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Isolation of complexes formed between insulin-like growth factor-binding protein-3 and transferrin from human serum

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Abstract: Insulin-like growth factors (IGFs) play an important role in the regulation of cell growth, differentiation and metabolism. The amount of free, biologically active IGFs is regulated by the IGF-binding proteins (IGFBPs). IGFBP-3 is the most abundant binding protein and it is known to interact with other circulating proteins, including transferrin (Tf). In order to elucidate the possible role of IGF/IGFBP-3 in the metabolism of iron, it is necessary to isolate IGFBP-3/Tf complexes. Several affinity-based techniques were employed. The results showed that only the double immunoprecipitation method with anti-Tf and anti-IGFBP-3 antibodies selectively separated the complexes from other molecular forms, such as monomers, oligomers or fragments of IGFBP-3 and Tf. The isolated complexes can now be used to investigate the relationship between IGF/IGFBP-3 and iron, both in structural and metabolic terms.

Keywords: IGFBP-3; transferrin; complex; isolation.

INTRODUCTION

Insulin-like growth factors (IGF-I and IGF-II), together with six IGF-binding proteins (IGFBP-1 to IGFBP-6), two IGF receptors (IGF-1R and IGF-2R) and insulin and its receptor (IR), comprise a complex system that plays an important role in the regulation of cell growth, differentiation and metabolism.[1,2] The pivotal role in the regulation of the IGF action have the IGFBPs. The most abundant in circulation is IGFBP-3.[3] It forms ternary complexes (150 kea) with IGF and an acid labile subunit (ALES).[4] The ternary complexes cannot cross the vascular barrier and they are assumed to be an IGF reservoir, releasing free, biologically active IGF upon mitogenic and/or metabolic request of the organism. IGFBP-3 is, in its native form, a glycoprotein (40–45 kDa), synthesized in the liver parenchymal cells.[5] Besides its ability to bind and regulate the activity of IGFs, it was

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also reported that IGFBP-3 has IGF-independent actions, *via* nuclear IGFBP-3 receptors,[6] and it may bind to other molecules, of which transferring is one.[7]

Transferrin (Tf) is a glycoprotein (70–80 kDa) functioning as the main iron transporter.[8] Regulation of the iron distribution in the organism affects the formation of hemoglobin, the functionality of enzymes (having iron as a cofactor) and redox processes.[9]

Since the IGF/IGFBP-3 axis promotes anabolic reactions, it would be important to investigate its possible role in iron metabolism and the significance of the formation of the IGFBP-3/Tf complex. In order to do so, the first step is to isolate the complexes from other molecular forms of either IGFBP-3 or Tf, and other blood constituents. In this work several approaches based on affinity interactions were tested.

EXPERIMENTAL

Serum samples

Serum samples were obtained from healthy individuals. All samples were taken from venous blood 12 h after fasting. The sera were kept frozen at –20 °C until used. This study was approved by the local ethical committee in the Institute for the Application of Nuclear Energy.

Immobilised metal-affinity chromatography (IMAC)

Iminodiacetic acid–agarose (IDA) was purchased from Sigma–Aldrich (Steinheim, Germany). A column was packed with 1 mL of gel and washed with distilled water. In order to saturate the column with ferric ions, 1.0 mL of 0.10 mol L^{-1} ferric chloride solution was applied. Unbound ions were washed with 5 mL of distilled water and 5 mL of 0.1 % acetic acid. Equilibration of the column was realized with dilution buffer: 0.05 mol L^{-1} MES (2-(*N*-morpholino)ethanesulphonic acid)/0.5 mol L^{-1} NaCl buffer pH 5.5.[10] Samples (100 µL of sera diluted in 900 µL of dilution buffer) were applied to column and circulated for 1 h to ensure maximal binding. Unbound material was washed out with 5 mL of MES buffer. Elution of the bound molecules was performed using 8 mL of 0.2 mol L^{-1} phosphate buffer (PB) pH 8.0. The column was regenerated using 10 mL of 0.1 mol L^{-1} borate/1.0 mol L^{-1} NaCl buffer pH 10.0 and 10 mL of distilled water, followed by saturation with ferric chloride. The collected fractions were analysed by electrophoresis and immunoblotting.

Immunoaffinity chromatography (IgY-C)

An IgY-12 column (1.2 mL of microbeads) was purchased from Beckman Coulter (Fullerton, USA, Proteomelab IgY-12 High Capacity Proteome Partitioning Kit). According to the manufacturer's description, the matrix with immobilised antibodies enables the binding of the 12 most abundant proteins from human plasma: albumin, IgG, IgA, IgM, α1-antitrypsin, haptoglobin, fibrinogen, orosomucoid, α_2M, HDL (apo A-I and apo A-II) and Tf. Samples were prepared by diluting 20 µL of sera with 480 µL of dilution buffer: 10 mmol L^{-1} Tris (tris(hydroxymethyl)aminomethane)–HCl/1.0 mol L^{-1} NaCl buffer pH 7.4, and incubated for 15 min at room temperature using a rotator.[11] Separation of the unbound proteins was performed by centrifugation for 30 s at 2000×*g*, followed by washing the column three times with dilution buffer. The bound proteins were eluted with 500 µL of elution buffer: 0.1 mol L^{-1} glycine–HCl, pH 2.5, for 3 min at room temperature using a rotator, separated from gel by centrifugation and immediately neutralised with 50 µL of 1 mol L^{-1} Tris–HCl buffer pH 8.0.

The elution was repeated three times. The column was neutralised with 500 μL of 2 mol L^{-1} Tris–HCl buffer pH 8.0 and washed with dilution buffer prior to the next chromatographic cycle. The collected fractions were analysed by electrophoresis and immunoblotting.

Lectin affinity chromatography (LAC)

Eleven agarose-immobilised lectins, purchased from Vector Laboratories (Burlingame, CA, USA), were packed in columns: SNA (*Sambucus nigra* agglutinin), LCA (*Lens culinaris* agglutinin), Con A (lectin from *Canavalia ensiformis*), PHA-E (*Phaseolus vulgaris* erythro-agglutinin), PHA-L (*Phaseolus vulgaris* leukoagglutinin), RCA-I (*Ricinus communis* agglutinin I), WGA (wheat germ agglutinin), succinylated WGA, ECL (*Erythrina cristagalli* lectin), MAL (*Maackia amurensis* lectin) and UEA (*Ulex europaeus* agglutinin). All buffers and hapten sugars were prepared following procedures recommended by the producer. The exact composition of each buffer was published elsewhere.[12] Serum samples (100 μL) diluted in 900 μL of the corresponding buffers were circulated through columns for 1 h. Unbound material was washed out with 20 mL of the dilution buffers. The elution of bound glycoproteins was performed with 7 mL of hapten sugar (0.2–0.5 mol L^{-1}, as suggested by the producer) in 0.1 M acetic acid, pH 3.0. The PHA-E and PHA-L columns were eluted using a 0.1 mol L^{-1} acetic acid solution. The collected fractions were immediately neutralised using 2 mol L^{-1} Tris–HCl buffer pH 8.9, dialysed against distilled water overnight at 4 °C and analysed by electrophoresis and immunoblotting.

Immunoelectrophoresis (IEP)

Immunoelectrophoresis (IEP) was performed in a standard manner.[13] Serum samples (5 μL) were applied in sample wells, and antibodies in channels (100 μL): polyclonal anti-Tf antibodies (INEP, Belgrade, Serbia), and polyclonal anti-IGFBP3 antibodies (DSL, Webster, TX, USA). The samples were incubated with antibodies in a humid chamber for 24 h at room temperature, to allow immunoprecipitation to occur.

Co-immunoprecipitation (Co-IP)

Immunoprecipitation of protein complexes was performed using Pierce® Co-Immunoprecipitation Kit (Pierce Biotechnology, Rockford, IL, USA). Following the manufacturer's instructions, AminoLink®Plus Coupling Resin (50 μL of 50 % slurry) was loaded into spin columns and 5 μg of anti-IGFBP-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 10 μg of anti-Tf antibodies (INEP, Belgrade, Serbia) were immobilised. Serum samples (10 μL) were diluted in 190 μL of the buffer: 2.5 mmol L^{-1} Tris/0.15 mol L^{-1} NaCl/1.0 mmol L^{-1} EDTA/1% NP-40/5 % glycerol, pH 7.4 and incubated overnight at 4 °C with a resin having immobilised anti-Tf antibodies. Separation of the unbound proteins was performed by centrifugation for 30 s at 2000×*g*, followed by washing the column three times with dilution buffer. The bound proteins were eluted with 110 μL of the elution buffer pH 2.8 (provided within the Kit), separated by centrifugation and immediately neutralised with 2 μL of 2 mol L^{-1} Tris–HCl buffer, pH 8.9. Specifically eluted anti-Tf immunoreactive proteins were further incubated with a resin having immobilised anti-IGFBP-3 antibodies, overnight at 4 °C. The removal of the unbound and the elution of the bound proteins followed the same procedure as previously. The collected fractions were analysed by electrophoresis, dot and immunoblotting.

Electrophoresis, dot and Western immunoblotting (WIB)

Protein fractions obtained by IMAC, IgY-C, LAC, and Co-IP were subjected to denaturing non-reducing sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS––PAGE) using 10 % gels.[14] The separated proteins were transferred to a nitrocellulose membrane and stained with Ponceau S.

The WIB procedure was performed using goat polyclonal anti-IGFBP-3 (DSL, Webster, TX, USA) and sheep polyclonal anti-Tf (INEP, Belgrade, Serbia) primary antibodies, followed by anti-goat secondary antibodies coupled to horseradish peroxidase (Biosource, Camarillo, CA, USA). The immunoreactive proteins were visualised with an enhanced chemiluminescence (ECL) reagent kit (Pierce, Minneapolis, MN, USA).[14] Molecular mass markers were obtained from BioRad Laboratories (Hemel Hempstead, UK). Recombinant IGFBP-3 (29 kDa, DSL, Webster, TX, USA) and Tf (INEP, Belgrade, Serbia) were used as protein markers.

The presence of IGFBP-3/Tf complexes was also confirmed by dot blotting, employing anti-IGFBP-3 and anti-Tf antibodies.

Ligand-binding assay

An in-house made matrix, CNBr-activated Sepharose 4B,[15,16] was used to immobilise sheep anti-Tf antibodies (INEP, Belgrade, Serbia). This immunoaffinity resin was employed to test the ability of IGFBP-3 complexed to Tf to bind the ligand.

Serum samples (100 μL) were incubated with ^{125}I-labelled IGF-II (^{125}I-IGF-II, 300 000 cpm) at 4 °C overnight, loaded onto a column packed with immunoaffinity resin and recirculated for 1 h. The column was left with the sample at 4 °C overnight and the next day, the sample was recirculated for a further 1 h. The unbound proteins were washed out with the dilution buffer: 0.05 mol L^{-1} PBS/0.15 mol L^{-1} NaCl, pH 7.2, and elution of the bound fraction was performed using the elution buffer: 0.1 mol L^{-1} glycine/HCl, pH 3.7. Fractions were immediately neutralised with 2 mol L^{-1} Tris–HCl buffer pH 8.9. The radioactivity and the A_{280} of the collected fractions were measured.

Ultrafiltration

Protein fractions containing IGFBP-3/Tf complexes were subjected to ultrafiltration, in order to concentrate samples and remove small proteins. Microcon YM-50 filters were used (Millipore Corporation, Billerica, USA).

RESULTS AND DISCUSSION

In order to isolate IGFBP-3/Tf complexes from human serum, several affinity-based techniques were employed. As IGFBP-3 and Tf are glycosylated[17,18] and IGFBP-3 is also phosphorylated,[3] methods based on the following interactions were applied: metal ion–amino acid or phosphate residue,[19] lectin–saccharide moiety[20–22] and antigen–antibody.[23]

In the IMAC method, all IGFBP-3 (Fig. 1A) and Tf (Fig. 1B) immunoreactive species were bound to the matrix: native IGFBP-3 (doublet at ≈45 kDa), an IGFBP-3 fragment (≈30 kDa),[24] oligomeric/complexed forms of IGFBP-3 (>80 kDa), as well as Tf (70–80 kDa) and complexes of Tf (>100 kDa).

In the IgY-C method,[25] the immobilised antibodies recognised and bound complexes of IGFBP-3 (>100 kDa), whereas native and fragmented IGFBP-3 species remained among the unbound proteins (Fig. 2A). There were also IGFBP-3 immunoreactive bands at ≈45 kDa and ≈70 kDa, which might have originated from dissociated complexes. Immunoblotting with anti-Tf antibodies (Fig. 2B) indicated binding of native Tf, a Tf fragment (≈40 kDa)[26] and Tf complexes. In the IgY-unbound fraction, anti-Tf antibodies recognised a protein at ≈70 kDa (Fig. 2B). It is known that Tf exists in several glycoforms[27] and the results of this

experiment suggested that the antibodies immobilised on the IgY–C matrix were unable to bind the Tf isoform that was less glycosylated.

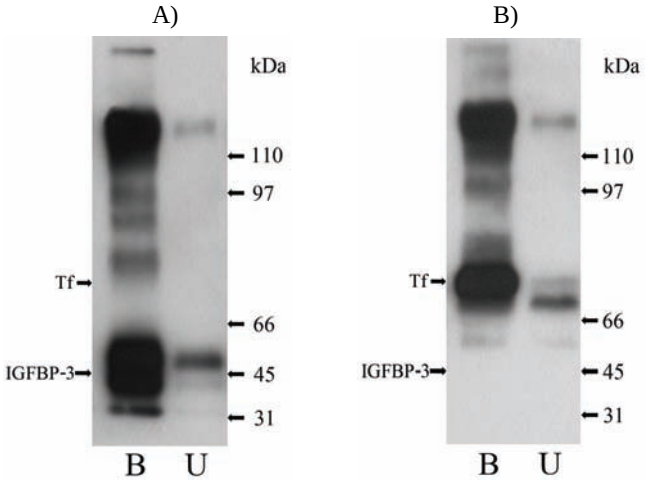


Fig. 1. Immunoblotting of the bound (B) and unbound (U) fractions of serum samples obtained after IMAC with A) anti-IGFBP-3 and B) anti-Tf antibodies. The positions of the IGFBP-3 monomer and Tf are indicated on the left side, whereas the positions of the molecular mass markers are indicated on the right side.

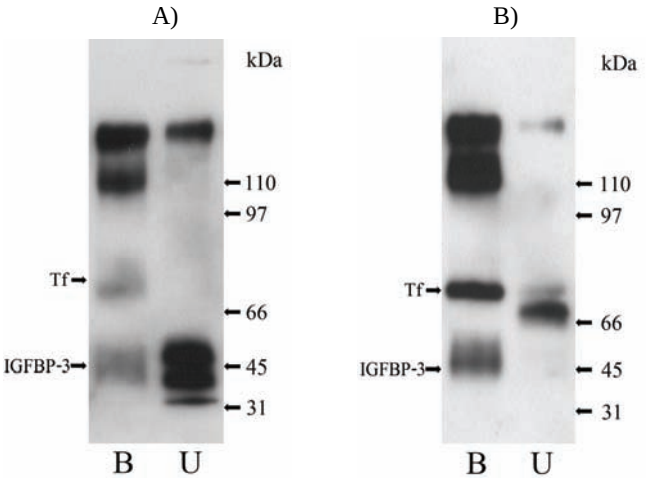


Fig. 2. Immunoblotting of the bound (B) and unbound (U) fractions of serum samples obtained after IgY-C with A) anti-IGFBP-3 and B) anti-Tf antibodies. The positions of the IGFBP-3 monomer and Tf are indicated on the left side, whereas the positions of the molecular mass markers are indicated on the right side.

Lectins are established as an important analytical tool in glycoproteomics. As both IGFBP-3 and Tf are glycoproteins, the LAC method was the next me-

thod of choice. The lectins used in this experiment were those that are known to interact with serum glycoproteins.[28,29] Of the eleven lectins that were employed in the LAC method, only six (PHA-E, RCA-I, Con A, WGA, SNA and LCA) bound native IGFBP-3 at ≈45 kDa (Fig. 3A) and five of them (except LCA) bound Tf at ≈80 kDa (Fig. 3B). Beside monomer forms (and in some cases fragments), higher molecular mass proteins that were both IGFBP-3 and Tf immunoreactive were not detected (except at the start). The tested lectins were, therefore, unable to bind IGFBP-3/Tf complexes.

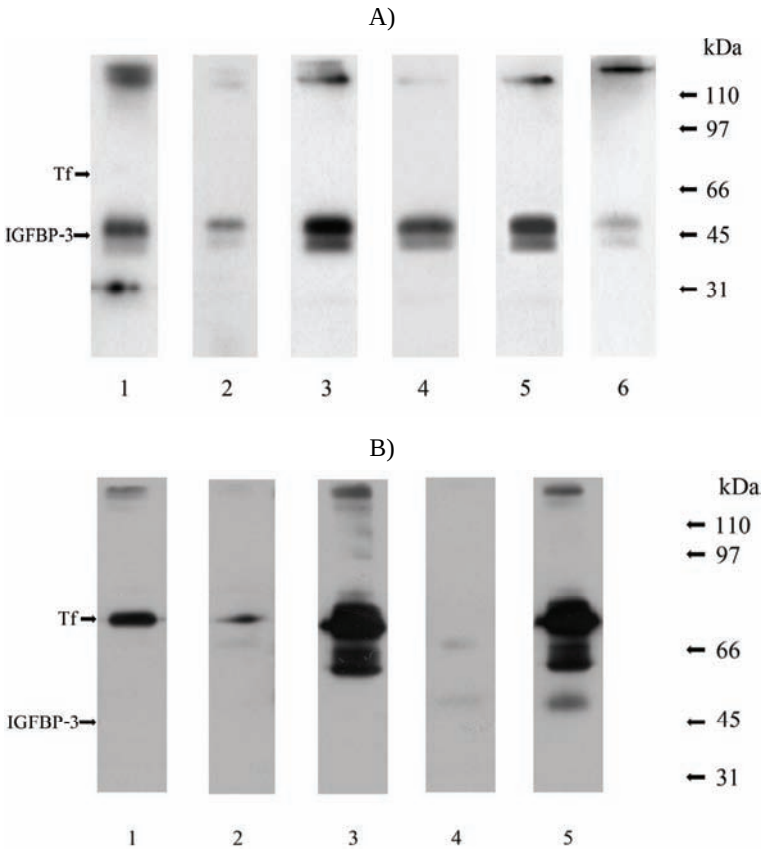
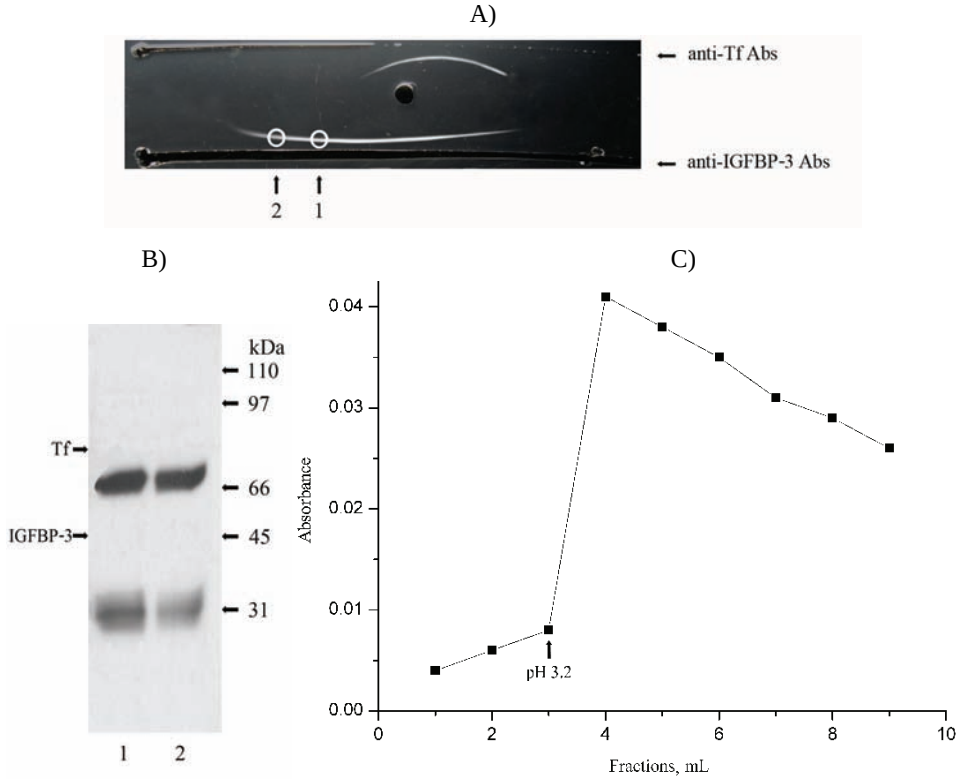


Fig. 3. Immunoblotting of the bound fractions of serum samples obtained after LAC with A) anti-IGFBP-3 and B) anti-Tf antibodies. The presented samples were eluted from: PHA-E-agarose (lane 1), RCA-I-agarose (lane 2), Con A-agarose (lane 3), WGA-agarose (lane 4), SNA-agarose (lane 5) and LCA-agarose (lane 6). The positions of the IGFBP-3 monomer and Tf are indicated on the left side, whereas the positions of the molecular mass markers are indicated on the right side.

To summarise the above results, none of the applied affinity-chromatography methods was able to selectively recognise IGFBP-3/Tf complexes and sepa-

rate them from the monomeric, oligomeric and fragmented forms of IGFBP-3 and Tf. As IgY-C seemed the most promising tool, other immunochemical techniques were tested for the isolation of the complexes: immunoelectrophoresis, preparative immunoaffinity chromatography and the immunoprecipitation method.[30]

In the IEP method, the idea was to analyse immunoprecipitated proteins. The IGFBP-3/Tf complexes were expected to travel at a rate different to those of their subunits. Since the concentration of Tf in serum[31] is much greater than the concentration of IGFBP-3,[32] the search for complexes was performed in the region where free Tf was not expected to be found, but where the reaction with anti-IGFBP-3 antibodies was positive. Two samples (Fig. 4A, spots 1 and 2), from the immunoprecipitating line formed by the interaction of serum with anti-IGFBP-3 antibodies were analysed. Immunoblotting with anti-IGFBP-3 antibodies revealed that an IGFBP-3 fragment and, probably, a dimer (≈70 kDa), were the only immunoreactive species in this part of the immunoprecipitated line (Fig. 4B). No band at the expected molecular mass of the IGFBP-3/Tf complexes (≈110 kDa) was observed.



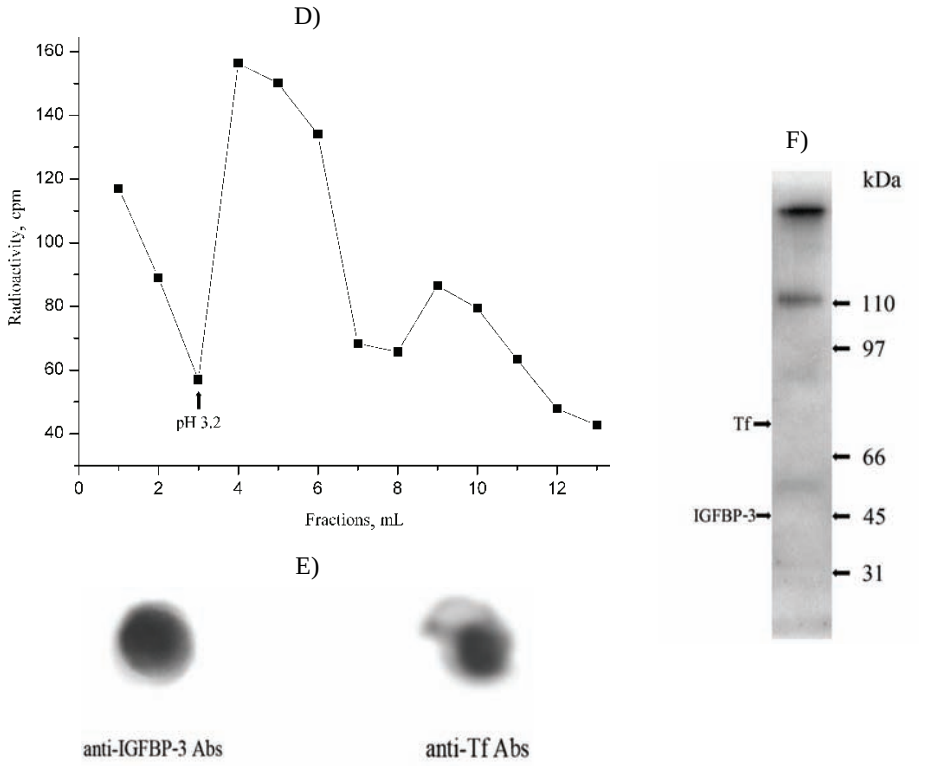


Fig. 4. A) IEP of a serum sample, with applied antibodies indicated on the right side; selected spots from the immunoprecipitating line (1 and 2) were subjected to B) immunoblotting with anti-IGFBP-3 antibodies; C) in preparative immunoaffinity chromatography, the amount of the eluted proteins (A_{280}) was measured. D) In preparative immunoaffinity chromatography, radioactivity of the eluted ligand-binding IGFBP-3/Tf complexes (cpm) was measured; E) dot blot and F) Western immunoblotting with anti-IGFBP-3 and anti-Tf antibodies of the isolated IGFBP-3/Tf complexes after co-immunoprecipitation. The positions of the IGFBP-3 monomer and Tf are indicated on the left side, whereas the positions of the molecular mass markers are indicated on the right side.

Preparative immunoaffinity chromatography is widely appreciated for the isolation and purification of immunoreactive species.[33] Since the commercial IgY matrix was able to bind IGFBP-3/Tf complexes, a matrix for the isolation of the anti-Tf antibody reactive molecules was prepared in-house, using CNBr activated resin.[34] The antibodies were coupled directly. The amount of bound immunoreactive proteins was, unfortunately, very small (Fig. 4C, A_{280}), probably due to an improper orientation of the antibodies. The yield of the bound proteins could possibly be improved by using spacer molecules, such as carbodiimide,[34] or protein A[35] in order to immobilise the antibodies *via* their C-terminal ends and leave their N-terminal, antigen-binding sites, free.[36] The same experiment was re-

peated with serum samples pre-incubated with ^{125}I-IGF-II. Radioactivity measurement (Fig. 4D, cpm) demonstrated that a certain amount of ^{125}I-IGF-II was bound to the immobilised anti-Tf antibodies, suggesting that IGFBP-3 in the IGFBP-3/Tf complexes retains its ability to bind ligand.

Finally, a co-immunoprecipitation method was employed, using a commercial activated resin to immobilise separately anti-IGFBP-3 and anti-Tf antibodies. The immunoprecipitation method is among the most specific techniques to isolate a target protein with the least amount of contaminating substances. In this study, a double immunoprecipitation was performed. Samples were allowed to interact with the immobilised anti-Tf antibodies in the first step and, after elution of the bound proteins, this fraction was allowed to interact with anti-IGFBP-3 antibodies. Due to the high affinity of antigen–antibody interactions, the elution necessitated the employment of rather harsh experimental conditions (pH 2.8). In order to test whether the IGFBP-3/Tf complexes had survived such conditions, their existence was investigated by dot blotting (Fig. 4E). The result demonstrated that complexes mostly resisted dissociation. The proteins that were eluted after the second step contained almost exclusively IGFBP-3/Tf complexes (Fig. 4F). Fragments (≈30 kDa) were present in minor amounts and they were completely removed by ultrafiltration.

CONCLUSION

Isolation of the IGFBP-3/Tf complexes from human serum was achieved by using an immunoaffinity method, whereas metal- and lectin-affinity techniques were unsuccessful. Among several immunochemical approaches, only the double immunoprecipitation method selectively separated the complexes from other molecular forms, such as monomers, oligomers or fragments of IGFBP-3 and Tf. A further study of the relationship between IGFBP-3, Tf and Tf saturation with iron is expected to elucidate the possible role of IGF/IGFBP-3 in iron metabolism.

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И З В О Д

ИЗОЛОВАЊЕ КОМПЛЕКСА ФОРМИРАНОГ ИЗМЕЂУ ВЕЗУЈУЋЕГ ПРОТЕИНА ЗА ИНСУЛИНУ СЛИЧНЕ ФАКТОРЕ РАСТА 3 И ТРАНСФЕРИНА ИЗ ХУМАНОГ СЕРУМА

ГОРАН МИЉУШ, МИОМИР ПЕТРОВИЋ и ОЛГИЦА НЕДИЋ

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Инсулину слични фактори раста (IGF) имају важну улогу у расту ћелија, њиховој диференцијацији и метаболизму. Количину слободног, биолошки активног IGF регулишу IGF везујући протеини (IGFBP). IGFBP-3 је присутан у највећој количини и има способност да интерагује са другим протеинима из циркулације, укључујући трансферин (Tf). Да би се утврдила могућа улога IGF/IGFBP-3 у метаболизму гвожђа, неопходно је изоловати ком-

плекс IGFBP-3/Tf. У овој студији је примењено неколико техника заснованих на афинитетним интеракцијама. Резултати су показали да само метода двоструке имунопреципитације са анти-Tf, а затим са анти-IGFBP-3 антителима, селективно одваја комплексе од осталих молекулских форми, као што су мономери, олигомери или фрагменти IGFBP-3 и Tf. Овако изоловани комплекс се може даље анализирати у циљу утврђивања односа између IGF/IGFBP-3 и гвожђа, у структурном и метаболичком смислу.

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REFERENCES

1. M. Annunziata, R. Granata, E. Ghigo, *Acta Diabetol.* **48** (2011) 1
2. D. Le Roith, *Exp. Diab. Res.* **4** (2003) 205
3. S. M. Firth, R. C. Baxter, *Endocr. Rev.* **23 (**2002) 824
4. R. C. Baxter, J. L. Martin, V. A. Beniac, *J. Biol. Chem.* **264** (1989) 11843
5. E. Arany, S. Afford, A. J. Strain, P. J. Winwood, M. J. P. Arthur, D. J. Hill, *J. Clin. Endocrinol. Metab.* **79** (1994) 1871
6. K. W. Lee, B. Liu, L. Ma, H. Li, P. Bang, H. P. Koeffler, P. Cohen, *J. Biol. Chem.* **279** (2004) 469
7. S. A. Weinzimer, T. B. Gibson, P. F. Collet-Solberg, A. Khare, B. Liu, P. Cohen, *J. Clin. Endocrinol. Metab.* **86** (2001) 1806
8. P. Aisen, C. Enns, M. Wessling-Resnick, *Int. J. Biochem. Cell. Biol*. **33** (2001) 940
9. P. T. Gomme, K. B. McCann, *Drug Discov. Today* **10** (2005) 276
10. M. Machida, H. Kosako, K. Shirakabe, M. Kobayashi, M. Ushiyama, J. Inagawa, J. Hirano, T. Nakano, Y. Bando, E. Nishida, S. Hattori, *FEBS J.* **274** (2007) 1576
11. O. Nedić, R. Masnikosa, *J. Chromatogr., B* **877** (2009) 743
12. R. Masnikosa, O. Nedić, I. Baričević, D. Lagundžin, *Biochimie* **92** (2010) 97
13. I. Roitt, J. Brostoff, D. Male, *Immunology*, Mosby, London, UK, 1996, p. 406
14. O. Nedić, R. Masnikosa, *Metab. Clin. Exp.* **57** (2008) 658
15. T. Korpela, K. Kurkijärvi, *Anal. Biochem.* **104** (1980) 150
16. B. J. Ryan, C. Ó'Fágáin, *BMC Biotechnol.* **7** (2007) 1
17. H. R. Bergen, J. M. Lacey, J. F. O'Brien, S. Naylor, *Anal. Biochem.* **296** (2001) 122
18. S. M. Firth, R. C. Baxter, *J. Endocrinol.* **160** (1999) 379
19. N. Imam-Sghiouar, R. Joubert-Caron, M. Caron, *Amino Acids* **28** (2005) 105
20. X. Wei, L. Li, *Briefings Funct. Genomics Proteomics* **8** (2008) 104
21. H. Geyer, R. Geyer, *Biochem. Biophys. Acta Proteins Proteomics* **1764** (2006) 1853
22. J. Zhao, D. M. Simeone, D. Heidt, M. A. Anderson, D. M. Lubman, *J. Proteome Res.* **5** (2006) 1792
23. J. M. McDowell, *Plant Immunity*, Humana Press, Blackburg, VA, 2011, p. 295
24. M. M. Weber, G. Spöttl, C. Gössl, D. Engelhart, *J. Clin. Endocrinol. Metab.* **84** (1999) 1679
25. L. Huang, X. Fang, *Methods Mol. Cell Biol.* **425** (2008) 41
26. D. Grenier, V. Goulet, B. Britigan, K. Nakayama, *Infect. Immun.* **72** (2004) 4351
27. V. Sanz-Nebot, E. Balaguer, F. Benavente, C. Neusüß, J. Barbosa, *Electrophoresis* **28** (2007) 1949
28. S. Miyamoto, *Curr. Opin. Mol. Ther.* **8** (2006) 507
29. M. V. Novotny, M. Madera, B. Mann, Y. Mechref, *J. Sep. Sci.* **31** (2008) 2722

30. J. A. P. Bons, E. C. H. J. Michielsen, D. de Boer, F. G. Bouwman, J. Jaeken, M. P. van Dieijen-Visser, M. E. Rubio-Gozalbo, W. K. W. H. Wodzig, *Clin. Chem. Acta* **387** (2008) 59
31. G. de Jong, J. P. van Dijk, H. G. van Eijk, *Clin. Chim. Acta* **190** (1990) 1
32. A. Juul, *Growth Horm. IGF Res.* **13** (2003) 113
33. I. Roy, K. Mondal, M. N. Gupta, *J. Chromatogr., B* **849** (2007) 32
34. W. Xu**,** Y. Luo, K. Huang**,** A. Deng, B. Zhai, H. Zhao, Y. Li, Z. Liang, *Afr. J. Food Sci*. **1** (2007) 24
35. M. Tichá, Z. Kučerová, J. Frýdlová, *J. Chromatogr., B* **800** (2004) 109
36. L. J. Lee, H. He, Y. Yuan, *Biotechnol. Bioeng.* **102** (2009) 891.