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Research Article

A 30-kDa fragment of insulin-like growth factor (IGF) binding protein-3 in human pregnancy serum with strongly reduced IGF-I binding

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Abstract. Proteolytic cleavage of insulin-like growth factor (IGF) binding protein (IGFBP)-3 during pregnancy is likely to have both IGF-dependent and -independent effects on maternal, placental and fetal growth and metabolism. A 30-kDa proteolytic IGFBP-3 fragment was isolated from third trimester pregnancy human serum and identified by N- and C-terminal amino acid sequence analysis and mass spectrometry to correspond to residues 1–212 of the parent protein. This fragment is the dominating IGFBP-3 immunoreactive species in pregnancy serum. The 30-kDa fragment was also detected in serum of non-pregnant women where it coexists with intact IGFBP-3. Using biosensor technology, (1–212)IGFBP-3 was found to have 11-fold lower affinity for IGF-I compared to intact IGFBP-3, while a 4-fold decrease in affinity was found for IGF-II. Tests with des(1–3)IGF-I suggest fast binding of IGF-I to the N-terminal region of IGFBP-3 and similar affinity to a slow binding site in the C-terminal region.

Keywords. IGFBP-3, proteolysis, IGF, pregnancy, biosensor, kinetics, amino acid sequence analysis, mass spectrometry.

Introduction

Insulin-like growth factors (IGFs)-I and -II bind with high affinity a family of six IGF binding proteins (IGFBP-1 to -6) present in body fluids in various proportions. Since the affinities of IGF-I and -II for IGFBPs are comparable with their affinities for the IGF type 1 receptor, IGFBPs have large impact on the mitogenic and metabolic effects of IGFs [1]. After the newborn period, large stores of circulating IGFs are bound to IGFBP-3 in a 150-kDa ternary complex including an acid labile subunit (ALS). In contrast, the fetus and the newborn display low expression of ALS and the IGFs circulate in a more readily available form as binary complexes with IGFBP-2 and -3 [2, 3]. In the ternary complex, IGF-I has prolonged clearance and is thought to be less available to the target tissues [4]. In addition, IGFBPs, as well as their proteolytic fragments, have IGF-independent effects on cell growth and metabolism [5–7]. IGFBP-3 interacts with receptor-like molecules on the cell surface [8], modulates

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IGF type 1 receptor function [9], and interacts with cytoplasmic or nuclear molecules [10].

Post-translational modifications such as glycosylation, phosphorylation and proteolysis frequently affect the binding properties of proteins. Maternal proteolysis of IGFBP-3 was demonstrated more than a decade ago in human [11, 12] and rodent [13, 14] pregnancies. However, the responsible protease(s) has not yet been identified. IGFBP binding affinity is reduced upon proteolysis resulting in increased IGF bioavailability. A finding in line with this notion is that inhibition of pregnancy-associated plasma protein-A (a protease cleaving IGFBP-4 and -5) results in retardation of fetal growth [15]. Experiments using semi-purified preparations of human pregnancy serum have indicated that the affinity of proteolytically cleaved IGFBP-3 for IGFs is reduced [16]. This potentially promotes maternal IGF actions supporting placental and fetal growth, although the ternary complex is not disrupted by IGFBP-3 proteolysis during pregnancy [17, 18].

All IGFBPs consist of conserved cysteine-rich globular N- and C-terminal domains connected by a linker region that shares little homology among IGFBPs. *In vitro* degradation of recombinant non-glycosylated IGFBP-3 by pregnancy serum suggests that cleavage occurs in the non-conserved linker region of the molecule [19]. Similarly, proteolytic cleavage of IGFBP-3 by plasmin and prostate-specific antigen results in larger fragments cleaved in the linker region of IGFBP-3 [19–21]. Alternative cleavage sites in the conserved C-terminal domain were also found [19, 21] but their susceptibility to cleavage *in vivo* has not yet been determined. Both the N- and C-terminal domains of the IGFBPs contain regions important for IGF binding. The amino acid sequence 215–232 in the C-terminal segment contains heparin and ALS binding sites as well as a nuclear localization site [22].

In the present study we have determined the N- and C-terminal amino acid sequence of a 30-kDa IGFBP-3 fragment isolated from human pregnancy and non-pregnancy serum. Biosensor analysis of interactions between this fragment and IGF-I, IGF-II, or des(1–3)IGF-I was carried out to determine the effect of IGFBP-3 proteolysis on IGF-binding kinetics. The results provide information important for identification of the responsible protease and the physiological role of IGFBP-3 proteolysis.

Materials and methods

Biacore equipment and reagents. The Biacore X instrument, sensorchip CM5 (research grade), HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005 % surfactant P20, pH 7.4), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and the amine coupling kit were purchased from Biacore AB (Uppsala, Sweden). Recombinant human IGF-I (rhIGF-I) was a gift from Genentech (South San Francisco, CA, USA) and rhIGF-II was a gift from KabiGen (Uppsala, Sweden). Glycosylated rhIGFBP-3 was from Upstate Biotechnology (Charlottesville, VA, USA). Monoclonal anti-IGFBP-1 and monoclonal anti-IGFBP-2 antibodies were from Diagnostic System Laboratories (Webster, Texas, USA) and Novartis, respectively.

Serum samples. Venous blood was obtained from healthy volunteers in the third trimester of pregnancy or from non-pregnant women and allowed to clot at low temperature (4°C for 2.5 h) to reduce *in vitro* proteolysis before centrifugation, pooling and storage at -20°C. The protocol has been approved by the regional ethical committee in Stockholm.

SDS-PAGE and electrotransfer. Proteins were separated using 12 % polyacrylamide gel electrophoresis (Hoefer SE 600, Pharmacia Biotech, Sweden) or gradient pre-cast gels (X Cell Mini-Cell, Novex, San Diego, Canada, or Criterion Cell, Bio-Rad, CA, USA). After SDS-PAGE, proteins were electrotransferred (Hoefer TE 70 SemiPhor, Hoefer Scientific Instruments, San Francisco, USA) onto Hybond-C nitrocellulose membrane or Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Uppsala, Sweden).

IGFBP-3 Western immunoblotting. The nitrocellulose membranes were washed for 20 min with Tris-buffered saline with 0.1 % Tween-20 (TBS-T) and then blocked with 3 % non-fat milk in TBS-T for 1.5 h. Incubation with primary antibody (Upstate Biotechnology, New York, USA) diluted 1:1000 with 3 % non-fat milk in TBS was performed at 4°C overnight. Membranes were again washed with TBS-T twice for 30 min before incubation with secondary antibody (anti-rabbit Ig, horseradish peroxidase linked $F(ab')_2$ fragment from donkey, Amersham) coupled with horseradish peroxidase diluted 1:5000 with TBS-T for 50 min. After incubation, membranes were washed three times for 5 min in TBS-T and 15 min in water. Bound antibody was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Silver staining. After separation by SDS-PAGE, proteins were stained with Instaview Silver Stain Kit (BDH, England) according to the manufacturer's instruction.

2-D PAGE of the 30-kDa IGFBP-3 fragment. The non-pregnancy serum or pregnancy serum, 10 µl, was mixed with 50 µl IPG buffer pH 3–10 (Amersham Pharmacia Biotech) and 140 µl 8 M urea. The samples were applied to 11-cm Immobiline DryStrip pH 3–10 (Amersham Pharmacia Biotech). Rehydration and isoelectric focusing were carried out at 20°C under non-reducing conditions for 15 h at 30 V, followed by 30 min at 200 V, and finally 6 h at 3000 V using an IPGphor Isoelectric Focusing Unit (Amersham Pharmacia Biotech). The gel strips were stored at –20°C. For separation in the second dimension, the IPGstrips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % SDS and a trace of bromophenol blue, applied to a 12 % separation gel and covered with 0.5 % agarose gel. Separation was carried out as described above. After 2-D PAGE and electrotransfer to PVDF membrane, the 30-kDa IGFBP-3 fragments were identified by Western immunoblotting.

Purification of intact IGFBP-3 and a 30-kDa proteolytic fragment. Purification was performed in four chromatographic steps (all chromatography material and instrumentation was from Amersham Pharmacia Biotech). Western immunoblotting and silver staining of gel bands were performed after each step. Acidic size exclusion chromatography was carried out to separate IGFBP-3 from the naturally occurring ternary complex. This step markedly improved the retention of IGFBP-3 material in the following IGF-I affinity step. Briefly, ternary complexes were disrupted by incubating a serum aliquot with an equal volume of 2 M acetic acid for 1 h at 25°C. The incubation mix was applied to a Sephadex G-50 column (0.5×30 cm) equilibrated with 1 M acetic acid at 0.5 ml/min and 4°C. Fractions containing IGFBP-3 immunoreactivity were collected and lyophilized. IGF-I containing fractions, defined by calibration with ^{125}I-labeled IGF-I mixed with serum, were excluded and the absence of IGF-I in IGFBP-3-

immunoreactive fractions was demonstrated by IGF-I RIA determinations.

The lyophilized fractions were resuspended in 50 mM HEPES, pH 7.5, and applied to an IGF-I affinity column (HiTrap NHS-activated HP prepared using 0.6 mg IGF-I according to the manufacturer's instruction). Washing was performed with 50 mM HEPES, pH 7.5, 2 ml/min at 4°C. IGFBP-3 fragments were eluted by 0.5 M acetic acid at 0.25 ml/min.

Proteins eluted from the IGF-I affinity column were further separated at 4°C by FPLC reversed-phase chromatography (ProRPC C1/C8 HR 5/10) equilibrated with 0.05 % trifluoroacetic acid (TFA). The proteins, dissolved in 0.5 M acetic acid, were injected at 0.3 ml/min. Separation was performed applying a gradient of 0–100 % acetonitrile in 0.05 % TFA. IGFBP-3-immunoreactive fractions were lyophilized and resuspended in 0.5 M acetic acid, pooled and further separated by SMART reversed-phase chromatography (μRPC C2/C18 SC 2.1/10) at 200 μl/min and room temperature in the same buffers used for FPLC reversed-phase chromatography. The acetonitrile concentration was increased 0–25 % in 2.5 min, 25–50 % in 12.5 min, and 50–100 % in 5 min.

N- and C-terminal amino acid sequence analysis. After electroblotting to PVDF membranes and detection by Coomassie staining, protein bands of expected size were cut out. For N-terminal sequence analysis, a Procise cLC or a Procise HT sequencer (Applied Biosystems) was used. For C-terminal sequence analysis, a Procise C instrument (Applied Biosystems) was employed and operated as described [23]. Two separate preparations of the 30-kDa IGFBP-3 fragments isolated from pregnancy serum were analyzed by N-terminal sequence analysis and a third preparation was analyzed by C-terminal sequence analysis. In addition, both the N- and C-terminal sequences were analyzed on a fourth preparation from pregnancy serum. A single preparation of the 30-kDa IGFBP-3 protein from non-pregnancy serum was analyzed by both N- and C-terminal sequence analysis.

Mass spectrometry. The purified 30-kDa IGFBP-3 fragment from pregnancy serum was subjected to mass spectrometric analysis to confirm and reinforce the results obtained by N- and C-terminal sequence analysis. Briefly, the 30-kDa fragment separated by SDS-PAGE was electroblotted onto a PVDF-membrane and stained with Coomassie. The IGFBP-3 band was excised and the protein extracted followed by in-solution trypsin digestion [24]. As opposed to in-gel digestion, extraction of intact proteins from PVDF membranes for digestion in solution reduces the risk for entrapment of large and/or hydrophobic peptides in the gel matrix. This procedure results in improved sequence coverage in the mass spectrometric analysis [24]. For analysis, a Voyager DE-Pro MALDI mass spectrometer (Applied Biosystems) and a nano-electrospray quadrupole time-of-flight tandem mass spectrometer (Micromass) were used.

Immobilization of IGFBPs on Biacore sensorchips. Equal volumes of NHS and EDC were mixed, and 35 μl of the mixture were injected over the surface of the sensorchip to activate the carboxymethylated dextran. IGFBP dissolved in 10 mM sodium acetate, pH 4.5 (15 μg/ml), was injected over the activated surface followed by addition of 35 μl ethanolamine to deactivate remaining carboxyl groups. The immobilization procedure was carried out at 25°C, 5 μl/min. A final binding corresponding to approximately 1000 response units (RU) was achieved. On each chip, the first of the two flow cells was used as an in-line blank reference. The carboxymethylated dextran in the reference cell was activated and deactivated as described above, but without any ligand bound.

Three IGFBP-3 sensorchips were prepared. Purified 30-kDa IGFBP-3 fragment from pregnancy serum was immobilized on one sensorchip, while intact IGFBP-3 purified from non-pregnancy serum was immobilized on a second chip. Finally, as a control for the possible effects of IGFBP-3 fragments co-purified with intact IGFBP-3, the rhIGFBP-3 was immobilized on a third sensorchip. In addition, sensorchips with immobilized IGFBP-1 and IGFBP-2 were prepared as controls to check for the possible presence of IGFBP-1 or IGFBP-2 on IGFBP-3 sensorchips using specific antibodies.

Kinetic analysis. All experiments were carried out at 25°C and with HBS-EP buffer at 10 μl/min. Analyte [IGF-I, IGF-II, or des(1–3)IGF-I], 35 μl diluted in HBS-EP buffer, was injected over the immobilized binding protein followed by a 5-min period with buffer alone passing over the surface. IGF-I and IGF-II at concentrations 3.13, 6.25, 12.5, 25, 50, and 100 nM were passed over each sensorchip. Due to a lower affinity of des(1–3)IGF-I to the 30-kDa IGFBP-3 fragment, higher concentrations were used to obtain reliable kinetic data (31.2, 62.5, 125, 250, 500, and 1000 nM). All kinetic assays were followed by an injection of 15 μl 0.1 M HCl to dissociate IGF from the binding protein. This regeneration procedure of the sensorchips was shown to not affect the binding of IGFs after multiple applications (data not shown). BIAevaluation 3.0 Software was used for data analysis. A 1:1 mass transfer curve-fitting model was used in the evaluation.

Since the quantity of immobilized IGFBP differed slightly, a simulation program (BIAsimulation Software 2.0) was used to compare the binding of IGFs to the three sensorchips. The mean on- and off-rates were employed for simulation using a prefixed concentration of 25 nM and an Rmax of 250 RU.

Statistics. The kinetic rate constants were from three independent experiments and the mean on- and off-rates were calculated with 95 % confidence intervals. Dissociation constants were calculated by dividing off-rates with on-rates to get K_d (M). Comparisons of on- and off-rates were made using Kruskal-Wallis One Way ANOVA on Ranks (SigmaStat, version 3.1, Chicago, USA). Student-Newman-Keuls method was used for all pairwise multiple comparison procedures. Differences were considered significant for $p<0.05$.

Results

Purification of a 30-kDa IGFBP-3 fragment and intact IGFBP-3

Western immunoblotting of pooled pregnancy serum demonstrated a single dominating band at 30 kDa, while pooled non-pregnancy serum contained IGFBP-3 immunoreactivity at both 40–43 and 30 kDa (Fig. 1). The quantity of intact 40–43-kDa IGFBP-3 relative to that of the 30-kDa IGFBP-3 varied in non-pregnancy serum among different individuals tested (data not shown). Occasionally very faint IGFBP-3 bands at 20 and 16 kDa were detectable in pregnancy serum (not visible in Fig. 1). On 2-D PAGE, 30-kDa IGFBP-3 from pregnancy and non-pregnancy serum migrated similarly displaying a train of seven spots, most likely an effect of various degrees of phosphorylation (data not shown).

After acidic size exclusion chromatography and IGF-I affinity chromatography, IGFBP-3-immunoreactive material was further separated by reversed-phase chromatography on a C1/C8 column. Both intact 40–43-kDa IGFBP-3 and the 30-kDa IGFBP-3 fragment eluted at 29–31 % acetonitrile. In the subsequent C2/C18 reversed-phase chromatography, IGFBP-3 immunoreactivity was detected only in two fractions eluting at 34–38 % acetonitrile (lanes 10 and 11, Fig. 1a, pregnancy serum and Fig. 1b, non-

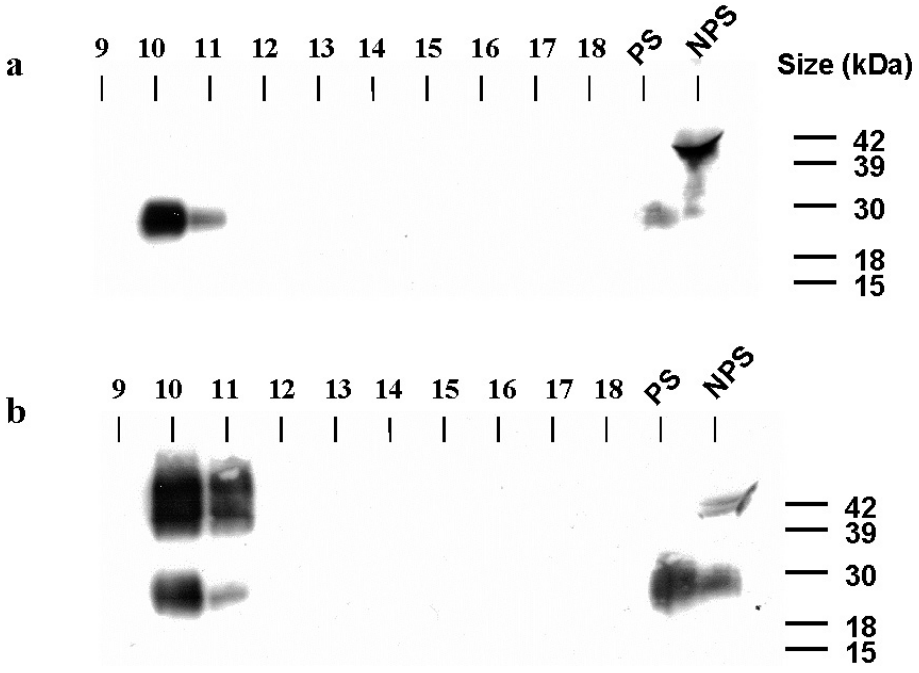

Figure 1. Western immunoblotting of material from the purification of insulin-like growth factor (IGF) binding protein (IGFBP)-3 from serum. Material obtained after acid exclusion chromatography, IGF-I affinity chromatography and FPLC C1/C8 reversed-phase chromatography was subjected to SMART C2/C18 reversed-phase chromatography and the fractions were analyzed: (*a*) pregnancy serum (*b*) non-pregnancy serum. Starting material was included as controls: pregnancy serum (PS) and non-pregnancy serum (NPS).

pregnancy serum). The purified material migrated identically to the starting material in SDS-PAGE, and silver staining revealed that the 30-kDa band in pregnancy serum, and 30- and 40–43-kDa bands in non-pregnancy serum, were the predominant protein species (Fig. 2). Electrophoresis under reducing conditions did not change the migration (Fig. 2). Recovery of IGFBP-3 during the purification was evaluated using Western immunoblotting and found to be in the range 50–70 % for each purification step.

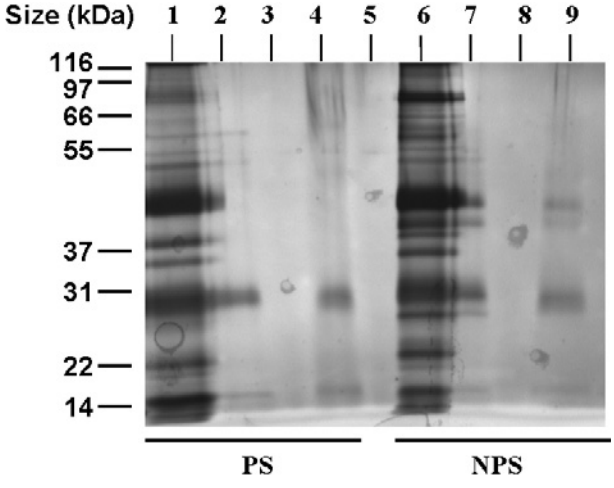

Figure 2. Silver staining of the IGFBP-3-immunoreactive material obtained in fraction 10 after C2/C18 reversed-phase chromatography. Lane 1: Molecular weight markers; lane 2: (1–212)IGFBP-3 from pregnancy serum, non-reduced conditions; lane 3: blank. lane 4: (1–212)IGFBP-3 from pregnancy serum, reduced conditions; lane 5: blank; lane 6: molecular weight markers; lane 7: intact and (1–212)IGFBP-3 purified from non-pregnancy serum under non-reduced conditions; lane 8: blank; lane 9: intact and (1–212)IGFBP-3 purified from non-pregnancy serum under reduced conditions.

Structural analysis of the 30-kDa fragment from pregnancy and non-pregnancy serum

N-terminal sequence analysis of the 30-kDa fragment isolated from pregnancy serum consistently showed the sequence GASSG that is identical to the N-terminal sequence of intact human IGFBP-3. C-terminal sequence analysis resulted in the determination of only one residue, a C-terminal asparagine. Since no further residues were detected, proline was anticipated in the next position, explaining the lack of further sequence information (proline is known to terminate C-terminal degradation under the standard conditions used in this experiment). The IGFBP-3 C-terminal sequence concluded at this stage, Pro-Asn-OH, corresponds uniquely to amino acids 211–212 and is therefore the likely C terminus of the 30-kDa fragment (Fig. 3).

Nine tryptic peptides of the (1–212)IGFBP-3 fragment were identified by mass spectrometry (Fig. 3). The first three peptides are located in the N-terminal region including the N terminus and correspond to residues 1–12, 61–66, and 67–84. The following five peptides are mainly located in the linker region corresponding to residues 85–95, 118–132, 138–144, 145–149, and 161–178. The last tryptic peptide is mainly located in the conserved C-terminal segment of the 30-kDa fragment, corresponding to residues 180–187.

Figure 3. The amino acid sequence of human IGFBP-3. Numbering corresponds to the full-length protein without the secretory signal peptide sequence. Underlined residues correspond to the conserved N- and C-terminal segments. The residues determined by N- and C-terminal amino acid sequence analysis of the 30-kDa fragment from pregnancy and non-pregnancy serum are shown in bold. Gray-shaded areas indicate tryptic peptides identified by mass spectrometry. The basic amino acid sequence 215–232 is highlighted using gray font and contains heparin, acid labile subunit (ALS) and IGF-I binding sites, and a nuclear localization site.

Subsequently, the 30-kDa fragment, co-purified with the intact form of IGFPB-3 from non-pregnancy serum, was N- and C-terminally sequenced. At this point, improvements of the C-terminal sequencing technique [23] allowed determination of the C-terminal sequence Ile-Pro-Asn-OH, corresponding to residues 210–212, which is identical to the finding for the pregnancy serum fragment (above). The N-terminal sequence was as before GASSG.

Kinetic analysis

Real time association (on-rate) and dissociation (off-rate) were recorded for the interactions of IGF-I, IGF-II, or des(1–3)IGF-I with three different IGFBP-3 preparations immobilized on Biacore sensorchips: purified (1–212)IGFBP-3 from pregnancy serum, purified intact IGFBP-3 from non-pregnancy serum or rhIGFBP-3 (Fig. 4). The mean on- and off-rates, and the dissociation constants are given in Table 1.

IGF-I affinity. IGF-I binding to native IGFBP-3 or rhIGFBP-3 displayed similar K_ds of approximately 0.25 nM. Binding of IGF-I to (1–212)IGFBP-3, native IGFBP-3 or rhIGFBP-3 displayed similar on-rates (Table 1). In contrast, the off-rate was 11-fold higher ($p<0.05$) for IGF-I binding to (1–212)IGFBP-3 as compared with IGF-I binding to native IGFBP-3. This increased off-rate of (1–212)IGFBP-3 results in a 11-fold increase in K_d as compared with native IGFBP-3. These differences are apparent in the simulated sensorgrams (Fig. 5).

IGF-II affinity. IGF-II binding to native IGFBP-3 and rhIGFBP-3 displayed a similarly high K_d as IGF-I binding, although with slower kinetics, *i.e.*, lower on- and off-rates. The change in kinetics of IGF-II after IGFBP-3 proteolysis follows the same pattern as that for IGF-I (Fig. 5). The on-rates were similar for IGF-II binding to (1–212)IGFBP-3 and native IGFBP-3. The off-rate for IGF-II to (1–212)IGFBP-3 was higher than that for IGF-II to native IGFBP-3 and rhIGFBP-3, resulting in a 4-fold higher K_d. Thus, IGF-II binding was not as affected by IGFBP-3 proteolysis as the IGF-I binding. The affinity of IGF-II for (1–212)IGFBP-3 was 2.5-fold higher than that of IGF-I (Table 1).

des(1–3)IGF-I affinity. The des(1–3)IGF-I association with native IGFBP-3 was more than 3-fold slower than the association of IGF-I to native IGFBP-3 or (1–212)IGFBP-3, suggesting that the first three amino acid residues in the N-terminal sequence of IGF-I are involved in fast association with the N terminus of IGFBP-3 (Table 1). The des(1–3)IGF-I dissociation from native IGFBP-3 was slower than dissociation of IGF-I from (1–212)IGFBP-3, suggesting that the stabilization of the IGF-I/IGFBP-3 complex is independent of the first three amino acid residues in the N-terminal sequence of IGF-I, but

Table 1. Mean on- and off-rates at 95% confidence interval (C.I.) from Biosensor measurements. Interactions of IGF-I, -II, or des(1–3)IGF-I with immobilized glycosylated rhIGFBP-3 (rhIGFBP-3 chip), IGFBP-3 purified from non-pregnancy serum (nIGFBP-3 chip) or (1-212)IGFBP-3 purified from pregnancy serum ((1–212)IGFBP-3 chip). Dissociation constants (K_d) were calculated by dividing off-rates by on-rates.

	On-rate $\times 10^6$ (mol^{-1} l s^{-1})	95% C.I.	Off-rate $\times 10^{-3}$ (s^{-1})	95% C.I.	Kd $\times 10^{-9}$ (mol l^{-1})
IGF-I					
rhIGFBP-3	**6.60**[b]	2.47–10.7	**1.62**	1.14–2.10	0.246
nIGFBP-3	**5.01**	0.109–10.1	**1.11**	0.883–1.33	0.221
(1-212) IGFBP-3	**4.87**	3.50–6.23	**12.3**[a]	6.58–18.0	2.52
IGF-II					
rhIGFBP-3	**3.33**	1.41–5.26	**0.620**[e]	0.471–0.769	0.186
nIGFBP-3	**2.41**	1.24–3.59	**0.589**[a]	0.485–0.693	0.244
(1-212)IGFBP-3	**2.74**[b]	1.97–3.50	**2.68**[bed]	2.52–2.84	0.978
Des(1-3)IGF-1					
rhIGFBP-3	**1.75**	1.39–2.11	**3.37**	3.13–3.61	1.93
nIGFBP-3	**1.45**[acd]	2.38–5.14	**2.77**[acd]	1.80–3.74	1.92
(1-212)IGFBP-3	**0.0213**[be]	0.0172–0.0254	**3.01**[b]	2.51–3.51	1.42

[a] $p<0.05$ *vs.* IGF-I interacting to nIGFBP-3, [b] $p<0.05$ *vs.* IGF-I binding to (1–212)IGFBP-3, [c] $p<0.05$ *vs.* des(1–3)IGF binding to (1–212)IGFBP-3, [d] $p<0.05$ *vs.* IGF-II binding to nIGFBP-3, [e] $p<0.05$ *vs.* IGF-II binding to (1–212)IGFBP-3.

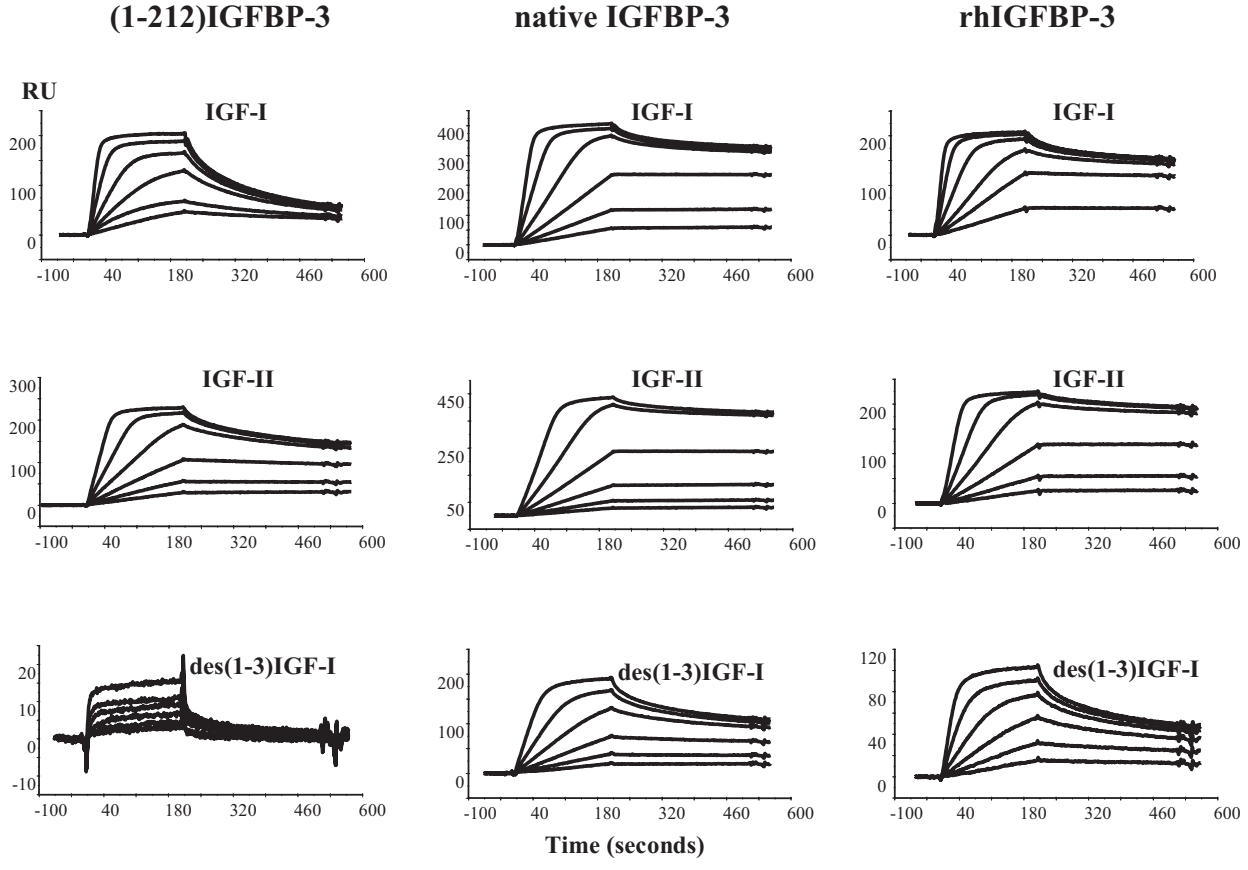

Figure 4. Biacore sensorgrams. Interactions of IGF-I, -II, or des(1–3)IGF-I with immobilized glycosylated rhIGFBP-3 (rhIGFBP-3 chip), intact IGFBP-3 purified from non-pregnancy serum (nIGFBP-3 chip) or 1–212 IGFPB-3 purified from pregnancy serum (1–212IGFBP-3 chip) was recorded at various concentrations of analytes: 3.13, 6.25, 12.5, 25, 50, and 100 nM for IGF-I and IGF-II and 31.2, 62.5, 125, 250, 500, and 1000 nM for des(1–3)IGF-I.

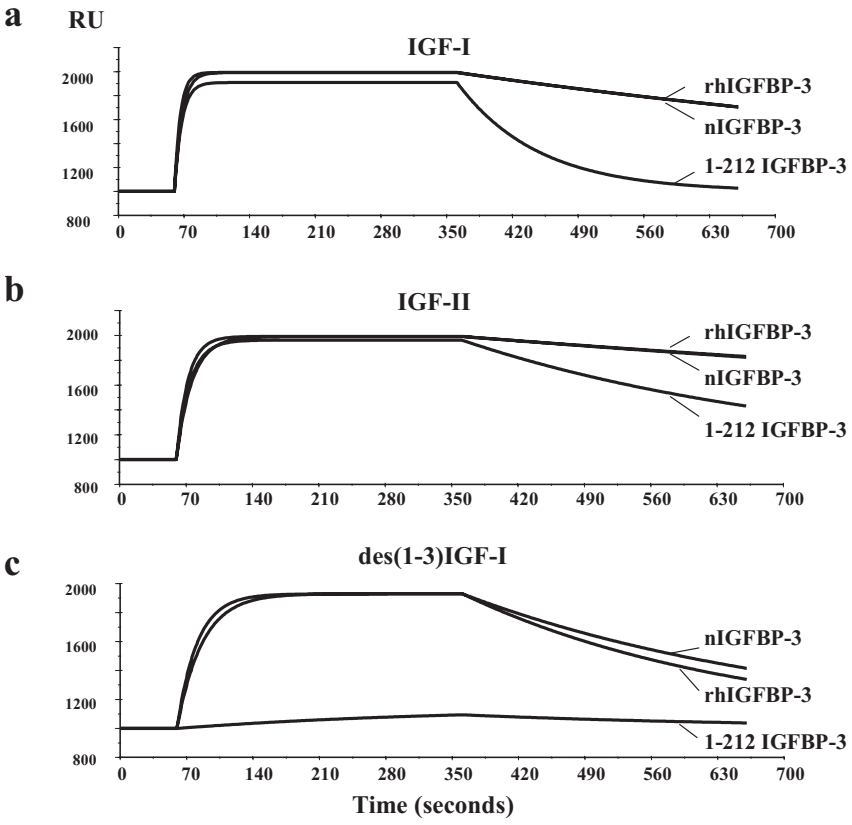

Figure 5. Biacore sensorgram modeling. The data shown in Figure 4 was used to construct an interaction model based on the same density of immobilized protein.

dependent on the C terminus of IGFBP-3. Furthermore, binding of des(1–3)IGF-I was almost completely lost when the distal C-terminal segment of IGFBP-3 was absent in (1–212)IGFBP-3, in accordance with the prediction that the loss of a rapid on- and off-rate binding site, as well as a slow on- and off-rate binding site, results in almost complete loss of binding.

Although SDS-PAGE and silver staining of IGFBP-3 material from pregnancy or non-pregnancy serum did not suggest that other proteins are present, it was still important to confirm that other IGFBPs, or their proteolytic fragments, did not contribute to IGF binding on the various IGFBP-3 sensorchips. To demonstrate this, we passed antibodies against IGFBP-1 and IGFBP-2 over the three IGFBP-3 sensorchips. In contrast to sensorchips with immobilized IGFBP-1 or IGFBP-2, no detectable binding of antibodies could be demonstrated.

Discussion

For the first time, the identity of a 30-kDa N-terminal IGFBP-3 fragment dominating in human pregnancy serum has been demonstrated and found to correspond to residues 1–212 of intact IGFBP-3. In non-pregnancy serum, in addition to intact IGFBP-3, we detected the same 1–212 fragment. Identification of the (1–212)IGFBP-3 fragment was by N- and C-terminal sequence analysis. The unexpected finding of a cleavage site in the conserved C-terminal domain of IGFBP-3 was further supported by mass spectrometry of tryptic peptides covering the sequence (1–212)IGFBP-3 from the N terminus to residue 187.

The IGF-binding kinetics of intact IGFBP-3 purified from serum of non-pregnant women in relation to the (1–212)IGFBP-3 fragment isolated from pregnancy serum was investigated. We show that IGFBP-3 proteolysis increase IGF-I bioavailability through an 11-fold decrease in (1–212)IGFBP-3 affinity for IGF-I and a 4.5-fold decrease in IGF-II affinity. In both cases, the decrease in binding affinity was almost entirely due to faster off-rates. Further kinetic studies of des(1–3)IGF-I binding to intact or (1–212)IGFBP-3 suggested that the N terminus of IGFBP-3 is responsible for rapid association of IGF, while the C-terminus of IGFBP-3 has similar IGF affinity but displays slower on- and off-rates.

Even though the purified (1–212)IGFBP-3 fragment has the same size as the 30-kDa fragment in pregnancy and non-pregnancy serum assessed by Western immunoblotting, we cannot exclude that other fragments of similar size contribute to the immunoreactivity. The characterization of

(1–212)IGFBP-3 from pregnancy serum challenges the results from *in vitro* proteolysis of non-glycosylated rhIGFBP-3 using pregnancy serum, demonstrating a large fragment cleaved in the non-conserved linker-region of the molecule [19]. The susceptibility of this region to proteolysis has also been shown by *in vitro* degradation of non-glycosylated rhIGFBP-3 using purified or recombinant proteases [22]. Absence of *N*-glycosylation in the linker-region of rhIGFBP-3 increases the susceptibility to proteolysis in this region (unpublished data). Furthermore, IGFBP-3 is not associated with IGF-I and ALS in a ternary complex in such *in vitro* experiments. Therefore, it is possible that potential cleavage sites in the linker region of IGFBP-3 are protected *in vivo*. Several proteases, including plasmin, thrombin and trypsin [25] cleave IGFBP-3 at several sites in the C-terminal segment of IGFBP-3, although the cleavage site now shown, between amino acids 212 and 213 of IGFBP-3, has not been reported. The importance of employing both N- and C-terminal amino acid sequence analysis is demonstrated in a study by Ständker *et al.* [26] who isolated two IGFBP-4 fragments from human hemofiltrate. C-terminal sequence analysis demonstrated that the isolated N-terminal IGFBP-4 fragment was eight amino acids shorter than predicted from the N-terminal sequence analysis of the C-terminal fragment. The C-terminal domain of IGFBP-3 containing the cleavage site between residues 212 and 213 is likely to be in an exposed position on the molecular surface as predicted from the 3-D structures of IGFBP-2 and -6 [27, 28]. In our study, the purified (1–212)IGFBP-3 fragment migrated as a 30-kDa fragment on SDS-PAGE, whether it was prepared under reducing conditions or not. Given the conserved pattern of cysteine residues in the N- and C-terminal domains of the IGFBPs, data from studies on recombinant IGFBPs including IGFBP-2, IGFBP-4 and IGFBP-6 [29–31] suggest that Cys^{186} and Cys^{213} in IGFBP-3 form a disulfide bond. If this is the case, cleavage of IGFBP-3 between residues 212 and 213 would generate two polypeptide segments interconnected by the Cys^{186} and Cys^{213} disulfide linkage and the product would appear at a higher mass position on a non-reducing SDS-PAGE than now observed. However, this is not the case here and several explanations are possible: IGFBP-3 may not follow the general pattern of disulfide bonding derived from studies on related IGFBPs; recombinant non-glycosylated proteins for which data are available may fold differently than endogenous proteins and give rise to different disulfide patterns; or activities by enzymes and substances with a reducing potential present in serum may result in separation of the two segments. Further studies are required to address this issue.

The amino acid sequence Pro^{211}-Asn^{212}-Cys^{213}-Asp^{214} in IGFBP-3 surrounding the cleavage site now shown is conserved in all IGFBPs except IGFBP-1. This suggests that the pregnancy IGFBP-3 protease may also degrade other IGFBPs. In accordance, pregnancy-associated plasma protein-A degrades both IGFBP-4 and -5 but not IGFBP-3 [32]. The conserved sequence motif in IGFBP-3 is not included in a consensus cleavage site for any known protease. Although the demonstration of the cleavage site does not give us a direct clue towards the nature of the pregnancy IGFBP-3 protease, the definition of the 30-kDa IGFBP-3 fragment is a prerequisite for structural–functional interpretations. The basic sequence 215–232 of IGFBP-3, close to the identified cleavage site, contains heparin, ALS and IGF-I binding sites as well as a nuclear localization site [22]. Interestingly, heparin has been reported to modulate IGFBP-3 proteolysis in pregnancy serum [19] as well as in non-pregnancy serum [33]. Durham *et al.* [34] demonstrated that prekallekrein degrades IGFBP-3 and that a peptide identical to the heparin-binding domain of IGFBP-3 inhibits IGFBP-3 binding to the protease. Proteolysis of IGFBP-5, which contains a basic amino acid sequence homologous to that in IGFBP-3, is also affected by heparin [35, 36]. Also, the proximity of the pregnancy serum IGFBP-3 cleavage site to the ALS binding site is notable. Although the ALS binding and ternary complex formation does not appear to prevent IGFBP-3 degradation, as already discussed, it may still affect the susceptibility to proteolysis of other regions in the molecule.

Proteolysis of IGFBPs has been shown to increase IGF bioactivity in various cell systems [22], and pregnancy serum is twice as potent at stimulating cell proliferation compared to non-pregnancy serum with identical IGF-I and -II content [37]. To understand how the bioavailability of circulating IGFs may be changed by IGFBP-3 proteolysis, we used biosensor technology to analyze the kinetics of IGF-I and -II interactions with (1–212)IGFBP-3 and intact IGFBP-3. Both native and recombinant IGFBP-3 revealed the same affinity for IGF-I as previously reported [38, 39]. We found an 11-fold reduction in affinity of IGF-I for the (1–212)IGFBP-3 pregnancy fragment compared to that for intact IGFBP-3, which is almost entirely due to a faster dissociation of IGF-I. Taking into account that the ternary complex is preserved in pregnancy [2, 17], this finding may explain that circulating stores of total IGF-I is maintained in pregnancy at the same time as IGF-I bioavailability is increased [14, 16, 37, 40]. The changes we detected in IGF-I and -II affinities are similar to those estimated

by Laserre and Binoux [16] using semi-purified IGFBP-3 fragments from pregnancy serum. Furthermore, these authors also observed a faster IGF dissociation using radiolabeled IGF-I and -II. Our data demonstrates that IGF-I bioavailability is more affected by proteolysis than IGF-II bioavailability. Whether this makes endocrine IGF-II a less important growth factor for placental and fetal growth in pregnancy has to be evaluated. In addition to effects on IGF-dependent actions, IGFBP-3 proteolysis may have impact on IGF-independent actions including IGFBP-3 induced insulin resistance [41, 42]. The generation of IGFBP-3 fragments lacking IGF binding affinity has been found to result in new biological activity [43]. Such potentially important IGF-independent activity of (1–212)IGFBP-3 needs to be evaluated.

To further characterize the structural and functional importance of the loss of the C-terminal segment in IGFBP-3 for IGF-I binding, we compared the binding kinetics of IGF-I with that of des(1–3)IGF-I, lacking the N-terminal tripeptide sequence Gly^1-Pro^2-Glu^3 [44]. It has been shown that Glu^3 in IGF-I, corresponding to Glu^6 in IGF-II, contributes to a binding patch that interacts with IGFBP-5 and most likely with IGFBP-3 (see review by Carrick *et al.* [45]). We have recently demonstrated that Ile^{56}, Leu^{80} and Leu^{81} contribute to a hydrophobic pocket in IGFBP-3 that is important for IGF-I binding [46]. From co-crystallization experiments of IGF-I and an IGFBP-5 peptide it is shown that Glu^3 in IGF-I form hydrogen bonds with His^{71} and Tyr^{50}, corresponding to Tyr^{57} in IGFBP-3 [47]. Taken together, these data suggest that fast association of IGF-I with IGFBP-3 is dependent on the N-terminal Gly-Pro-Glu sequence in IGF-I and the N-terminal region in IGFBP-3 including Ile^{56}, Tyr^{57}, Leu^{80} and Leu^{81}. IGF-I binding to (1–212)IGFBP-3 displayed a K_d of the same order as for des(1–3)IGF-I binding to intact IGFBP-3. However, slow on- and off-rates were seen in the latter case. This suggests that after fast association of the N-terminal domains of both peptides, the C terminus of IGFBP-3 is involved in stabilizing the complex independently of Gly^1-Pro^2-Glu^3. In accordance, when the fast on- and off- rate interaction as well as the slow on- and off-rate interaction were both lost in binding of des(1–3)IGF-I to (1–212)IGFBP-3, the affinity was largely reduced.

Our study was performed with immobilized binding protein since we experienced problems to obtain reliable data with IGFBP-3 or IGFBP-3 analogues in the mobile phase. This is in accordance with reports from other laboratories [38, 39]. This may in part explain some differences between our results and those of Galanis *et al.* [48] and Wong *et al.* [49] since these authors employed IGF-I immobilized to the sensor chip. Galanis *et al.* found similar association constants for IGF-I binding to intact and 1–88 IGFBP-3, which is in accordance with our results, as is their finding that IGF-I dissociated faster from 1–88 IGFBP-3 and from 165–264 IGFBP-3. However, IGF-I dissociated at least 10 000-fold faster from the two fragments than from intact IGFBP-3, while we found an 11-fold difference between (1–212)IGFBP-3 and intact IGFBP-3. Vorwerk *et al.* [39] have reported that IGF-I affinity for 1–97 IGFBP-3 is reduced 1000-fold compared to that for intact IGFBP-3, while they found that the on- and off-rates were equally affected. Together, these findings suggest that the proximal part of the C-terminal segment corresponding to residues 184–212 may add stability to IGF-I binding. Hence, the current evidences support a dual function of the N- and C-terminal binding sites in reaching the high affinity of IGF-I for IGFBP-3.

IGF-I and IGF-II is believed to equally contribute to normal fetal growth (reviewed in [50]). Recent findings of intrauterine growth retardation in patients with congenital severe primary IGF-I deficiency [51] or IGF-II deficiency due to imprinting disorders [52] demonstrate the importance of IGFs in human fetal growth. Our study addresses the regulation of IGF bioavailability in the maternal circulation. Maternal IGFs are important for placental growth and transfer of nutrients and thereby important for fetal growth. However, in a recent study in guinea pigs IGF-I was shown to be more important [53]. It is possible that our finding of increased bioavailability of IGF-I *versus* IGF-II due to IGFBP-3 proteolysis may explain this finding.

In conclusion, we have purified a 30-kDa IGFBP-3 fragment corresponding to residues 1–212 from human pregnancy serum where it dominates, and from non-pregnancy serum where it coexists with intact IGFBP-3. IGFs dissociates faster from the 1–212 fragment than from intact IGFBP-3. Interestingly, IGF-I binding was more affected than IGF-II binding. Future studies will be directed towards understanding of the physiological role of this IGFBP-3 fragment.

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