A Low Molecular Weight Insulin-Like Growth Factor Binding Protein from Rat: cDNA Cloning and Tissue Distribution of its Messenger RNA

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Rat serum contains two major forms of insulin-like growth factor (IGF) binding proteins (BPs) that have apparent mol wts of about 35,000 and 150,000. We have isolated a cDNA clone encoding an IGF-BP whose N-terminal sequence is completely homologous to the NH_2-terminal of the Buffalo rat liver cells-3A BP. The 270 amino acid mature protein has a predicted mol wt of 29,500. It contains a cysteine rich domain at each end of the molecule and an Arg-Gly-Asp (RGD) tripeptide motif near its C-terminus which suggests that this BP might associate with integrin cell surface receptors. The mature protein shares only partial homology with two published human IGF-BPs. Northern blot analysis shows that its mRNA is abundant in several fetal tissues, in adult brain, testes, ovaries, and kidney. Expression in the liver is high in fetal life but decreases to a barely detectable level in adulthood. However, upon hypophysectomy, the mRNA level increases at least 20-fold which suggests a hormonal regulation for the hepatic production of this small IGF-BP. (Molecular Endocrinology 3: 1053–1060, 1989)

INTRODUCTION

The presence of serum binding proteins (BPs) for insulin-like growth factors (IGFs) has been known for a long time (1–3). In rat and human serum, two major forms of IGF-BPs with apparent mol wts of about 35,000 and 150,000 have been identified (4, 5). The 150 kilodalton (kDa) form, which is under GH control, predominates in adult serum (5, 6). It is irreversibly broken down, upon exposure to dilute acids, to an active, stable form of about 50 kDa which also shows GH dependence (7, 8). The 30–35 kDa form which has been shown to be elevated in serum of fetuses, newborns as well as in plasma from hypopituitary individuals, may be inversely GH dependent (6, 9–11). Beside these two major forms, several other forms have been reported with apparent mol wts between 24,000 and 42,000 (12, 13). However, many of these forms have been shown to result from dissociation of the 150 kDa BP or to be the result of proteolytic degradation (14). In addition, IGF-BPs have been detected in cerebrospinal fluid, placenta, lymph, amniotic fluid, and conditioned medium from several cell lines (13, 15–20).

Multiple functions have been attributed to the BPs. For example: 1) they considerably increase the half-life of IGFs (21); 2) they protect the body against the insulin-like effect of free IGF (21, 22); 3) a binding protein produced by fibroblasts increases IGF binding to smooth muscle cell surfaces (23); 4) a purified fraction of the amniotic fluid BP enhances the response to IGF by fibroblasts and smooth muscle cells (24); 5) another group, using a purified preparation of the Buffalo rat liver cells-3A BP (BRL-BP) showed inhibition of IGF effects on fibroblasts (25); and 6) the purified amniotic fluid BP has been reported to occur in two forms with nearly identical amino acid composition and identical N-terminal sequence: one form was unable to bind to the cell surface of smooth muscle cells and inhibited IGF action whereas the other could bind to the cell surface and enhanced IGF action (26).

A first step towards an understanding of the various effects of BPs has been the cloning of the amniotic fluid BP by several groups (27–31) as well as the cloning of a subunit of the GH-dependent high molecular weight form of IGF-BP from human serum (32). We now report the isolation of a cDNA encoding another small molecular weight binding protein from rat.

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RESULTS

Cloning and Sequence Analysis of the cDNA Encoding the BRL-3A Binding Protein

Two λgt11 cDNA libraries prepared from adult rat liver mRNAs were screened with a mixture of two oligonucleotide probes corresponding to the NH_2-terminal sequence of the BRL-BP (33). Thirty-five positive clones were selected. As their insert size varied between 0.5 and 3 kilobases (kb), they were grouped by size and restriction pattern before selecting one representative from each group for partial sequencing. One clone encoded the NH_2 terminus of the BRL-BP and its complete sequence was determined (Fig. 1). This cDNA is incomplete. It shows a large open reading frame that goes from it's 5′-end to the termination codon at nucleotide (nt) 913 (see Fig. 1). This cDNA clone contained 301 base pairs of 3′-untranslated sequence but neither polyadenylation signal nor ATG initiation codon were present. Thus, the genomic *Eco*RI fragments containing the 5′- and 3′-end of the gene were isolated (Margot, J. B., manuscript in preparation). The determination of their sequence indicates that the AATAAA polyadenylation signal starts two bases downstream of the last deoxyadenosine of the cDNA clone (Fig. 1) and that the initiating methionine starts 18 bases upstream of the first base in the cDNA clone (boxed in Fig. 1).

The mature protein, whose first amino acid has been attributed to glutamic acid (see *Discussion*), is 270 amino acid long. This defines a 34 amino acid signal peptide whose sequence satisfies the consensus sequence for secreted proteins (34). The mature protein has a high proline and glycine content and has a total of 18 cysteines clustered at both ends of the molecule. The deduced amino acid sequence reveals an Arg-Gly-Asp (RGD) sequence at position 246–248 but no potential N-linked glycosylation sites are found in the predicted protein sequence.

Genomic DNA Analysis

Rat genomic DNA was digested with *Eco*RI, *Bam*HI, *Hin*dIII, and *Pst*I. Figure 2 shows the Southern blot after hybridization to the BRL-BP cDNA clone. Only a limited number of bands hybridize in each lane. For example, the *Eco*RI digest (lane E) reveals bands of 14, 4.8, and 3.7 kb and the *Hin*dIII digest (lane H) shows bands of 4 (doublet) and 3.3 kb. Furthermore, the intensity of the bands varies considerably. There is one internal *Hin*dIII site in the cDNA but there are no *Eco*RI, *Bam*HI, and *Pst*I sites present. Since our genomic clone is not complete yet, it is not yet possible to attribute the genomic bands to specific restriction sites. More data are therefore needed to determine whether it is a single gene spread over at least 12 kb or whether it is part of a gene family.

Tissue Specific Expression of the BRL-BP

Total RNA from different organs of an adult male rat was examined for BRL-BP expression by Northern blot analysis. Figure 3 shows that a good hybridization signal is found in kidney (K). The signal for brain (B) and testes (T) is even stronger (about 5-fold) but no signal is present in liver (L), skeletal muscle (M), and heart (H) after a 17-h exposure. A faint band can be detected in liver RNA after a 3-day exposure which explains that we were able to clone this gene from an adult rat liver cDNA library. Only a single RNA species of about 1.6 kb is seen in all tissues that transcribe the BRL-BP which indicates that the 5′-untranslated sequence is probably about 400 nt long and argues against a differential splicing mechanism as is the case for IGF-I or IGF-II (35–37). In the adult, the BRL-BP mRNA is also found in ovaries (see below) and in spleen (result not shown).

The expression of the BRL-BP was also examined in the tissues of a pregnant rat as well as in some fetal tissues. Figure 4 reveals that most tissues tested show the presence of the BRL-BP mRNA albeit to different extent. In placenta (lanes 1 to 3), it is expressed only weakly after 12 days of gestation. The level of the message increases sharply after 17 days and reaches a maximum after 20 days (1 day before birth). A similar pattern is seen in the uterus of a pregnant rat (lanes 8 and 9) but the very high placental expression already seen after 17 days of gestation is only reached after 20 days in the uterus. The BRL-BP is also expressed in the ovaries of both a pregnant and an adult female (lane 6 and 7, respectively). Umbilical cord and amnion (lanes 4 and 5, respectively), both at day 17 of gestation show only basal levels. The BRL-BP is also strongly expressed in fetal livers from day 12 until birth but is not found in fetal heart and only weakly detected in fetal muscle (results not shown).

Considering the high amount of transcripts found in adult brain, the mRNA expression of the BRL-BP was followed in this tissue during fetal development (see Fig. 5). Total brain RNA from 15- (lane 1), 17- (lane 2), and 20- (lane 3) day-old fetuses as well as brain RNA from a newborn (lane 4), an adult male (lane 5), and a pregnant rat after 17 days of gestation (lane 6) were probed with the cDNA. Figure 5 shows that the expression of the BRL-BP is already high at day 15 of gestation. It reaches a maximum at day 17 and thereafter decreases until adulthood but still remains considerable.

Regulation of the Liver Expression of the BRL-BP

The effect of GH deprivation upon the liver expression of the BRL-BP was determined by using hypophysectomized rats. The results (Fig. 6) indicate the presence of a 1.6 kb transcript in the liver of all three hypophysectomized rats tested (Fig. 6, *right panel*, lanes 4–6) whereas no signal is seen in the liver of three normal rats after an overnight exposure (Fig. 6, *right panel*, lanes 1–3). A 3-day exposure reveals at least a 20-fold

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CGG GGG AGG AGA AAG AAG GCA AGG AGG CGT CTC CCG CGC TCG CCA GGG CCG TGC CAC CTG      -40

                                                    Met Leu Pro Arg Leu Gly Gly
CCC GCT AGC TCG CCG CGC TAC GGT TTC CCA CTA GCC AAC ATG CTG CCG AGA TTG GGC GGC       21

Pro Ala Leu Pro Leu Leu Leu Pro Ser Leu Leu Leu Leu Leu Leu Leu Gly Ala Gly Gly
CCC GCG CTG CCG CTG CTC CTG CCG TCG CTG CTC TTG CTG CTG CTG TTG GGC GCG GGC GGT       81
                             1
Cys Gly Pro Gly Val Arg Ala Glu Val Leu Phe Arg Cys Pro Pro Cys Thr Pro Glu Arg
TGC GGT CCT GGG GTG CGC GCC GAG GTG CTG TTC CGC TGC CCA CCC TGC ACG CCC GAG CGT      141

Leu Ala Ala Cys Gly Pro Pro Pro Asp Ala Pro Cys Ala Glu Leu Val Arg Glu Pro Gly
CTG GCC GCC TGC GGA CCC CCA CCC GAC GCG CCC TGC GCC GAG CTG GTG CGA GAG CCC GGC      201

Cys Gly Cys Cys Ser Val Cys Ala Arg Gln Glu Gly Glu Ala Cys Gly Val Tyr Ile Pro
TGC GGT TGC TGC TCC GTG TGC GCA CGA CAG GAG GGC GAA GCT TGC GGC GTC TAC ATC CCG      261

Arg Cys Ala Gln Thr Leu Arg Cys Tyr Pro Asn Pro Gly Ser Glu Leu Pro Leu Lys Ala
CGC TGC GCC CAG ACG TTA CGC TGT TAC CCC AAC CCG GGC TCC GAG CTG CCC CTG AAG GCA      321

Leu Val Thr Gly Ala Gly Thr Cys Glu Lys Arg Arg Val Gly Ala Thr Pro Gln Gln Val
CTG GTC ACC GGC GCG GGT ACC TGT GAA AAG AGA CGC GTG GGC GCC ACC CCA CAG CAG GTT      381

Ala Asp Ser Glu Asp Asp His Ser Glu Gly Gly Leu Val Glu Asn His Val Asp Gly Thr
GCA GAC AGT GAG GAT GAC CAC TCG GAG GGA GGC CTG GTG GAG AAC CAT GTG GAC GGA ACC      441

Met Asn Met Leu Gly Gly Ser Ser Ala Gly Arg Lys Pro Pro Lys Ser Gly Met Lys Glu
ATG AAC ATG TTG GGA GGC AGC AGT GCT GGC CGG AAG CCC CCT AAG TCA GGC ATG AAG GAA      501

Leu Ala Val Phe Arg Glu Lys Val Asn Glu Gln His Arg Gln Met Gly Lys Gly Ala Lys
CTG GCT GTG TTC CGG GAG AAG GTC AAC GAG CAG CAC CGG CAG ATG GGC AAA GGT GCC AAA      561

His Leu Ser Leu Glu Glu Pro Lys Lys Leu Arg Pro Pro Pro Ala Arg Thr Pro Cys Gln
CAC CTC AGC CTG GAG GAG CCC AAG AAG CTG CGC CCA CCT CCT GCC AGG ACC CCT TGC CAG      621

Gln Glu Leu Asp Gln Val Leu Glu Arg Ile Ser Thr Met Arg Leu Pro Asp Asp Arg Gly
CAG GAG CTG GAC CAG GTC CTG GAG CGC ATC TCC ACC ATG CGC CTT CCG GAT GAT CGG GGT      681

Pro Leu Glu His Leu Tyr Ser Leu His Ile Pro Asn Cys Asp Lys His Gly Leu Tyr Asn
CCT CTG GAA CAT CTC TAC TCC CTG CAT ATC CCC AAC TGT GAC AAG CAT GGC CTG TAC AAC      741

Leu Lys Gln Cys Lys Met Ser Leu Asn Gly Gln Arg Gly Glu Cys Trp Cys Val Asn Pro
CTC AAA CAG TGC AAG ATG TCT CTG AAT GGA CAG CGT GGG GAG TGC TGG TGT GTG AAC CCC      801

Asn Thr Gly Lys Pro Ile Gln Gly Ala Pro Thr Ile Arg Gly Asp Pro Glu Cys His Leu
AAT ACT GGG AAG CCA ATC CAG GGA GCT CCC ACC ATC CGG GGA GAC CCC GAG TGC CAT CTC      861

Phe Tyr Asn Glu Gln Gln Glu Asn Asp Gly Ala His Ala Gln Arg Val Gln ***
TTC TAC AAC GAG CAG CAG GAG AAT GAT GGG GCT CAC GCC CAA AGG GTG CAG TAA ACC ACA      921

GCC AGT CGG TGC CTG GCT TCC CCA CCC CAA ACA CCA GCA GAA ATG GAG GGT GTC AGG GTG      981

ATG GGT GTG GAG GAT TTC CCA GTT TTG ACA CAT GTA TTT ATA TTT GGA AAG AGA CCA ACA     1041

CTG AGC TCA GAA GCC CCC CTG CGC CCC CCA GTG GCA GTT AAC CTG TAC CTC CGT TCC TGC     1101

TTC TAA TAG AGA GGG TGG TGG CAC TGG GGA TAC TGG GTA CAG GCT TGG GAA TGG GGA AAG     1161

AAA TTT TTA TTT TTG AAC CCC TGT GTC TCT TTT ACT TAA GAT TAA AGG AAG GAA A           1216
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Fig. 1. Nucleotide and Deduced Amino Acid Sequence of the BRL-BP

The sequence data obtained by the sequencing of genomic fragments is boxed. The first amino acid of the mature protein is numbered and the stop codon is marked by three asterisks. The RGD motif is underlined by a thick line. The polyadenylation signal AATAAA is found two bases downstream from the last deoxyadenosine of the sequence.

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increase in the level of liver BRL-BP mRNA between normal and hypophysectomized rats (not shown). However, GH treatment of hypophysectomized rats is not able to bring the concentration of RNA down to the level found in the liver of normal rats (Fig. 6, *right panel*, lanes 7–9). To ascertain that both hypophysectomy and GH treatment had been successfully carried out, the blot shown in Fig. 6 (*right panel*) was washed and rehybridized to a rat IGF-I cDNA probe. Figure 6 (*left panel*) shows that the 7.5 kb IGF message behaves as expected: it is abundant in the liver of normal rats (lanes 1–3); hypophysectomy has the effect of suppressing IGF message (lanes 4–6) and GH treatment is able to partially bring back up the level of expression (lanes 7–9). This shows that BRL-BP and IGF I message in the liver are inversely regulated by hypophysectomy. The transcription of the BRL-BP gene is not under pituitary control in the other adult tissues that express it. For instance, hypophysectomy has no effect upon BRL-BP mRNA level in testes, brain, kidney, skeletal muscle, or spleen (results not shown).

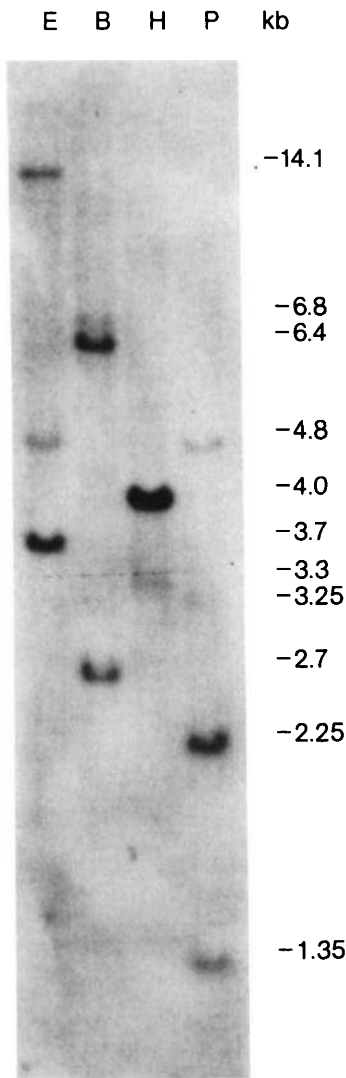

Fig. 2. Southern Blot of Genomic DNA

Ten micrograms of rat genomic DNA were digested with *Eco*RI (lane E), *Bam*HI (lane B), *Hin*dIII (lane H), and *Pst*I (lane P). The DNA was electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane. Hybridization to the BRL-BP cDNA clone was at 65 C in 5× Denhardt's buffer, 5× SSPE, 0.2% SDS, and 0.5 μg/ml denatured nonhomologous DNA. Washing was done at 65 C twice for 30 min in 2× SSC, 0.1% SDS, and once for 15 min in 0.1× SSC, 0.1% SDS. The fragment size is indicated in kilobases.

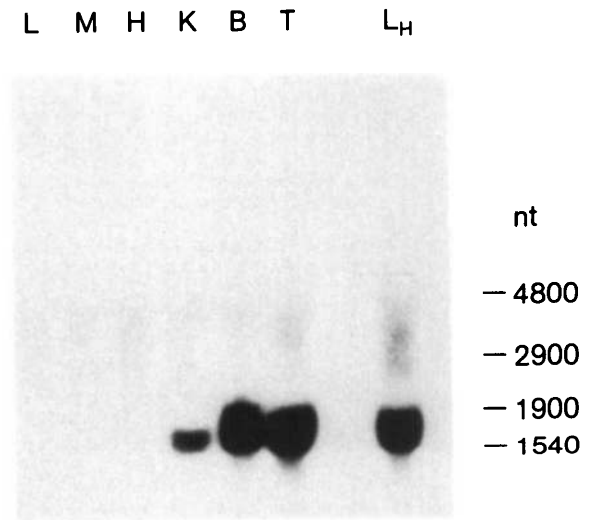

Fig. 3. Northern Blot Analysis of Total RNA from Various Adult Rat Tissues

Twenty micrograms of total RNA from liver (L), muscle (M), heart (H), kidney (K), brain (B), and testes (T) were glyoxylated and electrophoresed through a 1% agarose gel. The RNA was transferred to a nylon membrane and hybridized to the BRL-BP cDNA clone. The lane L_H is used as standard to allow a comparison of signals between blots. The sizes in the marker lane correspond to the sizes of prokaryotic and eukaryotic ribosomal RNAs.

DISCUSSION

A cDNA coding for the rat BRL-3A binding protein has been cloned. It encodes the amino acid sequence published by Mottola *et al.* (33) which was used to derive the oligonucleotide probes. These authors indicate that the N-terminal sequence of the mature protein starts with Leu-Phe-Arg. However, sequencing of the protein by two different groups showed that there were two additional amino acids: either Glu-Val-Leu-Phe-Arg (38) or X-X-Leu-Phe-Arg (18). Thus, lacking own protein sequence data, in accordance with von Heijne's (34) prediction for signal sequence cleavage sites, the first amino acid has been attributed to glutamic acid. The mature BRL-BP has a calculated mol wt of 29,500. However, the small nonreduced IGF-BP found in rat serum and supernatants of BRL-3A cells has an apparent mol wt of about 34,000 by sodium dodecyl sulfate (SDS) gel electrophoresis. This molecular weight discrepancy can be explained by the presence of intramolecular disulfide bridges as was shown for the amniotic fluid BP (29). The IGF-binding site of the protein has not been defined, although, for the amniotic fluid BP, a 21 kDa fragment containing the N-terminal region has been shown to bind IGF (39). In the BRL-BP, the clustering of cysteines defines two regions situated near the N- and C-terminus of the protein. These cysteine-rich domains are likely to play an important role for several reasons. Dot matrix sequence comparisons between the rat BRL-BP and the human amniotic fluid BP or the 53 kDa subunit of the high molecular weight human serum BP reveals that the homology between these three proteins is restricted to the cysteine-rich areas. The spacing of cysteines in all three proteins is conserved. The central region between the cysteine-rich domains is where amino acid replacements have occurred preferentially when one compares the rat and human BRL-BP (Binkert, C., submitted). Finally, cysteine-rich domains as well as the conserved spacing between cysteine residues appears crucial in providing ligand binding specificity to a variety of hormone receptors (40–44). So far we have not expressed the rat BRL-BP, but we have cloned and expressed the human

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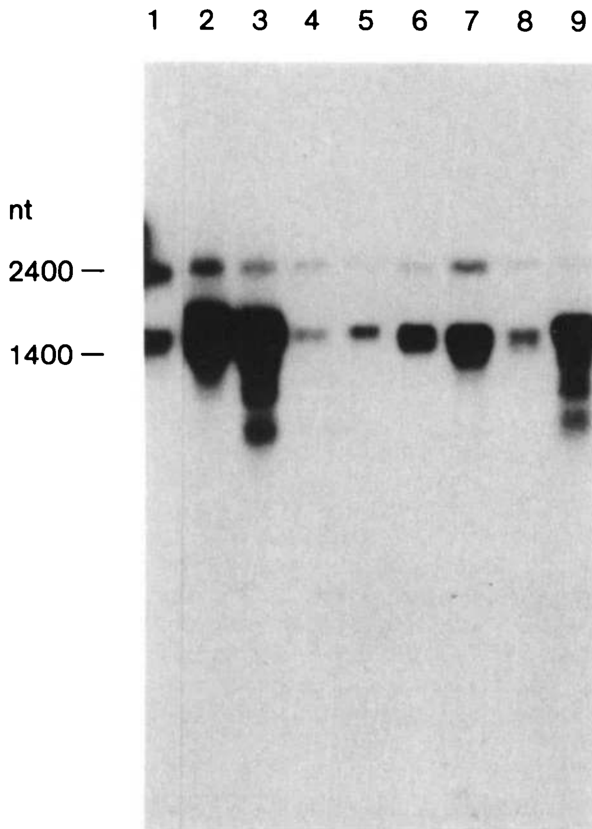

Fig. 4. Northern Blot Analysis of Total RNA from Fetal and Adult Rat Tissues

Twenty micrograms of total RNA from day-12 placenta (lane 1), day-17 placenta (lane 2), day-20 placenta (lane 3), day-17 umbilical cord (lane 4), day-17 amnion (lane 5), ovaries of a pregnant female after 20 days of gestation (lane 6), ovaries of a 12-week-old female (lane 7), day-17 uterus (lane 8), and day-20 uterus (lane 9) were electrophoresed as described in Fig. 3. The blots were simultaneously probed with the BRL-BP cDNA clone and with a partial cDNA coding for bovine pyruvate kinase which was used as internal control. This latter probe hybridizes to a 2.5 kb transcript. Size markers are from commercially available, synthetic RNAs.

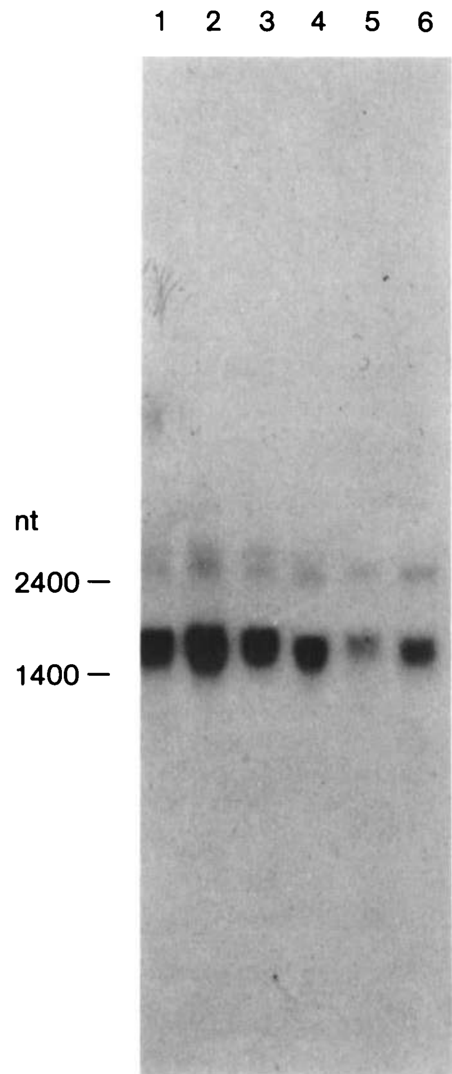

Fig. 5. Northern Blot Analysis of Total RNA from Fetal and Adult Rat Brains

Twenty micrograms of total brain RNA from day-15 fetuses (lane 1), day-17 fetuses (lane 2), day-20 fetuses (lane 3), 2-week-old newborn (lane 4), adult males (lane 5), and day-17 pregnant females (lane 6) were electrophoresed as described in Fig. 3. The BRL-BP cDNA clone and the pyruvate kinase cDNA were used as probes.

equivalent of this protein using the BRL-BP cDNA as probe. This human binding protein (IBP-2) is able to bind IGF-I and IGF-II specifically. (Binkert, C., submitted). Excluding a few deletions/insertions, rat and human BRL-BPs have over 90% homology at the amino acid level. On the other hand, the human equivalent to the BRL-BP is only homologous to less than 40% with the human amniotic fluid BP and to less than 45% with the 53 kDa subunit of the high molecular weight human serum BP. Furthermore, the latter protein has three potential N-glycosylation sites whereas the BRL-BP has no such sites. Thus, this cDNA encodes a new form of BP and is not the rat equivalent of the amniotic fluid BP as was previously thought (28, 30).

An important structural feature of the rat protein is the presence of an Arg-Gly-Asp (RGD) tripeptide. This motif is found within an amino acid sequence (IRGDP) that has been conserved as well in the human amniotic fluid BP which could imply some functional role. This motif appears to be essential for attachment to cell surface receptors (45). It is found in several matrix proteins, fibrinogen, and also in the human amniotic fluid BP. Its presence has been linked to the ability of these proteins to attach to cells and preliminary experiments have shown that a synthetic RGD peptide can competitively inhibit the attachment of the amniotic fluid BP to cell surfaces (28). The presence of specific integrin receptors offers the possibility for a tissue to autoregulate its IGF-binding capacity.

Southern DNA analysis carried out with different restriction enzymes reveals that only a few genomic fragments hybridize with the BRL-BP cDNA probe in

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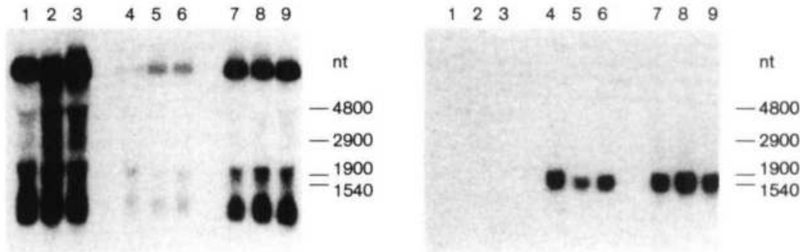

Fig. 6. Northern Blot Analysis of Total RNA from Rat Liver

Twenty micrograms of total liver RNA from three normal rats (lanes 1–3), from three hypophysectomized rats (lanes 4–6), and from three GH-treated hypophysectomized rats (lanes 7–9) were electrophoresed as described in Fig. 3. The blot was first hybridized to the BRL-BP cDNA clone (*right panel*), then washed, and rehybridized to an IGF I cDNA probe (*left panel*).

each of the digests. This result by itself does not suggest the presence of a single copy gene. However, the three genomic *Eco*RI fragments which hybridized to the cDNA have been isolated (Margot, J. B., manuscript in preparation). Hybridization and sequencing analysis indicate that the three *Eco*RI fragments carry all the sequences found in the cDNA and that no other gene with homology to the cDNA is present on these fragments. This suggests that it is a single copy gene. Taking into account species variations, the localized homology still found by dot matrix sequence comparisons between the rat BRL-BP and either the human amniotic fluid BP or the 53 kDa subunit of the high molecular weight human serum BP indicates that these IGF-BPs might be part of a gene family whose members have diverged.

The hepatic expression of BRL-BP mRNA is considerable in fetuses but barely detectable in adults. Although we have not yet correlated mRNA levels with protein levels, this pattern suggests that the BRL-BP gene is developmentally regulated. This developmental switch appears to be around or shortly after birth. This period corresponds to the time when IGF expression in the liver of the newborn becomes sensitive to GH (46, 47). Furthermore, in Laron-type dwarfism, a human disorder characterized by defective liver GH receptors (48), the serum of these patients does not contain any GH-dependent BP but has a high amount of the small form of IGF-BP (Zapf, J., personal communication). Thus, GH appears to suppress the expression of the small form of IGF-BP. This could explain the fact that, after hypophysectomy of normal adult rats, the small form of IGF-BP replaces the predominantly large mol wt form in serum (11, 49). Hypophysectomy was therefore chosen as a model of *in vivo* GH deprivation. Among all the tissues tested, the BRL-BP mRNA level in the liver was the only one which showed to be dependent upon hypophysectomy (an increase of at least 20-fold over the level found in liver of normal rats). Surprisingly, replacement of GH did not bring the level of the BRL-BP mRNA back down to the level found in normal liver. That GH treatment was effective was shown on one hand by the documented increase in body weight (see *Materials and Methods*) and on the other hand by the expected reappearance of the mRNA for IGF-I upon GH application (46, 47, 50). This means that GH alone cannot suppress BRL-BP mRNA expression in liver. There must be another hormonal factor acting independently or in conjunction with GH which is suppressing the BRL-BP mRNA production. Secondary factors to GH treatment like diabetes or high circulating IGF levels are known to stimulate the production of the small BP (Schmid, C., personal communication). However, these factors apparently did not play a role in the high level of BRL-BP still found upon GH treatment as glucose blood level of normal and GH treated rats was similar (see *Materials and Methods*) and as the amount of IGF I message in liver was still much lower in hypophysectomized rats than in normal rats. In addition, the serum half-life of IGF is much shorter in hypophysectomized than in normal adults (11).

The physiological importance of this differential hepatic regulation of the BRL-BP and the IGF-I message in the rat will remain unclear until the functional role of the different BPs and their influence on IGF activity will be better known. The similar pattern seen in fetal and hypophysectomized animals suggests a significance in physiology.

In adult rats, the BRL-BP mRNA is found in most adult tissues tested except heart and muscle. By analogy to humans, it is reasonable to assume that the rat also has an amniotic fluid BP whose expression parallels that found in man. In adult humans, the amniotic fluid BP mRNA is weakly expressed in liver and is absent in kidney, the only adult tissue so far tested (27). Thus these two proteins are coexpressed in liver but not in kidney. In fetal rats, BRL-BP mRNA is found in brain, placenta, skeletal muscle, amnion, and umbilical cord; whereas the amniotic fluid BP is only expressed in liver and placental membranes (27). These observations point to a different localization of the expression of both BPs and suggest a different biological function.

MATERIALS AND METHODS

Complementary DNA Isolation

The IGF-BP BRL-3A has been purified and characterized by Mottola *et al.* (33). They determined its NH_2-terminal sequence

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which was used to synthesize two 93 meric oligonucleotides according to the most frequent codon usage in rat proteins (51). Both oligomers were mixed (1:1), end labeled, and used to probe two rat liver cDNA libraries constructed in λgt11. The phages were lifted on Biodyne A nylon membranes (Pall Corporation, East Hills, NY) and hybridization was carried out at 50 C in 5× SSC. Approximately 10^6 plaques were screened from each library. Over 30 positive clones were picked from each library and a total of 35 remained positive after the second and third screen. Size as well as a similar restriction pattern were the criteria used to group all the clones whose inserts were long enough to code for the protein. Ten such groups were formed and the DNA insert from one representative of each group was isolated, subcloned into M13mp18, and partially sequenced (Sequenase kit, U.S. Biochemical Corp., Cleveland, OH). One clone (isolated four times) encoded a protein whose N-terminal sequence was identical to the BRL-BP of Mottola *et al.* (33).

Northern Analysis

Total RNA was extracted from different tissues according to Chomczynski and Sacchi (53) and its concentration was determined spectrophotometrically. The RNA to be analyzed was glyoxylated as described by Maniatis *et al.* (54). Twenty micrograms of RNA were loaded per lane and electrophoresed overnight at 30 V through a 1% agarose gel made in morpholinopropane sulfonic acid buffer. The RNA was blotted onto a Biodyne nylon membrane using 20× SSC and UV cross-linked. Hybridization and washing conditions were done according to Church and Gilbert (55). Hybridization was done at 65 C in 0.5 M $NaHPO_4$, pH 7.2, 1% BSA, 1 mM EDTA, 7% SDS. The blots were washed at 65 C twice for 5 min in 40 mM $NaHPO_4$, pH 7.2, 0.5% BSA, 1 mM EDTA, 5% SDS, and 8 times for 5 min in 40 mM $NaHPO_4$, pH 7.2, 1 mM EDTA, 1% SDS. The rat IGF-I cDNA corresponds to the genomic sequence between nt 2054 of exon 1 and nt 868 of exon 5 as reported by Shimatsu and Rotwein (35). All probes were labeled by random primer extension.

Treatment of Rats

Normal and hypophysectomized male Sprague Dawley rats were a gift of Dr. K. Muller (Ciba-Geigy AG, Basel, Switzerland). Normal rats weighed about 250 g whereas hypophysectomized rats were between 150 and 200 g. The weight over 1 month after surgery was followed as a criterion of successful hypophysectomy. Rats that had a daily weight increase below 0.5 g were chosen to be GH treated. GH replacement consisted in twice daily ip injections of 0.2 IU human recombinant GH (Nordisk, Gentofte A/S, 2820 Gentofte, Denmark) for 8 days. The weight increase of treated rats was 4.3 ± 0.8 g/day. Animals were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) before tissue removal for mRNA preparation. Blood was taken by aortic punction. Blood sugar of normal and GH-treated hypophysectomized rats were 12.5, and 10.6 mM, respectively.

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