Purification and Characterization of an Insulin-like Growth Factor II Variant from Human Plasma*

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An insulin-like growth factor II variant (IGF-II variant) was purified from Cohn fraction IV of human plasma by ion exchange, gel filtration, and reversed-phase high pressure liquid chromatography. The amino-terminal sequence of the first 35 amino acid residues showed a replacement of Ser-29 of IGF-II with the tetrapeptide Arg-Leu-Pro-Gly of IGF-II variant. Peptides isolated and sequenced after digestion with endoproteinase Asp-N and endoproteinase Glu-C disclosed no differences with the sequence predicted from an IGF-II variant cDNA clone isolated by Jansen, M., van Shaik, F. M. A., van Tol, H., Van den Brande, J. L., and Sussenbach, J. S. (1985) FEBS Lett., 179, 243–246. The molecular ion of intact IGF-II variant was 7809.4 mass units, as measured by plasma desorption mass spectrometry. This is in close agreement with the molecular ion of 7812.8 mass units calculated from the determined sequence and indicates the entire amino acid sequence had been accounted for. Binding of IGF-II variant to purified insulin-like growth factor I (IGF-I) receptors demonstrated a 2–3-fold lower affinity for this receptor compared with IGF-I or IGF-II. The dissociation constants for IGF-I, IGF-II, and IGF-II variant are 0.23, 0.38, and 0.80 nM, respectively. In a growth assay, the concentration of IGF-I variant required to stimulate the half-maximal growth of MCF-7 cells was 4 and 13 nM, respectively. Finally, the amount of IGF-II variant that can be purified by this method constitutes approximately 25% of the total IGF-II isolated from Cohn fraction IV of human plasma.

Insulin-like growth factor II (IGF-II) is a single-chain peptide of 67 amino acids that has a high degree of amino acid sequence homology with insulin-like growth factor I (IGF-I) and insulin (2). It is synthesized as a 180-amino-acid precursor and enzymatically processed to its mature form (3, 4). We and others have identified higher molecular weight forms of biologically active IGF-II (proIGF-II) in human plasma. Therefore, IGF-II does not have to be processed to its mature form before it is secreted (5–7). The concentrations of IGF-I and IGF-II in adult human serum range from 150 to 200 ng/ml and 600 to 800 ng/ml, respectively (8). Both are found predominantly in plasma complexes with a high molecular weight binding protein (9). IGF-I appears to be the principal mediator of pituitary growth hormone during adolescence, whereas IGF-II is thought to exert its effects primarily during fetal growth and development (reviewed in Refs. 10 and 11).

IGF-II binds with high affinity to the following two classes of membrane-bound receptors: 1) the type I IGF receptor whose structure is homologous to the insulin receptor and contains an intrinsic tyrosine kinase activity that is activated upon ligand binding (12–14), and 2) the type II IGF receptor which is a multifunctional protein with a monomeric structure that has been identified as the cation-independent mannose 6-phosphate receptor (15). In addition to binding IGF-II, the type II receptor has distinct binding sites for lysosomal enzymes containing mannose-6-phosphate moieties (16). Unlike the type I receptor, the type II receptor does not have a consensus tyrosine kinase domain (17).

An IGF-II variant cDNA has been isolated from a human liver cDNA library by Jansen et al. (1). This variant arose as a result of the insertion of nine nucleotides into the coding sequence at position 158 and would result in the replacement of amino acid Ser-29 with Arg-Leu-Pro-Gly. Based on the sequence of IGF-II genomic clones, it was determined that this sequence is derived from the 3' end of the intron located between exons 5 and 6 (referred to as exons 1 and 2 in Ref. 18). Zumstein et al. (6) have reported (as unpublished results) that they isolated the IGF-II variant described by Jansen et al. (1). These authors also isolated a 10-kDa variant of proIGF-II that contained a substitution of Cys-Gly-Asp for Ser-33 and had a carboxyl-terminal extension of 21 residues. This tripeptide insertion does not occur at an intron/exon junction. A variant form of IGF-II also has been isolated from chicken serum (19). In this report we describe the purification, characterization, receptor binding, and growth promoting properties of the IGF-II variant described by Jansen et al. (1) from a pool of human plasma.

EXPERIMENTAL PROCEDURES

Materials

Cohn fraction IV, paste was obtained from Hyland Laboratories. Bovine serum albumin, radi-immune assay-grade bovine serum albumin, was purchased from Sigma; n-octyl-β-D-glucopyranoside was purchased from Behring Diagnostics; recombinant IGF-I (rIGF-I) was purchased from IMCERA Bioproducts; sodium 225-Iodine and 131-I-
rIGF-I (2000 C/mmol) were from Amersham Corp.; purified human placenta IGF-I receptor protein was a generous gift of Dr. Yoko Fujita-Yamaguchi (Beckman Research Institute, City of Hope, Duarte, CA). Polyethylene glycol 6000 and acetonitrile were from EM Science; Sephadex G-50 superfine and SP-Sephadex C-25 were purchased from Pharmacia LKB Biotechnology Inc.; Spectra-Por 3 dialysis tubing from Spectrum Medical Industries; reverse-phase endoproteinase Asp-N and Glu-C from Boehringer Mannheim; trifluoroacetic acid, heptafluorobutyric acid (HFBA), constant boiling 6 N hydrochloric acid, and phenylisothiocyanate were from Pierce Chemical Co.; redistilled phenol was from International Biotechnologies; nitrocellulose was from Bio-Rad; electrophoresis reagents were purchased from British Drug House; and sequencing reagents from Applied Biosystems. Improved minimal essential medium (IMEM) and trace elements were from Biofluids. Bovine calf serum came from Gibco and tissue culture plasticware from Costar. All other chemicals were reagent grade.

Methods

Assay of IGF-II—IGF-II receptor binding activity in column fractions was determined through using a soluble rat placental membrane-derived IGF-II radioiodination assay (RRA). Briefly, a 5-μl sample of each fraction was dried with a Savant Speed-Vac concentrator and in 150 μl of extraction buffer (100 mM HEPES, pH 7.8, 120 mM NaCl, 1 mM EDTA, 1.2 mM MgSO4, 2.5 mM KCl, 5 mM glucose, 0.5% n-octyl-β-D-glucopyranoside, 1.0 mg/ml bovine serum albumin) and incubated with 13,000 cpm of 125I-IGF-II (80–120 μCi/ml) and 0.5 μg of n-octyl-β-D-glucopyranoside-solubilized rat placental membranes in a final volume of 400 μl of RRA buffer for 60 min at room temperature (0.15% aqueous 4% acetic acid). Receptor-bound ligand was precipitated from free ligand after addition of 100 μl of water containing 150 μg of bovine γ-globulin, 500 μl of 25% polyethylene glycol 6000 and centrifugation at 4,000 rpm for 30 min in a refrigerated bench top centrifuge. Pellets were counted for radioactivity in an LKB model 1272 gamma counter.

Purification of IGF-II—Cohn fraction IV, paste, 400 g, was extracted in 4000 ml of 2.0 M acetic acid, 0.075 M NaCl (extraction buffer). The extract was clarified by filtration and IGFs were adsorbed to 100 ml of SP-Sephadex C-25, equilibrated in extraction buffer, by stirring at 4°C for 60 min. The gel was allowed to settle for 30 min and the supernatant decanted. The gel was washed sequentially with 1500 ml of extraction buffer and 500 ml of extraction buffer containing 0.4 M NaCl. IGFs were eluted by washing the gel with 500 ml of 0.5 M Tris-Cl, pH 8.0, 2.0 M NaCl. The eluate was acidified immediately with glacial acetic acid to a final concentration of 1.0 M and dialyzed in Spectra-Por-3 dialysis tubing against 8000 ml of 0.1 M acetic acid for 36-72 h with at least three changes. The dialyzed proteins were concentrated by flash evaporation to 15 ml and applied to a 2.5 × 90-cm column of Sephadex G-50 (superfine) in 1.0 M acetic acid at a flow rate of 17 ml/h. Fractions were collected every 13 ml, and those containing IGF-II were pooled and pooled onto a 0.7 × 25-cm Aquapore butyl reversed-phase column equilibrated in 95% solvent A (0.1% trifluoroacetic acid in water), 5% solvent B (0.075% trifluoroacetic acid in acetonitrile). The column was washed until the absorbance at 215 nm returned to base line. Proteins were eluted from the column with a linear gradient of 5-25% solvent B over 5 min, an isocratic elution at 25 B for 10 min, a linear gradient from 25 to 30% solvent B over 20 min and isocratic elution at 30% solvent B for 15 min. Fractions containing IGF-II were dried in a Savant Speed-Vac concentrator and run on rpHPLC using the same conditions as above. IGF-I which coeluted with IGF-II was separated by chromatography on a 250 × 4.6-mm Aquapore RP-300 column using HFBA as the ion pairing agent (20). The column was equilibrated in 80% solvent A (0.2% HFBA in water) and 20% solvent B (0.15% HFBA in acetonitrile). The proteins were separated with a linear gradient of 35–40% B over 60 min.

SDS-PAGE—Aliquots of IGF-II and IGF-II variant were subjected to electrophoresis using the SDS-PAGE system of Laemmli (21). A 15% acrylamide, 0.4% N,N′-methylenebisacrylamide solution was polymerized in a Hoeffer mini-gel apparatus, and electrophoresis was carried out at a constant 200 volts until the bromophenol blue dye reached the end of the gel. Protein was visualized by staining the gel with 0.1% Coomassie Blue R-250, in 50% methanol, 10% glacial acetic acid and destaining with 9% glacial acetic acid, 5% methanol. Purification—Two aliquots of 1000-μmol each were digested with reverse-phase endoproteinase Asp-N at a 1:10 ratio of enzyme to protein in 50 mM NaH2PO4, pH 8.0, at 37°C for 18 h or with endoproteinase Glu-C at a 1:10 ratio of enzyme to protein in 25 mM ammonium carbonate, pH 7.8, at 25°C for 18 h. The disulfide bonds were subsequently reduced with 10 mM dithiothreitol at 37°C for 60 min and carboxymethylated with 22 mM iodoacetic acid at 25°C for 90 min. The resulting peptides were separated on an Applied Biosystems model 130A, microbore HPLC system with a 2.1 × 200-mm Applied Biosystems RP-300 column. Fractions containing IGF-I1 (70% B in water) were collected. Receptor-bound ligand was separated from IGF-binding proteins on Sephadex G-50 superfine and SP-Sephadex C-25 using a modified Elman chromagen. Cleaved amino acid derivatives were identified with an on-line Applied Biosystems model 120A PTH analyzer.

Mass Spectrometry—IGF-II variant was analyzed by plasma desorption mass spectrometry using a Bio Ion 20 (Bio Ion AB Uppsala, Sweden) instrument. Time-of-flight mass spectrometry and ion hundreds (22). A 100-pmol aliquot of IGF-II variant was applied to aluminaized Mylar which was previously electro-sprayed with 100 μg of nitrocellulose (23). The sample disk was spin-dried and washed with 20 μl of 0.1% aqueous trifluoroacetic acid (24). Spectra were collected for 20–24 h at 10 kV accelerating potential. Spectra were calibrated using hydrogen and sodium ions.

Characterization of IGF Receptor-binding Properties—IGF binding to IGF-I receptors was measured using human placenta-derived IGF-I receptors purified as previously described (25). Briefly, 12 ng of IGF-I receptor protein was incubated with 20,000 cpm of 125I-IGF-I (2000 Ci/mmol), 0.04–5 ng of either rIGF-I, IGF-II, or IGF-II variant in a final volume of 0.5 ml of RRA buffer at 4°C for 48 h. Receptor-bound ligand was separated from unbound ligand by the polyethylene glycol procedure as previously described (25). Pellets were washed once and counted as above. The data were analyzed by the method of Scatchard (26).

Growth Assays—The ability of IGF-II and IGF-II variant to stimulate the growth of MCF-7 cells was determined as previously described (27). Briefly, MCF-7 cells were plated in IMEM containing 5% bovine calf serum (sulfatase-treated and charcoal-stripped) at a density of 25,000 cells/well in 24-well cluster dishes. Twenty-four h later, the medium was removed and replaced with serum-free IMEM containing 2 μg/ml fibronectin, 2 μg/ml transferrin, 20 mM HEPES buffer, 292 μg/ml l-glutamine, and trace elements. After an additional 24 h, this medium was replaced with fresh serum-free IMEM or serum-free IMEM containing either IGF-II or IGF-II variant and the medium was refreshed again. Following 5 days of incubation, the dishes were removed from the cluster dishes with phosphate-buffered saline containing 0.02% EDTA and the number of cells/well was determined using a hemocytometer.

RESULTS

Insulin-like growth factors were adsorbed from an extract of Cohn fraction IV, with SP Sephadex C-25 using a modification of the procedure previously described (28). After washing the matrix with extraction buffer containing 0.4 M NaCl, the remaining proteins were eluted at pH 8.0 in 2.0 M NaCl, dialyzed, and concentrated by flash evaporation. IGF-II was separated from IGF-binding proteins on Sephadex G-50 (superfine) in 1.0 M acetic acid. The distribution of eluted protein and IGF-II receptor binding activity is presented in Fig. 1. IGF-II receptor binding activity is calculated as percent competition for 125I-IGF-II binding to solubilized rat placental membranes. The first peak of competing activity, which eluted in fractions 30–44, contained the acid-stable component of IGF-binding protein as determined by its ability to specifically bind 125I-IGF-II (data not shown). The second peak, containing the majority of the receptor binding activity (fractions 58–68), was pooled and purified further by rpHPLC (Fig. 2). Two peaks of receptor binding activity were identified, and they were designated I and II. Peak I contained most of the
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FIG. 1. Chromatography of Cohn IV extract on Sephadex G-50. The fraction that eluted from SP-Sephadex C-25 with 0.5 M Tris-HCl, pH 8.0, 2.0 M NaCl was applied to a 2.5 x 90-cm column of Sephadex G-50 (superfine) equilibrated in 1.0 M acetic acid at a flow rate of 17 ml/h, and 13-min fractions were collected. $V_0$ indicates the void volume of the column. —, $A_{280}$ ○○○, % competition in the RRA; — indicates which fractions (58–68) were pooled for rpHPLC.

FIG. 2. Reversed-phase HPLC of the IGF pool from the Sephadex G-50 column. Fractions 58–68 of the G-50 column (Fig. 1) were applied directly to a 0.7 x 25-cm Aquapore butyl rpHPLC column equilibrated in 95% buffer A (0.1% trifluoroacetic acid in water), 5% buffer B (0.075% trifluoroacetic acid in acetonitrile) at a flow rate of 2.0 ml/min. IGFs were eluted using the gradient described under "Experimental Procedures." The peaks designated I and II indicate the elution positions of the two fractions containing RRA active IGF-II; —, $A_{215}$. competing activity and eluted as a broad peak at 30% buffer B; peak II eluted approximately 4 min later. Peaks I and II were run on rpHPLC using the same conditions as above and then characterized by NH$_2$-terminal amino acid sequencing. This analysis revealed that peak I contained a 1:7 mixture of IGF-I and IGF-II, respectively. Rechromatography of peak I by rpHPLC, using HFBA as the ion pairing agent, resulted in two distinct peaks (Fig. 3). The first peak eluted at 33 min; amino acid analysis indicated that it had an amino acid composition similar to IGF-I (not shown). The second peak eluted in a broad region from 34 to 37 min. Limited NH$_2$-terminal sequence analysis of 1.2 nmol of this peak and 200 pmol of peak II (Fig. 2) showed only the expected sequence of IGF-II. These results indicate that the two preparations are not contaminated by IGF-I. The yield of apparently homogeneous preparations of IGF-II obtained from peak I and II were 96 and 34 µg, respectively, from 400 g of Cohn fraction IV. This procedure has been repeated twice with similar results.

Additional evidence for the apparent homogeneity of the IGF-II in peaks I and II was obtained by SDS-PAGE. Aliquots of peak I and II were subjected to electrophoresis in a 15% acrylamide, 0.4% N,N'-methylenebisacrylamide gel polymerized in a Hoeffer minigel apparatus using the buffer system previously described (17). Electrophoresis was carried out at a constant current of 200 volts until the marker dye reached the end of the gel. Lane 1, peak I; lane 2, peak I; lane 3, LKB CNBr fragments of myoglobin having molecular weights of 16,849; 14,404; 10,800; 8,159; 6,214; lane 4, Pharmacia LKB Biotechnology Inc. low molecular weight standards having molecular weights of 94,000; 67,000; 43,000; 30,000; 20,100 and 14,400.

IGF-II in peaks I and II was obtained by SDS-PAGE. Aliquots of peak I and II were subjected to electrophoresis in a 15% SDS-PAGE gel and stained with Coomassie Blue (Fig. 4). Only one Coomassie-stained band can be detected in either lane 1 or lane 2. The mobilities of these two peptides, relative to the 8.2-kDa myoglobin fragment band in lane 3, are similar to the mobility of the mature 7.5-kDa form of IGF-II. Amino-terminal sequencing of 1.6 nmol of peak II (Fig. 2) through the first 35 residues produced the expected amino acid sequence of IGF-II with the exception that Ser-29 was replaced with the tetrapeptide Arg-Leu-Pro-Gly (Fig. 5). The yield of phenylthiodyantoin-alanine and phenylthiodyantoin-tyrosine in the first cycle was 59%, and the repetitive...
**FIG. 5.** Amino acid sequence of IGF-II variant and alignment of proteolytically derived fragments with the sequence predicted by the cDNA. N-1, amino-terminal sequence obtained from intact IGF-II variant; A1–A4, sequence of endoproteinase Asp-N fragments; G1, sequence of endoproteinase Glu-C fragment. The tetrapeptide insertion at position 29 is underlined.

| Cycle | Residue | Amount (pmol) |
|-------|---------|---------------|
| 1     | A       | 728.7         |
| 2     | V       | 534.8         |
| 3     | R       | 245.7         |
| 4     | P       | 524.5         |
| 5     | S       | 283.0         |
| 6     | E       | 287.7         |
| 7     | T       | 277.2         |
| 8     | L       | 455.2         |
| 9     | ND'     | 26 F          |
| 10    | G       | 416.7         |
| 11    | G       | 456.6         |
| 12    | E       | 168.7         |
| 13    | L       | 317.2         |
| 14    | V       | 277.8         |
| 15    | D       | 104.2         |
| 16    | T       | 139.6         |
| 17    | L       | 236.2         |

* A double sequence is commonly observed during amino-terminal sequencing of IGF-II where approximately 25% of the sequence is the des-Ala form (2). The major sequence began with Ala-1 (~80%) and the minor sequence began with Tyr-2 (~20%). The major sequence, beginning with Ala-1, is shown above.

**TABLE I**

**Amino-terminal sequence results**

The amount of phenylthiohydantoin released is shown for each of the first 35 cycles.

| Cycle | Residue | Amount (pmol) |
|-------|---------|---------------|
| 1     | A       | 728.7         |
| 2     | V       | 534.8         |
| 3     | R       | 245.7         |
| 4     | P       | 524.5         |
| 5     | S       | 283.0         |
| 6     | E       | 287.7         |
| 7     | T       | 277.2         |
| 8     | L       | 455.2         |
| 9     | ND'     | 26 F          |
| 10    | G       | 416.7         |
| 11    | G       | 456.6         |
| 12    | E       | 168.7         |
| 13    | L       | 317.2         |
| 14    | V       | 277.8         |
| 15    | D       | 104.2         |
| 16    | T       | 139.6         |
| 17    | L       | 236.2         |

* Cysteine residues were not modified prior to sequencing. The cDNA sequence predicts cysteine residues at these cycles.

* ND, not determined.

yield was 92% based on leucine at cycles 8 and 17. Table I shows the amount of each cleaved residue through cycle 35. To determine whether there were any amino acid sequence differences between this peptide and the sequence predicted by the variant cDNA sequence, two aliquots (300 pmol each) of IGF-II variant were digested with either endoproteinase Asp-N, which cleaves at the amino-terminal side of aspartic acid, or endoproteinase Glu-C, which cleaves at the carboxy-terminal side of glutamic acid residues. The peptide fragments were subsequently reduced with dithiothreitol and cysteine residues converted to carboxyimidomethylcysteine with iodoacetamide. Four fragments from the endoproteinase Asp-N digest (A1–A4) and one fragment from the endoproteinase Glu-C digest (G1) were isolated using microbore rpHPLC and sequenced. Fig. 5 shows the alignment of these peptide fragments with the sequence predicted from the cDNA. The alignment of fragments A1, A2, and A3 was determined from the overlap of the amino-terminal sequence and fragment G1.
was aligned based on the overlap of the sequence with fragment A3. Fragment A4 was generated by anomalous cleavage of the protein by endoproteinase Asp-N. The NH2-terminal glutamic acid residue of A4 provides an overlap with fragment G1. This placement is consistent with the cDNA sequence and supported by the measured molecular ion of the intact protein (see below). The sequence containing Ser-29 could not be found in either the amino-terminal sequence of intact IGF-II variant or in any of the peptide fragments isolated after endoproteinase digestion, indicating the preparations were not a mixture of IGF-II and IGF-II variant.

Plasma desorption mass spectrometry was used to determine the mass of the IGF-II variant. Molecular ions corresponding to (M+H)+, (M+2H)+, and (M+3H)+ were detected at 7809.4, 3902.6, and 2601.6 mass units, respectively. The average measured mass of IGF-II variant, calculated from these molecular ions, was 7805.5 which is in close agreement with the mass, 7811.8, calculated from the predicted amino acid sequence. Similarly, the average measured mass for 7.5-kDa IGF-II was 7426.7 which also agrees closely with the calculated mass of 7421.8. These data support the alignment of the peptide fragments in Fig. 5 and exclude any carboxyl-terminal amino acid extensions that were not detected by sequence analysis. The results shown above identify it as the IGF-II variant described by Jansen et al. (1).

Since the mitogenic effects of IGF-II may be mediated through the type I IGF receptor (29, 30), it was important to compare the binding of IGF-II variant with IGF-I and IGF-II using this receptor system. Competition for 125I-rIGF-I binding to purified IGF-I receptors is shown in the inset. The dissociation constants (Kd) for rIGF-I and IGF-II binding were 0.24 and 0.38 nM, respectively, and are consistent with the observations of Casella et al. (31). However, when 125I-rIGF-I binding was competed for with the IGF-II variant its Kd was 0.80 nM, representing a 2-3-fold reduction in affinity. Since the IGF-I receptors used in this study were affinity purified, interaction of IGF-II variant with binding sites other than those on the IGF-I receptors was unlikely.

The ability of IGF-II and IGF-II variant to stimulate the growth of MCF-7 cells was tested in a serum-free defined medium to eliminate the interference of serum-derived growth factors and binding proteins in the assay. The concentration of IGF-II and IGF-II variant required for half-maximal stimulation of growth was 4 and 13 nM, respectively (Fig. 7). Additionally, IGF-II and IGF-II variant were only able to stimulate growth to a level that was 75 and 56%, respectively, of that achieved with 10% serum. These data are consistent with the 2-3-fold lower affinity of the IGF-II variant for the IGF-I receptor (shown above).

**DISCUSSION**

In this report we describe the purification and characterization of an IGF-II variant from a pool of human plasma. The existence of this IGF-II variant was first proposed by Jansen et al. (1) based on the nucleotide sequence of a cDNA clone isolated from a human liver library. More recently, IGF-II variant cDNA clones with a 9-base pair insertion at the same position were isolated from human placenta and hepatoma cell line (Hep G2) cDNA libraries (33). The amino acid sequence of the protein described here is in agreement with the sequence predicted from the variant cDNAs. Plasma desorption mass spectrometry confirms the mass calculated from the amino acid sequence analysis and, in contrast to the variant of proIGF-II isolated by Zumstein et al. (6), excludes any carboxyl-terminal amino acid extensions.

Analysis of receptor/ligand binding using purified IGF-I receptors indicates that the IGF-II variant binds to this receptor with a lower affinity than either IGF-I or IGF-II. These results are consistent with our observations that IGF-II variant is not as potent a mitogen as is IGF-II for MCF-7 cells. The results of site-directed mutagenesis studies established that tyrosine at position 24 and particular amino acid residues in the COOH-domain of IGF-I are involved in high affinity binding to the IGF-I receptor (34, 35) and may account for our findings since the IGF-II variant contains a tetrapeptide insertion in the same region. We propose that this structural modification is responsible for the functional differences between IGF-II and IGF-II variant.

The amount of IGF-II variant that can be purified from an acetic acid extract of Cohn fraction IV, using the procedures described above, is only 3-4-fold lower than the amount of IGF-I that can be purified from the same extract. This difference may not be an accurate reflection of the true concentration of the IGF-II variant in plasma since the relative yield throughout the purification procedure may not have been the same for both peptides. The development of a specific radioligand assay will allow us to determine the ratio of IGF-II variant/IGF-II in plasma and tissues of individuals. These results will determine whether IGF-II variant occurs at a low level in all individuals (33) or whether certain individuals are predisposed toward the production of relatively high levels of this variant.

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