Biochem. J. (1988) 251, 95–103 (Printed in Great Britain)

Insulin-like growth factors 1 and 2 in bovine colostrum

Sequences and biological activities compared with those of a potent truncated form

Geoffrey L. FRANCIS,*‡ Faye M. UPTON,* F. John BALLARD,* Kerrie A. McNEIL† and John C. WALLACE†

*C.S.I.R.O. Division of Human Nutrition, Kintore Avenue, Adelaide, South Australia 5000, and †Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5000, Australia

1. Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) together with a truncated form of IGF-1 were purified to homogeneity from bovine colostrum. 2. Two forms of IGF-1 were totally resolved from IGF-2 in the purification by h.p.l.c. involving cation-exchange and reverse-phase columns. 3. The complete amino acid sequences for all three forms of IGF were determined. The sequence of bovine IGF-1 was found to be identical with that of human IGF-1, and that of the variant lacked the *N*-terminal tripeptide Gly-Pro-Glu (−3N:IGF-1). Bovine IGF-2 was found to differ in three residues of the C-domain compared with human IGF-2, with serine, isoleucine and asparagine substituted for alanine, valine and serine respectively at positions 32, 35 and 36. 4. Protein synthesis in L6 rat myoblasts was stimulated and protein degradation inhibited in a co-ordinate response with all three IGFs. The relative potency in both processes was −3N:IGF-1 > IGF-1 > IGF-2. A similar order of potency was obtained for the stimulation of DNA synthesis by −3N:IGF-1 and IGF-1. The approximately 10-fold effect on biological activity of removing the *N*-terminal tripeptide is unexpected in view of current information on IGF-1 structure and function.

INTRODUCTION

The insulin-like growth factors IGF-1 and IGF-2, first isolated from human serum (Rinderknecht & Humbel, 1976), have been shown to stimulate a pleiotrophic growth response affecting rates of protein synthesis, protein degradation, DNA synthesis and the transport of substrates (Van Wyk, 1984; Ballard *et al.*, 1986). Both IGF-1 and IGF-2 have been shown to promote the growth of hypophysectomized as well as normal animals (Schoenle *et al.*, 1982, 1985; Hizuka *et al.*, 1986). The insulin-like growth factors have been isolated in a purified state from only a few species, including human, rat and the cow (Rinderknecht & Humbel, 1978*a*,*b*; Marquardt *et al.*, 1981; Rubin *et al.*, 1982; Francis *et al.*, 1986; Honegger & Humbel, 1986). Purification from human and bovine serum (Zumstein & Humbel, 1985; Blum *et al.*, 1986; Honegger & Humbel, 1986) and bovine colostrum (Francis *et al.*, 1986) has indicated that a family of minor forms may also exist *in vivo*.

Assessment of the importance of variant forms of IGF requires isolation of sufficient quantities for structural and biological studies. Previously we reported the partial sequence of a truncated form of bovine IGF-1 that co-purified with apparently authentic IGF-1 (Francis *et al.*, 1986). Sara *et al.* (1986) have also reported a truncated IGF-1 isolated from human brain that was only partly characterized owing to the small quantities obtained. We now report an improved purification procedure from bovine colostrum that has provided sufficient yields to complete the amino acid sequencing of this modified IGF-1. IGF-1 and IGF-2 purified at the same time were also sequenced. The effects of IGF-1, truncated IGF-1 and IGF-2 on protein synthesis, protein degradation and DNA synthesis in L6 myoblasts were compared to determine whether differences in biological potency occur between the different forms.

EXPERIMENTAL

Materials

Colostrum was collected before suckling on the day of parturition and supplied by Mr. B. Euwing, Mylor, South Australia, Australia. The colostrum was stored at −20 °C. [4,5-^{3}H]Leucine (specific radioactivity 40–60 Ci/mmol), [*Me*-^{3}H]thymidine (50 Ci/mmol), iodo-[2-^{14}C]acetic acid (56 mCi/mmol) and carrier-free Na^{125}I were purchased from Amersham International, Amersham, Bucks., U.K. Foetal bovine serum was obtained from Flow Laboratories, North Ryde, N.S.W., Australia. Sources for media, antibiotics and the L6 myoblasts have been given previously (Ballard, 1982). Bovine serum albumin (radioimmunoassay grade) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., heptafluorobutyric acid (sequencing grade) was from Beckman Instruments, Palo Alto, CA, U.S.A., trifluoroacetic acid (synthesis grade) was from Merck, Darmstadt, Germany, and 1,1,2-trichloro-1,2,2-trifluoroethane (A.R. grade), propan-1-ol and acetonitrile (h.p.l.c. grade) were from Mallinckrodt, Paris, KY, U.S.A. Solvents for h.p.l.c. were filtered through 0.22 μm-pore-size filters (Durapore type) supplied by Millipore Corp., Bedford, MA, U.S.A. Columns and packings for h.p.l.c. were purchased from Amicon, Danvers, MA, U.S.A., Millipore–Waters, Milford, MA, U.S.A., Pharmacia Pty. Ltd., Sydney, N.S.W.,

Abbreviations: IGF, insulin-like growth factor(s); IGF-1, insulin-like growth factor 1; IGF-2, insulin-like growth factor 2; −3N:IGF-1, des-Gly-Pro-Glu-IGF-1.

‡ To whom correspondence should be addressed.

Australia, and Brownlee Laboratories, Santa Clara, CA., U.S.A. Endoproteinase Glu-C was purchased from Boehringer Mannheim, North Ryde, N.S.W., Australia. Other materials used in the purification were obtained from suppliers listed previously (Francis *et al.*, 1986).

Measurement of IGF

Bovine IGF-1, −3N:IGF-1 and IGF-2 were each iodinated to a specific radioactivity of approx. 100–150 Ci/g by using the chloramine-T method as described by Van Obberghen-Schilling & Pouyssegur (1983). Separation of the labelled peptides from iodide that had not reacted and aggregated products was accomplished on a Sephadex G-100 column equilibrated with 50 mM-sodium phosphate buffer, pH 6.5, containing 0.2% (w/v) bovine serum albumin.

The bioassay for total IGF activity based on the stimulation of protein-synthesis activity in confluent L6 rat myoblasts has been described previously (Francis *et al.*, 1986). Briefly, this involved measuring the incorporation of [^{3}H]leucine into cell protein during a 17 h incubation. The activity is expressed as percentage stimulation above a buffer control.

Measurement of IGF-1 by radioimmunoassay was carried out as described previously (Read *et al.*, 1986) except that the monoclonal antibody used previously was replaced by a rabbit antiserum to IGF-1 kindly provided by Dr. L. Underwood and Dr. J. J. Van Wyk through the National Hormone and Pituitary Program of the National Institute of Diabetes, Digestive and Kidney Disease. Under the conditions of the assay 50% displacement of the tracer was achieved with the addition of 50 pg of IGF-1, 200 pg of −3N:IGF-1 and 2 ng of IGF-2 per tube.

Measurement of IGF-2 by competitive binding to L6 rat myoblasts was carried out essentially as described previously (Ballard *et al.*, 1986), except that the radioactive ligand used was ^{125}I-labelled bovine IGF-2. Under the conditions employed 25% displacement of bound tracer occurred with the addition of 1 μg of IGF-1, 250 ng of −3N:IGF-1 and 4 ng of IGF-2 per well. Half-maximum competition was achieved by the addition of 20 ng of IGF-2.

The protein content of IGF preparations was measured by their absorbance at 280 nm and quantified by using crystalline pig insulin as standard.

Purification of IGF

The purification was performed by following the method of Francis *et al.* (1986) as outlined below. Detailed descriptions are presented for steps that have been changed substantially.

Acid extraction. Approx. 4 litres of colostrum were centrifuged at 20000 *g* for 20 min at 5 °C. The infranatant (3.9 litres) was stirred with 0.33 vol. of 1,1,2-trichloro-1,2,2-trifluoroethane for 15 min at 5 °C before centrifugation at 3500 *g* for 15 min. The lipid-free supernatant (3.4 litres) was adjusted to pH 2.5 with acetic acid while being stirred vigorously before an equal volume of 1 M-acetic acid containing 250 mM-NaCl was added, and stirring was continued overnight at 5 °C. Acetonitrile was added at a final concentration of 10% (v/v) and the mixture stirred for a further 15 min, whereupon centrifugation at 20000 *g* for 20 min yielded a clear extract (4.5 litres). The large pellet contained more than 90% of the protein but little protein-synthesis-stimulating activity.

SP-Sephadex cation-exchange chromatography. This step was carried out as described previously (Francis *et al.*, 1986).

Concentration on silica C_{18}. The eluate from SP-Sephadex chromatography was adjusted to 0.1% (v/v) trifluoroacetic acid and 10% (v/v) acetonitrile and the pH to 2.1 with 6 M-HCl. This mixture was stirred overnight, and the precipitate that formed was removed by centrifugation at 20000 *g* for 20 min. The supernatant was pumped at a flow rate of 15 ml/min on to a 2.2 cm-diameter glass column containing 12 g of Amicon silica C_{18} chromatography medium equilibrated with 10% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The column was washed with the equilibration solution, and the IGFs were eluted with 100 ml of 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 2 ml/min.

Fractogel TSK gel-permeation h.p.l.c. The eluate was freeze-dried and redissolved in 60 ml of 1 M-acetic acid. The clear solution was chromatographed at a flow rate of 2 ml/min on a 5 cm diam. × 100 cm column of Fractogel TSK HW-55 (S) previously equilibrated with 1 M-acetic acid.

First reverse-phase h.p.l.c. Following the acid gel-permeation h.p.l.c., IGF-containing fractions that were eluted between 0.9 and 1.0 column volumes were adjusted to 0.13% (v/v) heptafluorobutyric acid and applied to an Amicon silica C_{18} h.p.l.c. column (22 mm diam. × 250 mm) equilibrated with 20% (v/v) acetonitrile in 0.13% (v/v) heptafluorobutyric acid. This concentration of heptafluorobutyric acid was maintained throughout. The IGFs were then eluted at a flow rate of 5 ml/min as a single peak of bioactivity by a two-step linear gradient, from 20% to 35% (v/v) acetonitrile in 10 min and then to 56% (v/v) acetonitrile over 210 min.

Second reverse-phase h.p.l.c. This step was carried out as described by Francis *et al.* (1986), whereon three peaks of protein-synthesis-stimulating activity were eluted by a two-step linear gradient to 44% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The largest of these peaks, containing more than 80% of the bioactivity, was selected for further purification.

Mono S cation-exchange chromatography. The pooled fractions were pumped directly on to a Pharmacia Mono S HR 5/5 column equilibrated with 10 mM-ammonium acetate buffer, pH 4.8, in 10% (v/v) acetonitrile. Protein, monitored by the absorbance at 280 nm, was eluted at a flow rate of 1 ml/min with a gradient of 10 mM- to 1 M-ammonium acetate buffer, pH 4.8, in 10% (v/v) acetonitrile, and fractions were assayed in the protein-synthesis bioassay and IGF-2 radio-receptor assay (Fig. 1). Two pools were taken for further purification; the first (pool A) contained the IGF-1 radioimmunoassay and protein-synthesis-stimulating activity and the second (pool B) contained all the IGF-2 radio-receptor assay activity.

Third reverse-phase h.p.l.c. Each pool was adjusted to 0.1% (v/v) trifluoroacetic acid and injected separately on to a Waters Nova-Pak C_{18} radial compression

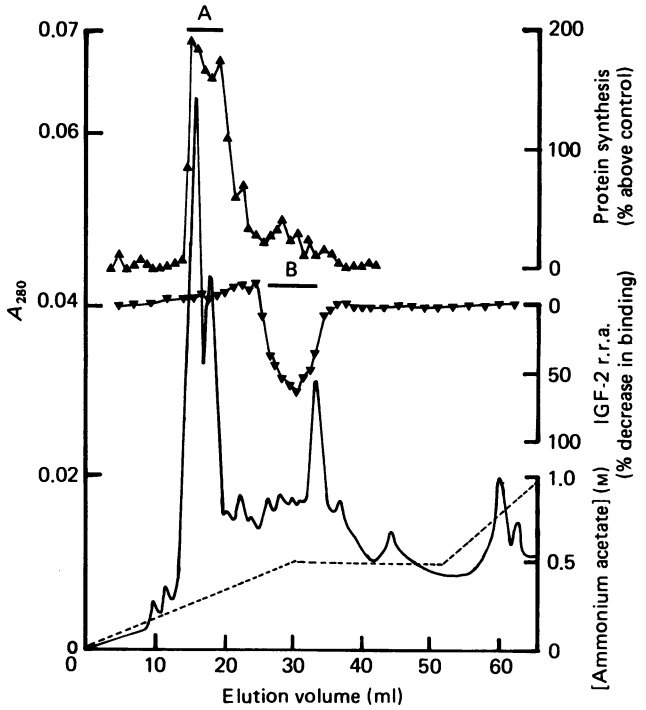

Fig. 1. Cation-exchange chromatography of the IGF pool from the second h.p.l.c. step

Elution of bioactivity (▲), IGF-2 radio-receptor assay (r.r.a.) activity (▼) and A_{280} (——) from a Pharmacia Mono S HR 5/5 column was achieved by a gradient of ammonium acetate buffer, pH 4.8, in 10% (v/v) acetonitrile (------). Fractions pooled for subsequent purification steps are indicated by solid bars (A and B).

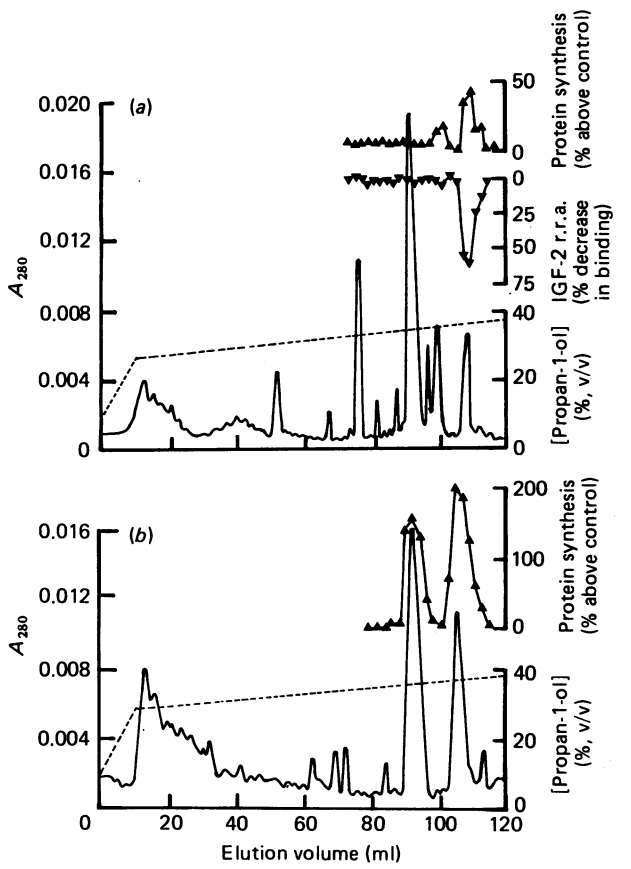

Fig. 2. Final reverse-phase h.p.l.c. step in the purification of (*a*) IGF-2 and (*b*) IGF-1 and −3N:IGF-1

Elution of protein monitored as A_{280} (——) and bioactivity (▲) or IGF-2 radio-receptor assay (r.r.a.) activity (▼) was achieved by a gradient of propan-1-ol in 0.13% (v/v) heptafluorobutyric acid (------).

cartridge (8 mm diam. × 100 mm) previously equilibrated with 10% (v/v) propan-1-ol in 0.1% (v/v) trifluoroacetic acid. All solvents used in this step contained 0.1% (v/v) trifluoroacetic acid. A two-step linear gradient, from 10% to 16.3% (v/v) propan-1-ol in 10 min and then to 19.8% (v/v) propan-1-ol over 157 min, was applied at a flow rate of 1 ml/min. The elution positions of IGF-1 and −3N:IGF-1 in this h.p.l.c. of pool A were identical, whereas for pool B IGF-2 was eluted at a slightly higher concentration of propan-1-ol (results not shown).

Fourth reverse-phase h.p.l.c. After adjustment of the pools to 0.13% (v/v) heptafluorobutyric acid, they were injected separately on to the same column as above but equilibrated with 10% (v/v) propan-1-ol. All solvents for this step contained 0.13% (v/v) heptafluorobutyric acid. The propan-1-ol concentration was stepped up to 27.5% (v/v) and the IGFs were eluted with a linear gradient to 41.5% (v/v) over 140 min at a flow rate of 1 ml/min. Fractions were assayed in the protein synthesis bioassay and IGF-2 radio-receptor assay (Fig. 2).

Sequencing of purified IGF

Approx. 3 nmol of IGF was dried in a polypropylene tube under vacuum and dissolved in 200 μl of denaturation buffer containing 6 M-guanidine hydrochloride, 0.5 M-Tris and 5 mM-EDTA with the pH adjusted to 8.5 with 6 M-HCl. Next 250 nmol of DL-dithiothreitol in 50 μl of denaturation buffer was added, and the solution was incubated under N_2 in the dark at 37 °C for 2 h. Reduction of the IGF was checked by chromatography of a sample of the reduction mixture containing 0.1 nmol of IGF on a Brownlee RP-8 (2 mm diam. × 30 mm) cartridge column equilibrated with 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. The IGF was eluted by a linear gradient of acetonitrile to 60% (v/v) in 0.1% (v/v) trifluoroacetic acid over 30 min. Under these conditions the reduced IGF was eluted approx. 6 min later than the oxidized form. To the reduced IGF was added 750 nmol of iodoacetic acid containing 5 μCi of iodo[2-^{14}C]acetic acid in denaturation buffer, and the mixture was incubated as before. Subsequently 2.5 μmol of iodoacetic acid dissolved in denaturation buffer and adjusted to pH 8.5 with 5 M-NaOH was added, and the incubation was continued for a further 2 h. The reaction was stopped by the addition of 10 μl of 2-mercaptoethanol, and the [^{14}C]carboxymethylated IGF was recovered by h.p.l.c. as above. Completion of the reaction was checked by analysis on h.p.l.c. as above, during which carboxymethylated IGF was eluted at a position intermediate between the reduced and the oxidized forms.

The [^{14}C]carboxymethylated IGF was dried under vacuum and dissolved in 100 μl of 10 mM-HCl, after which 400 μl of 0.1 M-ammonium bicarbonate buffer, pH 7.8, containing 2 mM-EDTA was added, followed by

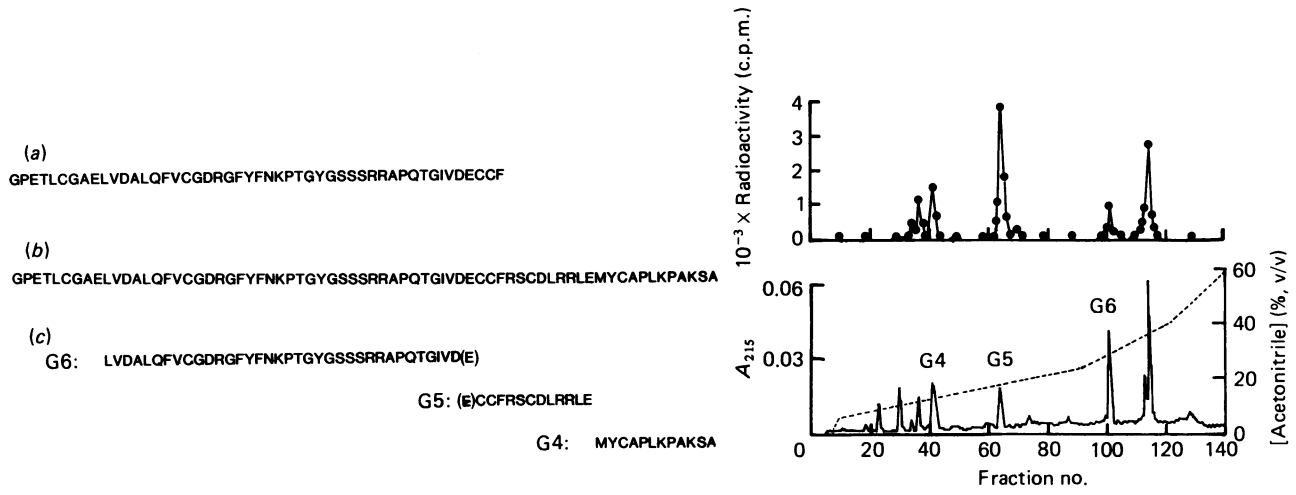

Fig. 3. Determination of the complete amino acid sequence of bovine IGF-1

N-Terminal sequencing (*a*) was used to establish the complete amino acid structure (*b*) in combination with sequencing of peptides obtained from endoproteinase Glu-C digests of [^{14}C]carboxymethylated IGF-1 (*c*). The peptides generated in (*c*) were separated on reverse-phase h.p.l.c. by a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (------) and pooled for sequencing on the basis of their A_{215} (——) and radioactivity (●).

0.5 μg of endoproteinase Glu-C dissolved in 10 μl of the same buffer. After incubation for 18 h the reaction was stopped by the addition of 5 μl of trifluoroacetic acid. The resulting peptides were separated on the RP-8 h.p.l.c. column as above but with a four-step linear gradient from 0 to 60% (v/v) acetonitrile in 0.10% (v/v) trifluoroacetic acid over 140 min as shown in the Results and discussion section. The flow rate was 0.5 ml/min. Fractions were pooled on the basis of their absorbance at 215 nm or their radioactivity.

Proteins and peptides were sequenced with the Applied Biosystems model 470A gas-phase sequencer by employing the methods described by Hunkapiller *et al.* (1983).

RESULTS AND DISCUSSION

Purification

Previously we reported the purification of bovine IGF-1 from colostrum and the partial amino acid sequence of this peptide (Francis *et al.*, 1986). Further investigation of the biological and receptor-binding properties of that material was complicated by unreliable yields and variable purity. The method described in the present paper overcomes these problems.

It has become apparent from our work and others (Hossenlopp *et al.*, 1986; Rosenfeld *et al.*, 1987) that it is extremely difficult to remove all traces of IGF-2 from preparations of IGF-1. Thus Rosenfeld *et al.* (1987) argued that the 5–20% cross-reactivity of IGF-1 for the IGF-2 receptor reported previously was probably due to a small but significant IGF-2 contamination. This conclusion was based on the finding that synthetic and recombinant-derived IGF-1 demonstrated an affinity for IGF-2 receptors only 1–10% of that exhibited by IGF-1 preparations purified from natural sources.

In the L6 myoblast IGF-2 radio-receptor assay used here the level of cross-reaction shown by purified IGF-1 was less than 0.5%. Moreover in a number of studies with other cell lines we have found a similar degree of competition of IGF-1 for IGF-2 binding (results not shown). Thus our preparations of IGF-1 behave similarly to recombinant and synthetic IGF-1 in this regard and appear to be free of IGF-2 contamination. Inclusion of the Mono S cation-exchange step (Fig. 1) facilitated the separation of IGF-1 from IGF-2, the resultant pool of IGF-1 being eluted well before the appearance of any activity detectable by the IGF-2 radio-receptor assay. Furthermore, IGF-1, assayed by both the protein-synthesis bioassay and the IGF-1 radio-receptor assay, was eluted earlier than IGF-2 under the conditions of the third and fourth h.p.l.c. steps.

The final h.p.l.c. step (Fig. 2*b*) clearly resolved the protein-synthesis-stimulating activity into two separate peaks. The first peak eluted possessed an *N*-terminal sequence identical with that of human IGF-1, but the second peak of bioactivity contained a truncated form of IGF-1 that lacked the *N*-terminal tripeptide Gly-Pro-Glu. The complete characterization of these peptides supports our earlier finding that the protein-synthesis-stimulating activity in bovine colostrum was due to the presence of two forms of IGF-1 (Francis *et al.*, 1986). The purity of IGF-1 and −3N:IGF-1 was established by amino acid sequence analysis of approx. 1 nmol of each peptide. Under the conditions employed the presence of a trace contaminant contributing between 2–5% of the phenylthiohydantoin amino acid yield in each of the first few cycles would have been detected. Also, the presence of an IGF-2 sequence was never detected during such *N*-terminal sequence analysis.

The purification of IGF-2 to homogeneity was also achieved in the final h.p.l.c. step with the recovery of approx. 20 μg of material from each purification (Fig. 2*a*). Several cycles of *N*-terminal sequence analysis were performed on batches of this material to establish purity as above for the other IGF. On no occasion was the presence of IGF-1 ever detected (see Table 1 below). This situation is a consequence of the last three steps of the purification procedure all resolving IGF-1 from IGF-2.

The amount of IGF-1 in the starting colostrum was estimated from analysis of the defatted acetic acid/acetonitrile extract. IGF-1 was separated from binding proteins by acid gel-permeation h.p.l.c. essentially as

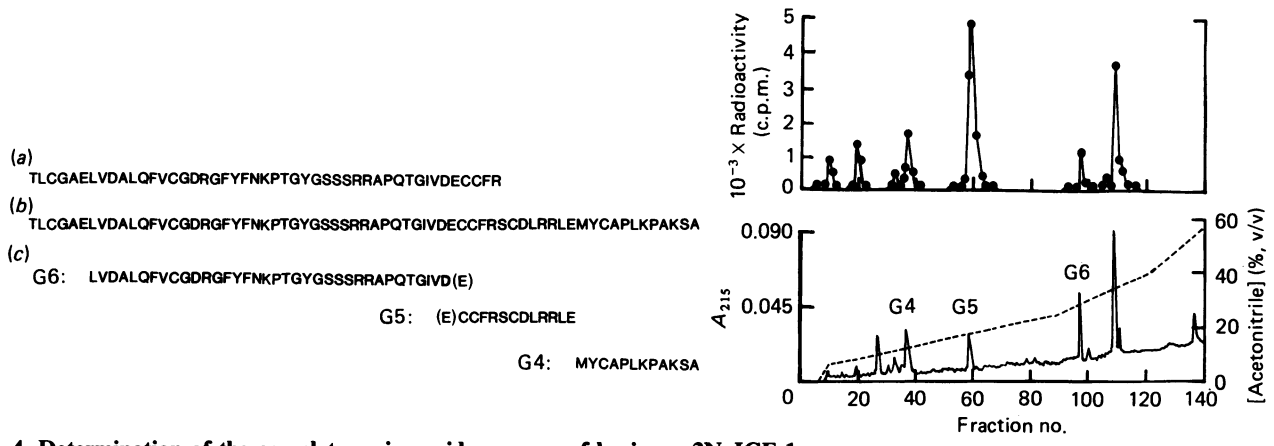

Fig. 4. Determination of the complete amino acid sequence of bovine −3N:IGF-1

N-Terminal sequencing (*a*) was used to establish the complete amino acid structure (*b*) in combination with sequencing of peptides obtained from endoproteinase Glu-C digests of [^{14}C]carboxymethylated −3N:IGF-1 (*c*). The peptides generated in (*c*) were separated on reverse-phase h.p.l.c. by a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (------) and pooled for sequencing on the basis of their A_{215} (——) and radioactivity (●).

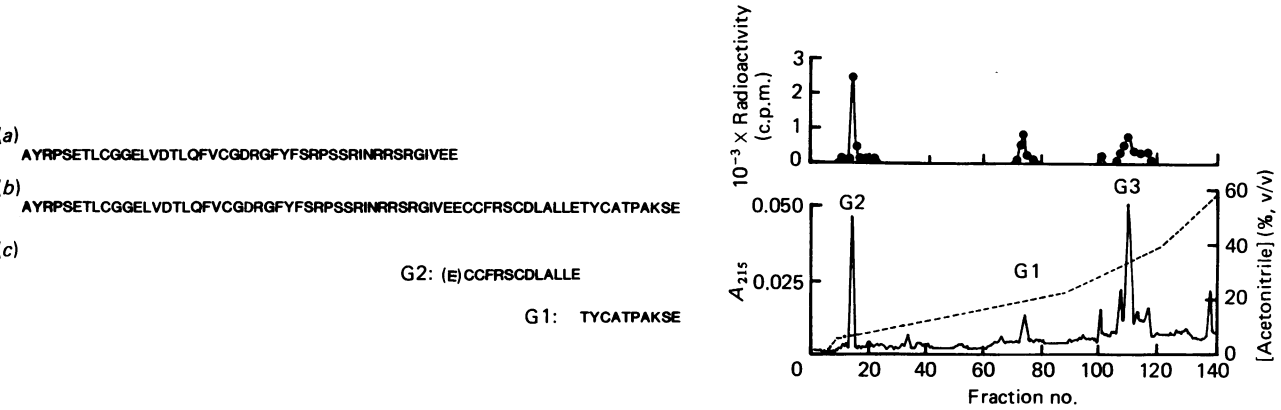

Fig. 5. Determination of the complete amino acid sequence of bovine IGF-2

N-Terminal sequencing (*a*) was used to establish the complete amino acid structure (*b*) in combination with sequencing of peptides obtained from endoproteinase Glu-C digests of [^{14}C]carboxymethylated IGF-2 (*c*). The peptides generated in (*c*) were separated on reverse-phase h.p.l.c. by a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (------) and pooled for sequencing on the basis of their A_{215} (——) and radioactivity (●).

described by Scott *et al.* (1985) and measured by radioimmunoassay. The amount found in the initial extract was 2 mg, from which 100 μg of IGF-1 and 50 μg of −3N:IGF-1 were purified, thus representing an overall yield of approx. 5–10%.

Sequence analysis

The sequence of bovine serum IGF-1 has been reported to be identical with that of human IGF-1 (Honegger & Humbel, 1986). We had previously obtained data from comparative binding studies of bovine, human and rat IGFs to membrane receptors and to antibodies against human IGF-1 suggesting that differences existed between bovine IGF-1 and human IGF-1 (Read *et al.*, 1986). These results, together with the reported heterogeneity of IGF-1 in human serum (Blum *et al.*, 1986), raised the possibility that the IGF-1 purified from bovine colostrum (Francis *et al.*, 1986) may have been different from the serum peptide. To answer this question [^{14}C]-carboxymethylated IGF-1 was sequenced from the *N*-terminus to residue 49 (Fig. 3*a*). The remainder of the sequence was determined from the analysis of peptides generated by digestion with endoproteinase Glu-C and separated on micro-bore h.p.l.c. (Fig. 3*c*). The peak G5 was selected for sequencing, as its higher specific radioactivity indicated that it was the predicted 12-amino-acid-residue peptide containing three ^{14}C-labelled cysteine residues. Correspondingly the low specific radioactivity of peak G6 suggested it was the 36-amino-acid-residue peptide containing only one labelled cysteine residue. Under the conditions employed endoproteinase Glu-C cleaved almost exclusively at the *C*-terminal side of glutamic acid residues. However, in the case of the sequence Asp-Glu at positions 45 and 46 it cut equally at the *C*-terminal side of both aspartic acid and glutamic acid to give the resulting peaks, G5 and G6, both of which on sequencing were found to contain two variants (Fig. 3*c*). The IGF-1 sequence was completed by the analysis of peak G4, which consisted of the *C*-terminal 12-amino-acid-residue peptide beginning with

Table 1. Sequence data for bovine IGF-2

The yield of phenylthiohydantoin amino acid derivatives at each cycle for the sequence analyses used to compile Fig. 5 is shown. The amount (pmol) of each derivative was calculated from the peak area relative to that of a standard mixture of phenylthiohydantoin amino acids. No correction was made for the efficiency of the cleavage step at each cycle.

	Yield of phenylthiohydantoin amino acids (pmol)				
	N-Terminal analysis		Endoproteinase Glu-C digest peptide		
Cycle no.	No. 1	No. 2	G1	G2	G3
1	A 1115	A 785/R 231	T 209/G 17	E 48/C 34/A 43	A 134/V 13/G 11
2	Y 1102	Y 1192/L 381/P 190	Y 282	C 77/ –/Y 15	Y 156/V 7
3	R 733	R 353/P 310/K 173	C 189/P 2	C 43/F 21	R 105/D 8/P 3
4	P 1344	P 450/E 268/H 117	A 212/V 4	F 26/R 17	P 94
5	S 677	S 292/Y 279	T 185	R 20/S 10	S 29/L 4
6	E 1013	E 392/L 187/I 74	P 136	S 10/C 17/P 8	E 80/Q 4
7	T 535	T 136/K 218	A 155	C 19/D 26/A 12	T 17
8	L 840	L 250/H 35	K 100	D 29/L 10/K 4	L 19/D 13/G 11
9	–	– /V 139/Q 58	S 81	L 14/A 10	C 43/L 21
10	G 1057	G 171/Y 110	E 63	A 10/L 12	G 50
11	G 1145	G 214/Q 187/L 16		L 16/ –	G 61
12	E 839	E 196/H 47/P 32		L 16/E 11	E 23
13	L 638	L 175/Q 134/P 44		E 13/ –	L 19
14	V 642	V 160/K 59			V 16
15	D 856	D 240/A 75			D 21
16	T 399	T 78/M 24/L 10			T 4
17	L 502	L 136/K 40			L 13
18	Q 566	Q 105/P 35			Q 6
19	F 343	F 64			F 9
20	V 312	V 87/I 18			V 10
21	–	–/Q 35			C 6
22	G 655	G 50/P 22			G 22
23	D 480	D 110/K 15			D 16
24	R 237	R 61/K 15			R 5
25	G 682	G 50/K 19			
26	F 223	F 47/V 21			
27	Y 194	Y 52			
28	F 206	F 56			
29	S 141	S 65			
30	R 170	R 20			
31	P 190	P 27			
32	S 120	S 29/A 13			
33	S 130	S 31			
34	R 122	R 17			
35	I 44	I 22			
36	N 54	N 10			
37	R 122	R 27			
38	R 136	R 16			
39	S 73	S 26			
40	R 129	R 14			
41	G 165	G 25			
42	I 74	–			
43	V 42	V 16			
44	E 23	E 21			
45	E 27	E 25			

methionine. Undigested [^{14}C]carboxymethylated IGF-1 was eluted coincident with the largest peak of absorbance. This and the other minor peaks were not characterized further. The resultant complete sequence of bovine IGF-1 from colostrum (Fig. 3*b*) is identical with that reported for IGF-1 purified from bovine serum (Honegger & Humbel, 1986). The mouse is the only other species for which the complete sequence of IGF-1 is known. The amino acid sequence for mouse IGF-1 was deduced from the cDNA sequence and is different from the human sequence at only three positions (Bell *et al.*, 1986). Clearly the amino acid structure of IGF-1 is highly conserved.

The complete sequence of the *N*-terminal des-tripeptide form of bovine IGF-1 was determined by employing the same techniques as described above for IGF-1 (Fig. 4). A very similar endoproteinase Glu-C digest pattern was seen and the h.p.l.c. peaks were chosen for sequencing accordingly (Fig. 4*c*). Sequencing revealed that the peptides eluted in h.p.l.c. peaks G4, G5 and G6 were

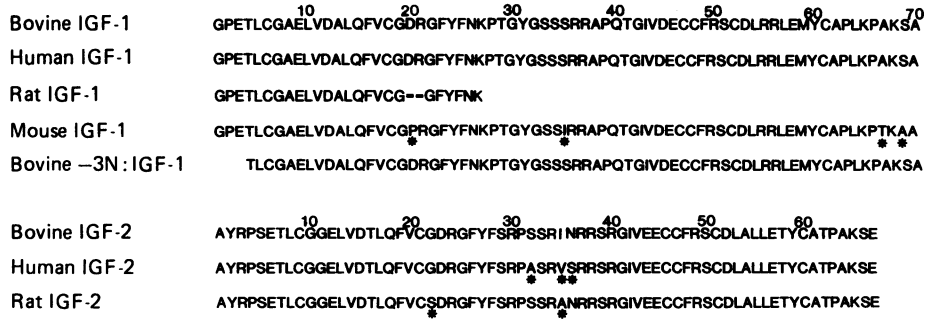

Fig. 6. Comparison of sequences for bovine, human, rat and mouse IGF-1 and IGF-2

The sequences for the different bovine IGFs are those determined in the present work, and those for human, rat and mouse IGF-1 are from Rinderknecht & Humbel (1978*a*), Rubin *et al.* (1982) and Bell *et al.* (1986) respectively. Sequences of human and rat IGF-2 are from Rinderknecht & Humbel (1978*b*) and Marquardt *et al.* (1981) respectively. Residues different from the bovine sequence are indicated by an asterisk and unknown residues are shown as a dash.

identical with those obtained with IGF-1 (compare Fig. 3*c* and Fig. 4*c*) and no differences from IGF-1 were found other than the loss of the *N*-terminal tripeptide (Fig. 4*b*). A truncated form of IGF-1 has also been reported in human foetal (Sara *et al.*, 1986) and adult brain (Carlsson-Skwirut *et al.*, 1986) and, although in both cases only partial sequences were reported, it seems likely that the truncated form is the same as reported here for the bovine peptide.

N-Terminal sequence analysis of bovine IGF-2 up to position 45 revealed three differences from that of human IGF-2 (Fig. 5*a*, Table 1 and Fig. 6). These changes at residues 32, 35, and 36 involved substitution of serine, valine and asparagine for alanine, valine and serine respectively. The remaining sequence of IGF-2 was obtained from analysis of the peptides G1, G2 and G3 generated by endoproteinase Glu-C digestion of [^{14}C]-carboxymethylated growth factor (Fig. 5 and Table 1). Peptide G1 consisted of the *C*-terminal decapeptide. G2 represented the next peptide from the *C*-terminus, but was actually a mixture of two peptides produced as a consequence of cleavage at consecutive glutamic acid residues 44 and 45 with equal frequency. This situation was also observed with IGF-1 and −3N:IGF-1, where the respective amino acids are aspartic acid and glutamic acid (see above). Sequencing of peptide G3, which probably represented undigested [^{14}C]carboxymethylated growth factor, allowed confirmation of the two remaining cysteine residues at positions 9 and 21.

The complete IGF-2 sequence differed from human IGF-2 by substitution at residues 32, 35 and 36 (Fig. 6) and from the previously reported bovine serum IGF-2 (Honegger & Humbel, 1986) by the replacement of isoleucine for serine at position 35. Our result was substantiated on two occasions (Table 1), and careful examination of the amino acid analysis data reported by Honegger & Humbel (1986) supports the presence of an extra isoleucine residue.

If in the cow only one IGF-1 gene exists, as is the case in the human (Brissenden *et al.*, 1984), generation of the truncated IGF-1 is most likely the result of post-translational modification. Alternatively it could arise from alternative splicing of the bovine IGF-1 mRNA, but this appears to be unlikely in the human owing to the absence of an intron–exon hinge region near the *N*-terminus of IGF-1 (Rotwein *et al.*, 1986). Isolation of apparently the same truncated form of IGF-1 from human brain (Sara *et al.*, 1986; Carlsson-Skwirut *et al.*, 1986) and bovine colostrum by different procedures suggests it arises by post-translational modification *in vivo*. More recently Carlsson-Skwirut *et al.* (1987) have reported the presence of truncated IGF-1 in human foetal serum and suggested that it is the major foetal form of IGF-1. Unfortunately, this was not verified by *N*-terminal sequencing. In order to resolve the question about the origin of the truncated IGF-1 we have processed 2 litres of bovine foetal serum by the purification procedure reported here and, although we recovered significant quantities of IGF-1 and IGF-2 in pure form, there was no indication of the presence of −3N:IGF-1 (results not shown). Hence −3N:IGF-1 does not appear to be generated during the isolation procedure, at least from serum.

Biological studies

The potencies of the isolated IGF to stimulate a co-ordinate growth response in L6 rat myoblasts were compared. At the highest concentrations tested all three peptides inhibited the basal rate of protein degradation by a maximum of 40% over the 4 h measurement period (Fig. 7*c*). The decreasing order of potency with the concentrations (ng/ml) required to give a half-maximal response in parentheses was:

−3N:IGF-1 (0.2) > IGF-1 (1.6) > IGF-2 (8.4)

The IGF preparations all stimulated protein synthesis in L6 myoblasts to the same extent (Fig. 7*b*). The concentrations (ng/ml) of peptide required for half-maximal stimulation in decreasing order of sensitivity were:

−3N:IGF-1 (1.5) > IGF-1 (12.6) > IGF-2 (66.8)

Hence each IGF induces a dual anabolic response on the protein metabolism of L6 myoblasts by inhibiting protein breakdown and stimulating protein synthesis. Although protein breakdown is about an order of magnitude more sensitive to the addition of the peptides under the respective measurement conditions, both processes show the same relative response, with −3N:IGF-1 the most potent and IGF-2 the least potent. The approximately 10-fold greater sensitivity of IGF-1 as compared with IGF-2 has been observed in a number of cell types and with a range of biological processes (Froesch *et al.*, 1985; Ballard *et al.*, 1986).

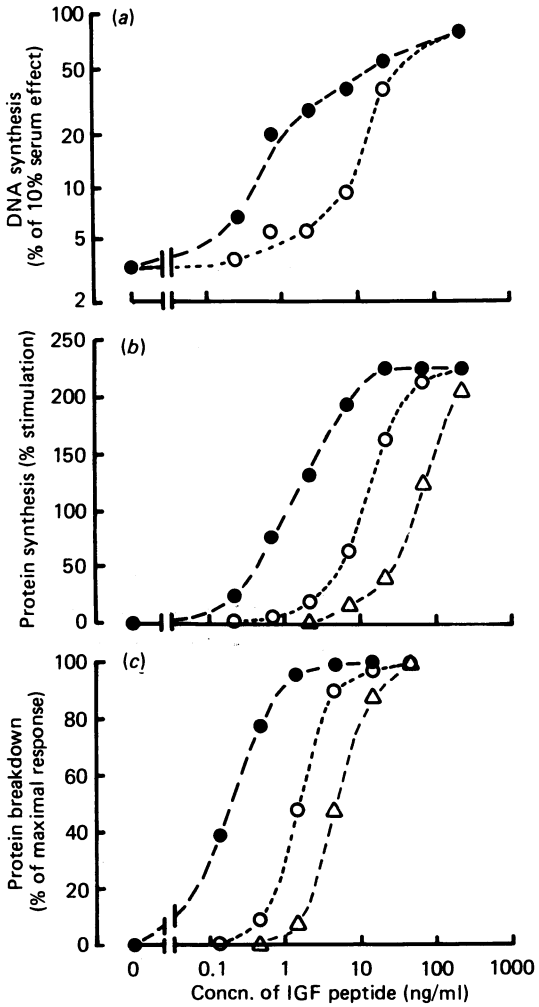

Fig. 7. Effects of different IGF peptides on (*a*) DNA synthesis, (*b*) protein synthesis and (*c*) protein breakdown

The symbols used are: ○, IGF-1; ●, −3N:IGF-1; △, IGF-2. Results represent triplicate measurements at each concentration.

Bovine IGF-1 and −3N:IGF-1 were tested for their mitogenic activity in L6 myoblasts and found to stimulate DNA synthesis by up to 80% of that observed with 10% foetal bovine serum (Fig. 7*a*). The truncated peptide was 3.5-fold more active than intact IGF-1 in stimulating DNA synthesis half-maximally, but even greater efficacy of −3N:IGF-1 compared with IGF-1 was observed at lower concentrations.

The region of the IGF-1 molecule important for receptor binding and mitogenic activity has been investigated by constructing various hybrids of insulin and IGF-1 (King *et al.*, 1982; De Vroede *et al.*, 1986; Tseng *et al.*, 1986). Using this approach King *et al.* (1982) found that the *C*-terminal octapeptide (D-domain) of IGF-1 when linked to the A-chain of insulin conferred increased growth-promoting activity relative to insulin. Moreover replacement of the A-chain of insulin with the equivalent homologous 21-amino-acid-residue peptide (A-domain) of IGF-1 led to an enhanced stimulation of DNA synthesis whereas substitution of the insulin B-chain by the equivalent *N*-terminal 29-amino-acid-residue peptide (B-domain) of IGF-1 led to diminished activity (Tseng *et al.*, 1986). These results agree with the report by De Vroede *et al.* (1986) that a chemically synthesized hybrid containing the A-chain of insulin and the B-domain of IGF-1 had diminished mitogenic activity compared with insulin. Thus it appears that the A- and D-domains of IGF-1 are important for mitogenic activity whereas the *N*-terminal B-domain is relatively less so. Also, Blundell *et al.* (1983) have proposed from computer graphic modelling that the *N*-terminal residues of IGF-1 are not involved in receptor binding. However, these results and predictions are at variance with our observations that loss of the tripeptide Gly-Pro-Glu from the B-domain markedly increases the biological activity of IGF-1. More extensive research is required to clarify the region of the IGF-1 molecule required for biological activity. A likely explanation of our observations is that interaction with the type 1 IGF-1 receptor is altered in some way by removal of the *N*-terminal tripeptide, the end result being an enhancement of the biological potency.

In conclusion it appears that a modified IGF-1 lacking the *N*-terminal tripeptide Gly-Pro-Glu is present in bovine colostrum and possesses enhanced biological activity compared with IGF-1. Although we could not identify this growth factor in bovine foetal serum, others have established the presence of a truncated IGF-1 in human brain (Carlsson-Skwirut *et al.*, 1986). If, as appears likely, it does not arise during the isolation procedure, we must conclude that similar proteolytic processing mechanisms operate in the mammary gland and in the central nervous system to produce the novel peptide.

We thank Mrs. Susan Dawe and Mrs. Marina Ross for helpful collaboration. We also express our appreciation to Stephen Rogers and Denise Turner for carrying out the sequence analysis. Support for this work from the National Health and Medical Research Council of Australia and the Reserve Bank Rural Credits Development Fund is gratefully acknowledged.

REFERENCES

Ballard, F. J. (1982) Biochem. J. **208**, 275–287
Ballard, F. J., Read, L. C., Francis, G. L., Bagley, C. J. & Wallace, J. C. (1986) Biochem. J. **233**, 223–230
Bell, G. I., Stempein, M. M., Fong, N. M. & Rall, L. B. (1986) Nucleic Acids Res. **14**, 7873–7882
Blum, W. F., Ranke, M. B. & Bierich, J. R. (1986) Acta Endocrinol. (Copenhagen) **111**, 271–284
Blundell, T. L., Bedarkar, S. & Humbel, R. E. (1983) Fed. Proc. Fed. Am. Soc. Exp. Biol. **42**, 2592–2597
Brissenden, J. E., Ullrich, A. & Francke, U. (1984) Nature (London) **310**, 781–784
Carlsson-Skwirut, C., Jornvall, H., Holmgren, A., Andersson, C., Bergman, T., Lundquist, G., Sjogren, B. & Sara, V. R. (1986) FEBS Lett. **201**, 46–50
Carlsson-Skwirut, C., Andersson, C. & Sara, V. R (1987) Acta Endocrinol. (Copenhagen) **114**, 37–40
De Vroede, M. A., Rechler, M. M., Nissley, S. P., Ogawa, H., Joshi, S., Burke, G. T. & Katsoyannis, P. G. (1986) Diabetes **35**, 355–361

Francis, G. L., Read, L. C., Ballard, F. J., Bagley, C. J., Upton, F. M., Gravestock, P. M. & Wallace, J. C. (1986) Biochem. J. **233**, 207–213

Froesch, E. R., Schmid, Chr., Schwander, J. & Zapf, J. (1985) Annu. Rev. Physiol. **47**, 43–467

Hizuka, N., Takano, K., Shizume, K., Asakawa, K., Miyakawa, M., Tanaka, I. & Horikawa, R. (1986) Eur. J. Pharmacol. **125**, 143–146

Honegger, A. & Humbel, R. E. (1986) J. Biol. Chem. **261**, 569–575

Hossenlopp, P., Seurin, D., Sergovia-Quinson, B. & Binoux, M. (1986) FEBS Lett. **208**, 439–444

Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J. & Hood, L. E. (1983) Methods Enzymol. **91**, 399–413

King, G. L., Kahn, C. R., Samuels, B., Danho, W., Bullesbach, E. E. & Gattner, H. G. (1982) J. Biol. Chem. **257**, 10869–10873

Marquardt, H., Todaro, G. J., Henderson, L. E. & Oroszlan, S. (1981) J. Biol. Chem. **256**, 6859–6865

Read, L. C., Ballard, F. J., Francis, G. L., Baxter, R. C., Bagley, C. J. & Wallace, J. C. (1986) Biochem. J. **233**, 215–221

Rinderknecht, E. & Humbel, R. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2365–2369

Rinderknecht, E. & Humbel, R. (1978*a*) J. Biol. Chem. **253**, 2769–2776

Rinderknecht, E. & Humbel, R. (1978*b*) FEBS Lett. **89**, 283–286

Rosenfeld, R. G., Conover, C. A., Hodges, D., Lee, P. D. K., Mistra, P., Hintz, R. L. & Li, C. H. (1987) Biochem. Biophys. Res. Commun. **143**, 199–205

Rotwein, P., Pollock, K. M., Didier, D. K. & Krivi, G. G. (1986) J. Biol. Chem. **261**, 4828–4832

Rubin, J. S., Mariz, I., Jacobs, J. W., Daughaday, W. H. & Bradshaw, R. A. (1982) Endocrinology (Baltimore) **110**, 734–740

Sara, V. R., Carlsson-Skwirut, C., Andersson, C., Hall, E., Sjogren, B., Holmgren, A. & Jornvall, H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4904–4907

Schoenle, E., Zapf, J., Humbel, R. E. & Froesch, E. R. (1982) Nature (London) **296**, 252–253

Schoenle, E., Zapf, J., Hauri, C., Steiner, T. & Froesch, E. R. (1985) Acta Endocrinol. (Copenhagen) **108**, 167–174

Scott, C. D., Martin, J. L. & Baxter, R. C. (1985) Endocrinology (Baltimore) **116**, 1094–1101

Tseng, L. Y.-H., Schwartz, G. P., Sheik, M., Chen, Z. Z., Joshi, S., Wang, J.-F., Nissley, S. P., Burke, G. T., Katsoyannis, P. G. & Rechler, M. M. (1986) Fed. Proc. Fed. Am. Soc. Exp. Biol. **45**, 1714

Van Obberghen-Schilling, E. & Pouyssegur, J. (1983) Exp. Cell Res. **147**, 369–378

Van Wyk, J. J. (1984) in Hormonal Proteins and Peptides (Li, C. H., ed.), vol. 12, pp. 82–125, Academic Press, Orlando

Zumstein, P. P. & Humbel, R. E. (1985) Methods Enzymol. **109**, part 1, 782–798

Received 8 June 1987/1 September 1987; accepted 25 November 1987