Interaction of Insulin-like Growth Factor II (IGF-II) with Multiple Plasma Proteins

HIGH AFFINITY BINDING OF PLASMINOGEN TO IGF-II AND IGF-BINDING PROTEIN-3*

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In the circulation, most of the insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and IGFBP proteases are bound in high molecular mass complexes of ≥150 kDa. To investigate molecular interactions between proteins involved in IGF-IGFBP complexes, Cohn fraction IV of human plasma was subjected to IGF-II affinity chromatography followed by reversed-phase high pressure liquid chromatography and analysis of bound proteins. Mass spectrometry and Western blotting revealed the presence of IGFBP-3, IGFBP-5, transferrin, plasminogen, prekallikrein, antithrombin III, and the soluble IGF-II/mannose 6-phosphate receptor in the eluate. Furthermore, an IGFBP-3 protease cleaving also IGFBP-2 but not IGFBP-4 was co-purified from the IGF-II column. Inhibitor studies and IGFBP-3 zymography have demonstrated that the 92-kDa IGFBP-3 protease has the class of serine-dependent proteases. IGF-II ligand blotting and surface plasmon resonance spectroscopy have been used to identify plasminogen as a novel high affinity IGF-II-binding protein capable of binding to IGFBP-3 with 50-fold higher affinity than transferrin. In combination with transferrin, the overall binding constant of plasminogen/transferrin for IGF-II was reduced 7-fold. Size exclusion chromatography of the IGF-II matrix eluate revealed that transferrin, plasminogen, and the IGFBP-3 protease are present in different high molecular mass complexes of ≥440 kDa. The present data indicate that IGFs, low and high affinity IGFBPs, several IGFBP-associated proteins, and IGFBP-proteases can interact, which may result in the formation of binary, ternary, and higher molecular weight complexes capable of modulating IGF binding properties and the stability of IGFBPs.

Both their mitogenic and metabolic effects are for the most part mediated through binding and activation of the IGF-I receptor (1). The interaction of IGFs with the IGF-I receptor is controlled by a family of six high affinity IGF-binding proteins (IGFBP-1 to IGFBP-6) exhibiting strong sequence homology. The cysteine-rich N- and C-terminal domains are conserved across all IGFBPs, and both domains appear to be involved in IGF binding (2, 3). In the circulation, the majority of the IGFs are sequestered into ternary 150-kDa complexes with either IGFBP-3 or IGFBP-5 and the 85-kDa acid-labile subunit, prolonging the half-life of IGFs, controlling their transcapillary transport to the target tissues, and functioning as a circulating reservoir of IGFs (4–6). The IGFBPs can also form binary complexes with IGFs. Limited proteolysis of IGFBP3 appears to be the major mechanism for the release of IGFs from binary and ternary IGFBP complexes, resulting in the generation of IGFBP fragments with reduced affinities for IGFs (7, 8). Several cation- and serine-dependent serum proteases, such as a disintegrin and metalloprotease (ADAM) 12S, matrix metalloprotease-3, α-kallikrein, plasmin, thrombin, and the pregnancy-associated plasma protein-A (PAPP-A), have been reported to cleave IGFBPs (9–15).

Recent studies have shown that IGFBP-3, IGFBP-5, and their IGF-I-IGFBP complexes interact also with other serum proteins. Thus, plasminogen (16), fibrinogen, fibrin (17), and fibronectin (18) bind to IGFBP-3, whereas plasminogen activator inhibitor-1 (19) and thrombospordin (20) were found to interact with IGFBP-5. Additionally, vitronectin has been reported to interact with IGFBP-2, -3, -4, and -5 (21). It is likely that the IGFBP interactions with proteins involved in wound healing processes are required to sequester IGFBPs at wound sites (17). Furthermore, IGFBP-3 has been demonstrated to bind to transferrin (22, 23), which may affect cell survival, the control of cell growth, or facilitate the cellular uptake of IGFBP-3 (24) via transferrin receptors.

In the present study, we have identified and characterized several serum proteins co-purified with IGFBP-3 and IGFBP-5 from an IGF-II affinity matrix. Analysis of their IGF-II and IGFBP-3 binding properties as well as size exclusion chromatography suggest the formation of high molecular mass protein complexes in the circulation that also contain IGFBP-3 proteolytic activity.

EXPERIMENTAL PROCEDURES

Materials—IGFBP-4 and IGFBP-5 were kindly provided by Dr. J. Zapf (University Hospital, Zurich, Switzerland). Nonglycosylated recombinant human IGFBP-3 was a kind gift from Dr. A. Sommer (Celtix, Santa Clara, CA). Recombinant human IGFBP-2 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and IGF-I and biotinylated IGF-II were from GroPep (Adelaide, Australia). IGF-I and

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† The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; RP-HPLC, reversed-phase high pressure liquid chromatography; ACN, acetonitrile; M6PR, mannose 6-phosphate receptor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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IGF-II were iodinated by the chloramine-T method (25). Transferrin, plasminogen, and antithrombin III were purchased from Sigma and α2-antiplasmin from Hematologic Technologies Inc. (Essex Junction, VT). Preparations were kindly provided by Dr. W. Müller-Esterl (Institute for Biochemistry II, Frankfurt, Germany). Proteins were labeled with Na\(^{125}\)I (Amersham Biosciences) using IODO-GEN (Pierce) as described (26). The hydroxamic acid-based metalloprotease inhibitor tumor necrosis factor α-activating protein inhibitor (TAPI) was prepared at Immunex Corporation (Seattle, WA) (27) and was a kind gift from Dr. S. Rose-John (University of Kiel, Germany). All chromatography media were purchased from Amersham Biosciences.

**Purification of Serum Proteins Eluted from IGF-II Affinity Columns**—Cohn fraction IV of human plasma was fractionated as described previously (28). In brief, Cohn fraction IV was resuspended in 2 mM acetic acid, pH 3.0, containing 0.075 mM NaCl and mixed with SP-Sephadex C-25 in a batch procedure to remove IGF-I and IGF-II. After adjustment to neutral pH the supernatant was passed over an IGF-II-Sepharose 4B affinity column (1 × 17 cm). The column was washed in reequilibration direction with 200 ml of phosphate buffer, pH 7.4 (0.05 mM sodium phosphate, 0.1 mM NaCl, 0.02% sodium azide), and with 100 ml of water before bound proteins were eluted with 0.1 M triethylammonium phosphate, pH 2.8. The elution of IGF-II was monitored by a competitive binding protein assay as described previously (28).

Subsequently, the fractions containing the bulk of eluted IGFBP-3 were applied to reversed-phase HPLC (RP-HPLC, C18 column, 1 × 50 cm, Serva, Heidelberg, Germany) in 0.1 M triethylammonium phosphate, pH 3.0, at a flow rate of 10 l/min, detected by UV absorption at 280 nm, and collected in fractions of 200 ml. Of each fraction 20 ml was analyzed for Western blotting or IGFBP-3 protease assay (see below), respectively.

**IGFBP-3 Protease Assay, IGFBP Zymography, and Western and Ligand Blots**—To examine fractions for IGFBP proteolytic activity, 10 μl of column fractions were incubated with [\(^{125}\)I]IGFBP-2, -3, or -4 (20,000–25,000 cpm) for 16 h at 37 °C in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 5 mM CaCl\(_2\). When indicated, protease inhibitor BS3 were included. The samples were subjected to SDS-PAGE (15% acrylamide) under nonreducing conditions and visualized by autoradiography. Radiolabeled IGFBP-3 zymography and ligand blotting of RP-HPLC fractions (12 μl) using monobiotinylated IGF-II were carried out as described previously (12, 29). For Western blot analysis, the HPLC fractions were separated by SDS-PAGE (10% and 15% acrylamide, respectively) transferred to nitrocellulose membranes (Bio-Rad). Polyclonal antibodies against transferrin (Dako, Hamburg, Germany, 1: 5,000), plasminogen (Dako, 1:100), antithrombin III (Dako, 1:1,000), and IGFBP-5 (R&D Systems Inc., 1:1,000) were used. The polyclonal antibody against prekallikrein, kindly provided by Dr. W. Müller-Esterl (30), was diluted 1:250. The polyclonal antibody against the extracellular domain of the IGF-II/mannose 6-phosphate receptor (IGF-II/M6P) has been described previously (31) (1:500). Anti-IGFBP-3 antisem (Upstate Biotechnology) was diluted 1:1,000. Immunoreactive bands were visualized with SuperSignal enhanced chemiluminescence detection system (ECL, Pierce).

**Surface Plasmon Resonance Interaction Analysis**—Interaction measurements were carried out as described previously (32) using IGF-II and IGFBP-3-coupled CMS sensor chips in a BIAdcore 3000 (Amersham Biosciences). Biosensor surfaces were coupled to final resonance value of ~300 response units for IGF-II and ~600 response units for IGFBP-3. A flow rate of 10 μl/min with HBS-P (10 mM HEPES, 150 mM NaCl, 0.005% Polysorbate 20, pH 7.4) as running buffer was used. For sensorgram analysis the 1:1 Langmuir binding model was used, and kinetic rate constants were calculated as described (33).

**Mass Analysis**—To identify eluted proteins, fractions were separated by SDS-PAGE and visualized by silver staining. Protein bands were excised and subjected to in-gel trypsin digestion using a modified trypsin solution (Promega). Matrix-assisted laser desorption ionization-time of flight mass spectrometry of peptide digests was carried out commercially at the Protein Chemistry Unit, Biomedical Helsinki, Finland, using a Bruker Daltonics Autoflex mass spectrometer (Bremen, Germany). For peptide fingerprint searches, the NCBI nr, Swiss Protein, and Mass Spectrometry Protein Sequence databases (MSDB) were used.

**Cross-linkage Analysis**—Cross-linking of [\(^{125}\)I]IGF-II to plasminogen was carried out as described previously (23). Plasminogen (0.5 μg) was incubated with [\(^{125}\)I]IGFBP-3 (150,000 cpm) in the presence or absence of unlabeled IGF-I or IGF-II (3 μM) for 1 h at 4 °C. Disuccinimidyl suberate (Pierce, 0.5 mM) was added, and the reaction mixture was further incubated for 15 min on ice. After the reaction was terminated, the samples were solubilized and analyzed by SDS-PAGE and autoradiography. To analyze the formation of protein-transferrin complexes in vitro, transferrin (200 ng) was incubated with or without single or multiple recombinant proteins (each at 200 ng), identified in the eluate of the IGF-II affinity matrix, in phosphate-buffered saline in a total volume of 20 μl. Bis(sulfo)succinimidyl)suberate (BS\(_2\), Pierce, 0.5 mM) was added and incubated for 30 min at room temperature. The reaction was terminated by the addition of 50 mM Tris/HCl, pH 7.4, for 15 min followed by transferrin immunoblotting.

**RESULTS**

**Multiple Non-IGFBP Plasma Proteins Bind IGF-II**—Human plasma Cohn fraction IV was subjected to IGF-II affinity chromatography, and the bound proteins were desorbed with SDS-PAGE and silver staining (A), IGF-II ligand blotting (B), and [\(^{125}\)I]IGFBP-3 protease activity (C). Increasing amounts of the eluted protein fraction (E1–E3) were incubated with [\(^{125}\)I]IGFBP-3 (20,000 cpm) for 16 h at 37 °C. Samples were analyzed by SDS-PAGE (15% acrylamide) and autoradiography. As control (co), buffer was incubated with [\(^{125}\)I]IGFBP-3 under identical conditions. The position of the molecular mass marker proteins is indicated.

![Fig. 1](image.png)
protein 1). The 43/45-kDa doublet in fraction 46 (24% ACN, protein 2) was identified as IGFBP-3 by IGF-II ligand blotting (Fig. 2C) and immunoblotting (Fig. 3). In fractions 50–53 and 53–57 (eluting at 27 and 30% ACN, respectively), proteins of ~92 and 70 kDa, respectively, were stained (Fig. 2B, proteins 4 and 3, respectively). When the same RP-HPLC fractions were tested by ligand blotting, prominent IGF-II-binding polypeptides of ~30/33 kDa and of 34 and 43/45 kDa were observed in fractions 36/37 and 46 to 50, respectively (Fig. 2C). Under these ECL exposure time conditions, the 92- and 70-kDa proteins in fractions 50–57 showed no IGF-II binding. These data suggest that the eluted 92- and 70-kDa polypeptides either exhibited low IGF-II binding affinity or bound through interaction with IGFBPs to the IGF-II column.

Silver-stained proteins were further analyzed by mass spectrometry. Protein 1 (Fig. 2B) could be identified as IGFBP-5, protein 2 as IGFBP-3, and protein 3 as transferrin (Table I). The mass analysis of the 92-kDa protein could not be assigned to a specific protein.

To confirm these results, Western blotting experiments were performed as shown in Fig. 3. The 32-kDa protein in fraction 36 represents IGFBP-5, and the immunoreactive doublet in fraction 46 was confirmed as IGFBP-3. This fraction contained also small amounts of the 29-kDa IGFBP-3 fragment. The 70-kDa protein in fraction 56 was verified as transferrin. Because it has been reported that both IGFBP-3 and IGFBP-5 bind to plasminogen migrating at a molecular mass of ~92/97 kDa (16, 34), fraction 53 was also tested for plasminogen immunoreactivity. This fraction contained 92-kDa plasminogen immunoreactive material with a slightly higher molecular mass than the purified standard (Fig. 3). Western blotting analysis of the protein fraction eluted from the IGF-II affinity column showed that additional non-IGFBP proteins such as antithrombin III, prekallikrein and the soluble IGF-II/M6PR bound to the matrix (Fig. 4).

Transferrin Affects the Association of Plasminogen to IGF-II and IGFBP-3—We used surface plasmon resonance spectroscopy to measure the rate constants and modulatory effects of transferrin, plasminogen, and IGFBP-3 in solution on binding to IGF-II or IGFBP-3 immobilized on the sensor surface. When transferrin was passed over the IGF-II biosensor surface, a low affinity binding ($K_D = 976$ nM) (23) was determined (Table II). In contrast, under these conditions plasminogen showed a high affinity for IGF-II with a $K_D$ value of 5.0 nM, which is comparable with the binding of IGFBP-3 to the IGF-II surface ($K_D = 3.6$ nM) (23). The affinity of plasminogen in combination with transferrin was ~7-fold lower for IGF-II ($K_D = 37$ nM) than for plasminogen alone. This was mostly due to a reduction of the association rate. The inclusion of IGFBP-3 in the plasminogen/transferrin mixture did not further affect protein binding to the IGF-II surface.

When IGFBP-3 was immobilized to the sensor, high affinity binding ($K_D = 1.3$ nM) of plasminogen was measured, which was about 54-fold higher than the binding of transferrin to IGFBP-3 (Table II). The combination of plasminogen and transferrin resulted in an overall binding constant, which is comparable with the $K_D$ value of plasminogen alone (1.0 versus 1.3 nM); however, this is higher than the binding affinity of transferrin alone (1.0 versus 69.9 nM). The increase in the association constant of the protein mixture to IGFBP-3 indi-
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With high efficiency to plasminogen (Fig. 5 and, less potently, by IGF-I. RP-HPLC fractions were additionally tested in an [125I]IGFBP-3 protease assay. The highest proteolytic activity was detectable in fraction 53, generating fragments of 21, 17, and most prominently 14 kDa (Fig. 6, 3rd lane). The protease in fraction 53 also cleaved [125I]IGFBP-2 into eight fragments (mainly 21 and 14 kDa) but showed almost no catalytic activity when [125I]IGFBP-3 was used as substrate (not shown).

To characterize the IGFBP-3 protease in fraction 53, the protease was isolated and characterized by size exclusion chromatography of IGF-II affinity matrix eluates. The highest proteolytic activity was detected in fraction 53 and was found to be associated with a polypeptide of ≈92 kDa (Fig. 8B). The binding of [125I]IGFBP-2 is specific and can be replaced by unlabeled IGF-II and, less potently, by IGF-I.

**IGFBP-Ileues Contain IGFBP Protease Activity**—To determine which fractions contained IGFBP-3 protease activity, the RP-HPLC fractions were additionally tested in an [125I]IGFBP-3 protease assay. The highest proteolytic activity was detectable in fraction 53, generating fragments of 21, 17, and most prominently 14 kDa (Fig. 6, 3rd lane). The protease in fraction 53 also cleaved [125I]IGFBP-2 into eight fragments (mainly 21 and 14 kDa) but showed almost no catalytic activity when [125I]IGFBP-3 was used as substrate (not shown).

To characterize the IGFBP-3 protease in fraction 53, the effects of protease inhibitors were tested. Serine and metalloprotease inhibitors were added to the protease assays. As shown in Fig. 7, the IGFBP-3 protease activity could be blocked almost completely (by 90%) as measured by remaining intact [125I]IGFBP-3 by densitometric evaluation of the autoradiographs by the serine protease inhibitor apronitin (1.5 μM, lane 4), whereas benzamidine (1 mM, lane 3) showed only a weak inhibition (by 20%). Furthermore, the addition of the specific naturally serine protease inhibitor antithrombin III (10 μg/ml, Fig. 7, lane 5) affected the IGFBP-3 proteolysis weakly (24% of nontreated controls), whereas another naturally circulating serine protease inhibitor, α2 antiplasmin, inhibited at low concentrations (5 μg/ml, lane 12) almost completely the cleavage of [125I]IGFBP-3. The metalloprotease inhibitors 1,10-phenanthroline (2 mM, Fig. 7, lane 8) or tumor necrosis factor α-activating protein inhibitor (TAPI, 0.5 mM, lane 9) showed no effects on [125I]IGFBP-3 proteolysis.

In a different experimental approach, the RP-HPLC fractions were analyzed by [125I]IGFBP-3 zymography. The highest proteolytic activity was observed again in fraction 53 and was found to be associated with a polypeptide of ≈92 kDa (Fig. 8A). Fig. 8B shows an [125I]IGFBP-3 zymography in the absence and presence of low molecular weight protease inhibitors capable to penetrate the gel. This confirms that the protease activity can be inhibited by the serine protease inhibitor apronitin, whereas the mixture of the metalloprotease inhibitors 1,10-phenanthroline and EDTA had no effect on the degradation of [125I]IGFBP-3.

**Transferrin Co-fractions with Plasminogen and the IGFBP-3 Protease by Size Exclusion Chromatography**—To verify that transferrin forms complexes with plasminogen and the IGFBP-3 protease, size exclusion chromatography of IGF-II matrix eluates was performed under physiological conditions on a Superdex 200 column. The elution profiles and the distribution of transferrin, plasminogen, and the [125I]IGFBP-3 protease in the eluted fractions are shown in Fig. 9, demonstrating that transferrin and plasminogen eluted in three peak fractions (fractions 8, 12, and 14, respectively). Comparison with the separation of protein standards of known relative molecular mass indicates that these peaks display molecular masses of >440 kDa (fractions 8 and 12) and 440 kDa (fraction 14), respectively. The majority of the [125I]IGFBP-3 protease activity was found in fraction 14 in the 440-kDa protein complex. These data are consistent with the conclusion that transferrin, plasminogen, and the [125I]IGFBP-3 protease are present in the same high molecular mass complex.

**TABLE I**

| Protein no. | Measured mass | Peptide sequence | Amino acids | Matched peptide |
|-------------|---------------|-----------------|-------------|----------------|
| 1           | 915.542       | IIAPFENR        | 177–184     | IGFBP-5        |
| 2           | 864.569       | PHPNLSK         | 65–71       | IGFBP-3        |
| 3           | 1322.792      | DSAGKFLKVPR     | 316–327     | Transferrin    |
Binding of IGFBP-3, plasminogen, and transferrin to IGFs and IGFBP-3

| Immobilized ligand | IGF-II | IGFBP-3 |
|-------------------|--------|---------|
|                   | $K_D$ (nM) | $k_a$ (1/μs) | $k_d$ (1/s) | $K_D$ (nM) | $k_a$ (1/μs) | $k_d$ (1/s) |
| IGFBP-3           | 3.8 ± 0.3 | 150 ± 10 | 1.5 ± 0.2 | 1.3 ± 0.2 |
| Plasminogen       | 5.0 ± 0.3 | 220 ± 10 | 1.1 ± 0.3 | 1.3 ± 0.2 |
| Plasminogen + IGFBP-3 | 5.6 ± 0.3 | 180 ± 10 | 1.0 ± 0.2 | 1.3 ± 0.2 |
| Transferrin       | 97.0 ± 0.3 | 2 ± 0.3 | 2.1 ± 0.4 | 7.0 ± 0.4 |
| Transferrin + transferrin | 37.0 ± 0.3 | 88 ± 5 | 3.2 ± 0.5 | 1.0 ± 0.2 |
| Plasminogen + transferrin + IGFBP-3 | 31.0 ± 0.3 | 66 ± 10 | 2.0 ± 0.4 | 1.0 ± 0.2 |

*a* Nonglycosylated human recombinant protein.

*b* Human recombinant protein.

FIG. 6. The fractions of IGF-II-interacting proteins contain IGFBP-3 protease activity. Aliquots of RP-HPLC fractions (shown for fractions 50–57) were incubated with [125I]IGFBP-3 (20,000 cpm) for 16 h at 37 °C. Samples were analyzed by SDS-PAGE (8% acrylamide) and autoradiography.

FIG. 7. Effects of protease inhibitors on IGFBP-3 protease activity in RP-HPLC fractions. Aliquots of RP-HPLC fraction 53 were incubated with [125I]IGFBP-3 (20,000 cpm) for 16 h at 37 °C. As a control (co), [125I]IGFBP-3 was incubated in the absence of RP-HPLC fraction 53 for 16 h at 37 °C with buffer (lanes 1, 6, and 9). The samples were incubated in the presence (lanes 3–5, 8, and 11) and absence (lanes 2, 7, and 10) of the protease inhibitors benzamidine (benz) (1 mM), aprotinin (apr) (1.5 μM), and antithrombin III (AT-III) (10 μg/ml), tumor necrosis factor α-activating protein inhibitor (TAPI) (0.5 mM), and a2-antiplasmin (2-AP) (5 μg/ml). The reaction products were separated by SDS-PAGE (12.5% acrylamide) and visualized by autoradiography.

FIG. 8. Co-purification of a 92-kDa aprotinin-sensitive IGFBP-3 protease. A, aliquots of RP-HPLC fractions were analyzed by [125I]IGFBP-3 substrate zymography. Relative migration positions of molecular mass marker proteins are indicated. B, the [125I]IGFBP-3 zymography gel was incubated either in the absence (co) or presence of aprotinin (apr) (1.5 μM) or 1,10-phenanthroline (phe) (2 mM)/EDTA (2 mM).
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Fig. 9. Size exclusion chromatography of protein fraction eluted from the IGF-II matrix. An aliquot of the protein fraction eluted from the IGF-II affinity matrix was separated on a Superdex 200-size exclusion column as described. A, the elution profile of the separation is shown. The fractions containing the standard proteins ferritin (440 kDa), albumin (66 kDa), and ribonuclease a (13.7 kDa), run under identical conditions, are indicated. B–D, analyses of fractions from the same separation experiment are shown. The experiment was repeated twice with identical results. B, 25 μl of each fraction (except 12.5 μl of fraction 14) was analyzed by Western blotting for transferrin. C, 50 μl of each fraction was analyzed by plasminogen immunoblotting. D, 20 μl of each fraction and 1 μl of the IGF-II matrix eluate (E) were incubated with [125I]IGFBP-3 (20,000 cpm) for 15 h at 37 °C. As a control (co), [125I]IGFBP-3 was incubated with buffer alone. The reaction products were separated by SDS-PAGE (15% acrylamide) and visualized by autoradiography.

FBP-associated proteins leads to the formation of different higher molecular mass complexes (≥440 kDa). These circulating complexes may function not only to protect IGFBPs from proteolysis and prevent the loss of IGFs by glomerular filtration but may also support the targeting of IGFs and/or IGFBPs, e.g., to wound sites (17), or modulate the migration, proliferation, and apoptosis of extravascular cells (22, 21).

Surface plasmon resonance spectrometry demonstrated that immobilized IGFBP-3 binds the co-purified plasminogen with high affinity (KD = 1.3 nM) exactly confirming the data reported previously by Campbell et al. (16). Surprisingly, plasminogen was also able to bind IGF-II with high affinity (KD = 5 nM) comparable with the binding affinities of IGFBP-3 for IGF-II (KD = 3.6 nM). The capability of plasminogen to bind IGF-II was confirmed by ligand blot analysis and [125I]IGF-II cross-linkage. Therefore, plasminogen may bind either directly to the IGF-II affinity matrix or indirectly in association with IGFBP-3. Plasminogen appears not to be unique in its ability to bind IGF-II and IGFBPs, because von Willebrand factor, a multifunctional plasma and extracellular matrix protein, has been reported also to bind IGF-II and IGFBPs directly (34). We have not tested whether the 70–75-kDa vimentin is present in the eluate of the IGF-II affinity matrix.

The biosensor measurements showed, additionally, that transferrin, a low affinity IGF-II-binding protein (KD = 831–976 nM; Ref. 23 and Table II) associating with IGFBP-3 with a moderate binding constant (KD = 70 nM), reduced the overall binding constant KD of the plasminogen/transferrin mixture for IGF-II 7-fold, whereas the interaction between plasminogen and IGFBP-3 was not affected. As demonstrated previously, in the presence of transferrin the overall affinity of IGFBP-3 for IGF-II is improved – 5-fold (23). Furthermore, the cross-linkage experiments with selected recombinant proteins provided evidence that at least the simultaneous presence of plasminogen, IGFBP-3, and IGF-II is required to reconstitute a 180–200-kDa transferrin-containing complex. In comparison with the gel filtration data, however, the IGF-II matrix eluate obviously contains other co-factors promoting the formation of high molecular mass transferrin-containing protein complexes. At present, however, the physiological significance of the intermolecular modulatory effects and factors regulating the interactions of IGFs, IGFBPs, and their associated proteins in the circulation are unclear and remain to be investigated.

In this report, we have described for the first time the co-purification of an IGFBP-3 protease with IGFBP-3 from human plasma. This protease was shown to cleave also IGFBP-2, but not IGFBP-4. Inhibitor studies and IGFBP-3 zymography suggest that the 92-kDa serine-dependent protease, which can be blocked completely by α2-antiplasmin, might represent plasmin or a plasmin-like protease. Other findings, however, argue against 92-kDa IGFBP-3 protease being identical with plasmin. First, Western blot analysis of the most active proteolytic RP-HPLC fraction detected only the presence of the ~97-kDa plasminogen form, representing presumably the proteolytic inactive Glu-plasminogen, but failed to visualize the 84-kDa Lys-plasminogen or the 60- and 25-kDa heavy and light chains of plasmin, which have been reported to co-purify with IGFBP-3.
in equimolar amounts from human citrate plasma (16). However, we cannot exclude that low amounts of the active IGFBP-3 protease, not detectable by silver staining or immunoblotting, may be sufficient to cleave the [125I]IGFBP-3 tracer. Of note, the exact determination of molecular masses of purified proteins and the comparison with published data estimated e.g. in different gel systems (Tricine/SDS versus SDS) is rather difficult. Therefore, it is unknown whether the 100-kDa protein in the total eluate fraction (Fig. 1A) was partially processed during the RP-HPLC purification to the ~92-kDa protein or whether the electrophoretic mobility of the same polypeptide varies after dilution in ACN. Second, the conversion of the inactive plasminogen to plasmin involved in the degradation of IGFBP-1, -3, and -5 and fibrin in the activation of matrix metalloproteases (9, 11, 35, 36) is tightly regulated and requires the presence of fibrin at sites of tissue injury. Therefore, the secondary activation of plasmin from the inactive plasminogen precursor co-purified with IGFBP-3 appears to be unlikely. Note that plasminogen appears to be partially cleaved to the 84-kDa form during the gel filtration step (Fig. 9B).

In the protein fraction eluted from the IGF-II matrix, only small amounts of immunoreactive IGFBP-3 fragments could be detected. Therefore, it can be speculated that either inhibitors of the IGFBP-3 protease were also co-purified or that the IGFBP-3 was protected from degradation. The former assumption is supported by the detection of the nonrelated protease inhibitor antithrombin III in the eluate, which might contain other natural serine protease inhibitors. On the other hand, Holly and collaborators (37, 38) have provided evidence for the presence of components protecting IGFBP-3 in normal serum from proteolysis. Their data suggest that the accessibility of the C-terminal basic heparin-binding domain of IGFBP-3 is necessary for proteolysis or for binding of an inhibitor. Plasminogen, prekallikrein, or transferrin, all co-purified with IGFBP-3, might be candidates to bind to the C-terminal basic domain (13, 16, 22) and protect IGFBP-3 from proteolysis. It is not known whether the IGFBP protease binds to the IGF-II affinity matrix through its interaction with IGFBP-3, IGFBP-associated proteins, or inhibitor complexes.

In summary, this study has provided further evidence for the complexity of components modulating the IGF/IGFBP system in the circulation. In addition to the classical IGFBPs, plasminogen, as shown in this study, and the soluble IGF-II/M6PR (39, 40) belong to the family of high affinity binding proteins for IGF-II. Whereas plasminogen interacts also with a high affinity constant with IGFBP-3, other IGFBP-3-associated proteins such as transferrin might be important to facilitate the formation of high molecular weight IGFBP-3 complexes with altered IGF binding properties. Second, an active 92-kDa serine-dependent IGFBP-3 protease has been co-purified with IGFBP-3 and IGFBP-3-associated proteins and appears to be a component in a 440-kDa high molecular mass complex. It has been reported that in serum the complexed IGFBP-3 protease shows a strong aprotinin and a2-antiplasmin sensitivity (37). The identity of the IGFBP-3 protease in the IGF-II matrix eluate and the inhibitors involved in IGFBP-3 proteolysis and in regulation of the susceptibility of IGFBP-3 to proteolysis in serum remain to be elucidated.

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