# Identification of Rat Cell Lines that Preferentially Express Insulin-Like Growth Factor Binding Proteins rIGFBP-1, 2, or 3

**Yvonne W.-H. Yang, Alexandra L. Brown, Craig C. Orlowski, Dale E. Graham, Lucy Y.-H. Tseng, Joyce A. Romanus, and Matthew M. Rechler**

Growth and Development Section
Molecular, Cellular and Nutritional Endocrinology Branch
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892

**The bioavailability and action of the insulin-like growth factors (IGFs) are determined by specific IGF-binding proteins (IGFBP) to which they are complexed. Complementary DNA clones have been isolated that encode three related IGFBPs: human IGFBP-1 (hIGFBP-1), human IGFBP-3 (hIGFBP-3), and rat IGFBP-2 (rIGFBP-2). IGFBP-1 and IGFBP-3 are regulated differently in human plasma, suggesting that they have different functions. In order to study the molecular basis of the regulation of the different IGFBPs, we have identified a panel of rat cell lines that express a single predominant binding protein and developed an assay strategy to distinguish the different binding proteins. Proteins in conditioned medium were examined by ligand blotting, and by immunoprecipitation and immunoblotting using antibodies to rIGFBP-2 and hIGFBP-1; RNAs were hybridized to cDNA probes for rIGFBP-2 and hIGFBP-1. 1) C6 glial cells and B104 neuroblastoma cells express an approximately 40 kilodalton (kDa) glycosylated binding protein that most likely represents rIGFBP-3, the binding subunit of the 150 kDa IGF: binding protein complex in adult rat serum. The C6 and B104 binding proteins do not react with antibodies to rIGFBP-2, and RNAs from C6 and B104 cells do not hybridize to cDNA probes for rIGFBP-2 or hIGFBP-1. 2) BRL-3A, Clone 9, and TRL12-15 cell lines derived from normal rat liver express rIGFBP-2, a 30 kDa nonglycosylated IGF-binding protein that is recognized by antibodies to rIGFBP-2 but not by antibodies to hIGFBP-1. RNAs from these cells hybridize to a rIGFBP-2 cDNA probe, but not to a hIGFBP-1 probe. 3) H35 rat hepatoma cells express a 30 kDa nonglycosylated IGFBP that is presumptively identified as rIGFBP-1. It does not react with antibodies to rIGFBP-2, but is recognized by polyclonal and monoclonal antibodies to hIGFBP-1. RNA from H35 cells hybridizes to a hIGFBP-1 cDNA probe, but not to a rIGFBP-2 probe. Expression of rIGFBP-1 by the H35 cell line has enabled us to establish and validate specific assays for this protein that allow us to study its regulation in intact rats. Identification of a panel of rat cell lines expressing specific IGFBPs should be useful in elucidating the molecular mechanisms of IGFBP regulation. (Molecular Endocrinology 4: 29–38, 1990)**

0888-8809/90/0029-0038$02.00/0
Molecular Endocrinology


## INTRODUCTION

The insulin-like growth factors (IGFs), IGF-I and IGF-II, are synthesized by multiple fetal and adult tissues, and circulate in plasma (1–3). They act by endocrine or paracrine mechanisms to promote cell growth and differentiation, and to stimulate the expression of differentiated cell functions. They exert their biological actions predominantly through IGF-I receptors and possibly through IGF-II/mannose δ-phosphate receptors.

Under virtually all circumstances, the IGFs do not exist as the free polypeptides, but instead are complexed to specific IGF-binding proteins (IGFBPs) that are not related to either type of IGF receptor (1). The role of the IGFBPs is unclear. Formation of IGF:BP complexes prolongs the half-life of IGFs in the circulation (1). IGFBPs protect against the potential hypoglycemic effects of the IGFs since the complex, unlike free IGFs, does not bind to insulin receptors (4). Purified binding proteins may act synergistically with IGFs (5, 6) or may inhibit their actions (5–9). The factors that determine whether the modulating effects of the binding protein are positive or negative are unknown. One possible explanation might be the existence of different IGFBPs having different functional capabilities.

Multiple IGFBPs have been identified in different bi-

ological fluids (1, 3, 10). Two IGFBPs have been purified from human sources: hIGFBP-3 (11), a glycosylated binding subunit that complexes with an acid-labile non-binding subunit (12, 13) and IGF-I or IGF-II to form the predominant 150 kilodalton (kDa) IGF:binding protein complex in adult human plasma, and hIGFBP-1 IGFBP-1, a low molecular weight IGF-binding protein purified from human amniotic fluid (14) and human placenta (15). A third IGFBP, rat IGFBP-2 (rIGFBP-2) has been purified from the BRL-3A rat liver cell line (16–18). The nucleotide sequences of cDNA clones for hIGFBP-1 (19–22), hIGFBP-3 (23), and rIGFBP-2 (24, 25), indicate that they represent three related but distinct proteins (3, 24, 26).

Human and rat serum contain a complex mixture of IGF-binding components (10). The complement of IGFBPs is profoundly altered in different physiological states such as GH deficiency and fasting (27–31). Distinctive regulation of individual binding proteins should provide clues to their unique biological roles. In the present study, we present evidence for the existence of three distinct IGFBPs in the rat: IGFBP-1, 2, and 3. We describe a strategy that differentiates these proteins and their respective mRNAs using a panel of rat cell lines that, under defined culture conditions, synthesize one predominant IGF-binding protein. These cell lines provide useful model systems to study the regulation of IGFBP gene expression.

## RESULTS

### Rat Cell Lines Express 30 kDa IGFBPs and an approximately 40 kDa cluster of N-Glycosylated IGFBPs

IGF-binding components in adult rat serum and serum-free conditioned media from different rat cell lines were examined by ligand blotting (32, 33). Samples were fractionated by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels under nonreducing conditions, the proteins transferred to nitrocellulose by electroblotting, and the blots incubated with $^{125}$I-IGF-II to identify the IGF-binding components (Fig. 1). As previously reported (33), the predominant IGF-binding component in adult rat serum is an approximately 40 kDa cluster of glycoproteins that is homologous to hIGFBP-3, the IGF-binding subunit of the 150 kDa IGF:BP complex in human serum (37, 38). A similar approximately 40 kDa cluster was observed in conditioned media from C6 glial cells and B104 neuroblastoma cells (Fig. 1, lanes 6 and 7).

BRL-3A cells express a 30 kDa IGF-binding protein, rIGFBP-2, that is immunologically related to the principal binding protein in fetal rat serum, but is not detectable in adult rat serum (16, 33, 39). A similar size IGF-binding protein is secreted by Clone 9 and TRL12-15 liver cells, and by the H35 rat hepatoma cell line (Fig. 1, lanes 1–4).[1]

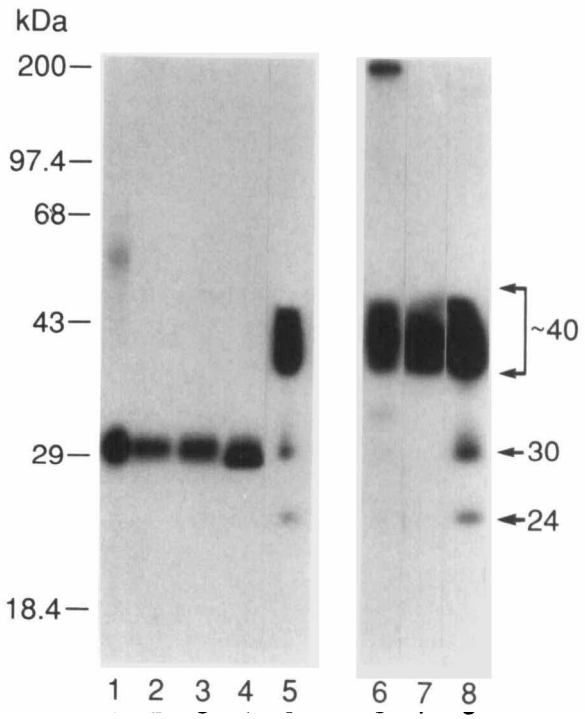


**Fig. 1.** Ligand Blot of IGF-II Tracer Binding to Media from Different Rat Cell Lines

Samples were electrophoresed on SDS-10% polyacrylamide gels under nonreducing conditions, and the proteins transferred to nitrocellulose. IGFBPs were identified by incubating the blots with IGF-II tracer followed by autoradiography. Lanes 1–5 are from one gel, lanes 6–8 from a second gel. Lane 1, purified rIGFBP-2; lane 2, Clone 9, 100 μl (equivalent of original medium); lane 3, TRL12-15, 100 μl; lane 4, H35, 20 μl; lane 5, adult rat serum, 2.5 μl; lane 6, C6, 100 μl; lane 7, B104, 100 μl; lane 8, adult rat serum, 2.5 μl. Samples were concentrated 10- to 40-fold using Centricon 30 Microconcentrators. The two autoradiographs were exposed for the same length of time. The positions of $^{14}$C-labeled molecular mass markers (in the presence of 0.1 M dithiothreitol) are indicated in kilodaltons on the *left*. The position and apparent molecular mass of specific IGF-binding proteins are indicated by *arrows on the right*. The high molecular mass (>200 kDa) IGF-binding activity in lane 6 may represent the extracellular domain of a truncated IGF-II/mannose 6-phosphate receptor (34–36).

The approximately 40 kDa binding protein cluster in adult rat serum is *N*-glycosylated (33, 38). As seen in Fig. 2, incubation with *N*-glycanase reduced the size of the approximately 40 kDa IGF-binding components in adult rat serum and C6 conditioned media to 34 kDa proteins, indicating that they contain *N*-linked oligosaccharides. The size of the 30 kDa IGF-binding proteins

[1] The H35 binding protein has a slightly faster electrophoretic mobility than the BRL-3A binding protein. This difference is not sufficiently large to reliably differentiate the two proteins.

in BRL-3A and H35 media was not decreased by *N*-glycanase treatment.[2]

### The 30 kDa IGF-Binding Proteins Differ in their Reactivity with Antibodies to rIGFBP-2 and hIGFBP-1

The ability of antibodies to rIGFBP-2 to recognize the IGF-binding proteins synthesized by the different rat cell lines was examined in immunoprecipitation and immunoblotting experiments (Figs. 3 and 4). Complexes of $^{125}$I-IGF-II with rIGFBP-2 purified from BRL-3A cell conditioned media or conditioned media from Clone 9 cells (not shown) were efficiently immunoprecipitated by antiserum 3695 to rIGFBP-2 (40) (Fig. 3). By contrast, no immunoprecipitation was observed using complexes formed from media conditioned by H35 hepatoma cells, C6 glial cells, or B104 neuroblastoma cells. Similar results were observed in immunoblotting experiments (Fig. 4): antiserum 3695 recognized rIGFBP-2 and the 30 kDa binding proteins from Clone 9 and TRL12-15 media, but did not recognize the 30 kDa binding protein in H35 medium. Thus, the 30 kDa IGF-binding proteins in BRL-3A, Clone 9, and TRL12-15 cells are immunologically related to each other, but different from the 30 kDa IGF-binding protein in H35 cells.

The preceding results did not distinguish whether the H35 binding protein was a variant of rIGFBP-2 containing modified antigenic determinants, or whether it represented a distinct binding protein, possibly the rat homologue of hIGFBP-1. The latter possibility was examined directly using polyclonal (41, 42) and monoclonal (43) antibodies to human IGFBP-1. The results are shown in Fig. 5. Polyclonal antibodies immunoprecipitated $^{125}$I-IGF:binding protein complexes formed with the H35 binding protein, but did not immunoprecipitate rIGFBP-2 complexes. Complexes formed with medium from the human HepG2 hepatocarcinoma cell line which expresses hIGFBP-1 (22, 44) gave comparable precipitation at 30-fold lower antibody concentrations (not shown), suggesting that the antiserum has higher affinity for hIGFBP-1 than for the heterologous rIGFBP-1. Similarly, in immunoblotting experiments, the monoclonal antibody 6303 to hIGFBP-1 reacted with a 30 kDa doublet in HepG2 and H35 conditioned media, but did not react with BRL-3A medium. These results demonstrate that the H35 binding protein is immunologically related to hIGFBP-1.

[2] Incubation with *O*-glycanase (with or without *N*-glycanase) caused no change in the electrophoretic mobility of the IGF-binding components in adult rat serum, or conditioned media from C6, BRL-3A, or H35 cells, whereas the size of a positive control protein ($\beta$TSH) was decreased by *O*-glycanase incubation (results not shown). We cannot exclude the presence of *O*-linked oligosaccharides too small to be detected by a change in electrophoretic mobility. Human IGFBP-3 has potential sites for *O*-glycosylation (23), but use of these sites has not been demonstrated. Human IGFBP-1 purified by Bohn and Kraus (15) contained 4.3% carbohydrate by chemical analysis.

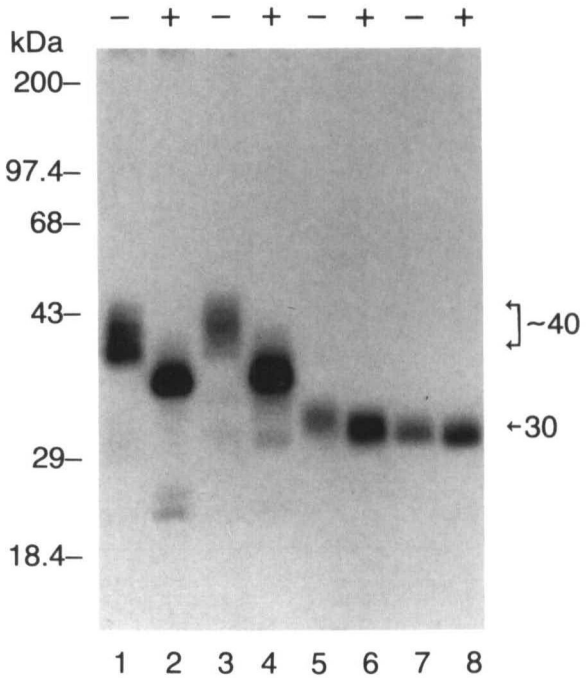


**Fig. 2.** Ligand Blot Showing the Effect of *N*- Glycanase on IGFBPs from Different Rat Cell Lines

Adult rat serum (lanes 1 and 2, 1 $\mu$l/lane), C6 medium (lanes 3 and 4, 50 $\mu$l/lane), BRL-3A medium (lanes 5 and 6, 2.5 $\mu$l/lane), and H35 medium (lanes 7 and 8, 5 $\mu$l/lane) were incubated with (+) *N*-glycanase as described in *Materials and Methods*. Control samples (−) were incubated under the same conditions in the absence of enzyme. Lanes 2 and 4 were incubated with 1 U/reaction, lanes 6 and 8 with 0.5 U/reaction. Samples were boiled, electrophoresed on an SDS-10% polyacrylamide gel, the proteins transferred to nitrocellulose, and the blot incubated with $^{125}$I-IGF-II as described. Positions of the approximately 40 kDa glycoprotein cluster and the 30 kDa proteins in samples not incubated with *N*-glycanase are indicated by *arrows on the right*. Although the size of the BRL-3A and H35 IGFBPs are not changed by *N*-glycanase, binding of $^{125}$I-IGF-II to enzyme-treated samples was consistently increased. The reason for this increase is not known.

### Hybridization of rIGFBP-2 and hIGFBP-1 cDNA Probes to RNAs from Different Rat Cell Lines

RNAs prepared from the same six cell lines plus a rat kidney epithelial cell line (NRK-52E) were hybridized to rIGFBP-2 and hIGFBP-1 cDNA probes (Fig. 6). The results support the assignments made from biochemical and immunological characterization. The rIGFBP-2 probe hybridized to an approximately 2 kilobase (kb) mRNA in total RNAs from BRL-3A, Clone 9, TRL12-15, and NRK-52E cells. No hybridization was observed to total RNA from H35, C6, or B104 cells. By contrast, using the hIGFBP-1 probe, hybridization to an approximately 2 kb mRNA only was observed using total RNA from H35 cells.

## DISCUSSION

Since virtually all of the IGF-I and IGF-II in plasma (33) and other biological fluids occurs complexed to specific

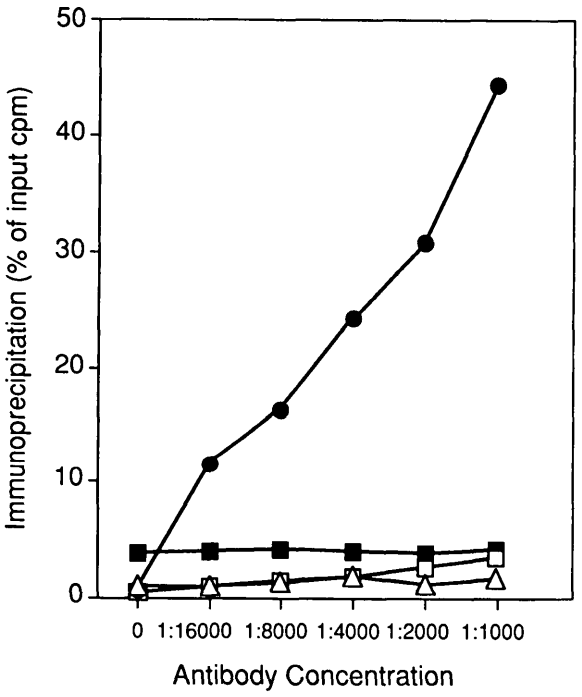


**Fig. 3.** Immunoprecipitation of Media from Different Rat Cell Lines Using Antiserum to rIGFBP-2

Purified rIGFBP-2 (*solid circles*), or media concentrated from H35 cells (2 ml equivalent of original medium; *solid squares*), C6 cells (0.5 ml; *open squares*), or B104 cells (1 ml; *open triangles*) were incubated with IGF-II tracer (400,000 cpm), and the unbound tracer removed by adsorption with activated charcoal. The charcoal supernate contained 25%, 19%, 42%, and 46% of input radioactivity, respectively, contrasted with 5.3% in the absence of added binding protein, indicating that 72–89% of the radioactivity in the charcoal supernate represented $^{125}$I-IGF:binding protein complex. Aliquots (1/15th) of the charcoal supernate were incubated with the indicated dilutions of antiserum 3695 or without antibody. Antibody complexes were precipitated with Pansorbin and the radioactivity recovered in the pellets quantitated. Precipitated radioactivity in the absence of added binding protein was less than 2% and has not been subtracted. Results obtained using preimmune serum were similar to those obtained with samples to which binding protein had not been added (not shown). Precipitated radioactivity in the absence of added antibody (5.3%) has been subtracted.

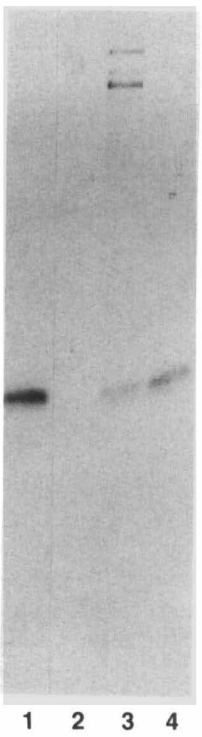


**Fig. 4.** Immunoblot of Media from Different Rat Cell Lines Using Antiserum to rIGFBP-2

Conditioned media from BRL-3A (10 μl, lane 1), H35 (30 μl, lane 2), TRL12-15 (50 μl, lane 3) and Clone 9 (200 μl, lane 4) cells were electrophoresed on SDS-10% polyacrylamide gels and the proteins transferred to nitrocellulose as described in *Materials and Methods*. The blot was incubated with antiserum 3695 (1:100 dilution). Proteins which bound immunoglobulins were identified using a biotinylated horseradish peroxidase staining procedure as described in *Materials and Methods*. The stained protein migrates as a 30 kDa protein.

IGFBPs, the IGFBPs are pivotally placed to determine the access of IGFs to different tissues, and their interactions with IGF receptors (1, 3). Based on protein purification and cDNA cloning, three distinct members of the family of IGFBPs have been identified: human IGFBP-1, human IGFBP-3, and rat IGFBP-2 (19–25). RIA of human serum suggests that hIGFBP-1 and hIGFBP-3 are oppositely regulated by GH and developmental age (27–29, 31, 45). This raises the possibility that each binding protein has a unique biological role.

To understand the molecular basis for the regulation of IGFBPs, we have turned to the rat because tissues as well as serum are readily available for study. However, of the three prototype IGFBPs, in the rat only IGFBP-2 has been cloned (24, 25), only IGFBP-2 and IGFBP-3 have been purified (16–18, 34, 35), and antibodies only are available for IGFBP-2 (16, 17, 39, 40). We reasoned that the identification of rat cell lines that predominantly express a single IGFBP would facilitate purification of the different binding proteins, generation of antibodies to them, as well as the isolation of cDNA clones encoding them.

To identify such cell lines, we developed an assay strategy that differentiates the rat IGF-binding proteins by biochemical and immunological properties of the proteins, and by molecular hybridization of their mRNAs. The results are summarized in Table 1. Ligand blotting in conjunction with *N*-glycanase digestion distinguished cells that express one of two classes of binding proteins: an approximately 40 kDa cluster of *N*-glycosylated binding proteins (exemplified by the C6 glial and B104 neuroblastoma cell lines), or 30 kDa proteins that are not *N*-glycosylated. The size and glycosylation of the approximately 40 kDa binding proteins in C6 and B104 cells suggested that they correspond to the IGF-binding subunit (rIGFBP-3) of the 150 kDa IGF: binding protein complex in adult rat serum (33, 37, 38). In support of this identification, the C6 and

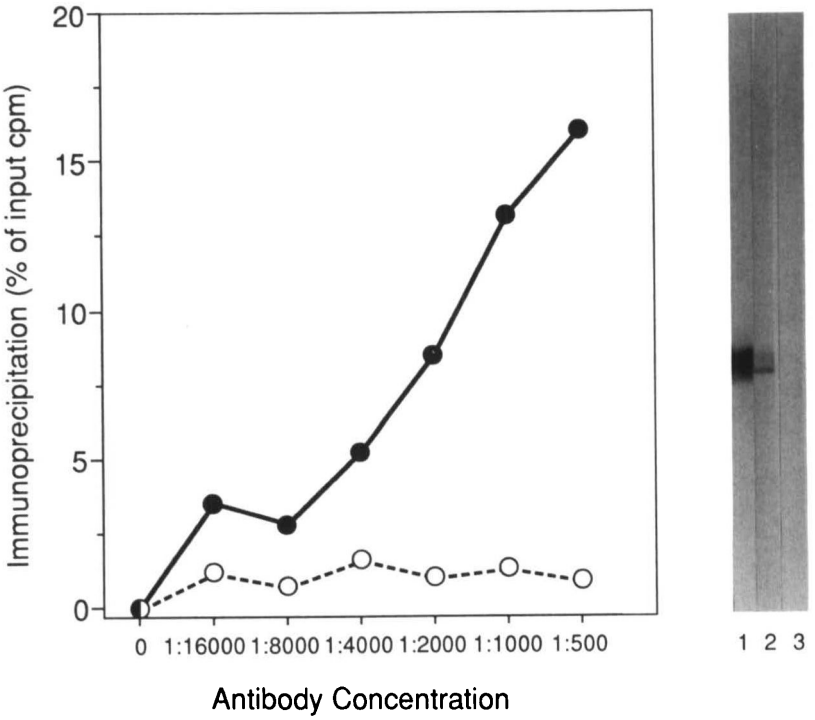


**Fig. 5.** Immunoreactivity of BRL-3A and H35 IGFBPs Using Antibodies to hIGFBP-1

*Left Panel*, Immunoprecipitation of purified rIGFBP-2 and H35 conditioned media using polyclonal antiserum to hIGFBP-1. Purified rIGFBP-2 (*open circles*) and H35 medium (*solid circles*; 0.5 ml) were incubated with $^{125}$I-IGF-II (200,000 cpm) overnight at 4 C, and the noncomplexed tracer removed with activated charcoal. Bound radioactivity in the charcoal supernate was 30% of input for BRL-3A medium and 25.5% for H35 medium compared to 16% in the absence of added binding protein. One-eighth aliquots of charcoal supernate were incubated with the indicated dilutions of antiserum, and radioactivity bound to antibody precipitated with Pansorbin as described in *Materials and Methods*. Precipitated radioactivity in the absence of added antibody (4.5–4.7%) has been subtracted. Using complexes formed from HepG2 medium (35% of input radioactivity) as a positive control, 27.9% of the input radioactivity was precipitated by a 1:500 dilution of the same antiserum (not shown). *Right Panel*, Immunoblot of BRL-3A and H35 binding proteins with monoclonal antibody to hIGFBP-2. Individual preparative gels were run for each binding protein using a comb equivalent to 15 strips, and electroblotted as described. The blots were cut into replicate strips, which were incubated individually with monoclonal antibody 6303 (1:500 for lane 1; 1:100 for lanes 2 and 3). Biotinylated horse anti-mouse immunoglobulin G was used as second antibody. Developed strips were arranged for photography. Samples equivalent to 50 $\mu$l original medium were examined in lane 1 (HepG2) and lane 2 (H35); 10 $\mu$l BRL-3A medium were examined in lane 3. Other monoclonal antibodies (6301, 6302, 6305) did not react with H35 medium; the polyclonal antiserum gave a faint band corresponding to that seen with 6303 (results not shown).

B104 binding proteins were not recognized by antibodies to rIGFBP-2, and mRNAs from C6 and B104 cells did not hybridize to cDNA probes for rIGFBP-2 or hIGFBP-1. In the absence of positive identification using hybridization probes or antisera specific for IGFBP-3, however, we cannot formally exclude the possibility that the IGF-binding proteins in C6 or B104 cells represent a novel glycosylated binding protein.

Immunological and molecular hybridization studies indicated that the 30 kDa IGFBPs include two groups of proteins: BRL-3A, Clone 9, and TRL12-15 cells express rIGFBP-2, whereas H35 cells express presumptive rIGFBP-1. The 30 kDa IGFBP in media from BRL-3A, Clone 9, and TRL12-15 cells was identified as rIGFBP-2 because it is recognized by antibodies to rIGFBP-2 but not by antibodies to hIGFBP-1. RNA from these cells hybridized to a rIGFBP-2 cDNA probe, but not to a hIGFBP-1 cDNA probe. The 30 kDa binding protein in H35 hepatoma cells did not react with antibodies to rIGFBP-2, nor did RNA from these cells hybridize to a rIGFBP-2 cDNA probe. The H35 binding protein, however, was recognized by polyclonal and monoclonal antibodies to human IGFBP-1, and H35 mRNA hybridized to a human IGFBP-1 cDNA probe, presumptively identifying it as rIGFBP-1.

Identification of rIGFBP-1, and the demonstration of its reactivity with antibodies and a cDNA probe for human IGFBP-1, have not previously been appreciated. Several factors may have contributed to the previous failure to identify rIGFBP-1: 1) rIGFBP-1 mRNA is expressed at low levels in adult liver and most fetal tissues (67). Fetal rat liver expresses high levels of rIGFBP-1 mRNA, but also expresses high levels of the related and similar-size rIGFBP-2 mRNA, making the identification less certain. 2) Antibodies to human IGFBP-1 exhibit lower reactivity with rat IGFBP-1. Thus, only the

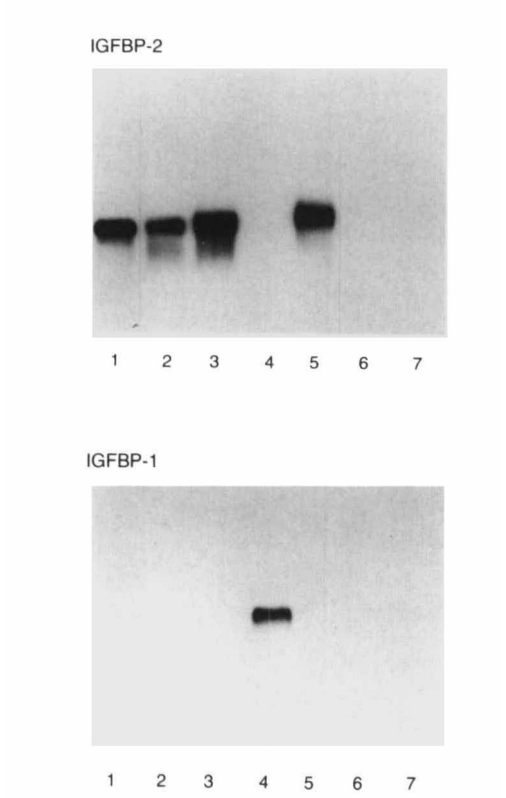


**Fig. 6.** Northern Blots of Total RNAs from Different Rat Cell Lines Hybridized to cDNA Probes for rIGFBP-2 (*top*) and hIGFBP-1 (*bottom*)

Lane 1, BRL-3A; lane 2, Clone 9; lane 3, TRL12-15; lane 4, H35; lane 5, NRK-52E; lane 6, C6; lane 7, B104. All lanes received 15 μg total RNA. Lane 1 (*top panel*) was from a different gel. The minor differences in migration of the RNAs hybridizing to the IGFBP-2 probe in lanes 2–5 reflect differences in the salt content of the samples; the positions of the ethidium bromide-stained ribosomal RNAs showed similar differences.

realization that the H35 cell line expresses rIGFBP-1 in high abundance and free of contamination by rIGFBP-2 or rIGFBP-3 made possible the unambiguous demonstration of this protein, and the recognition that antibodies and probes to human IGFBP-1 might be used to study rIGFBP-1 and its mRNA.

Our results with the H35 binding protein are consistent with the properties of the purified H35 IGF-binding protein reported by Mottola *et al.* (17). These authors observed that the H35 binding protein was similar in size to the BRL-3A binding protein, and was not glycosylated. They observed weak cross-reactivity of two polyclonal antibodies to rIGFBP-2 with the H35 binding protein in enzyme-linked immunosorbent and immunoblotting assays. Unlike our antibodies to rIGFBP-2 which do not inhibit $^{125}$I-IGF binding, their antibodies inhibited the binding of $^{125}$I-IGF-I and $^{125}$I-IGF-II to the purified H35 binding protein, suggesting that they recognize epitopes at or near the binding sites that may be more highly conserved in rIGFBP-1 and rIGFBP-2.

The presence of IGF-binding proteins was previously reported in C6 glial cells (36) and B104 neuroblastoma cells (46–48), but it was not possible to deduce the type of IGFBP from the gel filtration and affinity cross-linking experiments presented. In contrast to our results, Ocrant *et al.* (48) identified 23 and 27 kDa IGFBPs, but not an approximately 40 kDa cluster corresponding to rIGFBP-3, in ligand blots of B104 cell conditioned medium. Different conditions of cell propagation, possibly resulting in the selection of variant clones, may account for the differences in binding protein expression.[3]

It is intriguing that four liver-derived cell lines expressed 30 kDa rIGFBP-1 and rIGFBP-2, whereas two neural-derived cell lines expressed rIGFBP-3. This raised the possibility of tissue-specific expression of different classes of binding proteins. It seems unlikely however that rIGFBP-3 is a marker for native neural cells: both rIGFBP-2 and rIGFBP-3 are expressed in astroglial cell cultures from 1-day old rats (8), and hIGFBP-3 is expressed by dermal fibroblasts (49).

Expression of rIGFBP-2 and rIGFBP-1 by liver-derived cell lines is consistent with the presence of both of these mRNAs in fetal and neonatal rat liver (67). The H35 cell line was established from a minimal-deviation rat hepatoma (50), and continues to express a wide variety of proteins associated with normal liver (51). Although rIGFBP-2 was expressed in the three cell lines derived from normal liver, its expression is not restricted to liver. Immunoreactive IGFBP-2 is present in rat astroglial culture medium (8), and NRK-52E rat kidney epithelial cells express rIGFBP-2 mRNA. The MDBK bovine kidney cell line (52), murine glomerular mesangial cells [(53) and our unpublished results], and porcine smooth muscle cells (our unpublished results) also synthesize proteins related to rIGFBP-2.

The present results confirm that IGFBP-1 and IGFBP-3, as well as IGFBP-2, are expressed in the rat. We also have identified the human counterpart of rIGFBP-2 in a human rhabdomyosarcoma cell line and in human cerebrospinal fluid (54), and a cDNA clone encoding hIGFBP-2 has been isolated from a human liver library (55). It is quite likely that additional IGFBPs exist. One candidate is the 24 kDa nonglycosylated protein present in rat and human serum (10, 33), and in many cell lines (our unpublished results). We do not intend to suggest that all cells express one binding protein to the exclusion of all others, but rather that for simplicity we have first analyzed rat cell lines that express a predominant IGF-binding protein under our culture conditions.

The identification of cell lines expressing one of three IGFBPs in the rat should facilitate further studies investigating the regulation of expression of these proteins.

[3] The B104 cell lines used by Ocrant and colleagues (48) and by ourselves were obtained from R. Akeson (Children's Hospital, Cincinnati, OH). We also have examined a second line of B104 cells provided by R. Rosenfeld (Stanford, CA) that had been provided to him by R. Akeson and S. Chernausek. Although the 40 kDa glycoprotein cluster remains the predominant species, significant quantities of 24 and 28 kDa IGFBPs also were identified by ligand blotting.

**Table 1.** Rat IGF-Binding Proteins

| | IGFBP-3 | IGFBP-2 | IGFBP-1 |
|---|---|---|---|
| Protein | ~40 kDa glycosylated | ~30 kDa | ~30 kDa |
| Immunoreactivity | | | |
| IGFBP-2 | – | + | – |
| IGFBP-1 | – | – | + |
| RNA | | | |
| IGFBP-2 | – | + | – |
| IGFBP-1 | – | – | + |
| Cells | C6 Glia<br>B104 neuroblastoma | BRL-3A<br>Clone 9<br>TRL12-15 | H35 hepatoma |

Recognition that the H35 hepatoma cell line expresses IGFBP-1 at high levels has enabled us to demonstrate the specific cross-reactivity of heterologous antibodies and cDNA probes with this protein, validating the specificity of these reagents for studying the regulation of IGFBP-1 in the rat. Preliminary results indicate that IGFBP-1 (67) and IGFBP-1 mRNA (68) are profoundly increased in severely diabetic rats, similar to results for IGFBP-1 in human serum (56–58). Cell lines expressing rIGFBP-1, 2, and 3 should provide useful model systems for studying the molecular mechanisms of the regulation of expression of the IGFBP genes.

## METHODS

### Cell Lines

BRL-3A cells (ATCC CRL 1442) were propagated as previously described (40). The TRL12-15 cell line was derived from the livers of 10-day-old Fischer 344 rats (59), and provided by J. M. Rice (NCI, Frederick, MD). Clone 9 (60), a normal rat liver cell line, was obtained from the ATCC (CRL 1439). Rat neuroblastoma B104 cells (61) were obtained from D. Schubert (Salk Institute, La Jolla, CA) via R. Akeson (Children's Hospital Medical Center, Cincinnati, OH). C6 rat glial tumor cells [ATCC CCL 107] (36, 62) and NRK-52E cells (ATCC CRL 1571), an epithelial-like clone of normal rat kidney cells (63), were obtained from W. Kiess (NCI). The H4-EII-C3 differentiated rat hepatoma cell line (H35) was established from H35 Reuber hepatoma by H. C. Pitot (50) and provided by J. W. Koontz (University of Tennessee, Knoxville, TN). The human HepG2 hepatocarcinoma was established (64) and provided by B. B. Knowles (Wistar Institute, Philadelphia, PA).

### Cell Cultivation

B104 cells were grown in Ham's F-12/Dulbecco's minimum essential medium (MEM) (high glucose) (50:50) with 10% fetal bovine serum and passaged once a week (1:10). C6 glial cells were grown in Ham's F-10 medium containing 10% fetal bovine serum, and passaged once a week (1:10). Clone 9 cells were grown in Ham's F-12 medium containing 10% fetal bovine serum and passaged twice a week (1:6). TRL12-15 cells were grown in Williams' D medium containing 10% fetal bovine serum and passaged once a week (1:10). HepG2 cells were grown in Dulbecco's MEM containing 10% fetal bovine serum, and passaged once a week (1:5). H35 cells were grown in RPMI medium 1640 containing 10% fetal bovine serum, and passaged twice a week (1:4). NRK-52E cells were grown in Dulbecco's MEM with nonessential amino acids supplemented with 5% calf serum, and split 1:3 twice a week.

### Collection of Serum-Free Medium

Cells were grown to confluence in serum-containing medium, washed three times with PBS, and incubated with serum-free medium overnight. The medium was discarded and replaced with fresh serum-free medium (10 ml/56 $cm^2$ dish). Conditioned medium was harvested after 2 (C6 and B104) or 3 (BRL-3A, Clone 9, TRL12-15, H35) days, and centrifuged (3000 rpm for 20 min). Conditioned media were concentrated using Centricon 30 Microconcentrators (Dulbecco's PBS washing buffer). Media were stored at −20 C. Volumes of concentrated medium are expressed as equivalents of original conditioned medium, assuming 100% recovery after ultrafiltration.

### Antibodies

Polyclonal antiserum (245 VI) to BP-25 was raised in rabbits to hIGFBP-1 purified from human amniotic fluid (41) as previously described (42), and was kindly provided by R. Koistinen and M. Seppala (Department of Obstetrics and Gynecology, University of Helsinki, Helsinki, Finland). Mouse monoclonal antibodies to hIGFBP-1 (6301, 6302, 6303, and 6305) were kindly provided by E.-M. Rutanen [Minerva Institute for Medical Research, Kauniainen, Finland; (43)]. Antibody 3695 was raised in rabbits to purified rIGFBP-2 (37).

### Ligand Blotting

Samples were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions using a discontinuous buffer system as previously described (33). Proteins were transferred to nitrocellulose membranes by electroblotting, and the binding proteins identified by incubation with $^{125}$I-IGF-II (400,000 cpm/blot; 60–100 Ci/g) and autoradiography as previously described (32) and modified (33).

### Deglycosylation using *N*-Glycanase

Binding protein samples were concentrated using Centricon 30 Microconcentrators. Nonreduced samples were incubated with *N*-glycanase (Genzyme Corp., Boston, MA), 0.5–1 U/

reaction, as recommended by the manufacturer and described in (33). At the end of the reaction, an equal volume of SDS-gel sample buffer (twice concentrated) was added; the samples boiled and examined by ligand blotting.

### Immunoblotting

Samples containing IGF-binding proteins were electrophoresed on nonreducing SDS-polyacrylamide gels (10%), and transferred onto nitrocellulose as described for ligand blotting. The immunoblotting was performed using the immunoperoxidase staining technique and the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA) as previously described (33). In brief, the nitrocellulose membrane was incubated with 7% nonfat dry milk (Carnation Co., Los Angeles, CA) in PBS for 1 h at room temperature, followed by incubation with antibodies to rIGFBP-2 or hIGFBP-1. Next, the blot was incubated with biotinylated second antibody, followed by avidin-biotinylated horseradish peroxidase H complexes. Proteins of interest were identified using freshly prepared peroxidase substrate solution: 4-chloro-1-naphthol (Sigma, St. Louis, MO; 180 mg/10-ml ethanol), diluted in 50 ml 50 mM Tris-Cl (pH 7.4), 200 mM NaCl, to which $H_2O_2$ (Sigma, 60 $\mu$l, 30%) was added.

### Immunoprecipitation

$^{125}$I-IGF-II: binding protein complexes were prepared as previously described (18, 33). Aliquots were incubated with the indicated dilutions of antiserum 3695 to rIGFBP-2 or polyclonal antiserum to hIGFBP-1 in 0.4 ml microcentrifuge tubes (final volume, 0.2 ml) for 16 h at 4 C. Protein A-containing *Staphylococcus aureus* (Pansorbin Calbiochem Corp., San Diego, CA) was added to each tube and the incubation continued for 5–10 min at room temperature, after which the incubation mixture was centrifuged for 3 min, and the radioactivity in each pellet was determined. Precipitated radioactivity in samples incubated without antiserum or with preimmune serum did not differ significantly from each other.

### Northern Blot Hybridization

Total RNA was prepared by extraction with guanidine thiocyanate and precipitation with lithium chloride and ethanol (65). RNA (15 $\mu$g/lane) was electrophoresed on 1.5% agarose/formaldehyde gels in 1× MOPS buffer and blotted to GeneScreen (Dupont-NEN, Boston, MA) as previously described (65). The blots were hybridized with rIGFBP-2 or hIGFBP-1 cDNA probes. The rIGFBP-2 cDNA probe was a 531 bp *Sma*I-*Sac*I fragment corresponding to nucleotides 557-1087 of the cDNA clone described by Brown et al. (24). The probe was labeled with [$\alpha$-$^{32}$P]dCTP by random priming (66). The hIGFBP-1 insert (1443 base pairs) described by Julkunen *et al.* (21) was subcloned into the *Eco*RI site of pGEM3 and provided by O. Jänne. It was labeled by nick translation. The blots were hybridized with $1.2 \times 10^6$ cpm/ml at 50 C (50% formamide, 5× SSPE, 1% SDS), and washed in 0.1× SSPE at 60 C.

## Acknowledgments

We thank Nengjie Ge for assistance in the early ligand-blotting experiments, and N. Rao Thotakura and Peter Nissley for helpful discussions.

Received August 8, 1989. Revision received October 2, 1989. Accepted October 12, 1989.

Address requests for reprints to: Dr. Yvonne W.-H. Yang, National Institutes of Health, Building 10, Room 8D14, Bethesda, Maryland 20892.

A preliminary account of this work was presented at the 71st Annual Meeting of The Endocrine Society, 1989, Seattle, Washington.

## REFERENCES

1. Nissley SP, Rechler MM 1984 Insulin-like growth factors: biosynthesis, receptors, and carrier proteins. In: Li CH (ed) Hormonal Proteins and Peptides. Academic Press, New York and London, vol XII:127–203
2. Daughaday WH, Rotwein P 1989 Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev 10:68–91
3. Rechler MM, Nissley SP 1990 Insulin-like growth factors. Handbook of Experimental Pharmacology, in press
4. Zapf J, Hauri C, Waldvogel M, Froesch ER 1986 Acute metabolic effects and half-lives of intravenously administered insulinlike growth factors I and II in normal and hypophysectomized rats. J Clin Invest 77:1768–1775
5. Busby Jr WH, Klapper DG, Clemmons DR 1988 Purification of a 31,000-dalton insulin-like growth factor binding protein from human amniotic fluid. Isolation of two forms with different biologic actions. J Biol Chem 263:14203–14210
6. De Mellow JSM, Baxter RC 1988 Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts. Biochem Biophys Res Commun 156:199–204
7. Knauer DJ, Smith GL 1980 Inhibition of biological activity of multiplication-stimulating activity by binding to its carrier protein. Proc Natl Acad Sci USA 77:7252–7256
8. Han VK, Lauder JM, D'Ercole AJ 1988 Rat astroglial somatomedin/insulin-like growth factor binding proteins: characterization and evidence of biologic function. J Neurosci 8:3135–3143
9. Ritvos O, Ranta T, Jalkanen J, Suikkari A-M, Voutilainen R, Bohn H, Rutanen EM 1988 Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. Endocrinology 122:2150–2157
10. Hardouin S, Hossenlopp P, Segovia B, Seurin D, Portolan G, Lassarre C, Binoux M 1987 Heterogeneity of insulin-like growth factor binding proteins and relationships between structure and affinity: 1. Circulating forms in man. Eur J Biochem 170:121–132
11. Martin JL, Baxter RC 1986 Insulin-like growth factor-binding protein from human plasma. Purification and characterization. J Biol Chem 261:8754–8760
12. Baxter RC 1988 Characterization of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex. J Clin Endocrinol Metab 67:265–272
13. Baxter RC, Martin JL, Beniac VA 1989 High molecular weight insulin-like growth factor binding protein complex. J Biol Chem 264:11843–11848
14. Povoa G, Enberg G, Jornvall H, Hall K 1984 Isolation and characterization of a somatomedin-binding protein from mid-term human amniotic fluid. Eur J Biochem 144:199–204
15. Bohn H, Kraus W 1980 Isolation and characterization of a new placenta specific protein (PP12). Arch Gynecol 229:279–291
16. Lyons RM, Smith GL 1986 Characterization of multiplication-stimulating activity (MSA) carrier protein. Mol Cell Endocrinol 45:263–270
17. Mottola C, MacDonald RG, Brackett JL, Mole JE, Anderson JK, Czech MP 1986 Purification and amino-terminal sequence of an insulin-like growth factor-binding protein secreted by rat liver BRL-3A cells. J Biol Chem 261:11180–11188

18. Wang J-F, Hampton B, Mehlman T, Burgess WH, Rechler MM 1988 Isolation of a biologically active fragment from the carboxy-terminus of the fetal rat binding protein for insulin-like growth factors. Biochem Biophys Res Commun 157:718–726
19. Brewer MT, Stetler GL, Squires CH, Thompson RC, Busby WH, Clemmons DR 1988 Cloning, characterization, and expression of a human insulin-like growth factor binding protein. Biochem Biophys Res Commun 152:1289–1297
20. Brinkman A, Groffen C, Kortleve DJ, van Kessel AG, Drop SLS 1988 Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1). EMBO J 7:2417–2423
21. Julkunen M, Koistinen R, Aalto-Setala K, Seppala M, Janne OA, Kontula K 1988 Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA. FEBS Lett 236:295–302
22. Lee Y-L, Hintz RL, James PM, Lee PDK, Shively JE, Powell DR 1988 Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid from human HEP G2 hepatoma cells: predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors. Mol Endocrinol 2:404–411
23. Wood WI, Cachianes G, Henzel WJ, Winslow GA, Spencer SA, Hellmiss R, Martin JL, Baxter RC 1988 Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. Mol Endocrinol 2:1176–1185
24. Brown AL, Chiariotti L, Orlowski CC, Mehlman T, Burgess WH, Ackerman EJ, Bruni CB, Rechler MM 1989 Nucleotide sequence and expression of a cDNA clone encoding a fetal rat binding protein for insulin-like growth factors. J Biol Chem 264:5148–5154
25. Margot JB, Binkert C, Mary J-L, Landwehr J, Heinrich G, Schwander J 1989 A low molecular weight insulin-like growth factor binding protein from rat: cDNA cloning and tissue distribution of its messenger RNA. Mol Endocrinol 3:1053–1060
26. Rechler MM, Brown AL, Orlowski CC, Yang YW-H, Romanus JA, Chiariotti L, Bruni CB 1990 Characterization and cloning of a rat insulin-like growth factor binding protein. In: Raizada M, LeRoith D (eds) Molecular and Cellular Aspects of Insulin, IGFs and Their Receptors: Implications for the CNS. Plenum Publishing Corp., New York, in press
27. Baxter RC, Martin JL 1986 Radioimmunoassay of growth hormone-dependent insulinlike growth factor binding protein in human plasma. J Clin Invest 78:1504–1512
28. Povoa G, Roovete A, Hall K 1984 Cross-reaction of serum somatomedin-binding protein in a radioimmunoassay developed for somatomedin-binding protein isolated from human amniotic fluid. Acta Endocrinol (Copenh) 107:563–570
29. Drop SLS, Kortleve DJ, Guyda HJ, Posner BI 1984 Immunoassay of a somatomedin-binding protein from human amniotic fluid: levels in fetal, neonatal, and adult sera. J Clin Endocrinol Metab 59:908–915
30. Cotterill AM, Cowell CT, Baxter RC, McNeil D, Silinik M 1988 Regulation of the growth hormone-independent growth factor-binding protein in children. J Clin Endocrinol Metab 67:882–887
31. Busby WH, Snyder DK, Clemmons DR 1988 Radioimmunoassay of a 26,000-dalton plasma insulin-like growth factor-binding protein: control by nutritional variables. J Clin Endocrinol Metab 67:1225–1230
32. Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M 1986 Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding sites. Anal Biochem 154:138–143
33. Yang YW-H, Wang J-F, Orlowski CC, Nissley SP, Rechler MM 1989 Structure, specificity, and regulation of the insulin-like growth factor binding proteins in adult rat serum. Endocrinology 125:1540–1555
34. Kiess W, Greenstein LA, White RM, Lee L, Rechler MM, Nissley SP 1987 Type II insulin-like growth factor receptor is present in rat serum. Proc Natl Acad Sci USA 84:7720–7724
35. MacDonald RG, Tepper MA, Clairmont KB, Perregaux SB, Czech MP 1989 Serum form of the rat insulin-like growth factor II/Mannose 6-Phosphate receptor is truncated in the carboxyl-terminal domain. J Biol Chem 264:3256–3261
36. Kiess W, Lee L, Graham DE, Greenstein L, Tseng LY-H, Rechler MM, Nissley SP 1989 Rat C6 glial cells synthesize insulin-like growth factor (IGF-I) and express IGF-I receptors and IGF-II/mannose 6-phosphate receptors. Endocrinology 124:1727–1736
37. Baxter RC, Martin JL 1987 Binding proteins for insulin-like growth factors in adult rat serum. Comparison with other human and rat binding proteins. Biochem Biophys Res Commun 147:408–415
38. Zapf J, Born W, Chang J-Y, James P, Froesch ER, Fischer JA 1988 Isolation and $NH_2$-terminal amino acid sequences of rat serum carrier proteins for insulin-like growth factors. Biochem Biophys Res Commun 156:1187–1194
39. Romanus JA, Terrell JE, Yang YW-H, Nissley SP, Rechler MM 1986 Insulin-like growth factor carrier proteins in neonatal and adult rat serum are immunologically different: demonstration using a new radioimmunoassay for the carrier protein from BRL-3A rat liver cells. Endocrinology 118:1743–1758
40. Romanus JA, Yang YW-H, Nissley SP, Rechler MM 1987 Biosynthesis of the low molecular weight carrier protein for insulin-like growth factors in rat liver and fibroblasts. Endocrinology 121:1041–1050
41. Koistinen R, Huhtala ML, Stenman UH, Seppala M 1987 Purification of placental protein PP12 from human amniotic fluid and its comparison with PP12 from placenta by immunological, physiococochemical and somatomedin-binding properties. Clin Chim Acta 164:293–303
42. Koistinen R, Stenman U-H, Alfthan H, Seppala M 1987 Time-resolved immunofluorometric assay of 34-kDa somatomedin-binding protein. Clin Chem 33:1126–1128
43. Rutanen EM, Karkkainen T, Lundqvist C, Pekonen F, Ritvos O, Tanner P, Welin M, Weber T 1988 Monoclonal antibodies to the 27-34K insulin-like growth factor binding protein. Biochem Biophys Res Commun 152:208–215
44. Povoa G, Isaksson M, Jornvall H, Hall K 1985 The somatomedin-binding protein isolated from a human hepatoma cell line is identical to the human amniotic fluid somatomedin-binding protein. Biochem Biophys Res Commun 128:1071–1078
45. Hall K, Lundin G, Povoa G 1988 Serum levels of the low molecular weight form of insulin-like growth factor binding protein in healthy subjects and patients with growth hormone deficiency, acromegaly and anorexia nervosa. Acta Endocrinol (Copenh) 118:321–326
46. Orlowski CC, Chernausek SD, Akeson R 1989 Actions of insulin-like growth factor-I on the B104 neuronal cell line: effects on cell replication, receptor characteristics, and influence of secreted binding protein on ligand binding. J Cell Physiol 139:469–476
47. Sturm MA, Conover CA, Pham H, Rosenfeld RG 1989 Insulin-like growth factor receptors and binding protein in rat neuroblastoma cells. Endocrinology 124:388–396
48. Ocrant I, Pham H, Oh Y, Rosenfeld RG 1989 Characterization of insulin-like growth factor binding proteins of cultured rat astroglial and neuronal cells. Biochem Biophys Res Commun 159:1316–1322
49. McCusker RH, Camacho-Hubner C, Clemmons DR 1989 Identification of the types of insulin-like growth factor-binding proteins that are secreted by muscle cells *in vitro*. J Biol Chem 264:7795–7800
50. Pitot HC, Peraino C, Morse PA, Potter VR 1964 Hepatoma

in tissue culture compared with adapting liver *in vivo*. Natl Cancer Inst Monograph 13:229–242
51. Deschatrette J, Moore EE, Dubois M, Cassio D, Weiss M 1979 Dedifferentiated variants of a rat hepatoma: analysis by cell hybridization. Somatic Cell Genet 5:697–718
52. Szabo L, Mottershead DG, Ballard FJ, Wallace JC 1988 The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-1 for binding. Biochem Biophys Res Commun 151:207–214
53. Perfetti R, Conti FC, Elliott SE, Striker LJ, Striker GE, Mouse glomerular mesangial cells in culture produce insulin like growth factor-I binding proteins. Program of the 71st Annual Meeting of The Endocrine Society, Seattle, WA, 1989, p 275 (Abstract)
54. Romanus JA, Tseng L Y-H, Yang YW-H, Rechler MM 1989 The 34 kilodalton insulin-like growth factor binding proteins in human cerebrospinal fluid and the A673 rhabdomyosarcoma cell line are human homologues of the rat BRL-3A binding protein. Biochem Biophys Res Commun, 163:875–881
55. Binkert C, Landwehr J, Mary J-L, Schwander J, Heinrich G 1989 Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2). EMBO J 8:2497–2502
56. Suikkari AM, Koivisto VA, Rutanen EM, Yki-Jarvinen H, Karonen SL, Seppala M 1988 Insulin regulates the serum levels of low molecular weight insulin-like growth factor-binding protein. J Clin Endocrinol Metab 66:266–272
57. Brismar K, Gutniak M, Povoa G, Werner S, Hall K 1988 Insulin regulates the 35 kDa IGF binding protein in patients with diabetes mellitus. J Endocrinol Invest 11:599–602
58. Suikkari AM, Koivisto VA, Koistinen R, Seppala M, Yki-Jarvinen H 1989 Dose-response characteristics for suppression of low molecular weight plasma insulin-like growth factor-binding protein by insulin. J Clin Endocrinol Metab 68:135–140
59. Idoine JB, Elliott JM, Wilson MJ, Weisburger EK 1976 Rat liver cells in culture: effect of storage, long-term culture, and transformation on some enzyme levels. In Vitro 12:541–553
60. Weinstein IB, Yamaguchi N, Orenstein JM, Gebert R, Kaighn ME 1975 Mechanisms of chemical carcinogenesis analyzed in rat liver and hepatoma cell cultures. In: Gerschenson LE, Thompson EB (eds) Gene Expression and Carcinogenesis in Cultured Lines. Academic Press, New York, pp 441
61. Schubert D, Heinemann S, Carlisle W, Tarikas H, Kimes B, Patrick J, Steinbach JH, Culp W, Brandt BL 1974 Clonal cell lines from the rat central nervous system. Nature 249:224–227
62. Benda P, Lightbody J, Sato G, Levine L, Sweet W 1969 Differential rat glial cell strain in tissue culture. Science 161:370
63. DeLarco JE, Todaro GJ 1978 Epithelioid and fibroblastic rat kidney cell clones: epidermal growth factor (EGF) receptors and the effect of mouse sarcoma virus transformation. J Cell Physiol 94:335–342
64. Knowles BB, Howe C, Aden DP 1980 Human hepatocellular carcinoma cell lines secrete the major plasma proteins and Hepatitis B surface antigen. Science 209:497–499
65. Brown AL, Graham DE, Nissley SP, Hill DJ, Strain AJ, Rechler MM 1986 Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. J Biol Chem 261:13144–13150
66. Lowe Jr WL, Roberts Jr CT, Lasky SR, LeRoith D 1987 Differential expression of alternative 5′ untranslated regions in mRNAs encoding rat insulin-like growth factor I. Proc Natl Acad Sci USA 84:8946–8950
67. Ooi GT, Orlowski CC, Brown AL, Becker RE, Unterman TG, Rechler MM, Different tissue distribution and hormonal regulation of mRNAs encoding rat insulin-like growth factor binding proteins rIGFBP-1 and rIGFBP-2. Mol Endocrinol, in press
68. Unterman TG, Oehler DT, Becker RE 1989 Identification of a type I insulin-like growth factor binding protein (IGFBP) in serum from rats with diabetes mellitus. Biochem Biophys Res Commun 163:882–887