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Insulin-like growth factor II (IGF II) in human brain: Regional distribution of IGF II and of higher molecular mass forms

(somatomedins)

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ABSTRACT **Twenty-four distinct areas of human brain were analyzed for the presence of insulin-like growth factor (IGF). As reported for cerebrospinal fluid, only IGF II-like immunoreactivity, but no significant amounts of IGF I-like immunoreactivity, could be found. Upon gel permeation chromatography, two to five distinct size classes were separated on the basis of their immunoreactivity. The smallest component had an apparent molecular mass of 7.5 kDa, identical to the one of purified IGF II from human serum. Radioimmunoassays and a bioassay also gave results indistinguishable from those of serum IGF II. The highest amounts of IGF II-like immunoreactivity occur in the anterior pituitary—namely, 20–25 pmol equivalents/g of wet weight. This is up to 100 times more than in most other brain regions analyzed. The higher molecular mass immunoreactive species were partially characterized. After immunoaffinity purification, the 38- and 26-kDa species are active in a bioassay. Specific IGF-binding protein activity could be shown after purification of the 38- and 26-kDa species on an IGF-affinity column. The 13-kDa species released significant amounts of 7.5-kDa material. The results are interpreted as evidence for the presence of IGF II synthesized locally in human brain. The structure of the larger forms of IGF II-like immunoreactive material as well as the function of IGF II in brain are not yet known.**

Insulin-like growth factors (IGFs) I and II are peptides that contribute to the mitogenic properties of serum. Their amino acid sequences are homologous to the one of proinsulin (1, 2). The plasma concentration of IGF I (150–200 ng/ml) in adult man shows a marked growth hormone (GH) dependence (3), being elevated in acromegaly and decreased in hypopituitarism (3, 4). Furthermore, IGF I has been shown to induce skeletal growth in hypophysectomized rats (5). IGF I is identical with somatomedin C (6) and is now generally considered to be the main somatomedin in adolescent man—i.e., the growth factor under control of and mediating the effects of pituitary GH (7).

IGF II, on the other hand, affects skeletal growth only very little in the experimental model of the hypophysectomized rat (8). This is compatible with the poor correlation between the serum level of GH and IGF II in adult man (600–800 ng/ml) (9). These observations suggest that IGFs I and II have different biological functions. In fact, separate receptors for IGFs I and II have been described (10) and characterized (11). Some evidence in experimental animals points to a role for IGF II as a fetal growth factor that is replaced by IGF I around the time of birth (12). However, in man IGF II persists into adult life at a level 4 times higher than that of IGF I. Therefore, the proper function of IGF II in man remains a puzzle. In a previous communication (13) we reported that human spinal fluid contains immunoreactive 7.5-kDa and "big" IGF II (50–60 ng/ml) but no significant amounts of immunoreactive IGF I. In the work reported here, we investigated the presence and distribution of IGF II in human brain.

MATERIALS AND METHODS

Serum IGF Preparations. IGFs I and II were prepared from human serum as described (14). Preparations I/4 (IGF I) and 9 SE IV (IGF II) were used. IGFs I and II were labeled with ^{125}I by the chloramine-T method (15).

Preparation of Tissue Extracts. Human brains (3–6 hr postmortem) were dissected into specific regions (weight 1–3 g) and stored frozen (−80°C). The tissue was cut into small pieces (3–5 mm^3) while still lightly frozen and homogenized in ice in 2 vol of 1 M acetic acid/0.1% Triton X-100. This homogenate was sonicated twice for 5 s. A 100,000 × *g* supernatant was prepared and chromatographed on a column of Bio-Gel P-100 (150 × 1.5 cm) in 0.5 M acetic acid. Human liver tissue (4.2 g) was extracted by using the method described above. The liver had been perfused with 0.9% NaCl before storage in liquid nitrogen.

Immunoaffinity Purification. Lyophilized fractions were redissolved in 0.1 M sodium phosphate (pH 7.2) and applied to a Sepharose column to which either polyclonal antibodies to IGF II (15) or monoclonal antibody no. 43 (16) had been coupled. The latter antibody recognizes an epitope common to both IGFs I and II. Unbound proteins were washed off with 5 mM sodium phosphate, and adsorbed ones were washed with 1 M acetic acid.

Purification on IGF-Affinity Column. IGF I (1.1 mg) or IGF II (1 mg) was coupled to CNBr-activated Sepharose (Pharmacia). Samples to be applied were dissolved in 0.1 M sodium phosphate buffer (pH 7.4) and eluted with 1 M acetic acid.

Preparation of Synaptosomes. Motor cortex (11 g; 5.7 hr postmortem) was homogenized in 9 vol of 0.32 M sucrose/10 mM Tris buffer, pH 7.4, and processed as described by Guroff (17). The crude nuclear pellet and the final preparation of synaptosomes were extracted with 1 M acetic acid and an aliquot was chromatographed on Bio-Gel P-100 as described above.

Assays. Aliquots were assayed by RIA for IGF I or II by using polyclonal antibodies according to Zapf *et al.* (15) or by using monoclonal antibodies with antibody no. 73 for IGF II (18). Protein was estimated by using Coomassie blue (19).

For bioassays, aliquots were lyophilized after chromatography on Bio-Gel P-100 and determined in the fat cell assay (20). Pure IGF II served as a standard.

For binding protein assays (9), fractions (0.1 ml in 0.1 M sodium phosphate buffer, pH 7.4) were incubated at 4°C overnight with ^{125}I-labeled IGF II (5×10^4 cpm) in the pres-

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Abbreviations: IGF, insulin-like growth factor; GH, growth hormone.

ence or absence of an excess of unlabeled IGF. A solution of polyethylene glycol (1.5 ml, 30% containing 0.5 mg of gamma globulin per ml) was added. The mixture was centrifuged for 30 min at 1600 × *g* at 4°C. The supernatant was removed and radioactivity in the pellets was counted in a γ-counter. Specific binding was calculated by subtracting the cpm in the sample incubated with an excess of unlabeled IGF from the total cpm.

RESULTS

Pattern of Immunoreactivity After Bio-Gel P-100 Chromatography. In most extracts of different regions from brain two immunoreactive peaks were found, one of high molecular mass and one corresponding to the size of IGF (Fig. 1*A*). Pituitary or thalamic extracts had large amounts of additional high molecular mass immunoreactive peaks numbered I–IV, as illustrated in Fig. 1*C*. The apparent molecular masses of peaks I–IV in Fig. 1*C* were 38, 26, 13, and 9 kDa, respectively.

Amounts of Immunoreactive IGF II in Various Regions. The amounts of 7.5-kDa IGF II (Fig. 1*C*, peak V) found in different regions of the brain are listed in Table 1. By far, the highest amounts occurred in the anterior pituitary—namely, 146–190 ng/g of tissue (20–25 pmol/g). In most other regions the amounts varied between 0.2 and 1.0 pmol/g. In some regions of brain no 7.5-kDa IGF could be detected—notably in the hypothalamus. However, large amounts of the higher molecular mass immunoreactivity were found in the hypothalamus and the entire thalamic region. As will be discussed below, these higher molecular mass components could not be quantitated in the RIA because of nonparallel displacement curves (see Fig. 2*C*).

Since the liver is generally accepted as the major site of synthesis of IGF I (21), for comparison we determined IGF levels in perfused human liver by the same extraction procedure. One gram of liver tissue contained 22 ng equivalents of IGF I and 80.3 ng equivalents of IGF II. Hepatic IGF II was eluted from the Bio-Gel P-100 column at the same volume as serum IGF II. In liver, no higher molecular mass peaks were observed.

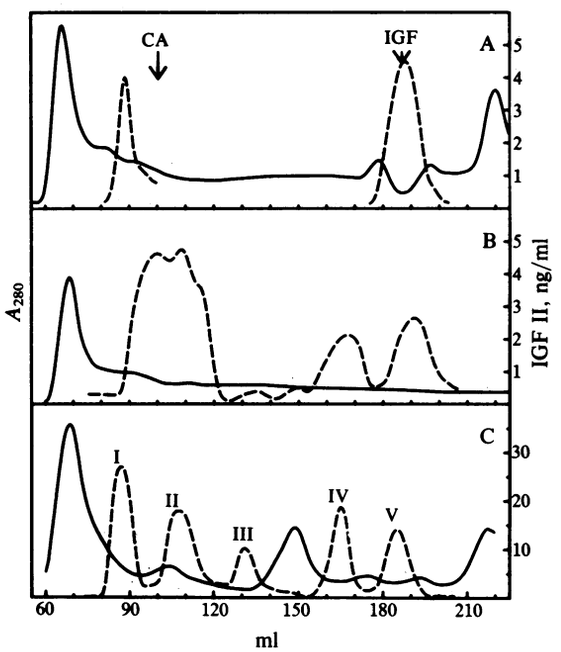

FIG. 1. Chromatography of acidic tissue extracts on Bio-Gel P-100. Solid line, absorbance at 280 nm; dashed line, ng equivalents in RIA for IGF II. The arrows point to the positions where carbonic anhydrase (CA) and IGF elute. (*A*) Chromatography of 100,000 × *g* supernatant prepared from 3.4 g of frontal cortex. (*B*) Acidic extract from synaptosomal preparation. (*C*) Chromatography of 100,000 × *g* supernatant prepared from anterior pituitary. Peak V elutes at the position of purified IGF.

Table 1. Distribution of immunoreactive IGF II in various regions of the human brain

Brain region	High molecular mass immunoreactivity in peak I	7.5-kDa IGF II, ng equivalents/ g of wet weight	Number of brains
Cortex	+	1–13*	1
Hippocampus	+	11	1
Forebrain nuclei	+	4–7†	1
Fornix	++	<1	1
Thalamus			
Anterior	++	8/15	2
Medial	++	6	1
Pulvinar	++	7/26	2
Hypothalamus	++	<1	2
Mammillary bodies	++	<1/2	2
Pituitary			
Anterior	++	146/190	5
Posterior	++	39/14	5
Periaqueductal gray	++	15/23	2
Substantia nigra	+	<1	4
Nucleus ruber	+	<1	4
Pons	−	2	1
Cerebellum	+	3/4	2

The amounts expressed as ng equivalents were determined by RIA for IGF II after Bio-Gel P-100 chromatography of tissue extracts. The numbers separated by slashes indicate the amounts from different experiments (separate extractions of different brains; 3–6 hr postmortem). The amounts of high molecular mass immunoreactivity are semiquantitative only due to the nonparallel displacement curves. +, <10 RIA ng equivalents; ++, >10 RIA ng equivalents; −, below the level of detection (<1 ng).

*The range of ng equivalents extracted from five distinct cortical regions (frontal and temporal lobes, primary motor and primary sensory areas, and striate area).

†The range of values found in separate extracts from putamen, globus pallidus, caudate nucleus, and amygdaloid nuclear complex.

Characterization of the 7.5-kDa Immunoreactive Peak. The lowest molecular mass immunoreactive peak (peak V, Fig. 1*C*) was eluted at the position of 7.5-kDa IGF purified from serum. Fig. 2*A* compares the competitive inhibition of binding of labeled IGF II to polyclonal antibodies with pure IGF from serum and with IGF purified from brain extracts. IGFs from the two sources gave parallel displacement curves. When the same fractions from peak V were analyzed by RIA for IGF I (Fig. 2*B*), no displacement of labeled IGF I occurred up to 30 ng equivalents of brain IGF. A comparison between serum IGF II and brain IGF II in RIA with polyclonal and monoclonal antibodies against IGF II (no. 73) revealed no difference among them. The binding constants of brain IGF II and serum IGF II for the monoclonal antibody no. 73 were in the same order of magnitude.

Subsequently peak V was further purified by immunoaffinity chromatography (Fig. 3*A*). When applied to an affinity Sepharose column in neutral buffer at pH 7.2, 80–85% of the immunoreactive material was bound and could be eluted with 1 M acetic acid. The result was the same irrespective of whether monoclonal (no. 43) or polyclonal antibodies to IGF II had been coupled to the column.

The bioactivity of the 7.5-kDa fraction obtained after immunoaffinity chromatography was tested in the rat fat cell assay. There was the same correlation of immunological to biological activity as with serum IGF II: 150 ng equivalents of brain IGF as determined by RIA had the same biological activity as 157 ng of our standard IGF II preparation.

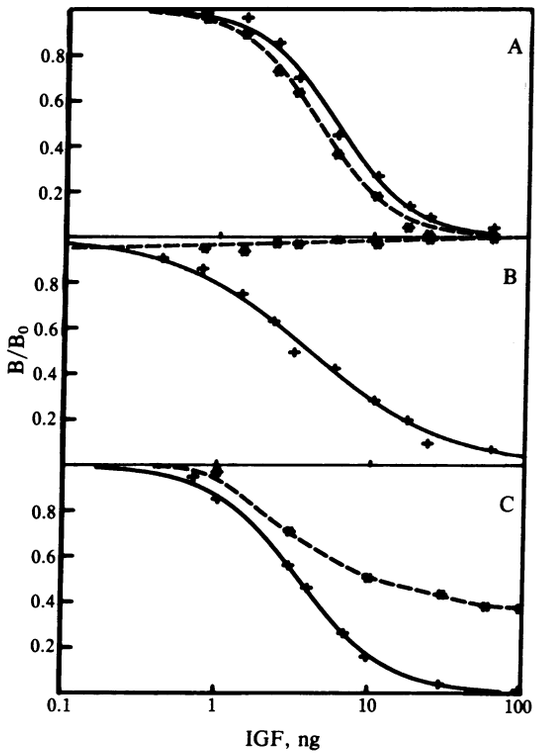

FIG. 2. Competitive inhibition of binding of ^{125}I-labeled IGF I or II by immunoreactive peaks. B/B_0, % bound (B) to free (B_0). (*A*) Dashed line, RIA for IGF II with purified human IGF II from serum; solid line, 0.1–100 ng RIA ng equivalents of peak V (Fig. 1*C*) assayed by RIA for IGF II. (*B*) Solid line, RIA for IGF I with purified IGF I from serum; dashed line, 0.1–100 RIA ng equivalents of peak V (Fig. 1*C*) assayed by RIA for IGF I. (*C*) Solid line, RIA for IGF II with purified IGF II; dashed line, 0.1–100 ng equivalents of peak I (Fig. 1*C*) assayed by RIA for IGF II.

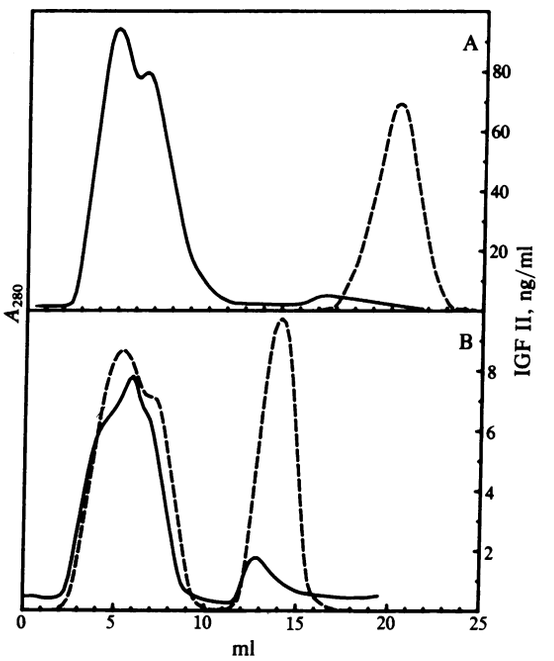

FIG. 3. Immunoaffinity chromatography of immunoreactive peaks after Bio-Gel P-100 chromatography. Lyophilized preparations of peaks I and V were suspended in 0.1 M sodium phosphate and chromatographed on an immunoaffinity column to which monoclonal antibody no. 43 had been coupled. Solid line, absorbance at 280 nm; dashed line, ng equivalents in RIA for IGF II. At 5 ml of elution volume, 5 mM sodium phosphate (pH 7.2) was added to wash off unadsorbed proteins. (*A*) Peak V of Fig. 1*C*. Acetic acid (1 M) was added at 13 ml. (*B*) Peak I of Fig. 1*C*. Acetic acid was added at 10 ml of elution volume.

Immunoreactive IGF II was also extracted from a cortical synaptosomal preparation. The final preparation of synaptosomes and the crude nuclear pellet were extracted with 1 M acetic acid and chromatographed on Bio-Gel P-100 (Fig. 1*B*). In the case of the synaptosomal extract there was a broad immunoreactive peak in the high molecular mass range. No immunoreactive material could be extracted from the crude nuclear pellet. There was 8–10 times more 7.5-kDa IGF II in the synaptosomal preparation per mg of protein than in the crude extract from motor cortex.

Some Properties of the Higher Molecular Mass Immunoreactive Peaks. Since the higher molecular mass peaks (peaks I–IV, Fig. 1*C*) were positive in RIA for IGF II, we also chromatographed them on the immunoaffinity column. The elution pattern of peak I is shown in Fig. 3*B*. Roughly one-third of the immunoreactive material bound to the affinity matrix and could be eluted with 1 M acetic acid.

When the higher molecular mass immunoreactive peak I or II obtained after immunoaffinity purification was assayed at several dilutions in the fat cell assay, the results were also positive. However, the ratio to the immunological activity could not be calculated due to the nonparallel displacement curves of these proteins in the RIA (Fig. 2*C*). Therefore, we can only roughly quantify these proteins (Table 1). In contrast to the 7.5-kDa IGF II (peak V) that did not crossreact in the RIA for IGF I, there was 15–25% crossreactivity of the immunoaffinity-purified fractions of peaks I and II in the RIA for IGF I. We tested the possibility that the 7.5-kDa IGF II could be released from either of the higher molecular mass immunoreactive peaks. After acid chromatography, pools of peaks I–III were prepared, lyophilized, and rechromatographed on the Bio-Gel P-100 column. Neither of peaks I and II released more than a few percent of the 7.5-kDa IGF II. Nearly the entire immunoreactivity of peak III, however, shifted to the elution volume of the 7.5-kDa IGF II. Upon immunoaffinity chromatography (monoclonal antibody no. 43), 30–40% of this immunoreactivity was bound and could be eluted with 1 M acetic acid. The flow-through material was subsequently analyzed for the presence of binding protein by incubating aliquots with labeled IGF II in the presence or absence of unlabeled IGF. A linear correlation between specific binding and amounts of protein was obtained. The specificity of this binding is shown in Fig. 4, which illustrates the elution pattern after chromatography under neutral conditions. Aliquots were incubated with ^{125}I-labeled IGF II in the presence or absence of an excess of unlabeled tracer and chromatographed on Sephadex G-100. A total displacement of the labeled tracer occurred in the sample containing the unlabeled IGF (dashed line). The other peak containing the bound ^{125}I-labeled IGF II eluted with an estimated size of 22–28 kDa. The lower value of 22 kDa was obtained by autoradiography after $NaDodSO_4$ gel electrophoresis and the higher one was obtained by relating its chromatographic behavior on Sephadex G-100 to that of marker proteins.

Another approach to test the higher molecular mass immunoreactive peaks for the presence of binding protein involved chromatography on an IGF-Sepharose affinity column to which equal amounts of IGF I or II had been coupled. There was no significant leakage of the coupled IGF (<2 ng/ml) when the column was rinsed with phosphate buffer or 1 M acetic acid. However, when peaks I–III were chromatographed under the same conditions, there was a pronounced overrecovery of immunoreactive material in the flow-through material. This is clearly due to release of affinity-bound IGF from the column. Inclusion of aprotinin (2.8

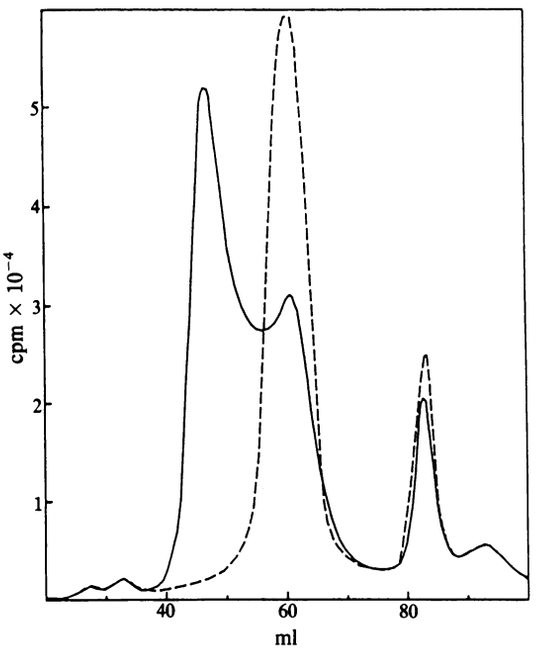

FIG. 4. Chromatography of fraction containing ^{125}I-labeled IGF II binding protein in the presence of an excess of unlabeled IGF. Peak III was pooled from several preparations and rechromatographed on Bio-Gel P-100. The immunoreactivity then eluting at the position of 7.5-kDa IGF was purified by immunoaffinity chromatography (monoclonal antibody no. 43). The material not binding to the coupled antibodies contained binding protein activity and was used for the experiment illustrated here. One of two aliquots (0.5 ml) was incubated for 1 hr with 3 μg of a partially purified preparation of IGF. Both samples were then incubated with labeled IGF II (5×10^5 cpm) for 20 hr at 4°C. Chromatography was carried out on Sephadex G-100 (150 × 0.9 cm) in 10 mM sodium phosphate (pH 7.2) and the radioactivity in each fraction (1 ml) was determined. The sample incubated with an excess of unlabeled IGF is represented by the dashed line; the other one is indicated by a solid line. The peak at 83 ml corresponds to ^{125}I; the one at 60 ml corresponds to ^{125}I-labeled IGF II. Under these conditions, carbonic anhydrase elutes at 45 ml of elution volume.

mg/ml) in the buffer was without effect, whereas phenylmethylsulfonyl fluoride (100 μM) reduced the amount of IGF released to ≈50%. Only the bound and acid-released fractions of peaks I–III were therefore analyzed in a binding protein assay. Serial dilutions of the affinity-chromatographed peaks I–III showed a linear relationship between specific binding of labeled IGF II and amounts analyzed.

DISCUSSION

Our studies demonstrate the presence of IGF II-like immunoreactivity in human brain. Since this organ cannot be perfused prior to extraction we have to consider the possibility that the measured IGF II was contributed by the vascular bed of the brain. The following findings argue against this possibility. (*i*) We find no IGF I that would have been coextracted. Though the serum IGF II is approximately 3–4 times higher than IGF I, the amounts of IGF I would still have been large enough to be measured. If as little as 5% of the total brain IGF were IGF I, we would have detected it by the RIA for IGF I. This has not been the case (Fig. 2*B*). Therefore, we conclude that only IGF II occurs in brain, as we have shown for cerebrospinal fluid (13). (*ii*) If the measured IGF II were derived from the vascular system, one would expect roughly equal amounts of IGF II in the anterior and posterior pituitary (Table 1). We have found a 4-fold difference. (*iii*) IGF II and higher molecular mass forms could be extracted from a synaptosomal preparation (Fig. 1*B*), implying granular storage of at least part of the IGF II. Preliminary immunofluorescent results in sections of monkey cerebral cortex showed a granular fluorescence in a small subpopulation of cortical neurons (unpublished observations). This is further evidence for the neuronal localization of the IGF immunoreactive material and its enrichment in synapses. (*iv*) In a clinical case of megalencephaly reported elsewhere (22), IGF II levels in frontal cortex of a 3-month-old child were roughly 10 times higher than those we report here for adults. However, the IGF II levels in plasma of that particular patient (187 ng/ml) were in the lower normal range for this age group. Therefore, no direct correlation seems to exist between the levels of serum and of brain IGF II. In conclusion, we believe that the amounts of IGF II measured in human brain cannot be accounted for by plasma IGF extracted from the vascular bed.

While the work described here was in progress, two papers on IGF in brain of experimental animals were published. Binoux *et al.* (23) reported the release of IGF and of carrier (binding) protein from rat brain explants in culture, whereas Sara *et al.* (24), using a radioreceptor assay, found a wide distribution of somatomedins in all regions of the central nervous system in the cat. However, neither assay method could discriminate between IGFs I and II, and no higher molecular mass forms were detected.

Our data show five size classes of IGF II immunoreactivity in brain. The smallest component (7.5 kDa) is indistinguishable from IGF II purified from serum (14) in its apparent size and its immunological and biological activity. However, only the determination of its primary structure by amino acid or nucleotide sequencing would establish or rule out its identity.

The highest amounts of the 7.5-kDa IGF II are found in the anterior pituitary, where there is 6 times more than in any other region and 50–100 times more than in pons or cerebellum. The values measured for the posterior part of the pituitary may be too high because of some contamination with anterior pituitary.

A comparison of the amount of IGF II extracted from liver to that from anterior pituitary shows that they are in the same range. In contrast to brain, the human liver had been perfused prior to extraction. The relatively high levels of 7.5-kDa IGF II in the anterior pituitary raise the question of whether the pituitary serves as a site of synthesis or storage (or both) of IGF II. The pituitary itself may be one of the functionally important target sites. In two recent reports on receptors for IGF in pituitaries, specific binding of IGF II was found to be greater than that of IGF I (25, 26). However, the postulated role for both IGFs I and II as stimulators of somatostatin release and inhibitors of GH release is difficult to reconcile with the poor correlation of serum concentrations of IGF II to those of GH (8). The GH-dependent IGF I would be a better candidate as a regulator of GH release. We were not able to demonstrate any significant amounts of IGF I in the pituitary but did demonstrate appreciable quantities of IGF II, including higher molecular mass forms tentatively interpreted as precursors. Therefore, we favor the idea that the main function of pituitary IGF II is not the regulation of GH release but rather some other trophic or regulatory effect. The pronounced variation in amounts of IGF II in different regions of the brain as well as the above-mentioned case of megalencephaly with severalfold higher amounts of IGF II also tend to point to a growth-regulatory function of IGF II in brain.

Part of the immunoreactive material from Bio-Gel P-100 peaks I–III could be bound to the matrix of an IGF-Sepharose column. Analysis of the material in the flow through is complicated by the fact that IGF is released from the matrix, presumably due to the presence of a protease. The affinity-

purified material was active in a competitive protein binding assay. This is evidence for the presence of a binding protein for IGF. At present we feel unable to state whether the described immunoreactivity, bioactivity, and binding protein activity are properties of the same molecule. IGF-like immuno- and bioactivities of peaks I and II are not released from the binding moiety as 7.5-kDa components, despite the acidic conditions of the extraction and gel permeation chromatography. In contrast, the immunoreactivity of peak III shifted to the elution volume of peak V after rechromatography. By means of immunoaffinity and IGF-Sepharose affinity chromatography this material could clearly be separated into an IGF II and a specific IGF II-binding protein fraction. The molecular mass of the complex of IGF-binding protein with IGF is estimated to range between 22 and 28 kDa. The stoichiometry and significance, however, remain to be elucidated. Thus, peaks I–III either contain an IGF-like substance and a binding protein not dissociable under these conditions or they contain a covalently linked molecule consisting of an IGF-like domain and a binding domain. If the latter possibility proves to be correct, the higher molecular mass peaks might represent a precursor of brain IGF II and of brain binding protein in analogy to other hormone precursors, such as the one for ocytocin-neurophysin I (27). Furthermore, peaks II–IV could contain consecutive stages of processing from precursor to product.

Several laboratories have described higher molecular weight forms of IGF. A precursor of rat IGF II of molecular weight 21,600 has been found as a cell-free translation product of mRNA isolated from a rat liver cell line (28). Somatomedin activity recovered from rat liver extracts has been described as a molecular weight 30,000 protein (29). The sequence of a human liver cDNA for IGF I codes for a molecule consisting of at least 156 amino acids (30, 31).

More recently, and after completion of the present studies, two reports have appeared on the nucleotide sequence of a cDNA clone encoding human prepro-IGF II and of the IGF II chromosomal gene (32, 33). The deduced preprohormone contains 180 amino acids and has a molecular weight of 20,100. The 89 amino acid COOH-terminal extension of pro-IGF II contains several potential cleavage sites for processing. In fact, we have found recently in human serum an intermediate between pro-IGF II and IGF II having a COOH-terminal extension of 20 amino acid residues (34). This intermediate form probably corresponds to the big IGF II found in cerebrospinal fluid (13).

Based on our assumption of a precursor–product relationship, the predominance of higher molecular mass forms in hypothalamus and thalamus may point to these regions as sites of biosynthesis. After local synthesis IGF precursors might be packaged into granules, processed, and transported to their final destination. These issues, particularly the structural relationship of the precursor forms to the 7.5-kDa IGF II and the mechanism of conversion, await further investigation. The intriguing question of the function of brain IGF as well as its relationship to the high concentration of IGF II in human plasma remains open.

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