Insulin-like growth factor-binding protein-3 (IGFBP-3), the major IGFBP in the circulation, sequesters IGF in a stable ternary complex with the acid-labile subunit. The high affinity IGF-binding site is proposed to reside within an N-terminal hydrophobic domain in IGFBP-3, but C-terminal residues have also been implicated in the homologous protein IGFBP-5. We have mutated in various combinations Leu\textsuperscript{77}, Leu\textsuperscript{80}, and Leu\textsuperscript{81} in the N terminus and Gly\textsuperscript{217} and Glu\textsuperscript{223} in the C terminus of IGFBP-3. All mutants retained immunoreactivity toward a polyclonal IGFBP-3 antibody, whereas IGF ligand blotting showed that all of the mutants had reduced binding to IGFs. Both solution IGF binding assays and BIAcore analysis indicated that mutations to the N-terminal region caused greater reduction in IGF binding activity than C-terminal mutations. The combined N- and C-terminal mutants showed undetectable binding to IGF-I but retained <10% IGF-II binding activity. Reduced ternary complex formation was seen only in mutants that had considerably reduced IGF-I binding, consistent with previous studies indicating that the binary IGF-IGFBP-3 complex is required for acid-labile subunit binding. Decreased IGF binding was also reflected in the inability of the mutants to inhibit IGF-I signaling in IGF receptor overexpressing cells. However, when present in excess, IGFBP-3 analogs defined as non-IGF-binding by biochemical assays could still inhibit IGF signaling. This suggests that residual binding activity of IGFBP-3 mutants may still be sufficient to inhibit IGF biological activity and questions the use of such analogs to study IGF-independent effects of IGFBP-3.

Insulin-like growth factors (IGFs)\textsuperscript{1} are potent mitogens that act through specific membrane receptors to promote cell proliferation and differentiation. They exhibit a broad range of effects on embryonic and fetal growth, development, and metabolism (1) and have been implicated in many diseases including cancer and other disorders such as neurodegeneration and osteoporosis (2–4). Their cellular effects are tightly controlled by a group of structurally related IGF-binding proteins (IGFBPs). Because IGFBPs have higher affinities for IGFs (K\textsubscript{D} = \textasciitilde 10\textsuperscript{-11} \textmu M) than does the type I IGF receptor (K\textsubscript{D} = \textasciitilde 10\textsuperscript{-8}–10\textsuperscript{-9} \textmu M), it is believed that IGFBPs achieve their regulation of IGFs through high affinity binding (5). Of the six known IGFBPs, IGFBP-3 and IGFBP-5 have the ability to form ternary complexes with IGFs and a 85-kDa glycoprotein, the acid-labile subunit (ALS) (6–8). The stable IGF ternary complexes prolong the half-life of circulating IGFs and act as a reservoir for the delivery of IGFs to the target tissues (9, 10). Apart from its role as a modulator of IGF actions, IGFBP-3 has also been found to possess other cellular functions independently of IGFs (11, 12), proposed to be mediated through an IGFBP-3 receptor (13, 14).

Based on sequence homology, all IGFBPs can be conceptually divided into three discrete domains: N-terminal, C-terminal, and central. There is a high degree of conservation in the N- and C-terminal domains, which are thought to be involved in IGF binding (11). The central domains, on the other hand, are unique in sequence among the six IGFBPs and are believed to serve as a hinge between the N- and C-terminal domains. Because most of the known posttranslational modifications (glycosylation, phosphorylation, and proteolysis) occur within the central domain, it is speculated that these modifications may participate in other diverse functions of IGFBPs (5).

Although the solution structures of both IGF-I and -II have been solved (15, 16), the full structures of IGFBPs have not been reported, thus hindering the understanding of the molecular interactions between IGFs and IGFBPs. In 1998, Kalus et al. (17) published a landmark paper on the solution structure of an IGFBP-5 N-terminal fragment, termed mini-IGFBP-5 (Ala\textsuperscript{40}–Ile\textsuperscript{92}), obtained by using NMR spectroscopy. It was revealed that mini-IGFBP-5 has a compact and globular three-dimensional structure that is uniquely folded. A single high affinity binding site for IGF-II was identified in mini-IGFBP-5 that comprises residues Val\textsuperscript{49}, Tyr\textsuperscript{50}, Pro\textsuperscript{62}, and Lys\textsuperscript{68}–Leu\textsuperscript{74}. Several hydrophobic residues including Val\textsuperscript{49}, Leu\textsuperscript{80}, and Leu\textsuperscript{74} expose their side chain into solution to form a hydrophobic patch on the surface of the mini-IGFBP-5. Because Leu\textsuperscript{73} and Leu\textsuperscript{74} of IGFBP-5 are conserved in IGFBP-3, it was predicted that these residues in IGFBP-3 would be among the common determinants for IGF binding. Several subsequent mutagenesis studies on the N termini of IGFBP-3 and -5 have provided evidence to support the existence of the putative N-terminal binding site (18–20). Mutations of some of the implicated residues in the N termini led to greatly reduced IGF binding, supporting the existence of a major IGF-binding site in the N-terminal regions of IGFBP-3.
In contrast to the N-terminal region, the role of the C-terminal region of IGFBPs in IGF binding is less well defined. Bramani et al. (21) previously reported that mutations at conserved residues Gly<sup>203</sup> and Gln<sup>209</sup> in the C-terminal region of rat IGFBP-5 decreased its binding to IGF-I. To further investigate the IGF binding function of these two residues, they combined mutations in both N- and C-termini and demonstrated a cumulative loss in the ability of the mutant to bind IGF-I (22). However, this study is inconsistent with previous reports that a total abolition of IGF binding could be demonstrated when mutations were made solely to the N terminus of IGFBP-3 (19) or IGFBP-5 (18), suggesting that the minimal IGF-binding site does not involve C-terminal residues. The involvement of C-terminal residues in the high affinity IGF-binding site of IGFBP-3 was recently reported by our laboratory. We showed that although discrete N-terminal (residues 1–88) and C-terminal (residues 185–264) fragments of IGFBP-3 bind IGFs separately, together they show cooperativity in the presence of IGFs to form high affinity binding complexes (23).

To define the high affinity IGF-binding sites in IGFBP-3 and to clarify the role and relative contribution of N- and C-termini in IGF binding, we used site-directed mutagenesis to generate a set of IGFBP-3 proteins with mutations made to either the N terminus or the C terminus or both. Characterization of these IGFBP-3 mutants by various biochemical and functional assays has demonstrated the importance of both N- and C-terminal residues in regulating IGF binary and ternary complex formation and IGF receptor activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human IGF-I was a gift from Genentech, Inc. (South San Francisco, CA). Recombinant human IGF-II was purchased from GroPep Pty. Ltd. (Adelaide, Australia). Human ALS was purified from serum as described previously (6, 24). Anti-hIGFBP-3 antisera (R-100) is a rabbit polyclonal antibody raised in house against the full-length human IGFBP-3. Heparin Hi-Trap columns were purchased from Amersham Biosciences. QuikChange site-directed mutagenesis kits were obtained from Stratagene (La Jolla, CA). MutaGeneic plasmids were transfected with plasmid DNA purified from CaCl<sub>2</sub> gradient to generate adenoviruses as previously described (26). High titer adenoviruses for wild-type and mutant hIGFBP-3 were generally obtained following three or four rounds of amplification. Wild-type and mutant IGFBP-3 were produced as secreted proteins in serum-free Dulbecco's modified Eagle's medium supplemented with 1 g/liter bovine serum albumin, 20 mM glutamine, and a mixture of protease inhibitors (500 units/ml aprotinin, 5 µg/ml α-macroglobulin, and 0.5 µg/ml leupeptin) from 450-cm<sup>2</sup> flasks of confluent 911 cells infected with high titer adenoviruses. Conditioned media were collected 48 h after infection, and proteins in the media were purified by either IGF-I affinity or heparin affinity chromatography (for mutants with very low IGF binding). Proteins bound to IGF-I affinity columns were eluted with 1 M NaCl, whereas proteins bound to heparin affinity columns were eluted by a stepwise NaCl gradient as described by Firth et al. (29). Further purification was achieved by reverse-phase HPLC as previously described (30). The proteins were quantitated by IGFBP-3 radioimmunoassay (RIA) using hIGFBP-3 antibody as described by Baxter and Martin (27). Purity of the proteins was assessed by silver staining after SDS-PAGE.

**IGF Ligand Blotting and IGFBP-3 Immunoblotting**—Briefly, 40 ng of purified wild-type or mutant protein was separated by 12% SDS-polyacrylamide gel under non-denaturing conditions and transferred onto Hybond-C Extra nitrocellulose (Amersham Biosciences) by electroblotting using a Multiphor II Novablot unit (Amersham Biosciences). After transfer, the blot was blocked for 2–3 h in Tris-buffered saline with 0.05% (v/v) Nonidet P-40 containing 1 g/liter bovine serum albumin, 20 mM glutamine, and a mixture of protease inhibitors (500 units/ml aprotinin, 5 µg/ml α-macroglobulin, and 0.5 µg/ml leupeptin) from 450-cm<sup>2</sup> flasks of confluent 911 cells infected with high titer adenoviruses. Conditioned media were collected 48 h after infection, and proteins in the media were purified by either IGF-I affinity or heparin affinity chromatography (for mutants with very low IGF binding). Proteins bound to IGF-I affinity columns were eluted with 1 M NaCl, whereas proteins bound to heparin affinity columns were eluted by a stepwise NaCl gradient as described by Firth et al. (29). Further purification was achieved by reverse-phase HPLC as previously described (30). The proteins were quantitated by IGFBP-3 radioimmunoassay (RIA) using hIGFBP-3 antibody as described by Baxter and Martin (27). Purity of the proteins was assessed by silver staining after SDS-PAGE.

**Cell Culture**—E1-transformed 911 human embryonic retinoblastoma cells (28) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 20 mM glutamine. NIH-3T3-IGFRI cells were maintained in the above medium plus 500 units/ml aprotinin, 5 µg/ml α-macroglobulin, and 0.5 µg/ml leupeptin from 450-cm<sup>2</sup> flasks of confluent 911 cells infected with high titer adenoviruses. Conditioned media were collected 48 h after infection, and proteins in the media were purified by either IGF-I affinity or heparin affinity chromatography (for mutants with very low IGF binding). Proteins bound to IGF-I affinity columns were eluted with 1 M NaCl, whereas proteins bound to heparin affinity columns were eluted by a stepwise NaCl gradient as described by Firth et al. (29). Further purification was achieved by reverse-phase HPLC as previously described (30). The proteins were quantitated by IGFBP-3 radioimmunoassay (RIA) using hIGFBP-3 antibody as described by Baxter and Martin (27). Purity of the proteins was assessed by silver staining after SDS-PAGE.

**Materials and Methods**

**Materials**—Recombinant human IGF-I and IGF-II were determined at a flow rate of 40 µl/min running...
buffer: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA), thereby minimizing mass transfer effects. Association proceeded for 300 s followed by 900 s for dissociation. IGFBP biosensor surfaces were regenerated with 10 mM HCl. For each binding curve, the response obtained using control surfaces (no protein coupled) was subtracted. Dissociation constants were derived using BIAevaluation 3.2 software and a 1:1 Langmuir binding model. This model describes a simple reversible interaction of two molecules in a 1:1 complex. Whereas two-site binding models have been used to describe the interaction between IGFs and IGFBP-3, this was not an appropriate model for analyzing the N- and C-terminal domain mutants. In addition, the low affinity binding component of the IGF-IGFBP-3 interaction only makes up ~10% of the total binding (33). All binding experiments were repeated at least twice, and biosensor chips coupled at different times yielded surfaces with comparable binding affinities. Experiments in the opposite orientation using IGFs were serum-starved for 24 h. Immediately prior to the experiment, the cells were washed with serum-free medium before being exposed for 5 min to either 2.5 ng/ml IGF-I or 10 ng/ml IGF-II that had been preincubated with or without either wild-type or mutant IGFBP-3 in molar ratios of 1:1, 1:5, and 1:10 (IGF:IGFBP-3) for 2 h at 22 °C. The reaction was quenched by the addition of 200 μl of cell solubilization buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 137 mM NaCl, 100 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na3VO4). To detect IGFRI tyrosine phosphorylation, 10 μl of cell lysate was separated by 8% SDS-PAGE under reducing conditions and immunoblotted with anti-phospho-IGF1Rα monoclonal antibody PY20 at a 1:1,000 dilution overnight at 4 °C. Phosphoproteins were visualized by ECL detection after incubating blots with horseradish peroxidase-conjugated anti-mouse secondary antibody at a 1:2,000 dilution. The same blot was then stripped with “RESTORE” stripping buffer (Pierce) and probed with IGFRI subunit antibody C-20 at a 1:2,000 dilution. The same blot was then stripped with “RESTORE” stripping buffer (Pierce) and probed with IGFRI subunit antibody C-20 at a 1:2,000 dilution. The corresponding sequences (residues 51–84 and 215–228) of IGFBP-3 are also shown, and residues that are conserved or homologous to IGFBP-5 are indicated in italics. Changes in specific amino acid residues are indicated for each IGFBP-3 mutant, unchanged residues are indicated by colons. On the right, the IGF binding function of each IGFBP-3 analog was assessed by IGF-I solution binding assay using culture medium of cells infected with recombinant adenoviruses expressing each mutant. The IGFBP-3 analogs were Q (Gln77), GG (Gly80-Gly81), QG (Gln80-Gln81), QQ (Gln77-Gln80-Gln81), S (Ser217), A (Ala223), SA (Ser217-Ala223), QSA (Gln77-Ser217-Ala223), and GGSA (Gly80-Gly81-Ser217-Ala223).

**FIG. 1.** The structure of IGFBP-3 and IGFBP-5 sequence is represented by the box at the top. The horizontal lines within the box represent the relative positions of the 15 curious residues that are conserved between IGFBP-3 and IGFBP-5. The gray regions correspond to the sequences shown for IGFBP-3 and IGFBP-5. Residues 44–77 in the N-terminal region and residues 201–214 in the C-terminal of IGFBP-5 are shown, residues implicated in IGF binding based on the studies of Kalus et al. (17) and Shand et al. (22) are shaded. The corresponding sequences (residues 51–84 and 215–228) of IGFBP-3 are also shown, and residues that are conserved or homologous to IGFBP-5 are indicated in italics. Changes in specific amino acid residues are indicated for each IGFBP-3 mutant, unchanged residues are indicated by colons. On the right, the IGF binding function of each IGFBP-3 analog was assessed by IGF-I solution binding assay using culture medium of cells infected with recombinant adenoviruses expressing each mutant. The IGFBP-3 analogs are Q (Gln77), GG (Gly80-Gly81), QG (Gln80-Gln81), QQ (Gln77-Gln80-Gln81), S (Ser217), A (Ala223), SA (Ser217-Ala223), QSA (Gln77-Ser217-Ala223), and GGSA (Gly80-Gly81-Ser217-Ala223).
mutants had reduced binding affinities for heparin. Further purification by reverse-phase HPLC showed that all of the mutant proteins eluted from the column at a similar retention time of 19 min (data not shown). Quantitation of the purified proteins by IGFBP-3 RIA showed that the recombinant wild-type and mutant proteins yielded displacement curves that were parallel to that of serum-derived IGFBP-3 (the standard in the RIA), suggesting that the epitope recognized by the hIGFBP-3 antibody was not significantly altered in the mutant proteins (Fig. 2A). Immunoblotting of the proteins with IGFBP-3-specific polyclonal antibody demonstrated that the recombinant IGFBP-3 analogs were similarly glycosylated because they appeared as doublet bands with apparent molecular masses of 42–45 kDa (Fig. 2B).

Mutations in hIGFBP-3 Decrease or Abolish Its Binding to Both IGF-I and -II—Effects of mutations in hIGFBP-3 on IGF binding were initially examined by IGF-I or IGF-II ligand blotting (Fig. 2, C and D). Mutations at either the N or C terminus caused varying degree of reduction in both IGF-I and -II binding compared with wild-type IGFBP-3. Reduction in IGF binding was more evident in N-terminal than in C-terminal mutants. The N-terminal mutants, GG and QQ, showed greater reduction in binding to IGF-I (Fig. 2C) than to IGF-II (Fig. 2D). Mutation of Leu77 to Gln (mutant Q) showed selectively decreased binding for IGF-I but in contrast appeared to bind IGF-II normally. The C-terminal mutants, S and A, showed a minimal effect on IGF binding by ligand blotting, whereas the combined mutant SA showed a cumulative effect in decreasing both IGF-I and IGF-II binding. The binding of the N-terminal mutant, QQ, and the combined mutants, QSA and GGSA, to both IGF-I and -II was undetectable by this technique.

Quantitative assessment of binding was obtained by solution IGF binding assays (Fig. 3) that in general confirmed the data.
 obtained by ligand blotting. The apparent binding activity of each mutant, relative to IGFBP-3, for IGF-I and -II was derived from its binding curve as described under "Experimental Procedures" and summarized in Table I. Clearly, neither mutations on the N terminus nor the C terminus alone could completely abolish IGF-I (Fig. 3, A and B) or IGF-II (Fig. 3, D and E) binding. The Gln<sup>217</sup> (Q) mutant had a ∼3-fold reduction in binding activity to both IGF-I and -II (Table I). Substituting Leu<sup>80</sup>-Leu<sup>81</sup> with glycine residues was more effective than glutamine residues in reducing IGF binding. Specifically, the GG analog resulted in 5- and 10-fold decreases in IGF-I and -II binding, respectively, whereas the QQ mutant had less than 2-fold reduction in IGF binding activity. Mutations at an individual C-terminal residue (Gly<sup>217</sup> to Ser or Gln<sup>223</sup> to Ala) caused only 2–3-fold decrease in binding activity, but the double mutations (SA) resulted in 5- and 10-fold decrease in binding to IGF-I and -II, respectively. More remarkably, only the combined N- and C-terminal mutants, QSA and GGSA, showed undetectable binding to IGF-I (Fig. 3C), with minimal binding to IGF-II detectable at high concentrations (> 50 ng/tube) of the IGFBP-3 analogs.

To obtain a kinetic evaluation of the IGF binding properties of the IGFBP-3 mutants, we conducted a biosensor analysis of binding kinetics of these proteins to IGF-I and -II (Fig. 4). The BIAcore analysis provides details of association (on) and dissociation (off) rate kinetic constants, from which equilibrium constants ($K_D$) can be derived. The results from the BIAcore biosensor measurements of the IGFBP-3 mutants are, for the most part, supportive of the findings by IGF ligand blotting and solution binding assays. We conclude that mutations at the N terminus generally caused more profound reduction in binding affinities than mutations at the C terminus (Table II). A slightly greater effect was seen in the A analog on IGF-I binding by BIAcore. A closer inspection of the BIAcore data revealed that the overall decreases in $K_D$ values observed for the N-terminal mutants were largely the result of the reduction in their $K_{on}$ values, whereas the decreases of $K_D$ values in C-terminal mutants mainly resulted from the increase in $K_{off}$ values (data not shown). Both BIAcore and solution binding assays showed that the two N- and C-terminal mutants, QSA and GGSA, did not bind IGF-I, but some residual binding to IGF-II could be detected. It is of note that binding to IGF-II could be detected for all analogs (Fig. 4, Table II).

Interestingly, by BIAcore analysis, glycine substitutions at Leu<sup>80</sup>-Leu<sup>81</sup> virtually abolished IGF-I binding (Fig. 4A), compared with only 5–6-fold reduction in activity measured by IGF-I solution binding assay (Fig. 3A). In contrast, substitution of these residues with glutamine only reduced binding by ∼5-fold, indicating that the effect of mutation on IGF binding is dependent on the type of substitution made. The most notable difference between the two assays is that binding to IGF-I by the triple N-terminal mutant, QQQ, was undetectable by BIAcore analysis, whereas in the solution binding assay, binding to IGF-I was detected with only 25-fold decrease in apparent binding activity. One possible explanation for the differences between the assay measurements in the case of GG and QQQ could be that a change in local structure of the mutants could have affected coupling such that inactivation of the mutants occurred during coupling to the biosensor chip.

**Ternary Complex Formation Is Reduced in Mutants with Decreased IGF Binding Affinity**—In the circulation, the majority of IGFBP-3 exists in the form of ternary complexes with IGFs and ALS. These stable complexes effectively prolong the circulating half-life of the protein and inhibit their access to target tissues and thereby regulate IGF bioavailability. In this study, we measured the ability of the mutants to form ternary complexes with IGF-I and ALS (Fig. 5). In the assay, IGF-I was added in 6-fold molar excess of the highest amount of IGFBP-3 used to facilitate binary complex formation between IGF-I and IGFBP-3. Among the N-terminal mutants, the Q and QQ analogs showed minimal effect on ALS binding, and once again, substitution of Leu<sup>80</sup>-Leu<sup>81</sup> with glycine residues (GG analog) had a greater effect than substituting with glutamine residues (QQ). Although the QQ analog was 1 log order of magnitude lower in IGF-I binding than the GG analog (Fig. 3A), these two analogs showed similar ALS binding ability when IGF-I is present in excess amounts (Fig. 5A).

Although mutation of Gly<sup>217</sup> and Gln<sup>223</sup> individually resulted in similar decreases in IGF-I binding activity (Fig. 3B), the loss of ALS binding was more pronounced in the S analog (Fig. 5B). Surprisingly, the combination of these two mutations in the SA analog caused less disruption to ALS binding than the single mutation in the S analog.

Although both the QSA and GGSA analogs showed undetectable binding to IGF-I, as analyzed by ligand blotting (Fig. 2C), solution binding assay (Fig. 3C), and BIAcore analysis (Table II), they were able to bind ALS with activities that were only 1–2 log orders of magnitude lower than wild-type IGFBP-3 (Fig. 5C). Unexpectedly, GGSA showed similar ALS binding in the absence of IGF-1, suggesting that the mutations may have caused a conformational change to the protein similar to that caused by IGF-I when bound to wild-type IGFBP-3 to facilitate ALS binding. This conformational change is not evident in the QSA analog, which could only bind ALS in the presence of IGF-I.

We have shown previously that wild-type IGFBP-3, at high concentrations (>20 ng/tube), can bind ALS weakly in the absence of IGF, thus indicating that the interaction between ALS and unoccupied IGFBP-3 is of very low affinity (29). Indeed all of the analogs show some weak binding to ALS in the absence of IGF-I with the exception of the QQ and GGSA analogs, which showed moderate binding to ALS alone (Fig. 5).

**Decreased IGF Binding Affinity Leads to Loss of the Ability of Mutants to Inhibit IGFR Signaling**—IGFs elicit their cellular effects through interaction with the IGFR, resulting in auto-phosphorylation of the receptor and the activation of intracellular signaling pathways. Because IGFBP-3 is able to bind IGFs and thereby prevents their access to the receptor, the affinity of the IGF-IGFBP-3 interaction should be reflected in the inhibition of receptor signaling. To investigate whether the loss of IGF binding affinity seen for the mutants was reflected in the loss of their ability to inhibit IGF-induced IGFR signaling, we assessed the relative ability of the QQQ, QA, and GGSA analogs to inhibit tyrosine autophosphorylation of the receptor in the IGFR-overexpressing 3T3 cells treated with either IGF-I at 2.5 ng/ml (Fig. 6A) or IGF-II at 10 ng/ml (Fig. 6B). At

<table>
<thead>
<tr>
<th>IGFBP-3 analog</th>
<th>Relative apparent activity compared to wild-type IGFBP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>0.36 &lt;0.01</td>
</tr>
<tr>
<td>GG</td>
<td>0.38 &lt;0.01</td>
</tr>
<tr>
<td>QQ</td>
<td>0.18 &lt;0.01</td>
</tr>
<tr>
<td>QQQ</td>
<td>0.62 &lt;0.01</td>
</tr>
<tr>
<td>S</td>
<td>0.04 0.07</td>
</tr>
<tr>
<td>A</td>
<td>0.30 0.59</td>
</tr>
<tr>
<td>SA</td>
<td>0.22 0.09</td>
</tr>
<tr>
<td>QSA</td>
<td>0.01 &lt;0.01</td>
</tr>
<tr>
<td>GGSA</td>
<td>0.01 &lt;0.01</td>
</tr>
</tbody>
</table>

$K_D$ values compared to wild-type IGFBP-3.
Fig. 4. Representative sensorgrams showing the association and dissociation profiles of 50 nM IGF-I (A–C) or IGF-II (D–F) injected over sensor surfaces immobilized with IGFBP-3 or analogs. The immobilized analogs with mutations in the N terminus, Q, QQ, GG, and QQQ (A and D); analogs with mutations in the C terminus, S, A, and SA (B and E); and analogs with mutations in both the N and C termini, QSA and GGSA (C and D) were compared with wild-type IGFBP-3 as indicated. Kinetic studies with a range of analyte (IGF-I or IGF-II) concentrations were determined at a flow rate of 40 μl/min to minimize mass transfer effects, allowing 300 s for association and 900 s for dissociation. Dissociation constants were derived using BIAevaluation 3.2 software and a 1:1 Langmuir binding model.

TABLE II

<table>
<thead>
<tr>
<th>IGFBP-3</th>
<th>K_D (nM)</th>
<th>Relative K_D</th>
<th>nW</th>
<th>Relative K_D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IGF-I</td>
<td>IGF-II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.80 ± 0.55</td>
<td>1.00</td>
<td>0.39 ± 0.11</td>
<td>1.00</td>
</tr>
<tr>
<td>Q</td>
<td>3.39 ± 0.47</td>
<td>0.24</td>
<td>3.87 ± 4.29</td>
<td>0.10</td>
</tr>
<tr>
<td>GG</td>
<td>NE</td>
<td>14.00 ± 13.34</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>QQ</td>
<td>3.78 ± 0.12</td>
<td>0.21</td>
<td>2.56 ± 1.60</td>
<td>0.15</td>
</tr>
<tr>
<td>QQQ</td>
<td>ND</td>
<td>4.21 ± 0.84</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>0.91 ± 0.04</td>
<td>0.88</td>
<td>0.77 ± 0.23</td>
<td>0.51</td>
</tr>
<tr>
<td>A</td>
<td>5.59 ± 0.57</td>
<td>0.14</td>
<td>0.73 ± 0.08</td>
<td>0.53</td>
</tr>
<tr>
<td>SA</td>
<td>2.60 ± 2.26</td>
<td>0.31</td>
<td>1.09 ± 0.21</td>
<td>0.36</td>
</tr>
<tr>
<td>QSA</td>
<td>ND</td>
<td></td>
<td>5.32 ± 0.18</td>
<td>0.07</td>
</tr>
<tr>
<td>GGSA</td>
<td>ND</td>
<td></td>
<td>NE</td>
<td></td>
</tr>
</tbody>
</table>

KD

The data shown are the means ± S.D.

* Relative K_D compared with wild-type IGFBP-3.

NE, very low binding affinity was detectable. However, kinetic parameters were not derived from the data.

ND, no binding was detectable.

equimolar concentration to IGF-I, IGFBP-3 inhibited the IGF-I-induced autophosphorylation of IGFRI by 80% (Fig. 6A). In contrast, the QQQ and SA analogs reduced IGFRI phosphorylation by 50 and 20%, respectively, whereas there was no inhibition by the GGSA analog when equimolar with IGF-I. IGFBP-3 and QQQ were equipotent in their inhibition when present in 5- or 10-fold molar excess of IGF-I, but the SA analog remained relatively inactive, whereas the GGSA analog inhibited 40% of the phosphorylation when present in molar excess. IGFBP-3-induced phosphorylation of IGFRI was inhibited in a dose-dependent manner by both IGFBP-3 and the SA analog (Fig. 6B). In contrast, both QQQ and GGSA were relatively inopment in inhibiting IGF-II action. The IGFBP-3 analogs in the absence of IGF-I or IGF-II had no effect on IGFRI phosphorylation.

DISCUSSION

There is considerable evidence to suggest that the high affinity binding of IGFs by IGFBPs involves the interaction of both the N- and C-terminal IGFBP domains. IGFBP fragments generated by proteolysis in vivo or in vitro and recombinant peptides representing N- and C-terminal domains invariably have reduced IGF binding (23, 34–36), leading to the proposal that proteolysis is a mechanism for modulating IGF bioavailability (5). Additionally, deletion of C-terminal structure of IGFBP-2, -3, -4, and -5 (29, 37–39) significantly reduced their IGF binding affinity, suggesting that the C-terminal domain is required for high affinity binding. Although some IGFBP fragments containing only the C-terminal region are known to retain affinity for IGF-I and -II (34, 40–44), Kalus et al. (17) reported that a C-terminal fragment of IGFBP-5 (residues 135–246) had no intrinsic binding affinity for either IGF-I or -II by BIAcore measurement, whereas an N-terminal domain fragment (residues 40–92) retained measurable affinity for IGFs. We have recently reported that isolated N-terminal (residues 1–88) and C-terminal (residues 185–264) fragments of IGFBP-3, which individually have greatly reduced IGF binding, together form complexes with IGF-I and -II with binding affinities comparable with the intact protein, demonstrating for the first time the cooperativity between the two regions in IGF binding (23).

X-ray crystallography would be the preferred method for predicting IGF binding determinants within these regions, but to date no full-length IGFBP crystal structure has been reported. This may be due to IGFBP aggregation at relatively low concentration, making crystals difficult to obtain (45). The crystal structure of the complex of IGF-I bound to “mini-IGFBP-5” does provide an important insight into the molecular interaction between IGFBPs and IGFs (46). The major advantage of mutagenesis over other techniques (e.g. isolated domain fragments) is that it may result in negligible disruption of the protein tertiary structure and so minimize the possibility that structural alterations could affect the functional properties of other regions within the protein (45). Furthermore, site-directed mutagenesis can be performed in full-length proteins, eliminating concerns of protein misfolding commonly found in recombinant fragments.

Imai et al. (18) mutated five residues (Lys68, Pro69, Leu70, Leu73, and Leu74) in the N-terminal region of IGFBP-5 and five homologous IGFBP-3 residues and reported that both mutants had >1000-fold reduction in IGF-I solution binding, although IGF-II binding was not studied. Buckway et al. (19) reported that substitutions of both Leu80 and Leu81 with Gly on IGFBP-3 led to nearly complete loss of binding for IGF-I or -II as measured by both solution binding and BIAcore analysis. In another study (20), six residues (Ile56, Tyr57, Arg75, Leu77, Leu78, and Leu81) in the putative IGF-binding region of IGFBP-3 were substituted with Ala, resulting in a >80-fold reduction in both IGF-I and -II solution binding. The mutant was subsequently used as a non-IGF-binding mutant in an investigation of the IGF-independent actions of IGFBP-3. The above studies are all consistent with the mini-IGFBP-5 structural model that indicated that the N-terminal region contains a high affinity IGF-binding site.

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In these studies, however, only residues in the N-terminal region were considered, whereas the role of the C-terminal region was not investigated. Recently, Shand et al. (22) generated a rat IGFBP-5 variant combining the five mutations in the N terminus described by Imai et al. (18) and two residues in the C terminus (Gly203 and Gln209) in an attempt to examine the relative contribution of two regions. The two N-terminal residues are conserved among all six IGFBPs and had previously been shown by the same group to be involved in IGF-I binding (21). By BIAcore measurements, a cumulative effect was seen for the combined N- and C-terminal mutations that led to a 126-fold reduction in IGF-I binding as measured by BIAcore, whereas mutations at either N- or C-terminal residues alone did not result in complete loss of affinity with the exception of the QQQ analog. The loss of IGF-I binding was particularly remarkable in the QSA mutant because neither the Q nor SA mutant alone had more than 5-fold reduction in activity. This finding also implies that both the N and C termini of IGFBP-5 are involved in high affinity IGF-I binding.

Our study is the first to use site-directed mutagenesis to target residues on both N- and C-terminal regions of IGFBP-3 and to investigate the importance of these residues in binary and ternary complex formation and the regulation of IGF signaling. Analysis of the two N- and C-terminal mutants (QSA and GGSA) by both solution binding assay and BIAcore biosensor measurement showed undetectable binding to IGF-I, whereas mutations at either N- or C-terminal residues alone did not result in complete loss of affinity with the exception of the QQQ analog. The loss of IGF-I binding was particularly remarkable in the QSA mutant because neither the Q nor SA mutant alone had more than 5-fold reduction in activity. This finding also implies that both the N and C termini of IGFBP-3 are critical in IGF-I binding, and the high affinity IGF-I binding can only be achieved through cooperativity between the two regions. Additionally, our BIAcore data agree with the previous IGFBP-5 study by Shand et al. (22) that showed that the N-terminal mutations generally lead to a slower $K_{on}$ rate, and the C-terminal mutations tend to have a faster $K_{off}$ rate relative to wild-type protein. Notably, none of the mutants resulted in a complete loss of affinity for IGF-II as measured by BIAcore, but mutations to the N terminus caused greater reduction in binding affinity than C-terminal mutations. This suggests that the N-terminal region plays a larger role in the association with the IGFs, whereas the C-terminal region is more involved in stabilization of the IGF-IGFBP complexes as previously implied for IGFBP-2 (32).

In addition to their reduced IGF binding affinities, the two N- and C-terminal mutants (QSA and GGSA) also showed reduced heparin binding, evident by their elution profile in the heparin affinity purification. Because the putative heparin binding motif resides in residues 219–226 in the C terminus of IGFBP-3 and the two variants had mutation at Gln223, it is likely that the mutation altered the heparin motif and led to reduced heparin binding. Moreover, mutation at Gly217 in these mutants might also contribute to the reduced heparin binding based on our previous finding that heparin binding was affected when residues adjacent to the C-terminal putative heparin binding motif were mutated (29). However, Shand et al. (22) reported that heparin binding was not affected, as demonstrated by heparin ligand blotting, by mutations at Gly217 and Gln223 in IGFBP-5, which correspond to Gly217 and Gln223 in IGFBP-3. Further investigation will be needed to clarify the discrepancy.

The results from the ALS binding assay were consistent with our previous observation that the IGF-IGFBP-3 binary complex is required for high affinity ALS binding (24). Reduced ternary complex formation seen for the mutants is a consequence of their reduced ability to form binary complex. Notably, this assay does not exactly reflect IGF binding affinity because IGF-I is added in excess to facilitate binary complex formation; therefore, only mutants with extremely low IGF binding affinity show a decrease in ternary complex formation. Interestingly, we observed that the GGSA mutant differed from other mutants in that it had considerable binding to ALS in the absence of IGF-I. A possible explanation is that the multiple mutations might have caused a degree of conformational change in the protein resulting in the exposure of ALS binding determinants similar to those exposed when IGFBP-3 is in complexes with IGFs.

To test the function of the IGFBP-3 analogs in modulating IGF signaling, we examined the ability of the QQQ, SA, and GGSA analogs to inhibit IGF-stimulated IGFRI autophosphorylation in IGFBP-3 overexpressing 3T3 cells. Despite a major reduction in IGF binding, the QQQ analog was surprisingly effective at inhibiting IGF-I stimulation of IGFRI phosphorylation, particularly when present at 5-fold molar excess to IGF-I, but only weakly inhibited IGF-II-stimulated IGFRI phosphorylation. Mutation of the C-terminal residues, Gly217 and Gln223, in the SA analog had very little effect on its ability to inhibit IGF-II-stimulated IGFRI phosphorylation relative to IGFBP-3 but had a profound effect on inhibiting IGF-I activity. GGSA, whose interaction with IGF-I and IGF-II was essentially undetectable biochemically, inhibited IGF-I- and IGF-II-induced IGFRI phosphorylation by 40 and 15%, respectively, when present in excess. Our data thus show that specific mutations in IGFBP-3 can have differential effects on IGF-I and IGF-II binding, and these may not translate into functional effects on IGF signaling. Analogs defined as non-IGF-binding appear to have some IGF inhibitory effect when used at higher concentrations, suggesting that these mutants may have residual IGF binding activity below the limit of detection.

It should be noted that the measurement of IGF binding
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differs remarkably: by our assay method, in which IGF-IGFBP complexes are immunoprecipitated, maximal binding (approximately 70%) was achieved at 2.5 ng of wild-type IGFBP-3 protein, whereas when charcoal is used to separate free from bound IGF tracer, 100 ng of IGFBP-3 is required for maximal binding (19). Thus assay methodology may significantly impact on the interpretation of binding data.

Within our own study, differences in IGF binding activity are seen between solution binding assays and BIAcore analysis. Although binding kinetics obtained from the BIAcore measurement provide more detailed information than the solution-based equilibrium binding assay, the reaction constants may not match those obtained from solution-based methods because of a variety of potential artifacts (47). For example, immobilizing a protein (IGFBP-3 in this case) to a surface could restrict its rotational freedom and diffusion properties, thus altering the reaction thermodynamics and binding kinetics. Biosensors also require that the reactant (IGF-I or -II) in solution be transported to and from the reaction surface in a rapid and uniform manner; otherwise, concentration gradients at the surface could affect the apparent rate constants (48). On the other hand, because the IGF solution binding curve of each mutant was not necessarily parallel to that of the wild-type protein, the apparent binding activities derived from solution binding assays may also be subject to a degree of error.

In summary, we have demonstrated that in IGFBP-3, Leu^{77}, Leu^{80}, and Leu^{81} in the N terminus and Gly^{217} and Gln^{223} in the C terminus are critical residues involved in high affinity IGF binding. Although the N terminus of IGFBP-3 contains a major binding site for IGFs, the C terminus also plays an indispensable role in forming high affinity stable complexes with IGFs. Our data strongly support the hypothesis that N- and C-terminal IGFBP-3 residues interact cooperatively in achieving high affinity IGF binding. More importantly, this study has shown that IGFBP-3 analogs defined as non-IGF-binding by biochemical assays can functionally inhibit IGF signaling when present in excess, and as such, the use of these analogs to study the IGF-independent effects of IGFBP-3 must be interpreted with caution.

**Fig. 6.** Inhibition of IGF-induced type I receptor phosphorylation by IGFBP-3. NIH-3T3-IGFRI cells were treated for 5 min with either 2.5 ng/ml IGF-I or 10 ng/ml IGF-II preincubated with IGFBP-3 in molar ratios of 1:1, 1:5, and 1:10. As controls, the cells were either untreated (UNT) or treated with IGF or IGFBP-3 alone. The cell lysates were prepared for SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody, as described under "Experimental Procedures." Representative blots are shown for cells induced with IGF-I (A) and IGF-II (B). The bands were quantitated by densitometry, and the results are expressed relative to the amount of phosphorylated IGFRI induced by IGF alone (striped bars). The means ± S.D. of phosphorylated IGFRI in untreated cells (pooled from four blots) are shown (hatched bars). The IGFBP-3 tested are wild-type IGFBP-3 (black bars), QQ analog (dark gray bars), SA analog (light gray bars), and GGSA analog (white bars).

affinities of mutant IGFBPs differs between laboratories. Many factors may contribute to these differences, including assay sensitivity and the experimental conditions of each method. For example, Imai et al. (18) found that the N-terminal IGFBP-5 or -3 mutants with five substitutions had >1000-fold reduction in IGF-I solution binding affinity, whereas Shand et al. (22) demonstrated only 60-fold reduction for the comparable rat IGFBP-5 mutant by BIAcore. Sensitivity of solution binding assay can also vary greatly with different protocols. The solution binding activity of the IGFBP-3 mutant, GG, was 5–10-fold decreased in this study, whereas it was measurable in the study of Buckway et al. (19). The sensitivity of these assays

**REFERENCES**

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