Isolation from Adult Human Serum of Four Insulin-like Growth Factor (IGF) Binding Proteins and Molecular Cloning of One of Them That Is Increased by IGF I Administration and in Extrapancreatic Tumor Hypoglycemia*

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We have isolated four insulin-like growth factor binding proteins (IGFBPs) from adult human serum by insulin-like growth factor (IGF) I affinity chromatography and high performance liquid chromatography. A 36-kDa binding protein (BP), not digestible with N-glycanase, is increased in patients with extrapancreatic tumor hypoglycemia and during IGF I administration in healthy adults. Its 38 NH2-terminal amino acids are identical to those of an IGFBP sequence derived from a human cDNA that cross-hybridizes with the rat IGFBP-2 cDNA. With probes encoding a NH2-terminal, COOH-terminal, and a middle region of this protein we have obtained three cDNA clones from a Hep G2 cDNA library; one encodes human IGFBP-2, and the other two presumably represent unspliced heteronuclear and alternatively spliced mRNA, respectively. A 28-30-kDa IGFBP represents a novel BP species in human serum. Its 38 NH2-terminal amino acids are not homologous to IGFBP-1, -2, or -3. It is not digestible with N-glycanase and does not bind 125I-IGF I. The NH2-terminal sequences of a 42/45- and a 31-kDa IGFBP are identical to that of human IGFBP-3. The 42/45-kDa proteins are two glycosylation variants of BP-3. The 31-kDa protein presumably is a degradation product of BP-3 that lacks the COOH terminus. It is likely that the different IGFBPs modulate autocrine and paracrine effects of IGFs on growth and metabolism in a different and specific manner.

Insulin-like growth factors (IGFs)1 are synthesized mainly in the liver but, in addition, in many other tissues and cell lines (1, 2). After release from their site of origin, IGFs are always found noncovalently linked to specific high affinity binding proteins (BP) (2). Their physiological role is not yet fully appreciated. In the circulation, IGFBPs prolong the half-life of the IGFs (3-5) and protect the organism against acute insulin-like actions of IGFs (4, 6). In the interstitial fluid they may regulate the bioavailability of locally secreted IGFs to IGF target cells (7,11).

Adult human serum contains two native IGFBP complexes. Upon gel permeation chromatography at neutral pH they elute at apparent molecular masses of 160-180 and 30-55 kDa (12). The large molecular weight complex is growth hormone-dependent (13, 14). It consists of three subunits: a glycosylated IGFBP, an acid-labile glycoprotein, and IGF (15). The IGFBP of this complex has recently been purified, and the corresponding cDNA has been cloned (16). The small molecular weight complex is inversely related to the growth hormone status (13). Ligand blot analysis of this complex yields a major protein band with an apparent molecular mass of 36 kDa (17). It is the predominant IGFBP in human fetal serum and in patients with Laron dwarfism and extrapancreatic tumor hypoglycemia (17). Furthermore, subcutaneous infusion of IGF I in healthy adults increases this 36-kDa protein in serum. The increase is prevented by simultaneous growth hormone administration (17).

We have isolated the 36-kDa IGFBP from adult human serum, determined its NH2-terminal amino acid sequence, and obtained cloned cDNA encoding the BP.1 In addition, we have purified three other IGFBPs from human serum and determined their NH2-terminal sequences.

MATERIALS AND METHODS

Sepharose-IGF I Affinity Column—60 mg of recombinant human IGF I (Ciba-Geigy AG, Basel, Switzerland) was dissolved in 20 ml of 0.1 m NaHCO3, pH 8.3, containing 0.5 m NaCl and coupled to CNBr-activated Sepharose 4B (4 g of dry gel) according to the protocol of the supplier. The gel was equilibrated with 100 mL of 0.01 m sodium phosphate buffer, 0.5 m NaCl, pH 6.5, in a 1.5 x 15-cm glass column (gel bed volume, 15 mL).

Purification of Serum IGFBPs—This was carried out according to a modified procedure of Martin and Baxter (18, 19). 1 liter of outdated human citrate plasma was stirred for 2 h at room temperature with 50 units (1 ml) of thrombin-calcium, filtered through cheesecloth and acidified. Dissociated IGF was removed with SP-Sephadex C-25. The pH was subsequently adjusted to 6.5 and the precipitate removed by centrifugation at 20,000 rpm for 30 min. The supernatant was pumped through the Sepharose-IGF I affinity column at 34 ml/h and the column washed with 500 mL of 0.05 m sodium phosphate buffer, 0.5 m NaCl, pH 6.5. BP was eluted with 40 ml of 0.5 m acetic acid, diazylzed 3 times against 2 liters of 0.1 m ammonium acetate, and

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2 The abbreviations used are: IGF, insulin-like growth factor; BP, binding protein(s); IGFBP, insulin-like growth factor binding protein(s); HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; kb, kilobase(s).
lyophilized. The lyophilized material (40 mg) was dissolved in 4 ml of 0.1 M heptfluorobutyric acid containing 20% (v/v) acetonitrile, and the insoluble material was removed by centrifugation at 10,000 "/g for 10 min. The clear supernatant was subjected to HPLC (two runs with 2 ml each) on a Nucleosil C18 column (Machery-Nagel, Düren, FRG) (19). Effluent fractions were dried in a Speed-Vac (Savant Instruments, Holtsville, NY) under vacuum up in 1 M acetic acid, and dried again. They were dissolved in 250 lU of H2O for ligand blot analysis (see below) and silver staining (20).

125I-IGF Ligand Blot Analysis—The method of Hoskenlopp et al. (21) was used with slight modifications (6, 19). 5-10 aliquots of the HPLC effluent fractions were subjected to electrophoresis on 15% SDS-polyacrylamide slab gels under nonreducing conditions. The 1 4 labeled material was transferred electrophoretically (2 h at 0.8 A) onto an Immobilon P membrane (Millipore Corp., Bedford, MA) as described by Matsudaira (23). The membrane was stained for 5 s with 0.1% Coomassie Blue R-250 in 50% methanol, destained in 50% methanol, 10% acetic acid for 5 min at room temperature, and then thoroughly rinsed in H2O. The membrane was air-dried, and the protein bands were cut out and stored at -20 °C. Amino acid sequence analysis was carried out as described by Yuen et al. (24).

Amino Acid Analysis—This was performed by automated Edman degradation using an Applied Biosystems model 470A protein sequenator (Foster City, CA) (25). Cysteine residues were identified after degradation using an Applied Biosystems model 470A protein sequenator. Cvsteine residues were identified after degradation using an Applied Biosystems model 470A protein sequenator. Replicates were performed, and the data were averaged.

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RESULTS

Relative Abundance of Different IGFBP Species in Human Serum—Fig. 1 shows a comparison of 125I-IGF II ligand blot signals for adult and fetal human serum. A broad band was observed in medium from Hep G2 cells. In normal adult human serum, the predominant IGFBP appears as a broad 42/45-kDa double band (doublet). A fainter narrow band is observed at 36 kDa. In fetal human serum and in the serum of Laron dwarfs (43),
were cultured to confluency in Dulbecco's modified Eagle's medium and supplemented with 10% (v/v) fetal calf serum and 2 mmol/liter L-glutamine in culture dishes with a diameter of 10 cm. The culture medium was poured off, and the cells were thoroughly rinsed with 3 ml human serum albumin instead of fetal calf serum and 2 mmol/liter L-glutamine and then kept in 10 ml of this medium for another 8 h at 37 °C. 5 ml of the conditioned medium was dialyzed extensively against 0.15 M NaCl, 0.02 M Tris-HCl buffer, pH 6.8, containing 4% SDS. 0.02 M NaEDTA and 24% (v/v) glycerol, and electrophoresed and processed as described under “Materials and Methods” and in Refs. 6 and 12. After incubation with ligand, the nitrocellulose membrane was exposed to the x-ray film for 24 h. Lane 1, conditioned medium from Hep G2 cells; lanes 2 and 3, two different serum pools from normal adults; lanes 4-6, fetal sera from gestational weeks 28, 31, and 34; lane 7, serum from a Laron-type patient.

The 36-kDa band predominates together with another band at 33 kDa. The latter corresponds to the predominant IGFBP binding band in culture medium from Hep G2 cells. Like serum, Hep G2 medium contains the 36-kDa band (appearing as a doublet) and the 42/45-kDa doublet.

Affinity Purification and HPLC of IGFBPs from Adult Human Serum—After affinity purification, the 125I-IGF II ligand blot pattern differs slightly from that of whole serum (Fig. 2). The 36-kDa band becomes more prominent relative to the 42/45-kDa doublet, and two additional bands appear which are seen in whole serum only after prolonged exposure to the x-ray film: a broad band between 28 and 31 kDa and a narrow band at 24 kDa. The 36-kDa protein is the most hydrophilic IGFBP with the longest retention time on HPLC (fractions 66-70, Fig. 2). It is not digestible with N-glycanase (Fig. 2, lower right panel). The silver stain shows a double band in fractions 66 and 67.

The most hydrophilic IGFBP (fractions 36-40, Fig. 3) has an approximate molecular mass of 30–31 kDa. It is followed by another BP (fractions 44-48, Fig. 3) with an approximate molecular mass of 28–30 kDa. In contrast to the 31-kDa protein, the 28–30-kDa protein is not digestible with N-glycanase (Fig. 4). It also differs by the gray color on the silver stain, whereas the preceding fractions stain brown (not shown). Neither the 31- nor the 28–30-kDa BP bind 125I-IGF I on ligand blots (not shown). The 24- and 45-kDa bands (fractions 50-55, Fig. 3) are not completely resolved. Digestion of the 42/45-kDa doublet with N-glycanase yields a single band at 37 kDa (Fig. 4).

Amino Acid Sequence Analysis of HPLC-purified IGFBPs—The 38 NH2-terminal amino acids of the 36-kDa protein (Fig. 5) are identical to those of an IGFBP whose amino acid sequence has been derived from a human cDNA obtained by cross-hybridization with the rat IGFBP-2 cDNA (31, 32). The 38 NH2-terminal amino acids of the HPLC-purified 31-kDa protein (Fig. 5) are the same as those of the recently cloned GH-dependent IGFBP (16), now termed IGFBP-3 (44).

The 30 NH2-terminal amino acids of the human serum 28–30-kDa IGFBP (Fig. 5) have not been reported before. However, most of the first 15 amino acids are shared with the NH2-terminal sequences of two IGFBPs purified from cerebrospinal fluid of adults and children, respectively (45). Furthermore, the 12 NH2-terminal amino acids recently reported for an IGFBP from conditioned medium of SV40-transformed human fibroblasts (46) are identical (except position 5) to those of the 28–30-kDa human serum BP.

The 42/45-kDa doublet yields a unique NH2-terminal amino acid sequence. It is identical to that of the 42-kDa band (Fig. 5) after transfer to an Immobilon (polyvinylidene difluoride) membrane. The 23 NH2-terminal amino acids are identical to those of the 31-kDa BP and of IGFBP-3 (16).

Nucleotide Sequence of the 36-kDa BP cDNA and Derived

![Fig. 2. Silver stain (upper panel) and 125I-IGF II ligand blot analysis (lower panel) of fractions 66–70 obtained by HPLC of affinity-purified IGF binding protein. Lyophilized fractions were dissolved in 250 μl of H2O. 20-μl aliquots were electrophoresed as described under “Materials and Methods” and the gels subjected to silver staining (30). 5-μl aliquots were used for ligand blot analysis (6, 19, 21). The lower right panel shows the ligand blot of pooled fractions 66–70 before and after N-glycanase digestion.](image-url)
Amino Acid Sequence—Three positive clones, 2-1, 2-2, and 5, were isolated from the Hep G2 cDNA library. Digestion of the plasmid prepared from clone 2-2 with BglII yielded a fragment of ~1.4 kb that hybridized with probes 1.33, 2.41, and 3.20. The sequence consists of 1414 nucleotides. An ATG codon (nucleotides 1–3) is preceded by 86 nucleotides and continues into an open reading frame of 328 amino acids. A hydrophobic region of 39 amino acids is followed by the 38 amino acids determined from the purified 36-kDa IGFBP (Fig. 5). The nucleotide sequence is terminated by a TAG stop codon at nucleotides 985–987. Obviously, nucleotides 1–984 encode an IGFBP precursor of 328 amino acids (~35 kDa) which contains a prepeptide region of 39 amino acids and the mature BP with 289 amino acids (~31 kDa). This sequence is identical to that reported by Binkert et al. (31) with the exception of two amino acids, a proline instead of a leucine in the prepeptide region at position 20 and an arginine instead of a cysteine at the carboxyl terminus at position 320.

The prepeptide region of the 36-kDa BP is particularly rich in leucine (31%), proline (26%), and glycine (18%) and contains 1 cysteine residue. 17 of the remaining 18 cysteine residues are clustered between residues 40–135 and 263–310. The deduced amino acid sequence also confirms the absence of N-glycosylation sites as suggested by the N-glycanase digestion experiment (Fig. 4). The COOH terminus contains an Arg-Gly-Asp sequence (residues 314–316), a recognition site for cell adhesion receptors (47, 48). The 5′-untranslated sequence of 86 nucleotides preceding the coding region of the BP messenger RNA starts to differ from that of Binkert et al. (31) at nucleotide ~54 and is shorter (86 as compared with 127 nucleotides). The TAG stop codon (nucleotides 985–987) is followed by a 3′-untranslated region (341 nucleotides) which is 10 nucleotides longer but otherwise identical to that of Binkert et al. (31) except for a T instead of a C at position 1000.

Sequencing of the two additional clones 2-1 and 5 yielded cDNAs related to that of clone 2-2 (not shown). Clone 5 has an insert, presumably an intron, of 1071 additional nucleotides. The insert starts with GT (nucleotides 682 and 683) and ends with AG (nucleotides 1751 and 1752). In clone 2-1, the open reading frame starts to differ from that of clone 2-2 at nucleotide 682. It ends with a TGA stop codon at nucleotides 922–924. Clone 5 probably represents incompletely spliced heteronuclear mRNA, clone 2-1 alternatively spliced mRNA.

Northern Blot of mRNA Encoding the 36-kDa Carrier Protein—Poly(A)+ RNA from human adult and embryonic liver, the Hep G2 liver cell line, the WRL embryonic liver cell line, and the kidney cell line 293 were hybridized with the purified cDNA insert of clone 2-2 (Fig. 6). Two transcripts of 1.6–1.8 kb and ~4.4 kb were found. Their distribution was unequal in different tissues; adult human liver yielded only a single transcript of 1.6–1.8 kb, whereas 4.4-kb mRNA was only detected in the WRL embryonic liver cell line. Fetal human liver contained predominantly the 1.6–1.8-kb transcript, and
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We have isolated four IGFBPs from human serum. The most hydrophobic BP with an approximate molecular mass of 36 kDa on 125I-IGF II ligand blots was of particular interest; its serum levels rise conspicuously during administration of IGF I to normal subjects, and it is increased in sera from patients with extraprancreatic tumor hypoglycemia (17). It is the major constituent of the native 50-kDa IGF carrier complex of human serum (17). Its isolation from human serum and its NH2-terminal amino acid sequence are reported here for the first time. 23 of the 24 NH2-terminal amino acids are identical to those of a rat IGFBP isolated from conditioned medium of BRL-3A rat liver cells (49), whereas the following 14 amino acids are lacking in the rat sequence. A cDNA encoding the rat protein has been obtained from an adult rat liver and a BRL-3A rat liver cell cDNA library (32, 50). Binkert et al. (31) have screened a fetal human liver cDNA library with this rat cDNA and have recognized a human cDNA that cross-hybrdizes with the rat cDNA. The NH2-terminal amino acid sequence of the 36-kDa BP that we isolated is identical to the sequence derived from this human cDNA. Therefore, it appeared likely that the 36-kDa BP was the same as the protein encoded by the cDNA of Binkert et al. (31). We, therefore, used the corresponding protein sequence (kindly provided by J. Schwander) to synthesize oligonucleotide probes encoding a NH2-terminal, a COOH-terminal, and a middle region and screened a Hep G2 cDNA library. Hep G2 cells release the 36-kDa IGFBP into the culture medium (Fig. 1). We identified three different cDNA clones. The derived amino acid sequence of clone 2-2 is identical to that reported by Binkert et al. (31) except for two amino acids (see “Results”). The protein is termed human IGFBP-2 (31, 44).

The NH2-terminal sequences of the 31-kDa and of the GH-dependent 42/45-kDa protein are identical to that of the recently cloned IGFBP-3 (16). The 42- and 45-kDa bands probably represent N-glycosylation variants of IGFBP-3 (Fig. 4) (18). Like the 42/45, the 31-kDa protein is N-glycosylated (Fig. 4). On fractionation of normal adult human serum by gel permeation chromatography it elutes together with the 42/45-kDa proteins in the 160–180-kDa carrier complex (17). A 31-kDa IGFBP whose NH2-terminal amino acid sequence is identical to that of a 42/45-kDa BP representing rat IGFBP-3 (51) has also been isolated from adult rat serum (19). Presumably, the rat and human 31-kDa proteins are degradation products of IGFBP-3 which lack the COOH terminus (17, 19). In contrast to the rat, the human 31-kDa protein is not completely digestible with N-glycanase (Fig. 4, fractions 39 and 40) although it yields a unique NH2-terminal amino acid sequence. Possibly the 31-kDa band contains a mixture of N-glycosylated and unglycosylated NH2-terminal fragments of IGFBP-3.

Both the NH2 and the COOH terminus of the IGFBPs are probably involved in IGF binding (52, 53). This explains why 125I-IGF II binds less well to the 31-kDa than to the 42/45-kDa BP.3 The lack of binding of 125I-IGF I to the 31-kDa protein indicates that both the NH2 and the COOH termini are required for binding of IGF I to IGFBP-3.

The similarity or identity between the NH2 termini of the 28–30-kDa serum BP and the BPs from human cerebrospinal fluid (45) or conditioned medium of SV40-transformed human fibroblasts (46) suggests that these proteins are closely related, if not identical. None of these sequences (up to amino acid 30 in the case of the 28–30-kDa serum BP) reveals any appreciable homology to the NH2 termini of human IGFBP-1 (54–58), IGFBP-2 (Ref. 31 and this paper), or IGFBP-3 (16). A prominent feature of the 28–30-kDa BP is that it does not bind 125I-IGF I on ligand blots. Preferential binding of IGF II has also been shown for the cerebrospinal fluid IGFBPs (45) and for the BP produced by the SV40-transformed human fibroblasts (46). In contrast to the 42/45-kDa BP (BP-3) and to the 36-kDa BP (BP-2), the 28–30- and the 31-kDa BPs are minor constituents in normal adult human serum. Like the 36-kDa BP, the 28–30-kDa BP is a component of the native 50-kDa IGF carrier complex (17). This complex also contains a 24-kDa BP (17). We did not obtain enough material of this protein to perform an amino acid sequence analysis. As judged from its app. molecular weight, it may represent the serum form of an IGFBP recently isolated from conditioned medium of human bone cells (59).

The sequence homology between the human IGFBP-2 and the other cloned IGFBPs is striking (Fig. 7). It is most pronounced in the NH2- and the COOH-terminal regions, whereas the middle regions are completely different. Most conspicuous, 17 of the 18 cysteine residues in human (Ref. 31 and this paper) and rat BP-2 (32, 50), human BP-1 (54–58), and human (16) and rat BP-3 (51) are found in identical positions. They are clustered in the NH2 and the COOH termini. This suggests the presence of binding epitopes in both of these regions (52, 53) and is compatible with the complete loss of IGF binding activity under reduced condi-

3 J. Zapf, unpublished observation.
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Fig. 7. Comparison of the amino acid sequences of human, rat, and bovine IGF binding proteins. The sequences have been aligned such as to give maximal homology. Sequences are taken from the following references: rat BP-2 from Refs. 32 and 49, human BP-3 from Ref. 16, human BP-1 from Ref. 53, and the bovine binding protein from MDBK kidney cells from Ref. 60.

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