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Identification of a Novel Binding Protein for Insulin-like Growth Factors in Adult Rat Serum

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Using gel filtration, ligand-affinity chromatography and reversed phase HPLC, four insulin-like growth factor-binding proteins (IGF-BPs) have been purified from adult rat serum. Sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) analysis revealed that all four proteins migrated as doublets at 45/37 (peak 1 in Fig. 4), 36/32 (peaks 2 and 5 in Fig. 4), 35/33 (peak 3 in Fig. 4) and 22/28 kDa (peaks 4a, 4b in Fig. 4), respectively under reducing conditions. N-terminal sequence analysis of the 45/37 kDa doublet showed that it is identical to the N-terminal of the 45 kDa rat IGF-BP whereas the 22/28 kDa doublet is the C-terminal truncated form of the 45 kDa species. The 35/33 kDa doublet has the same N-terminal sequence as that of the rat IGF-BP isolated from a BRL-3A cell line. However, the N-terminal sequence of the 36/32 kDa doublet is unique, although it may be related to the BRL-3A protein. The most abundant IGF-BP in adult rat serum corresponds to the 45 kDa species plus its C-terminal truncated forms, whereas the second most abundant IGF-BP is the novel protein detected in this study, while the least abundant species is the BRL-3A IGF-BP.

Insulin-like growth factors (IGFs) do not circulate in the blood as free hormones but bound to large protein carriers (1,2,3). The physiological function of the IGF-binding proteins (IGF-BPs) is not clear. It could serve as a carrier to prolong the biological half-life of the IGFs in circulation or act as a neutralization factor to prevent the IGFs from exerting their insulin-like effect since the circulating level of IGF-I is about a 100-fold higher than insulin (4). In normal adult human and rat serum, two IGF-BP complexes can be resolved by gel filtration chromatography at pH 8.0 into a 150 kDa and a 40 kDa species (1,2,3,5). The human 150 kDa complex has been further fractionated under acidic medium to yield three components, an IGF-binding subunit of 45 kDa, IGF-I and an 80 kDa acid-labile subunit which can only bind to the 45 kDa protein in association with IGF under neutral conditions (6). Both the 45 kDa IGF-BP and the 80 kDa acid-labile subunit are glycosylated. In contrast, the composition of the 40 kDa IGF-BP complex is not

Abbreviations: IGF: insulin-like growth factor, BP: binding protein, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, HPLC: high performance liquid chromatography, M_r: relative molecular size.

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clear. Studies conducted by others using adult rat serum have shown that it consisted of some C-terminal truncated fragments of the 45 kDa IGF-BP plus two IGF-BPs of 32 and 24 kDa (7,8). The chemical nature of these two smaller IGF-BPs is not known. They could be related to the IGF-BP isolated from a rat BRL-3A cell line (9) or the rat homolog of the human amniotic IGF-BP (10) purified from a HEP-G2 cell line (11) and placenta (12). In order to clarify the chemical nature of all of the IGF-BPs in adult rat serum, we undertook a systematic study to isolate all of the BPs from adult rat serum by ligand affinity chromatography with IGF-I, followed by reversed phase HPLC purification of the affinity purified proteins and SDS-PAGE as well as microsequence analysis of the recovered HPLC peaks. The analytical results showed that adult rat serum contains the intact 45 kDa IGF-BP plus its C-terminal truncated fragments, the BRL-3A IGF-BP, and a new IGF-BP whose N-terminal sequence has not been reported before.

MATERIALS AND METHODS

Materials

Adult rat serum was obtained from Chemicon (Temecula, CA). Recombinant IGF-I was generously supplied by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Sephacryl S-200 superfine was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). HPLC grade acetonitrile was from J.T. Baker Inc. (Phillipsburg, NJ). All other chemicals were reagent grade obtained from Sigma (St. Louis, MO).

Preparation of ligand-affinity chromatography column

Six milliliters (90 μmol of active ester sites) of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) was transferred to a 1.6 x 6 cm glass column sealed with a coarse fritted disk at the bottom. The gel was washed with 10 ml of cold water four times to remove the isopropanol and 6 ml of 0.1 M HEPES buffer, pH 7.4, containing 2 mg IGF-I (260 nmol) was added and the column rocked gently in the cold room overnight. To block the unreacted active sites, 1 ml of 1 M ethanolamine, pH 8.0 (1 mmol) was added and the mixture continued rocking for 24 hrs. The reacted gel was washed with ten column volumes each of 0.1 M HEPES buffer, 20 mM sodium phosphate plus 0.5 M NaCl, pH 7.4, followed by 0.5 M acetic acid, pH 3.0, and finally by 20 mM sodium phosphate plus 130 mM NaCl, pH 7.4 before application of the sample.

Isolation of rat IGF-BP

To remove most of the high molecular weight compounds and dissociate IGFs from their binding proteins, 250 ml of adult rat serum was mixed with 1 liter of cold water containing 35 g $(NH_4)_2SO_4$ in the cold room for 2 hrs to obtain a 5% saturated solution. The mixture was then dialyzed for 24 hrs in the cold room (Spectrapor No. 6 membrane tubing, 28.6 mm cylinder diameter, relative molecular weight (M_r) cutoff 1,000; Spectrum Medical Industries, Inc., Los Angeles, CA) against 14 liters of 30% (vol/vol) acetic acid with one change of the dialysis solvent. The retentate was centrifuged at 10,000 rpm to remove a copious white precipitate and the supernatant (~700 ml) lyophilized. The lyophilized material was dissolved in 200 ml 30% acetic acid and divided into four equal volumes for loading onto four 5 x 100 cm columns of Sephacryl S-200 superfine. Each column was eluted with 30% acetic acid at 20 ml/20 min and the column fractions monitored by UV absorption at 280 nm. A representative profile of one of the four columns is presented in Fig. 1. Based on the migration of reference proteins with known molecular weights in the Sephacryl S-200 gel filtration column, the column fractions were divided into three pools, the first consisting of proteins having M_r 60-30 kDa, the second 30-20 kDa and the third 20-10 kDa. Fractions corresponding to the respective molecular sizes from the four columns were pooled and then dialyzed against 4 liters of PBS buffer, consisting of 20 mM sodium phosphate, 130 mM sodium chloride, 0.02% sodium azide, pH 7.4. After dialysis the retentate was applied onto the IGF-I-coupled affinity column at 20 ml/hr through a peristaltic pump (Rainin Instrument Co., Emeryville, CA) in the cold room. After all the sample has been pumped through the column, the gel bed was washed with 200 ml PBS buffer containing 0.5 M NaCl at the same flow rate. The adsorbed proteins were eluted with 0.5 M acetic acid, pH 3.0, at 3.5 ml/hr and 1 ml fractions were collected. The IGF-BPs in the eluate fractions were located by UV absorbance at 280 nm.

The UV absorbing fractions recovered from the affinity column were pooled and after dilution with an equal volume of water pumped directly into a 0.7 x 25 cm Aquapore RP-300, 10 μ particle size, C_8 HPLC column (Brownlee Lab, Santa Clara, CA) at a flow of 3 ml/min. After loading the adsorbed proteins were separated in a Beckman 322 gradient HPLC system (Beckman, San Ramon, CA) by a linear gradient of 22-40% acetonitrile in 0.1% trifluoroacetic acid solvent system in 180 min at a flow of 3 ml/min. The column effluent was monitored by UV absorbance at 210 nm. The elution profiles from the 60-30 kDa and 30-20 kDa protein pools are shown in Figs. 2 and 3, respectively.

Characterization of rat IGF-BPs

The recovered IGF-BP peaks in the reversed phase HPLC fractions were subjected to microsequence analysis in a 470A gas-phase protein sequenator (Applied Biosystems, Inc., Foster City, CA) as described (13). In addition, the relative molecule size of each purified IGF-BP was determined by SDS-PAGE under reducing conditions (14).

RESULTS

Since it has been reported before that the IGF-BPs in adult rat serum exist in multiple molecular forms ranging from 24 to 45 kDa (7,8), we arbitrarily fractionated the proteins into three molecular size pools of 60-30, 30-20, and 20-10 kDa in Sephacryl S-200 superfine under acidic conditions (see Fig. 1). The IGF-BPs present in each molecular size pool were recovered by affinity chromatography using an IGF-I-coupled Affi-Gel 10 column (data not shown). The affinity purified IGF-BPs were then further fractionated to near homogeneity by reversed phase HPLC. As shown in Fig. 2, three peaks of IGF-BP were recovered from HPLC fractionation of the 60-30 kDa molecular size pool and data from N-terminal sequence analysis of each peak is presented in Table 1. Peak 1 showed an N-terminal sequence which is identical to the N-terminal sequence of

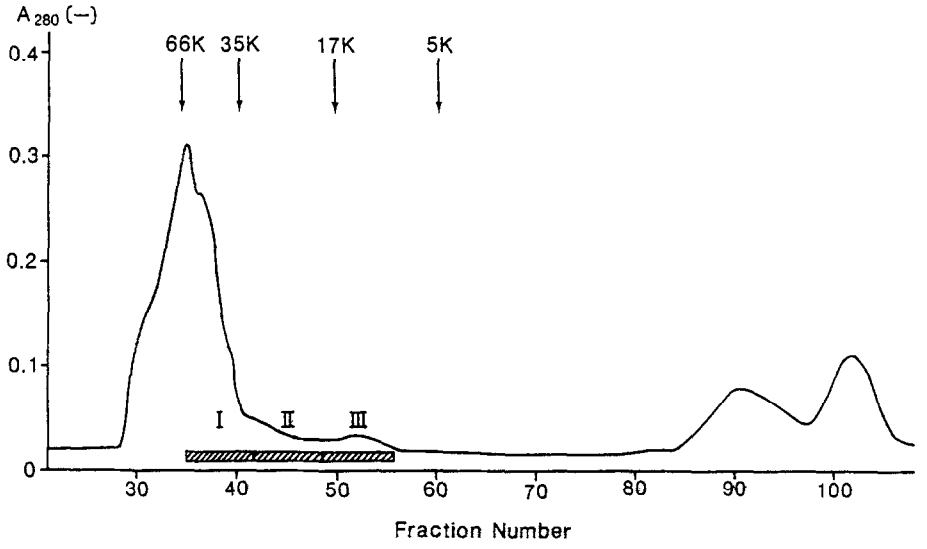

Fig. 1. Sephacryl S-200 superfine gel filtration chromatography of the adult rat serum proteins in 30% acetic acid (v/v). The recovered column fractions were arbitrarily divided into three molecular size pools of 60-30 (I), 30-20 (II) and 20-10 kDa (III) as denoted by the slashed bars. Arrows indicate elution positions of protein markers with known molecular weights.

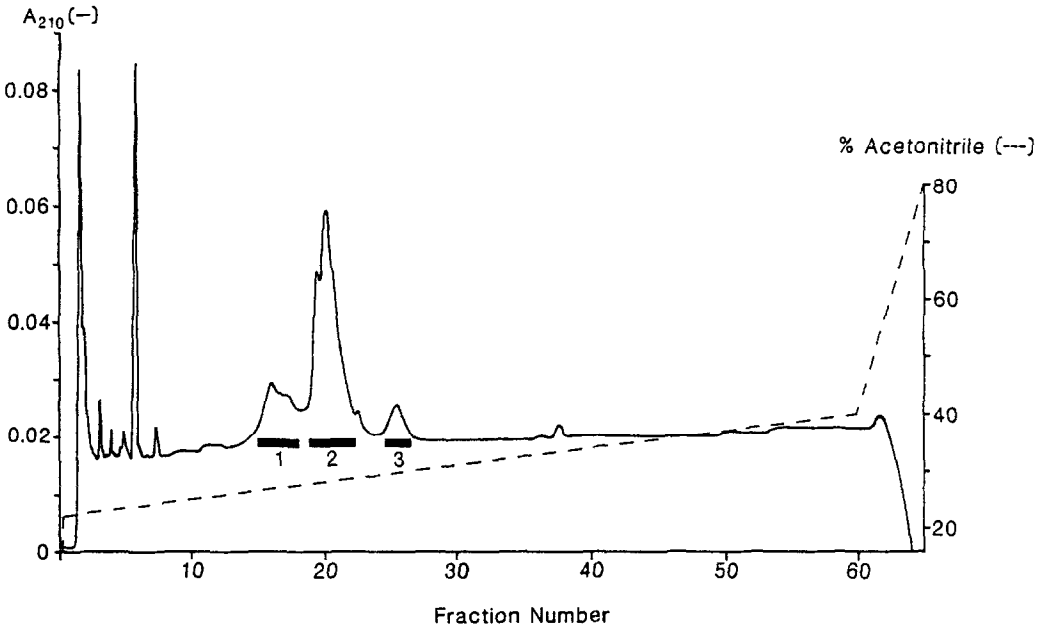

Fig. 2. Reversed phase HPLC purification of the M_r 60-30 protein pool (I) recovered from Fig. 1. Three peaks of IGF-BP (denoted by solid bars 1, 2, 3) were resolved in the HPLC chromatogram. The sharp peaks eluting before fraction 6 were due to column artifacts.

the 45 kDa rat IGF-BP that was reported before (7). In contrast, peak 2, which is the most abundant peak, showed a sequence which is different from any reported rat IGF-BP. Peak 3 showed an N-terminal sequence which is identical to the recently characterized rat IGF-BP identified in a BRL-3A cell line (15) and adult rat liver (16). Similarly, the 30-20 kDa molecular size pool yielded a large UV absorbing peak with a shoulder at the beginning of the HPLC chromatogram (peaks 4a and 4b) and two minor peaks at 28% (peak 5) and 33% (peak 6) acetonitrile in Fig. 3.

Table 1. N-terminal amino acid sequence of IGF-BPs isolated from adult rat serum

Peak*	Amino Acid Sequence†
	5 10 15 20 25 30 35 40
1	G A G A V G A G P V V R X E P X D A R A L
2	D E A I H X P P X S E E K L A R X R P P V G X E E L V R E P G X G X X A T X A L
3	E V L F R X P P X T P E R L A A X G P P P D A P X A E L V R
4a, b	G A G A V G A G P V V R X E P X D A R A L A
5	D E A I H X P P X S E E K L
6	No protein was detected

* Peaks 1, 2, 3 correspond to the IGF-BPs recovered from Fig. 2 and peaks 4a, 4b, 5 and 6 correspond to the IGF-BPs recovered from Fig. 3.
† X denotes a residue (most probably cysteine) which was not identified in the analysis.

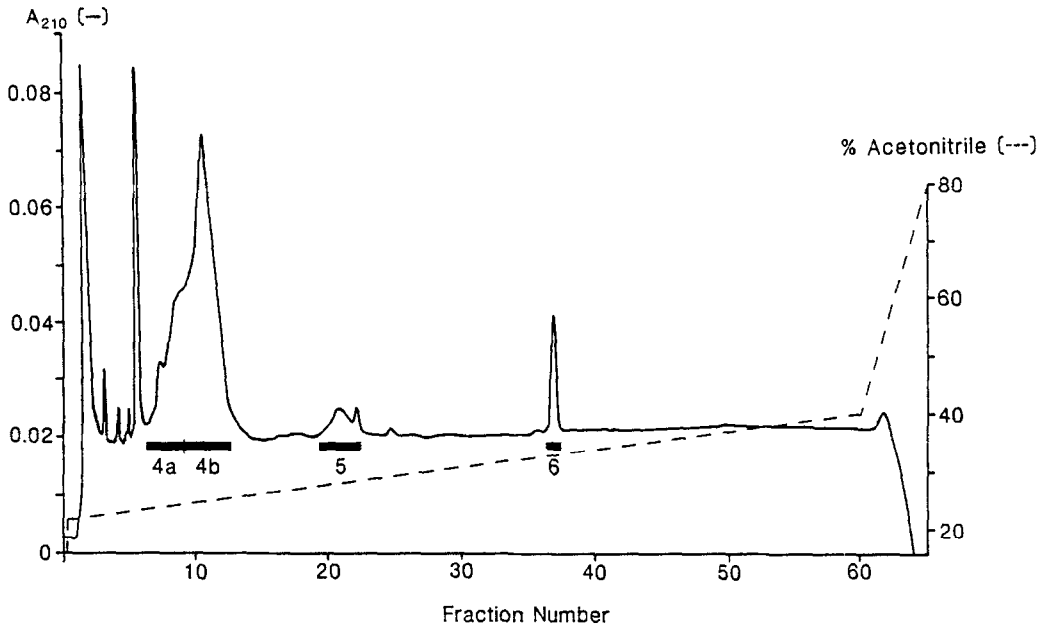

Fig. 3. Reversed phase HPLC purification of the M_r 30-20 protein pool (II) recovered from Fig. 1. Three peaks of IGF-BP (denoted by solid bars 4, 5, 6) were resolved in the HPLC chromatogram. The sharp peaks eluting before fraction 6 were due to column artifacts.

N-Terminal amino acid sequence analysis of peak 4a in Fig. 3 showed that it has the same N-terminal as peak 1 in Fig. 2 (see Table 1). The same amino acid sequence was found for peak 4b, whereas peak 5 yielded the same sequence as peak 2 in Fig. 2. Peak 6 did not contain any amino acids, suggesting it is an artifact from the HPLC column. The 20-10 kDa molecular size pool, when subjected to reversed phase HPLC purification, did not yield any UV absorbing peaks, indicating that there is no IGF-binding component that is <20 kDa in adult rat serum.

SDS-PAGE analysis of each of the isolated rat IGF-BP peaks under reducing conditions is shown in Fig. 4. Each of the proteins migrated as two bands on the gel as reported by others (7,8). Since N-terminal sequence analysis yielded only one amino acid sequence from each protein, the origin of the two bands might be due to different degrees of glycosylation and/or C-terminal truncation. The fact that peaks 4a and 4b have the same N-terminal sequence as peak 1 (see Table 1) and yet each migrates with two bands at 22/28 kDa and 22/27 kDa would suggest that they are the C-terminal truncated forms of the intact 45 kDa IGF-BP of peak 1. The IGF-BP present in peaks 2, 3 and 5 showed similar migrations on the SDS gel (32/36 kDa and 33/35 kDa) and yet they are separable by HPLC. Peak 3 has the same N-terminal sequence as the rat IGF-BP isolated from a BRL-3A cell line, whereas the N-terminal sequence of peaks 2 and 5 is unique, suggesting that it is a novel rat IGF-BP (see Table 1).

DISCUSSION

Using gel filtration, ligand affinity chromatography and reversed phase HPLC, four IGF-BPs have been purified from adult serum. N-terminal sequence determination together with SDS-

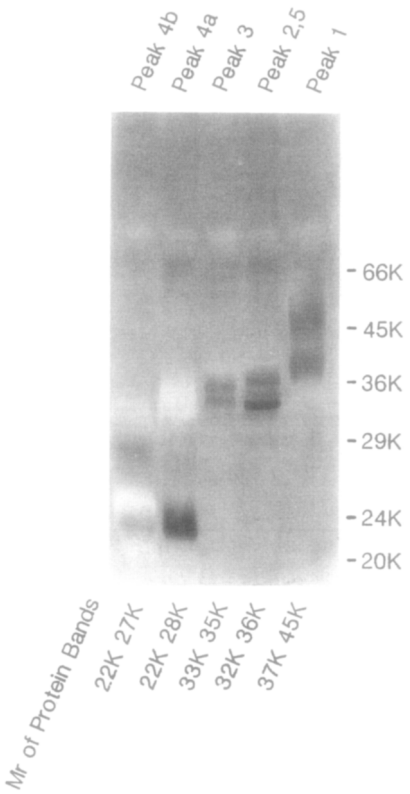

Fig. 4. SDS-PAGE analysis of the IGF-BP peaks purified by reversed phase HPLC. The analysis was performed under reducing conditions. Peaks 1, 2, 3 are from Fig. 2 and peaks 5, 4a, 4b are from Fig. 3. Note that all of the proteins exhibit two bands but only one N-terminal sequence was found in each protein. The numbers at the right correspond to the migration of the molecular weight markers.

PAGE analysis establish that the most abundant IGF-BP in adult rat serum is the C-terminal truncated form of the 45 kDa IGF-BP, although the intact form also exists in the serum. A small amount of the BRL-3A IGF-BP was also detected. However, the second most abundant IGF-BP present in adult rat serum actually corresponds to a new protein, whose amino acid sequence has not been reported. Based on homology alignment as shown in Table 2, the novel rat IGF-BP is closely related to the BP present in a BRL-3A cell line (15). In a previous report Yang et al. (8) have noted a BRL-3A related IGF-BP in adult rat serum based on its similar molecular size but non-crossreactivity with an antibody generated against the BRL-3A protein. But no amino acid sequence information was presented. The presence of two closely related IGF-BPs with similar molecular weights in adult rat serum is intriguing. Since the level of the BRL-3A protein is highly abundant in fetal rat serum (15), but decreases in adult serum, presence of another closely related BP in adult rat serum may suggest a switching of the regulatory signal to turn on the expression of the novel IGF-BP in adult rats. However, the true relation of the unique IGF-BP discovered in this study must await molecular cloning to determine its complete primary structure.

Table 2. Alignment of the N-terminal amino acid sequences of the rat IGF-BP present in peak 2 and the rat IGF-BP isolated from a rat BRL-3A cell line (15)*

	5	10	15	20	25	30	35	40
Peak 2 IGF-BP	D E A I H X P P X S E E K L A R X R P P V G - - X E E L V R E P G X G X X A T X A							
BRL-3A IGF-BP	E V L F R C P P C T P E R L A A C G P P P D A P C A E L V R E P G C G C C S V C A							

* Identical amino acids are boxed and gaps are inserted to maximize alignment homology.

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