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Insulin-like growth factor (IGF)-I binding to a cell membrane associated IGF binding protein-3 acid-labile subunit complex in human anterior pituitary gland

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Abstract

The binding characteristics of [^{125}I]insulin-like growth factor (IGF)-I were studied in human brain and pituitary gland. Competition binding studies with DES(1-3)IGF-I and R^3-IGF-I, which display high affinity for the IGF-I receptor and low affinity for IGF binding proteins (IGFBPs), were performed to distinguish [^{125}I]IGF-I binding to IGF-I receptors and IGFBPs. Specific [^{125}I]IGF-I binding in brain regions and the posterior pituitary was completely displaced by DES(1-3)IGF-I and R^3-IGF-I, indicating binding to IGF-I receptors. In contrast, [^{125}I]IGF-I binding in the anterior pituitary was not displaced by DES(1-3)IGF-I and R^3-IGF-I, suggesting binding to an IGF-binding site that is different from the IGF-I receptor. Binding affinity of IGF-I to this site was about 10-fold lower than for the IGF-I receptor. Using western immunoblotting we were also unable to detect IGF-I receptors in human anterior pituitary. Instead, western immunoblotting and immunoprecipitation experiments showed a 150-kDa IGFBP-3-acid labile subunit (ALS) complex in the anterior pituitary and not in the posterior pituitary and other brain regions. RT-PCR experiments showed the expression of ALS mRNA in human anterior pituitary indicating that the anterior pituitary synthesizes ALS. In the brain regions and posterior pituitary, IGFBP-3 was easily washed away during pre-incubation procedures as used in the [^{125}I]IGF-I binding experiments. In contrast, the IGFBP-3 complex in the anterior pituitary could not be removed by these washing procedures. Our results indicate that the human anterior pituitary contains a not previously described tightly cell membrane-bound 150-kDa IGFBP-3–ALS complex that is absent in brain and posterior pituitary.

Keywords: acid labile subunit, human brain, IGFBP-3, insulin-like growth factor binding proteins, insulin-like growth factor I, pituitary gland.

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The insulin-like growth factor (IGF) system comprises two IGFs (IGF-I and IGF-II), IGF-I and IGF-II receptors, and at least six distinct insulin-like growth factor binding proteins (IGFBPs) (Spagnoli and Rosenfeld 1997). In the central nervous system, IGF-I and IGF-II support the growth and differentiation of neurons and glial cells (Baskin *et al*. 1988; D'Ercole *et al*. 1996). The distribution of IGF-I and IGF-II receptors have been studied in both rodent and human brains (Bohannon *et al*. 1988; Lesniak *et al*. 1988; Kar *et al*. 1993; De Keyser *et al*. 1994; Wilczak *et al*. 2000). The IGF-I receptor is a heterotetrameric glycoprotein composed of two α and two β subunits linked by disulphide bounds (D'Ercole *et al*. 1996). The IGF-II receptor is a monomeric receptor with a striking extracellular domain made up almost exclusively of 15 cysteine-based repeats, and is identical to the cation-independent mannose-6-phosphate receptor (Morgan *et al*. 1987; Kornfeld 1992). IGFBPs play a crucial role

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Abbreviations used: ALS, acid-labile subunit; BSA, bovine serum albumin; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IGFBP-rP, insulin-like growth factor binding protein-related proteins; HRP, horseradish peroxidase; NaDOC, sodium deoxycholic acid; NP-40, non-idet P-40; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

in transporting IGFs in the circulation, cerebrospinal fluid, and across the capillary barrier to the target cells (Baxter 1994; Rosenfeld *et al*. 1999). IGFBP-3 and IGFBP-5, in addition to binding IGFs, also can associate with an acid-labile subunit (ALS), thereby further increasing the half-life of the IGFs (Twigg and Baxter 1998; Twigg *et al*. 1998). IGFBPs are also present at the level of the extracellular matrix or cell surface, where they can either enhance or inhibit the presentation of IGFs to their receptors (Spagnoli and Rosenfeld 1997; Clemmons 1998; Rechler and Clemmons 1998; Hwa *et al*. 1999). In addition, some of the IGFBPs, such as IGFBP-3, are also capable of mediating biological actions that are IGF independent (Clemmons 1998; Spagnoli and Rosenfeld 1997; Rechler and Clemmons 1998). More recently, a family of IGFBP-related proteins (IGFBP-rPs) has been identified that appear to have a 100-fold lower affinity for IGFs compared with IGFBPs. At present, the functional roles of these IGFBP-rPs remains elusive (Hwa *et al*. 1999). This study was undertaken to characterize the cell membrane-bound binding sites for IGF-I in human brain and pituitary gland.

Experimental procedures

Materials

Human recombinant [^{125}I]IGF-I was obtained from Du Pont de Nemours (Dreieich, Germany). [^{125}I] standards and [^{3}H] ultra films were obtained from Amersham Life Sciences (Amersham, Buckinghamshire, UK). IGF-I, Des1-3IGF-I and R^{3}-IGF-I were purchased from Gropep (Thebarton, Adelaide, Australia). Des1-3IGF-I is truncated IGF-I, missing the first three amino acids in the B domain, and R^{3}-IGF-I is an IGF-I analogue where the glutamate at position 3 has been replaced by arginine.

Recombinant human IGFBP-3 and the monoclonal antibody against the α-chain of human IGF-I receptor were purchased from Upstate (Lake Placid, NY, USA). Biotinylated anti-chicken IgG was obtained from Immuno Jackson Chemicals (Baltimore, MD, USA). The polyclonal antibody against human IGFBP-3 was obtained from Gropep (Thebarton, Adelaide, Australia). The polyclonal antibody against human ALS was purchased from DSL (Webster, TX, USA). Horseradish peroxidase (HRP)-streptavidin and HRP-conjugated goat-anti-rabbit were obtained from Bio-Rad (San Francisco, CA, USA). Normal rabbit serum was purchased from Zymed (San Francisco, CA, USA).

The human hepatoma cell line HepG2 was a gift from D. Hoekstra (Rijksuniversiteit Groningen, Groningen, the Netherlands). All cell culture plastic ware was obtained from Costar (Cambridge, MA, UKA). Dulbecco's modified Eagle's medium (DMEM) medium, antibiotics, all reagents for reverse transcription and all primers were from Life Technologies (Paisley, UK). Fetal calf serum was purchased from Bodinco (Alkmaar, the Netherlands). The High Fidelity PCR Master kit was obtained from Roche (Indianapolis, IN, USA) and the RNeasy® Mini Kit from Qiagen (Valencia, CA, USA). All other chemicals were of the highest grade commercially available.

Specificity of the antibodies

The antibody against human IGF-I receptor reacts specifically against a 19-residue synthetic peptide corresponding to amino acid residues 642–659 of the human IGF-I receptor α-subunit (Rosenweig *et al*. 1990). The polyclonal IGFBP-3 antibody used in this study was obtained from rabbits immunized with a synthetic peptide of unique sequence from the central domain of human IGFBP-3. The specificity of the antibody was confirmed using the antibody preabsorbed with excess of matched recombinant human IGFBP-3. Specificity of the immunoreactivity was also controlled by the incubation of tissue sections in 5% goat serum instead of primary antibodies; the immunohistochemical reactions were negative. For western blotting experiments, non-specific staining was determined by incubating parallel blots with non-immune rabbit serum instead of the primary antibody solution (Fig. 4b).

For immunoprecipitation experiments, non-specific binding was determined by incubating parallel blots with 3% bovine serum albumin (BSA) instead of the primary antibody solution. The non-specific binding of the anterior pituitary is shown in Fig. 5. The non-specific bands for serum and other brain samples were the same (not shown).

Tissues

Brains and pituitary glands were obtained from 10 patients without neurological or psychiatric diseases (five men and five women aged between 54 and 78 years). Pituitary glands and 0.5-cm thick blocks of brain tissue were dissected at 0–4°C, frozen rapidly by immersion in isopenthane–dry-ice, and stored at −80°C until used. Frontal cortex and white matter was obtained from the frontal gyri and cerebellum from one of the hemispheres. Care was taken to dissect comparable regions from the different individuals. The choroid plexus was obtained from the lateral ventricles. Post-mortem delay, defined as the time elapsed between death and freezing of the tissue sections, ranged between 7 and 14 h. An ethical committee approved the experiments on post-mortem brain.

[^{125}I]IGF-I binding studies

Frozen tissue blocks were mounted on a cryostat chuck, coated with embedding medium (OCT compound, Laboratory-Tek Products, Ontario, Canada) and serial sections, 10-μm thick, were cut at −20°C using a microtome-cryostat. Thereafter, sections were mounted on gelatine-coated glass slides, and dried overnight under vacuum. Sections were pre-incubated for 20 min at 20°C in 25 mM Tris/HCl (pH 7.4), containing 10 mM $MgCl_2$, and 0.1% BSA. The optimal conditions for the binding experiments have been described previously (De Keyser *et al*. 1994).

For competition experiments sections were incubated for 90 min with 0.1 nM [^{125}I]IGF-I and increasing concentrations of competitors IGF-I, DES(1-3)IGF-I, or R3-IGF-I. After incubation, the sections were washed three times for 1 min in the same buffer, to remove unbound ligand, and were then wiped from the slides using Whatman GF/B glass fibre filters. The level of radioactivity was determined in a gamma-counter. Binding isotherms were estimated by non-linear least-square curve fitting. The inhibition constants (K_i values) of IGF-I, DES(1-3)IGF-I and R^{3}-IGF-I were calculated by the method of Cheng and Prusoff (1973).

Autoradiographs were produced by incubating sections for 90 min at 20°C with 0.1 nM [^{125}I]IGF-I in the same buffer.

Incubating consecutive sections with 0.5 μM IGF-I, or 0.5 μM DES(1-3)IGF-I, or 0.5 μM R^3-IGF-I assessed non-specific binding. After incubation the sections were washed three times for 1 min in the same buffer and quickly dipped in distilled water. The sections were dried under a stream of cold air, placed in X-ray cassettes together with commercially available [^{125}I]-standards, and exposed to [^{3}H] ultra films for 4 days. The films were developed with a Kodak D19 developer at 4°C, and scanned with an Arcus plus scanner. The images were digitized and analysed by computer-assisted densitometry using NIH IMAGE analysis software. A calibration curve was generated by fitting of optical density and disintegrations per minute per milligram polymer values of the [^{125}I] standards. The regions of interest were sampled, and mean optical densities determined and converted into fmol/mg protein based on the experimentally determined relation between polymer and brain paste standards. Specific binding values were obtained by the subtraction of non-specific binding images from corresponding total binding images. All binding experiments were done in duplicate and repeated twice.

Preparation of homogenates

Frozen tissue blocks of approximately 1 g were homogenized in 10 mL ice-cold Tris/HCl buffer (25 mM, pH 7.4) containing serine-, cysteine-, aspartic- and metalloproteinase inhibitors, with an Ultraturrax and Potter Elvehjem homogenizer, and centrifuged at 50 000 *g* for 15 min. The pellets, containing the cell membranes, were resuspended in 10 mL of the same buffer as indicated above and centrifuged twice at 50 000 *g* for 15 min. The final pellet was suspended in 1 mL of the same buffer containing 10% glycerol (v/v), and stored at − 80°C. Protein concentrations were determined by the method of Lowry *et al*. (1951). To be sure that there is no blood contamination from the circulation, albumin was measured in homogenates from the anterior and posterior pituitary gland by using an albumin immune assay (BN2, Behring, Marburg, Germany). In all the samples we used in this study, the albumin fraction was not detectable.

Western immunoblotting and immunoprecipitation

For western immunoblotting experiments the homogenates were diluted and resolved in sample buffer, and ran both under native conditions [no sodium dodecyl sulphate (SDS), no mercaptoethanol and no boiling], and reducing conditions (0.25% mercaptoethanol, 0.5% SDS and boiling during 6 min). Human IGFBP-3 was used as a positive control and diluted in sample buffer containing 0.1% (w/v) SDS. Sixty micrograms of sample protein and 10 μL (0.001 μg/μL) human IGFBP-3 protein were loaded on the gel system and ran on 4–12% SDS–polyacrylamide gel electrophoresis (PAGE).

For immunoprecipitation experiments 50 μL of the homogenates were rehomogenized in 200 μL ice-cold 50 mM Tris-buffer containing 6.25 mM EDTA, 1% Triton-X-100 (pH 7.4) and the same protease inhibitors as indicated above. Homogenates were centrifuged at 3000 *g* for 10 min. Equal amounts of the supernatant (200 μL) were diluted in 200 μL detergent buffer [50 mM EDTA, 50 mM Tris pH 7.4, 0.2% sodium deoxycholic acid (NaDOC), 0.05% non-idet P-40 (NP-40), 0.5% SDS]. The suspensions (400 μL), 100 μL human serum (positive control for ALS) and 10 μL human IGFBP-3 (0.001 μg/μL) were then incubated with 5 μg polyclonal human IGFBP-3 antibody for 24 h at 4°C. The immunocomplexes were precipitated with agarose-G-beads at 4°C for 2 h. After washing, 3 times with low-salt washing buffer (50 mM Tris-HCl, 0.05% NaDOC, 0.1% NP40, pH 7.4) and twice with phosphate-buffered saline (PBS), the precipitated samples were resuspended in sample buffer containing 0.1% SDS and boiled for 6 min. Fifty microlitres of the samples were loaded on the gel system and separated under reducing conditions using 4–12% SDS–PAGE.

Proteins were transferred to nitrocellulose membranes and incubated overnight at 4°C with the primary antibody solution [chicken anti-IGF-I receptor (1 : 500) or rabbit anti-IGFBP-3 (1 : 1000) and or rabbit anti-ALS diluted (1 : 1000)] in Tris-buffered saline (TBS, pH 7.4) containing 0.5% milk powder. For the detection of the IGF-I receptor, the membranes were incubated with biotinylated second antibody solution (rabbit anti-chicken 1 : 600) in TBS containing 0,5% milk powder, for 2 h at room temperature. Finally, membranes were incubated with HRP-conjugated streptavidin (1 : 600) for 2 h at room temperature. For the detection of ALS and IGFBP-3, membranes were incubated with HRP-conjugated goat anti-rabbit solution (1 : 1000) in TBS containing 0.5% milk powder, for 2 h at room temperature. The complexes derived from western immunoblotting and immunoprecipitation experiments were visualized using freshly prepared diaminobenzidine (DAB, 30-mg/100 mL Tris buffer, pH 7.4 and 0.015% H_2O_2), and the opti-(4-chloro-1-naphthol) 4 CN staining method of Bio-Rad. Between all steps the nitro-cellulose membrane was rinsed in TBS for 30 min.

Immunohistochemical experiments

Frozen sections, 10-μm thick, were pre-incubated in 25 mM Tris/HCl (pH 7.4) containing 10 mM $MgCl_2$. Sections that were and were not pre-incubated were fixated for 15 min in 3% buffered formaldehyde and washed in PBS for 10 min. To exhaust endogenous peroxidase activity, sections were immersed for 10 min in 0.3% H_2O_2 in PBS prior to the first antibody incubation. Before the addition of first and secondary antibody solution, sections were incubated for 30 min at room temperature in normal goat serum to suppress non-specific antibody binding. Thereafter, sections were incubated in primary antibody solution: rabbit anti-IGFBP-3 (1 : 200) in PBS for 24 h at 4°C. Sections were then incubated with the secondary biotinylated antibody solution: goat anti-rabbit (1 : 200) in PBS for 120 min at room temperature. Finally, sections were incubated in HRP-conjugated streptavidin solution (1 : 200) in PBS for 90 min at room temperature and processed by the diaminobenzidine/H_2O_2 reaction (30 mg DAB/100 mL Tris buffer, pH 7.4) and 0.01% H_2O_2 and they were counterstained with 3% haematoxylin. Between all steps the sections were rinsed thoroughly with PBS.

RT-PCR

HepG2 cells were cultured in DMEM supplemented with 10% fetal calf serum, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Total RNA was isolated from either frozen anterior pituitary tissue (100 mg/sample) or HepG2 cells (10^7 cells/sample) using the RNeasy® Mini Kit and following the manufacturers' instructions. Single-stranded cDNA was synthesized from 5 μg total RNA using 2.5 μg Oligo $(dT)_{12-18}$ primer, 1000 units Superscript™ RT II, 20 μL first strand buffer, 10 mM dithiothreitol (DTT) and 0.5 mM dNTP in a total volume of 100 μL. The RNA sample and the Oligo (dT) primer were denatured

at 65°C for 15 min and placed on ice for 5 min before addition to the reaction tube. Reverse transcription was performed for 1 h at 37°C, and subsequently the samples were heated to 99°C for 5 min to terminate the reaction. PCR was performed with the High Fidelity PCR Master using 5 μL of the obtained cDNA and 7.5 pmol sense and antisense primer, according to the manufacturers' instructions. The final reaction volume was 50 μL. The tubes were incubated in a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, CT, USA) at 94°C for 5 min to denature the cDNA and primers. The cycling programme was 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for actin and 94°C for 30 s, 57°C for 30 s, 72°C for 50 s (7 min in the last cycle) for ALS. For each primer set and sample, an increasing number of PCR cycles (25–40) was performed with otherwise fixed conditions. The following primers were employed: actin: 5′-AACACCCCAGCCATGTAC-3′ (sense) and 5′-ATGTCACGCACGATTTCC-3′ (antisense, 254 bp amplified product) and ALS 5′-CTCAACCTCGGCTGGAATAG-3′ (sense) and 5′-CACAGGCTCTGCTCCTCAAT-3′ (antisense, 770 bp amplified product). In each experiment, water, RNA and cDNA alone, as well as cDNA with only sense or antisense primer, was used as a negative control to check for contamination and specificity. Ten microlitres of PCR products were separated on 2.0% agarose gels, stained with ethidium bromide, and photographed using a Polaroid DS34 Instant Screen Direct Camera (Hertfordshire, UK).

Results

[^{125}I]IGF-I binding

We first studied the IGF-I binding sites in human brain and pituitary gland by using radioligand binding techniques with [^{125}I]IGF-I and autoradiography. Before incubation with [^{125}I]IGF-I tissue slices were pre-incubated in buffer to remove endogenous IGFs and other soluble proteins. On adjacent tissue sections of frontal cortex (Fig. 1), cerebellum, choroid plexus, anterior and posterior pituitary, we performed [^{125}I]IGF-I competition binding experiments with IGF-I, DES(1-3)IGF-I and R^3-IGF-I. Both, DES(1-3)IGF-I and R^3-IGF-I bind to IGF-I receptors but have low affinity for IGFBPs (Ballard *et al*. 1987; Szabo *et al*. 1988; Tomas *et al*. 1991).

In all brain regions and choroid plexus, [^{125}I]IGF-I was equally displaced by IGF-I, DES(1-3)IGF-I, and R^3-IGF-I (Fig. 1). The curves were best fitted to a single-site-binding model. The mean K_i values ± SD were 1.0 ± 0.3 nM for IGF-I, 1.1 ± 0.2 nM for DES(1-3)IGF-I, and 1.2 ± 0.2 nM for R^3-IGF-I [$p = 0.74$ (Mann–Whitney *U*-test)]. In contrast, in the anterior pituitary, there was no displacement of [^{125}I]IGF-I binding with DES(1-3)IGF-I and R^3-IGF-I, and IGF-I had a 10-fold lower affinity [$K_i = 12.7 \pm 1.5$ nM, $p < 0.0001$ (Mann–Whitney *U*-test)] for this binding site than in brain regions and choroid plexus (Fig. 1).

We then compared autoradiographs obtained following incubation with [^{125}I]IGF-I in the absence and presence of 0.5 μM IGF-I, 0.5 μM DES(1-3)IGF-I, or 0.5 μM R^3-IGF-I. As shown in Fig. 2, binding of [^{125}I]IGF-I was equally displaced by all three competitors in cerebellum (not shown), frontal cortex, cerebral white matter, choroid plexus (not shown), and posterior pituitary. In the anterior pituitary (Fig. 2), [^{125}I]IGF-I binding was displaced by IGF-I, but not by DES(1-3)IGF-I or R3-IGF-I, indicating that [^{125}I]IGF-I labelled an IGF-binding site that was different from the IGF-I receptor. Non-specific binding of competition and autoradiographic experiments in the presence of IGF-I varied between 30 and 40% of the total binding. [^{125}I]IGF-I binding characteristics were not different between males and females. IGF-I receptor concentrations are shown in Table 1.

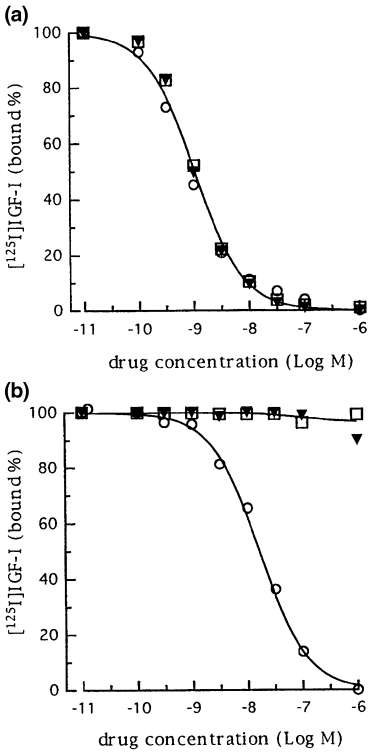

Fig. 1 Representative competition binding curves of IGF-I (○), DES(1-3)IGF-I (▼) and R^3-IGF-I (□) to 0.1 nM [^{125}I]IGF-I on microtome sections of human frontal cortex (a) and anterior pituitary (b). Computer analysis of the binding data revealed that the competition curves were best described by a one-component binding model. In frontal cortex, [^{125}I]IGF-I was equally displaced by IGF-I, DES(1-3) IGF-I and R^3-IGF-I. K_i values were 0.9 nM for IGF-I and DES(1-3) IGF-I, and 1.0 nM for R^3-IGF-I. In the anterior pituitary [^{125}I]IGF-I was displaced by IGF-I (K_i value of 12 nM) but not by DES(1-3)IGF-I and R^3-IGF-I.

IGF-I receptor and IGFBP-3-binding in homogenates from human pituitary and brain regions

To further examine the nature of the IGF-I binding site in the anterior pituitary, we performed western immunoblot analysis and immunoprecipitation using a monoclonal antibody against the human IGF-I receptor, and polyclonal antibodies against human IGFBP-3 and ALS. The antibody against

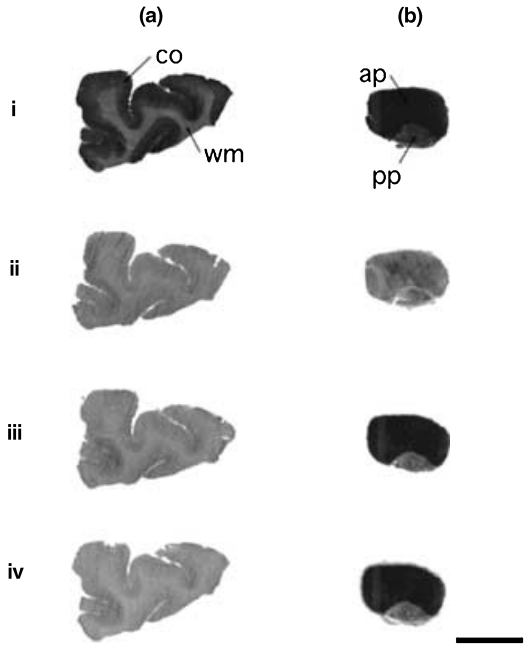

Fig. 2 Autoradiographs of 0.1 nM [^{125}I]IGF-I binding in the absence = total binding (i) and the presence of 0.5 μM unlabelled IGF-I = non-specific binding (ii), or 0.5 μM DES(1-3)IGF-I (iii), or 0.5 μM R^3-IGF-I (iv) to microscope slide-mounted sections of frontal cortex (a) and pituitary gland (b). In frontal cortex (co), white matter (wm), and posterior pituitary (pp) [^{125}I]IGF-I was equally displaced with IGF-I, DES(1-3) IGF-I and R^3-IGF-I. In the anterior pituitary (ap), [^{125}I]IGF-I was displaced with IGF-I, but not with DES(1-3)IGF-I and R^3-IGF-I. Scale bar = 1 cm.

Table 1 IGF-I receptor concentrations labelled by [^{125}I]IGF-I in human adult brain samples and pituitary gland in fmol/mg

Region	Male ($n = 5$)	Female ($n = 5$)	p*
Frontal cortex	24.6 ± 2.7	23.6 ± 3.7	0.84
Frontal white matter	8.4 ± 1.8	9.4 ± 2.7	0.69
Cerebellum			
Granule cell layer	12.6 ± 2.1	13.8 ± 4.2	0.84
Molecular layer	36.4 ± 5.7	38.2 ± 6.7	0.42
Deep white matter	9.0 ± 1.8	10.7 ± 3.0	0.39
Dendate nucleus	17.6 ± 2.7	20.0 ± 5.3	0.42
Choroid plexus	46.4 ± 8.1	43.4 ± 4.9	0.42
Pituitary gland			
Anterior lobe	ND	ND	–
Posterior lobe	23.8 ± 3.6	21.6 ± 2.7	0.42

Slide-mounted tissue sections were incubated with 0.1 nM [^{125}I]IGF-I. Autoradiograms were generated by exposing the slide-mounted tissue sections to [^{3}H] ultra film for 4 days, and subsequently quantified by computerized densitometry. Non-specific binding was determined in the presence of DES(1-3)IGF-I and or R3-IGF-I. ND; not detected. Data are the mean ± SD. *Mann–Whitney *U*-test.

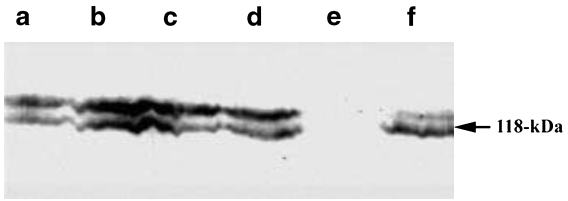

Fig. 3 Western immunoblot of brain samples and pituitary samples ran on 7.5% SDS–PAGE under reducing conditions using an antibody against the α-chains of the human IGF-I receptor. Bands were visualized using DAB. We found two bands of approximately 118 kDa corresponding to the α-subunits of IGF-I receptor into detergent-solubilized total cell membrane fraction (60 μg) of frontal cortex (a), choroid plexus (b), cerebellum (c), posterior pituitary gland (d) and cerebral white matter (f). We found no expression of IGF-I receptor α-subunits in pellet fractions of the anterior pituitary (e).

human IGF-I receptor reacts specifically against a 19-residue synthetic peptide corresponding to amino acid residues 642–659 of the human IGF-I receptor α-subunit (Rosenweig *et al*. 1990). In frontal cortex, choroid plexus, cerebellum, white matter, and posterior pituitary a double band of 118 kDa corresponding to the α-chains (LeRoith *et al*. 1995) of the IGF-I receptor were identified (Fig. 3). In the anterior lobe of the pituitary gland no reaction with anti-human IGF-I receptor was found (Fig. 3).

Western blotting was performed under different conditions. In the first approach pellet fractions of the anterior pituitary ran under denaturing and reducing conditions, and were stained for IGFBP-3. A single band of 40 kDa (Fig. 4a) was detected under these conditions. When the same samples ran under native conditions, and were stained for IGFBP-3 they revealed one band of 150 kDa (Fig. 4a). Purified IGFBP-3 was used as a positive control. With anti-IGFBP-3 we found one band of 40 kDa (Fig. 4a). Non-immune rabbit serum was used to determine the non-specific binding (Fig. 4b).

We performed immunoprecipitation experiments to visualize the components of the complex. Immunocomplexes of proteins with anti-IGFBP-3, revealed a band of approximately 40 kDa in anterior pituitary, posterior pituitary and frontal cortex, just as human IGFBP-3 that was used as a positive control (Fig. 5). Homogenates of the anterior pituitary immunoprecipitated with anti-IGFBP-3 and stained with anti-ALS revealed a 86-kDa band. The same band was present in human serum that contains ALS and was used as a positive control. This was not observed in the frontal cortex and the posterior pituitary (Fig. 5). Thus these results showed the presence of an IGFBP-3-ALS complex in the anterior pituitary. This complex was absent in the posterior pituitary and frontal cortex.

IGFBP-3 binding to cell membranes

To confirm the presence of IGFBP-3 in the anterior pituitary, we performed immunohistochemistry both with and without

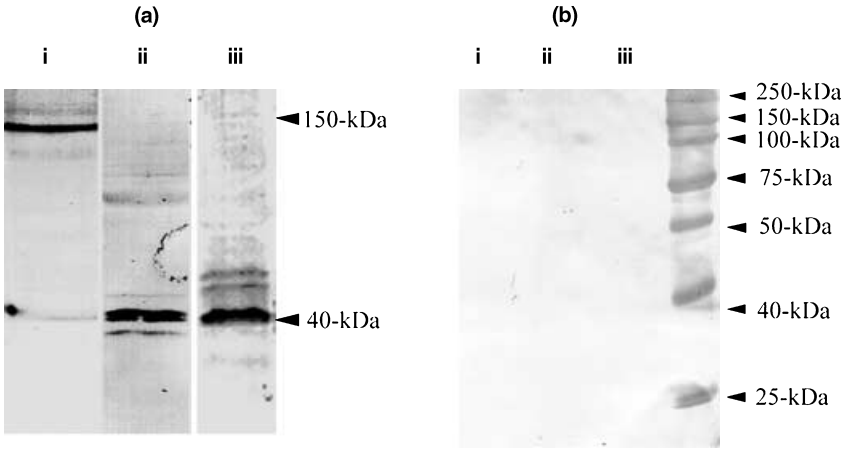

Fig. 4 Western immunoblots of anterior pituitary samples ran on 12% SDS–PAGE both under native conditions, and under reducing and denaturing conditions. Samples were stained for IGFBP-3 (a). Samples ran under native conditions revealed a 150-kDa-band (i) and samples that ran under reducing conditions revealed a single band of 40 kDa (ii). Human IGFBP-3 was used as a positive control and presents one band of 40 kDa (iii). In (b), non-specific binding was determined by incubating parallel blots with non-immune rabbit serum instead of anti-IGFBP-3. Samples ran under native conditions (i), denatured conditions (ii), and pure IGFBP-3 (iii) did not reveal non-specific bands. Protein marker is shown at the left. Bands were visualized using opti-4 CN.

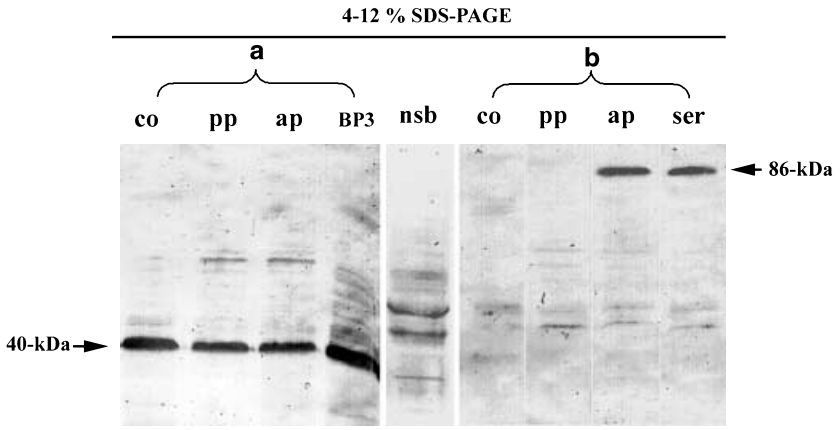

Fig. 5 Immunoprecipitation of proteins with anti-IGFBP-3 in anterior pituitary (ap), posterior pituitary (pp), frontal cortex (co), human IGFBP-3 (BP3) and serum (ser). Bands were visualized using opti-4-CN. Immunocomplexes stained for IGFBP-3 (a) showed one band of 40 kDa in anterior pituitary (ap), posterior pituitary (pp), frontal cortex (co) and for IGFBP-3 used as a positive control (BP3). Immunocomplexes stained for ALS (b) showed a single band of 86 kDa in anterior pituitary (ap) and serum (ser), but not in the posterior pituitary (pp) and frontal cortex (co). Non-specific bands (nsb) of the anterior pituitary are shown using BSA instead of the primary antibody.

pre-incubation in 25 mM Tris-buffer supplemented with 10 mM $MgCl_2$, using a polyclonal antibody against IGFBP-3. Without pre-incubation, IGFBP-3 was present in the anterior and posterior pituitary, and in the frontal cortex (Fig. 6). IGFBP-3 was located in anterior pituitary cells and in the posterior pituitary; IGFBP-3 was located in the pituicytes. In the brain regions, IGFBP-3 was located in neurons. Following pre-incubation in 25 mM Tris-buffer supplemented with 10 mM $MgCl_2$, IGFBP-3 was no longer detectable in the posterior pituitary and any of the brain regions, but was still present in the anterior pituitary.

ALS mRNA expression in anterior pituitary

In order to prove whether the ALS protein found in the anterior pituitary derives from the anterior pituitary itself or from the circulation, we performed RT-PCR on frozen anterior pituitary tissue of three patients. To date, it has not been shown that blood cells produce ALS. However, as it is not possible to wash tissue extensively before RNA isolation, we also tested samples of freshly isolated blood cells to exclude any possibility that mRNA of blood cells may falsify the results. The human hepatoma cell line HepG2 was used a positive control as it is known that ALS is highly expressed

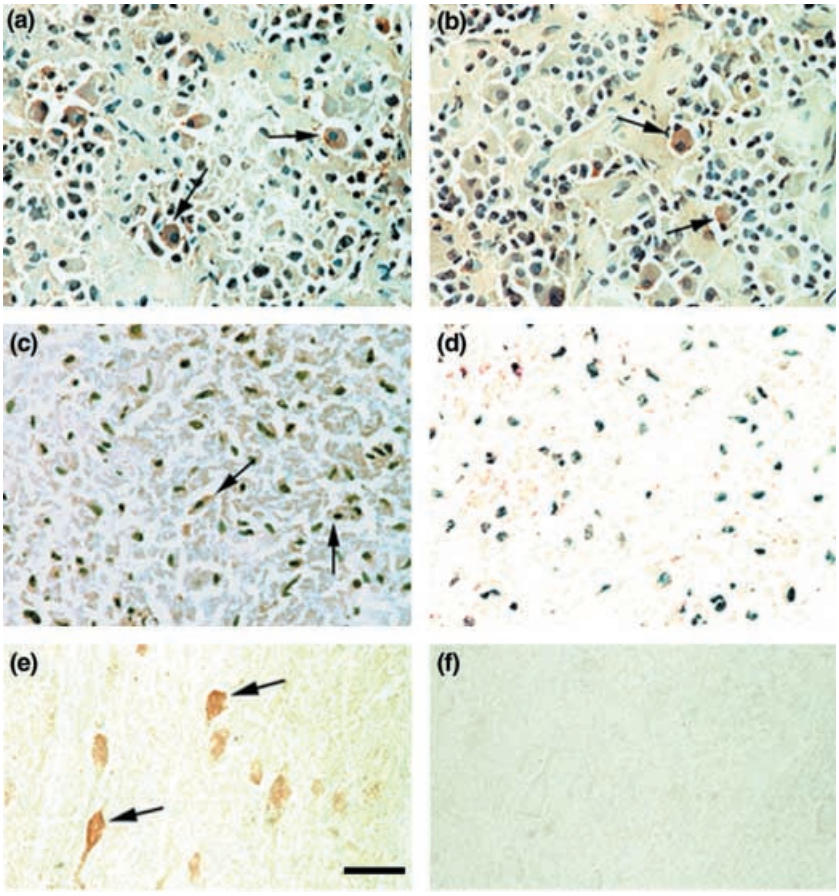

Fig. 6 Photomicrographs of IGFBP-3 immunohistochemistry in anterior (a, b) and posterior pituitary gland (c, d) and frontal cortex (e, f). Nuclei are counterstained with haematoxylin (dark blue). Prior to pre-incubation in Tris-buffer containing $MgCl_2$, IGFBP-3 was present on cells in anterior (a) and posterior pituitary (c), and on neurones in frontal cortex (e). After pre-incubation, expression of IGFBP-3 was no longer detectable in posterior pituitary (d) and in frontal cortex (f). In contrast, IGFBP-3 was still present on cells in anterior pituitary (b). Scale bars = 100 μm.

in liver (Scott and Baxter 1991). Furthermore, actin mRNA levels were used to standardize RNA contents of different samples.

The results show that ALS mRNA is detectable in the positive control HepG2 cells and the anterior pituitary (Fig. 7). When comparing the relative band intensities, the ALS mRNA level in the anterior pituitary ranged between 20% and 30% of the level in HepG2 cells. No specific signal for the ALS mRNA fragment was detected in the blood cell samples. The bands seen in blood cell samples are of smaller size and, therefore, are non-specific. This rules out ALS mRNA in the anterior pituitary being derived from contamination with blood.

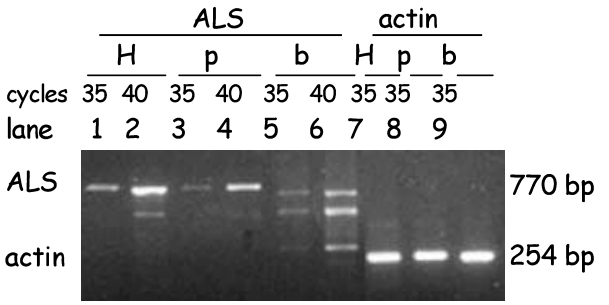

Fig. 7 RT-PCR was performed with RNA isolated from anterior pituitary tissue of three patients, blood cells and HepG2 cells. The PCR products were separated on a 2% agarose gel. One representative ethidium bromide-stained gel is shown. The amplified ALS fragment (770 bp) was detected in HepG2 cells (lanes 1 and 2) and anterior pituitary tissue (lanes 3 and 4) after 35 and 40 cycles but not in whole blood (lanes 5 and 6). Comparable amounts of the amplified actin fragment (254 bp, internal control) were detected in HepG2 cells (lane 7), anterior pituitary tissue (lane 8), and whole blood (lane 9) after 35 cycles. Names of the genes are indicated on the left and the corresponding fragment sizes on the right. H, HepG2 cells; p, anterior pituitary; b, blood cells.

Discussion

IGF-I binding sites are widely distributed throughout the human brain and pituitary gland (De Keyser *et al*. 1994). The brain contains not only IGF receptors but also the six different IGFBPs that have been identified (Spagnoli and Rosenfeld 1997; Rechler and Clemmons 1998). These IGFBPs are soluble proteins, and we showed that pre-incubation in a buffer solution (Tris/HCl containing 10 mM $MgCl_2$) readily abolished both the autoradiographic labelling

and immunohistochemical visualization of IGFBPs in slices of different brain regions (Wilczak *et al*. 2000). The binding characteristics of [^{125}I]IGF-I in the brain regions and posterior pituitary were compatible with binding to IGF-I receptors.

In the anterior pituitary, [^{125}I]IGF-I binding occurred to a binding site with a 10-fold lower affinity than the IGF-I receptor. Using western immunoblotting experiments with anti-IGFBP-3 under native conditions, we identified this binding site as a 150-kDa IGFBP-3-containing molecule. In this region, pre-incubation in buffer did not abolish IGFBP-3 immunoreactivity, indicating that this IGFBP-3-containing complex was tightly bound to the cell membranes. Additionally to these findings IGFBP-3 was completely complexed with ALS because we found no smaller proteins with anti-IGFBP-3. In contrast, when the same samples were analysed under high reducing and denaturing conditions, we identified a single 40-kDa IGFBP-3 band. Under these conditions the 150-kDa complex was dissociated.

Immunoprecipitation experiments confirmed the presence of a 150-kDa complex in the anterior pituitary. When proteins of the anterior pituitary were complexed with anti-IGFBP-3 and these immunocomplexes were stained for IGFBP-3, they showed one band of approximately 40 kDa, and when stained for ALS they showed an 86-kDa band. Immunocomplexes from the posterior pituitary and frontal cortex which were stained for ALS did not reveal an 86-kDa band, indicating the absence of ALS. When these immunocomplexes were stained for IGFBP-3, they revealed a band of approximately 40 kDa, indicating the presence of IGFBP-3 in both anterior and posterior pituitary and frontal cortex.

It has been shown previously that IGFBP-3 has cellular effects that are independent of IGF presence or action, and these effects are mediated by binding of IGFBP-3 to the cell surface, possibly to specific receptors (Mohseni-Zadeh and Binoux 1997; Young Lee and Rechler 1995; Rechler and Clemmons 1998). However, since IGF-I in association with IGFBP-3 prevents binding of IGFBP-3 to the cell membrane (Smith *et al*. 1992), the IGFBP-3 binding site in the anterior pituitary must be of a different composition.

The molecular weight of IGFBP-3 in its non-glycosylated form is 29 kDa, in its glycosylated state 40–44 kDa, and when it forms a complex with ALS, 140–150 kDa (Martin and Baxter 1992; Spagnoli and Rosenfeld 1997). IGFBP-3 is the predominant carrier protein of IGF-I in serum. The IGF-I–IGFBP-3 dimer forms a complex with ALS, and this ternary complex prolongs the serum half-live of IGF-I by many hours (Jones and Clemmons 1995; Spagnoli and Rosenfeld 1997; Rechler and Clemmons 1998). Once released from the complex, IGF-I leaves the circulation and enters target tissues with the aid of IGFBPs present on the cell surface or in the extracellular matrix.

By using RT-PCR in anterior pituitary and blood samples, we found the expression of ALS mRNA in the anterior pituitary but not in blood samples. Thus, our findings suggest that the human anterior pituitary gland expressed ALS mRNA and ALS protein and this ALS forms a complex with IGFBP-3 bound to the cell membrane. ALS is mainly synthesized by the liver, and the native ALS molecule appears in serum as a 84–86-kDa glycoprotein doublet (Baxter 1988; Baxter and Martin 1989; Baxter *et al*. 1989; Scott and Baxter 1991). Interestingly, it has been shown that follicular fluid ALS originates from the ovary and that ALS is also synthesized by the kidney (Dai and Baxter 1994; Wandji *et al*. 2000).

We were unable to detect IGF-I receptors in the anterior pituitary gland in this study. However, our *in situ* observations do not entirely exclude their presence *in vivo*. IGF-I receptor mRNA has been demonstrated by *in situ* hybridization in rodent anterior pituitary slices and cell cultures (Bach and Bondy 1992; Oomizy *et al*. 1998). It might be possible that the receptors were down-regulated by IGF-I, as has been shown for IGF-I receptors in rat pituitary cells (Yamamoto *et al*. 1993).

In conclusion, our findings show that in human anterior pituitary gland IGF-I binds to a not previously reported tightly membrane-bound IGFBP-3–ALS complex. The functional implication of the binding of IGF-I to this complex requires further experimentation at the cellular level.

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