Insulin-like Growth Factor (IGF)-binding Protein 5 Forms an Alternative Ternary Complex with IGFs and the Acid-labile Subunit*

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Up to 90% of circulating insulin-like growth factors (IGF-I and IGF-II) are carried in heterotrimERIC complexes with a binding protein (IGFBP) and a liver-derived glycoprotein known as the acid-labile subunit. IGFBP-3 is considered unique among the six well characterized IGFBPs in its ability to complex with the acid-labile subunit. However, a basic carboxyl-terminal domain of IGFBP-3, known to be involved in its interaction with the acid-labile subunit, is shared by IGFBP-5, suggesting the possibility of ternary complexes containing IGFBP-5. We now demonstrate using three independent methods that human IGFBP-5, when occupied by IGF-I or IGF-II, forms ternary complexes of approximately 130 kDa with the acid-labile subunit. IGFBP-3 competes with approximately twice the potency of IGFBP-5 for the formation of such complexes. No other IGFBP complexes with the acid-labile subunit itself or competes with IGFBP-5 for complex formation. As observed for IGFBP-3, ternary complexes containing IGFBP-5 form preferentially in the presence of IGF-I, even though IGFBP-5 has a preferential affinity for IGF-II over IGF-I. By size fractionation chromatography, serum IGFBP-5 co-elutes predominantly with ternary complexes. The demonstration of IGFBP-5-containing ternary complexes indicates an unrecognized form of IGF transport in the circulation and an additional mechanism for regulating IGF bioavailability.

The insulin-like growth factors (IGFs),1 which have both anabolic and mitogenic activity, play a critical role in cell and tissue growth regulation throughout life and in the maintenance of glucose homeostasis (1–3). They circulate in at least seven forms: unbound, in binary complexes with IGF-binding protein (IGFBP) and a liver-derived glycoprotein known as the acid-labile subunit. IGFs and the Insulin-like Growth Factor (IGF)-binding Protein 5 Forms an Alternative Ternary Complex with IGFs and the Acid-labile Subunit.

RESOURCES—Natural human IGFBP-1 (22), IGFBP-3 (23), IGFBP-6 (24), and ALS (25) were purified as described previously. Recombinant human (rh) IGFBP-2 was a gift from Sandoz, Basel, Switzerland. rhIGFBP-4 and rhIGFBP-5, derived from yeast expression systems (26), were purchased from Austral Biologicals, San Ramon, CA; rhIGFBP-5 was also generously donated by J. Zapf, Zürich, Switzerland. Human IGF-I and IGF-II were generous gifts from Genentech, South San Francisco, CA, and Kabi Peptide Hormones, Stockholm, Sweden, respectively. The rabbit anti-human ALS antiserum AL2/2, raised by immunization with serum-derived ALS, is indistinguishable in its characteristics from antisera AL3 (27). Rabbit anti-human IGFBP-5 antiserum was purchased from Upstate Biotechnology Inc., NY. Its stated cross-reactivity with human IGFBP-3 is <0.1%. Donkey anti-rabbit horseradish peroxidase was purchased from Amersham Australia Pty Ltd, Castle Hill, NSW, Australia. SuperSignal chemiluminescent substrate solutions (luminol/enhancer and stable peroxide) for enhanced chemiluminescence were purchased from Pierce.

Cross-linked IGFBP-5 tracer was prepared identically except that 2 μg of unlabeled rhIGFBP-5 in 1 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 1 g/liter bovine serum albumin for 2 h at 22 °C and cross-linked to disuccinimidyl suberate (0.25 mM final concentration) for 30 min, the reaction was terminated with Tris-HCl, pH 7.8 (50 mM final), and the tracer was purified by gel chromatography exactly as in previous studies (28). Cross-linked IGFBP-3 tracer was prepared identically except that 200 μCi of IGF-I (1 μg) was cross-linked to 8 μg of IGFBP-3. The resulting cross-linked tracers had estimated specific activities of 9 μCi/μg for IGFBP-5 and 18 μCi/μg for IGFBP-3 (higher specific activity due to more efficient cross-linking). The ALS tracer was iodinated to a specific activity of ~6 μCi/μg as described previously (27).
**Size Fractionation Experiments on Superose 12**—Samples were diluted to 200 μl in buffer containing 50 mM sodium phosphate, 0.15 M NaCl, 0.2 g/liter sodium azide, pH 6.5, and 10 g/liter bovine serum albumin. The IGF-I or IGF-II tracer or cross-linked IGFBP-5/IGF-I tracer (100,000 cpm) was added in 50 μl of the same buffer. After a 30-min incubation at 30 °C, 200 μl of the mixture was applied to a Superose 12 gel permeation column (Pharmacia) eluting at 1 ml/min in assay buffer with 1 g/liter bovine serum albumin. The albumin was added to the buffer to improve recovery of total radioactivity. Fractions were collected and counted, and the column was washed between runs, as described previously (28). The column was calibrated with IGF-I tracer (7.65 kDa, peaking in fraction 33), IGF-I tracer cross-linked to IGFBP-3 (30–50 kDa), which mainly eluted in fractions 25–27, peaking in fraction 26, and IGFBP-3 in ternary complex with IGF-I tracer and ALS, which eluted mainly in fractions 22–24, peaking in fraction 23. To test the effect of transient acidification of serum, samples of 50 μl were mixed with 25 μl of 2 M HCl for 30 min, then re-neutralized by the addition of 25 μl of 2 M NaOH. To control for the effect of ionic strength, an equivalent amount of pre-mixed acid and base was added to control serum samples.

**Immunoprecipitation Complex Formation Assay**—Increasing amounts of IGFBP-3 or IGFBP-5 (0.05–5 ng) were added to IGF-I or IGF-II tracer (25,000 cpm) and incubated at 22 °C for 2 h with or without 25 ng of ALS/tube, in a total of 300 μl buffer containing 50 mM sodium phosphate, 0.2 g/liter sodium azide, and 0.2 g/liter bovine serum albumin, pH 6.5. A rabbit anti-human ALS antiserum (28) affinity-purified on a column of protein A-Sepharose (Pharmacia) was added at 0.5 μl/tube in 25 μl of the same buffer to precipitate the complex. This antibody concentration had been previously shown to optimally bind the IGFBP-3 and IGFBP-5 ternary complexes formed. After a 1-h incubation at 22 °C, precipitating antibody (goat anti-rabbit immunoglobulin), was added as 25 μl of a 1:10 dilution. After 45 min, 1.0 ml of cold polyethylene glycol 6000 in 0.15 M NaCl was added, and after 10 min, each tube was centrifuged at 4000 rpm at 4 °C for 20 min, the supernatant decanted, and the pellet containing the precipitated complex counted in a gamma counter. For competition assays, IGF-I tracer cross-linked to IGFBP-5 was added at a constant amount of 10,000 cpm/tube to 25 ng of ALS. Where IGFBP-3 or IGFBP-5 was used in competition, increasing amounts were added over the range 0.05–50 ng/tube with or without IGF-I or IGF-II. IGF-I or IGF-II, when present, was added at 100 ng/tube. The mixture was incubated in a total of 300 μl of the phosphate buffer as used in the ternary complex formation assay. The conditions used for precipitation of the ternary complex and subsequent counting were exactly the same as for the immunoprecipitation assay described above.

**Affinity Labeling and SDS-Polyacrylamide Gel Electrophoresis**—All binding incubations were in a final volume of 150 μl of 50 mM sodium phosphate buffer, pH 6.5, with 1 g/liter bovine albumin. 125I-Labeled tracers were added at approximately 100,000 cpm per tube. Other tests were as indicated in the legend to Fig. 4. After a 2-h incubation at 22 °C, complexes were cross-linked and prepared for SDS-PAGE as described previously (29). Gel electrophoresis, fixation, destaining, and drying were performed as described previously (29). Destained gels were placed in contact with autoradiography film in a light tight cassette with intensifying screens at −70 °C for 6 h to 2 days.

**Demonstration of Serum-derived IGFBP-5 in Ternary Complexes**—Normal human serum (500 μl) was diluted 1:1 with 50 mM sodium phosphate buffer, pH 6.5, containing 0.2 g/liter sodium azide and then size-fractionated on a 1.6 × 30-cm column of Sephadex G-100 (Pharmacia) in the same phosphate buffer. Fractions of 1 ml were collected each 15 min. A 350-μl aliquot of each fraction was freeze-dried, then reconstituted in 75 μl of phosphate buffer. Samples were prepared for SDS-PAGE as described previously (29), except that standards and samples were heated to 65 °C for 3 min before electrophoresis. Linear 12% homogeneous polyacrylamide slab gels overlaid with 4% stacking gels were prepared according to the method of Laemmli (30). Electrophoresis was performed at 50 V for 3 h for stacking, then 100 V for 16 h for separating. Gels were then incubated in buffer (containing 47 mM Tris, 39 mM glycine, and 20% methanol) for 30 min and then transferred to nitrocellulose at 250 mM constant current and 40 V upper limit voltage. The gel was placed in radiolabel standards lane was removed, and the nitrocellulose membrane was then blocked in TBS (10 mM Tris, 0.15 M NaCl) with 0.1% Tween 20 and 5% skim milk for 4 h at 37 °C. Washes were performed in TBS, 0.1% Tween 20 three times each for 10 min. The membrane was then incubated with anti-human IGFBP-5 antibody at 1:1000 in TBS, 0.1% Tween 20 for 16 h at 22 °C. Tricarbonate washes were performed as before, followed by incubation of the membrane with anti-rabbit IgG horseradish peroxidase and detection with aminoethylcarbazole. To test the effect of transient acidification of serum, samples of 50 μl were mixed with 25 μl of 2 M HCl for 30 min, then re-neutralized by the addition of 25 μl of 2 M NaOH. To control for the effect ofionic strength, an equivalent amount of pre-mixed acid and base was added to control serum samples.

**Gel Chromatography Studies with Cross-linked Tracer**—Formation of a ternary complex containing IGFBP-5 was first demonstrated by a size shift on a Superose 12 high resolution gel permeation column. Initially, 125I-labeled-IGF-I (7.65 kDa) was covalently cross-linked using disuccinimidyl suberate to nonradioactive IGFBP-5 (28.5 kDa) to form a nondissociable complex, as in our previous studies with IGFBP-3 (28). This radioactive tracer, when incubated with normal human serum, showed an apparent increase in molecular mass from 40 kDa to ~130 kDa (Fig. 1A). When serum was first acidified to pH 3, then re-neutralized before combining with the cross-linked tracer, the molecular mass shift from 40 to 130 kDa did not occur, suggesting that the factor responsible is acid-labile (Fig. 1B). Similar results have previously been shown with 125I-labeled-IGF-I covalently bound to IGFBP-3 (28). The protein responsible for the size increase of IGFBP-3 was subsequently purified from serum and named ALS (25, 28). The existence of this protein had been first postulated almost a decade earlier (31, 32). To demonstrate the same protein was responsible for the size shift of the radioactive IGF-IGFBP-5 complex, increasing concentrations of electrophoretically pure serum-derived ALS (25) were incubated with covalent IGF-IGFBP-5 tracer and fractionated on the Superose 12 column (Fig. 1C). This resulted in a size shift similar to that seen after incubation with serum, indicating that pure ALS is able to increase...
 aliquots were analyzed on a Superose 12 column. Tracer alone is shown to have lower binding affinity for IGF-I. A smaller amount of binary complex than the other IGFBPs is formed due to its ability to form binary complexes only. IGFBP-6 formed a relatively small amount of binary complex relative to the other IGFBPs due to its lower binding affinity for IGF-I.

Fractionation positions of ternary complexes, binary complexes, and free IGF-I tracer, respectively. A, increasing formation of ternary complex with ALS plus increasing additions of IGFBP-5: ALS alone did not bind to IGF-I. B, comparison of ternary complex formation by IGFBP-3 and IGFBP-5: the IGFBP-3 complex eluted earlier. C, IGFBP-1 and IGFBP-2 (each 10 ng) and IGFBP-4 (10 ng) and IGFBP-6 (20 ng) (D) were able to form binary complexes only. IGFBP-6 formed a relatively smaller amount of binary complex than the other IGFBPs due to its lower binding affinity for IGF-I.

The size of IGFBP-5 bound to IGF-I by the formation of a complex containing all three proteins.

Competition for IGFBP-5 ternary complex formation by non-radioactive IGFBP-3 and IGF-I was demonstrated on the size fractionation column (Fig. 1D). The formation of a complex between covalent IGF-I-IGFBP-5 tracer and 500 ng of pure ALS was unaffected if the incubation mixture contained an excess (1 μg) of either IGF-I or IGFBP-3 alone, but if the two were present together, reduced formation of IGFBP-5 ternary complex was seen. This implies that binary complex formation between IGF-I and IGFBP-3 was required to compete with the IGF-HGFPB-5 tracer for binding to ALS and suggests that IGFBP-3 and IGFBP-5 react with a similar binding site on ALS.

**Gel Chromatography Studies with IGF Tracers**—To show that the IGFBP-5-containing ternary complexes could form from individual components in a noncross-linked state, 125I-labeled IGF-I or IGF-II tracer alone or after co-incubation with IGFBP-5 and ALS was subjected to size fractionation chromatography. Incubation with ALS alone had no effect on the apparent size of IGF-I tracer, but when IGFBP-5 was also added, an increase in size to ~130 kDa was seen (Fig. 2A). Incubation with IGFBP-3 instead of IGFBP-5, together with ALS, caused a slightly greater size increase, to approximately 150 kDa (Fig. 2B), as previously demonstrated (25). Identical results were seen when IGF-II was used in place of IGF-I (data not shown).

Apart from IGFBP-3 and IGFBP-5, none of the other well-characterized IGFBPs was able to form a ternary complex with ALS and either IGF-I or IGF-II. Incubation of IGF-I tracer with ALS and IGFBP-1 or IGFBP-2 (Fig. 2C) or with IGFBP-4 or IGFBP-6 (Fig. 2D) resulted in the formation of binary complexes only. Identical results were seen when the tracer was IGF-II (data not shown), except that IGFBP-6 forms more binary complex with IGF-II tracer than with IGF-I tracer, as expected from its much higher binding affinity for IGF-II than IGF-I (24).

**Immunoprecipitation of Radiolabeled Complexes—Quantitation** of the degree of ternary complex formation by IGFBP-5 was achieved using a rabbit anti-human ALS antisera to immunoprecipitate complexes containing radiolabeled IGF-I or IGF-II and unlabelled IGFBP-5. Using a constant amount of IGF-I or IGF-II and ALS, increasing IGFBP-5 caused increasing formation of immunoprecipitable complexes (Fig. 3A). IGFBP-3 was the only other IGFBP able to form complexes, which it appeared to do slightly more potently than IGFBP-5; neither IGFBP-1, -2, -4, nor -6 could form complexes with ALS, as assessed by this method (data not shown). Both IGFBP-3 and IGFBP-5 complexes with ALS formed more readily with IGF-I than with IGF-II tracer.

Competition studies were then performed to quantitate the relative ability of IGFBP-3 and IGFBP-5 to compete for IGF-I and IGF-II ternary complex, each in the presence and absence of IGF-I and IGF-II. The covalent complex of IGF-I tracer cross-linked to IGFBP-5 was precipitable in the presence of ALS (Fig. 3B). Neither IGFBP-5 nor IGFBP-3 was able to compete for complex formation in the presence of IGFBPs; in contrast, in the presence of excess unlabelled IGF-I or IGF-II, both IGFBP-5 and IGFBP-3 showed dose-dependent competition. In three experiments, the IGFBP concentration (mean ± S.D.) required for 50% inhibition of complex formation was 59 ± 1.5 ng/ml (IGFBP-3 + IGF-I), 88 ± 5.2 ng/ml (IGFBP-3 + IGF-II), 112 ± 1.7 ng/ml (IGFBP-5 + IGF-I), and 213 ± 3.5 ng/ml (IGFBP-5 + IGF-II).

**Electrophoretic Analysis of Complexes**—The size of IGFBP-5 complexes was compared with that of complexes containing IGFBP-3 by affinity labeling and SDS-PAGE. Ternary complex components were co-incubated then covalently cross-linked with diisuccinimidyl suberate and fractionated by SDS-PAGE. In the first study, 125I-labeled ALS was incubated then cross-linked with unlabelled IGF-I and IGFBP-5. Two radioactive bands were seen, corresponding to apparent molecular masses of approximately 135 and 110 kDa (Fig. 4). The addition of excess unlabeled ALS considerably decreased the intensity of the radioactive bands by competing with the labeled ALS. IGFBP-3 also formed two complexes with IGF-I and ALS of apparent molecular mass approximately 150 kDa and 135 kDa (Fig. 4). For both IGFBP-3 and IGFBP-5, the higher of the two bands is believed to represent the ternary complex between IGF, intact IGFBP, and ALS, whereas the lower band is be-

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**Fig. 2.** Formation of binary or ternary complexes containing radiolabeled IGF-I. IGF-I tracer (100,000 cpm) was incubated in a volume of 250 μl with ALS (500 ng) and IGFBPs as shown, and 200 μl aliquots were analyzed on a Superose 12 column. Tracer alone is shown in filled boxes in each graph. The arrows from left to right indicate the fractionation positions of ternary complexes, binary complexes, and free IGF-I tracer, respectively. A, increasing formation of ternary complex with ALS plus increasing additions of IGFBP-5: ALS alone did not bind to IGF-I. B, comparison of ternary complex formation by IGFBP-3 and IGFBP-5: the IGFBP-3 complex eluted earlier. C, IGFBP-1 and IGFBP-2 (each 10 ng) and IGFBP-4 (10 ng) and IGFBP-6 (20 ng) (D) were able to form binary complexes only. IGFBP-6 formed a relatively smaller amount of binary complex than the other IGFBPs due to its lower binding affinity for IGF-I.

**Fig. 3.** Immunoprecipitation of ternary complexes with ALS antibody. A, in the presence of increasing concentrations of IGFBP-3 or IGFBP-5, IGF-I or IGF-II tracers (25,000 cpm) formed a complex with ALS, which was precipitable by anti-ALS serum. Specific binding is shown. Nonspecific binding (radioactivity precipitated in the absence of ALS) did not exceed 12%. B, in the absence of IGFBPs, ~40% of cross-linked IGF-I-IGFBP-5 tracer (10,000 cpm) bound to 25 ng of ALS and was precipitable by ALS antiserum. Increasing concentrations of IGFBP-3 or IGFBP-5 alone had no effect on tracer binding to ALS, but in the presence of IGF-I or IGF-II (100 ng), IGFBP-3 and IGFBP-5 caused dose-dependent competition for tracer binding to ALS.
lieved to represent a complex in which the IGFBP is partly degraded to a smaller size. Because there is no evidence of this degradation when complexes are analyzed by gel permeation chromatography, it is believed to occur as a result of proteolytically nicked IGFBP-3 or IGFBP-5, losing its structural integrity when subjected to the denaturing conditions of SDS-PAGE (33). In the absence of IGFs, IGFBP-5 formed only a faintly visible band when co-incubated with ALS tracer, emphasizing the requirement of IGFs for significant binding of IGFBP-5 to ALS, as previously demonstrated for IGFBP-3 complexes (24, 29).

In an alternative affinity-labeling protocol, cross-linked IGF-I-IGFBP-5 tracer was incubated with unlabeled ALS and the mixture again reacted with disuccinimidyl suberate. In the presence of ALS, high molecular mass complexes were seen (Fig. 5) similar to those shown in Fig. 4 for ALS tracer. Again, covalent complexes containing IGFBP-3 appeared 15–20 kDa larger. Excess unlabeled IGF-I plus IGFBP-3 decreased the intensity of the radioactive IGFBP-5.

**Demonstration of Human Serum IGFBP-5 in Ternary Complexes**—After size fractionation of normal human serum on Sephadex G-100 and analysis of fractions by immunoblot (Fig. 6), intact human IGFBP-5 was present as a 30-kDa double (34) in fractions corresponding to the elution volume of gamma globulin (~150 kDa). In six separate experiments on normal adult serum, serum IGFBP-5 co-migrated with ALS immunoreactivity, consistent with its presence in complexes with ALS (Fig. 6). In these samples, immunoreactive IGFBP-5 was not readily detectable in fractions corresponding to binary-complexed or free IGFBP-5 (fractions 23–24) under the same conditions or in fractions corresponding to lower molecular masses. In some experiments, some of the immunoreactive IGFBP-5 corresponding to ternary complexes appeared in a proteolyzed form of ~18 kDa (not shown), as previously reported for serum IGFBP-5 (17). These studies indicate that the majority of IGFBP-5 exists in ternary-complexed forms in human serum rather than in binary-complexed or free forms.

**DISCUSSION**

Complexes of IGFBP-5 with ALS and IGF-I or IGF-II have been demonstrated using three independent methods: gel permeation chromatography, immunoprecipitation with ALS antiserum, and affinity labeling. Although the basic carboxyl-terminal domain of IGFBP-3 has been specifically implicated in ALS binding (16), the role of the corresponding domain in IGFBP-5 remains to be definitively demonstrated. This sequence, comprising 18 residues in the carboxyl-terminal region of both IGFBPs (IGFBP-3 [215–232] and IGFBP-5 [201–218]) is highly positively charged, with 10 basic and no acidic residues, and has been postulated to serve as a nuclear localization
signal for IGFBP-3 (35). In contrast, IGFBP-1, for example, has only two basic residues, and three acidic residues in the corresponding region, IGFBP-1 [183–200] (36).

Peptides corresponding to this basic domain have been shown to inhibit IGFBP-3 and IGFBP-5 binding to endothelial cells (37), and mutation of IGFBP-3 residues 228–232 (KGRKK) to the corresponding residues of IGFBP-1 (MGDEA) prevents IGFBP-3 cell association (16). The same mutation reduces ALS binding to IGFBP-3 by over 90% (16). The unique sharing of this basic structural domain involved in ALS binding, between IGFBP-3 and IGFBP-5, which are suggested to have evolved from a common gene (38), may account for their shared ability to form ALS complexes and indeed for other shared functions such as cell surface and matrix binding (37, 39) of these two proteins.

Whereas IGFBP-5 has a higher affinity for IGF-II than IGF-I (40), IGFBP-5 formed an ALS complex more potently with IGF-I than IGF-II. This parallels our previous findings for IGFBP-3 (25), where C and D domain residues of IGF-I were identified as contributing to the binding affinity for ALS (41). Further, the presence of either IGF-I or IGF-II was required for detectable IGFBP-5 binding to ALS; that is, IGFBP-5-ALS complexes formed to an unmeasurably low extent. This suggests that formation of a binary complex of IGF-I or IGF-II with IGFBP-5 produces a conformational change in IGFBP-5 and increases its affinity for ALS. We have similarly reported that human IGFBP-3 binding to ALS is unmeasurably weak in the absence of IGFs, although it can be stabilized by covalent cross-linking (25, 29). Other studies using rat proteins or nonglycosylated recombinant human IGFBP-3 have reported somewhat stronger formation of IGFBP-3-ALS complexes in vitro (42, 43).

We have demonstrated that IGFBP-5 in human serum is present in a predominantly high molecular mass form characteristic of a ternary complex with IGF-I or IGF-II and ALS. In some experiments, IGFBP-5 derived from ternary complexes appeared partially as an ~18-kDa proteolyzed form comparable to the 30-kDa form of IGFBP-3 that can be detected in 150-kDa complexes when analyzed similarly (44). Whether this proteolysis occurs in vivo or during the analysis is not fully understood; however, it is clear that even in the case of pregnancy, when all of the immunoreactive IGFBP-3 appears in a 30-kDa form by this analysis, it is still capable of carrying a normal concentration of serum IGFs in ternary complexes (33). The significance of limited IGFBP-5 proteolysis in ternary complexes therefore remains to be determined.

Our observation that much of the immunoreactive IGFBP-5 in serum co-elutes with immunoreactive ALS challenges the previous report that described serum IGFBP-5 only in fractions corresponding to the molecular mass of binary complexed IGFBP-5 or smaller (17). The possibility that IGFBP-3 was inadvertently detected in the present study is highly unlikely since the antiserum used was found to detect rhIGFBP-5 sensitively while showing no measurable cross-reactivity with IGFBP-3. In seeking an explanation for the discrepancy between the two results, the calibration of the Superose 6 column used in the earlier study may be called into question. This is suggested by the fact that Superose 6 has a fractionation range stated by the manufacturer to be 5–5000 kDa and an exclusion limit of 40,000 kDa. The elution profile described in the Superose 6-fractionated serum (17) is thus quite consistent with a molecular mass of ~100 kDa for IGFBP-5 in the first elution peak. In the study we describe with the Sephadex G-100 column, the immunoreactive IGFBP-5 in human serum eluted in fractions with similar mobility to bovine gamma globulin and was found repeatedly to co-elute with immunoreactive ALS. Furthermore, that IGFBP-5 circulates in ternary complexes to a significant extent is consistent with the demonstration of in vitro IGFBP-5 ternary complex formation, and competition between IGFBP-3 and IGFBP-5 for ALS would not be expected in human serum in vivo due to the excess ALS concentration in serum relative to IGFBP-3 and IGFBP-5.

Whatever the explanation for the previously published results, our in vitro evidence of ternary complex formation by IGFBP-5 (under conditions similar to the formation of IGFBP-3 ternary complexes), combined with the size distribution of endogenous serum IGFBP-5 corresponding to high molecular mass forms, together support the view that ternary complex formation by IGFBP-5 is a natural and physiologically significant phenomenon. Other less direct evidence for the existence of endogenous IGFBP-5 ternary complexes in serum is consistent with this finding. The highly significant association between IGFBP-3 and IGF concentrations seen in the human circulation under a variety of conditions is believed to be due predominantly to the stabilization of IGFBP-3 when occupied by IGF-I or -II in complexes with ALS (4, 5). A similar strong correlation between serum IGFBP-5 levels and IGF-I and -II has recently been reported (17). IGFBP-3 and IGFBP-5 levels show a parallel age-dependence (45), suggesting a commonality in their regulatory processes that would best be explained if IGFBP-5 is also complexed to a significant extent with ALS. Moreover, growth hormone (GH) therapy causes parallel increases in both IGF and IGFBP-5 levels in GH-deficient subjects (46), just as it also increases IGFBP-3 levels. An IGFBP-5 ternary complex in serum would be expected to be GH-dependent, as its existence and stability would depend on the GH-dependent protein ALS (27). In contrast, in a variety of studies, IGFBP-1, -2, -4, and -6 either have no association or an inverse relationship with GH and IGF levels (18–21). IGFBP-5 in ternary complexed form in serum may not have been detected until now due to limited availability of sensitive immunoassay methods, and the presumably smaller amount of ternary-complexed IGFBP-5 relative to IGFBP-3 complexes.

Circulating IGFBP-5 ternary complexes might serve to deliver IGFs to tissues in association with IGFBP-5. This could occur through the dissociation of ALS from the complexes, perhaps mediated by interaction with glycosaminoglycans (47); alternatively, there may be mechanisms such as limited proteolysis that increase the dissociation of IGFs from the complex while still in the vascular compartment. The possibility of IGFs complexed either to IGFBP-3 or IGFBP-5 reaching the tissues from the circulation raises the question whether these binary complexes would have different and possibly competing actions on cells. IGFBP-5 in a binary complex has been shown to act as a local extracellular IGF reservoir through binding to extracellular matrix and to potentiate IGF access to cells by binding to cell surfaces (48–50). In bone, IGFBP-5 binary complex is the major extracellular reservoir of IGFs and has been speculated to be partially serum-derived (51). IGFBP-3, in contrast, is inhibitory to IGF action in the majority of situations, although stimulatory effects are also well documented (52, 53). IGFBP-3 in general has an antiproliferative action in fibroblasts, breast cancer cells, and other cell types (54, 55) and also induces apoptosis (56). Although some of these effects occur independently of IGF receptors, they are not necessarily IGF-independent, since IGF-I acts in a receptor-independent manner to inhibit IGFBP-3 binding to cell surfaces (57). Serum-derived IGFBP-3 and IGFBP-5 complexes might be differentially targeted to various tissues or may compete at the same tissue sites.

Although ALS is predominantly found in serum, IGFBP-3 in skin interstitial fluid is found in 150-kDa complexes, implying
the presence of ALS (58), and both synovial fluid (59) and ovarian follicular fluid² contain high levels of immunoreactive ALS. Furthermore, ALS messenger RNA has been identified in at least two other non-hepatic tissue types: bone and renal cortex (60), both tissues that contain abundant IGFBP-5 (15, 61). It may therefore be speculated that, in addition to its role in the circulation, ALS functions at a tissue level by interacting with binary complexes containing either IGFBP-3 or IGFBP-5. Tissue IGFBP-5 ternary complexes containing ALS of non-hepatic origin might provide a local stable reservoir of IGFs and IGFBP-5, possibly involved in regulating local IGF actions. If ALS binds to a cell-association domain of IGFBP-5 as it does to IGFBP-3, locally produced ALS might block actions of these proteins that require interaction with cell surfaces.

In summary, we have made the novel observations that IGFBP-5, like IGFBP-3, is able to form heterotrimers by combining with IGF-I or -II and ALS, and that endogenous human serum IGFBP-5 is largely detectable in high molecular mass complexed forms. The demonstration of IGFBP-5-containing ternary complexes will demand a re-evaluation of current views on IGF transport in the circulation, release to the tissues, and regulation at the cellular level.

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REFERENCES
1. Hummel, R. E. (1990) Eur. J. Biochem. 190, 445–462
2. Le Roith, D. (1997) N. Engl. J. Med. 336, 633–640
3. Baxter, R. C. (1995) Metabolism 44, Suppl. 4, 12–17
4. Zapf, J. (1995) Eur. J. Endocrinol. 132, 645–654
5. Baxter, R. C. (1993) Trends Endocrinol. Metab. 4, 91–96
6. Guler, H.-P., Zapf, J., Schmid, C., and Froesch, E. R. (1989) Acta Endocrinol. 121, 753–758
7. Powell, D. R., Liu, F., Baker, B., Lee, P. D. K., Belsha, C. W., Brewer, E. D., and Hinta, R. L. (1995) Pediatr. Res. 38, 136–143
8. Jones, J. L., and Clemmons, D. R. (1996) Endocrinol. 133, 3–14
9. Blum, W. F., Albertsson-Wikland, K., Rosberg, S., and Ranke, M. B. (1993) J. Clin. Endocrinol. Metab. 76, 1610–1616
10. Lee, C. Y., Wu, H. B., Suh, D. S., and Rechler, M. M. (1997) J. Biol. Chem. 272, 1016–1021
11. Binoux, M., and Hossenlopp, P. (1988) J. Clin. Endocrinol. Metab. 67, 509–514
12. LeGoff, M. S., Saunders, H., Phuyal, J. L., and Baxter, R. C. (1993) J. Biol. Chem. 268, 1016–1021
13. LeRoith, D. (1997) J. Clin. Invest. 99, 1318–1324
14. Wood, W. I., Cachianes, G., Henzel, W. J., Winslow, G. A., Spencer, S. A., Hellmisa, R., Martin, J. L., and Baxter, R. C. (1989) J. Biol. Chem. 264, 1176–1185
15. Shimazaki, S., Shimonaka, M., Zhang, H. P., and Ling, N. (1991) J. Biol. Chem. 266, 10646–10653
16. Firth, S. M., Ganeshprasad, U., and Baxter, R. C. (1998) J. Biol. Chem. 273, 2631–2638
17. Mohan, S., Hamburthi, C., Dony, C., Lang, K., Srinivasan, N., and Baylink, D. J. (1995) J. Clin. Endocrinol. Metab. 80, 2638–2645
18. Lee, P. D. K., Conover, C. A., and Powell, D. R. (1993) Proc. Soc. Exp. Biol. Med. 204, 4–29
19. Clemmons, D. R., Snyder, D. K., and Busby, W. H. (1993) J. Clin. Endocrinol. Metab. 73, 725–733
20. honda, Y., Landale, E. C., Strong, D. D., Baylink, D. J., and Mohan, S. (1996)

*R. C. Baxter, unpublished data.