

# Transferrin binds insulin-like growth factors and affects binding properties of insulin-like growth factor binding protein-3

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**Abstract** In the circulation, most of the insulin-like growth factors (IGFs) are bound to a ternary 150 kDa complex with IGF-binding protein (IGFBP)-3 and the acid labile subunit. In the current study, we identify transferrin (Tf) by mass spectrometry, and immunoprecipitation as a component of a major IGF-binding fraction separated from human plasma. IGF ligand blotting, cross-linkage experiments and surface plasmon resonance spectrometry have been used to demonstrate the capability of Tf to bind IGFs specifically. In combination with Tf, IGFBP-3 showed a 5-fold higher affinity for IGF-II than IGFBP-3 alone. The data suggest that Tf may play an important role in regulating IGF/IGFBP-3 functions. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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Insulin-like growth factor-binding protein-3;  
Transferrin; Surface plasmon resonance spectroscopy

## 1. Introduction

The insulin-like growth factors (IGF-I and IGF-II) form complexes with six high-affinity IGF-binding proteins (IGFBPs) which modulate their activities [1]. IGFBP-3, a ~43/45-kDa glycoprotein, is most abundant in serum and sequesters both IGFs into ternary 150-kDa complexes with the 85-kDa acid-labile subunit (ALS). The 150-kDa complexes function as circulating reservoirs of IGFs, from which they are released either by proteolysis of IGFBP-3 or interaction of the ternary complex with cell-associated glycosaminoglycans [2]. Beside ALS, several other plasma proteins have recently been reported to interact specifically with IGFBP-3 and affect its binding properties. A disintegrin and metalloprotease-12 was found to bind and cleave IGFBP-3 in serum [3]. Other plasma proteins of 70, 100, and 150 kDa bind to IGFBP-3 [4] or form IGFBP-3-containing complexes with a

molecular mass above 200 kDa [5]. Furthermore, the inactive protease precursor plasminogen, and fibrin/fibrinogen bind to the basic C-terminal region of IGFBP-3 [6,7].

In this study, we report the identification of transferrin (Tf) as an IGFBP that was detected together with IGFBP-3 in a plasma fraction purified by affinity chromatography and reverse-phase high performance liquid chromatography (RP HPLC). The surface plasmon resonance technique was used to define the possible role of Tf in the IGF system.

## 2. Materials and methods

### 2.1. Reagents

IGF-I and IGF-II were purchased from GroPep, Adelaide, Australia. Glycosylated rhIGFBP-3 was kindly provided by Dr. A. Sommer (Celtrix, Santa Clara, CA, USA). Tf and protein-A agarose came from Sigma. Rabbit antiserum against rhIGFBP-3 was obtained from UBI (Lake Placid, NY, USA) and the polyclonal antibody against human Tf came from DAKO (Hamburg, Germany). IGF-I and IGF-II were iodinated by the chloramine T-method [8].

### 2.2. Purification and identification of new serum IGFBPs

Cohn fraction IV of human plasma was fractionated as described previously [9]. In brief, the purification scheme comprised the following steps: (i) suspension of Cohn fraction IV in 2 M acetic acid, pH 3.0, 0.075 M NaCl; (ii) removal of IGF-I and IGF-II with SP-Sephadex C-25 using a batch procedure; (iii) affinity chromatography over an IGF-II Sepharose 4B column at neutral pH and desorption of bound proteins with 0.5 M ammonium acetate, pH 2.8; (iv) HPLC chromatography over a Pro RPC 5/10 column (all chromatography media from Pharmacia, Freiburg, Germany) in 0.1 trifluoroacetic acid with a gradient from 0 to 44% acetonitrile (ACN). Fractions of 0.5 ml were collected and assayed for IGF-binding using a competitive binding protein assay [9] with [<sup>125</sup>I]IGF-I as tracer and for IGFBP-1, -2, and -3 using specific RIAs. The bulk of IGFBP-3 eluted at an ANC concentration between 20 and 21.5%. The highest peak of IGF-binding appeared at 27.7% ACN. It was further analyzed by SDS-PAGE, [<sup>125</sup>I]IGF-II ligand blotting [10], and silver staining. On Coomassie blue-stained protein bands a fingerprint analysis was performed: cleavage by trypsin, extraction of the resulting peptides from the gel, detection by mass spectrometry (Voyager-DE<sup>®</sup> PRO, Applied Biosystems, Foster City, CA, USA) and database search in SwissProt.010501 with the program MS-Fit/ProteinProspector (<http://pro.spectrosc.ucsf.edu>). Proteins of the main HPLC fraction were labeled with Na<sup>125</sup>I (Amersham Pharmacia Biotech, Freiburg, Germany) by IODOGEN (Pierce, Rockford, IL, USA).

### 2.3. Cross-linkage analysis

100 ng of holo-Tf (Sigma) dissolved in 35 µl of 20 mM NaPi buffer, pH 7.0, containing 20 mM NaCl were incubated with [<sup>125</sup>I]IGF-I or [<sup>125</sup>I]IGF-II (150 000 cpm) for 6–16 h at 4 °C. Disuccinimidyl suberate

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**Abbreviations:** IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; Tf, transferrin; ALS, acid-labile subunit; HPLC, high performance liquid chromatography; AD, activation domain

(Pierce) in dimethyl sulfoxide was added to a final concentration of 0.5 mM and further incubated for 15 min on ice. The reaction was stopped by addition of 5  $\mu$ l of 0.8 M Tris-HCl, pH 7.0, containing 8 mM EDTA. The samples were boiled in SDS-sample buffer and analyzed by SDS-PAGE (10% acrylamide) and autoradiography.

#### 2.4. Immunoprecipitation

Aliquots of the  $^{125}$ I-labeled 60-kDa fraction (100 000 cpm) in 60 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 5 mM iodoacetic acid (buffer A), were incubated either with anti-human Tf or IGFBP-3 antibodies for 3 h at 4°C. The immunocomplexes were precipitated by protein-A agarose, washed three times in buffer A, solubilized and analyzed by SDS-PAGE under non-reducing conditions.

#### 2.5. Yeast two-hybrid analysis

The full length human IGF-II cDNA was kindly provided by Dr. W. Rutter (San Francisco, CA, USA). The cDNAs encoding the prepro-IGF-II form of aa 1–180 and the mature (m) IGF-II form of aa 25–92 were amplified by PCR using primers IGF-II-F: 5'-GG-AATTCCGGATGGGAATCCCAATGGGG-3', IGF-II-R: 5'-CGG-GATCCCGTCACTTCCGATTGCTGGC-3', and mIGF-II-F: 5'-G-GAATTCCGGGCTTACCGCCCCAGTGAG-3', mIGF-II-R: 5'-C-GGGATCCCGTCACTCGGACTTGGCGGGGGT-3', respectively. The PCR products were cleaved subsequently with *Eco*RI and *Bam*HI and subcloned in frame with the Gal4-DNA-binding domain of vector pAS2-1 (Clontech). The human Tf cDNA (kindly provided by Dr. C. Körner, Göttingen, Germany) encoding aa 20–698 was amplified with primers TRF-F: 5'-GAAGATCTTCGTCCTGATAAACTGTG-3' and TRF-R: 5'-GAAGATCTTCTTAAGGTCTACGGAAAGT-3'. PCR products were cleaved by *Bgl*II and cloned in frame with the Gal4-activation domain (AD) into *Bam*HI restriction sites of vector pACT2 (Clontech). The two-hybrid analysis was carried out as described [11].

#### 2.6. Surface plasmon resonance interaction analysis

Surface plasmon resonance interaction analysis was carried out as described previously [10,12] using IGF-II-coupled CM5 sensor chips in a BIAcore 2000 biosensor (Amersham Pharmacia Biotech, Freiburg, Germany). Because of the high affinity of IGF-BPs for IGF-II and in order to minimize the influence of analyte re-binding during the dissociation period, flow rates ranging from 20 to 35  $\mu$ l/min were used for all interaction measurements. The kinetic rate constants were calculated exactly as described previously [12].

### 3. Results

Fractionation of human plasma Cohn fraction IV by affinity chromatography and subsequent RP HPLC yielded a major IGF-binding component that contained only small

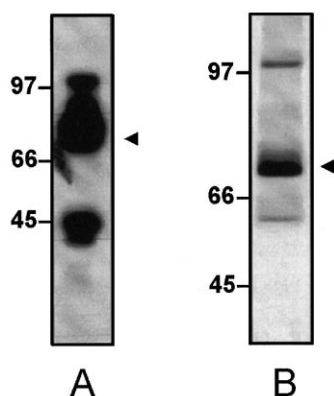


Fig. 1. IGF-II binding and protein composition of the serum fraction isolated by RP HPLC (27% ACN). 2  $\mu$ g of the fraction were separated on SDS-PAGE followed by [ $^{125}$ I]IGF-II ligand blotting (A), or visualized by silver staining (B). The position of the molecular mass marker and of Tf ( $\blacktriangle$ ) identified by mass spectrometry are indicated.

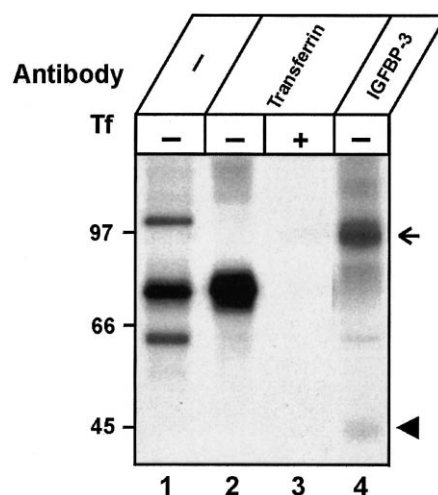


Fig. 2. Immunoprecipitation of Tf. Aliquots of the  $^{125}$ I-labeled major IGF-binding fraction from RP HPLC (100 000 cpm) were either directly solubilized (15 000 cpm; lane 1) or incubated with antibodies against Tf (lanes 2 and 3) or IGFBP-3 (lane 4). As indicated, 10  $\mu$ g non-labeled Tf was added. The precipitated proteins were analyzed by SDS-PAGE and autoradiography. The positions of the molecular mass marker proteins are indicated. ( $\blacktriangle$ ) IGFBP-3; ( $\blacktriangleleft$ ) IGFBP-3 dimer.

amounts of immunoreactive IGFBP-3, which could not account for the total IGF-binding capacity. Three major binding proteins of 100, 73, and a doublet of 45/43 kDa were detected in this fraction by IGF-II ligand blotting (Fig. 1A). The latter was identified as IGFBP-3 by immunoblotting (not shown). Analysis of the proteins of this fraction by SDS-PAGE and silver staining revealed three prominent bands at 100, 73, and 64 kDa (Fig. 1B). Fingerprint analysis identified the 73-kDa protein as Tf. When the  $^{125}$ I-labeled proteins of this fraction were incubated with antibodies against human Tf, the 73-kDa component was immunoprecipitated (Fig. 2). The precipitation of the  $^{125}$ I-labeled 73-kDa protein was inhibited by an excess of non-labeled Tf. Antibodies against human IGFBP-3 precipitated four proteins of 45, 64, 80, and 92 kDa from the  $^{125}$ I-labeled fraction (Fig. 2). The absence of the  $^{125}$ I-labeled 92-kDa protein in the starting material and the reduction of intensities of the 45-, and 92-kDa bands by the addition of unlabeled IGFBP-3 during precipitation suggest that the  $^{125}$ I-labeled 92-kDa protein represents an IGFBP-3 dimer.

The capability of Tf to bind IGFs was tested with different approaches. Cross-linking of [ $^{125}$ I]IGF-I or -IGF-II incubated with commercially available holo-Tf resulted in a labeled protein of approximately 80 kDa (Fig. 3A,B), which disappeared in the presence of an excess of the respective unlabeled IGF. Using the yeast two-hybrid system, no interaction between Tf and the mature or prepro-IGF-II was detected (not shown), indicating a low binding affinity. To determine the precise rate constants ( $k_a$ ,  $k_d$ , and  $K_D$ ) for the interaction between Tf and IGF-II, surface plasmon resonance spectroscopy was carried out with IGF-II immobilized on a sensor surface. When Tf was passed over the surface, a low-affinity binding ( $K_D = 831$  nM) of Tf to IGF-II was measured. For comparison, under the conditions used, a  $K_D$  value of 4.4 nM was estimated for the binding of IGFBP-3 to the IGF-II surface (Fig. 3C). Surprisingly, the affinity of IGFBP-3 in combination with Tf was

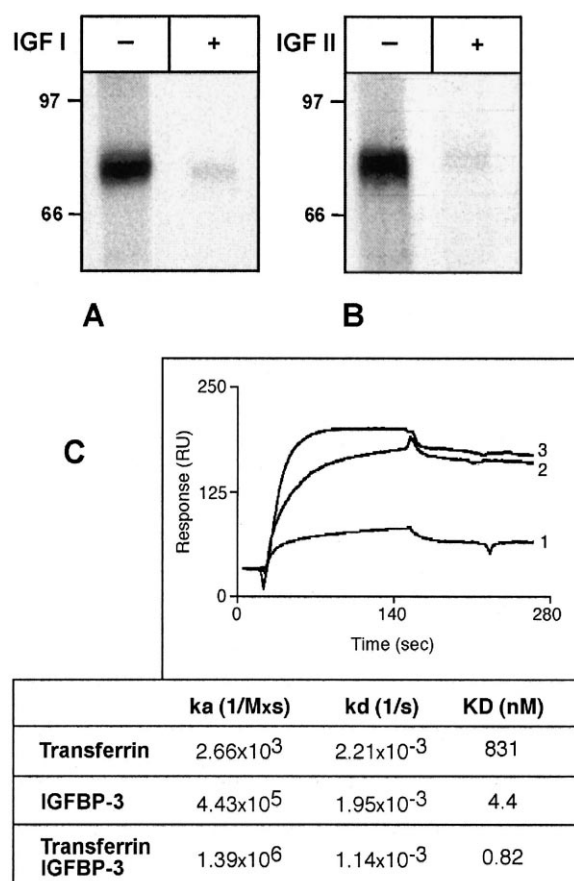


Fig. 3. Binding of Tf and IGFBP-3 to IGFs. A: Tf was incubated with [ $^{125}$ I]IGF I or (B) [ $^{125}$ I]IGF II in the absence (–) and presence (+) of unlabeled IGFs (3  $\mu$ M), cross-linked by the addition of 0.5 mM disuccinimidyl suberate and analyzed by SDS-PAGE and autoradiography. C: Tf (1), IGFBP-3 (2), or Tf+IGFBP-3 (3) were passed over IGF-II-coated chip surfaces for 2 min (association) before the chip surface was washed with buffer for 2 min (dissociation). The curves that are shown represent the sum of at least six binding cycles. The rate constants ( $k_a$  and  $k_d$ ), and the equilibrium constant  $K_D$  were calculated as described [12].

approximately five-fold higher for IGF-II ( $K_D = 0.8$  nM) than for IGFBP-3 alone.

#### 4. Discussion

Fractionation of human plasma Cohn fraction IV by the described procedure revealed the presence of various IGFBPs whose binding activities could not explained by the presence of immunoreactive classical IGFBPs. Tf was identified as a major component in the main IGF-binding fraction (eluted from the RP HPLC column at 27% ACN). Whereas two other proteins of 100 and 64 kDa in this fraction were also visualized by silver staining, IGFBP-3 was only detected by immuno- or IGF ligand blotting. However, the IGF-II ligand blot revealed that the 73- and 100-kDa but not the 64-kDa polypeptide bind IGF-II. The capability of Tf to bind IGF-I and IGF-II was confirmed by cross-linking experiments. In the course of the present study, Cohen and collaborators identified Tf as one of three proteins of 70, 100, and 150 kDa in human serum which bind IGFBP-3 with high affinity [4,13]. Whether the 100-kDa IGFBP-3-associated protein is also identical with the 100-kDa IGFBP found in this study re-

mains to be examined. The two-hybrid system was used to test the ability of Tf to bind IGF-II in yeast cells. Both co-transformation of the full length IGF-II cDNA fused to the Gal4-BD and the mature IGF-II fusion gene together with the Tf-Gal4-AD fusion gene failed to show any interactions. These data suggest a low binding affinity between the two polypeptides. Indeed, biosensor kinetic data revealed that the affinity of Tf for IGF-II was approximately 200-fold lower than that of IGFBP-3. It should be noted, that Wong et al. [14] using the same biosensor technique to measure the binding affinity constants of IGFBP-3 to IGF-II, calculated a  $K_D$  value of 23 pM. This higher affinity value is mainly due to differences in the dissociation rate with which IGFBP-3 elutes from the sensor surface. In contrast to Wong et al., a three-times higher flow rate was used in the present study to minimize the effect of re-binding of IGFBP-3 to the surface during the dissociation period. Although there may be discrepancies in the absolute kinetic rate constants under different experimental conditions, the surface plasmon resonance measurements are suitable for comparison of the interactions of Tf and IGFBP-3 with IGF-II. Thus, Tf may be classified as a new member of the IGFBP superfamily. However, except for the presence of three of the first four conserved cysteine residues occurring in the N-terminal domain of IGFBP-1 to -6 or of the IGFBP related proteins, which have been shown to have at least 100-fold lower affinities for IGFs [15], no significant structural similarities were found in Tf. Furthermore, Tf lacks conservation of the 'GCGCCXXC'-motif. Therefore, Tf, in addition to its function as the  $Fe^{3+}$ -carrier protein in serum [16], appears to be unique in its ability to bind IGFs and to interact with IGFBP-3. On the other hand, the physiological role of Tf in the IGF/IGFBP system is still unclear. Beside the fact that Tf is required for cell proliferation, survival and differentiation [17,18], it abolishes IGFBP-3-induced cell proliferation and apoptosis in different cell lines [13]. On the other hand, the  $Fe^{3+}$ -Tf complex might facilitate the transport of IGFs across the capillary wall by receptor-mediated transcytosis [19].

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