# Insulin-like Growth Factor I and Its Binding Proteins: A Study of the Binding Interface Using B-Domain Analogues[†]

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ABSTRACT: The biological activity of the insulin-like growth factors (IGF-I and IGF-II) is regulated by six IGF binding proteins (IGFBPs 1−6). To examine the surface of IGF-I that associates with the IGFBPs, we created a series of six IGF-I analogues, [His$^4$]-, [Gln$^9$]-, [Lys$^9$]-, [Ser$^{16}$]-, [Gln$^9$,Ser$^{16}$]-, and [Lys$^9$,-Ser$^{16}$]IGF-I, that contained substitutions for residues Thr$^4$, Glu$^9$, or Phe$^{16}$. Substitution of Ser for Phe$^{16}$ did not affect secondary structure but significantly decreased the affinity for all IGFBPs by between 14-fold and >330-fold, indicating that Phe$^{16}$ is functionally important for IGFBP association. While His$^4$ or Gln$^9$ substitutions had little effect on IGFBP affinity, changing the negative charge of Glu$^9$ to a positive Lys$^9$ selectively decreased the affinities of IGFBP-2 and -6 by 140- and 30-fold, respectively. Furthermore, the effects of mutations to both residues 9 and 16 appear to be additive. The analogues are biologically active in rat L6 myoblasts and they retain native structure as assessed by their far-UV circular dichroism (CD) profiles. We propose that Phe$^{16}$ and adjacent hydrophobic residues (Leu$^5$ and Leu$^{54}$) form a functional binding pocket for IGFBP association.

Compact hydrophobic regions are typically found at the interface of protein−protein complexes. These interface surfaces are composed of complementary hydrophobic determinants from both molecules and generally resemble the core of folded proteins (*1*−*3*). Functionally important hydrophilic residues are found at the periphery of the hydrophobic interfaces. They contribute to the binding affinity of complexed proteins through the formation of salt bridges and hydrogen bonds at the interface surface (*2*, *4*). With these general principles of protein−protein interaction in mind, we examined the surface of insulin-like growth factor I (IGF-I)[1] that is considered important for binding to IGF binding proteins (IGFBPs 1−6) and identified residues that could contribute to the binding surface. We then used directed mutagenesis to study the functional importance of those residues for association with the IGFBPs.

IGF-I and IGF-II are anabolic, single-chain polypeptides of 70 and 67 amino acid residues, respectively. Solution NMR studies (*5*−*7*) confirm that insulin and the IGFs have similar 3D structures, as predicted from their conserved hydrophobic core residues and an identical arrangement of disulfide bonds. Figure 1 shows the comparative amino acid sequences of human IGF-I, IGF-II, and insulin. Subtle evolutionary changes in the A- and B-domains of the IGFs allow them to associate with the six IGFBPs. In contrast, insulin does not bind the IGFBPs but self-associates to form dimers, tetramers, and hexamers in solution. Details of the IGFBP gene sequences, posttranslational modifications, and known physiological effects can be found in the excellent reviews published by Rechler (*8*), Kelley et al. (*9*), and Clemmons (*10*).

**B-chain/domain**

```
                B1          B10          B20          B30
Insulin:    FVNQHLCGSHLVEALYLVCGERGFFYTPKT
                 1          10          20
IGF-I:      GPETLCGAELVDALQFVCGDRGFYFNKPT
            1          10          20          30
IGF-II: AYRPSETLCGGELVDTLQFVCGDRGFYFSRPA
```

**C-domain**

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                30          40
IGF-I:      GYGSSSRRAPQT
                       40
IGF-II:     S--RVSRRSR
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**A-chain/domain**

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                A1          A10          A20
Insulin:    GIVEQCCTSICSLYQLENYCN
                       50          60
IGF-I:      GIVDECCFRSCDLRRLEMYCA
                       50          60
IGF-II:     GIVEECCFRSCDLALLETYCA
```

**D-domain**

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                       70
IGF-I:      PLKPAKSA
                       67
IGF-II:     T--PAKSE
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FIGURE 1: Comparative amino acid sequences of human insulin, IGF-I, and IGF-II. The underlined residues form helical regions (*5*−*7*).

The complete tertiary structure is not known for any of the IGFBPs or the IGF-IGFBP complexes; however, previous

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[1] Abbreviations: IGF, insulin-like growth factor; IGFBPs, insulin-like growth factor binding proteins; CD, circular dichroism.

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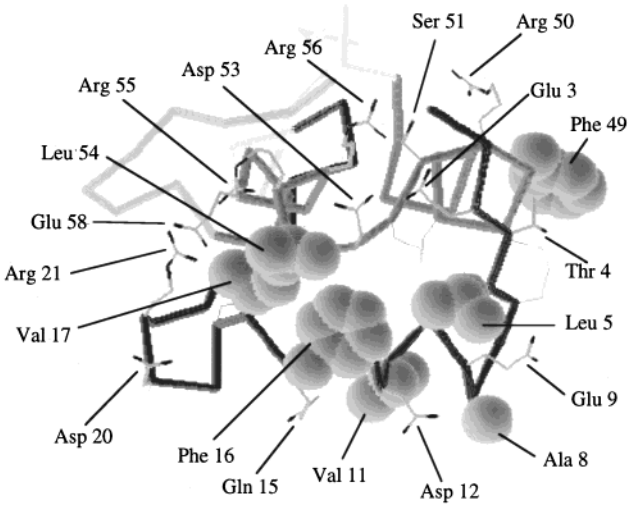


FIGURE 2: Three-dimensional structure of IGF-I showing the N-terminal region of the B-domain and closely aligned residues. This representation was created with RasMol, which was developed by Roger Sayle of Glaxo Corp. and the University of Edinburgh, Scotland. The coordinates are from the solution NMR structure of IGF-I (*6*) and are available from the Brookhaven Protein Data Bank, entered as PDB file 2gf1.

studies indicate that the IGF-I B-domain is unequivocally important for association of the IGFBPs (*11*−*13*). Substitution of various A-chain insulin residues for nonhomologous IGF-I or -II sequences demonstrate that the insulin tripeptide, $Thr^{A8}$-$Ser^{A9}$-$Ile^{A10}$, decreases the affinity of the IGFBPs when substituted for the analogous tripeptide, Phe-Arg-Ser, found in the IGFs (*12, 14*). These residues are located near the N-termini of the IGF B-domains and are generally thought to directly interact with the IGFBPs. The 3D structure of IGF-I (*6*) showing the N-terminal region of the B-domain and closely aligned residues is presented in Figure 2.

The solvent-exposed surface of the IGF-I B-domain, illustrated in Figure 2, contains a prominent hydrophobic patch formed by $Leu^5$, $Phe^{16}$, and $Leu^{54}$ (from the A-domain). If this region contributes to the protein−protein interface between the IGFBPs and IGF-I, then the large and centrally located $Phe^{16}$ residue (conserved as $Phe^{19}$ in IGF-II) may be critical for formation of the interface surface. We investigated this hypothesis by creating an IGF-I analogue containing a small, hydrophilic Ser substituted for Phe at residue 16.

Of the hydrophilic residues found on the surface of the molecule, $Glu^3$ (and the analogous $Glu^6$ of IGF-II) appears to be functionally important for IGFBP binding (*15*−*18*). This glutamate residue is adjacent to the hydrophobic patch formed by $Leu^5$, $Phe^{16}$, and $Leu^{54}$, as depicted in Figure 2. An additional glutamate residue ($Glu^9$ in IGF-I or $Glu^{12}$ in IGF-II) is also located at the periphery of this area. To assess the functional importance of $Glu^9$ for binding the IGFBPs, two IGF-I analogues were produced that contained substitutions for residue 9: [$Gln^9$]- and [$Lys^9$]IGF-I. Analogues containing mutations to both residues 9 and 16 ([$Gln^9$,$Ser^{16}$]- and [$Lys^9$,$Ser^{16}$]IGF-I) were further produced to determine the additive effects of these substitutions.

$Thr^4$, like $Glu^9$, is located at the periphery of the identified hydrophobic region and is in close proximity to $Phe^{49}$ (an A-domain residue thought to directly associate with the IGFBPs). We anticipated that the substitution of His, as found in most insulin peptides, for Thr at residue 4 might inhibit IGFBP association either because of its increased size or charge potential. An IGF-I analogue containing the $His^4$ substitution was produced to test this theory.

The six IGF-I analogues included in this study ([$Ser^{16}$]-, [$Gln^9$]-, [$Lys^9$],[$Gln^9$,$Ser^{16}$]-, [$Lys^9$,$Ser^{16}$]-, and [$His^4$]IGF-I) were assayed for their apparent affinity for each of the six IGFBPs. Far-UV circular dichroism (CD) spectroscopy was used as a screening procedure to assess structural changes, and the biological potency of these analogues was evaluated using rat L6 myoblasts.

## EXPERIMENTAL PROCEDURES

*Materials*. The Muta-Gene mutagenesis kit was purchased from Bio-Rad, South Richmond, CA. Synthetic oligonucleotides and other materials used for molecular biology were supplied by Bresatec Ltd., Adelaide, Australia. Recombinant hIGF-I was kindly supplied by GroPep Pty. Ltd., Adelaide, Australia. Radiolabeled IGF-I, iodinated to a specific activity of 30−40 Ci/g with chloramine T (*19*), was kindly provided by Spencer Knowles, CRC for Tissue Growth and Repair, Adelaide, Australia. L-[4,5-$^3$H]Leucine was purchased from Amersham Australia Pty. Ltd., Sydney, N. S. W., Australia.

Purified, lyophilized IGFBPs were kindly donated by Professor R. C. Baxter, Kolling Institute of Medical Research, Sydney, Australia (natural human IGFBP-3 and -6); Dr. S. Mohan, Pettis VA Medical Center, Loma Linda, CA (natural human IGFBP-4 and −5); Dr. G. Forsberg, KabiGen, Stockholm, Sweden (recombinant human IGFBP-1); and Dr. M. Rechler, NIH, Bethesda, MD (rat IGFBP-2, purified from BRL-3A culture supernatant).

*Analogue Production*. Recombinant DNA constructs encoding the IGF-I analogues were produced by the protocol described in the Muta-Gene mutagenesis kit (Bio-Rad, South Richmond, CA). The starting plasmid, originally described by Francis et al.. (*20*), was p[$Met^1$]-p$GH^{1-11}$-Val-Asn-IGF-I. An *Eco*RI−*Hind*III fragment cut from this plasmid was subcloned into pTZ18 for mutagenesis. The following oligonucleotides were used to generate changes (5′ to 3′): [$His^4$]IGF-I, AACGGCCCGGAACATCTGTGCGGTGCT; [$Gln^9$]IGF-I, CTGTGCGGTGCTCAGCTGGTTGACGCT; [$Lys^9$]IGF-I, CTGTGCGGTGCTAAACTGGTTGACGCT; [$Phe^{16}$]IGF-I, GACGCTCTGCAGAGCGTTTGCGGTGAC.

Two rounds of mutagenesis produced alterations in DNA coding for both residues 9 and 16, and all mutations were confirmed by DNA sequencing (*21*). After mutagenesis, plasmid DNA was digested with *Hpa*I−*Hin*dIII, ligated back into *Hpa*I−*Hin*dIII-cut starting plasmid, and transformed into *Escherichia coli* JM101 bacterial cells. Transformed colonies were grown on minimal agar containing 200 $\mu$g/mL ampicillin. Bresagen Ltd., Adelaide, Australia, then grew selected colonies in 2-L fermenters. Inclusion bodies were isolated and homogenized after induction with 250 $\mu$M isopropyl $\beta$-D-thiogalactoside (IPTG), as described by King et al. (*17*).

*Refolding and Purification of IGF-I Analogues*. Inclusion bodies containing the IGF-I fusion peptides were processed to purified IGFs by using the identical columns and conditions described by Shooter et al. (*22*). The purities of the IGF preparations were assessed by amino acid sequence analysis and mass spectrometry (*23*). Quantitation of analogues was performed by comparing analytical C4 HPLC absorbance profiles (measured at 215 nm) with profiles of a

reference recombinant human IGF-I preparation (*20, 22*), supplied by GroPep Pty. Ltd., Adelaide, Australia.

*Far-UV Circular Dichroism Spectroscopy*. CD measurements between 250 and 180 nm were made at room temperature on an Aviv 60DS CD spectropolarimeter calibrated with 0.6 mg/mL *d*-(+)-10-camphorsulfonic acid. Protein concentration was 0.1 mg/mL in 20 mM potassium phosphate buffer, pH 7.2. With a cell of 1 mm path length, spectra were recorded at 0.4 nm intervals and a scan rate of 2 nm/min.

*IGF-I Analogue Binding to IGFBPs1−6*. IGFBP affinities were assessed for the IGF-I variants by the charcoal binding assay described by Martin and Baxter (*24*) with the modifications suggested by Szabo et al. (*25*). Briefly, various concentrations of IGF-I or analogue, 10 000 cpm of $^{125}$I-labeled IGF-I (approximately 0.2 ng), and 10 ng of the appropriate IGFBP were suspended in a total volume of 0.25 mL of phosphate buffer [50 mM sodium phosphate and 2.5 g/L BSA (Sigma, radioimmunoassay grade), pH 6.5]. After incubation at 4 °C for 18 h, bound tracer was separated from free by the addition of 1.0 mL of activated charcoal suspension (phosphate buffer, as above, containing 5.0 g/L activated charcoal and 0.2 g/L protamine sulfate). Protamine sulfate was omitted from IGFBP-1 assays (*26*). After centrifugation (10000*g* for 3 min) to sediment charcoal and uncomplexed IGFs, 0.625 mL of supernatant was removed for $\gamma$-counting to determine quantities of bound $^{125}$I-labeled IGF. Results were expressed as the percentage of complexed $^{125}$I-labeled IGF-I in the absence of competing analogue. The experimental conditions resulted in the detection of between 1200 and 2200 cpm in control reaction tubes containing buffer, $^{125}$I-labeled IGF-I, and specific binding protein but no competing analogue. Values for nonspecific binding were less than 25% of control counts for all assays. Two competitive binding assays were conducted for each IGFBP, and IGFs were tested in triplicate at each dilution. Results from the IGFBP, assays were graphed with KaleidaGraph (Synergy Software) and lines connecting data points were drawn with the built-in Smooth curve-fit function. Concentrations of analogues that reduced tracer binding by 50% ($ED_{50}$ values) were read directly from the graphs.

*Biological Assays with Rat L6 Myoblasts*. Analogue affinities for the type 1 IGF receptor were measured with rat L6 myoblast cultures as previously described by Ross et al. (*27*). Briefly, a range of concentrations of unlabeled analogue and 0.2 ng of $^{125}$I-labeled IGF-I were added to confluent monolayers. The cultures were incubated at 4 °C for 18 h in a total volume of 0.5 mL of 0.1 M Hepes buffer, pH 7.6, with 5 g/L BSA. Binding was expressed as the percentage of that occurring in the absence of unlabeled IGF. The stimulation of protein synthesis in rat L6 myoblasts was measured as the incorporation of [$^3$H]leucine into cellular protein after incubation (18 h at 37 °C) and precipitation with trichloroacetic acid, as described by Francis et al. (*28*). Analogue affinities for the IGFBPs secreted by L6 myoblast were measured as described by Szabo et al. (*25*). Briefly, serum-free conditioned medium was collected after a 24-h incubation, centrifuged at 10000*g* for 5 min and filtered through a 0.2 $\mu$m filter. Binding was expressed as the percentage of bound $^{125}$I-labeled IGF-I in the absence of competing peptide. Results from the in vitro assays were graphed with KaleidaGraph as described for IGFBP assays.

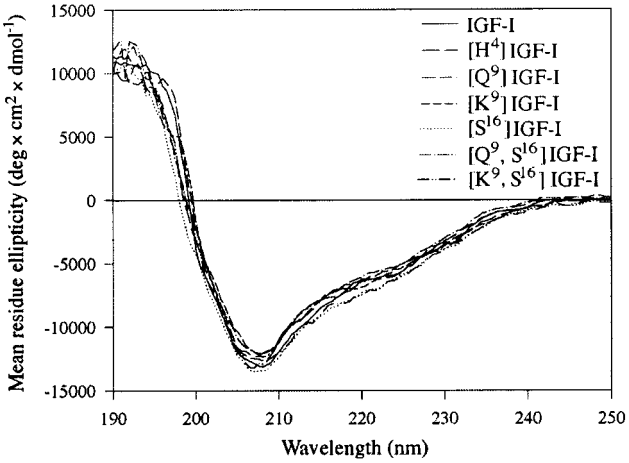


FIGURE 3: Far-UV CD spectra of IGF-I and IGF-I analogues.

## RESULTS AND DISCUSSION

*IGF-I Analogue Expression and Purification*. The IGF-I analogues were expressed as fusion proteins in transformed *E. coli* JM101 cells as described by King et al. (*17*). The fusion proteins had a N-terminal leader peptide that contained the first 11 amino acids of methionyl porcine growth hormone ([Met$^1$]pGH$^{1-11}$). The leader peptide ensured a predominance of correctly folded isomer during the oxidative folding process (*17, 23*). A Val-Asn dipeptide connected the leader sequences to IGF-I ([Met$^1$]pGH$^{1-11}$-Val-Asn-IGF-I); this dipeptide linker provided a convenient hydroxylamine cleavage site between Asn and the Gly$^1$ of IGF-I. The analogues were expressed as inclusion bodies with yields of approximately 0.2 g/L, and after folding, cleavage, and purification (*22*), all IGF-I analogues gave rise to single product peaks on analytical C4 HPLC that were consistent with the behavior of IGF-I standard (data not shown). N-Terminal peptide sequence analysis gave the expected sequences with approximate purities of >96%. Electrospray mass spectrometry gave mass values within ± 2 units of the calculated mass for all peptides (data not shown).

*CD Analysis of IGF-I Analogues*. Far-UV CD spectra of native IGF-I and the analogues are presented in Figure 3. Only minimal deviations in wavelength intensities were observed between the analogues and native protein, indicating that the introduced changes have had little effect on overall secondary structure. In contrast to these results, significant variations in wavelength intensities occur between the spectrum of IGF-I containing nonnative disulfide bonds and that of native peptide (*29*). The mismatched IGF is thought to have gross structural changes that greatly inhibit its binding to the type 1 IGF receptor (*23, 29*). Similarly, alanine substitution of residues located on the IGF-I B-domain α-helix (*29*) resulted in analogues that had CD profiles that varied significantly from that of native protein. Our results suggest that, for small, highly evolved proteins that have well-defined structures, a directed mutagenic approach for residue analysis may be more appropriate than alanine substitution. For instance, alanine substitution for Glu$^9$ in IGF-I would have resulted in an α-helix containing the N-terminal residues: Ala$^8$-Ala$^9$-Leu$^{10}$-Val$^{11}$. We considered it highly unlikely that these amino acids would form the partially solvent-exposed helix found in native IGF-I.

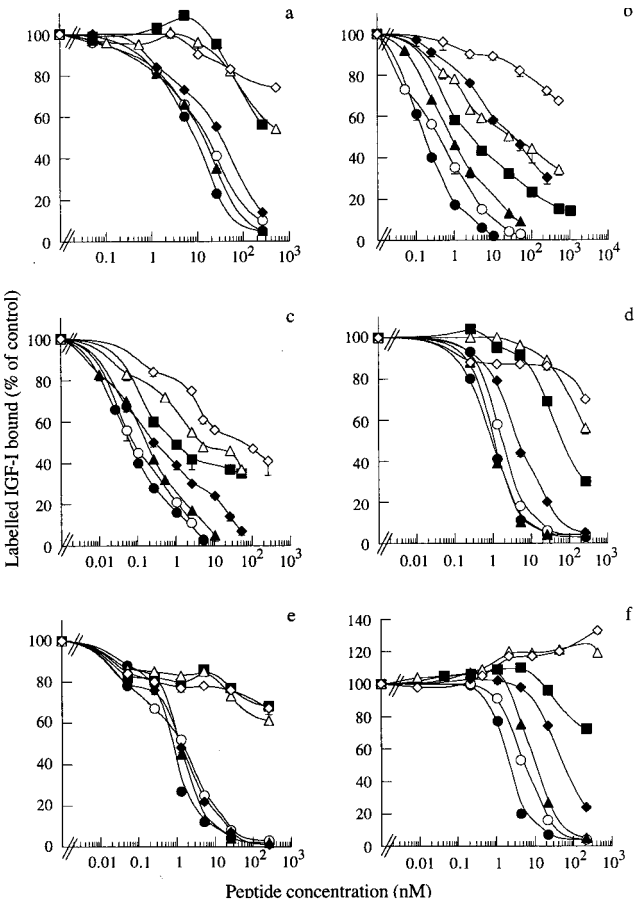


FIGURE 4: Binding of IGF-I analogues to IGFBPs 1−6: IGFBP-1 (a), IGFBP-2 (b), IGFBP-3 (c), IGFBP-4 (d), IGFBP-5 (e), and IGFBP-6 (f). The symbols represent competition of $^{125}$I-labeled IGF-I binding to the IGFBPs by IGF-I (●), [His$^4$]IGF-I (○), [Gln$^9$]-IGF-I (▲), [Lys$^9$]IGF-I (◆), [Ser$^{16}$]IGF-I (■), [Gln$^9$,Ser$^{16}$]IGF-I (△) and [Lys$^9$,Ser$^{16}$]IGF-I (◇). Assays were performed twice for each binding protein and data points for IGFBPs 1, 4, 5, and 6 are the means of triplicate determinations at each peptide concentration from two individual experiments. The IGFBP 2 and -3 graphs are representative; displayed data points are the means of triplicate determinations at each concentration from one experiment. The SEM values are indicated by descending bars when larger than the displayed symbols.

*Binding to IGFBPs 1−6*. The analogues' affinities for purified IGFBPs 1−6 were determined with competitive binding assays. Results from these assays are presented in Figure 4. The concentrations of analogues that reduced tracer binding by 50% ($ED_{50}$ values) were further determined and are included in Table 1. Discussed below are the rationales behind the amino acid substitutions and the results from competitive binding studies with IGFBPs 1−6.

*Rationale for Residue Substitution Phe$^{16}$ to Ser$^{16}$*. Previously published studies by Jansson et al. (*29*) indicate that alanine substitution of Phe$^{16}$ results in a significant reduction in the helical content of IGF-I, a 50-fold reduced affinity to IGFBP-1, and a 37-fold reduction in type 1 IGF receptor affinity. The loss of both structure and function of this variant led the authors to conclude that the aromatic side chain of residue 16 is critical for IGF-I integrity. Insulin-like substitutions involving both Gln$^{15}$ and Phe$^{16}$ are apparently less detrimental to structure, as might be expected from their conserved structural motifs. While not characterized by CD analysis, the insulin-like [Tyr$^{15}$,Leu$^{16}$]IGF-I variant has a slightly increased affinity for the type 1 IGF receptor (124%) and a 9-fold increased affinity for the insulin receptor (*15*). Competitive binding studies with [Tyr$^{15}$,Leu$^{16}$]IGF-I further indicate that this area may be selectively important for IGF binding to IGFBP-4 and -5 (*12*).

The residue analogous to Phe$^{16}$ in IGF-I (insulin B$^{17}$) is not strictly conserved as Leu in all insulin peptides; fish sequences have various hydrophobic residues at this position (Leu, Val, or Phe) and three hystricomorph insulins contain Ser (*30*). CD analyses of two of these highly substituted rodent insulins (coypu and casiragua) indicate that they are structurally similar to bovine insulin (*31*). Further, the biological activities of these insulins more closely resemble the IGFs as they have a decreased metabolic potential and are more potent in mitogenic assays (*32*). We therefore predicted that the Phe$^{16}$ to Ser substitution would produce an IGF-I analogue with native structure and function. We also reasoned that if Phe$^{16}$ contributes to the hydrophobic interface between the IGFBPs and IGF, then the selection of a nonhydrophobic residue for substitution would cause greater disruption to the IGF-IGFBP binding surface.

*[Ser$^{16}$]IGF-I Binding Assays with IGFBPs 1−6*. Competitive binding studies with the [Ser$^{16}$]IGF-I analogue demonstrated a significantly reduced affinity for all IGFBPs. These results were generally consistent with those reported (*12*) for the [Tyr$^{15}$,Leu$^{16}$]IGF-I analogue in that IGFBP-4 and -5 had a much greater loss of affinity (83-fold and >330-fold, respectively) compared to IGFBP-2 and -3, both of which had a 14−15-fold loss of affinity. While Tyr$^{15}$ and Leu$^{16}$ substitutions resulted in a modest 2-fold decrease in binding for IGFBP-1, the [Ser$^{16}$]IGF-I mutant decreased affinity for IGFBP-1 by >30-fold. Further, the affinity of IGFBP-6 was reduced by more than 90-fold with the Ser$^{16}$-substituted analogue.

The significant decrease in apparent affinities resulting from the substitution of Ser for Phe at residue 16 indicates that this residue is functionally important for IGFBP association. The variability of binding detected among the IGFBPs 1−6 suggests that evolutionary divergence within their IGF binding surfaces could affect their specific association with the various IGF-I analogues, as suggested by Oh et al. (*13*).

*Rationale for Residue Substitution Glu$^9$ to Gln$^9$ and Lys$^9$*. The relevance of the N-terminal end of the B-domain α-helix for IGFBP binding has not previously been evaluated. We chose to examine the contribution of Glu$^9$, the second residue of the α-helix. This negatively charged residue is in a position where it may stabilize the end of the helix through electrostatic interactions with the positive dipole (*33*). His$^{B10}$ (the analogous insulin residue) at this position does not enhance helical stability but has been conserved in most insulins as it is critical for the formation of zinc hexamers. We chose to investigate Glu$^9$ with Gln and Lys substitutions. These amino acids were chosen largely in response to early structural studies that suggested IGF-I could form dimers (*6*); we reasoned that the substitution of Glu$^9$ with Gln and Lys (rather than His) would allow a thorough assessment of this residue without presenting further opportunities for self-association. Coypu and casiragua hystricomorph insulins both have Gln at the analogous B$^{10}$ position. These natural insulin variants are predominantly monomeric in nature and are structurally similar to bovine insulin (*31*).

Table 1: Biological Potencies of the IGF-I Analogues and Their Relative Affinities for IGFBPs 1−6[a]

| | IGF-I | [H$^4$]IGF-I | [Q$^9$]IGF-I | [K$^9$]IGF-I | [S$^{16}$]IGF-I | [Q$^9$,S$^{16}$]IGF-I | [K$^9$,S$^{16}$]IGF-I |
|---|---|---|---|---|---|---|---|
| | | | | L6 Myoblasts | | | |
| Protein synthesis | 1.0 (2.6 nM ± 0.1) | 1.8 ± 0.3 | 0.87 ± 0.06 | 0.24 ± 0.01 | 3.0 ± 0.1 | 1.4 ± 0.1 | 0.59 ± 0.03 |
| Type 1 IGF receptor | 1.0 (0.55 nM ± 0.02) | 1.88 ± 0.01 | 0.72 ± 0.02 | 0.21 ± 0.03 | 1.29 ± 0.01 | 0.59 ± 0.01 | 0.32 ± 0.01 |
| IGFBP | 1.0 (0.67 nM± 0.02) | 0.72 ± 0.06 | 0.55 ± 0.06 | 0.14 ± 0.02 | 0.05 ± 0.01 | 0.071 ± 0.004 | 0.01 ± 0.007 |
| | | | | IGFBP | | | |
| (r)hIGFBP-1[b] | 1.0 (8.1 nM ± 0.7) | 0.55 ± 0.03 | 0.68 ± 0.04 | 0.23 ± 0.04 | < 0.034 | < 0.017 | < 0.017 |
| rIGFBP-2[c] | 1.0 (0.18 nM ± 0.02) | 0.45 ± 0.04 | 0.25 ± 0.09 | 0.007 ± 0.002 | 0.07 ± 0.01 | 0.005 ± 0.001 | < 0.0004 |
| hIGFBP-3[d] | 1.0 (0.08 nM ± 0.02) | 0.83 ± 0.14 | 0.38 ± 0.15 | 0.26 ± 0.05 | 0.065 ± 0.03 | 0.02 ± 0.01 | 0.002 ± 0.001 |
| hIGFBP-4[d] | 1.0 (0.92 nM ± 0.02) | 0.53 ± 0.03 | 0.89 ± 0.02 | 0.22 ± 0.01 | 0.012 ± 0.01 | < 0.004 | < 0.004 |
| hIGFBP-5[d] | 1.0 (0.65 nM ± 0.03) | 0.45 ± 0.11 | 0.61 ± 0.07 | 0.53 ± 0.08 | < 0.003 | < 0.003 | < 0.003 |
| hIGFBP-6[d] | 1.0 (2.1 nM ± 0.1) | 0.40 ± 0.02 | 0.21 ± 0.01 | 0.033 ± 0.007 | < 0.011 | < 0.006 | < 0.006 |

[a] The table values are expressed as a ratio of the $ED_{50}$ for IGF-I to the $ED_{50}$ of the IGF analogue except for receptor binding, which are a ratio of $ED_{30}$ values. The nanomolar concentrations of IGF-I that have been used for comparison are given in parentheses. The relative potencies are the mean ± SD, $n = 2$. [b] Recombinant human. [c] Natural rat. [d] Natural human.

*[Gln$^9$]- and [Lys$^9$]IGF-I Binding Assays with IGFBPs 1−6*. IGFBP-1, -3, and -4 responded to mutations at residue 9 in a similar fashion; they had marginally reduced affinities for [Gln$^9$]IGF-I and each had a 4−5-fold reduced affinity for [Lys$^9$]IGF-I compared to native peptide. Further, the association of IGFBP-5 was only slightly affected by either Lys$^9$ or Gln$^9$ substitutions (affinity for IGFBP-5 decreased approximately 2-fold for both analogues). IGFBP-2 and -6 were the most sensitive to residue 9 substitutions; they had 4−5-fold reduced affinities for [Gln$^9$]IGF-I and each had a significantly decreased affinity for [Lys$^9$]IGF-I compared to native protein (140- and 30-fold, respectively, for IGFBP-2 and -6).

The substitution of Glu$^9$ with either Lys or Gln should decrease the thermostability of the N-terminal end of the helix (*33*) and may introduce subtle structural changes that affect IGFBP association. However, the significant decrease in apparent affinities noted only with IGFBP-2 and -6 for [Lys$^9$]IGF-I suggest that the introduced positive charge may directly inhibit binding of these IGFBPs. The results from substitution of Glu$^9$ are interesting because they, like the Ser$^{16}$ substitution, can distinguish between the binding patterns of IGFBP-1 and -2, which are generally considered to be identical (*12*). They are further of interest because they only marginally affect the association characteristics of IGFBP-5, in contrast to previous studies that predict a larger binding pocket and very stringent requirements for IGFBP-5 binding (*12*).

*Effects of Mutations to both Glu$^9$ and Phe$^{16}$*. The effects of mutations involving both the N- and C-terminal ends of the α-helix ([Gln$^9$,Ser$^{16}$]- and [Lys$^9$,Ser$^{16}$]IGF-I) appear from the competitive binding assays (Figure 4) to be approximately additive, as would be expected if the mutational effects were independent (*34*).

*Rationale for Residue Substitution Thr$^4$ to His$^4$*. The functional importance of residues located within the extreme N-terminal region (residues 1−3) of IGF-I has been evaluated in some detail. Deletion analysis (*16*) has demonstrated that the removal of Gly$^1$ and Pro$^2$ has little effect on IGFBP affinity. Glu$^3$ is, however, important for IGFBP−IGF complex formation as shown by both substitution (*17*) and deletion analysis (*16*). Thr$^4$ is not aligned with Glu$^3$; instead it points toward the opposite side of the molecule where it is juxtaposed with Phe$^{49}$ from the A-domain. The 3D model of IGF-I, presented in Figure 2, shows that a distance of less than 4 Å separates the Cα atom of Phe$^{49}$ from the γ-methyl of Thr$^4$, a distance that would allow hydrophobic interactions between the two residues. Because Phe$^{49}$ belongs to the A-domain tripeptide (Phe-Arg-Ser) that is thought to directly interact with IGFBPs (*12*, *14*), we anticipated that the introduction of His at position 4 would either functionally or sterically inhibit IGFBP binding.

*[His$^4$]IGF-I Binding Assays with IGFBPs 1−6*. The substitution of His for Thr at position 4 resulted in only marginally decreased affinities for the IGFBPs. These decreased affinities probably reflect small changes to the presentation of the preceding Glu$^3$ residue. While the results with [His$^4$]IGF-I are consistent with reported alanine substitutions for this residue (*15*), from the 3D model it is difficult to visualize how the closely aligned Phe$^{49}$ residue could be in a position to interact with the IGFBPs. A further substitutional analysis of the Phe-Arg-Ser residues in IGF-II indicated that changing Arg to Glu had only a small effect on IGFBP affinity (*14*). These results lead us to speculate that the apparent loss of affinity seen when the Thr-Ser-Ile insulin residues replace Phe-Arg-Ser in IGF is a result of the serine to isoleucine substitution. In addition to replacing a residue that may contribute to the IGFBP binding surface, this bulky hydrophobic residue could either (1) protrude directly onto the IGFBP binding surface on the face of the IGFs or (2) form van der Waals contacts to anchor the N-terminal B-domain residues. Alternatively, isoleucine in this position may sterically impede movement of the N-terminal residues. A decreased mobility of the N-terminal residues of the B-domains, resulting from either steric hindrance or hydrophobic interactions, may reduce IGFBP affinity if conformational changes occur when IGFBP−IGF complexes form. Solution NMR studies of IGF-I and -II indicate that both molecules have flexible N-terminal residues (*6*, *7*). Moreover, this flexibility may well be important for IGFBP association. It is interesting to note that NMR studies of insulin monomers indicate that the analogus isoleucine, Ile$^{A10}$, forms interchain NOE (nuclear Overhauser effect) contacts with residues Asn$^{B3}$, Gln$^{B4}$, and His$^{B5}$ (*5*). The close relationship between these A- and B-chain residues in insulin is thought to determine in part the orientation of the N-terminal region of the B-chain (*5*).

*Biological Activity Assessed with Rat L6 Myoblasts*. While the primary objective for generating this series of analogues was to better define the IGF-IGFBP interface, we wanted an overall picture of the biological activities of these analogues to reinforce our interpretation of the IGFBP assay

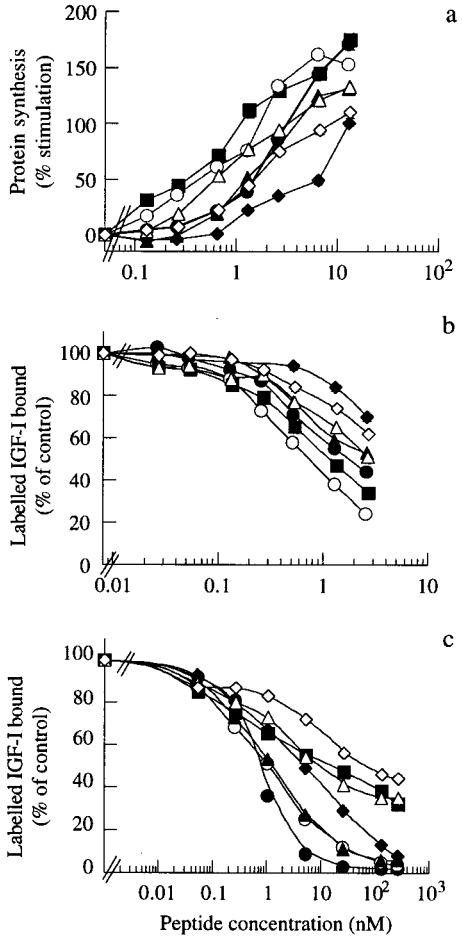


FIGURE 5: Biological potency of IGF-I analogues measured in rat L6 myoblasts: stimulation of protein synthesis (a), type 1 IGF receptor binding (b), and binding to secreted IGFBPs (c) by IGF-I and variants (symbols are the same as in Figure 4). Values for receptor binding and secreted IGFBPs are the means of triplicate determinations in two individual experiments at each peptide concentration. Protein synthesis values are the average of triplicate samples from three cultures. The SEM values are indicated by descending bars when larger than the displayed symbols.

results and to alert us to any inconsistencies. The in vitro activities of the IGF-I analogues were assessed by use of the rat L6 myoblast cell line, previously characterized by Ballard et al. (*35*). The results from assays measuring the stimulation of protein synthesis, binding to the type 1 IGF receptor, and analogue affinity for secreted IGFBPs are presented in Figure 5.

*Protein Synthesis*. Each of the analogues significantly stimulated protein synthesis in rat L6 myoblasts, as depicted in Figure 5a. At the highest concentration tested (12.8 nM), IGF-I resulted in a 171% increase in protein synthesis compared to control cultures. The concentrations of peptides required to give half the maximal stimulation seen with IGF-I ($ED_{50}$ values) were determined and their relative potencies are included in Table 1.

*Type 1 IGF Receptor Binding*. Protein synthesis in L6 myoblasts results from peptide association with the type 1 IGF receptor (*35*). [$Lys^9$]IGF-I reduced receptor binding of labeled IGF-I by 30% at the highest concentration tested (2.6 nM), as illustrated in Figure 5b. To enable a comparison between all analogues for receptor binding, we chose to determine relative $ED_{30}$ values; these are recorded in Table 1.

Each of the mutations affected type 1 IGF receptor affinity such that both [$His^4$]- and [$Ser^{16}$]IGF-I had marginally increased affinities while analogues having substitutions to $Glu^9$ had reduced receptor affinities. The results from substitution of $Glu^9$ parallel those observed with various insulin analogues; substitution of $His^{B10}$ in insulin with a negatively charged Asp both prevents the formation of zinc hexamers and increases insulin receptor affinity 2-fold (*36*). Also, substitution of insulin $His^{B10}$ with a more positively charged Lys residue results in a corresponding decrease in receptor affinity (*37*). Our results indicate that the type 1 IGF receptor, like the homologous insulin receptor, has an apparent higher affinity for peptides that contain a negatively charged residue at the start of the B-domain helix. These increased affinities may reflect additional ionic interactions with the receptors or they may be due, in part, to the predicted increased stability of the N-terminal ends of the helices.

*L6 Myoblast-Secreted IGFBPs*. The analogues' affinities for the IGFBPs contained in culture supernatant were assessed and results from these assays are presented in Figure 5c. The concentrations of analogues that reduced tracer binding by 50% ($ED_{50}$ values) relative to IGF-I are listed in Table 1. A variety of parameters are altered with the inclusion of culture supernatant into the assay system, all of which may affect the binding profiles seen with the various analogues when compared to those observed with purified IGFBPs. For instance, varied salt concentrations, different pH values, and secreted proteases could each be expected to influence the assay results. In addition, cultured cells are known to secrete a mixture of IGFBPs depending on culture conditions and cell type. Previous studies indicate that L6 myoblasts secrete primarily IGFBP-4 (*38*, *39*). Within cell culture systems, IGFBP-4 has been shown to sequester IGF-I peptides, thereby limiting their availability for receptor binding (*40*, *41*). When L6 myoblasts were used to analyze the biological activity of IGF analogues, those peptides with reduced affinity for IGFBP-4 and unaltered affinity for the type 1 IGF receptor would generally be expected to show an increase in protein synthesis compared to native IGF-I.

Protein synthesis in L6 myoblasts was approximately proportional to receptor binding activity for those analogues containing single substitutions at residue 4 or 9 ([$His^4$]-, [$Gln^9$]-, and [$Lys^9$]IGF-I), as indicated from the comparative values listed in Table 1. Peptides containing the $Phe^{16}$ to Ser substitution ([$Ser^{16}$]-, [$Gln^9$,$Ser^{16}$]-, and [$Lys^9$,$Ser^{16}$]IGF-I) stimulated protein synthesis to a greater degree than might have been expected from their relative affinities for the receptor. Those analogues containing the $Phe^{16}$ to Ser substitution further demonstrated significantly decreased affinities for the IGFBPs found in the culture supernatant, as shown in Figure 5c. From these results, we would expect IGFBP-4, the predominant form of IGFBP produced by L6 myoblasts, to also have a reduced affinity for $Ser^{16}$-substituted peptides. Competitive binding assays conducted with purified IGFBP-4 confirmed these predictions, as illustrated in Figure 4d.

*Conclusions*. Results from these and previous studies can help define the functional binding epitope of IGF-I that associates with the IGFBPs. Analyses of other protein−protein complexes (*42*−*44*) indicate that only a small number

of residues contribute to the free energy of binding, usually between 3 and 10 residues from each protein. The functionally important residues are generally located in a continuous patch and have centrally located hydrophobic surfaces. Our substitutional analysis of Phe[16] shows that this residue is important for association of all IGFBPs. By extrapolation, Phe[16] and other closely located hydrophobic residues (Leu[5] and Leu[54]) may form a functional binding pocket. Of these residues, Leu[54] would seem an obvious target for further analysis as various amino acids (Ile, Lys, Arg, and Ser) are structurally tolerated at this position in fish and hystricomorph insulins (*30*). Mutational analysis of Leu[5] may be more problematic as evolutionary pressures have ensured that this residue is strictly conserved throughout all insulin and IGF peptides.

Hydrophilic residues contiguous to the hydrophobic binding pocket are thought to control the recognition process and, when functionally active, form salt bridges or hydrogen bonds between ligands to stabilize the complexes. Mutational analysis of Glu[3] in IGF-I (*17*) indicates that this residue is functionally important for IGFBP binding. While Thr[4], Asp[12], Gln[15], Arg[55]-, and Arg[56] all surround the putative binding pocket on the front face of IGF-I, this and previous studies suggest that these residues are not functionally critical for IGFBP binding (*22*, *29*, *45*). Substitutional analysis of Glu[9] indicates that the N-terminal end of the α-helix may be selectively important for binding IGFBP-2 and -6. The 3D model of IGF-I (Figure 2) shows that the hydrophilic residues Ser[51], Asp[53]-, and Asp[20] are located in close proximity to either Glu[3] or Phe[16]. The highly variable nature of the analogous insulin residues (*30*) and the position of these residues on the face of the IGFs suggest that they would be interesting targets for further analysis.

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