Binding Sites and Binding Properties of Binary and Ternary Complexes of Insulin-like Growth Factor-II (IGF-II), IGF-binding Protein-3, and Acid-labile Subunit*

(Received for publication, June 26, 1997, and in revised form, August 12, 1997)

Ryuji Hashimoto†, Mayumi Ono, Hiroyuki Fujiwara, Nobuyuki Higashihashi, Makoto Yoshida, Tomoko Enjoh-Kimura, and Katsu-ichi Sakano

From the Basic Technology Research Laboratory, Daiichi Pharmaceutical Co., Ltd., 16-13, Kitakasai 1-chome, Edogawa-ku, Tokyo 134, Japan

We have examined regions of rat IGF-binding protein-3 (IGFBP-3) important for complex formations using two kinds of deletion mutants, three kinds of chimeric molecules between rat IGFBP-3 and rat IGFBP-2, and a synthetic peptide (41 residues, Glu22-Ala28) derived from rat IGFBP-3. Solid-phase binding assays using 96-well microtiter plates were designed to quantify the relative binding affinities. It was found that not only the IGFBP-3 derivatives with the amino-terminal, cysteine-rich domain (N domain) but also the synthetic peptide maintained affinity for IGF-II. Ternary complex formation was observed with full-length IGFBP-3 and chimera IGFBP, the carboxyl-terminal cysteine-rich domain (C domain) of which was derived from IGFBP-3, unlike the mutants lacking the C domain and the chimeric IGFBPs, the C domain of which was derived from IGFBP-2. These results were confirmed by affinity crosslinking experiments. Furthermore, the IGFBP-3 derivatives that possessed the C domain of IGFBP-3 bound to the acid-labile subunit, even in the absence of IGFs. Finally, we observed sites in IGF-II important for the ternary complex formation using various IGF-II mutants. These IGF-II mutants, which contained a substitution of Tyr27 for Leu, had extremely reduced activity. These results strongly suggest that: 1) the N domain, containing at least Glu22-Ala28, of rat IGFBP-3 is important for binding to IGF-II; 2) the C domain of IGFBP-3 is essential for binding to the acid-labile subunit both in the presence and absence of IGF-II; and 3) Tyr27 of IGF-II is important for the ternary complex formation.

Insulin-like growth factor (IGF) I and -II contain three disulfide bonds and have both amino acid sequence and tertiary structural homology with each other and with insulin (1–4). Unlike insulin, IGFs bind to a family of specific IGF-binding proteins (IGFBPs) that regulate IGF function in blood and body fluids (5, 6). There are six kinds of IGFBPs, designated IGFBP-1 to IGFBP-6, which have related primary structures (7). Except for IGFBP-6, all human and rat IGFBPs contain 18 cysteines, consistent with the IGFBP-1 to IGFBP-5 molecules, usual 12 cysteines are conserved (8), and IGFBP-7 binds IGFs with specific affinity (9). Although IGFBP-7 contains a total of 18 cysteines, consistent with the IGFBP-1 to IGFBP-5 molecules, the carboxyl-terminal domain contains only one homologous cysteine position.

When IGFs are bound to IGFBP-3, an acid-labile subunit (ALS) can bind to the IGF/IGFBP-3 complex to form an IGF/IGFBP-3/ALS 150-kDa ternary complex (10, 11). A recent study also identified a binary complex of IGFBP-3/ALS in rat serum (12, 13).

NEW solid-phase binding assays that can be performed easily using 96-well microtiter plates. Using these assays, we evaluated the binding properties of various mutants, chimera, and a synthetic peptide derived from rat IGFBP-3 to analyze IGFs/IGFBP-3 and IGFs/IGFBP-3/ALS structure-function relationships.

EXPERIMENTAL PROCEDURES

Materials—A rat pancreas cDNA (agt 11) library of adult Sprague-Dawley females was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The prokaryotic expression vector pTrc99A, pGEX-2T, DEAE-Sepharose CL-6B resin, SP-Sepharose F.F. resin, and NHS-activated HiTrap affinity column were from Pharmacia Biotech, Inc. (Uppsala, Sweden). Protein A affinity resin (PROSEP-A) was from Bioprocessing, Ltd. (Durham, United Kingdom) and DEAE-5PW 75 × 7.5 mm inside diameter was from Tosoh (Tokyo, Japan). A YM-10 column (250 × 4.6 mm inside diameter) was from YMC Co.,

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† To whom correspondence should be addressed. Tel.: 81-3-3650-0151; Fax: 81-3-5696-8336.

‡ The abbreviations used are: IGF, insulin-like growth factor; rIGF, recombinant IGF; IGFBP, IGF-binding protein; ALS, acid-labile subunit; HRP, horseradish peroxidase; PhoA, phosphatase A; pAb, polyclonal antibody.
FIG. 1. Schematic illustrations of IGFBP-3 derivatives. The N domain (Gly–Cys–99), L domain (Ala–Pro–136), and C domain (Cys–Glu–265) of IGFBP-3 are shown as a solid box, dashed box, and shaded box, respectively. For the chimera IGFBPs, the L domain and C domain of IGFBP-2 include positions Glu–Pro–171 and Cys–Glu–265, respectively, using IGFBP-2 numbering.

| N domain | L domain | C domain |
|----------|----------|----------|
| 1        | 89       | 186      |
| N domain |          |          |
| 83       |          |          |
| 41 residue peptide | chimera-322 | chimera-332 |
|          | chimera-332 | chimera-332 |

Ld. (Kyoto, Japan). A maleimide-activated horseradish peroxidase kit and discuccimidyl suberate were purchased from Pierce. 2.2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate and microtiter plates (96-well) were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD) and Costar (Cambridge, MA), respectively. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (anti-rabbit IgG-HRP), a biotinylation kit, and 125I-labeled human recombinant IGF-II (2000 Ci/mmol) were obtained from Amersham Corp. All other chemicals were of the highest quality commercially available.

Recombinant human IGF-II and IGF-II mutants of [Leu27]rIGF-II, [Leu27]rIGF-II, and [Arg27]Arg27]rIGF-II were expressed in *Escherichia coli* MC1061 using constructs and purification procedures as described previously (22). A double mutant of [Leu27, Leu43]rIGF-II was constructed by identical procedures using synthetic oligonucleotides. Oligonucleotides were synthesized by an Applied Biosystems model 390A synthesizer, purified as described previously (24), and sequences were confirmed by the dideoxynucleotide chain termination method (25) using a 7-DEAZA sequencing kit (Takara Shuzo, Kyoto, Japan).

**Preparations of Rat IGFBP-3 and Its Mutants Expressed in E. coli or Synthesized Chemically—Cloning of IGFBP-3 and IGF-II/IGFBP-2 DNA was performed by polymerase chain reaction with reference to known cDNA sequences (26, 27) using rat pancreas cDNA library (invitrogen) as a template. Considering the significant homology among all rat IGFBPs, IGFBP-3 contained the three major domains: an amino-terminal (Gly–Cys–99) and carboxyl-terminal domain (Cys–Glu–265), rich in cysteine residues; and an linkage-domain (Ala–Pro–136) with no significant homology. IGFBP-3 mutants designated N–L domain (Gly–Pro–98) and N domain (Gly–Ser–98) were constructed by polymerase chain reaction methods using the rat IGFBP-3 cDNA as a template. A chimera protein whose N, L, and C domain were derived from IGFBP-3, IGF-II, and IGFBP-2, respectively, was named as chimera-322. In this way, chimera proteins designated chimera-322, chimera-332, and chimera-333 were constructed by mixing polymerase chain reaction-derived fragments from the N, L, and C domains using appropriate rat IGFBP-3 and rat IGFBP-2 DNA clones as templates. All IGFBP-3 derivatives are illustrated in Fig. 1.

For the expression of IGFBP-3, N–L domain, chimera-322, chimera-332, and chimera-333 in *E. coli*, the phosphatase A (PhoA) signal sequence was chemically synthesized and introduced into the Nov-HindIII site of pTrc99A, upstream of the clone, which were not derived from their own signal sequences. These expression vectors were transformed into *E. coli* UT5600. The cells were grown at 37 °C in a LB medium supplemented with 50 μg/ml ampicillin. After isopropyl-1-thio-β-D-galactopyranoside induction (final concentration, 0.3 mM), the cells were cultured an additional 1 h and then collected by centrifugation at 6000 rpm for 20 min. The periplasmic fluid was prepared by osmotic shock (28) from the cells was adjusted to pH 7.2 with phosphate-buffered saline and applied to an IGF-II coupled affinity resin column prepared from an NHS-activated HiTrap affinity column according to the manufacturer’s recommendations (29) and further purified by reverse-phase high-performance liquid chromatography as described above.

Two of the six disulfide bond pairs included in the N domain of IGFBP-3 were determined by peptide mapping procedures that involved trypsin digestion, reverse-phase high-performance liquid chromatography, and amino-terminal amino acid sequence analysis of isolated peptide fragments (data not shown). By referring to the identified disulfide bonds of Cys–Glu–99 and Cys–Glu–265, a 41-residue peptide derived from positions Glu–Ala–29 was synthesized by an Applied Biosystems model 431A peptide synthesizer using the selective S-S formation procedures (30).

**Binding Sites and Properties of IGF Complexes**—Eighty ml of rat serum were dailized against 50 mM Tris-HCl, pH 8.2, and chromatographed on a DEAEd-Sephrose CL-6B column and an IGF-II/IGFBP-3 complex affinity column essentially as described previously with some modifications (11, 31). For this study, IGF-II was conjugated to an NHS-activated HiTrap affinity column, and recombinant IGFBP-3 was used instead of serum-derived IGFBP-3. Finally, the ALS was purified on a DEAEd-Sephrose CL-6B column (75 × 7.5 mm inside diameter) using a linear gradient from 0.1 to 0.5 μl NaCl in 10 mM sodium phosphate buffer, pH 8.0. Fractions containing ALS were collected and dialyzed against 50 mM sodium phosphate buffer, pH 6.5, by ultrafiltration.

**Determinations and Analyses of Purified Proteins**—The purity of each purified IGFBP-3, N–L domain, N domain, chimera-322, chimera-332, chimera-333, the 41-residue peptide, and ALS was confirmed by SDS-polyacrylamide gel electrophoresis in the Laemmli buffer system (32) under reduced conditions followed by Coomassie Brilliant Blue staining. All of the protein concentrations were determined using an AccuTag amino acid composition analysis column according to the manufacturer’s instructions (Waters, Milford, MA). Amino acid sequences of all purified proteins were also confirmed by sequencing using an Applied Biosystems 476A gas phase sequencer.

**Modifications of IGF-II and ALS for Binding Assays—HRP-IGF-II was prepared using a maleimide-activated HRP kit according to the manufacturer’s recommendations (Pierce). Briefly, IGF-II was reacted with N-succinimidyl-3-ethylthioacetate to introduce a free sulfhydryl into the primary amine and was then conjugated with maleimide-activated HRP. ALS was biotinylated on primary amines using a biotinylation kit according to the manufacturer’s recommendations (Amersham Co.).**

**Preparations of Rabbit Anti-IGFBP-3 and Anti-41 Residue Peptide Polyclonal Antibodies—**Five injections (0.4 mg each) of IGFBP-3 or the 41-residue peptide were administered s.c. to five male rabbits at 2-week intervals. Each polyclonal antibody (anti-3P aAb and anti-Pep pAb, respectively) was purified from the antisera using a PROSEP-A affinity column according to the manufacturer’s recommendations and dialyzed against 50 mM sodium phosphate buffer, pH 6.5.

**Characterization of Binding and Complex Formation Properties of IGFBP-3 Mutants, 41-residue Peptide, and IGF-II Mutants Using Solid-phase Binding Assays**—All solid-phase binding assays described were carried out with 96-well microtiter plates using binding components as illustrated in Fig. 2. For all assays, the plates were coated with proteins in 50 mM sodium phosphate buffer, pH 6.5, and blocked with the same buffer containing 1% (w/v) bovine serum albumin. All washes were done...
with the same phosphate buffer containing 0.03% (v/v) Tween 20. Binding proteins were diluted in the phosphate buffer containing 0.25% bovine serum albumin and 0.03% (v/v) Tween 20. After coating, all steps were performed at room temperature.

For the HRP-IGF-II/IGFBP-3 competitive binding assay (Fig. 2A), 50 μl of 150 ng/ml IGFBP-3 were immobilized at 4 °C overnight. The wells were washed and blocked for 2 h at room temperature. The wells were washed, and 25 μl of a 1:6000 dilution of HRP-IGF-II and 25 μl of each competitor were added simultaneously to each well. The plates were incubated for 2 h and washed, and 100 μl of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Kirkegaard & Perry Laboratories) were added. After 20 min, the absorbance of the reaction product at 405 nm was read using a Vmax microplate reader (Molecular Devices, Menlo Park, CA).

To study complex formation with ALS, indirect immobilization procedures using biotinylated ALS were performed. Wells were precoated with 50 μl of 1 μg/ml streptavidin, and unbound sites were blocked. After washing, 50 μl of 200 or 50 ng/ml biotinylated ALS were added and incubated for 2 h to immobilize ALS indirectly. To observe the relative affinities of the IGFBP-3 derivatives to form ternary complexes (Fig. 2B; the HRP-IGF-II/IGFBP-3/ALS solid-phase binding assay), 50 μl of 200 ng/ml biotinylated ALS were used. After indirect immobilization of ALS, 25 μl of a 1:1000 dilution of HRP-IGF-II and 25 μl of various concentrations of IGFBP-3 derivatives were incubated for 2 h to form the ternary complex. The quantity of the formed complex in the well was measured as described above. To detect the binding of IGFBP-3 derivatives to ALS in the absence of IGFs (Fig. 2C; the IGFBP-3/ALS solid-phase binding assay), various concentrations of IGFBP-3 derivatives were incubated with the immobilized biotinylated ALS. After washing the wells, the quantity of the formed complex was detected by incubation with 50 μl of 3 μg/ml anti-BP-3 pAb for 2 h, followed by washing and 50 μl of a 1:1000 dilution of anti-rabbit IgG-HRP for 2 h. To observe the ability of the IGF-II mutants to form the ternary complexes (Fig. 2D), the Igfs/IGFBP-3/ALS solid-phase binding assay, 50 μl of 50 ng/ml biotinylated ALS were immobilized indirectly to avoid detection of the IGFBP-3/ALS complex in the absence of IGFs. After indirect immobilization of ALS, 25 μl of various concentrations of IGFBP-3 mutants and 25 μl of 50 ng/ml IGFBP-3 were incubated for 2 h. The formed ternary complexes were detected using the anti-BP-3 pAb and anti-rabbit IgG-HRP system as described above.

Characterizations of Complex Formation between 125I-labeled IGF-II, the 41-Residue Peptide, and IGFBP-3 Mutants by Affinity Cross-linking—Each IGFBP-3 mutant or the 41-residue peptide was incubated with the immobilized biotinylated ALS. Following an overnight incubation, 30 μl of 1.5 mM disuccinimidyl suberate were added, and the reaction mixture was further incubated on ice for 15 min. The cross-linking reaction was terminated by adding 60 μl of 1 M Tris-HCl buffer, pH 8.5. Proteins were precipitated by trichloroacetic acid at a final concentration of 20% and applied to a 12% SDS-polyacrylamide gel electrophoresis gel under reducing conditions, followed by autoradiography.

**RESULTS**

Preparation of Recombinant IGFBP-3 Mutants, the 41-residue Peptide, and Rat Serum ALS—IGFBP-3, two kinds of deletion mutants, three kinds of chimera IGFBPs, the 41-residue peptide, and rat serum ALS were prepared as described under “Experimental Procedures,” and their purity was confirmed by reverse-phase high-performance liquid chromatography (data not shown) and by SDS-polyacrylamide gel electrophoresis (Fig. 3). The chimera IGFBPs were expressed using the same expression vector as the IGFBP-3, but all chimera IGFBPs showed doublet bands. The doublet bands were also detected by Western blotting using anti-BP-3 pAb and by far Western ligand blotting using HRP-IGF-II (data not shown). From the results of amino-terminal amino acid sequence analyses, it was found that all chimera IGFBPs contained the expected amino-terminal sequence of Gly-Ala-Gly-Ala-Val-Gly-Ala as the primary sequence and an unexpected minor Gly-Ala-Val-Gly-Ala sequence. The minor sequence is probably due to different processing of the PhoA signal sequence, resulting in the doublet bands. Recombinant rat IGFBP-3 expressed in *E. coli* did not show such doublet bands nor the minor sequence, however, it is possible that IGFBP-3 may have contained the minor sequence at undetectable levels.

Characterizations of IGF-II/IGFBP-3 Binary Complex Formation Using IGFBP-3 Mutants and the 41-residue Peptide—Using the HRP-IGF-II/IGFBP-3 solid-phase competitive binding assay, the relative affinities of five IGFBP-3 mutants and the 41-residue peptide to IGF-II were calculated from their displacement curves (Fig. 4) and compared with that of IGFBP-3, which was set to 100% (Table I). The chimera IGFBPs retained over 40% of the relative affinity, but the isolated N-L domain or N domain showed much lower affinity. A three-fold difference in affinity was observed between N-L domain and N domain. In the case of the 41-residue peptide, although it showed only 0.008% affinity relative to IGFBP-3, an anti-Pep pAb inhibited HRP-IGF-II binding to immobilized IGFBP-3. To verify the IGFBP-3 mutants and the 41-residue peptide binding to IGF-II, affinity cross-linking procedure was performed using 125I-labeled IGF-II. The IGFBP-3, N-L domain, N do-
main, and the 41-residue peptide all showed bands consistent with cross-linking specifically to 125I-labeled IGF-II (Fig. 5). The negative control using only ALS did not form a complex band. These results are consistent with the conclusion that the N domain of IGFBP-3, and at least a portion of the 41-residue peptide (position Glu52-Ala92), is important for binding to IGFBP-3.

**Characterizations of Binding Activities of IGFBP-3 Derivatives to ALS in the Presence or Absence of IGFs**—Using the HRP-IGF-II/IGFBP-3/ALS solid-phase binding assay, activities for the ternary complex formation of IGFBP-3 mutants and the 41-residue peptide could be compared. In this assay, indirect immobilization of ALS was adopted because direct immobilization of ALS or biotinylated ALS did not give a measurable absorbance using 25 ng/ml of IGFBP-3 (data not shown). IGFBP-3 showed concentration-dependent ternary complex formation between 0.1 and 10 nM, the same range as the competitive binding assay shown in Fig. 4. However, neither the N + L domain, N domain, nor the 41-residue peptide, which all had low affinity for IGF-II, were observed to form ternary complexes (Fig. 6A). Of the chimeras, only chimera-323 showed ternary complex formation (Fig. 6B). The activities of the chimeric IGFBPs for the ternary complex formation were also examined by affinity cross-linking experiments using 125I-labeled IGF-II and ALS (Fig. 7). The bands derived from the binary complex with 125I-labeled IGF-II were detected with IGFBP-3 and all chimeric IGFBPs, whereas bands derived from the ternary complex were detected only with IGFBP-3 and chimera-323. Because chimeras-323, which contains only the C domain from IGFBP-2, did not show activity, this suggests that the L domain is not involved for ternary complex formation.

To detect the IGFBP-3/ALS binary complex, the indirect immobilization procedure was adopted, similar to the HRP-IGF-II/IGFBP-3 solid-phase competitive binding assay. Competitive inhibition of HRP-IGF-II binding to immobilized IGFBP-3 (A, IGFBP-3 ●, N + L domain ▲, N domain ▼, 41-residue peptide ◆), and anti-Pep pAb (○); B, IGFBP-3 (●), chimera-322 (○), chimera-323 (▲), and chimera-332 (◆). After subtraction of nonspecific binding, which was obtained when no IGFBP-3 was immobilized, the percentages of maximum specific binding (B/B0) were plotted, and the data are expressed as the mean (bars, S.D.) of three determinations.

**TABLE I**

| IGFBP-3 derivatives | Relative affinity* (% of IGFBP-3) |
|---------------------|----------------------------------|
| N + L domain        | 12.5                             |
| N domain            | 4.2                              |
| 41-residue peptide  | 0.008                            |
| Chimera-322         | 40                               |
| Chimera-323         | 63                               |
| Chimera-332         | 100                              |

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*Relative affinities were determined from the competitive binding inhibition data shown in Fig. 4.

IGF-II/IGFBP-3/ALS solid-phase binding assay. Various concentrations of IGFBP-3 were added to the wells, and the IGFBP-3/ALS binary complex formed was detected by anti-BP-3 pAb followed by anti-rabbit IgG-HRP. Preliminary experiments confirmed that the anti-BP-3 pAb could bind to all chimeric IGFBPs equally by Western blot analysis (data not shown). As shown in Fig. 8, IGFBP-3 and chimera-323 bound to ALS in a concentration-dependent manner. The other chimeric IGFBPs (chimera-322 and chimera-392) had essentially no binding to ALS. These results strongly suggested that the C domain of IGFBP-3 plays an important role for the binding of IGFBP-3 to ALS in the presence and absence of IGFs.

**Characterizations of Ternary Complex Formation Mediated by IGF-II Mutants**—The relative affinities of four IGF-II mutants to IGFBP-3 were determined by the HRP-IGF-II/IGFBP-3 solid-phase competitive binding assay from their displacement curves shown in Fig. 9A and compared with that of IGF-II, which was set to 100%. The affinities of [Leu27]rIGF-II, [Leu43]rIGF-II, [Arg54, Arg55]rIGF-II, and [Arg27, Leu32, Arg54]rIGF-II for IGFBP-3 were 100, 108, 25, and 100%, respectively, relative to IGF-II. The values from the first three mutants are similar to those reported previously, i.e., 70, 100, and 37%, respectively (16). The ability to form ternary complexes with these IGF-II mutants were examined with the IGFs/IGFBP-3/ALS solid-phase binding assay (Fig. 9B). The [Leu43]rIGF-II and [Arg24, Arg54]rIGF-II showed almost the same activities relative to IGF-II. However, the two mutants with substitution...
with lanes 5 and 6, chimera-323 (1.5 ng); lanes 7 and 8, chimera-332 (1.5 ng) with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) ALS (0.6 μg). Bands derived from the ternary complex (A), the binary complex (B), and 125I-labeled IGF-II (C) are shown with arrowheads.

of Tyr27 to Leu showed markedly less ternary complex formation, suggesting that Tyr27 of IGF-II contributes to the binding energy in the ternary complex.

DISCUSSION

To ensure optimal folding of the recombinant IGFBP-3 and the mutant, the expression system with PhoA signal sequence was used with the exception of the N domain molecule. All of the expressed proteins were purified by IGF-II coupled affinity column chromatography, a binding property that requires correct folding and permitted comparison of relative affinities. The IGFBP-3 derivatives used in this study were highly purified except for chimera IGFBPs. Each chimera IGFBP contained a mature form and a minor percentage with two amino acids deleted from the amino terminus. Although the reason is unclear, it is possible that the Ala2 of each chimera IGFBP was deleted from the amino terminus. Although the reason is unclear, it is possible that the Ala2 of each chimera IGFBP was deleted from the amino terminus. The Ala2 of each chimera IGFBP was deleted from the amino terminus. The Ala2 of each chimera IGFBP was deleted from the amino terminus.

IGFBP-3, the N + L domain, and the N domain showed 12.5 and 4.2% affinities for IGF-II, respectively, relative to full-length IGFBP-3. Previous studies showed that a 30-kDa IGFBP-3 in pregnancy plasma has 20-fold lower affinity for IGF-I than normal plasma IGFBP-3 (33), and a carboxyl-terminal truncated 31-kDa IGFBP-3 from rat serum also has lower affinity for IGF-I (34). The truncated IGFBP-3 resulted from proteolysis by IGFBP-3 proteases and reduced its affinity for IGFs, thereby facilitating dissociation of the complexes and hence increasing the bioavailability of the IGFs (33). Results obtained from the binding of IGFBP-3 deletion mutants to IGF-II are in good agreement with that of proteolyzed and truncated form of IGFBP-3. Furthermore, we found that the 41-residue peptide (position Glu52-Ala85) could bind to IGF-II. In a previous study, the amino-terminal 88 amino acids of human IGFBP-3 showed low affinity for IGF-I (20). The 41-residue peptide is the smallest fragment of the IGFBP-3 derivatives found to bind to IGFs. This peptide was designed to obtain two S-S bonds, which were identified by peptide mapping procedures. The remaining four S-S bonds in the N domain could not be determined because of the cysteine-rich region at positions Cys42-Gly-Cys44-Cys45.
Leu-Thr-Cys48. Therefore, we prepared one peptide that contained the S-S bonds. Although the 41-residue peptide showed 1/500th of the affinity for IGF-II relative to the N domain, the 41-residue peptide whose disulfide bonds were reduced and alkylated did not show any binding affinity (data not shown).

Thus, the structure maintained by the S-S bonds are thought to be important for the binding affinity. However, compared to the relative affinities of chimera-332 (100%) and the N + L domain (12.5%) for IGF-II, the presence of only the N domain is important but not sufficient for full binding. For example, the I and C domains possibly play important roles for conformational stabilization of the N domain.

For ternary complex formation, it was found that only the chimera IGFBP, which possessed the C domain derived from IGFBP-3 (chimera-323), showed ternary complex formation activity in the HRP-IGF-II/IGFBP-3/ALS solid-phase binding assay. Thus, the mutants lacking the C domains, such as the N + L domain, the N domain, and the 41-residue peptide, were not active in the affinity cross-linking experiment, and it was found that the bands derived from the ternary complex were observed using only IGFBP-3 and chimera-323. Although complexes between 125I-labeled IGF-II and ALS were not observed (Fig. 5, lane 5), bands derived from the binary complex of 125I-labeled IGF-II and ALS were detected in the presence of IGFBP-3 or chimera-323 as shown in Fig. 7. A previous study also showed that such a complex was detected by affinity cross-linking in the presence of IGFBP-3 (10). It is speculated that IGF-II does not have a significant binding affinity for ALS but may be positioned near ALS in the ternary complex such that the cross-linker can link IGF-II and ALS in the presence of IGFBP-3. From the observation that IGFBP-3 and chimera-323 can form the ternary complex but that the N + L domain and chimera-332 cannot, it is likely that the C domain of IGFBP-3 is necessary for binding to ALS. The results of the IGFBP-3/ALS solid-phase binding assay also suggest that the C domain of IGFBP-3 is important for binding to ALS, even in the absence of IGF-II. In this study, we did not perform an affinity cross-linking experiment to observe chimera IGFBPs binding to ALS. To clarify the binding of IGFBP-3 to ALS, only the C domain expressed in E. coli should be used in the solid-phase binding assay and affinity cross-linking experiments.

Previously, we confirmed the three-dimensional structure of recombinant IGF-II (4) and identified the binding sites of IGF-II for their receptors (22, 23) and for IGFBPs (16) using various IGF-II mutants. We also observed IGF-II sites important for the ternary complex formation using IGF-II mutants. Previous studies revealed that Tyr27 of IGF-II is important for binding to insulin and IGF-I receptors (22) and not for binding to the IGF-II/cation-independent mannose 6-phosphate receptor, and that the Tyr27 residue is structurally positioned at the opposite side from a binding region for IGFBPs, which include Glu6, Phe48, Arg49, and Ser50 (4). In this study, the results of the IGFs/IGFBP-3/ALS solid-phase binding assay suggest that mutation of Tyr27 to Leu does not support ternary complex formation. Similar results have been obtained by other groups using IGF-I mutants in which Tyr27 substituted with Leu ((Leu24)rIGF-I) reduced the ternary complex formation activity (21). Tyr24 of IGF-I is also important for binding to insulin and IGF-I receptors (35). Therefore, the Tyr27 residue of IGF-II, which is important for binding to insulin and IGF-I receptors, interacts only weakly with ALS and is on a side opposite to the Glu6, Phe48, Arg49, and Ser50 residues, which bind the amino-terminal domain of IGFBP-3, within positions Glu52–Ala59. Additionally, the carboxyl-terminal domain of IGFBP-3 binds to ALS, even in the absence of IGF-II.

Acknowledgments—We thank Drs. M. Furusawa, J. F. Perdue, and Y. Fujita-Yamauchi for valuable advice, discussions, and continuous encouragement. We also thank Y. Nagano, M. Nishimura, and K. Sato for excellent technical assistance.

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