Characterization of somatomedins from human fetal brain: Identification of a variant form of insulin-like growth factor I

(fetal growth/central nervous system)

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ABSTRACT   A fetal form of somatomedin (insulin-like growth factor) that crossreacts in the fetal brain radioreceptor assay has been proposed to exist in humans. Using this assay to monitor activity during purification, we have isolated a variant form of insulin-like growth factor I (IGF-I) from human fetal brain tissue. The variant IGF-I showed potent crossreaction in the fetal brain radioreceptor assay and stimulated DNA synthesis in fetal brain cells in vitro. Structural analysis revealed the variant IGF-I to have a truncated NH₂-terminal region compared to IGF-I isolated from serum. An additional peptide, which displayed less potent crossreaction in the fetal brain radioreceptor assay, was also isolated from the human fetal brain. Partial amino acid sequence analysis revealed identity to insulin-like growth factor II.

The somatomedins, or insulin-like growth factors, are a family of growth-promoting peptide hormones (1). Insulin-like growth factors I and II (IGF-I and IGF-II) have been isolated and characterized from adult human plasma (2, 3). Somatomedins A and C are identical to IGF-I, disregarding possible deamidation differences (4, 5). An additional, human fetal form of somatomedin has been proposed (6, 7). This suggestion was based upon low or undetectable levels of immunoreactive IGF-I or IGF-II in human fetal serum (7, 8), in spite of fetal tissues displaying a high concentration of somatomedin receptors (9) and somatomedins having a growth-promoting action on fetal cells in vitro (10, 11). Additionally, early in gestation the somatomedin receptor in human fetal membranes showed characteristics incompatible with its classification as a type I or II receptor, thereby suggesting the presence of an additional fetal peptide (8, 9). Accordingly, a radioligand–receptor assay utilizing the fetal receptor as matrix and IGF-I as ligand was developed (7). By use of this radioreceptor assay, elevated levels of somatomedins were detected in the fetal circulation. These values fell with maturation. Around birth, there was proposed to be a switch from production of fetal to adult forms of somatomedins (7, 8). Attempts were made to purify the fetal somatomedin from medium conditioned by human brain cells. Gel filtration revealed a peak of activity corresponding to a molecular weight of about 6000; material in this peak crossreacted in the fetal radioreceptor assay but was not detected by antibodies directed against IGF-I and only slightly by IGF-II antibodies (unpublished data). Since medium sufficient for complete characterization could not be harvested, an alternative source for purification was sought. The present study describes the purification and characterization of peptides with fetal radioreceptor assay activity from human fetal brain tissue.

MATERIALS AND METHODS

Preparation of Cytosol from Human Fetal Brain Tissue. Immediately after legal prostaglandin abortion, fetuses were stored in sterile 0.9% NaCl at 4°C. Within 12 hr, the whole fetal brain minus cerebellum was removed and stored at −70°C until use. Five volumes of 50 mM Tris Cl (pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride was added to frozen brain tissue which was then homogenized with a Polytron for 1 min. The homogenate was centrifuged at 50,000 × g for 15 min, and the supernatant was collected and stored at −85°C until use. Approximately 150 brains from fetuses at 12–20 weeks of gestation were used.

Purification. Activity was monitored by fetal brain radioreceptor assay, and protein was determined by the method of Kalb and Bernlohr (12) or by following the absorbance at 280 nm or 215 nm. All glassware was siliconized and procedures were carried out at 4°C. All reagents were of analytical grade.

Acid precipitation and Sephadex G-50 chromatography. As inhibitors of proteolysis, 0.2 mM phenylmethylsulfonyl fluoride and 0.1 μM pepstatin were added to cytosol during thawing. Cytosol protein was precipitated by lowering the pH to 5.5 with 1 M acetic acid. After centrifugation at 16,000 × g for 30 min, the supernatant was further acidified to 0.1 M acetic acid, stirred overnight at 4°C, and then centrifuged again. The supernatant was concentrated 3 times by rotary evaporation, dialyzed for 20 hr with 3 changes of 10 volumes of 0.1 M acetic acid (Spectra/Por 6 dialysis membrane, molecular weight cut-off 1000; Spectrum Medical Industries, Los Angeles), further concentrated by rotary evaporation, and then chromatographed on a column (3 × 150 cm) of Sephadex G-50 fine (Pharmacia) equilibrated with 0.1 M acetic acid. Markers for calibration were Blue Dextran and CoCl₂. The column was operated at a flow rate of 24 ml/hr.

Affinity chromatography. Amniotic fluid somatomedin carrier protein coupled to CNBr-activated Sepharose 4B (Pharmacia) was a generous gift from K. Hall and G. Póvoa (Karolinska Hospital). Pooled active fractions from the Sephadex G-50 column were concentrated, dialyzed overnight against 5 volumes of 50 mM Tris Cl (pH 7.7), and applied to the affinity column (6 × 20 mm) equilibrated in the same buffer. The column was washed with 3 ml of buffer and 3 ml of water. Activity was eluted with 6 ml of 1 M acetic acid, and the eluate was frozen.

Cation-exchange FPLC. Active fractions from the affinity-chromatography step were pooled, concentrated, and applied to a fast protein liquid chromatography (FPLC) system using an HR 5/5 column of Mono S (Pharmacia) equilibrated with 10 mM ammonium acetate, pH 6.5/10% acetonitrile. Elution was at a flow rate of 1.0 ml/min with a linear gradient of ammonium acetate, pH 6.5/10% acetonitrile.

Abbreviations: IGF-I and IGF-II, insulin-like growth factors I and II; RRA-IGF-I, radioreceptor assay using ¹²⁵I-labeled IGF-I; u, unit(s).

Reversed-phase HPLC. Active material after lyophilization was resuspended in 30% acetic acid and applied to an Ultropac C_{18} TSK ODS-120T column (4.6 × 200 mm, particle size 5 μm; LKB, Bromma, Sweden). Elution was with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min.

Structural Analysis. *Amino acid composition.* Samples were hydrolyzed for 24 hr at 110°C with 6 M HCl/0.5% phenol in evacuated tubes. Liberated amino acids were converted to phenylthiocarbamoyl derivatives by coupling with phenylisothiocyanate and then were identified by reversed-phase HPLC (Waters Associates) on a C_{18} column (Spherisorb S3 ODS-2; 3 μm; Phase Separations, Queensferry, UK), using an acetonitrile gradient in a sodium phosphate buffer as described (13).

Amino acid sequence. Samples of native peptide for sequence analysis were degraded in an Applied Biosystems 470A gas-phase sequencer (14).

Fetal Brain Radioreceptor Assay (Fetal Brain RRA-IGF-I). This assay was performed as described (7). Human fetal brain plasma membrane was used as matrix. Purified IGF-I was iodinated by the lactoperoxidase method (15) and used as ligand. IGF-I and IGF-II, prepared as described, with protein content and purity assessed by amino acid composition and NH_2-terminal analysis (4), were generous gifts from G. Enberg (Karolinska Hospital). Values were expressed in relation to a human reference serum standard given an arbitrary value of 1 unit (u)/ml.

Bioassay for Growth-Promoting Activity. Biological activity was assessed by determining the incorporation of [^{3}H]thymidine into fetal rat brain cell DNA (16). A primary cell suspension was prepared from fetal rat brains on day 18 of gestation. After incubation in a serum-free medium containing [^{3}H]thymidine for 20 hr at 37°C, DNA was extracted from the cells (16) and the incorporation of [^{3}H]thymidine into DNA was determined.

RESULTS

The purification of peptides with fetal brain RRA-IGF-I activity from human fetal brain cytosol is summarized in Table 1. After acid precipitation to separate somatomedins from their binding proteins, cytosol was subjected to gel filtration on Sephadex G-50. The column was developed with 0.1 M acetic acid. Fetal brain RRA-IGF-I activity was eluted as two peaks corresponding to apparent molecular weights of 12,000 and 4000, respectively. The material in the lower molecular weight peak (pool B) was further purified by affinity chromatography. Activity was eluted from this column with 1 M acetic acid. This eluate was then concentrated and chromatographed on Mono S (Fig. 1). Two peaks of material with fetal brain RRA-IGF-I activity were eluted from the column. Peaks I and II were lyophilized and further
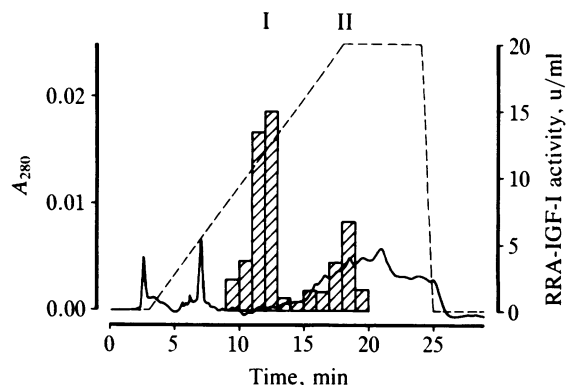

FIG. 1. Cation-exchange FPLC (HR 5/5 Mono S column) of the active material eluted from the affinity-chromatography column. Elution was with a linear gradient of 10–300 mM ammonium acetate in 10% acetonitrile at pH 6.5 (broken line) at 1 ml/min. Fetal brain RRA-IGF-I activity, eluted as peaks I and II (fractions 12–13 and 18–19, respectively), is shown by the hatched bars.

purified by reversed-phase HPLC using an Ultropac C_{18} column. The elution pattern for each, given in Fig. 2, showed a single peak of fetal brain RRA-IGF-I activity at approximately 37% acetonitrile, with the active material from peak I (peptide I) eluted slightly before the active material from peak II (peptide II). The specific activities of peptides I and II were approximately 50,000 and 5000 u/mg of protein, respectively. The protein content was estimated by acid hydrolysis. The purity of these peptides was confirmed by NH_2-terminal analyses. This purification has been reproduced in three separate preparations.

The crossreaction of peptides I and II, as well as of purified IGF-I and IGF-II, in the fetal brain RRA-IGF-I is given in Fig. 3. Prior to assay, the protein content of all peptides was determined by acid hydrolysis, and their purity, by NH_2-terminal analyses. Peptide I was approximately 5 times more potent than IGF-I and 10 times more potent than IGF-II. Peptide II was equipotent to IGF-II. In this assay, peptides I and II have specific activities of 50,000 and 5000 u/mg, respectively, whereas IGF-I and IGF-II purified from serum have specific activities of 10,000 and 5000 u/mg, respectively. Biological activity monitored by the fetal brain cell bioassay was maintained during the purification. Peptide I added to the incubation medium at 0.5 ng/ml [t = 2.46, degrees of freedom (df) = 7, P < 0.05), 2.5 ng/ml (t = 4.69, df = 7, P < 0.01), and 5 ng/ml (t = 9.40, df = 7, P < 0.001) caused a significant dose-dependent stimulation of [^{3}H]thymidine incorporation into fetal brain cell DNA. A 36% increase over basal [^{3}H]thymidine incorporation was observed at 5 ng of peptide I per ml of incubation medium.

The amino acid compositions of peptides I and II are,

Table 1. Purification of fetal brain RRA-IGF-I activity from human fetal brain cytosol

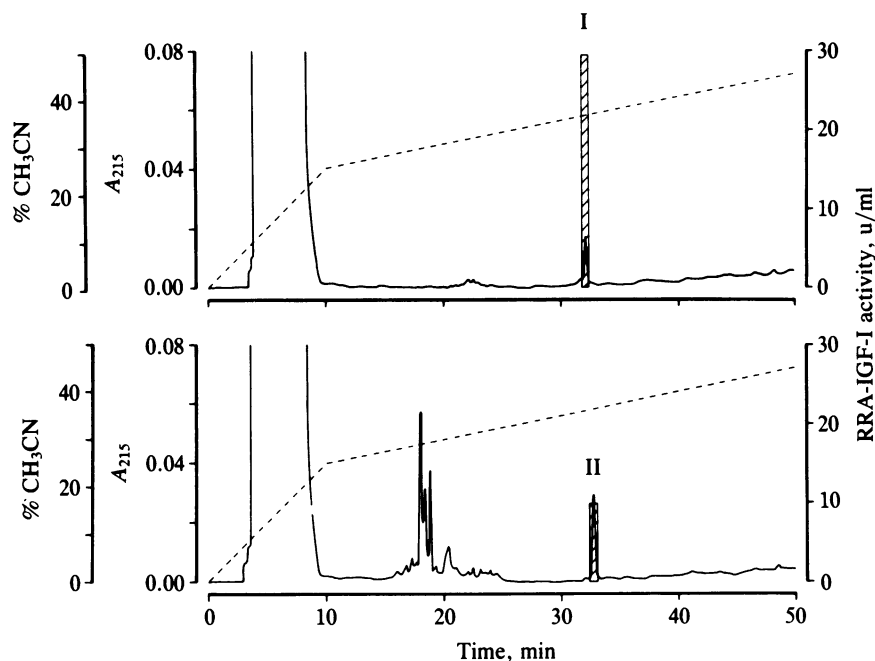| Step	Specific activity, u/mg	Fold purification	Recovery, %
Cytosol	0.04	1.0	100
Acid precipitation	0.07	1.8	60
Sephadex G-50			
Pool A	0.20	5.0	30
Pool B	0.50	12.5	12
Pool B			
Affinity chromatography	56	1,400	12
FPLC Mono S	—*	—*	11
HPLC Ultropac			
Peptide I	50,000	1,250,000	5
Peptide II	5,000	125,000	5

*Protein content not determined.

FIG. 2. Reversed-phase HPLC (Ultropac C_{18} TSK ODS 120T) chromatography of peaks I (*Upper*) and II (*Lower*) derived from cation-exchange FPLC (see Fig. 1). Elution was with a gradient of 0–45% acetonitrile in 0.1% trifluoroacetic acid (broken line). Fetal brain RRA-IGF-I activity (peptides I and II) is shown by the hatched bars.

within experimental error, almost identical to the amino acid compositions of IGF-I and IGF-II, respectively (Table 2). However, gas-phase sequence degradations revealed NH_2-terminal differences between peptide I and IGF-I (Fig. 4). The NH_2-terminal threonine of peptide I aligned with position 4 of IGF-I. With this alignment, identical amino acid residues were observed, so that positions 1–29 in peptide I were identical with positions 4–32 of IGF-I. The degradations also revealed homogeneity of the preparations. Residues 1–11 of peptide II were identical to residues 1–11 of IGF-II (Fig. 4). The small amounts of material (125 pmol of peptide I and 55 pmol of peptide II) limited the extent of structural analysis.

DISCUSSION

Previous studies of somatomedins in the human fetus led to the hypothesis that a fetal form of somatomedin could be detected by the fetal brain radioreceptor assay (7). Using this assay for identification during purification, we have isolated and characterized a variant form of IGF-I from the human fetal brain. This peptide displayed more potent crossreac-
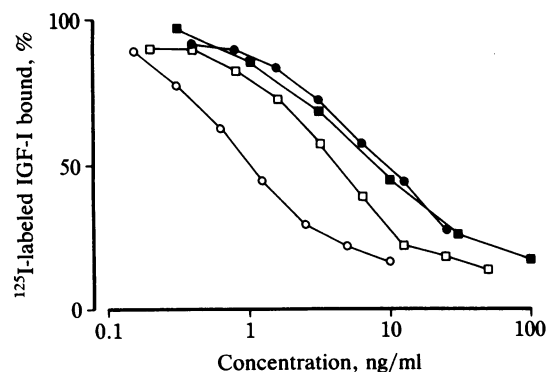

FIG. 3. Crossreaction in the fetal brain RRA-IGF-I, expressed as percentage of maximal binding (total specifically bound, 23%). Data represent inhibition of binding of ^{125}I-labeled IGF-I to human fetal brain plasma membranes (330 μg of protein per ml) by peptides I (o) and II (●), IGF-I (□), and IGF-II (■).

tivity than IGF-I and IGF-II in the fetal brain radioreceptor assay. Similarly, the variant IGF-I stimulated fetal brain cell proliferation *in vitro* at concentrations less than those previously observed for purified IGF-I and IGF-II (11). Structural analysis revealed this variant form to have a truncated NH_2-terminal region compared to the M_r 7500 IGF-I isolated from adult human plasma (2). The NH_2-terminal amino acid residue, threonine, in the variant form aligns with position 4 of IGF-I. The amino acid sequence, followed up to position 29, was identical with IGF-I from position 4 to 32. It is unlikely that this truncated NH_2-terminal region resulted from the purification procedure, which is less harsh than other purification methods resulting in the isolation of the M_r 7500 IGF-I. Furthermore, the NH_2 terminus of the variant

Table 2. Amino acid compositions of peptides I and II, obtained in this work, compared to the known compositions of IGF-I and IGF-II

Residue	No. of residues per mol			
	Peptide I	Peptide II	IGF-I	IGF-II
Asx	3.9	3.1	5	3
Glx	6.0	5.5	6	7
Ser	6.0	4.6	5	7
Gly	6.1	3.8	7	5
Thr	2.8	3.8	3	4
Ala	5.3	4.7	6	5
His	0.0	0.0	0	0
Pro	3.3	2.9	5	3
Arg	6.3	8.6	6	8
Tyr	1.7	3.4	3	3
Val	3.1	4.5	3	4
Met	0.1	0.1	1	0
Cys	ND	ND	6	6
Ile	0.9	0.9	1	1
Leu	5.6	7.1	6	6
Phe	3.5	4.0	4	4
Lys	1.9	1.1	3	1

Compositions of IGF-I and IGF-II are from refs. 2 and 3. ND, not determined.

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a
  B Domain
    Peptide I (1–26)        T L – G A E L V D A L Q F V – G D R G F Y F N K P T
    IGF-I (1–29)          G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T

  C Domain
    Peptide I (27–29)    G Y G
    IGF-I (30–41)        G Y G S S S R R A P Q T

b
  B Domain
    Peptide II (1–11)    A Y R P – E T L – G G
    IGF-II (1–11)        A Y R P S E T L C G G
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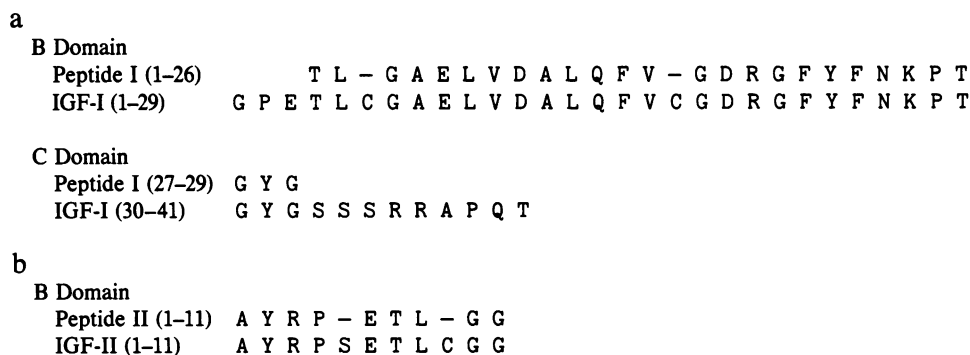
FIG. 4. (*a*) Proven parts of the amino acid sequence of peptide I compared to the sequence of IGF-I. (*b*) NH₂-terminal sequence of peptide II compared to the NH₂-terminal sequence of IGF-II. Dashes represent unidentified amino acid residues. IGF-I and IGF-II sequences are from refs. 2 and 3.

was found to be homogeneous. The NH_2 terminus of IGF-I is not an intron/exon hinge region (17), and therefore the truncated variant form is unlikely to be due to alternative splicing of mRNA coding for IGF-I. There is no evidence of a second IGF-I gene, and although sequence analysis identified only 29 amino acid residues, their identity with the sequence of IGF-I, together with the similarity of amino acid composition, suggests that the variant IGF-I probably arises from posttranslational modification of the prohormone form of IGF-I. However, only complete sequence analysis of the variant IGF-I can resolve this question.

IGF-II was also isolated from the human fetal brain. Sequence analysis was performed on the first 11 amino acid residues, which were identical to those of the M_r 7500 IGF-II from plasma (3). However, variant forms of IGF-II have also been isolated (18, 19). Although one IGF-II variant appears to arise by alternative splicing of mRNA (18), the amino acid substitution in the M_r 10,000 variant pro-form of IGF-II is not located at a known intron/exon hinge region (19). As these amino acid substitutions occur at positions 29 and 33 of IGF-II, respectively, it was not possible to determine whether the fetal brain IGF-II was one of the variant forms. The presence of a variant form of IGF-I indicates different processing or gene expression in the human fetal brain. In spite of failure to detect immunoreactive IGF-I in the adult human brain (20) or IGF-I gene expression in the fetus (21) the presence of the variant in either the adult nervous system or other fetal tissues remains to be investigated.

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