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Purification and characterization of insulin-like growth factor II (IGF II) and an IGF II variant from human placenta

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Abstract

In order to purify variant IGF II peptides from human placenta, we have developed a purification procedure combining heparin affinity chromatography and cation-exchange, reversed-phase and size-exclusion HPLC. Two peptides were purified, both having apparent M_r values of ca. 7300 Da as evaluated by SDS-PAGE. N-Terminal sequencing revealed IGF II and an IGF II variant in which Ser^{29} was replaced by the tetrapeptide Arg-Leu-Pro-Gly. The final yield of variant IGF II was about eight-fold lower than that of IGF II. Both pure peptides were functionally active as they bound to type I and type II IGF receptors from ovine and human placental membranes, as determined by crosslinking experiments and displacement curve studies.

1. Introduction

Insulin-like growth factor I (IGF I) and insulin-like growth factor II (IGF II) are single chain polypeptides that are structurally similar to pro-insulin. They were initially purified from a Cohn fraction of human serum [1,2]. Human IGF I and II consist of 70 and 67 amino acids, with molecular masses of 7649 and 7471 Da, respectively. Their sequences are identical for 62%. They both bind to type I and type II IGF receptors with different affinities [3]. Both variant and precursor forms exist besides these two main IGFs. An N-terminal truncated IGF I found in human brain [4,5] and in other species [6,7] as well as an N-terminal truncated IGF II found in human blood [8] probably result from alternative processing of pro-IGFs. Human IGF II forms with an apparent molecular mass >7500 Da have been described in human serum [9,10] and in spinal fluid [9]. Zumstein et al. [11] purified a 10 kDa pro-form of IGF II in which Ser^{33} was replaced by a Cys-Gly-Asp sequence. Since there is only a single copy of the human IGF II gene [12], and since Ser^{33} is not located at an intron/exon hinge region, this substitution may result from an allelic variation, the frequency of which remains to be determined by genetic studies. Finally, variant forms of human IGF II in which Ser^{29} is replaced by a Cys residue [13] or an Arg-Leu-Pro-Gly sequence [14,15] have been purified from a Cohn fraction IV of human plasma. This latter form was first predicted by Jansen et al. [16], who isolated a variant IGF II cDNA from a human liver cDNA library. This variant IGF II cDNA was subsequently isolated from a human placenta cDNA

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library [17]. The existence of an alternative splice acceptor site in the intron preceding exon 8 of the IGF II mRNA precursor accounts for the insertion of 9 nucleotides found in the variant cDNA sequence and for the replacement of a Ser residue with an Arg-Leu-Pro-Gly tetrapeptide. Schofield and Tate [18] demonstrated that the mRNA coding for this variant IGF II was present as a low but constant fraction of the major IGF II mRNA species in a number of adult and fetal organs and cell lines. IGF II and variant IGF II mRNA have also been found to be present in human fetal ovary and uterus [19]. Nevertheless, the biological effects of the variant IGF II peptides are still unknown.

Human placenta is a well-known source of IGF II activity [20]. In the present paper however, it was chosen for the first time for the isolation and characterization of multiple forms of IGF II peptides. This report describes the purification of wild type IGF II and the Ser^{29} variant IGF II from the human placenta and their ability to bind to the IGF receptors from both human and ovine placental membranes.

2. Experimental

2.1. Materials

Human recombinant IGF I (rIGF I) and IGF II (rIGF II) were from Genzyme (Boston, MA, USA) and bovine pancreatic insulin from Sigma (St. Louis, MO, USA). The IGF peptides were iodinated by the chloramine T method [21], separated from aggregated material and free $Na^{125}I$ (Amersham, UK) on a Sephadex G-50 column (Pharmacia LKB, Sweden) and eluted with 0.1 *M* NH_4HCO_3 containing 0.1% (w/v) IGF-free bovine serum albumin (BSA). The specific activity was 80–140 μCi/μg. Microsomal placental membranes were prepared as previously described [22] using human term placentae kindly provided by Dr. F. Ferré (Paris, France) or ovine term placentae from Dr. Mirmant (Magny les Hameaux, France).

2.2. Assays for IGFs

IGF II was monitored during purification by a specific radioreceptor assay (RRA) using ovine placental membranes [23] and ^{125}I-rIGF II as a tracer. Briefly, an aliquot of each fraction was dried in a SpeedVac concentrator and incubated overnight at 4°C with 15 μg membrane protein and 4000–6000 cpm ^{125}I-rIGF II in 0.3 ml of binding buffer [100 m*M* Tris-HCl, 0.25 *M* NaCl containing 0.25% (w/v) BSA, pH 7.5]. Non-specific binding (NSB) was determined with excess (100 ng) unlabelled rIGF II. The bound and free tracer was separated by adding 1.25 ml of a mixture containing 13% polyethylene glycol (PEG 6000) and 0.1% (w/v) bovine γ-globulin, followed by centrifugation for 30 min at 4000 *g*. The supernatant was removed and the radioactivity in the pellet was counted in a CG 4000 Counter (Kontron-Intertechnique, St. Quentin-en-Yvelines, France). Results corrected for NSB are expressed as microgram equivalent IGF II (μg Eq. IGF II). The same method was used for receptor-binding studies with ovine or human placental membranes using ^{125}I-rIGF I or ^{125}I-rIGF II as a tracer. The concentration of IGF I was measured by use of a specific radio immunoassay (RIA) with ^{125}I-rIGF I as a tracer and IGF I antiserum from NIH (batch UBK 487) [24]. This antiserum had ca. 1% cross-reactivity with IGF II, and minimally cross-reacted with human insulin at very high concentrations (up to 10^{-6} *M*).

2.3. Extraction from crude material

An acid placental extract (EAP, batch 8901) prepared from pooled human placentae was obtained from IMEDEX (Chaponost, France) [25]. EAP (400 ml corresponding to 7 kg placental tissue) was suspended in 20 l of demineralized water and adjusted with acetic acid and solid NaCl to a final concentration of 2 *M* acetic acid and 0.075 *M* NaCl. The material was ultrafiltered (flow-rate 30 ml/min) on a CH2 concentrator connected to a S1Y30 spiral cartridge (cut-off 30 000 Da) and diafiltered (flow-rate 10 ml/

min) on the same system connected to a S1Y3 (cut-off 3000 Da) spiral cartridge (concentrator and cartridges, Grace Amicon, Beverly, MA, USA). The resulting material was lyophilized and suspended in 400 ml of 10 m*M* Tris-HCl 7.0 containing 0.02% NaN_3 (Tris buffer). A large amount of insoluble protein was removed by centrifugation for 10 min at 4000 *g*.

2.4. Heparin affinity chromatography

The supernatant was loaded onto an 400 × 50 mm I.D. heparin-Ultrogel column (IBF Biotechnics, Villeneuve-La-Garenne, France). Unretained material was washed from the column by rinsing with 1.5 l of Tris buffer and proteins eluted with a linear gradient of NaCl (0–3 *M*) for 22 h at a flow-rate of 125 ml/h. Fractions were collected every 15 min and immediately acidified by addition of 0.2 ml of acetic acid. Protein content was determined by the method of Bradford using BSA as the standard [26]. The eluates containing RRA-IGF II activity were pooled and dialysed in Spectrapor 3 bags (Spectrum Medical Industries, Los Angeles, CA, USA) against 0.05 *M* acetic acid–sodium acetate pH 5.0 (buffer A) prior to HPLC purification.

2.5. High-performance liquid chromatography

HPLC purification was performed using a Waters HPLC system (Millipore Waters, Milford, MA, USA) consisting of a Model 510 solvent delivery system with two pumps, a Model 680 automated gradient controller and a Model 486 absorbance detector. The HPLC system was connected to a Tandon micro-computer and chromatograms were registered and integrated using the PC Integration Pack software from Softron (Kontron, St. Quentin-en-Yvelines, France).

Dialysed material was pumped onto a 250 × 22 mm I.D. hydroxy ethyl methacrylate (HEMA) 300 Sulfobutyl cation-exchange (particle size 10 μm, pore size 250 Å) HPLC column (Interchim, Asnières, France). The column was washed with buffer A and peptides were eluted with a linear gradient of 0–1.2 *M* NaCl in buffer A for 60 min (flow-rate 3.6 ml/min), followed by a linear gradient of 1.2–2 *M* NaCl in buffer A for 5 min, and isocratic elution with 2 *M* NaCl in buffer A for 20 min.

The IGF II fractions with highly increased specific activity were pooled and further purified by reversed-phase HPLC. Two solvent gradient systems were used (designated I and II) with a 250 × 4.6 mm I.D. Vydac C_{18} column, type 201 TP 5 μm (Chrompack, Middleburg, Netherlands) at a flow-rate of 0.7 ml/min. In system I, solvent A was 10 m*M* KH_2PO_4 in 50 m*M* $NaClO_4$, pH 7.0 and solvent B was 60% (w/v) acetonitrile (Prolabo) in solvent A, as described by Zumstein and Humbel [27]. Material was eluted with a gradient of 10–30% solvent B for 10 min followed by 30–60% solvent B for 55 min and 60–100% solvent B for 10 min. In system II, solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) (Baker, Deventer, Netherlands) and solvent B was 60% (w/v) acetonitrile in solvent A. The following gradients were used: system IIA: 10–35% solvent B for 10 min, 35–65% solvent B for 50 min, and 65–100% solvent B for 10 min. System IIB: 10–40% solvent B for 10 min, 40–70% solvent B for 50 min, and 70–100% solvent B for 10 min.

The final purification step was size-exclusion chromatography on a Sep protein column (250 × 20 mm I.D., particle size 5 μm, pore size 300 Å; Shandon, Eragny, France) at a flow-rate of 3.5 ml/min. Fractions (1.05 ml) containing peptide material were dried in a SpeedVac concentrator, suspended in 100 μl of acetonitrile–water (40:60, w/v) containing 0.1% (v/v) TFA and injected by use of a type 7125 injection valve (Rheodyne, Cotati, CA, USA) equipped with a 500-μl sample loop.

Purity was assessed by SDS-PAGE followed by silver staining (Silver staining kit, Bio-Rad, Richmond, CA, USA) and by N-terminal sequencing. Sequence analyses were performed at the Service central d'Analyses (CNRS, Vernaison, France) with a gas-phase protein mi-

crosequenzer (Model 470 A) from Applied Biosystems (Foster City, CA, USA) connected to a Model 120 A phenylthiohydantoin amino acid analyser (Applied Biosystems).

2.6. Covalent crosslinking of ^{125}I-IGFS

Affinity crosslinking with disuccinimidyl suberate (DSS) (Pierce Chemical Co, Rockford, IL, USA) was performed as previously described [28,29] with minor modifications. Human and ovine microsomal placental membranes (60–80 μg) were incubated overnight at 4°C with 300 000 cpm radiolabelled IGF peptides with or without an excess of unlabelled ligand in a final volume of 250 μl of 0.2 *M* phosphate buffer, pH 7.4. The membranes were then centrifuged for 5 min at 4000 *g* and the supernatants were discarded. The pellet was resuspended in 150 μl of phosphate buffer and 10 μl of DSS (dissolved in dimethysulfoxide) was added to a final concentration of 0.5 m*M*. The mixture was incubated for 15 min on ice, and the reaction was stopped by adding 1 ml of 1 m*M* EDTA in 10 m*M* Tris-HCl pH 7.4. The membranes were centrifuged for 5 min at 4000 *g* and the pellets were solubilized and boiled for 5 min in 0.0625 *M* Tris-HCl pH 6.8 containing 3% SDS, 10% glycerol, 0.005% bromophenol blue and 1% dithiothreitol (DTT). Each sample was subjected to SDS-PAGE [30] using a 16 × 18 × 0.1 cm slab-gel apparatus (Hoefer, San Francisco, CA, USA). The gels were stained with Coomassie blue R 250, dried, and exposed to Kodak X-omat RP films with a quanta III intensifying screen (Dupont Cronex, Wilmington, DE, USA) at −80°C.

3. Results

3.1. Purification of placental IGF II and variant IGF II

The purification yields and specific activities are shown in Table 1. Ultrafiltration increased the specific activity 11.3-fold with 80% recovery of the IGF II material. The protein and IGF II activity elution profiles after heparin affinity chromatography are shown in Fig. 1. Five percent of the total IGF II activity did not bind to heparin. Retained IGF II activity was eluted in fractions 15–31, with a maximum in fraction 21 (0.5 *M* NaCl). After correction for cross-reacting IGF II in the IGF I RIA, no IGF I activity was found in any fraction. Fractions 20–31 (specific activity 1.68 μg Eq. IGF II/mg protein) were pooled, desalted and chromatographed on the cation exchanger (Fig. 2). The highest amount of IGF II activity was eluted in fraction 44 followed by a tail until fraction 86. Fractions 34–64 contained 80% of the IGF II activity. However, the specific activity was only 3.6-fold increased over the preceding step and no IGF II peptide could be purified to homogeneity from these pooled fractions by using the purification procedure described here. Fractions 65–86 which had a 44-fold increased specific activity (74.1 μg Eq. IGF II/mg protein) over the heparin-eluted material were further purified by reversed-phase HPLC using system I. The IGF II activity was eluted as two fractions (A and B) (Fig. 3). Fraction A was eluted at 31.1–33.1% acetonitrile, while the more hydrophobic fraction B was eluted at 35.7–43.2% acetonitrile. Fraction A and fraction B were separately rechromatographed on an identical column using gradient elution system IIA and IIB respectively. The IGF II activity corresponding to fraction A (970 μg Eq. IGF II/mg protein) was found at 32.5% acetonitrile while fraction B (768 μg Eq. IGF II/mg protein) eluted at 33.4% acetonitrile (data not shown). A and B were run separately on the HPLC size-exclusion system (Fig. 4A), each of them eluting as a sharp symmetric peak. The final yield of peptides IGF II A and IGF II B was 10.8 and 1.33 μg Eq. IGF II/mg protein, respectively, starting from 400 ml of EAP. Their homogeneity was assessed by SDS-PAGE. Aliquots of A and B and of rIGF II (standard) were electrophoresed in a 15% SDS-PAGE gel (Fig. 4B). The apparent M_r of rIGF II was ca. 7600 Da, peptides A and B migrated slightly faster as single bands with an apparent M_r of ca. 7300 Da. This slight difference may be due to the presence of a methylation site in rIGF II.

Table 1
Specific IGF II activities at each purification step

Purification step	RRA-IGF II activity (μg Eq.)	Specific activity (μg Eq. IGF II/mg protein)	Purification factor
EAP (crude extract)[a]	1130	0.0653	1
Ultrafiltration[a]	900	0.738	11.3
Heparin-Ultrogel[a]	526	1.68	25.7
HEMA 300 SB cation exchanger (2 *M* eluted material)[b]	68.6	74.1	1130
Vydac 201 TP System I[b]			
Fraction A	36.8	190	2910
Fraction B	9.11	138	2110
System II[b]			
Fraction A	20.3	970	14900
Fraction B	1.89	768	11800
Size exclusion HPLC Sep prot 300[b]			
Peak A	10.8	1110	17000
Peak B	1.33	1090	16700

[a] Protein content determined by the method of Bradford [26].
[b] Protein content estimated by the area of HPLC peaks. Calibration with BSA.

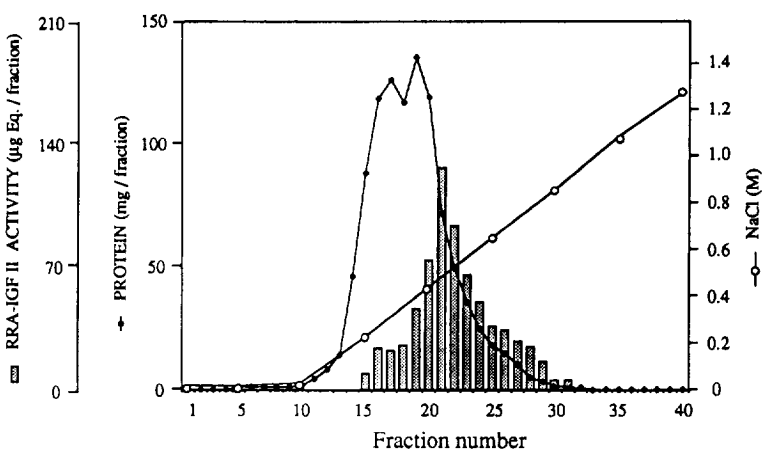

Fig. 1. Affinity chromatography of human placental extract on heparin-Ultrogel A4R. Acid placental extract (400 ml) was ultrafiltered and lyophilised as described in Experimental. Lyophilised powder was suspended in 10 m*M* Tris-HCl pH 7.0 and applied to a 400 × 50 mm I.D. column. Non-adsorbed proteins were washed out by rinsing for 16 h with 1.5 l of Tris buffer. Adsorbed material was eluted with a linear gradient of NaCl at a flow-rate of 125 ml/h. Fractions were collected every 15 min. The vertical bars denote the IGF II activity in each fraction. Fractions 20–31 were pooled for reversed-phase HPLC.

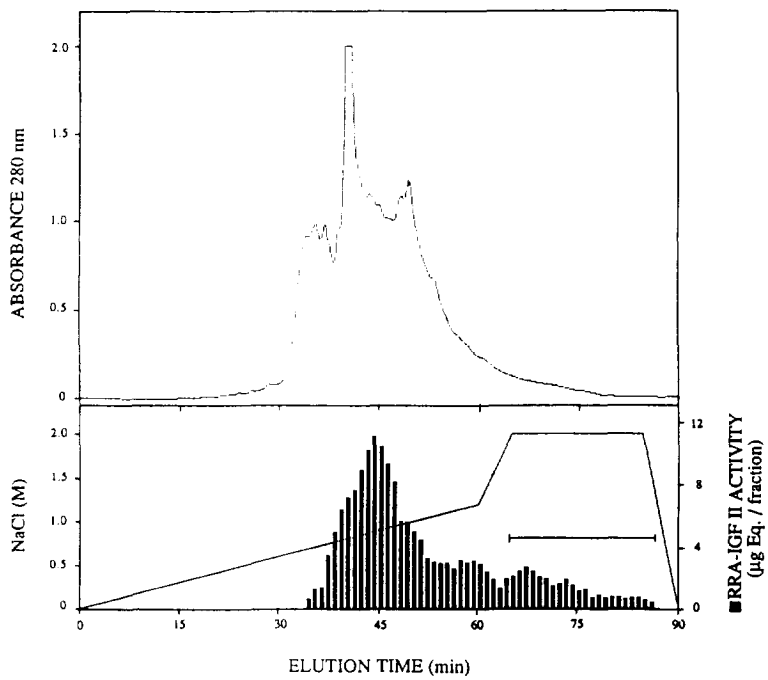

Fig. 2. Cation-exchange HPLC profiles of protein (upper panel) and IGF II activity (lower panel) of one third of the pooled IGF II fractions from the heparin column. The eluent was 2 *M* NaCl in 0.05 *M* acetic acid–sodium acetate, pH 5.0. Flow-rate was 3.6 ml/min. The vertical bars denote the IGF II activity in each fraction. Fractions 65-86 were pooled for further purification.

3.2. Amino acid sequence analysis

Samples (200 pmol) of each peptide were sequenced from the amino terminus (Table 2). The NH_2 terminal sequence of peptide A was similar to that of IGF II [1]. Peptide B was identical to the variant IGF II purified by others from human plasma, in which Ser 29 is replaced by the tetrapeptide Arg-Leu-Pro-Gly [14]. Consistent with previous reports [1,8], as much as 15–25% of both A and B lacked the N-terminal alanine, and thus tyrosine was the first amino acid residue.

3.3. Crosslinking experiments

Samples of ovine placental membranes were incubated and covalently crosslinked with pure ^{125}I-radiolabelled peptides A (wild type IGF II) and B (Ser^{29} variant IGF II) or ^{125}I-rIGFs prior to SDS-PAGE (Fig. 5A). A single band with a molecular mass of 250 kDa was observed when the ^{125}I-radiolabelled peptides A or B were used as ligands (lanes 4 to 9) as well as with ^{125}I-rIGF II (lanes 1 to 3). With excess unlabelled rIGF II this band completely disappeared, but it was always present with insulin excess of up to 10 μg. This is consistent with a ^{125}I-IGF–type II IGF receptor complex [28,31]. The low or undetectable content of type I IGF receptors in ovine placental membranes was confirmed by the absence of any binding of ^{125}I-IGF I. There was a similar binding of ^{125}I-IGF II like peptides to the type II IGF receptor in human placental membranes (Fig. 5B). In addition, ^{125}I-peptide A, ^{125}I-peptide B, ^{125}I-rIGF II, as well as ^{125}I-rIGF I bound to a 135 kDa receptor complex thought to be a blend of the α-subunit of the type I IGF receptor and of the α-subunit of the insulin receptor, since they both are present in human placental tissue [32]. The bound radioactivity was specifically displaced by 200 ng unlabelled rIGF II or 10 μg unlabelled insulin. A 270 kDa band exhibiting identical binding specificities

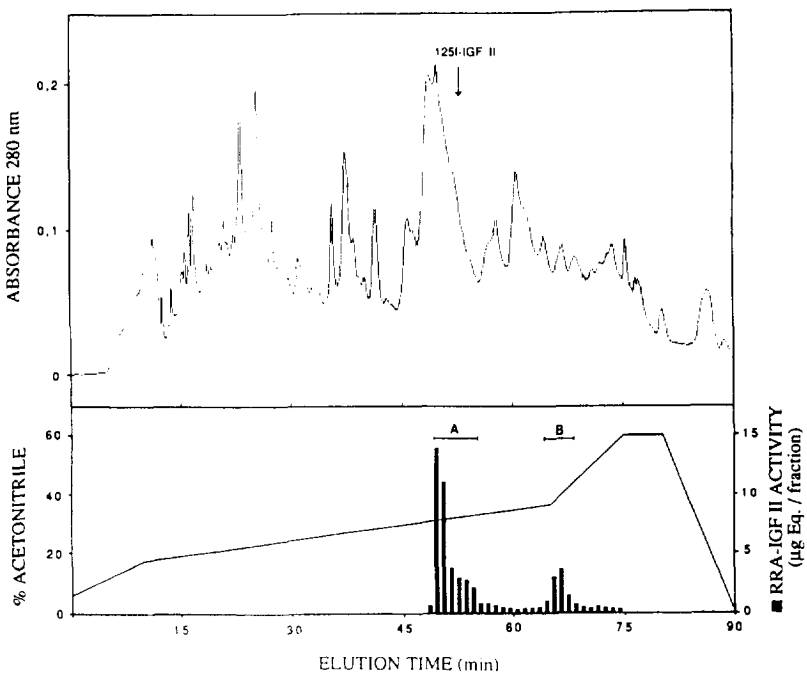

Fig. 3. Reversed-phase HPLC profiles of protein (upper panel) and IGF II activity (lower panel) of the IGF II fractions from cation-exchange HPLC. The elution position of the ^{125}I-radiolabelled rIGF II as a marker is indicated by the arrow. The vertical bars denote the IGF II activity in each fraction. |-A-| and |-B-| indicate the two pools which were taken for further purification.

may be most likely a dimeric form of the 135 kDa band, since it disappeared when lower concentrations of DSS were used (data not shown).

3.4. Displacement curve studies

The specific binding of purified peptides A and B to the type II IGF receptor in ovine placental membranes was measured by competitive binding using ^{125}I-rIGF II as a ligand. The maximal specific binding of ^{125}I-rIGF II was 52%, whereas the binding of ^{125}I-rIGF I was less than 4%. Peptide A, peptide B and rIGF II all competed with ^{125}I-rIGF II (Fig. 6A). Half maximal displacement (IC_{50}) was obtained with 0.68–0.70 ng of unlabelled peptide, showing that the Ser^{29} variant as well as the extracted and recombinant IGF II had similar affinity for the type II IGF receptor. ^{125}I-rIGF II was not displaced from ovine placental membranes by up to 10 ng rIGF I per tube. The maximal specific binding of ^{125}I-rIGF II to human placental membranes was 18%, and 23% for ^{125}I-rIGF I. The three IGF II peptides gave parallel displacement curves for ^{125}I-rIGF I, with significantly less affinity than rIGF I (Fig. 6B). The Ser^{29} variant had the lowest affinity, with an IC_{50} of 3.3 ng, as compared to 1.9 ng for the recombinant and extracted IGF II, and 0.62 ng for rIGF I.

4. Discussion

We have purified IGF II and a Ser^{29} variant of IGF II from a human placenta pool. This is, to our knowledge, the first report of the purification of IGF peptides from human tissue extracts rather than blood fractions. The placenta was chosen as a convenient source of IGF II peptides because of its richness in IGF II material [20,33] and its ready availability. With a mean of 2820 ng Eq. IGF II per ml, EAP had a high IGF II content compared to human serum, which contains 600–700 ng IGF II per ml. No IGF II peptide could be purified from EAP by using the

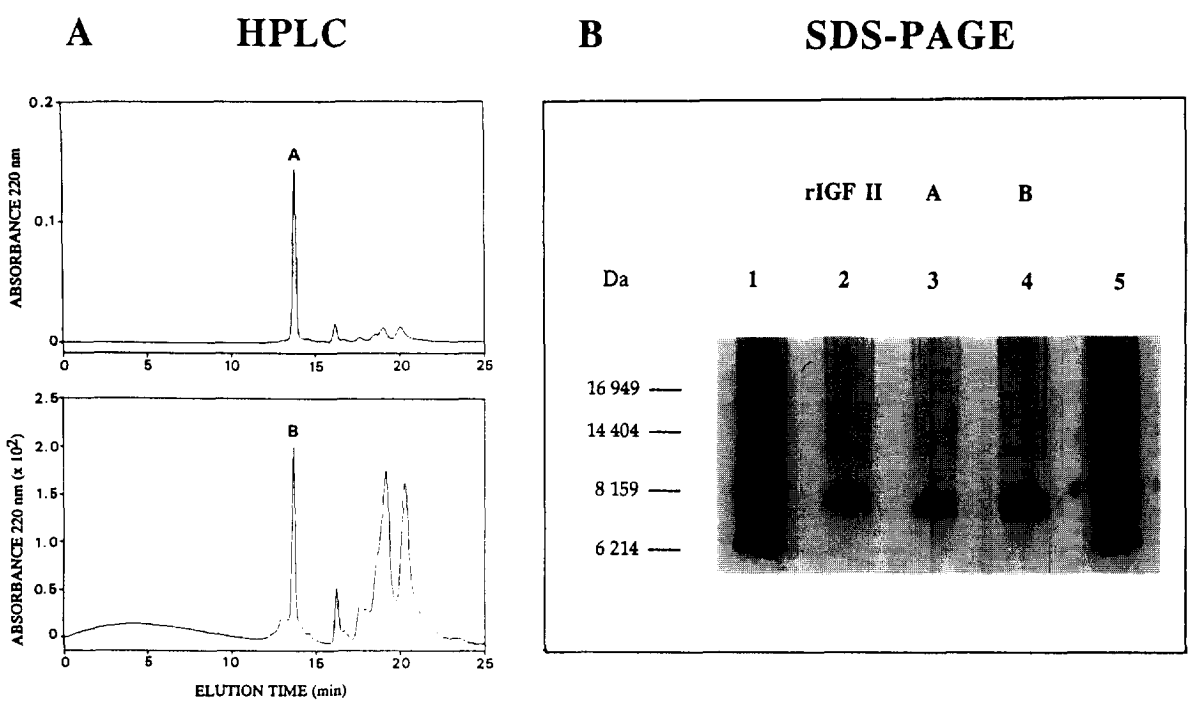

Fig. 4. Final purification of reversed-phase HPLC fractions A and B as evaluated by (A) size exclusion HPLC and (B) SDS-PAGE. Fractions A and B were each chromatographed on a Sep Prot 300 column (A). The eluent was acetonitrile–water–TFA: 40:60:0.1. Flow-rate was 3.5 ml/min. The peaks designated A and B indicate the elution positions of the two fractions containing the RRA-IGF II activity. Samples attributable to peaks A and B were electrophoresed separately in a 15% SDS-PAGE and silver stained (B). Lanes 1 and 5: myoglobin fragments (Electran, BDH); lane 2: rIGF II (GENZYME); lane 3: A; lane 4: B.

conventional IGF II purification procedures. An efficient purification procedure for placental-derived IGF II peptides was thus developed.

Three extraction methods were initially evaluated for primary extraction. Hydrochloric acid–ethanol extraction according to Daughaday et al. [34] gave a mean of 102% recovery of the IGF II activity with a 1.5-fold increased specific activity over the crude extract. Using the formic acid–acetone extraction according to Bowsher et al. [35], a mean of 48.2% of the IGF II activity was recovered with a 3.2-fold increase of specific activity. As ultrafiltration enabled a 11.3-fold increase of specific activity and a low loss of IGF II activity, it was preferentially chosen.

Until now, only little advantage has been taken of the heparin-binding property of IGF II [36]. One of the major advantages of preparative heparin affinity chromatography for the purification of IGF II peptides from EAP was the complete removal of contaminating IGF I activity, which was not retained on the heparin matrix. Both pH and salt gradients were used for further purification on the HEMA 300 sulfobutyl cation-exchanger. pH-Step gradients with ammonium acetate buffers, which are generally used for chromatography on Sulfopropyl columns, caused a large loss of IGF II activity and did not lead to reproducible separations. Thus, a sodium chloride gradient at pH 5.0, which gave more satisfactory results, was preferred. The material eluting at a concentration of 0.4–0.9 *M* NaCl after heparin chromatography was shown to be highly heterogeneous in IGF II activity when chromatographed on the HPLC cation exchanger. Partial characterization of the discarded fractions (34–64) indicated that they did not contain peptide IGF II B any more. How-

Table 2
N-Terminal sequences of peptides IGF II A and IGF II B

Peptide IGF II A						Peptide IGF II B					
Cycle	Amino acid	Yield (pmol)	Cycle	Amino acid	Yield (pmol)	Cycle	Amino acid	Yield (pmol)	Cycle	Amino acid	Yield (pmol)
1	Ala	194	23	Asp	3.7	1	Ala	43.0	23	Asp	1.8
2	Tyr	45.0	24	Arg	9.4	2	Tyr	18.4	24	Arg	2.7
3	Arg	45.8	25	Gly	6.1	3	Arg	12.7	25	Gly	0.9
4	Pro	60.4	26	Phe	8.0	4	Pro	19.5	26	Phe	2.0
5	Ser	11.0	27	Tyr	8.1	5	Ser	3.8	27	Tyr	1.9
6	Glu	32.9	28	Phe	5.1	6	Glu	11.5	28	Phe	1.3
7	Thr	11.3	29	Ser	[a]	7	Thr	4.5	**29**	**Arg**	**2.2**
8	Leu	35.1	30	Arg	2.2	8	Leu	10.4	**30**	**Leu**	**2.0**
9	–[b]		31	Pro	4.0	9	–		**31**	**Pro**	**1.3**
10	Gly	37.7	32	Ala	3.7	10	Gly	8.9	**32**	**Gly**	**1.2**
11	Gly	[a]	33	Ser	[a]	11	Gly	[a]	33	Arg	1.5
12	Glu	15.0	34	Arg	2.2	12	Glu	4.5	34	Pro	0.9
13	Leu	21.9	35	Val	2.5	13	Leu	6.6	35	Ala	1.2
14	Val	20.6	36	Ser	0.5	14	Val	6.1	36	–	
15	Asp	13.6	37	Arg	2.2	15	Asp	3.8	37	Arg	0.4
16	Thr	6.5	38	Arg	[a]	16	Thr	2.3	38	Val	0.6
17	Leu	14.0	39	Ser	[a]	17	Leu	3.6	39	Ser	[a]
18	Gln	14.1	40	–		18	Gln	3.9	40	Arg	0.4
19	Phe	13.5	41	–		19	Phe	3.3			
20	Val	11.3	42	Ile	0.8	20	Val	3.1			
21	–		43	Val	0.5	21	–				
22	Gly	8.7	44	Glu	0.5	22	Gly	1.9			

[a] Not accurately quantified.
[b] –: Unidentified amino acid.
The variant sequence in peptide IGF II B is indicated in bold.

ever, IGF II material with the same physicochemical properties as peptide IGF II A was found in addition to a more hydrophylic IGF II material (data not shown). The latter, which may contain glycosylated precursor forms of IGF II [37], should be further investigated. Reversed-phase HPLC of fractions 65–86 at a neutral pH enabled separation of peptides IGF II A and IGF II B. The significant loss of IGF II activity after this purification step may be due to the neutral pH, which is not suitable when IGF II is relatively pure: contrary to most peptides and proteins, whose denaturation increases with decreasing pH, IGFs are stable at an acidic pH. Thus reversed-phase HPLC was carried out under acidic conditions by using TFA as an ionic modifier in protein–peptide interactions. Since peptide IGF II B was found to be more hydrophobic than peptide IGF II A, gradient system IIB was used so that peptide IGF II B elution occurred in the gentle slope rather than in the final steep slope of the gradient, which may lead to a lower separation efficiency. Size-exclusion HPLC provided complete purification. The addition of 40% acetonitrile to the mobile phase enabled to avoid strong adsorption phenomena on the column matrix. The final yield of Ser^{29} variant IGF II was about 8-fold lower than for IGF II in this experiment and was up to 5-fold lower in some experiments using either the same placental extracts or purification procedure (final yield range from 4 experiments: IGF II = 8.92–15.3 μg; Ser^{29} variant IGF II 1.33–3.12 μg). This result is in good accordance with the 4:1 IGF II/Ser^{29} variant IGF II ratio isolated from a human plasma pool [14]. However, depending

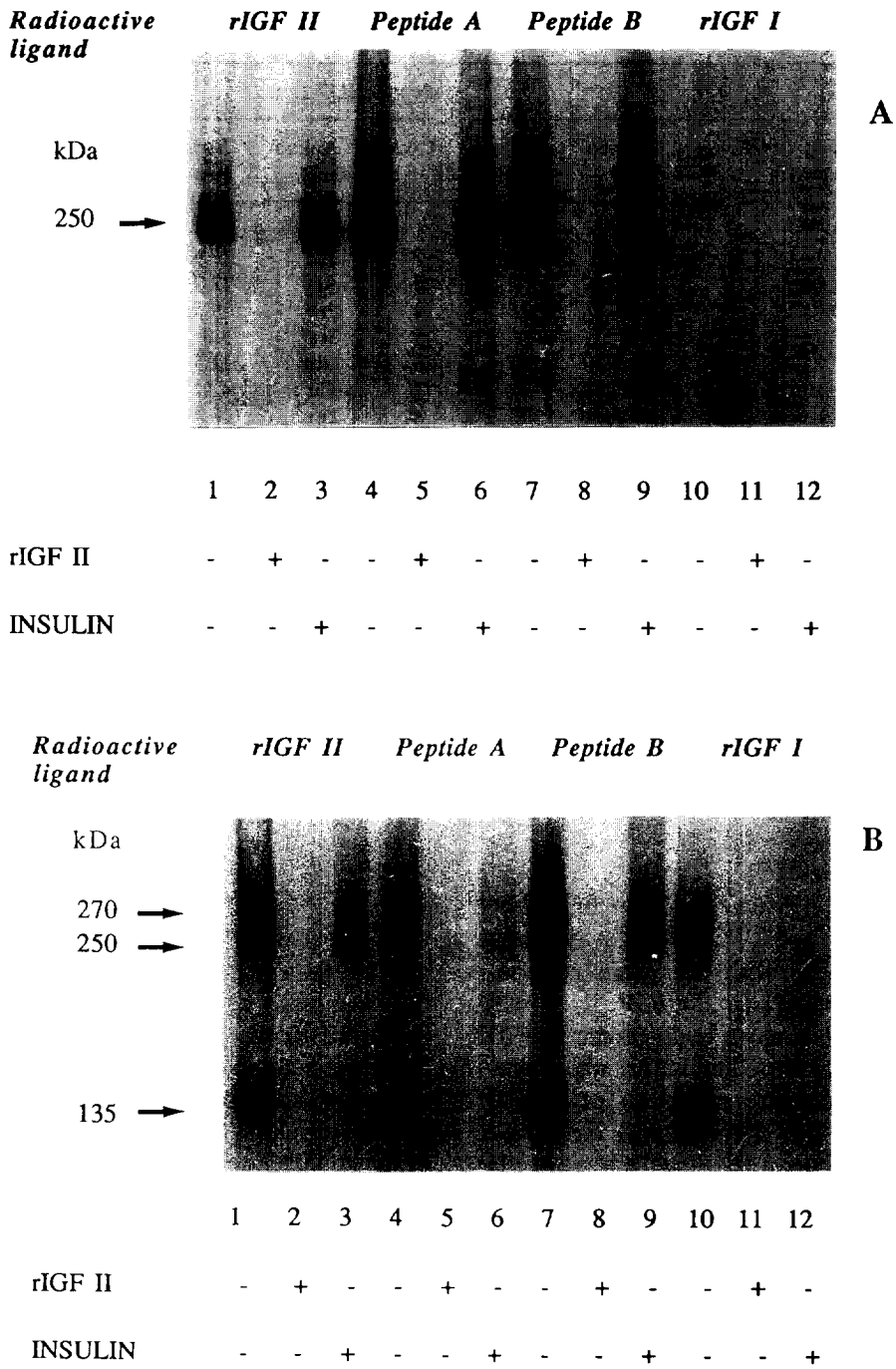

Fig. 5. Covalent crosslinking of radiolabelled IGF peptides to ovine placental membranes (A) and human placental membranes (B) in the presence or absence of excess unlabelled rIGF II (200 ng) or insulin (10 μg). Electrophoresis was carried out under reducing conditions (see Experimental). The markers used were myosin 200 000 Da, β-galactosidase 116 250 Da, phosphorylase b 97 400 Da, BSA 66 200 Da, ovalbumin 42 700 Da (Bio-Rad). Lanes 1, 2, 3: ^{125}I-rIGF II; lanes 4, 5, 6: ^{125}I-IGF II peptide A; lanes 7, 8, 9: ^{125}I-IGF II peptide B; lanes 10, 11, 12: ^{125}I-rIGF I.

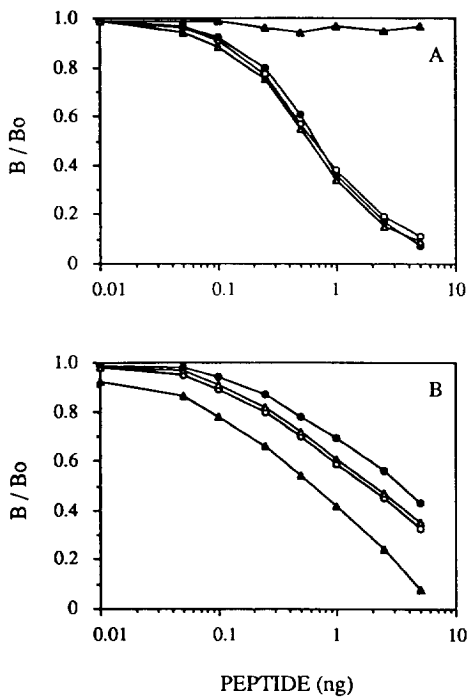

Fig. 6. Competitive binding curves. (A) Displacement of ^{125}I-rIGF II binding to ovine placental membranes. (B) Displacement of ^{125}I-rIGF I binding to human placental membranes. Each point corrected for NSB is the mean of a triplicate determination. (△) rIGF II, (▲) rIGF I, (○) IGF II peptide A, (●) IGF II peptide B.

on the experimental conditions, purification could lead to unequal loss or enrichment and this ratio cannot be determined from the final purification yields. Since no monoclonal antibodies to the variant IGF II are available, the exact ratio of each peptide in biological fluids has not yet been determined.

Binding experiments were carried out on both ovine placental membranes, which contain only type II IGF receptors, and human placental membranes, which contain both type I and type II IGF receptors [22]. This was confirmed in our membrane preparations by crosslinking studies. The ovine placental membranes showed no difference in the affinities of IGF II and variant IGF II. This result is in agreement with that reported by Lüthi et al. [38] who found that variant IGF II had a 10% higher affinity for BHK type II IGF receptor overexpressing cells than did IGF II. The small difference could be due to the use of different assay systems. The variant IGF II was less potent at inhibiting the binding of ^{125}I-rIGF I to human placental membranes than were IGF II or IGF I. Hampton et al. [14] using type I IGF receptors purified from human placenta, and Lüthi et al. [38] using the HIGR1 cell line which expresses only the type I IGF receptor, showed that variant IGF II is bound to a lower extent than IGF II. Thus, the difference in the affinities of IGF II and variant IGF II for human placental membranes should reflect a lower affinity of variant IGF II for the type I IGF receptor.

Site directed mutagenesis experiments indicate that the B–C hinge region of IGFs is important for peptide binding to the type I IGF receptor [39]. The structural modification of the Ser^{29} variant IGF II, which is located in this region, is probably responsible for its lower affinity for the type I IGF receptor. The biological potency of both placental IGF II and Ser^{29} variant IGF II is currently under investigation.

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