

Interaction of Insulin-Like Growth Factor-Binding Protein 2 with α 2-Macroglobulin in the Circulation

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Abstract Insulin-like growth factors (IGFs) play active role in mitogenic and metabolic processes. In the peripheral circulation, they are mostly bound to specific IGF-binding proteins (IGFBPs). Proteolysis of IGFBPs releases free, active IGFs. IGFBP-2 is the second most abundant of the six binding proteins and its concentration increases in catabolic states. The possible interaction between IGFBP-2 and other proteins in the circulation was investigated in this study. Our results showed that IGFBP-2 associates with α 2-macroglobulin (α 2M), a protease inhibitor. Formation of IGFBP-2/ α 2M complexes most likely contributes to the regulation of IGFBP-2 proteolysis and, thus, the activity of IGFs.

Keywords IGFBP-2 · α 2-macroglobulin · Protein complexes · IGFBP-2 proteolysis

Abbreviations

α 2M	Alpha 2-macroglobulin
ConA	<i>Canavalia ensiformis</i> lectin
IGF	Insulin-like growth factor
IGFBP	IGF-binding protein
LCA	<i>Lens culinaris</i> agglutinin
PHA-E	<i>Phaseolus vulgaris</i> erythroagglutinin
IGF-R	IGF receptor
RCA-I	<i>Ricinus communis</i> agglutinin I

SNA	<i>Sambucus nigra</i> agglutinin
Tf	Transferrin

1 Introduction

The insulin-like growth factor (IGF) system plays crucial role in cellular growth, and metabolism, affecting all phases of cell development such as proliferation, differentiation, migration and survival [8, 12]. IGF-I and IGF-II, their receptors (IGF-1R and IGF-2R), the insulin (IR) and the hybrid receptor (IR/IGF-1R) and six IGF-binding proteins (IGFBPs) are members of the IGF system [8, 12].

Free IGFs exert their actions through binding to specific receptors. They are mostly inactive when attached to IGFBPs but can be released by proteolysis. Thus, IGFBPs increase the half-life of IGFs and regulate the intensity of their activity [12]. The affinities of IGFBPs and IGF receptors for IGFs are similar [13, 19]. IGFBP-3 is the major IGF-binding protein in the circulation of healthy people, followed by IGFBP-2, the physiological role of which still remains to be fully elucidated [2, 5].

IGFBP-2 is a 36 kDa protein, not post-translationally modified, and it is not known to form complexes with other proteins in the circulation [6, 27]. The concentration of IGFBP-2 in blood ranges from 370 to 550 μ g/L but does not exhibit diurnal variation or changes related to daily food intake [8, 14]. While it is synthesized in almost every cell in the body, IGFBP-2 in the peripheral circulation is mostly derived from the liver [20]. Circulating IGFBP-2 levels increase several times in acute or chronic pathophysiological states, such as shock, extreme fasting [28], hypoxemia, malignancy [15] or after trauma. In these situations IGFBP-2 may become the major IGF-binding

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protein [1, 21]. Many hormones and growth factors regulate IGFBP-2 expression. The expression of IGFBP-2 is influenced by growth hormone, IGFs, follicle-stimulating hormone, transforming growth factor- β , estradiol, glucocorticoids and insulin [11].

In this study we have investigated whether IGFBP-2, up to now known to exist only as a separate molecular entity, forms complexes with other proteins in the circulation. Thus, IGFBP-3 is known to bind to transferrin (Tf) [25] and IGFBP-1 to α 2-macroglobulin (α 2M) [26]. In order to analyse possible interactions of IGFBP-2 with other blood proteins, immunoaffinity and lectin-affinity methods were used.

2 Materials and Methods

2.1 Serum Samples

Sera were obtained from venous blood of healthy adult volunteers ($n = 20$, 10 males and 10 females, 28–56 years old) after fasting for 12 h. Sera were stored frozen at $-20\text{ }^{\circ}\text{C}$ until used. This study was approved by the local ethical committee in the Institute for the Application of Nuclear Energy.

2.2 Immunoaffinity chromatography

In order to detect possible IGFBP-2 complexes with the eleven most abundant serum proteins, an immunoaffinity matrix (Beckman Coulter, Fullerton, USA) with immobilised antibodies against these proteins was used. These included antibodies against albumin, Tf, IgG, IgA, IgM, HDL apolipoproteins I and II, haptoglobin, α ₁-antitrypsin, α ₁-acid glycoprotein and α 2M. The matrix (1.2 mL) was incubated with 0.5 mL of serum diluted 1:25 in 10 mM Tris-HCl/1.5 M NaCl buffer, pH 7.4, at room temperature for 15 min using a rotator. Unbound proteins were removed by centrifugation for 30 s at $2,000\times g$ and the matrix was washed three times with the dilution buffer. Proteins bound to immobilised antibodies were eluted with 0.1 M Gly-HCl buffer, pH 2.5, in three 0.5 mL portions. Eluates were separated by centrifugation as described. Eluted fractions were neutralised with 50 μ L of 1 M Tris-HCl buffer, pH 8.0. The matrix was then neutralised with 0.6 mL of 2 M Tris-HCl buffer, pH 8.0, and three times washed with the dilution buffer before application of the next sample.

2.3 Lectin-Affinity Chromatography

Agarose-immobilised lectins (Vector, Burlingame, USA) were used to isolate serum glycoproteins that might have

interacted with IGFBP-2: SNA (*Sambucus nigra* agglutinin), LCA (*Lens culinaris* agglutinin), Con A (lectin from *Canavalia ensiformis*), PHA-E (*Phaseolus vulgaris* erythroagglutinin) and RCA-I (*Ricinus communis* agglutinin I). Equilibration and elution buffers for each matrix were prepared following procedures recommended by the producer. Matrices (2.0 mL) were packed into columns and 1.0 mL of sera diluted 1:10 in the appropriate buffer was circulated through each column at room temperature for 1 h. Unbound proteins were removed with 20 mL of dilution buffer. The bound glycoproteins were eluted with 7 mL of hapten sugar (0.2–0.5 M, as suggested by the producer) in 0.1 M acetic acid, pH 3.0. PHA-E-bound glycoproteins were eluted with 0.1 M acetic acid. Eluted fractions were neutralised with 2 M Tris-HCl buffer pH 8.9 and dialysed against distilled water overnight at $4\text{ }^{\circ}\text{C}$. Matrices were neutralised with 20 mL of the appropriate dilution buffer before application of the next sample.

2.4 Immunoprecipitation

A preactivated matrix, AminoLink[®]Plus Coupling Resin (Pierce Biotechnology, Rockford, USA) was used to immobilise separately antibodies against IGFBP-2 (Santa Cruz Biotechnology, Santa Cruz, USA; goat), α 2M (AbD Serotec, Kidlington, Oxford, UK; rabbit) and Tf (INEP, Belgrade, Serbia; goat). The matrix (50 μ L of 50 % slurry) was incubated with 0.2 mL of serum diluted 1:20 in the buffer supplied by the manufacturer, at $4\text{ }^{\circ}\text{C}$ overnight. Unbound proteins were removed by centrifugation for 30 s at $2,000\times g$ and the matrix was washed three times with the dilution buffer. Proteins bound to immobilised antibodies were stripped with the elution buffer (pH 2.5) in three 50 μ L portions. Eluates were separated by centrifugation as described. Eluted fractions were neutralised with 10 μ L of 1 M Tris-HCl buffer pH 8.0. The matrix was then neutralised with 0.1 mL of 2 M Tris-HCl buffer pH 8.0 and washed three times with the dilution buffer before application of the next sample.

2.5 Immunoblotting

The presence of a specific protein in fractions obtained after immuno- and lectin-affinity chromatography, and immunoprecipitation was detected by immunoblotting. SDS-PAGE (10 % gel) under reducing conditions was followed by electrotransfer to nitrocellulose membrane and immunoblotting [18]. The primary antibodies employed were the same as those used for immunoprecipitation. HRP-coupled secondary antibodies were anti-goat IgG (Biosource, Camarillo, USA; swine) and anti-rabbit IgG (AbD Serotec, Kidlington, Oxford, UK; sheep). Immunoreactive proteins were visualised with enhanced

chemiluminescence (ECL) reagent (Pierce Biotechnology, Rockford, USA). Molecular markers were from Bio-Rad Laboratories (Hertfordshire, UK).

3 Results

An immunoaffinity matrix with immobilised antibodies against eleven abundant serum proteins was used to assess possible interactions with IGFBP-2. Unbound proteins and those bound to the matrix were analysed by immunoblotting with anti-IGFBP-2 antibody. In Fig. 1 it can be seen that IGFBP-2 was detected among proteins bound to the matrix. Three immunoreactive species were distinguished: a band of mass between the 31 and 45 kDa markers, corresponding to the IGFBP-2 monomer, and two other bands at masses ≥ 100 kDa. The same proteins were detected in the unbound fraction, together with an IGFBP-2 fragment (mass between the 21 and 31 kDa markers) and an immunoreactive species close to the 66 kDa marker. These data suggested the existence of a complex between IGFBP-2 and at least one other serum protein.

Some serum proteins are simple polypeptides, while others are glycoproteins. IGFBP-2 is a simple protein, without carbohydrate moiety and alone it does not bind to

lectins. However, after interacting with some other serum (glyco)proteins, an IGFBP-2 complex may or may not bind to lectins depending on the nature of its partner in complex formation—whether it is a glycoprotein or simple protein. In the second experiment, lectin-affinity chromatography followed by immunoblotting was employed to investigate whether IGFBP-2 interacted with glycoproteins. Five lectin-bound matrices were used and they all interacted with glycoprotein(s) that formed complexes with IGFBP-2 (Fig. 2). The SNA- and Con A-bound fractions contained monomer IGFBP-2, three immunoreactive species of higher masses and traces of IGFBP-2 fragments. These protein profiles resembled the IGFBP-2 immunoreactive profile exhibited by the serum itself (i.e. the unbound fraction in Fig. 1). RCA-I-, LCA- and PHA-E-bound fractions contained IGFBP-2 monomer and traces of IGFBP-2 complexes and fragments.

Since it is known that IGFBP-3 forms complexes with Tf and IGFBP-1 with $\alpha 2M$, and that both Tf and $\alpha 2M$ are glycoproteins, the next experiment was directed towards detecting possible interactions between IGFBP-2 and Tf or $\alpha 2M$. Serum samples were subjected to immunoprecipitation using matrix-immobilised anti-IGFBP-2, anti-Tf or anti- $\alpha 2M$ antibodies. Proteins specifically bound to these matrices were eluted and analysed by immunoblotting. Immunoblotting with anti-IGFBP-2 antibody (Fig. 3,

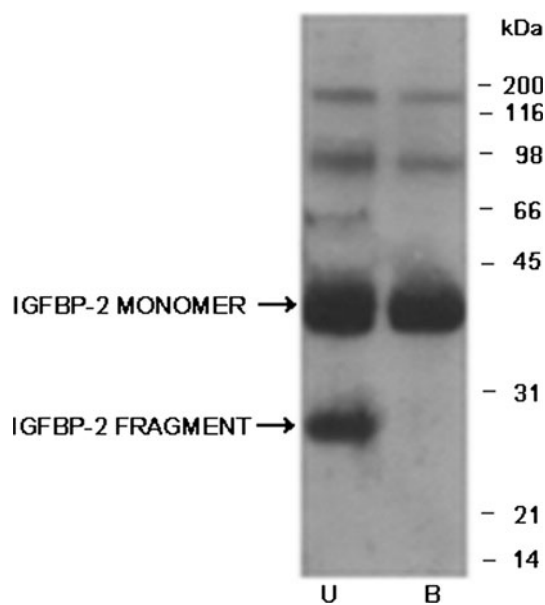


Fig. 1 Immunoblot of the immunoaffinity matrix unbound (*U*) and bound (*B*) IGFBP-2 species. Serum samples were allowed to interact with a matrix with immobilised antibodies against eleven most abundant serum proteins in 10 mM Tris-HCl/1.5 M NaCl buffer, pH 7.4, at room temperature for 15 min; specifically bound proteins were eluted with 0.1 M Gly-HCl buffer, pH 2.5 and subjected to SDS-PAGE (10 % gel) under reducing conditions (200 V, 45 min) followed by electrotransfer to nitrocellulose membrane (100 V, 1 h) and immunoblotting with anti-IGFBP-2 antibody (1: 1,000 dilution)

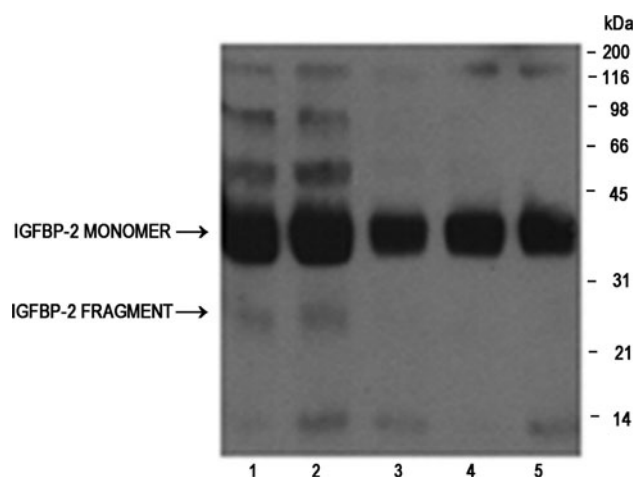


Fig. 2 IGFBP-2 immunoblot of protein fractions specifically eluted from immobilised lectins: SNA (1), Con A (2), RCA-I (3), LCA (4) and PHA-E (5). Immobilised lectins were incubated with serum samples in buffers recommended by the producer for each lectin, at room temperature for 1 h; specifically bound glycoproteins were eluted with hapten sugar (0.2–0.5 M, as suggested by the producer) in 0.1 M acetic acid, pH 3.0 or, in the case of PHA-E, bound glycoproteins were eluted with 0.1 M acetic acid; eluted fractions were subjected to SDS-PAGE (10 % gel) under reducing conditions (200 V, 45 min) followed by electrotransfer to nitrocellulose membrane (100 V, 1 h) and immunoblotting with anti-IGFBP-2 antibody (1: 1,000 dilution)

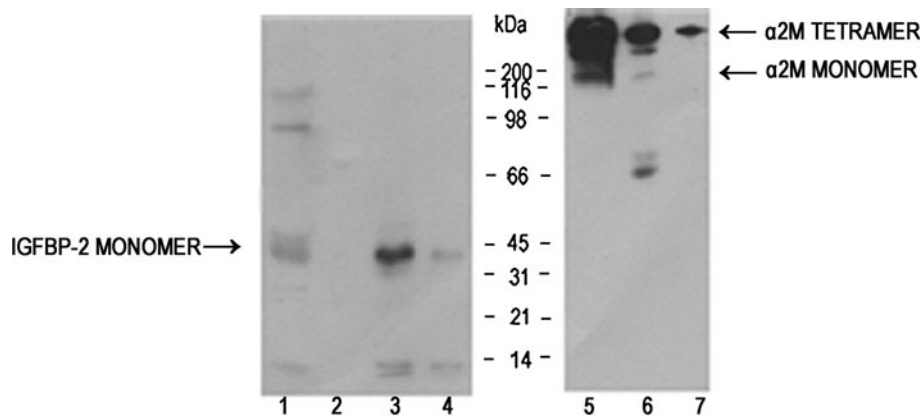


Fig. 3 Immunoblot of the IGFBP-2 (1–4) and α_2 M (5–7) species obtained by immunoprecipitation with anti-IGFBP-2 antibody (4, 7), anti-Tf antibody (2) or anti- α_2 M antibody (3, 6). Samples 1 and 5 are untreated sera. Immunoprecipitation was performed with matrices with separately immobilised antibodies against IGFBP-2, α_2 M and Tf. Matrices were incubated with serum samples in a buffer supplied

by the manufacturer, at 4 °C overnight; specifically bound proteins were eluted with a supplied buffer pH 2.5 and subjected to SDS-PAGE (10 % gel) under reducing conditions (200 V, 45 min) followed by electrotransfer to nitrocellulose membrane (100 V, 1 h) and immunoblotting with anti-IGFBP-2 antibodies (1: 1,000 dilution) or anti- α_2 M antibody (1: 5,000)

samples 1–4) detected the presence of immunoreactive species in eluates obtained from matrices with immobilised anti-IGFBP-2 or anti- α_2 M antibody. IGFBP-2 was not detected in the eluate from anti-Tf resin. According to these results, IGFBP-2 formed complexes with α_2 M but not with Tf. In order to confirm this finding, immunoblotting with anti- α_2 M antibody was performed (Fig. 3, samples 5–7). α_2 M immunoreactive protein of high molecular mass was detected in the eluate obtained from the matrix with immobilised anti-IGFBP-2 antibody, indicating that this antibody could bind IGFBP-2/ α_2 M complexes besides its natural ligand. α_2 M immunoblotting performed after elution of proteins precipitated with anti- α_2 M antibodies revealed a presence of α_2 M fragments. According to literature data, α_2 M may cleave into several fragments. The fragment at approximately 60 kDa is the major one [10]. α_2 M fragments were not precipitated with anti-IGFBP-2 antibody, implicating that only intact α_2 M is capable of binding IGFBP-2 in a complex.

All serum samples exhibited similar immunoblotting profiles. There were differences between samples in the relative abundance of immunoreactive proteins, but without any specific pattern related to age or gender.

4 Discussion

Our results have demonstrated that IGFBP-2 in the peripheral circulation forms complexes with α_2 M but not with Tf. One prerequisite for complex formation is the existence of appropriate structural elements in each subunit allowing interaction to occur. It is not known which protein

sequences in IGFBP-1 and IGFBP-3 are directly involved in their respective binding to α_2 M and Tf. However, a unique feature of IGFBP-1 and IGFBP-2 is the presence of an RGD sequence that enables them to interact with integrins [9, 22], while IGFBP-3 was found to possess a metal-binding domain [23].

The formation of a specific protein association is often connected with performance of a unique physiological role. α_2 M is a large multi-domain protein (a tetramer of approximately 190 kDa subunits) known to inhibit many proteases, but also able to bind smaller proteins in a non-covalent, reversible manner, thereby regulating their rate of degradation [7, 24]. Proteolysis of binding proteins liberates ligands, which can then exert their functions. Circulating IGFBP-2 inhibits IGF actions [27], so IGFBP-2 proteolysis is a regulatory step in the balance between bound (inactive) and free (active) IGFs.

A number of pathophysiological states are characterised by elevated concentrations of IGFBP-2 [1, 15, 21, 28]. Increased proteolytic activity most often accompanies these situations [3]. A study on IGFBP-1/ α_2 M complexes demonstrated that α_2 M protects IGFBP-1 from chymotrypsin in a dose-dependent manner [26]. Plasmin [16], calpain [4] and matrix metalloproteases [17] are known to degrade IGFBP-2, especially in severe catabolic illness. A complex relationship between the relative abundance of IGFBP-2, its proteases and protease inhibitors, including α_2 M, and the degree of their interaction may be expected to regulate the activity of IGFs.

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