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Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2)

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Insulin-like growth factors bind with high affinity to specific binding proteins in extracellular fluids. To identify structural characteristics of IGF-binding proteins that might define their physiological roles, we determined the complete primary structure of a novel human IGF-binding protein (IGFBP-2) from a cloned cDNA. The cDNA encodes a 328 amino acid IGF-binding protein precursor which contains a 39-residue signal peptide. The mature 289 amino acid IGFBP-2 has a predicted M_r of 31 325. Chinese hamster ovary (CHO) cells stably transformed with the IGFBP-2 cDNA secreted a 36 kd protein which bound, with different affinities, IGFII and IGFI, but did not bind insulin. The predicted protein sequence of this IGF-binding protein shares extensive amino acid homology (>85%) with the IGF-binding protein secreted by rat BRL-3A cells, but <40% homology with human IGFBP-1. Therefore IGFBP-2, and not IGFBP-1 as previously suggested, represents the human homologue of the rat BRL-BP (αIGFBP-2). Moreover, from alignment of the predicted protein sequences of IGFBP-1 and IGFBP-2, extensive conservation of the distribution of cysteine residues is observed. Although the overall amino acid homology shared by these proteins is not high, we suggest that they represent a family of structurally related human IGFBPs. Southern blot analysis of human DNA demonstrates that IGFBP-2 is encoded by a single-copy gene, different from that of IGFBP-1.

Key words: IGF-binding protein/IGFBP-2/human fetal liver/cDNA/rat BRL-3A binding protein equivalent

Introduction

Insulin-like growth factors I and II (IGF-1 and -II) are polypeptide hormones with structural homology to proinsulin (Rinderknecht and Humbel, 1978a,b). IGF-1 mediates the growth-promoting effects of growth hormone (GH) in cartilage and bone formation. The role of IGF-II is less well understood. It might have some importance in the regulation of fetal development. Both IGFs have insulin-like effects (Froesch *et al.*, 1985; Jakobovits, 1986). Most of the growth and mitogenic effects of the IGFs are mediated by the IGF-I receptor. Both IGFs also bind to the IGF-II receptor and cross-react with the insulin receptor with a 100 times lower affinity than insulin (Rechler and Nissley, 1985). The total serum concentration of the IGFs in humans is >100 times higher than the postprandial insulin serum peaks and therefore could cause hypoglycaemia (Guler *et al.*, 1987). The presence of binding proteins that bind >99% of the IGFs present in serum inhibits IGF–insulin receptor interactions and protects the organism from hypoglycaemia (Froesch *et al.*, 1985). These binding proteins prolong the half-life of the IGFs and have in addition been shown to either inhibit or stimulate the growth-promoting effect of the IGFs on cells in culture (Moses *et al.*, 1976; Zapf *et al.*, 1985; Elgin *et al.*, 1987; Ritvos *et al.*, 1988; Rutanen *et al.*, 1988). These findings led to the hypothesis that IGF activity at the tissue level could be regulated not only by the concentration of IGF and the number of receptors, but also by the presence of binding proteins.

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ATT CGG GGC GAG GGA GGA GGA AGA AGC GGA GGA GGC GGC TCC CGC TCG CAG GGC CGT GCA      - 56

                                                                          Met
CCT GCC CGC CCG CCC GCT CGC TCG CTC GCC CGC CGC GCC GCG CTG CCG ACC GCC AGC ATG       3

Leu Pro Arg Val Gly Cys Pro Ala Leu Pro Leu Pro Pro Pro Pro Leu Leu Pro Leu Leu
CTG CCG AGA GTG GGC TGC CCC GCG CTG CCG CTG CCG CCG CCG CCG CTG CTG CCG CTG CTG      63
                                                                  1
Pro Leu Leu Leu Leu Leu Leu Gly Ala Ser Gly Gly Gly Gly Gly Ala Arg Ala Glu Val
CCG CTG CTG CTG CTG CTA CTG GGC GCG AGT GGC GGC GGC GGC GGG GCG CGC GCG GAG GTG     123

Leu Phe Arg Cys Pro Pro Cys Thr Pro Glu Arg Leu Ala Ala Cys Gly Pro Pro Pro Val
CTG TTC CGC TGC CCG CCC TGC ACA CCC GAG CGC CTG GCC GCC TGC GGG CCC CCG CCG GTT     183
                                                          40
Ala Pro Pro Ala Ala Val Ala Ala Val Ala Gly Gly Ala Arg Met Pro Cys Ala Glu Leu
GCG CCG CCC GCC GCG GTG GCC GCA GTG GCC GGA GGC GCC CGC ATG CCA TGC GCG GAG CTC     243

Val Arg Glu Pro Gly Cys Gly Cys Cys Ser Val Cys Ala Arg Leu Glu Gly Glu Ala Cys
GTC CGG GAG CCG GGC TGC GGC TGC TGC TCG GTG TGC GCC CGG CTG GAG GGC GAG GCG TGC     303
                                                          80
Gly Val Tyr Thr Pro Arg Cys Gly Gln Gly Leu Arg Cys Tyr Pro His Pro Gly Ser Glu
GGC GTC TAC ACC CCG CGC TGC GGC CAG GGG CTG CGC TGC TAT CCC CAC CCG GGC TCC GAG     363

Leu Pro Leu Gln Ala Leu Val Met Gly Glu Gly Thr Cys Glu Lys Arg Arg Asp Ala Glu
CTG CCC CTG CAG GCG CTG GTC ATG GGC GAG GGC ACT TGT GAG AAG CGC CGG GAC GCC GAG     423
                                                        120
Tyr Gly Ala Ser Pro Glu Gln Val Ala Asp Asn Gly Asp Asp His Ser Glu Gly Gly Leu
TAT GGC GCC AGC CCG GAG CAG GTT GCA GAC AAT GGC GAT GAC CAC TCA GAA GGA GGC CTG     483

Val Glu Asn His Val Asp Ser Thr Met Asn Met Leu Gly Gly Gly Gly Ser Ala Gly Arg
GTG GAG AAC CAC GTG GAC AGC ACC ATG AAC ATG TTG GGC GGG GGA GGC AGT GCT GGC CGG     543
                                                        160
Lys Pro Leu Lys Ser Gly Met Lys Glu Leu Ala Val Phe Arg Glu Lys Val Thr Glu Gln
AAG CCC CTC AAG TCG GGT ATG AAG GAG CTG GCC GTG TTC CGG GAG AAG GTC ACT GAG CAG     603

His Arg Gln Met Gly Lys Gly Gly Lys His His Leu Gly Leu Glu Glu Pro Lys Lys Leu
CAC CGG CAG ATG GGC AAG GGT GGC AAG CAT CAC CTT GGC CTG GAG GAG CCC AAG AAG CTG     663
                                                        200
Arg Pro Pro Pro Ala Arg Thr Pro Cys Gln Gln Glu Leu Asp Gln Val Leu Glu Arg Ile
CGA CCA CCC CCT GCC AGG ACT CCC TGC CAA CAG GAA CTG GAC CAG GTC CTG GAG CGG ATC     723

Ser Thr Met Arg Leu Pro Asp Glu Arg Gly Pro Leu Glu His Leu Tyr Ser Leu His Ile
TCC ACC ATG CGC CTT CCG GAT GAG CGG GGC CCT CTG GAG CAC CTC TAC TCC CTG CAC ATC     783
                                                        240
Pro Asn Cys Asp Lys His Gly Leu Tyr Asn Leu Lys Gln Cys Lys Met Ser Leu Asn Gly
CCC AAC TGT GAC AAG CAT GGC CTG TAC AAC CTC AAA CAG TGC AAG ATG TCT CTG AAC GGG     843

Gln Arg Gly Glu Cys Trp Cys Val Asn Pro Asn Thr Gly Lys Leu Ile Gln Gly Ala Pro
CAG CGT GGG GAG TGC TGG TGT GTG AAC CCC AAC ACC GGG AAG CTG ATC CAG GGA GCC CCC     903
                                                        280
Thr Ile Arg Gly Asp Pro Glu Cys His Leu Phe Tyr Asn Glu Gln Gln Glu Ala Cys Gly
ACC ATC CGG GGG GAC CCC GAG TGT CAT CTC TTC TAC AAT GAG CAG CAG GAG GCT TGC GGG     963

Val His Thr Gln Arg Met Gln ***
GTG CAC ACC CAG CGG ATG CAG TAG ACC GCA GCC AGC CGG TGC CTG GCG CCC CTG CCC CCC    1023

GCC CCT CTC CAA ACA CCG GCA GAA AAC GGA GAG TGC TTG GGT GGT GGG TGC TGG AGG ATT    1083

TTC CAG TTC TGA CAC ACG TAT TTA TAT TTG GAA AGA GAC CAG CAC CGA GCT CGG CAC CTC    1143

CCC GGC CTC TCT CTT CCC AGC TGC AGA TGC CAC ACC TGC TCC TTC TTG CTT TCC CCG GGG    1203

GAG GAA GGG GGT TGT GGT CGG GGA GCT GGG GTA CAG GTT TGG GGA GGG GGA AGA GAA ATT    1263

TTT ATT TTT GAA CCC CTG TGT CCC TTT TGC ATA AGA TTA AAG GAA GGA AAA GTA A          1318
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Fig. 1. Nucleotide and deduced amino acid sequence of human IGFBP-2. The first residue of the mature protein is indicated by 1. The RGD motif and the imperfect polyadenylation signal are underlined. The two A residues at the 3′ end represent the start of the poly(A) tract.

Two major forms of binding protein are detected by radioligand binding studies in adult human serum: a 150 kd form which is growth hormone regulated, and a smaller 35 kd form (Hardouin *et al.*, 1987). The biosynthesis of a small binding protein, termed IGFBP-1, originally purified from amniotic fluid is not GH dependent. The complete amino acid sequence of this 28 kd IGFBP-1 was deduced from cDNA clones of human placenta, decidua and HEPG2 origin (Brewer *et al.*, 1988; Brinkman *et al.*, 1988; Grundmann *et al.*, 1988; Julkunen *et al.*, 1988; Lee *et al.*, 1988). By virtue of their IGF binding characteristics, ontogenetic regulation, molecular mass and partial amino terminal sequence homology, it was suggested that the IGFBP-1 was the human equivalent of the rat IGF binding protein produced by a rat liver cell line (BRL-3A) (Mottola *et al.*, 1986; Brinkman *et al.*, 1988; Lee *et al.*, 1988).

In this paper we report the isolation and characterization of clones from a human fetal liver cDNA library that encode a human IGF-BP, which we call IGFBP-2. Sequence comparison of IGFBP-1, IGFBP-2 and the rat BRL-BP reveals that IGFBP-2 is a novel human IGF-BP and that it represents the human equivalent of the rat BRL-3A BP. The mature IGFBP-2 has a predicted mol. wt of 31 kd, is encoded by a single copy gene and has a higher affinity for IGF-II than IGF-I. From Northern blot analysis it appears that both of these binding proteins exhibit different patterns of tissue-specific expression.

Results

Isolation, characterization and deduced primary protein structure of human IGF-BP cDNA clones

We have previously determined the nucleotide sequence of the cDNA and the deduced amino acid sequence of the rat IGF-BP found in BRL-3A cell-conditioned medium (Lyons and Smith, 1985; Mottola *et al.*, 1986; Margot *et al.*, 1989). This cDNA was used for screening of a fetal human liver cDNA library. The nucleotide sequence of a cross-hybridizable 1.43 kb cDNA insert is shown in Figure 1. This sequence has an open reading frame of 984 nucleotides which begins with an ATG codon 118 nucleotides from the extreme 5′ terminus. This initiation codon is flanked by nucleotides matching the criteria for a translation initiation site as determined by Kozak (1987). The open reading frame is terminated by an amber codon (TAG) and followed by 330 nucleotides of 3′-untranslated sequence containing an imperfect polyadenylation signal (ATTAAA).

The open reading frame of this cDNA has the capacity to encode a protein of 328 amino acids with a calculated M_r of 35 000. The first 39 amino acids form a signal peptide, as shown by N-terminal sequencing of the secreted recombinant protein (H.P.Kocher, unpublished results). The mature protein, called IGFBP-2, consists of 289 amino acids and has a calculated M_r of 31 325. No potential N-linked glycosylation sites are present in the predicted protein sequence.

Protein sequences of the rat BRL-BP and the human IGFBP-1 and IGFBP-2 were aligned to maximize homologies (Figure 2). Comparison of the BRL-BP with IGFBP-2 reveals an overall homology of >85%. In contrast, the human IGFBP-2 and IGFBP-1 exhibit <40% predicted amino acid homology. Nevertheless, all three BPs have several features in common: they share not only a cysteine-rich region at their amino terminus, but the spacing between homologous cysteine residues is also conserved. Furthermore, the three IGF-BP sequences share the RGD motif (Ruoslahti and Pierschbacher, 1986; Hynes, 1987) within the conserved pentapeptide sequence, IRGDP. The amino- and carboxy-terminal regions share the highest degree of homology. No stretch of conserved amino acids between the two human BPs is >5 residues. Although the mature IGFBP-2 is >85% homologous with the rat BRL-BP, it is

```
          1                                                                  80
IBP-2     EVLFRCPPCT PERLAACGPP PVAPPAAVAA VAGGARMPCA ELVREPGCGC CSVCARLEGE ACGVYTPRCG QGLRCYPHPG
BRL-BP    ---------- ---------- -D-                --- ---------- ------Q--- -----I---A -T-----N--
IBP-1      APWQ-A--S A-K--L- -- VS-                S-S -VT-SA---- -PM--LPL-A ----ATA--A R--S-RAL--

          81                                                                 157
IBP-2     SELPLQALVM GEGTCEKRRD AEYGA   SP EQVADNGDDH SEGGLVENHV DSTMNMLGGG GSAGRKPLKS GMKELAVFRE
BRL-BP    -----K---T -A-------    V--   T- Q----SE--- ---------- -G----- -- S------P-- ----------
IBP-1     EQQ--H--TR -Q-A-VQES- -SAPHAAEAG SPESPESTEI T-EE-LD-FH LMAPSEEDHS ILWDAISTYD -S-A-H-TNI

          158                                                                237
IBP-2     KVTEQHRQMG KGGKHHLGLE EPKKLRPPPA RTPCQQELDQ VLERISTMRL PDERGPLEHL YSLHIPNCDK HGLYNLKQCK
BRL-BP    --N------- --A- --S-- ---------- ---------- ---------- --D------- ---------- ----------
IBP-1                           --W       KE--RI--YR -V-SLAKAQE TS    G-EI SKFYL---N- N-F-HSR--E

          238                                                  289
IBP-2     MSLNGQRGEC WCVNPNTGKL IQGAPTIRGD PECHLFYNEQ QEACGVHTQR MQ
BRL-BP    ---------- ---------P ---------- ---------- --ND-A-A-- V-
IBP-1     T-MD-EA-L- ---Y-WN--R -P-SPE---- -N-QMYF-V- N
```

Fig. 2. Comparison of the deduced amino acid sequences of the mature forms of human IGFBP-1, IGFBP-2 and the rat BRL-BP. The numbering of the amino acids is according to the IGFBP-2 sequence. A dash indicates sequence identity with IGFBP-2. Cysteine residues are marked with a triangle. (IGFBP-1 and IGFBP-2 are represented as IBP-1 and IBP-2 in the figure).

19 amino acids longer. This results from four in-frame deletions in the BRL-BP cDNA between amino acids 23 and 38, 99 and 103, and at positions 135 and 172, based on the amino acid numbering of IGFBP-2. IGFBP-1 contains two major in-frame deletions, as well as minor deletions and insertions, when compared with IGFBP-2. As in BRL-BP, the N-terminal stretch between amino acids 23 and 38 is not present in IGFBP-2. Another extended deletion is located in the area between residues 155 and 190. Due to the low degree of homology in this region, the deletion cannot be positioned exactly. Around residue 100 there appears to be an insertion of three amino acids, while four amino acids are deleted around residue 212. The 11 extreme C-terminal residues, including one cysteine, are absent in IGFBP-1.

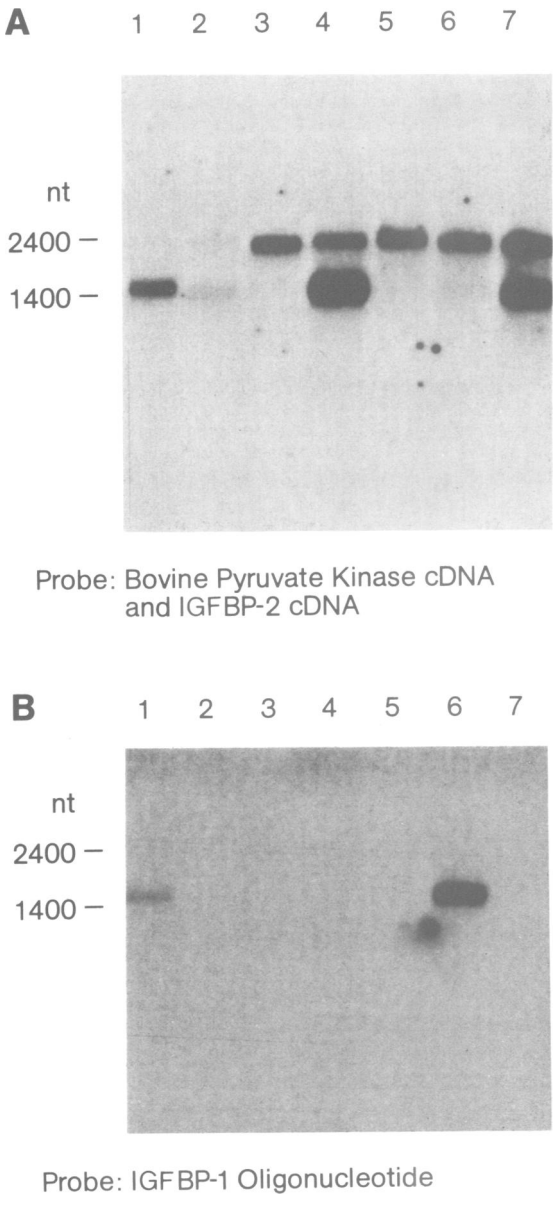

Fig. 3. Northern blot analysis of poly(A$^+$) RNA isolated from human liver (**lane 1**), human brain (**lane 2**) and the human cell lines HeLa (**lane 3**), Jurkat (**lane 4**), Namalwa (**lane 5**), HEPG2 (**lane 6**) and embryonal kidney 293 (**lane 7**). RNA (1 μg) was electrophoresed through a 0.8% agarose–formaldehyde gel, transferred to a Hybond-N membrane and hybridized with IGFBP-2 and bovine pyruvate-kinase cDNAs (**A**) or an IGFBP-1-specific 33meric oligodeoxynucleotide (see text; **B**). Position and size of RNA standards is indicated.

Analysis of IGFBP-1 and IGFBP-2 mRNA

To determine the size and tissue distribution of the mRNA encoding the IGFBP-2 protein, a Northern blot hybridization experiment was performed using poly(A)$^+$ RNA isolated from human brain and liver tissue as well as from the human cell lines HPEG2, HeLa, the T-helper cell line Jurkat, the embryonic kidney cell line 293 and the B lymphoma Namalwa. The complete IGFBP-2 cDNA and an IGFBP-1-specific 33meric oligodeoxynucleotide (Brinkman *et al.*, 1988; nt 792–824) were used as probes along with bovine pyruvate-kinase cDNA as a control. A single transcript of ~1500 nt could be detected with the IGFBP-2 cDNA (Figure 3A) in liver (lane 1), brain (lane 2), Jurkat (lane 4), and kidney 293 cells RNA (lane 7). This suggests that the 1.43 kb cDNA clone may represent a full-length cDNA. The 2400 nt transcript in Figure 3A results from hybridization with the bovine pyruvate-kinase cDNA. Another RNA of ~1500 nt could be detected with the IGFBP-1-specific oligodeoxynucleotide (Figure 3B), though the tissue distribution of the two IGFBP mRNAs was very different. IGFBP-1-specific RNA was only detected in HEPG2 cells and adult liver tissue (Figure 3B, lanes 1 and 6).

Genomic Southern blot analysis

To determine the number of IGFBP-2 genes present in the human genome, Southern blot analysis was performed. Human placenta DNA was digested to completion with restriction endonucleases *Bam*HI, *Eco*RI and *Hin*dIII. Following gel electrophoresis and transfer to nitrocellulose, the DNA was probed with the ^{32}P-labelled IGFBP-2 cDNA. In the digests, a 10 kb *Bam*HI, a 6.5 kb *Eco*RI and a 4 kb

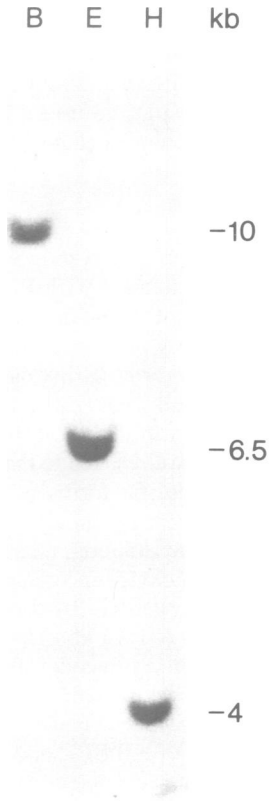

Fig. 4. Southern blot of genomic DNA. Human placental DNA (10 μg) was digested to completion with *Bam*HI (B), *Eco*RI (E) or *Hin*dIII (H). The fragments were electrophoresed through a 0.6% agarose gel, transferred to a nitrocellulose membrane and hybridized with the IGFBP-2 cDNA. Fragment size is indicated in kb.

*Hin*dIII fragment strongly cross-hybridized with the IGFBP-2 cDNA under stringent hybridization conditions (Figure 4). These data are consistent with the presence of only one IGFBP-2 gene in the human haploid genome. Preliminary cloning and sequencing data support these findings (C. Binkert *et al.*, unpublished results).

IGFBP-2 specifically binds IGF

To investigate the binding of IGF to the protein encoded by the IGFBP-2 cDNA, we co-transfected CHO cells with the plasmid pSV2-neo and the eukaryotic expression vector pXMT containing the complete coding region of the IGFBP-2 cDNA either in correct (pXMT+) or opposite orientation (pXMT−). After selection, cells were allowed to condition medium in the absence of serum. To detect proteins with IGF-binding activity, the conditioned medium as well as umbilical cord serum and mock-transfected CHO conditioned medium were separated by PAGE, electroblotted onto nitrocellulose sheets and exposed to [^{125}I]IGF-1. The results are shown in Figure 5. In medium conditioned with CHO cells transfected with pXMT+ (lane 1) and in human serum (lane 3) a band of M_r 36 000 emerged. No signal was detected in media conditioned by mock-transfected CHO cells (lane 4) or by cells transfected with pXMT− (lane 2).

To determine the ability of IGFBP-2 expressed by CHO cells to bind IGF-II and its relative affinities for IGF-I and IGF-II and insulin, competitive binding studies were performed. The results of these experiments are illustrated in Figure 6. They show that IGFBP-2 specifically binds IGF-II because the binding can be competed with only 5-fold excess of unlabelled IGF-II (lane B). The binding can also be inhibited by an excess of unlabelled IGF-I, but IGF-I has a lower affinity than IGF-II. A 25-fold excess of unlabelled IGF-I is required to obtain an effect comparable to that of a 5-fold excess of unlabelled IGF-II (see Figure 6, compare B and E). With a 100-fold excess of cold insulin no inhibition of [^{125}I]IGF-II binding was detected (lane F).

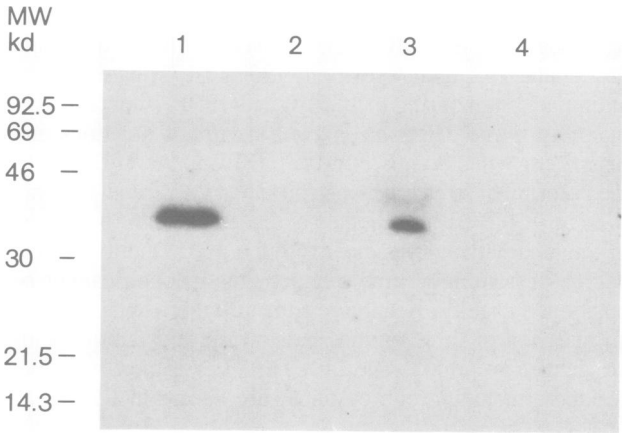

Fig. 5. Western blot analysis of media conditioned by transfected CHO cells. Medium (30 μl) conditioned by untransfected (**lane 4**), pXMT+ (**lane 1**) or pXMT− (**lane 2**) transfected CHO cells was electrophoresed on polyacrylamide gels under non-reducing conditions. Proteins were then transferred to nitrocellulose membranes and exposed to [^{125}I]IGF-I. To **lane 3**, 20 μl of human umbilical cord serum was applied. Position of protein mol. wt standards is indicated.

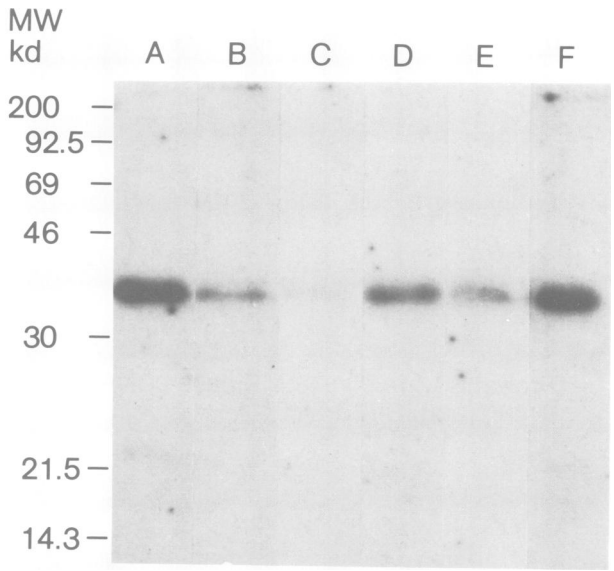

Fig. 6. Western blot analysis of medium conditioned by pXMT+ transfected CHO cells. The binding of 1 ng [^{125}I]IGF-II was competed with 0 ng (**lane A**), 5 ng (**lane B**), 25 ng (**lane C**) or unlabelled IGF-II, 5 or 25 ng (**lanes D** and **E**) of cold IGF-I or 100 ng (**lane F**) of unlabelled insulin. The blot was prepared as in Figure 5. Position of protein mol. wt standards is indicated.

Discussion

Two forms of IGF-binding proteins with mol. wts of ~30 kd have been described: the human IGFBP-1 isolated from amniotic fluid, and the rat BP purified from conditioned medium of BRL-3A cells (Lyons and Smith, 1986; Mottola *et al.*, 1986; Brewer *et al.*, 1988; Brinkmann *et al.*, 1988; Grundmann *et al.*, 1988; Julkunen *et al.*, 1988; Lee *et al.*, 1988). The partial homology of their N-terminal amino acid sequences and the similar mol. wts led to the hypothesis that these BPs were species-specific forms of the same IGF-binding protein.

In this paper we report the isolation, characterization and expression of human cDNA clones encoding an IGF-binding protein homologous to the rat BRL-3A form, but different from the human IGFBP-1. A 1.43 kb cDNA clone from a human fetal liver cDNA library encodes a 289 amino acid mature IGF-BP, IGFBP-2, with a calculated M_r of 31 325 and a 39 amino acid signal peptide (Figure 1). The homology shared by the mature forms of IGFBP-2 and the rat BRL-BP is >85%, even if a 14 amino acid deletion and three shorter deletions comprising a total of five amino acids in BRL-BP are considered. Both proteins are different from IGFBP-1, with which they share <40% amino acid homology.

Despite the distinct amino acid sequence, spacing between all 18 cysteine residues of IGFBP-1 is conserved in IGFBP-2 and its rat homologue (Figure 2). Eleven of the 18 conserved cysteines are located within the 75 N-terminal amino acids, a region shown to be involved in IGF-binding by IGFBP-1 (Huhtala *et al.*, 1986). Cysteine-rich regions also appear to define ligand specificity in the binding domain of hormone receptors that bind low density lipoprotein, epidermal growth factor, nerve growth factor, insulin or IGF-I (Yamamoto *et al.*, 1984; Ebina *et al.*, 1985; Ullrich *et al.*, 1985, 1986; Johnson *et al.*, 1986; Gill *et al.*, 1987). The C terminus, as well, is characterized by an increased cysteine content and carries in addition an RGD motif embedded in a conserved pentapeptide. This homology around the RGD motif implies some structural or functional relevance. It is known that BPs can stimulate or inhibit IGF-mediated cell proliferation. It has been shown that IGFBP-1 potentiates the action

of IGFs, in stimulating the proliferation of human fibroblast and muscle cells (Elgin *et al.*, 1987). This effect seems to be related to the association of the BP with the cell surface (Clemmons *et al.*, 1986). It is tempting to speculate that BPs can bind to a cell surface receptor by virtue of their RGD sequence which is recognized by RGD-directed receptors, also called integrin receptors (Ruoslahti and Pierschbacher, 1986; Hynes, 1987). The overall carboxy-terminal sequence homology shared by the three BPs, and the conserved IRGDP pentapeptide of IGFBP-1 and IGFBP-2, may represent structural features that determine cell surface binding.

The lowest level of sequence similarity exhibited by the three BPs is located in the central region of the proteins. In IGFBP-1 this region is rich in proline, glutamine, serine and threonine amino acid residues and is flanked by the positively charged amino acid histidine. It has been shown for other proteins that these so-called internal PEST sequences play a role in the relative instability of proteins *in vivo* (Rogers, 1986). In contrast to IGFBP-1, a PEST sequence could not be detected in the protein sequence of either IGFBP-2 or BRL-BP (Julkunen *et al.*, 1988).

In addition to the limited sequence similarity between the two human IGF-BPs, further differences were detected. The recombinant IGFBP-2 expressed in CHO cells was found to have a higher affinity for IGF-II than for IGF-I and did not bind insulin (Figure 6), while IGFBP-1 has been shown to have a higher affinity for IGF-I than for IGF-II (Baxter *et al.*, 1987).

Analysis of mRNA size and level in various tissues and cell lines revealed a different pattern of expression for IGFBP-1 and IGFBP-2. A 1.5 kb mRNA was detected for IGFBP-2 in adult liver, brain as well as in Jurkat and kidney 293 cells. No hybridization signal could be detected with this cDNA in Northern blot analysis of HeLa, Namalwa and HEPG2 RNA. Using an IGFBP-1-specific probe, however, mRNA was only detected in HEPG2 cells and adult liver. This clearly indicates different patterns of tissue-specific expression for both IGFBP-1 and IGFBP-2.

Comparing the results of Southern blotting experiments performed with the IGFBP-2 cDNA as a probe (Figure 4) with those of Brinkman *et al.* (1988), who used the IGFBP-1 cDNA as a probe, shows that IGFBP-1 and IGFBP-2 are encoded by two separate single-copy genes in the human genome. We suggest that the products of these two genes represent structurally related human IGFBPs. The distinct patterns of expression, together with the differences in the primary protein structure and the relative affinities for IGF-I and IGF-II, leads us to propose that these binding proteins provide an additional opportunity to control IGF activity at the site of local IGF-BP production.

Materials and methods

Isolation of cDNA clones and nucleotide sequence determination

To isolate the human equivalent of the IGF-BP secreted by rat BRL-3A cells, a fetal liver cDNA library (Clontech, HL 1005) was screened. Independent phage cDNA clones (2.5×10^5) were hybridized with the 1.2 kb *Eco*RI cDNA fragment encoding the BRL-BP secreted by rat BRL-3A cells. Fourteen cross-hybridizable clones were obtained. The cDNA used as a probe was previously isolated in our lab (Margot *et al.*, 1989). It was radiolabelled using random hexanucleotide priming (Feinberg and Vogelstein, 1983). Screening procedures were identical to those previously described (Heinrich *et al.*, 1984). The cross-hybridizing cDNA fragments were cloned into M13mp18 in both orientations. The single-stranded form of one orientation was disrupted randomly by sonication, hybridized to its complementary strand and treated with S1 nuclease. DNA fragments of 200–500 bp in length were ligated into M13mp18 and the nucleotide sequence was determined according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Preparation of poly(A$^+$) RNA and Northern blot analysis

Poly(A$^+$) RNA was prepared from the human CD4$^+$ helper T-cell line Jurkat K16 (Gilles and Watson, 1980), the human Burkitt lymphoma B-cell line Namalwa, HeLa (ATCC), the human liver cell line HEPG2 (ATCC), and the human embryonic kidney cell line 293 (ATCC) as described previously (Maniatis *et al.*, 1982). The concentration of RNA was determined by measurement of the optical density OD_{260}.

Human brain and human liver poly(A)$^+$ RNA was obtained from Clontech (cat. no. 6510-1 and 6516-1), 1 μg poly(A$^+$) RNA was subjected to electrophoresis in 0.8% agarose–formaldehyde gels, and blotted onto Hybond-N membranes (Amersham) as described (Thomas, 1980). After 2 h at 80°C the filters were hybridized as described by Church and Gilbert (1984). The random primer extension method (Fineberg and Vogelstein, 1983) was used to label the cDNA probes with [α-^{32}P]dATP. The IBP-1-specific 33meric oligodeoxynucleotide was kinased with [γ-^{32}P]dATP. Its sequence was 5′-GTACATTAAAATACATCTGGCAGTTGGGGTCTC-3′.

Expression and Western blot analysis

A cDNA fragment encoding the human precursor IGFBP-2 was cloned in both orientations into the eukaryotic expression vector pXMT which was derived from p91023(B) (Wong *et al.*, 1985). The recombinant vectors were linearized and co-transfected with the circular plasmid pSV2-neo (Southern and Berg, 1982) into Chinese hamster ovary (CHO) cells using the scrape loading method (Fechheimer *et al.*, 1987). After 2 weeks of selection in α^+ medium containing 1 mg/ml G418 + 10% fetal calf serum (FCS), selection conditions were switched to α^- medium containing 10% dialysed FCS. After a further 2 weeks, dihydrofolate reductase positive colonies were used to condition medium. Culture supernatants were separated on SDS–PAGE under non-reducing conditions and Western blot analysis was carried out according to Hossenlopp *et al.* (1986). The Western blots were incubated with human [^{125}I]IGF-I or [^{125}I]IGF-II. Recombinant human IGF-I and IGF-II were labelled with ^{125}I to a specific activity of ~10^6 c.p.m./5 ng (Zapf *et al.*, 1980). Human insulin was purchased from Sigma (cat. no. 1029S).

Southern blot analysis

High mol. wt human placenta DNA was digested with restriction endonucleases *Bam*HI, *Eco*RI and *Hin*dIII (Boehringer Mannheim) according to the supplier's conditions. The fragments were separated in a 0.6% agarose gel and transferred to a nitrocellulose filter according to the method of Southern. The filter was hybridized to the ^{32}P-labelled 1.43 kb *Eco*RI cDNA fragment encoding IGFBP-2 and washed at 65°C with 0.1 × SSC + 0.5% SDS for 45 min.

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Note added in proof

We have adapted the nomenclature for insulin-like growth factor binding proteins decided upon at an IGF-binding protein meeting in Vancouver in June 1989.

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