FEBS Letters 518 (2002) 124–128 FEBS 26050

Isolation and characterization of circulating fragments of the insulin-like growth factor binding protein-3

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Received 13 February 2002; revised 3 April 2002; accepted 4 April 2002

First published online 10 April 2002

Edited by Judit Ovádi

Abstract Proteolysis of insulin-like growth factor binding protein-3 (IGFBP-3), the major carrier of IGFs in the circulation, is an essential mechanism to regulate IGF bioavailability. To analyze naturally occurring IGFBP-3 fragments a peptide library established from human hemofiltrate was screened. Three IGFBP-3 fragments were detected with apparent molecular masses of 34, 16, and 11 kDa. Mass spectrometric and sequence analysis identified the 16 and 11 kDa peptides as glycosylated and non-glycosylated N-terminal fragments spanning residues Gly^1–Ala^{98} of IGFBP-3. Both the circulating forms and those secreted from IGFBP-3^{1-98} overexpressing cells bound IGF. Additionally, two smaller fragments (IGFBP-$3^{139-157}$ and IGFBP-$3^{139-159}$) were identified in the hemofiltrate. The data indicate that proteolysis of circulating IGFBP-3 occurs in the variable domain at residues alanine 98, phenylalanine 138, glutamine 157, and tyrosine 159. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Insulin-like growth factor binding protein-3; Circulating fragment; Peptide library; Glycosylation

1. Introduction

The bioavailability of the insulin-like growth factors, IGF I and IGF II, is modulated in the circulation and extracellular environment by six different IGF binding proteins (IGFBP-1–IGFBP-6) that bind IGFs with high affinities [1,2]. In human serum the majority of IGFs circulate as 150 kDa complex consisting of IGFBP-3, an approximately 85 kDa acid-labile subunit and IGF I or IGF II [3]. The ternary complexes function as circulating reservoirs of IGFs and prolong the half-lives of IGFs by preventing their clearance [4]. IGFBP-3, the most abundant IGFBP, is found in serum as a characteristic doublet of approximately 43–45 kDa consisting of two differentially glycosylated forms [5].

IGFs can be released from bi- and ternary IGF–IGFBP complexes by limited proteolysis of IGFBPs resulting in the generation of fragments with reduced affinities for IGFs [6]. In pregnancy serum IGFBP-3, -4, and -5 proteases have been characterized and identified as disintegrin metalloprotease ADAM 12S and PAPP-A [7–12]. Furthermore, elevated IGFBP-3 protease activity has been shown under catabolic and disease states, e.g. in patients with acute or chronic renal failure or in diabetes [13–15]. The isolation of naturally occurring fragments of IGFBP-4 and -5 in human hemofiltrate (HF), the identification of cleavage sites, and their IGF binding properties [16,17] support the physiological significance of IGFBP proteolysis.

In the present study we report on the purification and biochemical characterization of IGFBP-3 fragments from human HF allowing the delineation of IGFBP-3 protease cleavage sites.

2. Materials and methods

2.1. Chromatographic isolation of IGFBP-3 fragments

A peptide library was generated from 10 000 l of HF as described recently [18]. The lyophilized reversed phase (RP) fractions of human HF were tested for IGFBP-3 immunoreactivity. Immunoreactive fractions were pooled and subjected to further RP chromatography on a C18 Prepak cartridge (300×47 mm inside diameter (i.d.), 30 nm, 15–20 μm, YMC, Schernbeck, Germany). Proteins were eluted using a linear gradient (eluent A: 20:80 v/v MeOH/H_2O, 0.1% TFA; eluent B: 0.1% TFA in 100% MeOH) at a flow rate of 40 ml/min. The most intensive IGFBP-3 immunoreactive fractions were pooled and separated by size-exclusion chromatography (140×920 mm i.d., Superdex prepgrade 75, Pharmacia, Freiburg, Germany). The buffer used for the isocratic elution was 200 mM ammonium formiate, pH 7.4, with a flow rate of 100 ml/min. Immunoreactive fractions were further enriched using cation-exchange chromatography (column of 150×30 mm i.d., Fractogel TSK Sp650 S, Merck, Darmstadt, Germany). Elution of proteins was performed using a linear NaCl gradient (buffer A: 20 mM NaH_2PO_4, pH 5.0; buffer B: buffer A containing 1.5 M NaCl; flow rate: 10 ml/min; gradient: 0–50% buffer B within 40 min). Final purification of immunoreactive fractions was carried out on an analytical RP-C18 column (250×4.6 mm i.d., 30 nm, 5 μm, ODS AQ, YMC). Elution of proteins was achieved with a flow rate of 0.7 ml/min using a linear gradient (30–50% B within 60 min) with the above-mentioned RP solvents.

2.2. SDS–PAGE and Western immunoblotting

Lyophilized aliquots of high performance liquid chromatography (HPLC) fractions were reconstituted in non-reducing sample buffer,

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Abbreviations: IGF, insulin-like growth factors; IGFBP, IGF binding protein; bIGF, biotinylated IGF; ESI–MS, electrospray ionization–mass spectrometry; MALDI–MS, matrix-assisted laser desorption ionization–mass spectrometry; RP-HPLC, reversed phase high performance liquid chromatography

0014-5793/02/$22.00
PII: S0014-5793(02)02673-X

separated by TRICINE–SDS–PAGE [19], transferred to nitrocellulose membranes (Trans-Blot, Bio-Rad, Munich, Germany) and incubated with rabbit anti-IGFBP-3 antiserum (Upstate Biotechnology, Lake Placid, NY, USA; 1:1 000) followed by anti-rabbit IgG coupled to alkaline phosphatase (Sigma) or goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) (Dianova, Hamburg, Germany; 1: 10 000). Reactive bands were visualized using a bromochloroindolyl phosphate/nitrotetrazolium substrate system (Sigma) or the SuperSignal enhanced chemiluminescence (ECL) detection system (Pierce Chemicals, Rockford, IL, USA).

2.3. Ligand blotting

After transfer of polypeptides to nitrocellulose, membranes were incubated with phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 followed by incubation with 20 ng/ml mono-biotinylated IGFs (mono-bIGFs; GroPep, Adelaide, Australia). IGF binding peptides were visualized by streptavidin-coupled HRP (Jackson Immunoresearch, West Grove, PA, USA; 1:10 000) and ECL.

2.4. Peptide analytics

Mass determination of peptides was performed by electrospray ionization–mass spectrometry (ESI–MS) using a Sciex API III quadrupole mass spectrometer (Sciex, Perkin-Elmer, Langen, Germany) or by matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS; Voyager-DE STR, Perseptive Biosystems, Framingham, MA, USA) as described previously [20,21]. The purity of isolated IGFBP-3 peptides was investigated by capillary zone electrophoresis and N-terminal sequencing was performed by conventional Edman degradation as described recently [20].

2.5. Carbohydrate analysis

Peptides were incubated with neuraminidase (1 mU/μl) and/or *N*-glycosidase (1 mU/μl) (Roche Diagnostics, Mannheim, Germany) at 37°C overnight and the reaction mixture was analyzed using an HPLC–ESI–MS method as described recently [20]. Alternatively, the polypeptides were transferred to nitrocellulose, blocked and incubated with biotinylated concanavalin A (Sigma; 10 μg/ml). The glycosylated polypeptides were visualized by streptavidin-coupled HRP and ECL.

2.6. Generation of IGFBP-3$^{1-98}$

Human IGFBP-3 cDNA in pBSK was a gift from J. Zapf (Zurich, Switzerland). A 1.2 kb fragment was subcloned using flanking *Bgl*II sites into *Bam*HI predigested expression vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany). The mutant IGFBP-3$^{1-98}$ cDNA was generated by site-directed mutagenesis (QuikChange, Stratagene) using the primer1 (5′-CGCCTGCGCGCCTAAGATCTGCCAGCGCCG-3′) and primer2 (5′-CGGCGCTGGCAGATCTTAGGCGCGCAGG-CG-3′) to introduce a stop codon (TAA) at the corresponding triplet coding tyrosine residue 99 (TAC) and an additional *Bgl*II site for restriction analysis. Oligonucleotides were synthesized by Nucleic Acids Products Supply (Göttingen, Germany). The mutation was verified by sequencing.

2.7. Cell culture and transfection

The embryonic rat liver cell line RLC-18 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The cells were transfected with 10 μg of plasmid DNA using FUGENE 6 (Roche, Mannheim, Germany). Stable colonies selected for the resistance against neomycin (Invitrogen) were isolated and screened for IGFBP-3 fragment expression in media conditioned for 48 h by IGFBP-3 Western blotting.

3. Results

Fractions of a peptide bank generated from 10 000 l of HF [18,21] were tested for the presence of circulating fragments of IGFBP-3. In the first step, peptides of the HF bound to a cation-exchange column were eluted stepwise with eight buffers of increasing pH (Fig. 1A) followed by fractionation of the resulting pH pool eluates by RP-HPLC. When aliquots of these RP fractions were analyzed by immunoblotting, three prominent IGFBP-3 immunoreactive polypeptides of approximately 34, 16, and 11 kDa were detected in fraction 23–26 of pH pool 2 (not shown) and in fractions 23 and 24 of pH pool 3 (Fig. 1B). All three IGFBP-3 fragments bound IGF II as shown by ligand blotting (Fig. 1C).

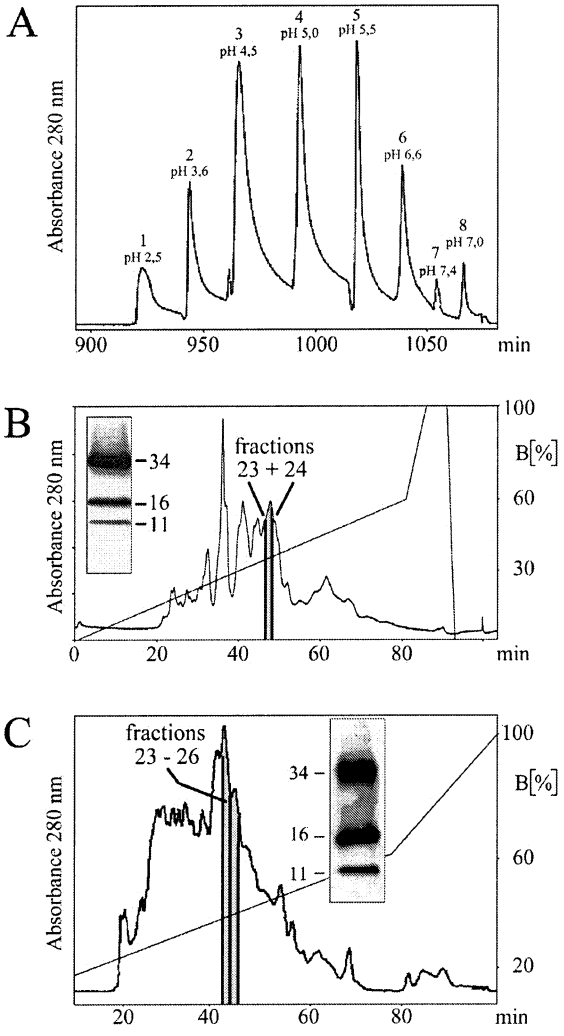

Fig. 1. IGFBP-3 immunoblot-screening in fractions of a peptide library. A: After cation-exchange chromatography, bound peptides of HF were batchwise eluted with increasing pH (pH pool fractions 1–8). B: IGFBP-3 immunoreactive material was detected in fractions derived from pH pool eluate 2 and 3. The RP-HPLC fractionation profile of the peptides from pH pool eluate 3 is shown. The fractions 23 and 24 containing IGFBP-3 fragments (black bar) with apparent molecular masses of 34, 16, and 11 kDa (insert) are marked. C: Aliquots of each fraction collected by RP-HPLC chromatography derived from pH pool 2 eluate were also tested by IGF II ligand blotting. The IGFBP-3 immunoreactive peptides of 34, 16, and 11 kDa in the fractions 23–26 (black bars) bound also IGF II (insert).

The immunoreactive IGFBP-3 fragment with an apparent molecular mass of 16 kDa was subsequently purified by the use of RP, size-exclusion, and cation-exchange chromatography (data not shown) accompanied by IGFBP-3 immunoblotting. A final analytical RP-chromatography resulted in a single peak (Fig. 2A). N-terminal sequence analysis of this fraction revealed GASSAGLGPVVRXEPX (X, unidentified amino acids) proving the isolation of the N-terminal fragment of human IGFBP-3. By MALDI–MS analysis (not shown) and ESI–MS (Fig. 2B) the exact molecular mass of the puri-

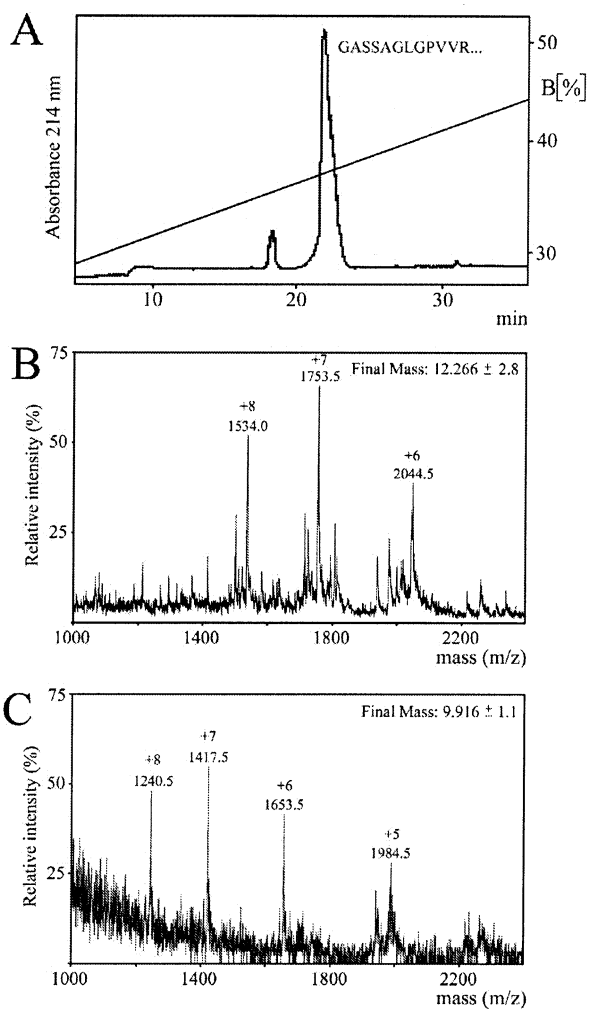

Fig. 2. A: Isolation of the 16 kDa IGFBP-3 fragment by analytical RP-HPLC chromatography followed by Edman degradation. The resulting N-terminal amino acid sequence is given. B: The ESI–MS spectrum of the apparent 16 kDa N-terminal IGFBP-3 fragment revealed a molecular mass of 12 266 ± 2.8 Da. C: The ESI–MS spectrum of the deglycosylated IGFBP-3 fragment revealed a molecular mass of 9 916 ± 1.1 Da.

fied IGFBP-3 fragment was calculated to be 12 266 ± 2.8 Da. Neuraminidase treatment of this fraction resulted in a decrease in molecular mass of 582 Da to 11 685 ± 3.6 Da indicating the removal of two *N*-acetyl neuraminic acid residues. Further treatment with *N*-glycosidase F yielded in a polypeptide of 9 916 ± 1.1 Da (Fig. 2C) that was identical with the theoretical mass of disulfide bridged N-terminal IGFBP-3 fragment (residues 1–98) with an Ala residue in position 5. This result was supported by the observation that reduction and amido alkylation of this IGFBP-3 fragment increased the molecular mass to 10 607 ± 9.9 Da corresponding to 12 alkylated cysteine residues (mass increase of 58 Da for each cysteine). The ESI–MS spectrum of the isolated IGFBP-3 fragment with an apparent molecular mass of 11 kDa showed also a molecular mass of 9 916 Da (not shown) suggesting that the circulating 11 kDa IGFBP-3 fragment represents the deglycosylated 16 kDa IGFBP-3 fragment. IGFBP-3 immunoblot analysis of media from rat liver carcinoma cell line RLC-18 expressing the truncated human IGFBP-3^{1-98} revealed the presence of 16 and 11 kDa IGFBP-3 fragments (Fig. 3) demonstrating that the different electrophoretic mobilities of the fragments were due to posttranslational modifications. This observation was supported by the finding that the binding of the lectin concanavalin A was restricted to the 16 kDa but not to the 11 kDa IGFBP-3 fragment (Fig. 4). Additionally, when the fraction 24 of pH eluate 3 was treated with *N*-glycanase, the mass of the majority of the 16 kDa IGFBP-3 fragment was reduced to 11 kDa (not shown). Ligand blotting experiments showed that both IGF I and IGF II bound to the 16 and 11 kDa IGFBP-3 fragments with similar intensity (Fig. 4). Two additional IGFBP-3 fragments comprising the amino acid residues 139–157 and 139–159, were identified by system-

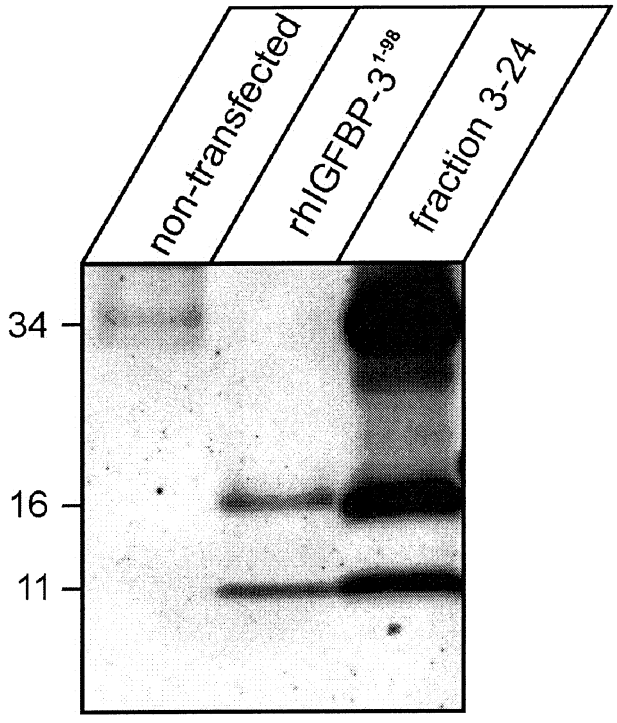

Fig. 3. IGFBP-3 immunoblot of recombinant IGFBP-3^{1-98}. Aliquots of media from RLC-18 cells transfected with the human IGFBP-3^{1-98} cDNA and of the fraction 24 of pH pool 3 eluate were analyzed by Tricine SDS–PAGE and IGFBP-3 immunoblotting.

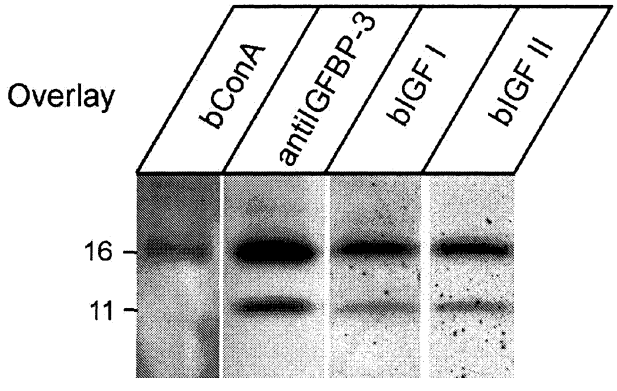

Fig. 4. Glycosylation and IGF binding of IGFBP-3 fragments. Equal aliquots of fraction 24 of pH pool 2 eluate were separated by Tricine SDS–PAGE, transferred to nitrocellulose and analyzed either by concanavalin A (bConA), bIGF I, bIGF II, or anti-IGFBP-3 overlay and ECL.

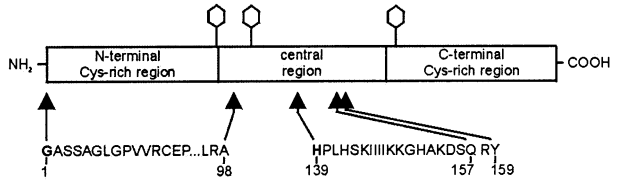

Fig. 5. Schematic diagram of the IGFBP-3 structure and proteolytic cleavage sites as detected in human HF.

atic sequence analysis of peptides derived from the HF pH pool 7 fraction.

4. Discussion

In the present study, the isolation and biochemical characterization of circulating IGFBP-3 fragments derived from in vivo proteolysis are described. Five IGFBP-3 fragments were identified by immunoblotting (34, 16, and 11 kDa fragments) and systematic peptide sequencing (2–2.5 kDa) of fractions from a peptide library generated from human HF (Fig. 5). The exact mass and cleavage sites of the 34 kDa IGFBP-3 fragment could not be determined. Treatment of a fraction containing the 34 kDa fragment with *N*-glycanase under non-reducing conditions decreased the size of the fragment to a major band of 26 kDa and a minor band of 22 kDa on SDS–PAGE analysis (B. Kübler, unpublished results). Consistent with the findings that the mass of an N-linked carbohydrate chain is approximately 4–5 kDa [5], our data suggest that the 34 kDa IGFBP-3 fragment contains all three *N*-glycosylation sites (N^{89}, N^{109} and N^{172}) which are not fully accessible to *N*-glycanase. Zapf et al. [22] have also reported on the isolation of a 30–32 kDa IGFBP from adult rat serum with the N-terminal sequence of IGFBP-3 which has been assumed to be a degradation product digestable with *N*-glycanase to a 26 kDa form.

Both the 16 and 11 kDa fragments contain the N-terminus and comprise the amino acid residues 1–98 of IGFBP-3. Whereas the 16 kDa IGFBP-3 fragment contains an N-linked bi-antennary carbohydrate chain with two *N*-acetyl neuraminic acid residues, the 11 kDa fragment represents a non-glycosylated circulating IGFBP-3^{1-98} fragment. Proteolytic modifications of IGFBPs are thought to be a principle mechanism for regulating IGF availability and IGF-independent functions of IGFBPs. Serum IGFBP-3 proteolysis has been reported in severe illness, after surgery, in cancer patients, in diabetes, and during pregnancy [8,15,23,24]. A disintegrin and metalloprotease, presumably ADAM 12 S, is the most likely candidate for the IGFBP-3 protease in pregnancy serum [9,10]. However, several other neutral proteases such as the prostate specific antigen (PSA), plasmin, thrombin, matrix metalloproteases (MMPs) as well as the acidic proteases cathepsin D and L have been reported to function as IGFBP-3 proteases in vitro [25–29]. Sequence analysis revealed that IGFBP-3^{1-97} is a major cleavage product of PSA, plasmin and thrombin [25,26] and IGFBP-3^{1-99} is formed by MMP-1 [27]. The isolation of the IGFBP-3^{1-98} fragment from HF suggests that either none of these proteases are involved in the proteolysis of circulating IGFBP-3, or the half-lives of the generated fragments are significantly shorter. However, it can not be excluded that the IGFBP-3^{1-98} fragment represents an intermediate proteolytic product which might be further modified by other endo- or carboxy peptidases. It is currently unknown whether different serum proteases with distinct cleaving site specificities are responsible for proteolysis of IGFBP-3 during pregnancy and catabolic states. The isolation of the non-glycosylated IGFBP-3^{1-98} fragment was surprising and its origin is unclear because aspartylglucosaminidases hydrolyzing complete carbohydrate chains from glycoproteins are localized intracellularly [30] whereas successive terminal deglycosylation of the intact or IGFBP-3 fragment would be accompanied by rapid clearance form the circulation.

Both the glycosylated and non-glycosylated IGFBP-3^{1-98} bound IGFs which is consistent with the findings of other laboratories that (i) N-terminal IGFBP-3 fragments bind IGFs with reduced affinities [31,32], and (ii) glycosylation of the intact IGFBP-3 is not essential for IGF binding [5]. However, the IGFBP-3^{1-97} fragment generated by in vitro proteolysis by plasmin expresses also IGF-independent bioactivities such as inhibition of insulin receptor autophosphorylation [31], and FGF-activated mitosis [33], or the stimulation of glucose uptake in microvessel endothelial cells [34]. Whether the isolated IGFBP-3^{1-98} fragment is biologically active too is not known. Although the objective of this study was to isolate and identify circulating IGFBP-3 fragments generated in vivo, it will be interesting to investigate the modulation of IGF activity by IGFBP-3 fragments in biological assays. The ability to express the IGFBP-3^{1-98} fragment in rat liver cells is an initial step to examine its potential biological functions. Another question raised by our data concerned the role of IGFBP-3 proteolysis in patients with end stage renal disease used as source of the HF.

Acknowledgements: We thank Ulrike Schrameck for excellent technical assistance. This work was supported by the German government BMBF Grant 0311139.

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