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Strategy for identifying circulating fragments of insulin-like growth factor binding proteins in a hemofiltrate peptide bank

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Abstract

A differentiated strategy was established to isolate circulating forms of the six human insulin-like growth factor binding proteins (IGFBPs). As starting material we used our peptide bank, a comprehensive blood plasma peptidoma generated from human blood filtrate. The peptides were initially identified in the fractions of the hemofiltrate peptide bank by their immunoreactivity, their capacity to bind the insulin-like growth factors (IGFs), and their molecular masses determined by polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS). Fractions revealing both immunoreactivity and IGF-binding capacity were analyzed by direct sequencing of immunoreactive bands from a Coomassie-stained gel. Further purification of the IGFBP peptides was performed by consecutive chromatographic steps guided by sensitive MALDI–MS. Using this strategy, different fragments of IGFBP-3, -4, and -5 were identified and a fragment of IGFBP-4 was purified to homogeneity. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The insulin-like growth factors (IGFs) are known to stimulate cell growth and differentiation in a variety of tissues. They are able to bind with high affinity to the six related insulin-like growth factor binding proteins (IGFBPs), which play an integral role in modulating IGF actions in either an inhibitory or stimulatory manner [1].

Besides these effects of modifying IGF activity, other intrinsic activities of the IGFBPs have been recently described [2,3].

The structure of the IGFBPs consists of the conserved and cysteine-rich N- and C-terminal domains connected by a non-conserved central region. The N- and the C-terminal regions are thought to be IGF-binding domains [4] and proteolysis of intact IGFBPs by different proteases in vitro often yielded two main fragments corresponding to these two domains [5,6].

Some proteolytic fragments of plasma and matrix proteins exhibit important biological activities, which are often independent of functional properties of the parent molecule, e.g. the origin of the potent angiogenesis inhibitors angiostatin or endostatin from plasminogen or collagen XVIII, respectively [7,8]. In this context, recent studies have shown that proteolytic fragments of different IGFBPs reveal biological functions [9].

Therefore, the aim of this study was to identify new circulating IGFBP fragments. The starting material for the screening for novel IGFBP fragments

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was the comprehensive peptide bank of circulating human peptides generated from human hemofiltrate [10]. Human hemofiltrate is obtained during treatment of patients suffering from chronic renal failure and was shown to be an excellent source for the purification of circulating regulatory peptides with M_r<20 000 [11–13].

For the isolation of novel IGFBP fragments, we developed a strategy of characterizing different biochemical and immunochemical parameters before starting the purification. Initially, identification of the IGFBPs in the fractions of the peptide bank was performed using immunoblotting with polyclonal IGFBP antibodies in combination with IGF-binding studies and mass determination by matrix-assisted laser desorption ionization mass spectrometry (MALDI–MS) and polyacrylamide gel electrophoresis (PAGE). Fractions showing both IGFBP immunoreactivity and IGF-binding capacity were subsequently chromatographed by consecutive HPLC steps. The purification of selected IGFBP peptides was guided by MALDI–MS.

Using this strategy, we were able to identify several different IGFBP fragments in the peptide bank and purified a novel fragment of IGFBP-4 to homogeneity.

2. Experimental

2.1. Peptide bank from human hemofiltrate

Circulating human peptides were separated into 400 fractions in a standardized two-step procedure from an initial amount of up to 10 000 l hemofiltrate [10]. In brief, for peptide extraction, the blood filtrate was immediately cooled to 4°C, adjusted to a pH of 2.7, and the peptides were bound to a cation-exchanger followed by batch elution. The first subfractionation was performed by stepwise batch elution using a cation-exchanger (200×250 mm, Merck, Darmstadt, Germany, Fractogel TSK SP 650(M), 100 nm, 40–90 μm) and seven buffers with increasing pH (pH 3.6–9.0). The resulting pH pools were fractionated in the second separation step by reversed-phase (RP) chromatography using a Source RPC-column (125×200 mm, Pharmacia, Freiburg, Germany, 15 μm). Fractions were collected and aliquots were lyophilized and used for the experiments.

2.2. Purification of fractions with liquid chromatography

Further purification of selected fractions from the peptide bank was achieved using RP and cation-exchange HPLC. As RP solvents in gradient elution, 0.1% trifluoroacetic acid (solvent A) and 80% (v/v) acetonitrile containing 0.1% trifluoroacetic acid (solvent B) were used. Twenty m*M* sodium phosphate buffer, pH 3.0 (solvent A) and 20 m*M* sodium phosphate buffer, 1 *M* NaCl, pH 3.0 (solvent B) were the solvents in the cation-exchange chromatography.

First, the selected fractions were applied to a preparative Vydac RP-C_{18} column (300×47 mm, Hesperia, CA, USA, 30 nm, 15–20 μm). For further purification an analytical Nucleosil C_{18} column (250×20 mm, Machery–Nagel, Düren, Germany, 30 nm, 5 μm) was used. The following fractionation step was carried out by cation-exchange chromatography (125×10 mm column, Biotek, Östringen, Germany, Parcomer PepKat Pro Q, 100 nm, 7 μm). Further purification was performed using an analytical Vydac RP-C_4 column (250×20 mm, 30 nm, 5 μm). In the following RP step, an analytical C_{18} column (250×10 mm, YMC, Schernbeck, Germany, 12 nm, 5 μm) was used. An analytical Vydac C_{18} column (250×10 mm, 30 nm, 5 μm) served for final purification.

2.3. Peptide analysis

MALDI mass determination was carried out either using a LaserTec RBT II (PerSeptive Biosystems, Freiburg, Germany) as described previously [14] or using a Voyager-DE STR (PerSeptive Biosystems, Framingham, MA, USA) equipped with a 2 m flight tube and a 337 nm nitrogen laser. Measurements with the Voyager-DE STR were performed in reflector mode. Positive ions were accelerated at 25 kV and up to 256 laser shots were accumulated in an automated measurement. The spectra were calibrated externally for each sample plate yielding in a mass

accuracy better than 0.1%. α-Cyano-4-hydroxycinnamic acid (CHC, Sigma–Aldrich, Deisenhofen, Germany) was used as matrix with 6-desoxy-L-galactose (Sigma–Aldrich) as co-matrix.

Peptide sequencing was performed on a 473 A gas-phase sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin amino acids using the fast cycle protocol as recommended by the manufacturer.

2.4. Sodium dodecyl sulfate (SDS)-PAGE and immunoblotting

Aliquots of the peptide bank fractions corresponding to 100 ml equivalent of hemofiltrate (HF) were lyophilized, reconstituted in sample buffer, and subjected to a non-reducing SDS-PAGE according to Schägger and von Jagow [15] in Mini-Protean systems (Bio-Rad, Munich, Germany) at 150 V for 2 h. Molecular mass standards of 4000–210 000 (SeeBlue, NOVEX, Offenbach/Main, Germany) were used. The separated peptides were transferred to a hydrophilic polyvinylidene difluoride (PVDF) membrane at 7 V for 2 h using a semi-dry electrophoretic transfer unit (Bio-Rad). The membrane was blocked with 5% powdered skimmed milk in Tris-buffered saline (TBS; 10 m*M* Tris, 150 m*M* NaCl, pH 8.0) containing 0.05% Triton X-100 at 4°C overnight or for 1 h at room temperature.

Electroblotted and blocked PVDF membrane was incubated with polyclonal rabbit recombinant human (rh) IGFBP antisera (Upstate Biotechnology, Lake Placid, NY, USA) diluted to 1:1000 in TBS containing 0.05% Triton X-100 at 4°C overnight (or for 2 h at room temperature). The membrane was washed in TBS containing 0.05% Triton X-100 and then incubated with antirabbit IgG coupled to alkaline phosphatase (Sigma, Deisenhofen, Germany) in the identical buffer. Afterwards, the PVDF membrane was washed first in TBS containing 0.05% Triton X-100, then in TBS, and finally with 10 m*M* Tris, 10 m*M* NaCl, and 10 m*M* $MgCl_2$, pH 9.5.

Bands were developed using a bromochloroindolyl phosphate/nitro blue tetrazolium substrate system as recommended by the manufacturer (Sigma).

2.5. IGF-I-binding assay

IGF-I-binding assays were performed with radiolabeled IGF-I (PeproTech, London, UK) iodinated by the chloramine T method. Briefly, 20 000 cpm of [^{125}I]-IGF-I were incubated with aliquots of the peptide bank corresponding to 10 ml hemofiltrate equivalent or with 50 ng of rh IGFBP-4 (kindly provided by Dr. Kurt Lang, Boehringer Mannheim, Penzberg, Germany) as a control for 22 to 24 h at 4°C in a total volume of 300 μl of assay buffer [0.1 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.44 m*M* $NaHCO_3$, 0.02% Triton X-100, 0.1% BSA, pH 6.0]. Bound and free IGF-I were separated by addition of 500 μl 40% (w/v) polyethylene glycol 4000 in assay buffer. The samples were mixed vigorously, incubated for 30 min at room temperature, and centrifuged at 4°C at 2000 *g* for 25 min. The supernatant was removed and the samples were centrifuged again at 4°C at 2000 *g* for 20 min. The bound radioactivity in the final pellet was measured by γ-counting (1470 Wallac Wizard, Pharmacia, Turku, Finland).

Nonspecific binding was determined by adding a 500-fold excess of unlabeled IGF-I and subtracted from the total bound radioactivity to determine specific binding.

3. Results and discussion

3.1. Strategy

Human hemofiltrate was recently described as a valuable source for the isolation of regulatory peptides with M_r<20 000 [11–13]. The systematic establishment of a peptide bank prepared from up to 10 000 l of human blood filtrate [10] enables us to isolate regulatory peptides occurring in subpicomolar amounts in human plasma. Up to the present time, many hundreds of different circulating peptides from the hemofiltrate peptide bank have been identified by systematic isolation and sequence analysis [16]. This number should grow enormously with the actual increasing progress in mass spectrometric and chromatographic techniques.

The conventional approach of monitoring the

purification of a peptide by detecting immunoreactivity in the selected fractions was supplemented by two additional techniques, molecular mass analysis by MALDI–MS and IGF-binding capacity.

In the attempt to isolate a typical binding protein which binds to the IGFs, it appears to be obvious to test the binding properties of the fractions revealing immunoreactivity. The mechanism of IGF-binding is proposed to depend on two binding sites within the insulin-like growth factor binding proteins (IGFBPs), one representing the N-terminal domain and the other the C-terminal domain [4]. Fragments of the IGFBPs corresponding to these two domains have been shown to bind the IGFs with reduced affinity, but they have in part lost the IGF-binding capacity [9,17]. Therefore, IGFBP fragments without affinity for the IGFs, or smaller fragments with only weak immunoreactivity, could not be detected using this strategy. Nevertheless, the present procedure should be of special advantage in the case of isolating fragments of binding proteins, since isolation of peptides showing cross-reactivity to the polyclonal IGFBP antisera used could be reduced. Furthermore, purification of non-IGFBP peptides was prevented by direct sequence analysis of fractions showing both immunoreactivity and IGF-binding capacity.

During the purification procedure, the selected peptides were monitored using MALDI–MS, which represents a fast and sensitive detection system. MALDI–MS was recently shown to be a suitable method for monitoring the purification of bioactive peptides from complex biological mixtures if their molecular masses are known [14]. Therefore, the molecular masses corresponding to the detected IGFBP fragments were determined by MALDI–MS referring to the estimated masses obtained from SDS-PAGE.

3.2. Isolation of an IGFBP-4 fragment

To obtain a general overview of the immunoreactivities occurring in the fractions of the hemofiltrate peptide bank, Western immunoblot analysis was performed. The fractions were generated in a standardized two-step procedure including stepwise batch elution from a cation-exchanger with seven buffers of increasing pH (Fig. 1A) and subsequent fractionation of the resulting pH pools by RP chromatography; an example is shown for pH pool 3 (Fig. 1B).

Using IGFBP-3, -4 and -5 antisera, several immunoreactivities in the molecular mass range of 8000–30 000 were observed in pooled fractions of the peptide bank. Performing IGFBP-4 immunoblotting, two significant immunoreactive bands with molecular masses of about 11 000 and 14 000 were detectable in the pooled fractions 26–30 and 31–35 from pH pool 3 (Fig. 1C) and pH pool 4 (Fig. 1D), respectively. Besides the two significant immunoreactivities a few minor immunoreactive bands of higher molecular masses were observed in the same fractions. The prominent M_r 14 000 immunoreactivity from pH pool 3 (Fig. 1C) was selected for further immunological and biochemical characterization. First, IGFBP-4 immunoreactivity (Fig. 2A) and IGF-binding capacity (Fig. 2B) were determined within the single fractions 25–34 of pH pool 3. Besides a significant immunoreactive band of approximately M_r 11 000 in the fraction 27, strong immunoreactivity in the range of M_r 14 000 was observed in the fractions 28, 29, and 31 (Fig. 2A). The IGF-binding experiments showed a significant specific IGF-I-binding in the fractions 27–30 (Fig. 2B), which was in accordance with the results obtained from the IGFBP-4 immunoblotting.

Fraction 29 from pH pool 3 showing both a strong IGFBP-4 immunoreactivity in the range of M_r 14 000 and significant IGF-I-binding capacity was selected for further purification. N-terminal sequence analysis of the immunoreactive band confirmed the occurrence of an IGFBP fragment. The generated MALDI mass spectrum showed two masses in the range of 13 000–14 000 (Fig. 2C). Since the molecular mass of 13 483 was already known to represent a fragment of the fibrinogen A α-chain (unpublished data), the molecular mass of 13 200 was assigned to the IGFBP-4 fragment.

In order to achieve purification of the selected peptide, six further chromatographic steps using RP-HPLC and cation-exchange chromatography were necessary (Fig. 3A–F). At first, a preparative RP step using a C_{18} column was performed (Fig. 3A). In the next purification step, fractions containing the molecular mass of 13 200 were loaded onto an analytical C_{18} column (Fig. 3B). To achieve further separation of the selected fractions, analytical cation-

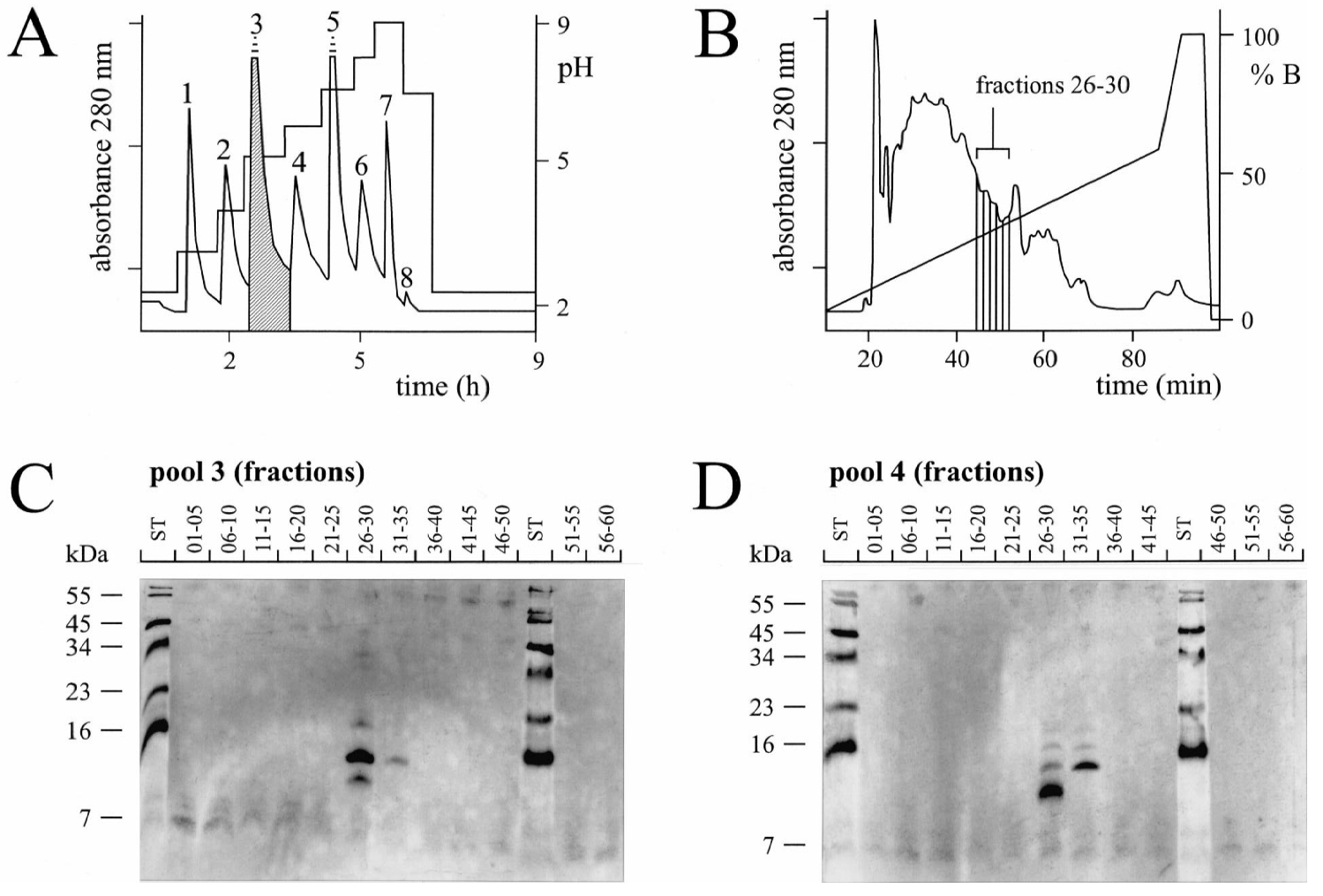

Fig. 1. Initial screening for IGFBP fragments in pooled fractions of the hemofiltrate peptide bank by immunoblotting. The peptide bank was generated in a standardized two-step procedure. First, stepwise batch elution of the crude peptide extract from human hemofiltrate was performed by cation-exchange chromatography using seven buffers increasing the pH with each one (A). The resulting pH pools were further fractionated by preparative RP-HPLC as shown here for pH pool 3 (B). The generated fractions were used as starting material for the screening by immunoblotting, examplarily shown for IGFBP-4. Significant IGFBP-4 immunoreactivities with molecular masses in the range of 11 000 and 14 000 were detectable in the pooled fractions 26–30 and 31–35 from pH pool 3 (C) and pH pool 4 (D). The prominent 14 000 immunoreactivity in the fractions 26–30 from pH pool 3 was selected for further characterization and purified. kDa=kilo daltons.

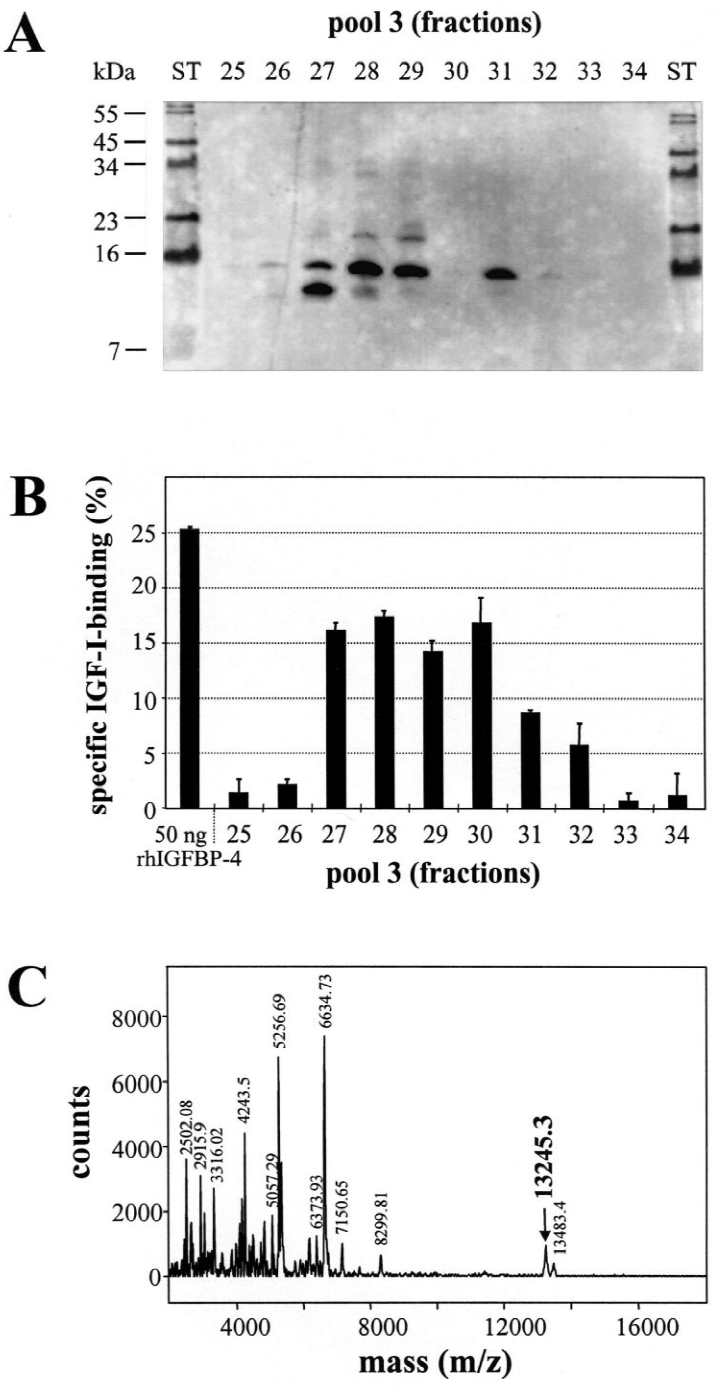

Fig. 2. (A) Immunoblotting of the single fractions 25–34 from pH pool 3 corresponding to the immunoreactive bands in Fig. 1C. A strong IGFBP-4 immunoreactivity with an apparent mass of 14 000 was detected in fractions 28 and 29. (B) IGF-binding analysis of the same fractions. Significant specific IGF-I-binding occurred in the fractions 27–30 from pH pool 3, which correlates to the results obtained from IGFBP-4 immunoblotting. (C) MALDI–MS spectrum of the complex peptide mixture of fraction 29 from pH pool 3 containing an immunoreactive IGFBP-4 fragment. The corresponding mass of 13 200 is marked with an arrow. This fraction was selected for further purification. Note: contaminant peptide of M_r 13 483 (fragment of human fibrinogen Aα).

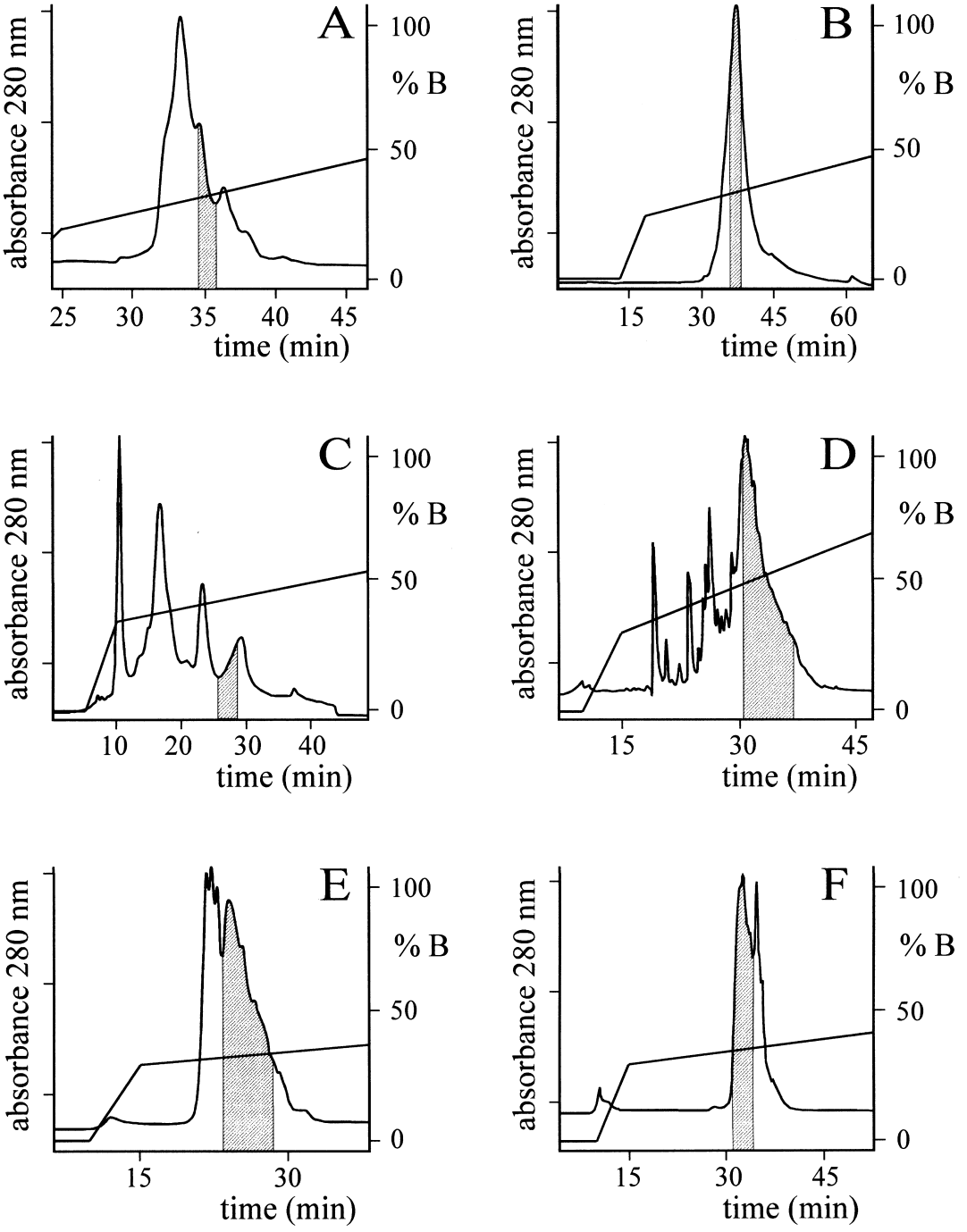

Fig. 3. Purification of an immunoreactive IGFBP-4 fragment by consecutive chromatographic steps guided by MALDI–MS. (A) Preparative RP chromatography to perform further purification of the selected fraction 29 from pH pool 3 containing an immunoreactive IGFBP-4 fragment with an apparent mass of 13 200 (as shown in Fig. 2C). The resulting fractions revealing the molecular mass of 13 200 are marked. (B) Analytical RP chromatography of the marked fractions from (A). (C) Cation-exchange fractionation of the marked area from (B) containing the molecular mass of 13 200. (D–F) Further purification steps using analytical RP columns monitoring the IGFBP-4 peptide by MALDI–MS. The last separation step resulted in the purification of an N-terminal IGFBP-4 fragment with a molecular mass of 13 200.

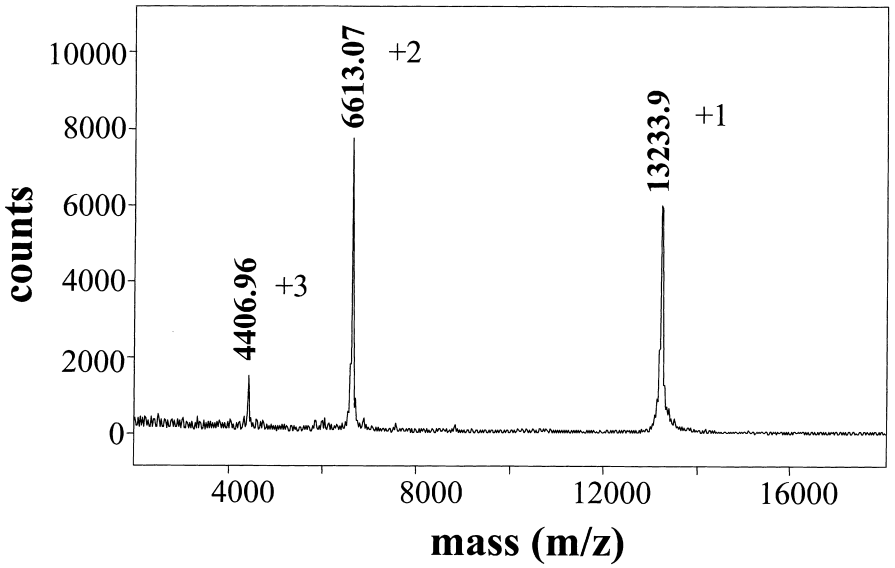

Fig. 4. MALDI–MS analysis of the purified IGFBP-4 fragment revealed a molecular mass of 13 233 within the experimental error of <0.1%. Sequence analysis by conventional Edman degradation confirmed the purification of an N-terminal IGFBP-4 fragment.

exchange chromatography was used (Fig. 3C) and the generated fractions were again analyzed with respect to their molecular masses. In the subsequent purification step, analytical RP chromatography was carried out using an RP-C_4 column (Fig. 3D). Final purification of the peptide corresponding to the molecular mass of 13 200 was obtained by two further analytical RP chromatographic steps (Fig. 3E–F). The MALDI mass spectrum of the purified peptide (Fig. 4) revealed a molecular mass of 13 233 within the experimental error of <0.1%. N-terminal sequence analysis by conventional Edman degradation resulted in the sequence DEAIHXPPXSEE, which corresponds exactly to the partial sequence of human IGFBP-4 (1–12) [18]. These data demonstrate the purification of a new N-terminal fragment of IGFBP-4.

In conclusion, only two different significant immunoreactivities with apparent molecular masses of 11 000 and 14 000 were detectable by IGFBP-4 immunoblotting. Since proteolysis of the IGFPBs into two main products, the N- and the C-terminal regions, is described in vitro and the M_r 14 000 immunoreactivity was shown to correspond to the N-terminal fragment of IGFBP-4, the M_r 11 000 immunoreactivity could represent a C-terminal fragment. Due to the cut-off of the hemofilters used (M_r 20 000), peptides with M_r>20 000 were present in the fractions of the hemofiltrate peptide bank only at lower concentration or completely abroad. Therefore, high-molecular mass fragments, e.g. glycosylated forms, or intact IGFBPs with molecular masses >25 000 were not expected to occur in sufficient amounts.

4. Conclusions

Identification of circulating fragments of the IGF binding proteins gives insight into specific proteolysis of the IGFBPs and facilitates the detection of proteases responsible for their degradation. Proteolysis of the IGFBPs is thought to be essential for regulating the bioavailability of the IGFs. The generated fragments show reduced or lost binding affinity for the insulin-like growth factors. Moreover, they partly exert IGF-independent activities on proliferation and differentiation of cells and tissues.

We established a combined strategy to identify circulating fragments of the insulin-like growth factor binding proteins from our peptide bank generated from human hemofiltrate. This strategy includes

identification of IGFBP fragments by means of immunoreactivity and IGF-binding capacity, by direct sequencing of immunoreactive bands, and subsequent MALDI–MS guided purification of the peptides using HPLC. As a proof of the concept, we were able to isolate circulating fragments of IGFBP-4 and IGFBP-5 [19]. Further studies will focus on the functional relevance of the IGFBP peptides isolated from hemofiltrate.

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