The insulin-like growth factors (IGFs) are transported by a family of high-affinity binding proteins (IGFBPs) that protect IGFs from degradation, limit their binding to IGF receptors, and modulate IGF actions. The six classical IGFBPs have been believed to have no affinity for insulin. We now demonstrate that IGFBP-7/mac25, a newly identified member of the IGFBP superfamily that binds IGFs specifically with low affinity is a high-affinity insulin binding protein. IGFBP-7 blocks insulin binding to the insulin receptor and thereby inhibiting the earliest steps in insulin action, such as autophosphorylation of the insulin receptor β subunit and phosphorylation of IRS-1, indicating that IGFBP-7 is a functional insulin-binding protein. The affinity of other IGFBPs for insulin can be enhanced by modifications that disrupt disulfide bonds or remove the conserved COOH terminus. Like IGFBP-7, an NH2-terminal fragment of IGFBP-3 (IGFBP-3(1–87)), also binds insulin with high affinity and blocks insulin action. IGFBPs with enhanced affinity for insulin might contribute to the insulin resistance of pregnancy, type II diabetes mellitus, and other pathological conditions.

Insulin-like growth factors (IGFs)-I and -II are structurally related to insulin, sharing approximately 50% amino acid homology with insulin in the A- and B-chain regions but retaining a connecting peptide, as well as a carboxyl-terminal extension (1). The anabolic and mitogenic actions of IGFs are mediated largely through the type 1 IGF receptor, which, like the insulin receptor, is a heterotetrameric, membrane-spanning tyrosine kinase (2).

It has been accepted dogma that, unlike insulin, the IGFs bind to a family of binding proteins (IGFBPs) with high affinity and specificity (3–5). The six well characterized IGFBPs have significant sequence homology, including a GCGCCCXC motif as part of 10–12 conserved cysteines and six conserved cysteines at the carboxyl terminus. We have recently characterized two additional secreted proteins with lower affinity for IGFs (IGFBP-7/mac25 and IGFBP-8/CTGF) and two additional potential members of the IGFBP superfamily (6–9). The structure of IGFBP-7 revealed the presence of amino-terminal conserved sequences, including 11 cysteines, but lower sequence homology in the normally well-conserved COOH terminus. IGFBPs 1–6 are believed to function as carrier proteins for IGFs, delaying degradation of IGF peptides and increasing their half-lives and inhibiting IGF access to receptors (10–14).

Proteolysis of IGFBPs, as observed during pregnancy, results in IGFBP fragments with decreased affinity for IGFs and, thereby, promotes enhanced access of IGFs to their receptors (15, 16). Recent studies have demonstrated that several of these IGFBPs, especially IGFBP-3, may also be capable of directly inhibiting cell growth in an IGF-independent manner (17–23). Both intact IGFBP-3 and IGFBP-3 proteolytic fragments have been shown to be capable of blocking the mitogenic effect of insulin (24, 25). Whether these effects reflect inhibition of insulin signaling pathways or a direct effect of IGFBP-3 and its fragments on cell growth remains unclear.

We have, therefore, reevaluated the ability of IGFBPs 1–6, IGFBP-7, and IGFBP-3 proteolytic fragments to bind insulin and have demonstrated that IGFBP-7 and NH2-terminal fragment of IGFBP-3 (IGFBP-3(1–87)) not only bind insulin specifically but also modulate insulin binding to its receptor and subsequently inhibiting insulin-stimulated autophosphorylation of the insulin receptor β subunit and phosphorylation of IRS-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—High performance liquid chromatography-purified hIGFBP-1 from human amniotic fluid was kindly provided by Dr. D. R. Powell (Baylor College of Medicine, Houston, TX) (26). Recombinant human IGFBP-3 (rhIGFBP-3), a nonglycosylated 29 kDa core protein expressed in Escherichia coli cells, was the generous gift of Celtrix, Inc. (Santa Clara, CA) (27). Recombinant human IGFBP-2, -4, -5, and -6 were purchased from Austral Biologics (San Ramon, CA). The cDNA for IGFBP-7 (mac25) was cloned, and baculovirus recombinants were made as described in (7). IGF-I was purchased from Bachem California (Torrance, CA). IGF-II was kindly provided by Lilly. Bovine insulin was purchased from Sigma. [Gln6, Ala7, Thr18, Leu19, Leu27] IGF-II (Torrance, CA). IGF-I and -II were kindly provided by DSL (Webster, TX). Hormone and 125I-prolactin were kindly provided by DSL (Webster, TX). 125I-labeled growth hormone and 125I-prolactin were kindly provided by DLS (Webster, TX). NaC125I was obtained from Amersham Corp. Iodination was performed by a modification of the chloramine-T technique to specific activities of 350–500 μCi/μg for IGF-I and -II. Reagents used for SDS-polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA).

**Cell Culture**—NIH-3T3 cells overexpressing the human insulin receptor were kindly gifted from Dr. C. T. Roberts, Jr. (Dept. of Pediatrics, Oregon Health Sciences University) and grown in Dulbecco's modified Eagle's medium + 10% fetal calf serum + 500 μg/ml geneticin at 37 °C.
HCl, pH 6.8, 40% glycerol, and 2.3% SDS) with or without 100 mM suberate was added to a final concentration of 0.5 mM. After cross-linking for 15 min at 4 °C, the samples were subjected to 12% SDS-PAGE and autoradiography. B, IGFBPs 1–7, at the concentrations indicated in the figure, were incubated with a mixture 125I-IGF-I (10,000 cpm) and 125I-IGF-II (10,000 cpm) (top), or 125I-insulin (20,000 cpm) (bottom) and cross-linked as in Fig. 1A.

with 5% CO2. High Five cells were maintained in ExCell 405 Media (JRH Biosciences, Lenexa, KS) at 27 °C. The wild-type baculovirus (AcNPV) was obtained from Invitrogen Inc. (Carlsbad, CA). All tissue culture media and components were purchased from Life Technologies, Inc.

Affinity Cross-linking—Aliquots of reagents plus binding buffer (50 mM Tris-HCl, pH 7.4) were incubated with 125I-insulin (20,000 cpm), 125I-IGF-I (20,000 cpm), or 125I-IGF-II (20,000 cpm) with or without indicated reagents, at the concentrations indicated under “Results” and in Figs. 1–3 for 16 h at 4 °C. At the end of incubation, the cross-linking reagent disuccinimidyl suberate (Pierce) was added to a final concentration of 0.5 mM. After cross-linking for 15 min, the samples were subjected to 12% SDS-PAGE and autoradiography. Bands were quantified by densitometry, as calculated by a densitometer (Bio-Rad).

Generation and Purification of Recombinant IGFBP-3, 1–87—Polymerase chain reaction was used to add a FLAG epitope sequence (DYKDDDK) and a new stop codon immediately following amino acid 87 of the mature IGFBP-3. After sequencing, this fragment was subcloned into the baculovirus expression vector pFASTBAC1 (Life Technologies, Inc.) and transfected into SF9 insect cells, and viral recombinants were identified by immunoblotting with the anti-FLAG M2 antibody (Eastman Kodak, New Haven, CT). Recombinant protein was purified from the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over the media of High Five cells after infecting at an m.o.i. of 3 for 3 days at 27 °C. The media was collected, and the protein was purified over

FIG. 1. Affinity cross-linking of IGFBP with 125I-insulin or 125I-IGFs. A, 2–200 pmol of IGFBP-7 were incubated with 125I-insulin (20,000 cpm) for 16 h at 4 °C. At the end of incubation, disuccinimidyl suberate was added to a final concentration of 0.5 mM. After cross-linking for 15 min at 4 °C, the samples were subjected to 12% SDS-PAGE and autoradiography. B, IGFBPs 1–7, at the concentrations indicated in the figure, were incubated with a mixture 125I-IGF-I (10,000 cpm) and 125I-IGF-II (10,000 cpm) (top), or 125I-insulin (20,000 cpm) (bottom) and cross-linked as in Fig. 1A.

Insulin Receptor Binding Assay—20,000 cpm of 125I-insulin were preincubated with unlabeled IGF-I, IGF-II, and insulin at concentrations indicated in the table and then incubated with 50 μg of microsomal placentatal crude membranes in 500 μl of binding buffer (50 mM Tris-HCl, 0.5% BSA, pH 7.4) for 16 h at 4 °C. Samples were centrifuged at 10,000 × g for 10 min at 4 °C. After samples were centrifuged, pellets were counted in a γ-counter.

Specificity of IGFBP binding to 125I-insulin. Autoradiogram of 125I-insulin (A), 125I-prolactin (B), and 125I-labeled growth hormone (C) cross-linked to 20 pmol of IGFBP-1, -3, or -7, as in Fig. 1.

**RESULTS**

Affinity Cross-linking of IGFBP 1–7—To evaluate the affinity of IGFBPs 1–7 for IGFs and insulin, the affinity cross-linking was performed with 125I-IGF-I + 125I-IGF-II or 125I-insulin. Affinity cross-linking of IGFBP-7 with 125I-insulin shows that IGFBP-7 binds insulin in a dose-dependent manner (Fig. 1A). As shown in Fig. 1B, top panel, the expected sizes of individual IGFBPs coupled to 125I-insulin were detected on the SDS-PAGE gel. In a similar way, Fig. 1B, bottom panel, showed 125I-insulin bound to IGFBPs 1–7 with proper size of bands. This data demonstrates that IGFBPs 1–6 also bind insulin but with significantly reduced affinity compared with their affinities for IGFs. In contrast, the ratio of insulin:IGF binding for IGFBP-7 was approximately 500-fold higher than for IGFBPs 1–6 by densitometry analysis.

To establish the specificity of IGFBP binding to insulin, we attempted to cross-link 125I-IGF-I, -3, and -7 with 125I-prolactin (Fig. 2B) and 125I-labeled growth hormone (Fig. 2C), as well as with 125I-insulin (Fig. 2A). Although these IGFBPs failed to bind the other proteins, binding of 125I-insulin was confirmed. Furthermore, analysis of competitive affinity cross-linking revealed
that the relative affinities of IGFBP-7 are: insulin ≥ IGFs ≥ [QAYLL] IGF-II (an IGF-II analog whose affinity for IGFBPs 1–6 is normally 100-fold less than that of native IGF-II). In contrast, affinities for IGFBP-3 are: IGFs ≫ insulin > [QAYLL] IGF-II (Fig. 3, A and B). The binding of IGFBP-3 or -7 to 125I-IGFs or 125I-insulin was competitively displaced by both IGFs and insulin, suggesting that the insulin binding site may be at, or near, the IGF binding site. IGFBP-7 was found to have similar affinity for IGFs and insulin (IC50 = 20–34 nM). Previous studies have suggested that IGF binding sites reside on both the NH2- and COOH-terminal regions of the IGFBP-3 molecule; each binding site is individually capable of low-affinity binding, whereas high-affinity binding of IGFs requires the presence of both binding domains. Taken together, our data suggest that the low affinity of IGFBP-7 for IGFs and the relatively high affinity for insulin, compared with those of IGFBPs 1–6, can be attributed to the lack of conserved amino acid sequence and cysteines at the COOH terminus of IGFBP-7 (only 1 of the 6 cysteines is conserved) although IGFBP-7 retains 11 of the 12 cysteines found in the IGFBPs at the NH2 terminus. High-affinity binding of IGFBPs to IGFs thus appears to require proper structural configuration involving both NH2- and COOH-terminal ligand-binding domains.

Reduction or Fragmentation of IGFBPs Enhances the Affinity for Insulin—Insulin binding to IGFBPs can be further demonstrated by use of WLB. It appears that insulin binding to IGFBPs is much less effective in WLBs, especially in the absence of DTT compared with that seen in solution binding, as assessed by affinity cross-linking (Fig. 1). This presumably reflects changes in the tertiary structure of IGFBPs during SDS-PAGE. However, WLB demonstrated that reduction of

![Image](https://example.com/image1.png)

**FIG. 3.** Competitive affinity cross-linking of IGFBP-7 and -3. A, 125I-insulin was cross-linked to 20 pmol of IGFBP-7 (top) or IGFBP-3 (bottom) alone or in the presence of unlabeled IGF-I or insulin. B, 125I-IGF-II was cross-linked to 20 pmol of IGFBP-7 (top) or 1 pmol of IGFBP-3 (bottom) alone or in the presence of unlabeled IGF-II, [QAYLL] IGF-II, or insulin. Cross-linking was performed as in Fig. 1.

![Image](https://example.com/image2.png)

**FIG. 4.** Western ligand blot of IGFBP-1, -3, and -7. IGFBP-1, -3, and -7 at the concentrations indicated in the figures with or without 100 mM DTT were processed by SDS-PAGE. A, 12%; B and C, 15%.

![Image](https://example.com/image3.png)

**FIG. 5.** Characterization of IGFBP-3 (1–87). A, the purified FLAG-tagged IGFBP-3 (1–87) was subjected to SDS-PAGE (15%) and then to immunoblot with anti-M2 antibody. IGFBP-3 and IGFBP-3 (1–87) were subjected to SDS-PAGE with or without DTT, and electroblotted onto filters, which were incubated with 125I-insulin (B) or 125I-IGFs (C).
IGFBPs -1, -3, and -7 with DTT resulted in a marked decrease in affinity for IGF but increased affinity for insulin (Fig. 4). These data indicate that disulfide bonds and the tertiary structure of IGFBP molecules are, at least in part, responsible for the relatively low affinity for insulin and high affinity for IGF. To further test this hypothesis, we expressed human IGFBP-3(1–87) in a baculovirus system, which was epitope-tagged with the FLAG sequence at the COOH end (Fig. 5A). While the affinity for IGF was greatly diminished in IGFBP-3(1–87), the affinity for insulin was significantly increased in IGFBP-3(1–87), compared with that of intact IGFBP-3 (Fig. 5B and C). Reduction of IGFBP-3(1–87) with DTT resulted in a modest increase in affinity for insulin, suggesting that disulfide bonds within the IGFBP-3(1–87) fragment still limit, at least in part, the binding affinity for insulin. These results suggest that disulfide bonds between the NH2 and COOH termini result in a ternary structure for the IGFBPs that confers high affinity for IGFs. To enhance the affinity for insulin, the IGFBP-3 molecule must be modified by either disrupting the ternary structure (under reducing conditions) or by eliminating or modifying the COOH-termini domain (IGFBP-3(1–87) or IGFBP-7). A schematic model is depicted in Fig. 6.

IGFBP-7 and IGFBP-3 1–87 Modulate Insulin Binding to Its Receptor and Inhibit Insulin Action—We next investigated the biological relevance of IGFBP-7 binding to insulin. Insulin receptor binding assays were performed using125I-insulin and crude human placental microsomal membranes in the presence or absence of unlabeled insulin, IGFBP-3, or IGFBP-7. IGFBP-3, at concentrations as high as 300 pmol, did not block insulin binding. The specific binding of 125I-insulin to human placental insulin receptors was inhibited, however, by IGFBP-7 (60% by 100 pmol, and 90% by 300 pmol) (Fig. 7), indicating that IGFBP-7 can compete with insulin receptors for binding of insulin. Inhibition of insulin binding to placental membranes by IGFBP-7 was further confirmed by affinity cross-linking studies (data not shown). Thus, 100–300 pmol of IGFBP-7, but not IGFBP-3, inhibit insulin binding to placental membranes. This discrepancy between IGFBP-3 and IGFBP-7 action presumably reflects the higher affinity of the latter protein for insulin, as suggested in Fig. 1. Although care must be exercised in estimating affinities from cross-linking experiments, it appears likely that IGFBP-7 binds insulin more effectively than does IGFBP-3 although with lower affinity than that exhibited by the insulin receptor.

The effect of IGFBPs on insulin receptor signaling pathways, such as receptor autophosphorylation and IRS-1 phosphorylation, was tested. IGFBP-3(1–87) and IGFBP-7 blunted insulin-stimulated autophosphorylation of the insulin receptor β subunit in a dose-dependent manner, with 55 and 80% inhibition at concentrations of 100 pmol of IGFBP-3(1–87) or IGFBP-7, respectively (Fig. 8A). IGFBP-3 was ineffective at these concentrations, as predicted by the inability of IGFBP-3 to inhibit insulin binding to placental membranes. Similarly, the insulin-stimulated phosphorylation of IRS-1 was inhibited 65 and 85% by 100 pmol of IGFBP-3(1–87) or IGFBP-7, respectively (Fig. 8B). These differences did not reflect reduced concentrations of IRS-1 among samples tested, as demonstrated by IRS-1 immunoblots. Taken together, our data demonstrate that IGFBP-7...
and IGFBP-3(1–87) have the ability to bind insulin and subsequently inhibit insulin binding to the insulin receptor, resulting in the inhibition of insulin-stimulated autophosphorylation of the insulin receptor β subunit and phosphorylation of IRS-1.

**DISCUSSION**

Current dogma proposes that a major difference between insulin and IGFs is that only IGFs have the ability to bind IGFBPs 1–6 with high affinity. In addition, we have recently shown that IGFBP-7 also binds both IGF-I and IGF-II but with at least 5–25-fold lower affinity than do the other IGFBPs. However, it has been shown that several IGFBPs may play roles in regulating insulin action. IGFBP-1 production is suppressed by insulin and carbohydrate and stimulated by hypoglycemia, suggesting that IGFBP-1 might play a counterregulatory role in glucose homeostasis. Proteolysis of IGFBP-3 fragments, but not intact IGFBP-3, inhibited the mitogenic effect of insulin, suggesting that proteolyzed IGFBP-3 fragments may have the ability to regulate insulin signaling pathways, despite having significantly reduced affinity for IGFs. In the present studies, we have tested the ability of IGFBPs to bind insulin and regulate insulin action. We demonstrate that IGFBPs have the ability to bind insulin, and that fragmented IGFBP-3 has increased affinity for insulin, resulting in the inhibition of insulin action. Our further studies have revealed that IGFBP-3 fragments, either generated by plasmin digestion or present in normal human urine, bind insulin with relatively high affinity, supporting the physiological relevance of IGFBP-3 fragments on insulin action. 

Thus, recent studies have demonstrated that the current view of IGFBPs must be modified by the following observations: 1) IGFBP proteolysis results in peptide fragments with decreased affinity for IGFs (15, 16); 2) additional related proteins with low affinity for IGFs indicate the existence of an IGFBP superfamily (7, 9); 3) IGFBPs can bind insulin, as well as IGFs, and the affinity for insulin can be increased by modification of the protein by reduction of disulfide bonds or proteolysis of the protein; and 4) IGFBPs can, under certain conditions, inhibit insulin binding to its receptor and reduce the resulting stimulation of receptor and IRS-1 phosphorylation.

Immunoblot studies with antibodies specific for IGFBP-7 have demonstrated the presence of the mature protein in the conditioned media of a number of cell lines, as well as in cerebrospinal fluid, urine, amniotic fluid, and serum (31). Quantitative studies await the development of radioimmunoassays. Proteolytic fragments of IGFBP-3, as well as other IGFBPs, were originally identified in pregnancy serum but subsequently have been found in a wide variety of clinical conditions, including critically ill patients, patients with catabolic disease, diabetes mellitus, or noninsulin-dependent diabetes mellitus (15, 16, 33–38). Many of these diseases are characterized by insulin resistance of varying degrees, as measured by decreased insulin receptor tyrosine kinase activity and defective insulin receptor signaling (39–42). It is tempting to speculate that this insulin resistance might reflect increased concentrations of IGFBP-3 fragments or IGFBPs with enhanced affinity for insulin, such as IGFBP-7. These proteins may compete with insulin receptors for peptide binding, much as IGFBPs sequester IGF peptides from IGF receptors. These observations thus support a novel model for the interactions of the IGF and insulin pathways and suggest a potential new approach to our understanding of the pathophysiology of insulin resistance.

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IGFBPs Inhibit Insulin Receptor Activation

**Inhibition of Insulin Receptor Activation by Insulin-like Growth Factor Binding Proteins**

Yoshitaka Yamanaka, Elizabeth M. Wilson, Ron G. Rosenfeld and Youngman Oh


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