because little binary (IGF-IGFBP-3) complex forms, but the affinity of ALS for this complex is not reduced (14). In the presence of IGF-I, the deletion variants that had low affinities for IGF-I (decreased by 20–60 fold) showed very low or no binding to ALS, which is consistent with the requirement of an IGF-IGFBP-3 complex for ALS binding. rhIGFBP-3-[250KED → RGD], which had a 4-fold decrease in IGF-I affinity, displayed normal ALS binding affinity compared with rhIGFBP-3. In contrast, rhIGFBP-3[252KGRKR → MDGEA] showed markedly impaired ALS binding function, attributable to a loss of affinity for ALS, even though its IGF-I binding is relatively normal. These results specifically implicate IGFBP-3 residues 228–232, but not 253–255, in the interaction with ALS.

The two mutations, therefore, have quite distinct effects on the protein-protein interactions within the ternary complex. The KED → RGD mutation has altered the capacity of IGF-BP-3 to bind to IGF-I without affecting the affinity of ALS. This implies that when the binary complex between rhIGFBP-3-[250KED → RGD] and IGF-I has formed, the mutation has no bearing on the structural integrity of the ALS binding site, because the affinity of ALS remains unchanged. In contrast, the KGRKR → MDGEA mutation appears to disrupt the ALS binding site, as ALS affinity for this variant was decreased. ALS binding is known to be sensitive to increasing ionic strength (25, 43), and the KGRKR → MDGEA mutation introduces a significant charge reversal in this region of IGFBP-3, suggesting that interaction between key charged residues may be important. Although binding determinants in the ALS structure have not been elucidated, there is a region of acidic residues in the amino-terminal region of human ALS (25DDDAD) (44) which might interact with the highly basic region in IGF-BP-3, and preliminary molecular modeling studies suggest that within the leucine-rich repeating region of ALS, there may be a surface with an accumulation of negative charge.

In summary, this study has shown that the protein-protein and protein-cell interactions of IGFBP-3 are complex and involve distinct domains of the protein. The structural integrity of the IGF-I binding site is disrupted significantly by deletion of either the central or carboxyl-terminal region of IGFBP-3, but more specific mutations of the carboxyl-terminal region can reduce IGF-I binding. The IGF-I and ALS binding sites are functionally distinct as shown by contrasting the binding characteristics of the 253KGRKR variant, with decreased IGF-I binding.
but normal ALS affinity, and the 228MDGEA variant, with near normal IGF binding and greatly reduced ALS affinity. Finally, although gross deletions affected the ability of IGFBP-3 to bind to IGF-I and consequently ALS, the deletion of amino acid residues 89–184 did not alter its interaction with the cell surface. We therefore conclude that the carboxyl-terminal region and in particular, 228KGRKR, is essential for this function. The availability of IGFBP-3 mutants with selective reduction in the affinity for IGFs, on the one hand, and reduced binding to ALS and the cell surface on the other, will provide powerful tools to help elucidate further the dual roles of IGFBP-3 as a transporter of IGFs and a regulator of cell function.

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