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# The expression of insulin-like growth factor binding proteins is tissue specific during human fetal life and early infancy

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Funk B, Kessler U, Eisenmenger W, Hansmann A, Kolb HJ, Kiess W. The expression of insulin-like growth factor binding proteins is tissue specific during human fetal life and early infancy. *Acta Endocrinol* 1992;127:107–14. ISSN 0001–5598

The insulin-like growth factors (IGFs) are bound to multiple IGF binding proteins (IGFBPs) that are present both in the circulation and in extracellular fluids. There are at least six different IGFBP species that have been fully characterized in terms of molecular structure and amino acid sequence. The tissue distribution and local production of these proteins as well as the regulation of IGFBP production in different tissues have not been elucidated. We have studied the distribution of multiple IGFBP species in protein extracts from human kidney, skeletal muscle, lung, liver and brain by ligand blotting employing [<sup>125</sup>I]IGF-2 as the radiolabeled hormone. Five distinct IGFBP species with a respective molecular weight of 43, 38, 34, 30 and 20 kDa were detected on the ligand blots in tissues from human fetuses and infants (23 weeks of gestation till 24 months of postnatal age). The 34 kDa species and a 30–32 kDa IGFBP species were predominant in brain, whereas a 30 kDa IGFBP species was mainly detected in skeletal muscle. Immunoblotting experiments using an anti IGFBP-2 antiserum showed that the 34 kDa IGFBP species from human brain was presumably related to IGFBP-2. We conclude that IGFBPs are differentially expressed in different tissues throughout human fetal life and early infancy. Local production or accumulation of the different IGFBPs could modulate IGF action at a local level or alternatively have differential functions during development.

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The insulin-like growth factors (IGF-1 and -2) are polypeptides which belong to the insulin family of polypeptide hormones that also include proinsulin and relaxin. The IGFs are potent mitogens for cells in culture. IGF-1 is thought to mediate the action of growth hormone in vivo. Although the amino acid sequence and molecular structure of IGF-2 have also been characterized, its biologic action and physiologic role in vivo are still elusive (1–4). In the circulation, the IGFs are bound to specific binding proteins (IGFBPs) that are produced by the liver. IGFBPs are also synthesized in many other tissues and are present in many body fluids like amniotic, cerebrospinal and seminal fluid. IGFBPs are released from many different cells in culture (2, 4–7). There are at least six major classes of IGFBPs that have been fully characterized. Molecular cloning and biochemical techniques have helped to elucidate the structure and evolution of these molecules (2, 4–20). However, the function of these proteins is not completely understood: it is hypothesized that the IGFBPs serve as a reservoir for IGF storage prolonging the biologic half life of the IGFs (2, 5–7, 21, 22). It has also been suggested that the IGFBPs function as major modulators of IGF action either as inhibitors (13, 23) or enhancers (21, 22) of IGF biologic effects. It is important to note that different

IGFBP species are released from different cell types in culture, supporting the notion that different tissues might synthesize or retain different molecular forms of IGFBPs in vivo. The IGFBPs might then exert differential effects in various tissues during organ development and tissue growth (7, 24). We have therefore studied the expression of different molecular forms of IGFBPs in multiple tissues from human fetuses and infants.

## Materials and methods

### *Tissue collection*

Tissues (kidney, brain, liver, lung, testis, ovary, spleen, thymus, muscle, heart) were collected from twelve aborted or stillborn fetuses and infants who had died suddenly (sudden infant death syndrome) or by accident. The time interval between death and removal of tissues at the Institute of Forensic Medicine or at the Institute of Pathology, University of Munich, Munich, Germany, was between 4 and approximately 24 h. Tissues were removed aseptically, diced, rinsed carefully to remove residual blood, and immediately frozen in liquid nitrogen. The study protocol had been approved by the Ethics

Committee of the Children's Hospital, University of Munich, Germany.

#### *Tissue extraction*

Approximately 20 mg of thawed tissue was placed in 1.5 ml plastic tubes and weighed. 0.4 ml of 20 mmol/l TRIS HCl, 2% Triton X-100, pH 7.4, and 0.1 ml Laemmli buffer containing 2% SDS (25) was added, and the tissues homogenized for 3 min with a Polytron homogenizer (Polytron PT 10-35, Bachofer, Reutlingen, Germany). The samples were boiled for 5 min and then incubated overnight at 4°C. The protein homogenates were then boiled again for 1 min and centrifuged for 5 min at 10 000 g in a Mikrorapid/K centrifuge (Hettich, Tuttlingen, Germany). Aliquots of the supernatants were stored at -70°C until further analysis. No visible pellet was seen after extraction. The protein content of the tissue extracts was determined using the BCA protein assay (Pierce Chemical Co, cat. no. 23225, Rockford, IL). Dilutions of bovine serum albumin were used as protein standards.

#### *Immunoblotting*

Aliquots of the tissue extracts amounting to 0.2 mg protein per organ examined were subjected to SDS-polyacrylamide gel electrophoresis (12.5% acrylamide bis) under non-reducing conditions using the discontinuous buffer system of Laemmli (25). Prestained molecular weight markers were used as follows; lysozyme 14300, beta-lactoglobulin 18400, carbonic anhydrase 29000, ovalbumin 43000, bovine serum albumin 68000, phosphorylase B 97400, myosin 200000 (BRL, Eggenstein, Germany, prestained protein molecular weight standards, cat. no. 60411A). The electrophoresed samples were transferred onto nitrocellulose (Schleicher & Schuell, cat. no. 401180, Dassel, Germany) by electroelution. Immunoblotting was then performed using a specific antiserum against the human IGFBP-2 (polyclonal rabbit anti human IGFBP-2 antiserum, a kind gift from Dr David Clemmons, Chapel Hill, North Carolina) following an earlier described protocol (26, 27). The antiserum was used at a final dilution of 1:1000. An immunoperoxidase staining kit (Vectastain ABC Kit, Vector Laboratories, Inc, Burlingame, CA) was used to detect specific protein bands on the nitrocellulose filters. After drying overnight, the nitrocellulose sheets were photographed.

#### *Ligand blotting*

Aliquots (200 µg) of the tissue extracts were subjected to SDS-PAGE (12.5% acrylamide bis) under non-reducing conditions as described by Laemmli (25). Proteins were then transferred onto nitrocellulose filters by electroelution as described for the immunoblotting procedure. Ligand blotting was carried out essentially as described

by Hossenlopp et al. (28). In brief, the nitrocellulose filters were preincubated in buffer containing 1% bovine serum albumin (BSA), and subsequently incubated with [<sup>125</sup>I]IGF-2 (Amersham, Braunschweig, Germany) diluted in 0.15 mol/l NaCl, 10 mmol/l TRIS-HCl, 1% BSA, 0.1% Tween-20, pH 7.4 (20 ml/filter/bag), rinsed extensively in 0.15 mol/l NaCl, 10 mmol/l TRIS-HCl, 0.1% Tween, pH 7.4 buffer, and then in 0.15 mol/l NaCl, 10 mmol/l TRIS-HCl, pH 7.4, and dried overnight. Autoradiographs were obtained by exposing the filters to Kodak X-OMAT films (Eastman Kodak, Rochester) using enhancer screens (Du Pont, Newton, CT) at -70°C. In a subset of experiments, strips of the nitrocellulose filters containing one lane of electrophoresed proteins were cut and the strips incubated with [<sup>125</sup>I]IGF-2 and increasing amounts of unlabeled IGF-2 or IGF-1 (recombinant human IGF-1 and -2, kindly provided by Dr Anna Skottner, Kabi, Stockholm, Sweden, and Dr K Mueller, Ciba Geigy, Basel, Switzerland) according to Hossenlopp et al. (28).

## Results

#### *Detection of IGF BPs in human tissue extracts*

When protein extracts from human skeletal muscle, brain, liver, kidney and lung tissues were analyzed by SDS-PAGE and ligand blotting employing [<sup>125</sup>I]IGF-2 five distinct radiolabeled protein species with an approximate molecular weight of 43, 38, 34, 30 and 20 kDa respectively were detected (Figs 1-3). The intensity of the radiolabeled bands varied among the tissues studied: the 34 kDa band was predominant in brain tissue, while a 30 kDa band was predominant in skeletal muscle protein extracts and all five IGFBP species were variably detected in kidney, liver and lung extracts. The IGFBP profiles from the ligand blot experiments with brain extracts in Fig. 1 and Fig. 2 resemble those reported for cerebrospinal fluid (20, 29). The apparent molecular weight of the IGFBP species found in human tissue extracts by ligand blotting experiments corresponds well with the molecular weight of IGFBP species commonly detected in human plasma and serum (5, 9, 24, 28-31). Lung, liver, kidney, and muscle protein extracts from all 12 fetuses and infants were analyzed by ligand blotting employing [<sup>125</sup>I]IGF-2 in a parallel fashion as shown in one representative blot in Fig. 1 (upper panel). Brain extracts were analyzed from seven infants. Serum samples from a six-year-old healthy boy and alternatively from a 30-year-old healthy man were analyzed in parallel for comparison reasons as indicated in each Figure.

#### *Competition of unlabeled IGF with <sup>125</sup>I-IGF binding to IGF BPs in human tissue extracts*

When tissue extracts were analyzed by SDS-PAGE and the ligand blotting experiment using [<sup>125</sup>I]IGF-2 was

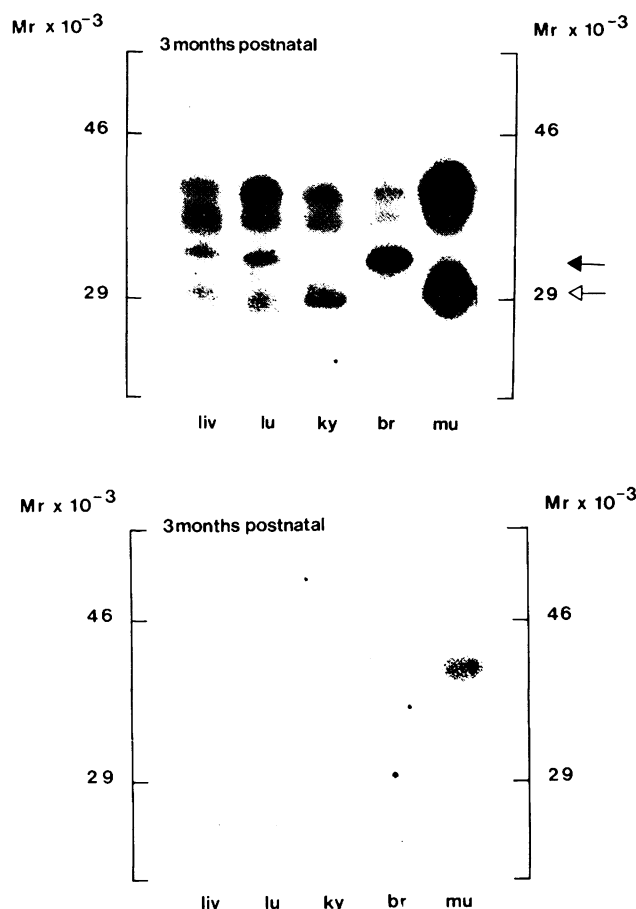


Fig. 1. Analysis of IGF binding proteins in protein extracts from human tissues (3 months old infant, liv=liver, lu=lung, ky=kidney, br=brain, mu=skeletal muscle) by ligand blotting. Proteins were extracted and analyzed by SDS-PAGE and ligand blotting using [ $^{125}$ I]IGF-2 as described in Methods. In lower panel an excess (300 nmol/l) of unlabeled human recombinant IGF-2 was present during the incubation of the blot with [ $^{125}$ I]IGF-2.

performed in the absence (Fig. 1, upper panel) or presence (Fig. 1, lower panel) of excess unlabeled IGF-2 (300 nmol/l) labeling of all bands was greatly diminished or disappeared completely in the presence of the unlabeled ligand (Fig. 1, lower panel). It is concluded that the radiolabeled bands detected by ligand blotting represent proteins that bind tracer amounts of [ $^{125}$ I]IGF-2 and that binding of [ $^{125}$ I]IGF-2 to the IGFBP species is saturable.

#### Expression of IGF binding proteins in human brain during early infancy

When protein extracts from brain tissue from infants of different age (41 weeks gestation till 24 months postnatal) were analyzed using SDS-PAGE and ligand blotting using [ $^{125}$ I]IGF-2 as the radioligand, again the 34 kDa species was the predominant band detected by

autoradiography of the blots. Again, the IGFBP profiles from the ligand blotting experiments with brain extracts resemble those reported for cerebrospinal fluid (20, 29). The 30–32 kDa IGFBP species in brain extracts might correspond to a similar molecular weight IGFBP species in cerebrospinal fluid described by Rhogani et al. (20). There was some variation in the intensity of the bands detected in the blots; however, no obvious trend of the intensity of the labeling throughout early infancy (3 months postnatal to 24 months postnatal) could be detected using the ligand blotting technique (Fig. 2).

#### Expression of IGF binding proteins in skeletal muscle during human fetal life and early infancy

Protein extracts from skeletal muscle (psoatic) were prepared from human fetuses (20 till 26 weeks gestation) and infants (neonate, 41 weeks gestation, and 2 till 24 months postnatal); proteins were analyzed using SDS-PAGE and ligand blotting employing [ $^{125}$ I]IGF-2 as the radiolabeled ligand. Autoradiography of the blots showed that the characteristic binding protein species in all skeletal muscle tissues examined was a 30 kDa protein which was present throughout fetal life (Fig. 3, upper panel) and throughout early infancy (Fig. 3, lower panel). The 34 kDa IGFBP species which predominated in brain was virtually absent in the muscle extracts. However, a 30–32 kDa band was seen in prenatal muscle tissues. This band corresponds in size with the 30–32 kDa IGFBP which was also detected in brain extracts. Again, there was great variation of the intensity of the bands throughout the time period studied, and no obvious developmental change was detected.

#### Characterization of a 34 kDa IGF binding protein in human brain tissue extracts

To further characterize the predominant IGF binding protein species from human brain tissues, protein extracts from human brain (female infant, six months of age) were analyzed by SDS-PAGE and Western blotting using a specific anti IGFBP-2 antiserum. For comparison human sera were analyzed in parallel (Fig. 4). A specific protein band with an approximate molecular weight of 30–34 kDa was detected both in human sera and in brain extracts, whereas no such band was seen in extracts from human skeletal muscle. The 30–34 kDa protein was not detected when non-immune control serum from normal rabbits was used in the Western blotting experiment instead of the antiserum (Fig. 4, right panel). It can be speculated that the 30–34 kDa IGF binding protein species in the Western blotting experiments might correspond to the 34 kDa band detected in the ligand blotting experiments, and that thus the 34 kDa IGF binding protein measured in brain tissues is



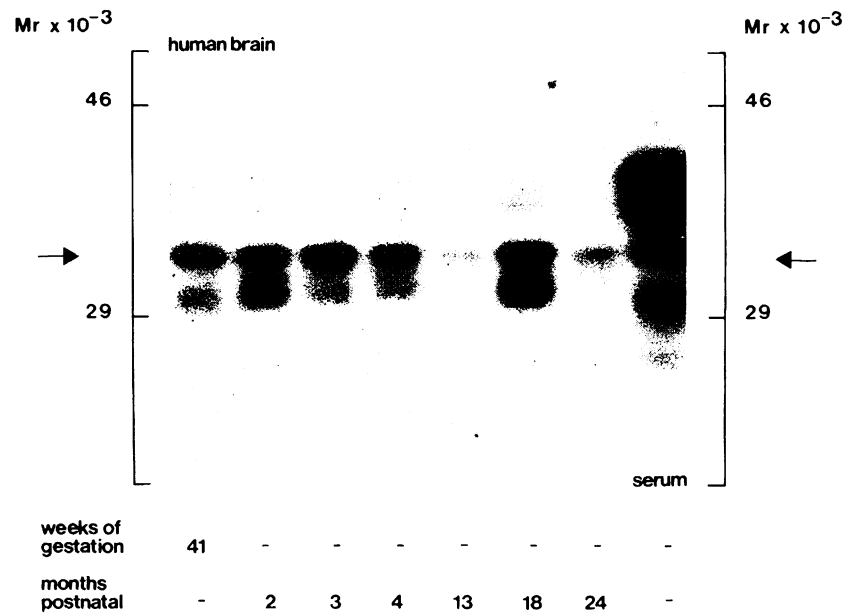


Fig. 2. Expression of IGF binding proteins in brain tissue during early infancy. Brain tissues from seven infants were analyzed. For comparison reasons serum (2  $\mu$ l) from a healthy male adult was analyzed in parallel. Proteins were extracted and ligand blotting was performed as in Fig. 1. The arrow points to the predominant 34 kDa IGF binding protein species in human brain extracts.

immunologically related to IGFBP-2. In a subset of experiments, aliquots of protein extracts from human brain were analyzed by SDS-PAGE, the proteins were transferred onto nitrocellulose paper by electroelution, the nitrocellulose filters were cut, and the filters were incubated with [ $^{125}$ I]IGF-2 in the presence or absence of increasing concentrations of either unlabeled IGF-1 or IGF-2. Labeling of IGFBP species at 34 and 30 kDa was inhibited completely by co-incubation of the radiolabeled IGF-2 with 0.3 nmol/l unlabeled IGF-2 or 3–30 nmol/l unlabeled IGF-1 (Fig. 5, both panels). Thus, unlabeled IGF-2 was approximately 10-fold more potent than IGF-1 in competing for [ $^{125}$ I]IGF-2 binding to IGFBP species expressed in human brain tissue. Interestingly, in three separate experiments, very low concentrations of both IGF-1 and IGF-2 actually increased binding of [ $^{125}$ I]IGF-2 to the IGFBP species (Fig. 5, both panels).

#### Characterization of IGF binding proteins from human skeletal muscle

When protein extracts from human skeletal muscle tissues were subjected to SDS-PAGE, transferred onto nitrocellulose paper and ligand blotting using [ $^{125}$ I]IGF-2 was performed in the absence or presence of increasing concentrations of unlabeled IGF-1 or IGF-2, unlabeled IGF-2 competed at low concentrations (0.3 nmol/l) for binding to the 30 kDa IGF binding protein species of skeletal muscle, whereas IGF-1 only at high concentrations (30 nmol/l) was able to displace the radiolabeled hormone. An additional radiolabeled band of approxi-

mately 40 kDa presumably represents a non-specific (high capacity, low affinity) protein since even very high concentrations of unlabeled IGFs did not compete for binding of the radiolabeled IGF-2. Interestingly, very low concentrations of unlabeled IGF-1 and IGF-2 again actually increased the intensity of the radiolabeled IGFBP-IGF-band (Fig. 6, both panels).

#### Discussion

At least six different IGFBP species have been cloned and characterized at a molecular level (5–20). However, little is known about the ontogeny, the tissue distribution and local function of these classes of proteins (2, 5–7). We have measured the expression of IGFBP species by ligand blotting experiments employing [ $^{125}$ I]IGF-2 as the radiolabeled ligand in multiple tissues from human fetuses and infants (23 weeks gestation till 24 months postnatal). Differential expression of five distinct IGFBP species with a molecular weight of 43, 38, 34, 30 and 20 kDa respectively was detected. These radiolabeled protein species correspond in size with the IGFBP species described in the literature (for review, see 5–8, 30). The 34 kDa protein was preferentially seen in brain tissues at all ages studied, whereas in skeletal muscle tissue the 30 kDa IGFBP species predominated. It is clear from this study that [ $^{125}$ I]IGF-2-ligand blotting can be used to study IGFBP expression in tissue extracts. Contrary to expectations, the IGFbps are not degraded by intra- or

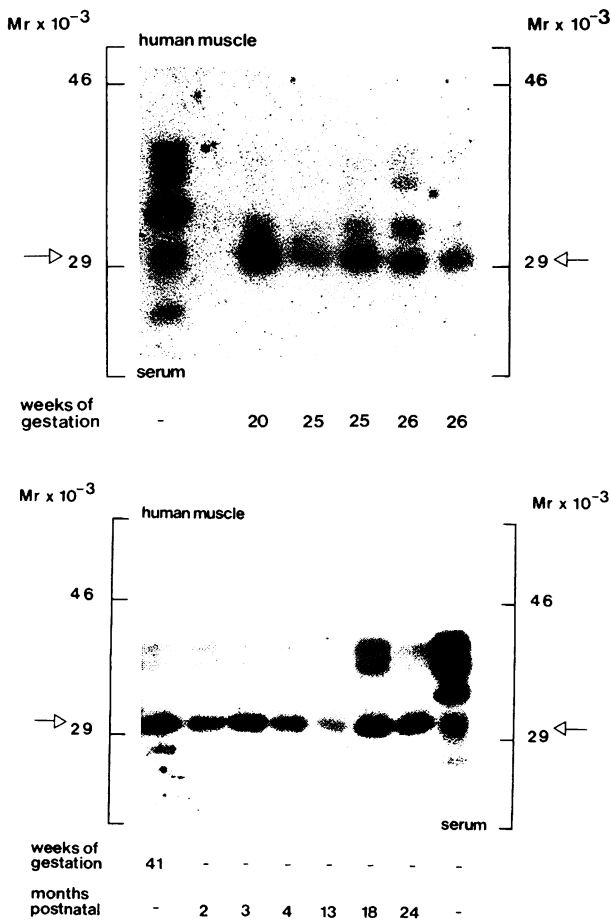


Fig. 3. Expression of IGF binding proteins in skeletal muscle during human fetal life and early infancy. Proteins were extracted and ligand blotting was performed as described in Methods. In upper panel, skeletal muscle tissues from five human fetuses (gestational age: 20–26 weeks) were examined. For comparison reasons, serum (2  $\mu$ l) from a six-year-old healthy boy was also examined. In lower panel, skeletal muscle tissues from seven infants (age range: newborn day one–24 months) were studied. For comparison reasons serum (2  $\mu$ l) from a healthy male adult was also blotted. The arrow points to a 30 kDa IGF binding protein species that is predominant in protein extracts from human skeletal muscle tissues.

extra-cellular proteases under the conditions of the experiments presented in this study. The appearance of different species of IGFbps in protein extracts from different organs on ligand blots can be explained by the following: (a) There might be differences in local production of IGFbps, as the occurrence of mRNAs for specific IGFbps in some and their absence in other tissues has been reported (11–17). (b) A residual amount of blood contaminating the tissues might vary among the tissues studied. However, all tissues have been carefully rinsed to remove blood as extensively as possible, contamination of the samples with blood seems unlikely, and might mainly lead to changes in the abundance of IGFBP-3 related species, since IGFBP-3 is the main IGFBP in the

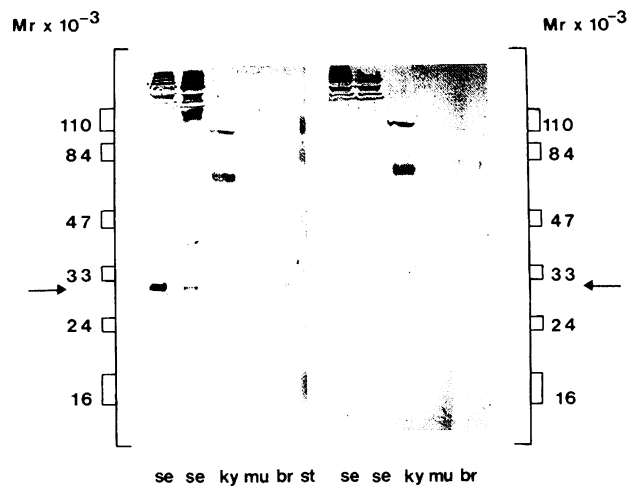


Fig. 4. Immunoblotting of IGFBP-2 in human serum and tissue extracts. Adult human serum (5  $\mu$ l and 2  $\mu$ l, se) or tissue extracts from kidney (ky), skeletal muscle (mu) and brain (br) were analyzed by SDS-PAGE, and immunoblotting employing anti IGFBP-2 antiserum (upper panel) or non-immune rabbit serum (lower panel) was performed. The arrow points to a 31–34 kDa protein band corresponding in size to IGFBP-2. Molecular weight markers as described in Methods are shown in lane (st).

circulation (5–7). (c) Preferential retention of some IGFBP species over others in different organs might lead to different patterns of IGFbps in the organs studied. (d) Local production of proteases that are capable of specifically cleaving the IGFbps could alter the appearance of the IGFBP species on ligand blots. In fact, specific proteases that cleave IGFBP-3 in plasma and serum of pregnant women have been found (31).

There was considerable variation in the amount of IGFbps present at various ages in brain and muscle; however, no obvious developmental pattern of IGFBP expression was measured in the two organs. It is well known that small molecular weight IGFbps (i.e. IGFBP-1) are mainly expressed in amniotic fluid and during fetal life (for review, see 5, 7), whereas IGFBP-3 is the main IGF carrier during postnatal life in the human (1–4, 9). The ontogeny of rat and porcine serum IGFbps has also been elucidated (32, 33). The failure to detect a developmental pattern of IGFBP expression in our study could be explained by the age of the fetuses and infants studied, i.e. developmental changes could occur earlier or later during development in the tissues examined. Alternatively, the ligand blotting method might not be sensitive enough to detect small changes in IGFBP expression. Since ligand blotting cannot be considered to be a quantitative technique, no definitive answer can be given as to whether or not there might be quantitative differences in IGFBP expression at different ages. The large variation and scatter of IGFBP expression could be due to interindividual variation in IGFBP expression or due to some unknown factors influencing the detection

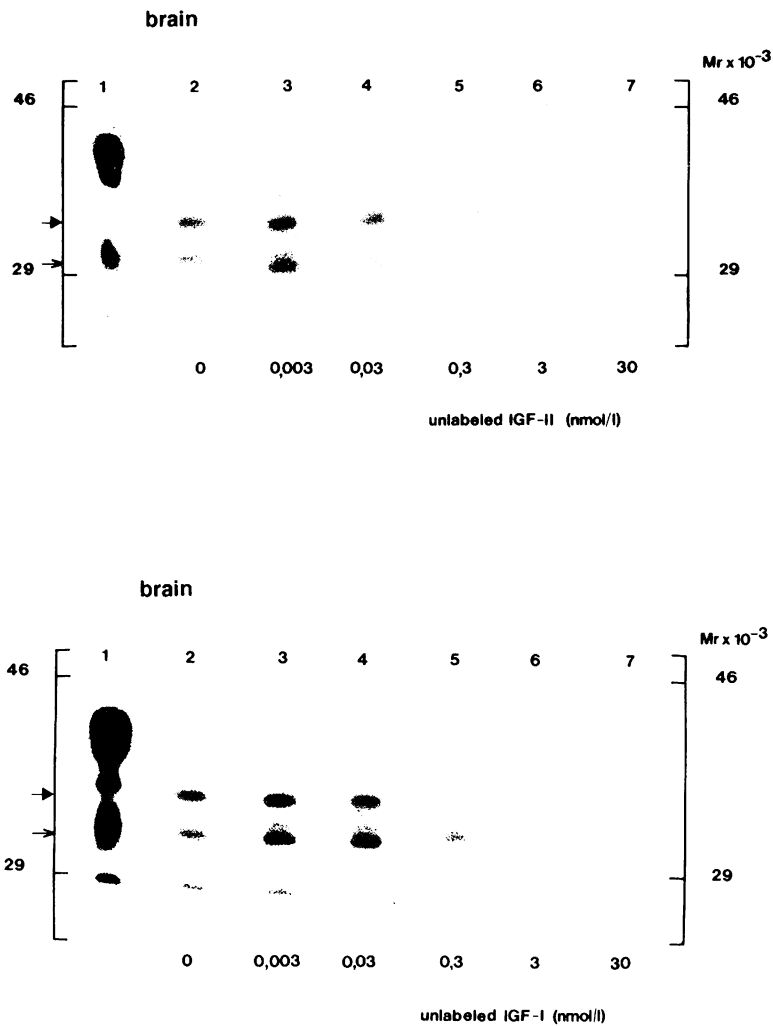


Fig. 5. Competition of [<sup>125</sup>I]IGF-2 labeling of IGFBPs in brain extracts by increasing concentrations of IGF-2 (upper panel) and IGF-1 (lower panel). Ligand blotting was performed as described in Methods. The filled arrows point to the 34 kDa IGFBP species that predominates in brain, the lower arrow points to the 30 kDa binding protein species that predominates in muscle tissue. Lane 1 contains 2  $\mu$ l of serum from a healthy adult male for comparison.

of IGFBPs in ligandblots. Among these, proteolytic degradation post mortem might be a predominant cause of variation.

By immunoblotting using an IGFBP-2 antiserum we have detected an IGFBP with a molecular weight above 30 kDa in human brain tissue extracts. This protein might correspond to the 34 kDa protein detected in brain tissue by ligand blotting. It thus seems likely that the 34 kDa IGFBP predominantly expressed in brain is related to IGFBP-2. Specific IGFBPs of similar size have been isolated from both cerebrospinal fluid and brain tissue (14, 20, 29). It has been reported in the literature that these brain specific IGFBPs might preferentially bind IGF-2 and only weakly recognize IGF-1 (20, 29). Since both the 34 kDa and a 30 kDa IGFBP measured in our experiments seem to bind IGF-2 with higher affinity than

IGF-1 (IGF-2 displaces [<sup>125</sup>I]IGF-2 binding at ten times lower concentrations than IGF-1, Figs. 5 and 6 and data not shown), it is also possible that these IGFBP species are related proteins. It is unclear why very low concentrations of unlabeled IGFs actually increased the binding of [<sup>125</sup>I]IGF-2 to the IGFBP species in these experiments.

We hypothesize that preferential expression or retention of IGFBPs in different human tissues might reflect differential functions of the different IGFBP species. It is difficult, however, to relate the different IGFBP species detected by ligand blotting techniques to the known and well characterized IGFBPs. Therefore, no definitive assignment of specific IGFBP species as to their relationship with the IGFBP species detected in the ligand blotting experiments is attempted. Local homeostasis between the IGFs and their binding proteins might

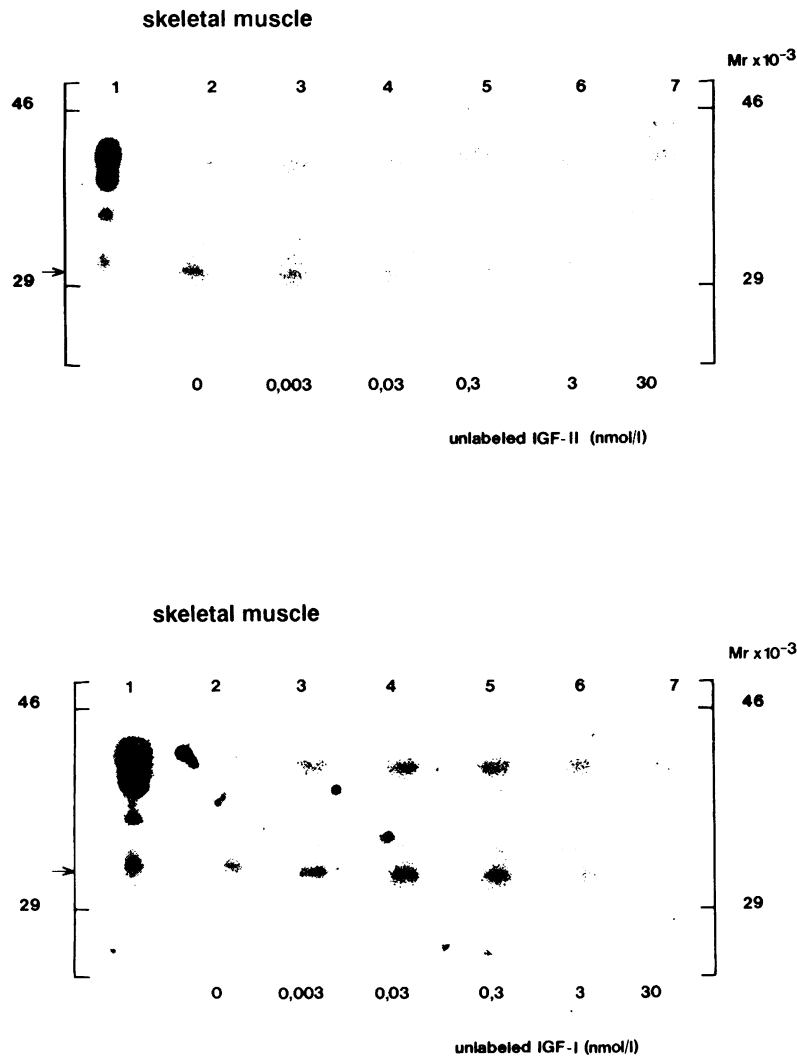


Fig. 6. Competition of [ $^{125}$ I]IGF-2 labeling of IGFBPs in muscle extracts by increasing concentrations of IGF-2 (upper panel) and IGF-1 (lower panel). Ligand blotting was performed as described in Methods. The arrow points to a 30 kDa IGF binding protein species that is predominantly detected in skeletal muscle tissue. Lane 1 contains 2  $\mu$ l of serum from a healthy adult male for comparison.

provide sensitive mechanisms of regulation. Further molecular characterization of the different IGFBP species present in different tissues at different time points of development is needed.

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