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Isolation from human cerebrospinal fluid of a new insulin-like growth factor-binding protein with a selective affinity for IGF-II

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In biological fluids IGF-I and IGF-II are bound to specific, high-affinity binding protein (BPs). Two human BPs have been isolated, one from serum, which is GH-dependent, the other from amniotic fluid (AF BP), and their cDNAs have recently been cloned. We report here the isolation of another, new species from cerebrospinal fluid (CSF) where this BP predominates. The protein was purified to homogeneity by a four-step procedure: gel filtration, chromatofocusing, hydrophobic-interaction chromatography and reverse-phase chromatography. Thereafter, SDS-polyacrylamide gel electrophoresis gave an M_r of 34 000 (non-reduced), chromatofocusing gave an isoelectric point of 5.0, and its affinity for IGF-II (3×10^{10} M^{-1}) was 10 times that for IGF-I. The N-terminal amino acid sequence of the first 15 residues determined in a BP preparation from the CSF of children was Leu-Ala-Pro-Gly-(/)-Gly-Gln-Gly-Val-Gln-Ala-Gly-Ala-Pro-Gly. A similar sequence was found for adult CSF, apart from residues 12 and 13 (-Leu-Leu-). These are highly analogous with the sequences starting from residue 69 of the GH-dependent BP, and from residue 61 of the AF BP. The new BP isolated is therefore related to, but distinct from, the other human BPs.

Insulin-like growth factor; IGF-binding protein; Protein purification; Amino acid sequence; Cerebrospinal fluid

1. INTRODUCTION

Over the past few years we have identified several forms of IGF-binding protein (BP) with different physico-chemical properties, regulation and affinities for IGF-I and IGF-II [1-4]. In man, five forms with M_r values of 41 500, 38 500, 34 000, 30 000 and 24 000 are identifiable by ligand blotting. All these forms are present in all biological fluids, but the relative proportions vary with tissue of origin [2,3,5]. The 41.5 and 38.5 kDa BPs, whose synthesis appears to be under the control of growth hormone (GH) [3,4], represent two forms of the same protein which has been purified from serum and the cDNA of which has recently been cloned [6,7]. The circulating forms of the 34 and 30 kDa BPs appear to be negatively controlled by GH [3,4]. The BP corresponding to the 30 kDa form has been purified from amniotic fluid [8,9], the placenta [10] and Hep G2 human hepatoma culture medium [11,12]. In addition, several groups have cloned its cDNA [13-16]. We noted [4] that in immunoblotting experiments the 34 kDa BP was immunologically unrelated both to the 41.5 and 38.5 kDa BPs which are recognized by the anti-GH-dependent BP antibody [17] and to the 30 kDa BP which is recognized by the anti-amniotic fluid BP antibody [18].

We had previously reported that the 34 kDa BP was predominant in cerebrospinal fluid (CSF) and that is was probably responsible for the selective affinity for IGF-II of the BPs extracted from this fluid [2,19]. We therefore undertook to isolate and purify the BP from CSF.

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2. MATERIALS AND METHODS

2.1. *Biological samples and peptides*

CSF samples collected during neuroradiological examination of adults and during treatment of hydrocephaly in children gave us two 700 ml pools. Pure IGF-I (prep. I/4) and IGF-II (prep.

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II/5) were generously provided by Dr R.E. Humbel (Zurich, Switzerland). IGF-II was iodinated using the chloramine T method, with subsequent purification by gel filtration.

2.2. *Ultrafiltration*

Membranes with a cut-off of 10000 were used (Centiprep, Amicon, USA).

2.3. *Gel filtration*

A 1.5 × 90 cm column of Ultrogel AcA54 (IBF, France) equilibrated with 0.025 M bis–Tris-HCl, 0.1 M ammonium sulphate, pH 7.4, was used. Chromatography was performed at 4°C at a flow rate of 15 ml/h (1 ml/fraction).

2.4. *Chromatofocusing*

A Mono P anion-exchanger column (HR 10/20, FPLC system, Pharmacia, Sweden) equilibrated with 0.025 M bis–Tris-HCl, pH 6.5, was used. The proteins were eluted in a gradient of pH 6.5–4.0 with polybuffer 74-HCl, pH 4.0, (10%, v/v) at a flow rate of 0.5 ml/min and collected at 4°C in 1 ml fractions.

2.5. *Hydrophobic-interaction chromatography*

A phenyl-Superose column (HR 10/10, FPLC system) equilibrated with 0.025 M bis–Tris-HCl, 1 M ammonium sulphate, pH 6.5, was used. The proteins were eluted within 45 min in a descending ionic strength gradient, from 1 to 0 M $(NH_4)_2SO_4$, at a flow rate of 1 ml/min and collected at 4°C in 1 ml fractions.

2.6. *Reverse-phase chromatography*

A 4.6 × 250 mm column of C8 Aquapore RP 300 (Brownlee Labs, USA) with an HPLC system (Gilson, France) was used. The column was equilibrated with an aqueous solution of 0.1% (v/v) trifluoroacetic acid (TFA). The proteins were eluted within 30 min in a linear gradient from 0 to 100% isopropanol, 0.1% TFA at a flow rate of 1 ml/min and collected at 4°C in 0.5 ml fractions.

2.7. *Characterization and assays*

The proteins were assayed according to Bradford [20].

2.7.1. SDS-polyacrylamide gel electrophoresis and ligand blotting

Electrophoresis was performed on 11% acrylamide slab gels (15 × 15 × 0.1 cm) according to Laemmli [21]. Gels were stained either with Coomassie blue or silver [22].

Ligand blotting was performed as previously described [23]. After SDS-PAGE (without reducing agent, except in the case of the markers), the proteins were electroblotted onto a nitrocellulose sheet and the BPs identified using ^{125}I-labelled IGF-II as probe.

Binding activity assay was performed as described [24]. The samples were gel filtered in 1 M CH_3COOH, 0.15 M NaCl, 0.1% BSA in order to separate the BPs from the IGFs. The relative BP concentration was assessed by comparing the binding to ^{125}I-labelled IGF-II with that of a reference CSF BP preparation arbitrarily assigned a value of 1 U BP/ml. Samples were studied at 6 concentrations, each in duplicate. Incubation time was 24 h. Charcoal was used for the separation step.

2.7.2. Competitive-binding experiment [19]

This was performed using an aliquot of the purified CSF BP preparation. Before assaying it was submitted to acidic gel filtration in order to separate the BPs from residual endogenous IGFs. The amount of BP binding approximately 25% ^{125}I-labelled IGF-II was determined and thereafter displacement curves were established using increasing concentrations of IGF-I and IGF-II, each in duplicate.

2.7.3. N-terminal amino acid sequence

All sequence data were obtained using either an Applied Biosystems 470 A or 477 A protein sequencer with an on-line 120 A PTH analyser.

3. RESULTS

3.1. *Purification scheme*

3.1.1. Step 1

Samples of CSF (100 ml initial volume) were concentrated to 3 ml by ultrafiltration and incubated overnight at 4°C with ^{125}I-labelled IGF-II, then gel filtered at pH 7.4 (fig.1). The ^{125}I-IGF-II–BP complexes eluted in a peak with a K_d of 0.40 corresponding to material with an apparent M_r of 40000. The elution profile of the radioactivity and that of the 34 kDa BP analysed by ligand blotting were superimposed. This BP appeared as a single band in adults and as a doublet in children. The fractions corresponding to the summit of the peak were pooled, concentrated by ultrafiltration and rechromatographed under the same conditions to eliminate the maximum amount of albumin and 41.5 and 38.5 kDa BPs. The eluate of the second gel filtration was desalted and concentrated by ultrafiltration in 0.025 M bis-Tris-HCl, pH 6.5.

3.1.2. Step 2

The material obtained from the seven gel filtrations (corresponding to the 700 ml initial CSF) was applied to the Mono P anion exchanger and then chromatofocused as described above (fig.2). The ^{125}I-IGF-II–BP complexes eluted in a single peak around pH 5. Binding activity measurements of aliquots precipitated with 85% ammonium sulphate and submitted to acidic gel filtration showed maximum binding activity to correspond to the radioactivity peak.

3.1.3. Step 3

The chromatofocusing eluate was brought to high ionic strength (1 M ammonium sulphate in bis-Tris, pH 6.5) in preparation for hydrophobic-

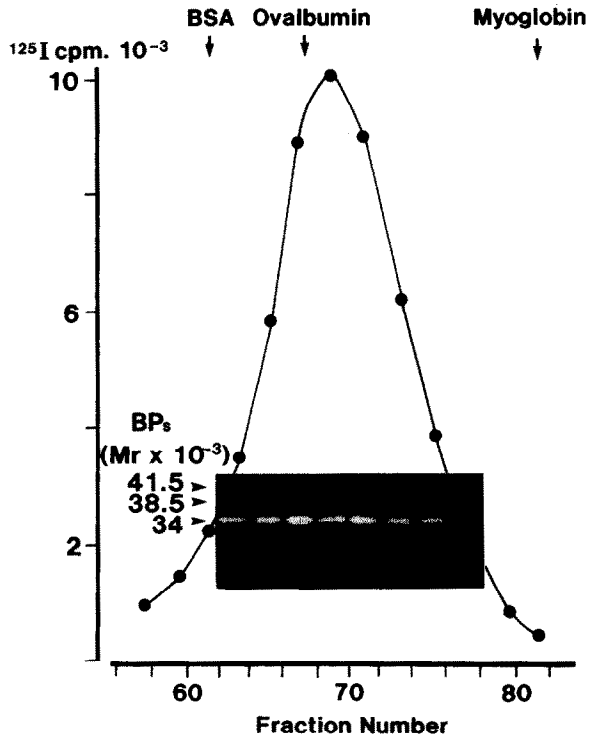

Fig.1. Gel filtration on Ultrogel AcA54 at pH 7.4 of the CSF concentrated by ultrafiltration. The material (100 ml equivalent of CSF) had previously been incubated with ^{125}I-labelled IGF-II. The radioactivity profile corresponds to the elution zone of the IGF-BP complex. An aliquot of each fraction was analysed by SDS-PAGE and ligand blotting.

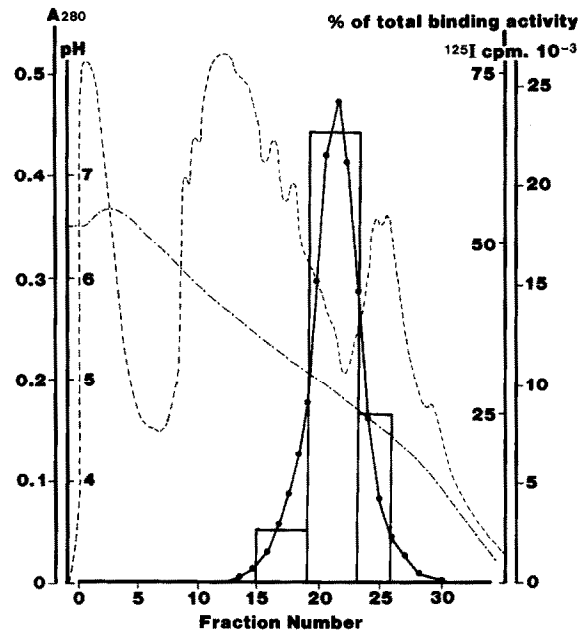

Fig.2. Chromatofocusing of the partially purified IGF-binding protein on a Mono P column (FPLC system). The material applied was that obtained from gel filtration (see section 3). The radioactivity peak corresponds to the elution peak of the IGF-BP complexes. The bars represent binding activity measured in aliquots of pooled fractions.

interaction chromatography. After being kept overnight at 4°C, the sample was centrifuged (50 000 × *g* for 30 min) and the supernatant chromatographed on the phenyl-Superose column as described above.

3.1.4. Step 4

TFA (0.1% final) was added to the eluate from step 3 which was then subjected to reverse-phase chromatography as described above (fig.3). The absorbance profile at 254 nm revealed a homogeneous peak at 60% isopropanol. This material was rechromatographed, yielding a single peak in the same position. IGF-II eluted at 55% isopropanol.

3.2. *Protein content and BP recovery*

The total protein content (average of the two purifications) was 330 mg in the starting material (700 ml CSF), 8 mg in the material collected after gel filtration and 1 mg after step 3. In view of the minute quantities of material available, total protein content was not determined after the final step. Binding activity in the starting material was 450 U. BP recovery after reverse-phase chromatography was estimated at 2% and 5% for the two purifications, respectively.

3.3. *Characterization*

The purity of the protein obtained after the final chromatography was demonstrated by SDS-PAGE followed by ligand blotting and silver staining (fig.3). The BP isolated from the CSF of children migrated as a doublet with an estimated molecular mass of 34 kDa (non-reduced), which was the same as the doublet seen with ligand blotting in the starting material. The BP purified from adult CSF gave a single broad band which migrated slightly faster (~32 kDa).

In chromatofocusing, the purified BP eluted at pH 5.0.

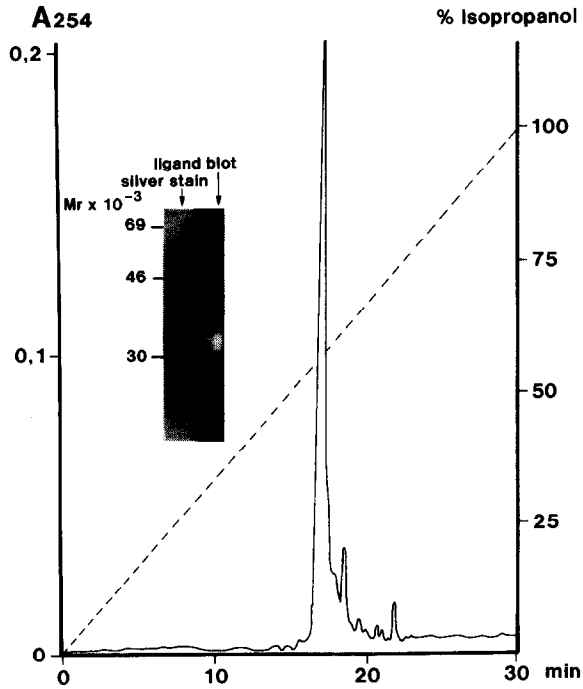

Fig.3. Isolation by reverse-phase chromatography (C8 Aquaphore, HPLC) of the IGF-binding protein. The peak corresponds to material obtained after 2 successive runs. An aliquot of the material was analysed by SDS-PAGE.

The competitive-binding experiment done with the purified BP and ^{125}I-IGF-II showed that IGF-II had a competitive potency 10 times that of IGF-I

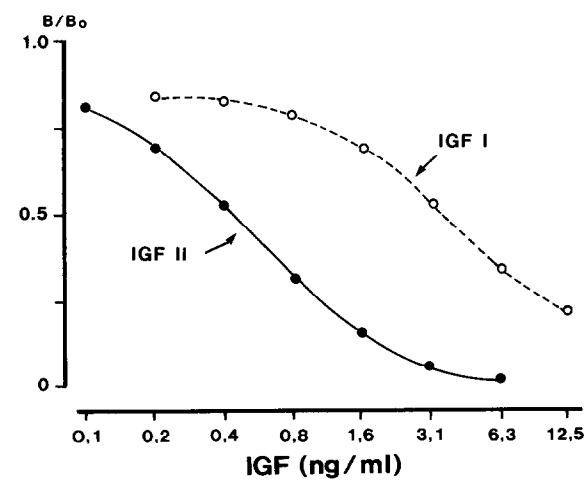

Fig.4. Competitive inhibition by IGF-I and IGF-II of the binding of ^{125}I-labelled IGF-II to the IGF-binding protein purified from CSF.

Table 1

N-terminal amino acid sequences of the IGF-binding protein purified from cerebrospinal fluid

Step number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CSF BP (children)	L	A	P	G	/	G	Q	G	V	Q	A	G	A	P	G
CSF BP (adults)	/	/	P	G	K	G	Q	G	V	Q	A	L	L	P	G
GH-dependent BP	69	P	S	P	D	E	A	R	P	L	Q	A	L	L	D	G
AF BP	61	A	L	P	G	E	Q	Q	P	L	H	A	L	T	R	G
BRL-3A BP (rat)	63	P	N	P	G	S	E	L	P	L	K	A	L	V	T	G

The two sequences determined for the BPs isolated from the CSF of children and adults were shown. (/) Used where no amino acid could be deduced. The CSF BP sequences have been aligned for purposes of comparison with the analogous regions of the GH-dependent BP [7], the amniotic fluid BP [13–16] and the BRL-3A BP [27]

(fig.4). Scatchard analysis of binding data gave linear plots and the calculated affinity constants were 3×10^{10} M^{-1} for IGF-II and 3×10^{9} M^{-1} for IGF-I.

3.4. *Amino acid sequence*

The N-terminal ends (first 15 residues) of the two BPs isolated, one from CSF of adults and the other from CSF of children, were sequenced (table 1). The sequence for the BP of children was obtained at a level of 50 pmol and, with the exception of step 5, was easily identifiable. The sample from the adults was identifiable at low level (below 20 pmol), and presented some amino acid contaminants at steps 1, 2, 6, 7, 8 and 14. Nonetheless, apart from residues 1 and 2, the sequence could be deduced without ambiguity.

4. DISCUSSION

In this study a purification scheme was established for isolating the IGF-binding protein form predominant in CSF. Only small quantities of IGF were available and to this extent we selected 'non-affinity' approaches to purify the BP. The four-step procedure yielded a protein with an M_r of 34 000 (non-reduced) and a pH$_i$ around 5. The elution of the BP at 60% isopropanol in the reverse-phase step suggests that the BP is strongly hydrophobic.

This BP is distinguishable from the two ohter human BPs previously isolated in the following ways: (i) its molecular mass: under our electro-

phoretic conditions, the GH-dependent BP [6] appears as a doublet of 42.5–38.5 kDa and the BP isolated from amniotic fluid (AF BP) [8,9] has an estimated M_r of about 30000 [3,4]; (ii) its pH_i which is slightly less acidic than that of the AF BP around 4 [9]; (iii) as mentioned at the beginning of this article, it possesses no epitopes recognizable by the specific polyclonal antibodies to the other two BPs [4]; (iv) its selective affinity for IGF-II which is ten times that for IGF-I, whereas purified preparations of the GH-dependent BP and AF BP have only slight differences in their affinities for IGF-I and IGF-II [6,25]. Nevertheless, the specific ability to bind IGFs, to the exclusion of all other peptides, including insulin, implies some structural analogy between the various BPs. This has been confirmed for the BPs purified to date in man and in other species (review in [26]). It is also true for the CSF BP as shown by the amino acid sequence data.

The sequences obtained for the two purified BP preparations were identical in children and adults, apart from two residues in positions 12 and 13. At present it is impossible to know whether the differences are due to sample origin or sequence variability. If different variants of CSF BP exist in adults and children, they may be in different proportions, and sample preparation could result in the isolation of one particular form.

Comparison of the amino acid sequences determined in our two BP preparations with the sequences known for the other BPs shows analogous regions in sequences starting from residue 69 of the GH-dependent BP (6 identical residues), from residue 61 of the AF BP (6 identical residues) and from residue 63 of a rat BP (5 identical residues) whose cDNA has recently been cloned from a BRL-3A liver cell line [27]. This rat BP corresponds to one we identified by ligand blotting as a 32 kDa species in rat liver culture media and serum where it is predominant in the fetus [5].

The CSF BP therefore appears to be related to the others. The question now arises whether or not the sequence really is at the N-terminal end of the protein, as the homology we have found with the other three BPs applies to zones beyond the 60th residue. The possibility of N-terminal end proteolysis during purification cannot be excluded. However, the data from SDS-PAGE and the competitive-binding experiment strongly favour integrity of the structure of the CSF BP and, moreover, an N-terminal sequence identical to the one determined in the CSF BP from children has been found in a BP purified from a human fetal lung fibroblast cell line (Ballard, J., personal communication). Studies under way to clone its cDNA should provide answers to this question. Nonetheless, the data presented here are consistent with the conclusion that the BP isolated from CSF is a new species which is related to, but distinct from, the other human BPs purified to date. Its preponderance in CSF and its selective affinity for IGF-II suggest that it plays some particular role in nervous tissue.

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