



J. Serb. Chem. Soc. 74 (7) 707–716 (2009) JSCS–3869 JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS UDC 618.36+612.6:612.349.8:576.314 Original scientific paper

IGFBP-1 forms associated with placental cell membranes

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(Received 9 March, revised 7 April 2009)

Abstract: Fetal growth *in utero* depends on the proper development and function of the placenta. Insulin-like growth factors (IGFs) are critically involved in placental development. During pregnancy, an IGF-binding protein, IGFBP-1, which is produced by maternal decidua, plays an important role in the control of the bioavailability of IGFs. It has recently been proposed that cleavage of decidual IGFBP-1 by matrix metalloproteases is a novel mechanism in the control of placental development. The presence of IGFBP-1 in solubilized placental cell membranes, *i.e.* its association with the membranes, was detected in an earlier work. Herein, it is shown that IGFBP-1 from the solubilized membranes forms dimers, as well as high molecular mass complexes. IGFBP-1 dimers preferably contain the non-phosphorylated form of IGFBP-1. The high molecular mass forms are polymers of IGFBP-1 or its complexes with other membrane proteins. Dimerization of IGFBP-1, together with its association with the placental cell membrane, could serve as an additional mechanism of the regulation of IGF availability to the type 1 IGF receptors.

Keywords: IGFBP-1 dimers; placenta; IGFBP-1 phosphoisoforms.

INTRODUCTION

The insulin-like growth factor (IGF) axis is an important regulator of fetal growth and it has been suggested that IGF-I and -II may, in part, mediate this effect by promoting proper placental development and function.¹ For IGFs to exert their effects, they must interact with cell-surface receptors, principally the type 1 IGF receptor (IGF1R).² IGF-II can also bind to the type 2 IGF receptor (IGF2R) and insulin receptor isoform A (IR-A).² Ligand access to these receptors is controlled by a family of six IGF binding proteins (IGFBPs 1–6).^{2,3} IGFBPs have similar or higher affinities for IGFs than IGF1R, suggesting that the formation of IGF–IGFBP complexes is favored over the formation of IGF–IGF1R complexes. Acting through this mechanism, IGFBPs would sequester



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doi: 10.2298/JSC0907707M

IGFs and act as inhibitors of IGF actions.⁴ In certain physiological situations, IGFBPs can enhance biological actions of IGFs, which was shown for IGFBP-3.³ However, there are many physiological situations (*e.g.*, pregnancy), in which IGFs must be released from IGFBPs, principally through decreasing their affinities for IGFs. This could be realized by the binding of IGFBPs to extracellular matrix components or by post-translational modifications of IGFBPs.⁴

Immunohistochemical studies showed IGFBP-2 in the syncityotrophoblast cells of the placenta.⁵ IGFBP-1 and IGFBP-3 are produced in abundance by the maternal-fetal interface.⁵ IGFBP-1 was found to associate with the membrane of placental trophoblast cells.⁶ It is thought that IGFBP-1 regulates the biological activity of IGFs within the local environment of the human placenta by modulating their interaction with the IGF1R.⁷ The role of IGFBP-1 in the placenta is not yet elucidated and the mechanisms involved are under vigorous research. Post-translational modifications, including phosphorylation,⁸ proteolysis,⁹ and polymerization,¹⁰ have been shown to alter the affinity of IGFBP-1 for IGF-I. Three phosphorylation sites have been identified in human IGFBP-1: Ser 101, 119 and 169.11 When human IGFBP-1 purified from amniotic fluid or a cell culture supernatant is analyzed by non-denaturing gel electrophoresis, one non-phosphorylated (np-IGFBP-1) and four phosphorylated isoforms (p-IGFBP-1) can be identified.¹² In the circulation of non-pregnant women, IGFBP-1 almost exclusively exists as p-IGFBP-1, which has high affinity for IGFs. During pregnancy, however, IGFBP-1 becomes dephosphorylated by placental alkaline phosphatase to np-IGFBP-1 and lesser phosphorylated isoforms, which have a reduced affinity for IGF-I. Whereas p-IGFBP-1 is inhibitory, np-IGFBP-1 stimulates the action of IGF-I.8

Sakai and co-workers¹⁰ demonstrated the formation of covalently linked multimers of IGFBP-1 by a tissue transglutaminase *in vitro*, as well as by a fibroblast membrane extract. They also detected the polymerized forms of IGFBP-1 in human amniotic fluid. These findings prompted the herein described examination of the association/polymerization of the IGFBP-1 protein associated with placental cell membranes.

EXPERIMENTAL

Materials

Na¹²⁵I was supplied by Isotope (Budapest, Hungary). Human IGF-I and des(1–3)IGF-I (IGF-I lacking the three amino acid residues at the *N*-terminus) were from GroPep (Adelaide, Australia). Affinity purified goat polyclonal anti-human IGFBP-1 antibodies were from DSL (Webster, USA). Horse radish peroxidase-conjugated (HRP) anti-goat IgG was supplied by Biosource (Camarillo, USA). Enhanced chemiluminescence (ECL) detection reagents were the products of Amersham Biosciences (Aylesbury, UK) and MXB films and developing reagents were from Kodak (Paris, France). All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). ¹²⁵I–IGF-I and ¹²⁵I–des(1–3)IGF-I were prepared using the chloramine T method.¹³

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Human placental tissue was obtained from uncomplicated pregnancies at term, according to protocols approved by the local ethical committee. The tissue was collected in ice cold 0.10 M phosphate buffered saline (pH 7.4) and brought to laboratory within 60 min. After washing the placenta free of blood, the amniotic and chorionic membranes were dissected away. The placental tissue was minced and homogenized in a 0.25 M sucrose solution with protease inhibitors. After a short centrifugation to remove the cell debris, the supernatant was centrifuged at 18000 x g for 30 min. The pellet was resuspended and solubilized by Triton X-100. The solubilized membranes were recovered in the supernatant after centrifugation at 100,000 x g for 90 min. The detailed procedure was described in a previous article.¹⁴

Gel filtration

Solubilized membranes (2 mg of membrane protein) were incubated with ¹²⁵I–IGF-I or ¹²⁵I–des(1–3)IGF-I (1 pmol, 10⁶ cpm) at 4 °C overnight. In some cases, the solubilized membranes were incubated with the tracers in the presence of high concentrations of unlabeled IGF-I or des(1–3)IGF-I (4 µg). The samples were then subjected to gel filtration on a Sephadex G-100 column, exactly as described previously.¹⁵ In some cases, the solubilized membranes (45 mg of membrane proteins) were applied on the column without prior incubation with the tracer and the collected fractions were employed in electrophoresis and in binding assays with ¹²⁵I-labeled IGF-I and des(1–3)IGF-I. In a separate experiment, fractions from 9 to 15 were pooled and the pool was designated as peak 1 proteins. The binding assays commenced by incubating aliquots (50 µL) of the fractions obtained after gel filtration with ¹²⁵I–IGF-I or ¹²⁵I–des(1–3)IGF-I (10⁵ cpm) and were performed as described earlier.¹⁶ The protein concentrations were determined using the Bradford protein assay¹⁷ with ovalbumin standards.

Gel electrophoresis and blotting

Aliquots of the fractions obtained from gel filtration were subjected to both non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE, in 8 % gel) and to non-denaturing PAGE (in 10 % gel). The samples were mixed with an equal volume of sample buffer (0.125 M Tris-HCl, 4 % SDS, 20 % glycerol, 0.010 % bromphenol blue, pH 6.8) and boiled for 7 min before being applied onto the gels. The non-denaturing PAGE was performed following the procedure for SDS PAGE,¹⁸ except that the SDS was omitted from all the buffers, including the sample buffer. The proteins were transferred to Immobilon P membranes (Millipore, Billerica, USA) and probed with anti-human IGFBP-1 antibodies as described previously.¹⁸ The immunoreactive proteins were visualized using HRP-conjugated anti-goat IgG antibodies and an ECL detection system, followed by autoradiography. Before the ligand blotting, the solubilized membranes and peak 1 proteins were subjected to SDS PAGE using an 8 % gel and then the membranes were probed with ¹²⁵I–IGF-I (2.5×10⁶ cpm in 50 mL of 0.010 M Tris-HCl, 0.15 M NaCl, pH 7.4) and visualized by autoradiography as described previously.¹⁹

Immunoradiometric assay (IRMA) of IGFBP-1

The IGFBP-1 concentration in the fractions eluted from the Sephadex G-100 column was determined by a DSL (Webster, USA) Total IGFBP-1 IRMA kit.

RESULTS AND DISCUSSION

The proteins which interacted with $^{125}I-IGF-I$ and with $^{125}I-des(1-3)IGF-I$ were detected after separation by gel filtration of the solubilized placental cell membranes pre-incubated with the tracers (Fig. 1). The first peak (peak 1), which

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appears at the void volume (V_0) of the column, is known to contain complexes of IGF1R with ¹²⁵I-labeled IGF-I or des(1–3)IGF-I, whereas the second peak contains ¹²⁵I–IGF-I/IGFBP-1 complexes.¹⁵ Peak 2 is missing in the elution profile of the solubilized membranes pre-incubated with ¹²⁵I–des(1–3)IGF-I, because it has a markedly reduced affinity for IGFBPs.²⁰ Peak 3 represents unbound ¹²⁵I-labeled ligand.



Fig. 1. Two identical samples of solubilized membranes (2 mg of total membrane protein) were incubated with 1×10⁶ cpm of ¹²⁵I–IGF-I (full circles) and ¹²⁵I–des(1–3)IGF-I (empty circles) before being applied to a Sephadex G-100 column ((1.8×60) cm) and eluted using 0.050 M sodium phosphate buffer, pH 7.5, containing 0.10 M NaCl and 0.10 % Triton X-100. The flow rate was 20 mL/h. Following the pre-elution of 20 mL, 2 mL fractions were collected and the radioactivity in each was measured. The positions of molecular mass markers (Tg-thyroglobulin, OA-ovalbumin) are indicated. The results from representative experiments are illustrated.

The specificity of ligand binding to peak 1 and peak 2 proteins was analyzed by gel-filtration of the solubilized membranes, which were pre-incubated with 125 I-labeled ligands in the presence of high concentrations of unlabeled ligands. The 125 I-des(1–3)IGF-I binding in peak 1 was specific, as this peak disappeared upon gel filtration in the presence of 4 µg of either unlabeled IGF-I or des(1– -3)IGF-I (results not shown). This pattern of cross-reactivity reflects the fact that des(1–3)IGF-I binds to IGF1R with a similar affinity to the full length IGF-I.²¹ In contrast, 125 I–IGF-I bound to the proteins in peak 1 could not be completely displaced by unlabeled des(1–3)IGF-I, whereas unlabeled IGF-I reduced the binding of the ligand to the level of non-specific binding (Fig. 2). In other words, a

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proportion of 125 I–IGF-I binds to the protein(s) of peak 1 to which des(1–3)IGF-I does not bind.



Fig. 2. Solubilized membranes (2 mg of total membrane protein) were incubated with 1×10⁶ cpm of ¹²⁵I–IGF-I, without competing ligand (full circles) or in the presence of unlabeled competitor: IGF-I (empty circles), des(1–3)IGF-I (asterisks), applied to a Sephadex G-100 column and eluted as described (see legend under Fig. 1).

To investigate whether IGFBP-1 was also present among the proteins of high molecular mass in peak 1, the concentration of IGFBP-1 in the fractions eluted after chromatography of 45 mg of solubilized membrane proteins was measured immunoradiometrically. The results are shown in parallel with the graph that represents the binding of ¹²⁵I–IGF-I in fractions eluted after the preparative gel filtration (Fig. 3). The highest IGFBP-1 concentrations, according to the DSL To-tal IGFBP-1 IRMA, were detected in fractions 24–28, with the maximal value in fraction 26 (above 230 ng/mL), coinciding with peak 2. Fractions that belonged to peak 1 also contained IGFBP-1. In other words, IGFBP-1 eluted within V_0 , among the other high molecular mass proteins, such as IGF1Rs, IGF2Rs and IRs.¹⁴

To examine the possibility that IGFBP-1 from placental cell membranes existed in the form of a high molecular mass species, the fractions of peak 1 and peak 2 were analyzed by SDS PAGE, followed by immunoblotting using affinity purified polyclonal anti-human IGFBP-1 antibodies (Fig. 4). Several molecular forms of IGFBP-1 from the solubilized placental cell membranes were visible on the immunoblots (Fig. 4). The 60 kD-band on the blot in Fig. 4 most probably originated from dimeric IGFBP-1, which peaked in fractions 18 to 21. The existence of covalently linked monomers of IGFBP-1, which formed dimers follow-

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ing exposure to pure tissue transglutaminase, was reported.¹⁰ Monomeric IGFBP-1 (molecular mass of 29 kD) bands were seen at the bottom of the gel, with the greatest abundance in fractions eluting after fraction 21. Some of these fractions also showed the greatest IGFBP-1 concentration in IRMA (Fig. 3). However, the concentration of IGFBP-1 in the fractions in which IGFBP-1 was present mostly as a dimer was low. In other words, the antibodies used in IRMA poorly recognized the dimeric IGFBP-1 forms. According to the immunoblot in Fig. 4, high molecular mass proteins (of approximately 150 and 220 kD) that were immunoreactive with anti-IGFBP-1 antibodies were also detected (gel filtration fractions 12 to 15). These proteins are either polymers of IGFBP-1 or its complexes with other proteins from placental cell membranes.



Fig. 3. Solubilized membranes (45 mg of total membrane protein) were chromatographed on a Sephadex G-100 column as usual, but without pre-incubation with ¹²⁵I–IGF-I. The binding of ¹²⁵I–IGF-I in each fraction was measured by ligand binding assay. The result from a representative experiment is illustrated. The concentration of IGFBP-1 in the fractions, measured by DSL Total IGFBP-1 IRMA, is shown in parallel (ng/mL).



Fig. 4. The fractions eluted from the Sephadex G-100 column were analyzed by SDS PAGE (8 % gels) followed by immunoblotting using affinity purified goat polyclonal anti-human IGFBP-1 antibodies. The blots are of two gels that were run simultaneously in one electrophoretic unit. Molecular mass markers (in kD) are shown on the left.

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A further step was to check whether np-IGFBP-1 and p-IGFBP-1 were differently distributed between the peak 1 and peak 2 proteins. Non-denaturing PAGE followed by immunoblotting for IGFBP-1 was undertaken to analyze the phosphoisoform pattern of IGFBP-1 in the fractions eluted after gel filtration (Fig. 5). The results presented in Fig. 5 clearly show that the fractions contained different isoforms of IGFBP-1. The band positions of p- and np-IGFBP-1 isoforms upon non-denaturing PAGE were established knowing that the phosphorylated forms, due to their charge density, migrate towards the anode faster than the np-IGFBP--1.22 The same pattern of bands was obtained by others upon non-reducing PAGE of IGFBP-1 from human amniotic fluid.^{22,23} np-IGFBP-1 was mostly present in fractions number 18 to 21, which were the same fractions that contained the dimeric form of IGFBP-1 (see Fig. 4). These results strongly suggest that the dimeric form of IGFBP-1, associated with the placental cell membranes, consists almost exclusively of the np-IGFBP-1 isoform. These findings are in accordance with those obtained by others, who observed that np-IGFBP-1 polymerized more rapidly and to a greater extent than phosphorylated isoforms.¹⁰ Monomeric IGFBP-1, however, mostly contained phosphorylated isoforms of IGFBP-1 (fractions 24 to 28). Fraction 26, which had the greatest amount of IGFBP-1 in IRMA and which bound the most ¹²⁵I-labeled IGF-I when pre-incubated with the tracer (see Fig. 1), showed the strongest bands on the immunoblots following non-denaturing electrophoresis.



Fig. 5. The fractions eluted from the Sephadex G-100 column were applied to two 10 % gels, which were simultaneously subjected to non-denaturing PAGE, and then to immunoblotting for IGFBP-1. The upper arrow denotes the position of np-IGFBP-1. The two lower arrows indicate the positions of the phosphorylated isoforms of IGFBP-1.

The ligand binding properties of IGFBP-1 forms were also examined in this study. The affinity of IGFBP-1 isoforms present in the solubilized placental membranes and in peak 1 for ¹²⁵I-labeled IGF-I was assessed using ligand blot. As can be seen in Fig. 6, the monomeric IGFBP-1 was the only protein able to bind ¹²⁵I–IGF-I, giving a strong single band. The binding of ¹²⁵I-labeled IGF-I to the IGFBP-1 dimer or to some other high molecular mass proteins could not be demonstrated. The binding of the tracer to IGF1R was also lacking. It is generally believed that polymeric forms of IGFBP-1 do not bind the ligand,¹⁰ but some of the present results indicate that a proportion of ¹²⁵I–IGF-I bound to the

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protein(s) of peak 1 to which des(1–3)IGF-I did not bind, most probably IGFBP-1. Although the ligand blot result suggests the monomeric form of IGFBP-1 as the sole molecular species able to bind the ligand, the technique might not be as sensitive as gel filtration of the pre-labeled placental membrane proteins.



Fig. 6. The solubilized placental membrane proteins and proteins eluted in peak 1 after gel filtration on Sephadex G-100 were analyzed by 8 % SDS PAGE followed by ligand blotting using ¹²⁵I– –IGF-I. Lanes 1 and 2 contained solubilized membranes and lanes 3 and 4, peak 1 proteins (pooled fractions 9 to 15 from preparative gel filtration). Molecular mass markers (in kD) are shown on the left. The arrow denotes the position of monomeric IGFBP-1.

These results are in accordance with those presented in Fig. 1, where fractions containing dimers of IGFBP-1 resided between peaks 1 and 2. These results, however, may be taken into account in the light of the finding of others who demonstrated that the formation of multimers of IGFBP-1 led to a reduction or loss of IGF-I binding.¹⁰ Since the ability of IGFBP-1 to polymerize was also shown to be related to the loss of its capacity to inhibit IGF-I actions, it was suggested that this may be one mechanism by which phosphorylation of IGFBP-1 may enhance its inhibitory potential.¹⁰ The deletion of twenty C-terminal amino acids from the IGFBP-1 molecule was also reported to result in a loss of IGF binding and the formation of dimeric IGFBP-1 molecules.²⁴

Tissue transglutaminase is an enzyme which is widely distributed in many tissues and organs and which has been localized to the cytoplasm,²⁵ cell surface²⁶ and extracellular matrix.²⁷ Polymerization of IGFBP-1 is catalyzed not only by the isolated enzyme, but also by fetal fibroblast cell cultures and their membrane extract.¹⁰ The expression of tissue transglutaminase at the embryo-maternal interface was recently discovered,²⁸ hence, this enzyme might be responsible for the presence of the IGFBP-1 dimers detected in this study.

Relevant literature data were sought in order to find protein candidates that might associate with the IGFBP-1 in the placental cell membranes. In human plasma, α_2 -macroglobulin (α_2 -M) forms high molecular mass complexes with IGFBP-1, it preferentially associates with p-IGFBP-1 and these complexes can still bind IGF-I.²⁹ On the other hand, the high molecular mass complexes of IGFBP-1 detected in this study consisted almost exclusively of np-IGFBP-1. Fur-



thermore, these forms bound only small amounts of ¹²⁵I-labeled IGF-I. α_2 -M was detected in placental trophoblast.³⁰ It is known that some anti-IGFBP-1 antibodies recognize IGFBP-1 even when it is engaged in a complex with α_2 -M, but some cannot.²⁹ The association of IGFBP-1 with α_2 -M is suggested to be of low affinity, resulting in the dissociation of the complex during gel filtration,²⁹ whereas the high molecular mass complexes of IGFBP-1, which were immunedetected in the present experiments, survived the 7-min heating with SDS prior to SDS PAGE. An association of IGFBP-1 with α_2 -M still cannot be ruled out.

When the phosphoisoforms of IGFBP-1 were separated by anion exchange chromatography, the fractions that contained phosphorylated forms inhibited IGF-I actions, whereas those that were enriched in np-IGFBP-1, potentiated IGF-I actions.²² Taking this into account, it can be presumed that phosphorylation is the major factor modulating the affinity of IGFBP-1 for IGFs. It is suggested that the generation of non-phosphorylated and phosphorylated isoforms of IGFBP-1 and their association with cell membranes are involved in the regulation of IGF availability to IGF1R. Dimerization of IGFBP-1 may be a reversible process, catalyzed by membrane-associated tissue transglutaminase. Since IGFBP-1 and IGF1R couple for the ligand, increased binding of IGF-I to the IGF1R could be achieved through increased sequestering of high-affinity p-IGFBP-1 in dimers. The biological significance of dimeric IGFBP-1 forms has yet to be revealed.

CONCLUSIONS

Previously, the presence of IGFBP-1 in solubilized placental cell membranes was detected, *i.e.* its association with the membranes. Herein, it is reported that IGFBP-1 forms dimers, which preferably contain np-IGFBP-1. Polymerization of IGFBP-1 *in vitro* can be catalyzed by tissue transglutaminase, which was recently detected at the embryo-maternal interface. This enzyme might be responsible for the presence of the IGFBP-1 dimers detected in this study.

Acknowledgments. This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia (Grant No. 143019).

ИЗВОД

ФОРМЕ ИГФБП-1 У МЕМБРАНАМА ПЛАЦЕНТНИХ ЋЕЛИЈА

РОМАНА МАСНИКОСА, БОГОВИД ЖИВКОВИЋ и ОЛГИЦА НЕДИЋ

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Унутарматерични раст фетуса зависи од правилног развоја и ваљаног функционисања плаценте. Инсулину-слични фактори раста (IGF) су важни фактори за развој плаценте. За време гестације, измењени ендометријум материце (децидуа) синтетише IGF-везујући протеин-1 (IGFBP-1), који контролише биолошко дејство IGF молекула. Разградња IGFBP-1 пореклом из децидуе, дејством металопротеаза из матрикса, сматра се новим механизмом контроле развића плаценте. У нашем ранијем раду утврдили смо присуство IGFBP-1 у солубилизованим мембранама ћелија из плаценте, што смо објаснили везивањем IGFBP-1 за

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ћелијске мембране. У овом раду показано је да IGFBP-1 из солубилизованих мембрана образује димере, као и комплексе великих молекулских маса. Димери IGFBP-1 претежно садрже нефосфориловане молекуле мономерног IGFBP-1, док облици IGFBP-1 великих молекулских маса представљају агрегате IGFBP-1, и/или његове комплексе са другим мембранским протеинима. Димеризација IGFBP-1, као и његова асоцијација са ћелијским мембранама из плаценте, могла би да представља додатни механизам за регулацију доступности IGF молекула за тип 1 IGF рецептора.

(Примљено 9. марта, ревидирано 7. априла 2009)

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