Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Land, H., Grez, M., Ruppert, S., Schmale, H., Rehbein, M., Richter, D., & Schütz, G. (1983) *Nature (London) 302*, 343-344.
Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149-2154.
Nicolas, P., Delfour, A., Boussetta, H., Morel, A., Rholam, M., & Cohen, P. (1986) *Biochem. Biophys. Res. Commun. 140*, 565-573.
Parish, D. C., Tuteja, R., Alstein, M., Gainer, H., & Loh, Y. P. (1986) *J. Biol. Chem. 261*, 14392-14397.
Rholam, M., Nicolas, P., & Cohen, P. (1986) *FEBS Lett. 207*, 1-6.

Identification and Purification of Truncated Insulin-like Growth Factor I from Porcine Uterus. Evidence for High Biological Potency†

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ABSTRACT: We report the completion of the purification of uterine-derived growth factors (UDGF) described previously by this laboratory [Ikeda, T., & Sirbasku, D. A. (1984) *J. Biol. Chem. 259*, 4049-4064]. During isolation, the mitogenic activity was monitored by using the human MCF-7 breast cancer cells in serum-free Ham's F12 and Dulbecco's modified Eagle's medium (1:1, v/v) containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 200 μg/mL bovine serum albumin, and 10 μg/mL human transferrin. This medium sustained growth for several days in response to a single addition of growth factor. The isolation of UDGF began with acetic acid extraction followed by sulfopropyl-Sephadex chromatography, Bio-Gel P-10 molecular sieve fractionation, and a series of reverse-phase high-pressure liquid chromatography separations. Purifications [[(1.0-8.5) × 10^6]-fold] of three mitogens (5-20 ng each) were achieved. The mitogens were shown by protein microsequencing to be DES 1→3 to DES 1→6 forms of insulin-like growth factor I (truncated IGF-I). An M_r estimated by ^{125}I labeling, urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and autoradiography was consistent with a DES 1→3(4) N^{α} truncation. Immunoadsorption and radioimmunoassay confirmed immunological properties equivalent to IGF-I. Radioreceptor assays showed truncated IGF-I was functionally equivalent to recombinant IGF-I. The ED_{50} values of DES 1→3 IGF-I and recombinant IGF-I for MCF-7 cell growth were 0.8-6.0 and 30-150 pg/mL, respectively. With Balb/c 3T3 mouse fibroblasts, the ED_{50} of DES 1→3 IGF-I was 100 times lower than that of IGF-I. We conclude that the major acid-stable low-M_r mitogenic activities isolated from uterus are very potent forms of truncated IGF-I capable of stimulating growth of epithelial and mesenchymal cells.

Previously, this laboratory has shown that extracts of uterus contained estrogen-inducible growth factor activities for breast cancer cells in culture (Sirbasku, 1978; Sirbasku et al., 1981; Ikeda et al., 1982). When extracts were prepared at neutral pH, one of the activities identified was an M_r 70000 protein (Sirbasku et al., 1981) later purified to homogeneity and identified as transferrin (Riss et al., 1986; Riss & Sirbasku, 1987a). In contrast, acid extraction of uterus yielded lower M_r activities that were heat stable and readily assayed with breast, pituitary, or uterine cancer cells (Ikeda et al., 1984b).

The purification of an acid-stable low M_r mitogenic activity from uterus had been described by Ikeda and Sirbasku (1984). This material, designated UDGF,[1] was obtained in milligram quantities per kilogram of lyophilized powder of pregnant sheep uteri after a 150-fold purification. The M_r of UDGF was estimated between 3700 and 7200. The ED_{50} was measured in nanomolar concentrations. Although moderatively active, the ED_{50} of this preparation was from 10 to 1000 times greater than those of other well-characterized mitogens (Barnes & Sirbasku, 1987a,b; Ogasawara & Sirbasku, 1988; Karey & Sirbasku, 1988).

Analytical data obtained by a variety of methods initially indicated UDGF had been purified to near homogeneity (Ikeda & Sirbasku, 1984). Nevertheless, additional evaluation by protein chemistry methods confirmed this preparation was not

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[1] Abbreviations: UDGF, uterine-derived growth factor; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; TGFα, transforming growth factor α; SP-Sephadex, sulfopropyl-Sephadex; RP-HPLC, reverse-phase high-pressure liquid chromatography; F12/DME, a 1:1 (v/v) mixture of Ham's F12 nutrient medium and Dulbecco's modified Eagle's medium (high glucose concentration formulation) containing 2.2 g/L sodium bicarbonate; PBS, Dulbecco's phosphate-buffered saline, pH 7.2; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2; TFA, trifluoroacetic acid; FBS, fetal bovine serum; RIA, radioimmunoassay; Tf, human transferrin; BSA, bovine serum albumin, fraction V; PMSF, phenylmethanesulfonyl fluoride; cpm, counts per minute; cpd, cell population doublings; ELISA, enzyme-linked immunosorbent assay; RRA, radioreceptor assay; SFM-3T3, serum-free defined medium for 3T3 cell growth.

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homogenous but instead contained a number of fragments of known tissue and serum proteins.[2] These peptides shared similar M_r and charge properties. Later isoelectric focusing studies confirmed UDGF was heterogeneous (Sirbasku et al., 1985). Some of the peptides present appeared to possess nonspecific mitogenic activity with the rat mammary tumor cells.[2] These and other problems with the original rat mammary cell assay have been described (Riss et al., 1986; Riss & Sirbasku, 1987a; Ogasawara & Sirbasku, 1988).

To further characterize the putative acid-stable mitogens of uterus, we developed new serum-free assays for measurement of mitogenic activities by both cell number increase and incorporation of tritium-labeled thymidine into DNA using the MCF-7 human breast cancer cells (Riss et al., 1986; Ogasawara & Sirbasku, 1988; Karey & Sirbasku, 1988). This line was maintained in serum-free F12/DME supplemented only with HEPES, Tf, and BSA. Under these conditions, the MCF-7 cells responded to picomolar to nanomolar concentrations of a number of growth factors including IGF-I, IGF-II, insulin, EGF, TGFα, and bFGF.

By use of this bioassay, the isolations of three acid-stable mitogens from porcine uterus have been completed. The fold purifications required were (1–8.5) $\times 10^6$, yielding fractions with ED_{50} values in the 0.1–1.0 pM range. The factors were proven to be truncated forms of IGF-I by N^{α} microsequencing, M_r estimation, immunoadsorption, RIA, and RRA. The isolation and properties of uterine-derived truncated IGF-I will be described next. A preliminary report of these results has been presented (Ogasawara et al., 1987).

Experimental Procedures

Cell Culture. The MCF-7 cells (Soule et al., 1973) were obtained from the American Type Culture Collection (Rockville, MD). They were monitored every other month for mycoplasma contamination by using the MycoTect detection kit (GIBCO, Grand Island, NY) and found free of infection. The stock cultures were grown and passed as described (Ogasawara & Sirbasku, 1988).

The Balb/c 3T3 cells were obtained from the American Type Culture Collection and stock cultures grown and passed as described by Riss et al. (1988).

Cell Growth Assays. All cell growth assays were done at 37 °C in a carbon dioxide/air atmosphere incubator. The MCF-7 cell number growth assay has been described (Riss et al., 1986; Ogasawara & Sirbasku, 1988; Karey & Sirbasku, 1988). The assays were done in F12/DME supplemented with 15 mM HEPES (pH 7.2), 10 μg/mL Tf, and 200 μg/mL BSA (hereafter referred to as Tf/BSA). Growth in Tf/BSA without other additions was designated C_0 and in 8 days was usually 0.5–1.5 cpd over day zero controls (i.e., the number of cells determined within 3 h of the initial seeding). The data were converted to cpd by using the averages of triplicate dishes on day zero and the averages of cell numbers in other dishes at the end of the experiments. In most experiments, positive growth controls (C_{INS}) of 100 ng/mL to 10 μg/mL insulin in Tf/BSA were included. The ED_{50} values were calculated as half the difference between insulin-stimulated growth (C_{INS}) and proliferation in Tf/BSA alone (C_0). One unit of activity was defined as the amount of protein required to achieve ED_{50}.

In experiments measuring MCF-7 growth by incorporation of [*methyl*-^{3}H]thymidine into DNA, the assays were done as described by Riss et al. (1986). The notations C_0, C_{INS}, and units have the same meaning as with the cell number assay.

Growth assays with Balb/c 3T3 cells to measure IGF-I specific activity were done in SFM-3T3 serum-free defined medium as described by Riss et al. (1988).

RP-HPLC Chromatography. The Vydac RP-HPLC C_4 (0.46 × 25 cm, 5-μm particle size, 300-Å pore size) and C_{18} (0.46 × 25 cm, 5-μm particle size, 300-Å pore size) columns were obtained from Separations Group (Hesperian, CA). The RP-HPLC μBondapak column [0.39 × 30 cm, 10-μm particle size, irregular 125 (50–300) Å average pore size, part number P/N 84176] was purchased from Waters Associates (Milford, MA). Care was taken to use new or almost new columns during the final stages of purification. This precaution lowered yields. The RP-HPLC separations were done with a Rainin Rabbit HP apparatus equipped with a variable-wavelength monitor and computer-regulated gradient developing capacity.

Bioassay of Chromatography Samples. The fractions from conventional chromatography were diluted ≥1000-fold into Tf/BSA for assay of biological activity. Fractions from RP-HPLC were diluted ≥1000-fold into the assay or added to 100 μL of BSA fraction V (5.0 mg/mL) and dried in a Speed Vac concentrator. The dried samples were reconstituted in Tf/BSA and added to the growth assays to 5000–20 000-fold dilutions. Samples for cell number assay were sterilized by passage through 0.22-μm pore diameter filters (Corning). Samples used in RRA were dissolved in binding buffer (Furlanetto & DiCarlo, 1984).

Protein Microsequencing. Automated sequence analysis of UDGF samples was performed on a Model 470A amino acid sequencer (Applied Biosystems, Foster City, CA) with the 03RPTH software program as described (Ikeda et al., 1987).

M_r Estimated by Urea–SDS–PAGE and Autoradiography. The M_r of purified UDGF was estimated after iodination by the method of Samsoondar et al. (1986). A sample of UDGF containing 90 000 units (≈90 ng of protein) was lyophilized in a microcentrifuge tube, 20 μL of 0.5 M sodium phosphate buffer (pH 7.5) added to dissolve the sample, followed by 5 μL of 0.5 mCi of carrier free sodium [^{125}I]iodide and 10 μL of freshly prepared aqueous chloramine T (5.0 mg/mL). After 2 min at room temperature, the iodination reaction was terminated by addition of 20 μL of sodium metabisulfite (1.0 mg/mL). A solution (100 μL) of 20% (w/v) sucrose, 1% (w/v) potassium iodide, and 0.1% (w/v) blue dextran was added before chromatography on a 1 × 18 cm Sephadex G-10 column equilibrated and eluted with PBS containing 0.1% (w/v) BSA (PBS/BSA). The first peak of radioactivity that eluted from the column was dialyzed against PBS in a Spectrapor 3 membrane (MWCO 3500). Aliquots were stored at −20 °C until analyzed by urea–SDS–PAGE.

The recombinant 71 amino acid human IGF-I (Burleigh & Meng, 1986) and recombinant human IGF-II used in these studies were either gifts from Dr. B. Daniel Burleigh, IMC Pitman-Moore (Northbrook, IL) or were supplied under a research contract by IMCERA Bioproducts, Inc. (Terre Haute, IN). Recombinant IGF-I was iodinated as described (Karey & Sirbasku, 1988). The specific activity of ^{125}I-IGF-I was usually 100–150 μCi/μg. This procedure was used to prepare the ^{125}I-IGF-I for the RIA and the RRA with MCF-7 cells. The iodinated product was confirmed to specifically bind the α subunit of the type I $\alpha_2\beta_2$ (IGF-I) receptor of Balb/c 3T3 cells by using a chemical cross-linking method (Karey et al., 1988).

The M_r of ^{125}I-UDGF was compared to that of ^{125}I-labeled recombinant IGF-I and unlabeled standard proteins by urea–SDS–PAGE (Burr & Burr, 1983) followed by formaldehyde fixation, staining, and autoradiography (Steck et al.,

[2] D. A. Sirbasku, unpublished data.

1980). The iodinated protein samples were added to the electrophoresis buffer containing 8 M urea and 1% SDS and boiled for 3–5 min before analysis. Electrophoresis was done for 20 h at room temperature (constant ≈50 V) after which time the gel was fixed and stained. Finally, the gels were washed extensively with a solution of 25% (v/v) ethanol, dried under vacuum, and exposed to Kodak X-ray film (X-OMAT AR XAR5) to localize the radioactivity.

Polyclonal Antibodies to Recombinant Human IGF-I. Antibodies against recombinant IGF-I were raised by the method of Furlanetto et al. (1977). A total of 400 μg of IGF-I was conjugated to 800 μg of ovalbumin by using glutaraldehyde. After extensive dialysis (Spectrapor 3 membrane), the sample was mixed well (1:1, v/v) with complete Freund's adjuvant (Sigma). Female New Zealand white rabbits received the amount of complex corresponding to 70 μg of IGF-I at multiple sc injection sites. The second and third injections (62.5 μg/animal) were made in incomplete Freund's adjuvant (1:1, v/v) at 7 and 12 weeks after the first, respectively.

Two weeks after the last antigen challenge, blood was collected from the ear vein, and after 24 h at 4 °C, the serum was assayed by ELISA methods for the presence of antibody (Riss & Sirbasku, 1989). Immulon II microtiter dishes (Dynatech Laboratories, Alexandria, VA) were coated with 1.0 ng of recombinant IGF-I, blocked by incubation with excess BSA, and incubated with PBS dilutions of preimmune and immune sera. Alkaline phosphatase conjugated to goat anti-rabbit IgG secondary antiserum and a disodium *p*-nitrophenyl phosphate substrate were used to identify specifically bound anti-IGF-I antibodies. Antiserum dilutions of 4750–10000-fold detected 0.2 absorbance units changes at 410 nm after 1 h.

Immunoabsorption of UDGF. Freeze-dried powder of protein A–Sepharose CL-6B was swollen in 0.1 M sodium phosphate buffer, pH 7.2, and washed three times in the same buffer. The swollen gel was incubated with 5–6 times the volume of undiluted preimmune or immune serum at 4 °C overnight with shaking. Thereafter, the gels were collected by centrifugation, the supernatants discarded, and finally the gels washed several times with PBS. Immunoadsorptions of UDGF and IGF-I were done with 500 μL of gel and 300 μL of sample at 4 °C overnight with shaking. The gel was packed by centrifugation, the supernatants were collected, diluted with Tf/BSA, and filter-sterilized through 0.22-μm pore diameter filters, and biological activity was measured.

RIA of UDGF and IGF-I. The amount of truncated IGF-I in selected conventional chromatography and RP-HPLC fractions was estimated by RIA methods (Furlanetto & Marino, 1987) using recombinant IGF-I as standard and rabbit polyclonal antibody raised as described above.

Protein Determinations. The concentrations of protein in tissue extracts were measured with a kit purchased from Bio-Rad Laboratories using BSA as standard. Concentrations in chromatography steps preceding RP-HPLC were estimated by A_{280nm} assuming 0.6 unit equaled 1.0 mg/mL protein. During RP-HPLC, concentrations were estimated from peak area comparisons to known amounts of BSA. An estimation of the protein present in purified samples also was derived from the quantity (picomoles) of amino acid residues found during protein sequencing; this measure assume a 30–50% yield. To better quantify the amount of growth factor in various preparations, the IGF-I-like material was determined by RIA as described above.

RRA Measurement of UDGF versus IGF-I. The method described by Furlanetto and DiCarlo (1984) was used to measure the specific displacement of ^{125}I-IGF-I binding to MCF-7 cell surface receptors by recombinant IGF-I and UDGF.

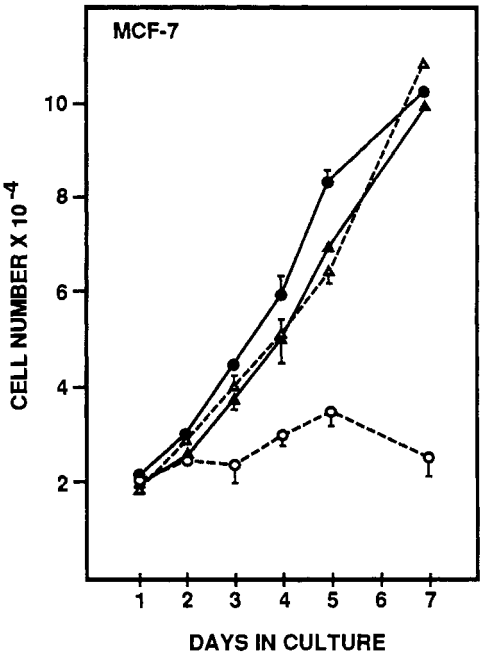


FIGURE 1: Growth of the MCF-7 cells in Tf/BSA supplemented with insulin (10 μg/mL, open triangles), uterine extract (25 μg/mL, solid circles), and pituitary extract (25 μg/mL, solid triangles) was compared to that in Tf/BSA only (open circles). The tissue extracts were prepared by homogenizing 1.0 g/3.0 mL of 1.0 M acetic acid with a Tekmar tissue mizer. The soluble fraction was collected by centrifugation at 100000*g* for 45–60 min at 4 °C. The supernatants were dialyzed against PBS, precipitates removed by centrifugation, and the supernatants assayed. The data points represent the means of triplicate plates (±SD, bars).

RESULTS

Growth of the MCF-7 Cells in Response to Insulin and Tissue Extracts. A single addition of acetic acid extracted protein from porcine uterus caused logarithmic MCF-7 cell growth for at least 7 days (Figure 1). In dose–response studies, the ED_{50} of the uterine extracts ranged between 5 and 15 μg/mL (data not shown). Acid extracts of pituitary tissue were shown previously (Ikeda et al., 1984a) to be mitogenic for MCF-7 and T-47D (Keydar et al., 1979) human breast cancer cells as measured by [*methyl*-^{3}H]thymidine incorporation. In this study, a single addition of a saturating concentration of insulin caused the same rate of cell proliferation as 25 μg/mL of either uterine or pituitary extract (Figure 1).

Purification of UDGF from Porcine Tissue. The steps of the isolation through Bio-Gel P-10 were carried out at 4–7 °C. All RP-HPLC steps were done at room temperature. Between separation procedures, the samples were stored at either 4 or −17 °C. Table I summarizes the quantification of protein and mitogenic activity (ED_{50} and units) at each step.

Step 1. Porcine uterus was collected from multiparous sows, washed with tap water, and stored at −20 °C; the activity was stable for many months. A total of 1.0 kg of frozen tissue was cut into 2.5-cm cubes and placed in saline to thaw and remove residual blood. The washed tissue was passed through a meat grinder and homogenized with a Tekmar tissue mizer (Cincinnati, OH) at a ratio of 1.0 g of tissue to 3.0 mL of 1.0 M acetic acid containing 20 μg/mL pepstatin A. The homogenization was done for a total of 4 min with 150–200-g samples of tissue. The debris was removed by centrifugation at 10000*g* for 30 min and the supernatant (pH 3.3) passed

Table I: Summary of the Purification of Truncated IGF-I from Porcine Uterus

steps	total protein	volume (mL)	protein (concn/mL)	ED_{50} (concn/mL)	total units	yield (%)
(1) initial steps						
crude extract	17 200 mg	2620	6.6 mg	8.52 μg	2.0×10^6	100
SP-Sephadex	327 mg	14	23.4 mg	187 ng	1.7×10^6	87
Bio-Gel P-10 A	8.47 mg	33	0.26 mg	8.0 ng	1.0×10^6	52
Bio-Gel P-10 B	2.29 mg	43	53 μg	2.8 ng	8.2×10^5	41
(2) RP-HPLC purification of Bio-Gel P-10 pool A						
μBondapak C_{18}	72.4 μg	12.8	5.55 μg	41.4 pg	1.1×10^6	53
first Vydac C_4 (eluted with acetonitrile)[a]	0.96 μg	2.4	0.40 μg	2.8 pg	4.2×10^5	21[b]
second Vydac C_4 (eluted with 2-propanol)						
fraction 27	0.28 μg	1.2	0.23 μg	3.1 pg	91 200	4.5
fraction 28	0.23 μg	1.2	0.28 μg	11 pg	30 600	1.5
Vydac C_{18} of C_4 fraction 27						
fraction 92	9.13 ng	0.6	15.2 ng	1.4 pg	6600	0.3
fraction 93[c]	9.13 ng	0.6	15.2 ng	1.4 pg	6300	0.3
fraction 94	11.1 ng	0.6	18.5 ng	2.9 pg	3840	0.2
Vydac C_{18} of C_4 fraction 28						
fraction 56[c]	4.22 ng	0.6	7.0 ng	0.8 pg	5160	0.2
(3) RP-HPLC purification of Bio-Gel P-10 pool B						
μBondapak C_{18}	3419 ng	9.6	356 ng	21.7 pg	157 000	7.8
Vydac C_4 fraction 44[c]	85 ng	1.0	85 ng	1.0 pg	81 000	4.0

[a] Only half of the preceding fraction from the μBondapak column was applied to this Vydac C_4. [b] This yield represents only half of the total sample. [c] These fractions were used to obtain amino acid sequences (Table II). The protein concentration in each sample was estimated from peak height or from protein sequencing data. The ED_{50} calculations based on these approximations were evaluated again by RIA for IGF-I as shown in Table IV.

through glass wool. This extract was neutralized (about 80 mL of 50% sodium hydroxide) and 100 μM PMSF added immediately. A large precipitate formed after neutralization and was removed by centrifugation at 30000*g*. The supernatant was acidified to pH 4.7 (about 100 mL of glacial acetic acid) and dialyzed (Spectrapor 3 membranes) for 2–3 days against frequent changes of 0.1 M acetic acid containing 20 μg/mL pepstatin A, and the precipitate was removed by centrifugation at 30000*g*. This fraction (pH 4.0) was designated the crude extract.

Step 2. A total of 50 g of dry SP-Sephadex C-25 was added to 0.2 M sodium chloride for 5 min followed by extensive washing with distilled water in a Büchner funnel. The resin was then swollen in 0.1 M acetic acid for 24 h at room temperature, transferred to a 5.2 × 20 cm glass column, and washed extensively with 0.1 M acetic acid before the crude extract was applied at a flow rate of 250 mL/h. The column was washed subsequently with the acetic acid until the absorbance at 280 nm reached background. The material bound to the SP-Sephadex was eluted (250 mL/h) with 0.2 M sodium acetate (pH 8.3), concentrated by ultrafiltration (Amicon, YM-2 membrane), and dialyzed against 0.1 M acetic acid containing 20 μg/mL pepstatin A.

Step 3. The active fraction from SP-Sephadex was applied to a Bio-Gel P-10 column equilibrated and eluted with acetic acid (Figure 2). Two peaks of mitogenic activity were separated at $M_r \leq 10\,000$ and were collected separately to form pool A and pool B for further purification.

Step 4. The purification of Bio-Gel P-10 pool A was pursued next. Half of the total sample of pool A was applied to a μBondapak C_{18} RP-HPLC column. The protein and activity were eluted with an acetonitrile gradient (Figure 3A). The active fractions (shaded area) were pooled and applied to a Vydac C_4 RP-HPLC column also eluted with an acetonitrile gradient (Figure 3B). Although further resolution was observed, additional fractionation was suggested by the presence of a major absorbing peak eluting immediately after the activity. The active fractions (shaded area) were pooled and applied to a Vydac C_4 column eluted with 2-propanol (Figure 3C). Mitogenic activity (shaded area) appeared in two fractions (27 and 28); these were stored separately.

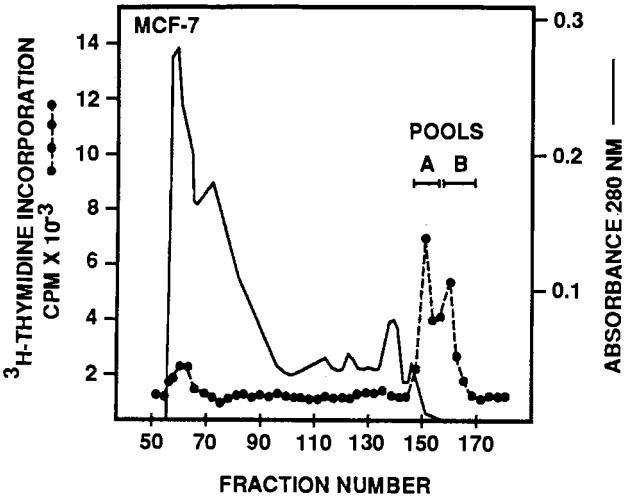


FIGURE 2: Elution profile of protein and mitogenic activity from the Bio-Gel P-10 column. The active fraction from SP-Sephadex was applied to a 2.5 × 120 cm column equilibrated and eluted with 0.1 M acetic acid. Fractions (3.7 mL) were collected each 15 min. Absorbance at 280 nm was monitored continuously. Each fraction was assayed for activity. Fractions 145–154 were combined into pool A and fractions 155–167 into pool B.

The experiment in Figure 4A shows the final purification of fraction 27 material on a Vydac C_{18} column. Fractions 92–94 contained significant mitogenic activity. Approximately half of fraction 93 was subjected to N^α sequencing. The first four cycles of Edman degradation produced several amino acids, including those expected of DES 1→3 IGF-I. Beginning with cycle five, the amino acid sequence of IGF-I (Rinderknecht & Humbel, 1978a) was confirmed by the next 7–10 residues (Table II).

The experiment in Figure 4B shows the final Vydac C_{18} RP-HPLC purification of material from fraction 28. Fraction 56 contained the majority of activity and was subjected to N^α sequencing. As found with fraction 27 material, the data were consistent with the presence of a DES 1→3 to DES 1→6 form of IGF-I (Table II). The overall purifications of the truncated forms of IGF-I from Bio-Gel P-10 pool A were [(1–8) × 10^6]-fold.

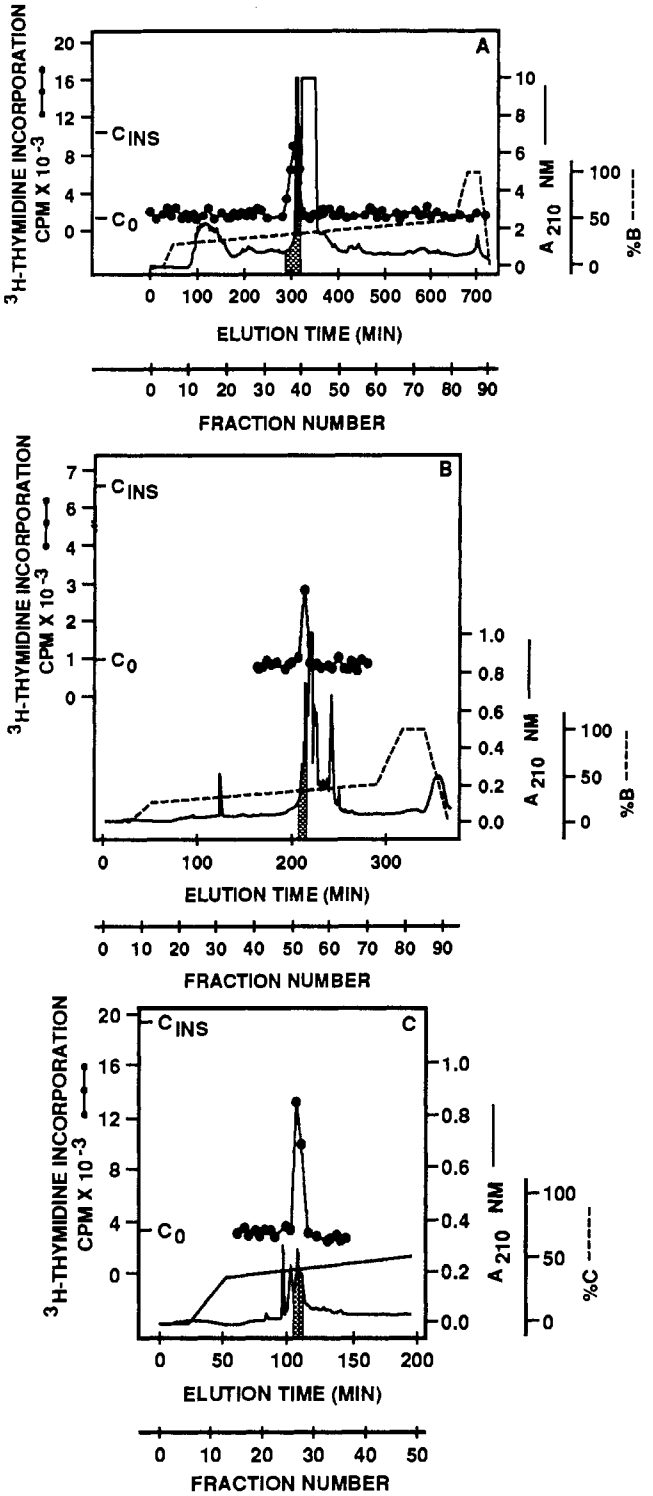


FIGURE 3: RP-HPLC separations of Bio-Gel P-10 pool A material. (A) Pool A was lyophilized and reconstituted in 2 mL of 0.1% TFA, and half of the sample was applied to a μBondapak C_{18} column equilibrated with solution A (0.1% TFA). The column was eluted with a 20–50% (v/v) linear gradient of solution B (0.1% TFA plus 90% acetonitrile) over 600 min. The flow rate was 0.2 mL/min; fractions were collected every 8 min. Absorbance was monitored at 210 nm. Mitogenic activity was estimated by the [*methyl*-^{3}H]thymidine assay. (B) Active fractions from panel A (shaded area) were diluted 1:4 with solution A and applied directly to a Vydac C_4 column equilibrated with solution A. The column was eluted with a linear gradient of 20–40% (v/v) solution B at a flow rate of 0.3 mL/min. Fractions were collected every 4 min. (C) Active fractions from panel B (shaded area) were diluted 1:4 and applied to the Vydac C_4 column equilibrated with solution A and eluted with a linear gradient of 35–75% solution C (0.1% TFA containing 60% 2-propanol). The flow rate was 0.3 mL/min; fractions were collected every 4 min. Two active fractions (27 and 28, shaded areas) were further purified separately.

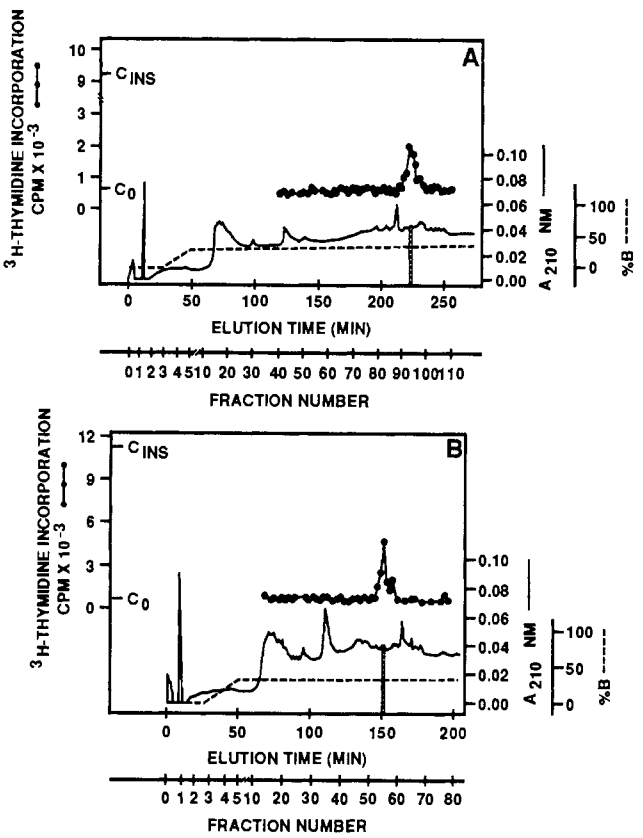


FIGURE 4: Purification of pool A fractions 27 and 28. (A) Fraction 27 from Figure 3C was diluted 1:4 with solution A and applied to a Vydac C_{18} column equilibrated with solution A. The column was eluted with a linear gradient of 30–40% solution B. The flow rate was 0.3 mL/min; fractions were collected every 2 min. The mitogenic activity was estimated by the [*methyl*-^{3}H]thymidine assay. (B) Fraction 28 from Figure 3C was diluted 1:4 with solution A and applied to a Vydac C_{18} column equilibrated with solution A. The column was eluted with a linear gradient of 32–42% solution B. The flow rate was 0.3 mL/min; fractions were collected every 2 min. Mitogenic activity was found with the [*methyl*-^{3}H]thymidine assay.

Table II: Partial Amino Acid Sequences of UDGF Fractions Isolated by RP-HPLC

	1				5				10				15				20			
human IGF-I[a]	G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	C	G	D	R	G
pool A fraction 93[b,c]				X	X	X	X	A	E	L	V	D	A	L	X	F	V	X				
pool A fraction 56				X	X	X	X	A	E	L	V	D	A	L	Q	F	V	X				
pool B fraction 44				I	L	X	G	A	E	L	V	D	A	L	Q	F	V	X	G	D	R	G
human IGF-II[d]	A	Y	R	P	S	E	T	L	C	G	G	E	L	V	D	T	L	Q	F	V	C	S	D	R	G

[a] See Rinderknecht and Humbel (1978a) for the complete sequence of IGF-I. [b] Underlined residues represent tentative assignments. [c] The X denotes sequencing cycles when either more than one or no amino acid residue was found. [d] See Rinderknecht and Humbel (1978b) for the complete sequence of IGF-II.

Step 5. The purification of Bio-Gel P-10 pool B material was pursued next. The lyophilized sample of pool B was reconstituted in 0.1% TFA and applied to a μBondapak C_{18} RP-HPLC column. Elution with acetonitrile yielded a single area of activity corresponding to a small peak of material absorbing at 210 nm (Figure 5A). The active fractions (shaded area) were pooled and applied to a Vydac C_4 RP-HPLC column (Figure 5B). Several absorbance peaks were separated that contained no biological activity. The single fraction (44) that contained activity was analyzed by N[α] sequencing and showed the presence of DES 1→3 IGF-I (Table II). The overall purification of DES 1→3 IGF-I from the Bio-Gel P-10 pool B was (8.5×10^6)-fold.

Estimate of UDGF M_r. At the SP-Sephadex step of isolation, the M_r of UDGF activity was estimated by Superose

Table III: Summary of the Purification of Truncated IGF-I for M_r Estimation by Urea–SDS–PAGE and Autoradiography

steps	total protein	volume (mL)	protein (concn/mL)	ED_{50} (concn/mL)	total units	yield (%)
(1) Vydac C_{18} eluted with acetonitrile						
fractions 24–26	540 ng	1.8	300 ng	7.8 pg	69 300	3.4
fractions 28–30	10.3 μg	1.8	5.72 μg	40.8 pg	252 000	12.4
(2) Vydac C_{18} eluted with 2-propanol						
fraction 17	350 ng	0.6	583 ng	3.8 pg	90 000	4.4
fraction 18	164 ng	0.6	275 ng	26.1 pg	6 300	0.3

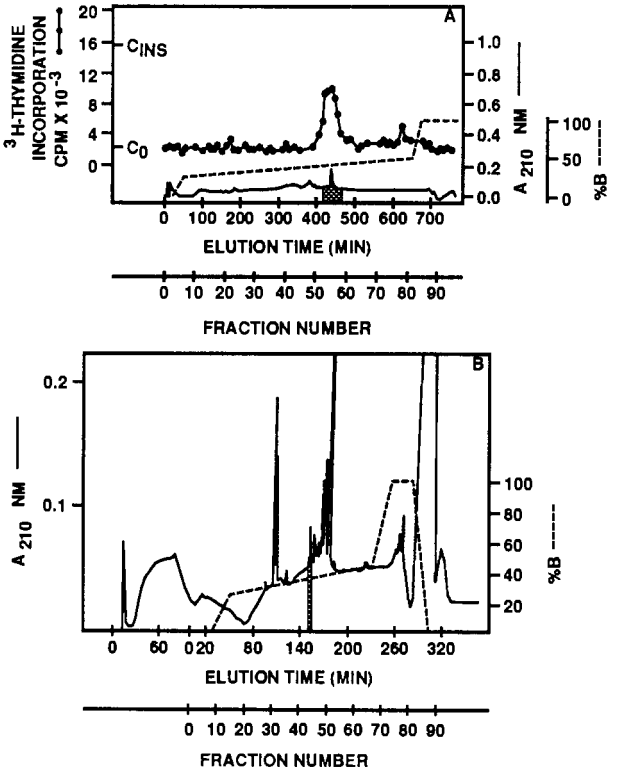


FIGURE 5: RP-HPLC separations of Bio-Gel P-10 pool B material. (A) The lyophilized sample of Bio-Gel P-10 pool B was reconstituted in solution A and applied to a μBondapak column equilibrated with solution A. The column was eluted with a linear gradient of 25–50% solution B at a flow rate of 0.2 mL/min. Fractions were collected every 8 min. Mitogenic activity (shaded area) was found with the [*methyl*-^{3}H]thymidine assay. (B) The fractions corresponding to the shaded area (8 mL) in panel A were diluted 1:2 with solution A and applied to a Vydac C_4 column equilibrated with solution A and eluted at 0.3 mL/min with a linear gradient of 25–45% solution B. Fractions were collected every 3.5 min. The mitogenic activity was localized to fraction 44 (shaded).

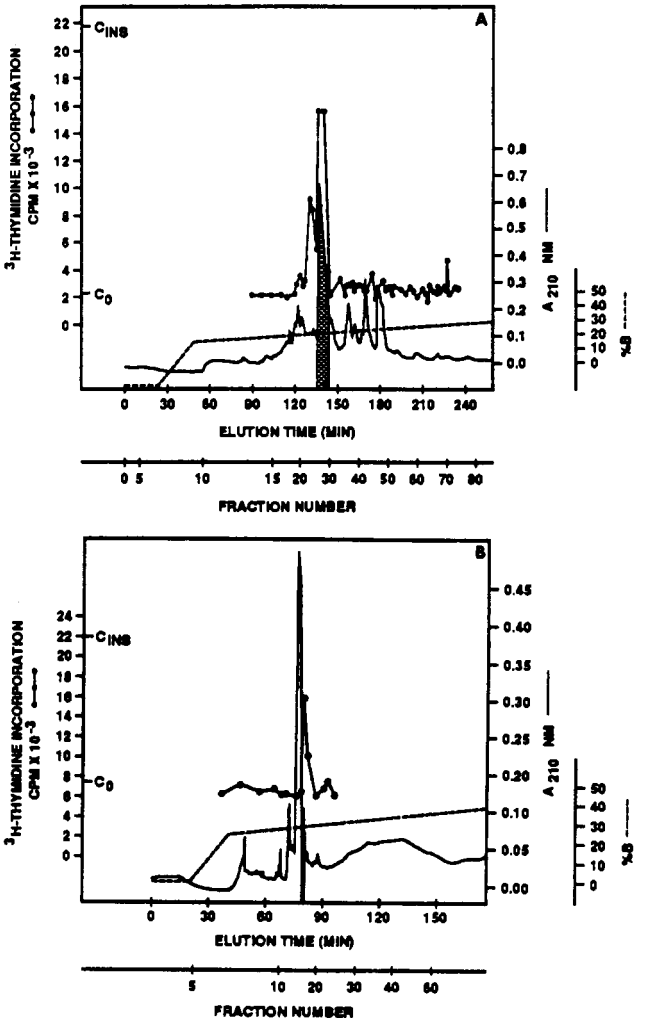


FIGURE 6: RP-HPLC purification of UDGF for M_r estimation by iodination, urea–SDS–PAGE, and autoradiography. Half of the sample from the μBondapak separation of Bio-Gel P-10 pool A (Figure 3A) was diluted 1:4 with solution A and applied to a Vydac C_4 column equilibrated with solution A and eluted at 0.3 mL/min with a linear gradient of 30–40% solution B. Fractions were collected every 2 min. Activity was found in fractions 28–30. (B) Fractions 28–30 from panel A were diluted 1:4 with solution A and applied to a Vydac C_{18} column equilibrated with solution A and eluted at 0.3 mL/min with a linear gradient of 25–50% solution C. Fractions were collected every 2 min. The mitogenic activity was found in fractions 17–19.

12 molecular sieve chromatography in PBS. The activity eluted in the volume corresponding to M_r 6500 ± 500 (data not shown). To better estimate the M_r of UDGF versus that of 71 amino acid recombinant IGF-I, the remaining half of the sample from the μBondapak fractionation of Bio-Gel P-10 pool A (Figure 3A) was purified by an abbreviated method. The sample was applied to a Vydac C_{18} column and eluted with an acetonitrile gradient (Figure 6A). The active fractions 24–26 and 28–30 were pooled separately. The latter pool contained 3–4 times more total activity than the former (Table III). Fractions 28–30 were applied to another Vydac C_{18} column that was eluted with a 2-propanol gradient (Figure 6B). The activity eluted in fractions 17–19. Fraction 17 contained 70–90% of the activity and showed an ED_{50} of 3.8 pg/mL (Table III). This fraction yielded approximately 350 ng of protein, which was considerably greater than the amounts obtained by the more lengthy methods summarized in Table I.

Fraction 17 UDGF sample was ^{125}I-labeled and analyzed by urea–SDS–PAGE and autoradiography (Figure 7). The migration of labeled UDGF was compared to that of proteins of known M_r and to that of ^{125}I-labeled recombinant 71 amino acid IGF-I. A plot of migration versus M_r showed UDGF migrated somewhat faster than recombinant IGF-I (data not shown). The M_r of UDGF was 500 less than IGF-I. The data were consistent with UDGF being truncated by a removal of a small number (i.e., three or four) of residues. However, the absolute M_r for recombinant IGF-I was estimated by urea–SDS–PAGE to be 5300, while that of UDGF was estimated at 4800. Others (Smith et al., 1987) have noted also that the

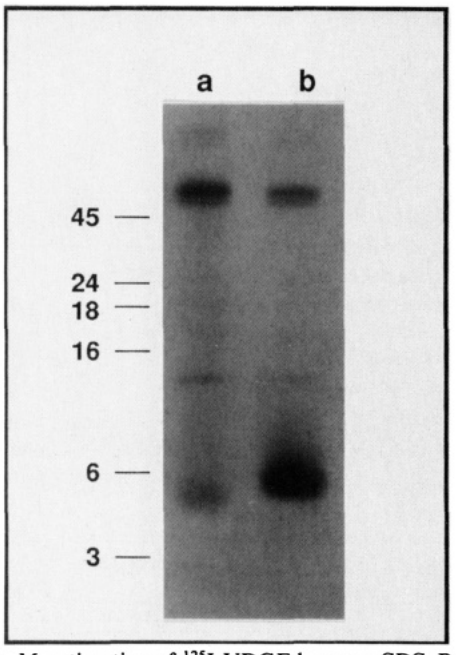


FIGURE 7: M_r estimation of ^{125}I-UDGF by urea–SDS–PAGE and autoradiography. Iodinated UDGF (20000 cpm, lane a) and iodinated recombinant IGF-I (12000 cpm, lane b) were prepared as described under Experimental Procedures. The arrows show the migration positions of standard prestained proteins including ovalbumin (45 kDa), α-chymotrypsinogen (24 kDa), β-lactalbumin (18 kDa), lysozyme (16 kDa), bovine trypsin inhibitor (6 kDa), and insulin B chain (3 kDa).

M_r of IGF-I estimated by SDS–PAGE was significantly lower than 7649 expected from the chemical composition (Rinderknecht & Humbel, 1978a). Also, two iodinated bands were found in the autoradiogram (Figure 7) at M_r higher than that of IGF-I or UDGF. These macromolecules were present in the quenching and chromatography mixtures and became iodinated by residual chloramine T activity.

Immunoadsorption of UDGF and IGF-I by Antibodies to Recombinant IGF-I. To determine if UDGF shared antigenic properties with IGF-I, rabbit polyclonal antibodies to recombinant IGF-I were bound to protein A–Sepharose, and this gel was incubated with both UDGF and recombinant IGF-I. The UDGF used in this study came from fraction 93 used to obtain an N^{α} amino acid sequence (Table II). As controls, the same amount of protein A–Sepharose was coated with preimmune serum and used in parallel incubations. The UDGF activity (105 units) was completely absorbed by the immune serum coated gel (Figure 8A). The similar experiment with 50 units of recombinant IGF-I (Figure 8B) showed a reduction of activity by incubation with immune serum coated gel, but not complete elimination. However, 105 units of truncated IGF-I (UDGF) was 105 pg of growth factor, while 50 units of recombinant IGF-I was 3.5 ng. Removal of 35 times more recombinant IGF-I was expected to be less efficient.

Identification of UDGF by RIA. Samples from the final stages of RP-HPLC purification were evaluated by RIA for IGF-I content. Fraction 44 (N^{α} sequenced, Table II) from the purification of Bio-Gel P-10 pool B was compared to recombinant IGF-I and active fractions from the first Vydac C_4 column isolation step of the pool A growth factor. The growth factors from both pool A and pool B generated curves that were parallel to that of recombinant IGF-I, indicating either identity or very close similarity (Figure 9A). By comparison, unlabeled recombinant IGF-II and unlabeled insulin were 10^2 and 10^4 times less effective competitors, respectively, than recombinant IGF-I and displayed B/B_0 plots that were not parallel with standard recombinant IGF-I (Figure 9B). Since IGF-I and IGF-II have similar amino acid sequences within residues determined in our study (Table II), these data provide important additional evidence that UDGF was not equivalent to IGF-II.

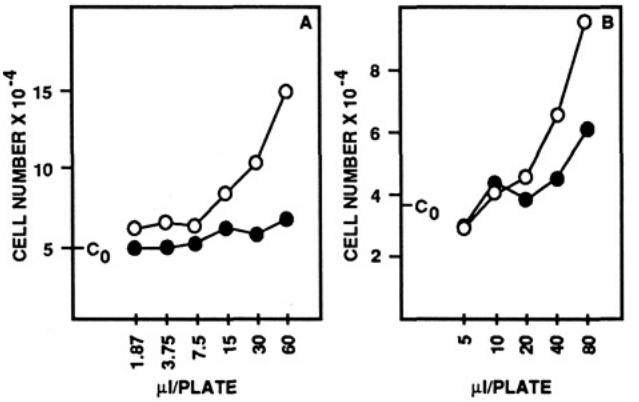


FIGURE 8: Immunoadsorption of UDGF and recombinant IGF-I by rabbit polyclonal antibodies to recombinant IGF-I. The experiment was carried out as described under Experimental Procedures. (A) Purified UDGF (105 units/300 µL) from fraction 93 of the final Vydac C_4 column was incubated with protein A–Sepharose coated with preimmune serum (open circles) and the same amount of gel coated with immune serum (solid circles). The supernatants (microliter) were assayed for growth activity by the cell number method. (B) Recombinant IGF-I (50 units/300 µL) was incubated with protein A–Sepharose coated with preimmune serum (open circles) and with the same amount of gel coated with immune serum (solid circles).

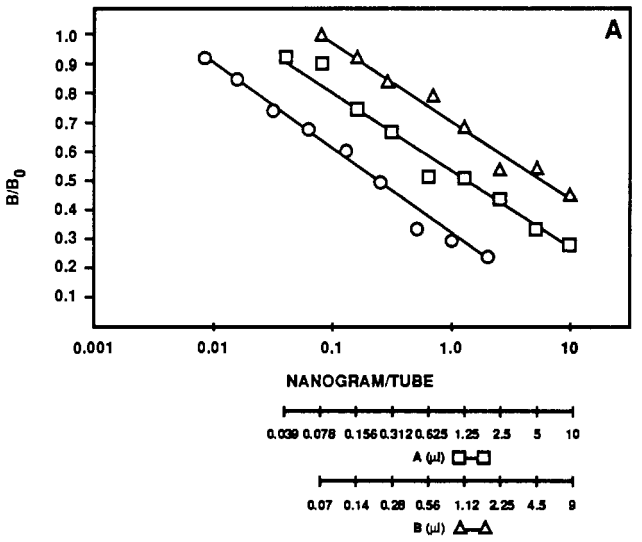
Table IV: ED_{50} Estimations of Truncated IGF-I Growth Promoting Activity

fractions analyzed by RIA for IGF-I	ED_{50} estimation with MCF-7 cells (pg/mL)
Bio-Gel P-10 pool A	
Bio-Gel P-10 pool A fraction	0.6
first Vydac C_4 column	
pooled fractions 53 and 54	1.0
final Vydac C_{18} column	
fraction 93 (sequenced)	1.4
fraction 56 (sequenced)	0.8
Bio-Gel P-10 pool B	
final Vydac C_4 column	
fraction 44 (sequenced)	0.7

Comparison of the Biological Potency of UDGF and Recombinant IGF-I. Because the ED_{50} data presented in Table I were based upon protein determination methods that were subject to considerable error (i.e., peak area and N^{α} sequencing), another method of quantifying was required to compare the biological potency of truncated IGF-I (UDGF) to that of recombinant IGF-I.

Several fractions from the purification of Bio-Gel P-10 pool A were analyzed by RIA for IGF-I. These assays all yielded lines parallel to recombinant IGF-I when B/B_0 versus concentration (log scale) plots were constructed (data not presented). The concentrations in these samples were used to estimate the biological potency of UDGF (truncated IGF-I) versus that of recombinant material with the MCF-7 cells. The ED_{50} values of truncated IGF-I purified from pool A were 0.6–1.4 pg/mL whether the material was derived from an early RP-HPLC step or from fractions used to obtain partial amino acid sequences (Table IV).

When pool B DES 1→3 IGF-I (fraction 44) was assayed for biological activity versus recombinant IGF-I, the ED_{50} values were 0.7 and 40 pg/mL, respectively (Figure 10). Comparison of the RIA-based ED_{50} data in Table IV with those obtained by peak area estimations or from protein se-



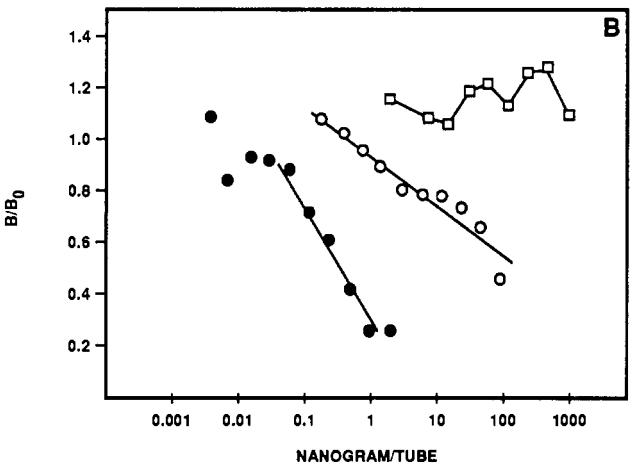


FIGURE 9: Identification of UDGF as IGF-I by RIA. (A) Fraction 44 from the final step of the isolation of Bio-Gel P-10 pool B was compared by RIA to recombinant IGF-I. Other samples (see Figure 3B, fractions 53 and 54) from the purification of pool A material were included for comparison. The curves generated by standard unlabeled IGF-I competition are shown by circles, while those of unlabeled fraction 44 are shown as triangles and those of unlabeled fractions 53 and 54 combined are shown as squares. Duplicate incubations were used to calculate each data point. (B) Comparison of recombinant IGF-I (solid circles), recombinant IGF-II (open circles), and insulin (squares) measurement by RIA. The designation B_0 represents the cpm of ^{125}I-IGF-I bound to the antibody without unlabeled IGF-I added; B is cpm in the presence of increasing concentrations of unlabeled factors.

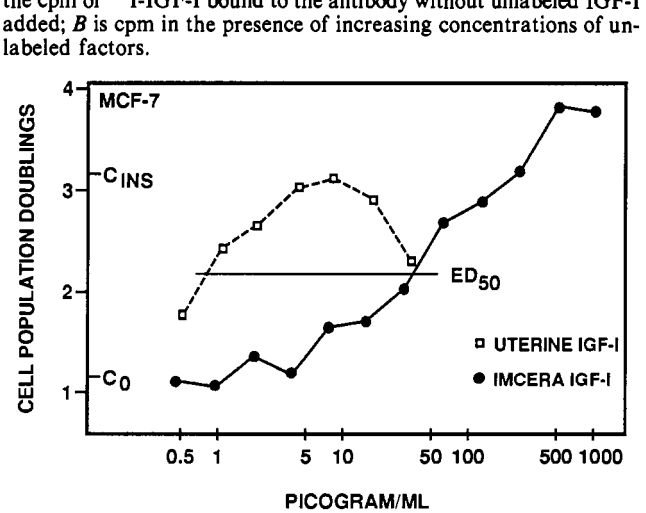


FIGURE 10: Growth of the MCF-7 cells promoted by uterine-derived IGF-I and IMC (IMCERA) recombinant human IGF-I. The assay was done by the cell number method. The data points are the averages of duplicate determinations of cell number.

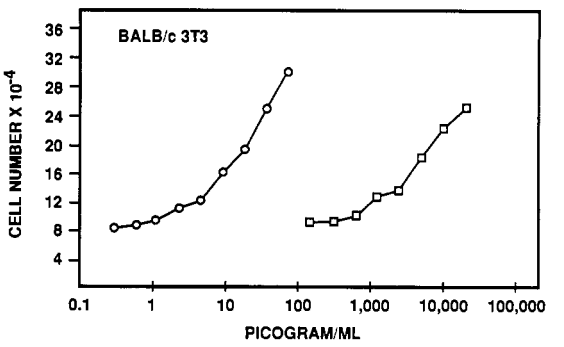


FIGURE 11: Growth of the Balb/c 3T3 cells in SFM-3T3 supplemented with uterine-derived IGF-I (open circles) or recombinant IGF-I (open squares) for 4 days. The truncated IGF-I preparation used in this experiment was from the large-scale (6.48 kg) preparation carried through the final Vydac C_{18} isolation step using 2-propanol elution of the activity. The data points represent the averages of duplicate cell number determinations.

Table V: Large-Scale Isolation of UDGF

steps	total protein[a]	volume[a] (mL)	total units[a]
crude extract preparation[b] (tissue weight)			
14, 1.11 kg	ND	ND	ND
15, 2.03 kg	ND	ND	ND
16, 2.07 kg	ND	ND	ND
17, 1.27 kg	ND	ND	ND
SP-Sephadex			
14	406 mg	20	ND
15	809 mg	55	ND
16	818 mg	25	ND
17	362 mg	21	ND
Bio-Gel P-10			
14	9.46 mg	5	ND
15	21.7 mg	10	ND
16	18.1 mg	5	ND
17	9.60 mg	5	ND
first Vydac C_{18} RP-HPLC[c] (acetonitrile gradient eluted)			
14, 16	ND	7.8	460 200
15, 17	ND	8.4	1 302 000
second Vydac C_{18} RP-HPLC[d] (2-propanol gradient eluted)			
fraction A	496 ng	4.8	427 000
fraction B	1440 ng	6.0	1 020 000

[a] ND = not determined. [b] The numbers 14–17 indicate individual batch numbers of the tissue processed as summarized in Table I for similar 1-kg preparations. The 1-kg preparation described in Table I was tissue batch 13. [c] Tissue batches 14 and 16 were pooled to carry out the first Vydac C_4 separation as were batches 15 and 17. [d] Total protein is the amount of IGF-I determined by RIA.

quencing (Table I) confirmed a remarkable agreement.

To further evaluate the biological potency of truncated IGF-I versus the recombinant 71 amino acid factor, the effects of each were compared with Balb/c 3T3 cells. Truncated IGF-I was ≥100 times more potent than the recombinant material (Figure 11). A single addition of 75 pg/mL truncated IGF-I caused more growth than 20 ng/mL recombinant IGF-I.

Large-Scale Isolation of UDGF. To compare receptor binding properties of recombinant IGF-I versus the truncated factor, it was essential to isolate larger amounts of UDGF. A total of 6.48 kg of tissue was used in a modified isolation procedure. A summary of the large-scale isolation is presented in Table V.

The isolation was modified to a five-step procedure that yielded only 10–20% homogeneous truncated IGF-I (data not presented). Four batches of tissue (14–17) were taken separately through the same first three isolation steps used in the

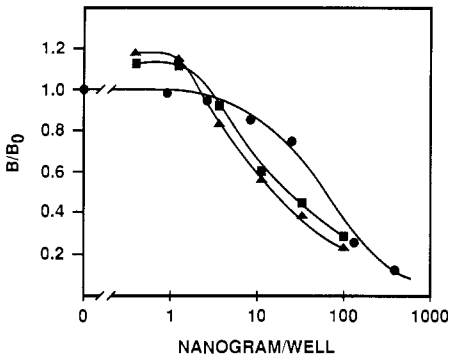


FIGURE 12: RRA comparison of the efficiency of competition for ^{125}I-IGF-I binding to MCF-7 type I receptors by UDGF samples from large-scale pool A (squares) and pool B (triangles) as well as by recombinant IGF-I (circles). The samples were those from Table V (second Vydac C_{18} RP-HPLC column). Data points are the averages of duplicate incubations at each concentration.

1.0-kg preparation including crude extract preparation (step 1), SP-Sephadex ion-exchange chromatography (step 2), and Bio-Gel P-10 molecular sieve chromatography (step 3). The first modification in the original procedure was not to pool Bio-Gel P-10 fractions A and B separately; these were combined. Further, the Bio-Gel P-10 fractions from tissue batches 14 and 16 were pooled, as were batches 15 and 17. The total units of activity in each pooled preparation is shown in Table V. The second modification was to apply the two pools separately to a Vydac C_{18} column equilibrated with TFA. The column was eluted with a linear gradient of acetonitrile as described in the legend of Figure 4A. One major peak of activity was eluted in both cases (data not shown). The third modification was to complete the isolation by applying the most mitogenic fractions from the first Vydac C_{18} RP-HPLC of tissue batches 14 and 16 (combined) and batches 15 and 17 (combined) (see Table V) to a Vydac C_{18} column equilibrated with 0.1% TFA and eluted with a linear gradient of 15–45% (v/v) of a solution of 0.1% TFA containing 60% (v/v) 2-propanol. The fractions were assayed for mitogenic activity. The results with pooled tissue batches 15 and 17 were the same as found with batches 14 and 16. Activity pools labeled A and B were identified and stored separately (data not shown).

The concentrations of IGF-I in pools A and B were determined by RIA. The plots of B/B_0 versus concentration were parallel to that of recombinant IGF-I, confirming the presence of materials that were immunologically equivalent (data not shown). Table V shows the total amounts of IGF-I and units of activity in both pools A and B. By use of the RIA quantification data, the ED_{50} values of both pools A and B were shown to be identical and within the 1–3 pg/mL range expected for truncated IGF-I prepared by the smaller scale methods (data not shown).

RRA Comparison of UDGF and Recombinant IGF-I. The UDGF from pools A and B of the large-scale preparation was used to conduct an RRA comparison to recombinant IGF-I binding to the type I receptor (Figure 12). Specific binding of ^{125}I-labeled IGF-I to MCF-7 cell monolayers was competed 50% by 44 ng/well unlabeled recombinant IGF-I. Pool A and pool B samples likewise competed for labeled IGF-I binding; the 50% competition level required 21 and 15 ng/well, respectively. The binding displacement curves with unlabeled pool A and pool B samples were very similar to those of unlabeled recombinant IGF-I.

Discussion

Since the first report of the characterization of UDGF from lyophilized powder of pregnant sheep uterus (Ikeda & Sirbasku, 1984), several changes in methodology were required to complete the isolation. The modifications included using fresh (porcine) tissue rather than lyophilized and powdered uteri, inclusion of protease inhibitors during the initial extraction/chromatography steps, elimination of the 95 °C heating procedure, which generated peptides of known proteins, and finally application of RP-HPLC methods to achieve greater resolution of the mixtures.

Another essential modification was the development of a new serum-free assay in which the nutritional requirements of the MCF-7 cells were satisfied sufficiently to allow continuous cell growth for several generations in response to picomolar concentrations of growth factors (Ogasawara & Sirbasku, 1988; Karey & Sirbasku, 1988). Random peptides produced by limited proteolysis or heat/acid fragmentation of known tissue and serum proteins were not mitogenic in this assay nor did they show significant interference with growth factor stimulated proliferation.[2] Ample evidence of this fact was provided by RP-HPLC experiments in which only one or at most a few fractions showed mitogenic activity, while as many as 20 other UV-absorbing peaks were observed. In every case, RP-HPLC fractions that showed biological activity were proven by purification and sequencing to be defined mitogenic species.

Application of these modifications showed that homogeneous products required considerably more isolation effort than recognized before. Whereas the previous paper (Ikeda & Sirbasku, 1984) described a 150-fold isolation of mitogenic activity, the newer methods reported here proved that final enrichments of (1.0–8.5) × 10^6 were necessary to achieve products for N^α sequencing. Although the final products showed M_r values within the 3700–7200 range estimated by our previous study (Ikeda & Sirbasku, 1984), it was clear that the mitogens isolated were different than expected. The data obtained caused reassessment of several aspects of our previous paper (Ikeda & Sirbasku, 1984). A summary of the new conclusions is presented next.

Growth Factor Forms in Uterus. The data presented in this paper showed that all of the acid-stable mammary cell mitogenic activities isolated from uterus were truncated forms of IGF-I. The mitogenic activities present in each fraction of the column separations were monitored by an assay designed to detect picogram–nanogram per milliliter concentrations of IGF-I, IGF-II, insulin, EGF, TGFα, and bFGF. Nevertheless, the acidic methods used throughout this study eliminated some factors from consideration. For example, bFGF, which has been found in many tissues (Gospodarowicz, 1987), was unstable under acidic conditions (Gospodarowicz & Cheng, 1986). Our previous studies with COMMA-D mouse mammary cells in serum-free defined medium confirmed the presence of a mitogen(s) in neutral extracts of porcine uterus (Riss & Sirbasku, 1987b). Under those conditions bFGF might have been the activity measured. However, in studies not presented,[3] acetic acid extracts of porcine uterus contained 3–4 times more total units of activity than those made at neutral pH, implying that even if bFGF was present, it did not represent a majority.

Recently, DiAugustine et al. (1988) reported the presence of EGF-specific mRNA in mouse uterus. By use of immunological methods, a high-M_r acid-stable form of this protein was identified in different types of cells of that tissue and in uterine luminal fluid. These studies indicated a 2-fold induction of EGF mRNA by estrogens, but did not demonstrate that the corresponding protein was increased by steroid

[3] M. Ogasawara and D. A. Sirbasku, unpublished data.

treatment or that the high-M_r factor was biologically active. Our study of mitogens in uterine luminal fluid (Leland et al., 1983) had shown also that estrogen-induced accumulations contained mammary/pituitary tumor cell growth factors. A partial characterization of this material indicated an activity consistent with members of the insulin-like family.[2] Although we recognize that biologically active EGF/TGFα mitogens from uterus may have been lost during the isolation reported here, we believe this unlikely because similar RP-HPLC methods have been used by this laboratory to isolate a mitogenic M_r 15000 form of TGFα from porcine pituitaries (Riss & Sirbasku, 1989).

Other studies by Murphy et al. (1987) indicated that IGF-I was estrogen-induced in rat uterus as measured by specific mRNA production and RIA measurements of the protein. Their data implied that IGF-I might serve as a local regulator or estromedin (Sirbasku, 1981; Ikeda et al., 1982) functioning in the steroid hormone promoted growth of uterus. Although characterization of the molecular properties of the IGF-I was not described, the RIA data suggested an extraordinarily high concentration of mitogen in this tissue (i.e., 700–800 μg/kg). These concentrations rank uterus as a richer tissue source of IGF-I than any other known (D'Ercole et al., 1984; D'Ercole & Underwood, 1987). Our study indicated that no more than 5–10 μg/kg was present. In fact, Schwander et al. (1983) and D'Ercole et al. (1984) have concluded that IGF-I was present in tissues in only small amounts compared to plasma concentrations. Others have examined the amount of IGF-I-specific mRNA in a variety of tissues (Mathews et al., 1986; Hynes et al., 1987; Roberts et al., 1987) and have concluded that liver was the major site of biosynthesis of this factor. The data describing the exceptionally high IGF-I concentrations in uterus (Murphy et al., 1987) may reflect problems of measurement by RIA. *It should be noted that truncated IGF-I has not been established to be estrogen inducible.* These studies await development of RIA and Northern blot (mRNA identification) methods for specifically measuring truncated IGF-I versus the intact 70 amino acid form in uterine extracts.

The amount of immunoreactive IGF-I in any tissue may in part be derived from residual blood (D'Ercole & Underwood, 1987). We expected that at least some of the porcine-truncated IGF-I isolated in this study came from blood contamination. One indication that all the uterine-derived IGF-I was not from this source came from the lack of identification of IGF-II. This mitogen was usually found in plasma at higher concentrations than IGF-I (Zapf et al., 1981; Daughaday et al., 1981; Hintz & Liu, 1982). Isolation of IGF-II has been accomplished under acidic conditions by conventional chromatography methods (Rinderknecht & Humbel, 1978b) and under acidic conditions by RP-HPLC (Marquardt et al., 1981; Greenstein et al., 1984). When IGF-II was present in tissue/cell extracts, it was readily purified by the same RP-HPLC methods employed in this study (Karey et al., 1987). For example, extracts of outdated human platelets were shown to contain two mitogens for MCF-7 cells in serum-free Tf/BSA. These were purified to homogeneity, and partial amino acid sequencing was used to confirm IGF-I and IGF-II primary structures. The absence of IGF-II in uterine preparations was taken to indicate minimal blood contamination. Further evaluation of IGF-II production by porcine uterus awaits Northern blot analysis for specific mRNA. Also, it may be that the IGF-II was present in a truncated biologically inactive form. Nevertheless, it can be stated with certainty that the forms of IGF-I from uterus were highly mitogenic and all truncated at the N^{α} position compared to IGF-I isolated from serum (Rinderknecht & Humbel, 1978a; Klapper et al., 1983).

Another potential problem with the quantification of IGF-I in uterine extracts was its propensity to associate with serum- and tissue-derived proteins to form complexes that cause discrepancies in RIA measurements (Furlanetto, 1982; Daughaday et al., 1987). If carrier or binding proteins are not separated carefully from IGF-I before RIA, their presence causes a marked overestimation of IGF-I.

Structural and Other Similarities to Intact IGF-I. The N^{α} amino acid sequence data and M_r estimations presented in this paper suggested that the factors from uterus were DES 1→3 to DES 1→6 IGF-I. Because only nanograms of the factors could be isolated per kilogram of tissue, a complete amino acid sequence could not be determined. Although we cannot conclude that the only modifications in uterine-derived IGF-I were at the N^{α} terminus, estimation of M_r by urea–SDS–PAGE suggested that truncation at the carboxyl terminus was minimal. Further confirmation of structural similarities between intact and truncated IGF-I came from RIA data showing parallel B/B_0 plots and RRA data demonstrating very similar receptor binding characteristics.

Also, it should be noted that the microsequencing data allowed us to identify a DES 1→3 form of IGF-I as well as leaving open the possibility that as many as six residues might have been removed from the N^{α} terminus (Table II). If truncation included residue 6, which is a half-cystine, this would disrupt the disulfide bond between residues 6 and 48 in the intact structure. Further studies are in progress using solid-phase peptide synthesis methods to prepare DES 1→3 to DES 1→6 IGF-I for structure/function studies.

Other Characterizations of Truncated IGF-I. Our observations correlated well with work by others characterizing new forms of IGF-I. Sara et al. (1986) isolated DES 1→3 IGF-I from human fetal brain. Also, Francis et al. (1986) isolated this same form from bovine colostrum. In addition, DES 1→3 IGF-I has been chemically synthesized (Ballard et al., 1987). As was found with uterine-derived truncated IGF-I, the other studies indicated that removal of N^{α} amino acids resulted in a substantial increase in biological potency.

Sara et al. (1986) demonstrated that DES 1→3 IGF-I was 5 times more potent than intact IGF-I in an RRA competition of ^{125}I-labeled IGF-I binding to glial cells. They concluded that truncated IGF-I showed a higher affinity for the type I receptor than the intact 70 amino acid form. However, a study of the domain of IGF-I required for association with carrier proteins showed the N^{α} region was important (De Vroede et al., 1986). This observation led Ballard et al. (1987) to examine receptor binding affinities of intact and DES 1→3 IGF-I and to postulate that increased biological activity of the truncated form was due to the lack of association with binding (inactivating) proteins. These studies were supported by more recent data from this group (Szabo et al., 1988) confirming a role of the terminal three amino acids in binding to carrier protein. Since MCF-7 and many other kinds of cells in culture secrete IGF-I-binding proteins (Adams et al., 1984; Clemmons et al., 1986; Dickson et al., 1986; Mottola et al., 1986), and at least some of these caused inactivation, this mechanism might explain the high potency of DES 1→3 IGF-I from uterus.

Our data showed that the relative biological potencies of truncated IGF-I and recombinant IGF-I failed to correlate with receptor binding affinity. Uterine DES 1→3 IGF-I was 35–70 times more potent as a mitogen but only 2–3 times more effective than recombinant IGF-I in an RRA with MCF-7 cells. These results were consistent with DES 1→3 IGF-I

being highly active primarily because of lack of association with binding proteins. However, more study of this issue is required because the mitogenic assays and the binding competition assays were carried out under different conditions that might have influenced the results.

Cell Type Specificity. The previous study (Ikeda & Sirbasku, 1984) indicated that UDGF promoted growth of mammary, pituitary, and uterine-derived cells but not 3T3 mouse fibroblast cells. These observations led to the conclusion that UDGF was specific for cells from estrogen-responsive tissues. The identification of UDGF as truncated IGF-I required another evaluation of these data, especially in view of the well-established effects of insulin-like factors on mesenchymal origin cells (Clemmons & Van Wyk, 1981, 1985) including the Balb/c 3T3 line (Clemmons et al., 1980). For this purpose, we developed a new serum-free assay method for IGF-I with 3T3 cells (Riss et al., 1988). The previous assays of UDGF with 3T3 cells were done in medium deficient in several essential nutrients and growth factors required for IGF-I mitogenic action. As presented in this paper, it was clear that UDGF (truncated IGF-I) was mitogenic for 3T3 cells when conditions were properly optimized.

Formation of Truncated IGF-I. It was noteworthy that all three mitogens isolated from the uterus were truncated forms of IGF-I. This fact raised several possibilities with regard to the origins of this highly active growth factor.

It is possible that truncated IGF-I was formed by limited proteolysis of the intact species during the isolation process. This might have happened even though precautions were taken to restrict protease action in the early extraction steps. However, if this was the origin of the truncated IGF-I, the removal of at least three amino acids from the intact structure must be essentially complete because no significant amounts of the intact Rinderknecht and Humbel (1978a) structure were found.

Alternatively, if truncated IGF-I was a natural product of uterus, several processing mechanisms are possible. Truncated IGF-I might be formed by an unusual processing of the high-M_r precursor of IGF-I (Jansen et al., 1983) or by limited and specific proteolysis of the 70 amino acid form isolated from plasma (Rinderknecht & Humbel, 1978a). Although still speculative, it is possible that synthesis of the processing enzymes could be under estrogen control. Other possible origins of this factor might be from expression of a IGF-I gene in uterus that is inactive in liver or by a novel mechanism of tissue-specific processing of common IGF-I mRNAs. In any case, the identification of such a high specific activity mitogen in uterus indicates that further study of the biosynthesis and function of this factor in regulation of growth of this tissue is warranted.

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REFERENCES

Adams, S. O., Kapadia, M., Mills, B., & Daughaday, W. H. (1984) *Endocrinology 115*, 520–526.

Ballard, J. F., Francis, G. L., Ross, M., Bagley, C. J., May, B., & Wallace, J. C. (1987) *Biochem. Biophys. Res. Commun. 149*, 398–404.

Barnes, D. W., & Sirbasku, D. A., Eds. (1987a) *Methods Enzymol. 146*, 3–417.

Barnes, D. W., & Sirbasku, D. A., Eds. (1987b) *Methods Enzymol. 147*, 3–444.

Burleigh, D. B., & Meng, H. (1986) *Am. Biotechnol. Lab. 4*, 48–53.

Burr, F. A., & Burr, B. (1983) *Methods Enzymol. 96*, 239–244.

Clemmons, D. R., & Van Wyk, J. J. (1981) *J. Cell. Physiol. 106*, 361–367.

Clemmons, D. R., & Van Wyk, J. J. (1985) *J. Clin. Invest. 75*, 1914–1918.

Clemmons, D. R., Van Wyk, J. J., & Pledger, W. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6644–6648.

Clemmons, D. R., Elgin, R. G., Han, V. K., Casella, S. J., D'Ercole, A. J., & Van Wyk, J. J. (1986) *J. Clin. Invest. 77*, 1548–1556.

Daughaday, W. H., Trivedi, B., & Kapadia, M. (1981) *J. Clin. Endocrinol. Metab. 53*, 289–294.

Daughaday, W. H., Kapadia, M., & Mariz, I. (1987) *J. Lab. Clin. Med. 109*, 355–363.

D'Ercole, A. J., & Underwood, L. E. (1987) *Methods Enzymol. 146*, 227–233.

D'Ercole, A. J., Stiles, A. D., & Underwood, L. E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 935–939.

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.

DiAugustine, R. P., Petrusz, P., Bell, G. I., Brown, C. F., Korach, K. S., McLachlin, J. A., & Teng, C. T. (1988) *Endocrinology 122*, 2355–2363.

Dickson, R. B., Huff, K. K., Spencer, E. M., & Lippman, M. E. (1986) *Endocrinology 118*, 138–142.

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.

Furlanetto, R. W. (1982) *J. Endocrinol. Metab. 54*, 1084–1086.

Furlanetto, R. W., & DiCarlo, J. N. (1984) *Cancer Res. 44*, 2122–2128.

Furlanetto, R. W., & Marino, J. M. (1987) *Methods Enzymol. 146*, 216–226.

Furlanetto, R. W., Underwood, L. E., Van Wyk, J. J., D'Ercole, A. J. (1977) *J. Clin. Invest. 60*, 648–657.

Gospodarowicz, D. (1987) *Methods Enzymol. 147*, 106–119.

Gospodarowicz, D., & Cheng, J. (1986) *J. Cell. Physiol. 128*, 475–484.

Greenstein, L. A., Nissley, S. P., Moses, A. C., Short, P. A., Yang, Y. W.-H., Lee, L., & Rechler, M. M. (1984) in *Cell Culture Methods for Molecular and Cell Biology* (Barnes, D. W., Sirbasku, D. A., & Sato, G. H., Eds.) Vol. 1, pp 111–138, Alan R. Liss, New York.

Hintz, R. L., & Liu, F. (1982) *J. Clin. Endocrinol. Metab. 54*, 442–446.

Hynes, M. A., Van Wyk, J. J., Brooks, P. J., D'Ercole, A. J., Jansen, M., & Lund, P. K. (1987) *Mol. Endocrinol. 1*, 233–242.

Ikeda, T., & Sirbasku, D. A. (1984) *J. Biol. Chem. 259*, 4049–4064.

Ikeda, T., Liu, Q.-F., Danielpour, D., Officer, J. B., Iio, M., Leland, F. E., & Sirbasku, D. A. (1982) *In Vitro 18*, 961–979.

Ikeda, T., Danielpour, D., & Sirbasku, D. A. (1984a) *J. Cell. Biochem. 25*, 213–229.

Ikeda, T., Danielpour, D., Galle, P. R., & Sirbasku, D. A. (1984b) in *Cell Culture Methods for Molecular and Cell Biology* (Barnes, D. W., Sirbasku, D. A., & Sato, G. H., Eds.) Vol. 2, pp 217–241, Alan R. Liss, New York.

Ikeda, T., Lioubin, M. N., & Marquardt, H. (1987) *Biochemistry 26*, 2406–2410.

Jansen, M., van Schaik, F. M. A., Ricker, A. T., Bullock, B., Woods, D. E., Gabbay, K. H., Nussbaum, A. L., Sussenbach, J. S., & Van den Brande, J. L. (1983) *Nature (London) 306*, 609–611.

Karey, K. P., & Sirbasku, D. A. (1988) *Cancer Res. 48*, 4083–4092.

Karey, K. P., Marquardt, H., & Sirbasku, D. A. (1987) *Proc. Am. Assoc. Cancer Res. 28*, 54.

Karey, K. P., Riss, T. L., Burleigh, D. B., Parker, D., & Sirbasku, D. A. (1988) *In Vitro Cell. Dev. Biol. 24*, 1107–1113.

Keydar, I., Chen, L., Karby, S., Weiss, R., Delarea, J., Radu, M., Chaichik, S., & Brenner, H. J. (1979) *Eur. J. Cancer 15*, 659–670.

Klapper, D. G., Svoboda, M. E., & Van Wyk, J. J. (1983) *Endocrinology 112*, 2215–2217.

Leland, F. E., Kohn, D. F., & Sirbasku, D. A. (1983) *Biol. Reprod. 28*, 1243–1255.

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

Mathews, L. S., Norstedt, G., & Palmiter, R. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9343–9347.

Mottola, C., MacDonald, R. G., Brackett, J. L., Mole, J. E., Anderson, J. K., & Czech, M. P. (1986) *J. Biol. Chem. 261*, 11180–11188.

Murphy, L. J., Murphy, L. C., & Friesen, H. G. (1987) *Mol. Endocrinol. 1*, 445–450.

Ogasawara, M., & Sirbasku, D. A. (1988) *In Vitro Cell. Dev. Biol. 24*, 911–920.

Ogasawara, M., Marquardt, H., & Sirbasku, D. A. (1987) *Proc. Am. Assoc. Cancer Res. 28*, 58.

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

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

Riss, T. L., & Sirbasku, D. A. (1987a) *In Vitro Cell. Dev. Biol. 23*, 841–849.

Riss, T. L., & Sirbasku, D. A. (1987b) *Cancer Res. 47*, 3776–3782.

Riss, T. L., & Sirbasku, D. A. (1989) *J. Cell. Physiol.* (in press).

Riss, T. L., Ogasawara, M., Karey, K. P., Danielpour, D., Stewart, B. H., & Sirbasku, D. A. (1986) *J. Tissue Cult. Methods 10*, 133–150.

Riss, T. L., Karey, K. P., Burleigh, B. D., Parker, D., & Sirbasku, D. A. (1988) *In Vitro Cell. Dev. Biol. 24*, 1099–1106.

Roberts, C. T., Lasky, S. R., Lowe, W. L., Seaman, W. T., & LeRoith, D. (1987) *Mol. Endocrinol. 1*, 243–248.

Samsoondar, J., Kobrin, M. S., & Kudlow, J. E. (1986) *J. Biol. Chem. 261*, 14408–14413.

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

Schwander, J. C., Hauri, C., Zapf, J., & Froesch, E. R. (1983) *Endocrinology 113*, 297–305.

Sirbasku, D. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3786–3790.

Sirbasku, D. A. (1981) *Banbury Rep. 8*, 425–443.

Sirbasku, D. A., Leland, F. E., & Benson, R. H. (1981) *J. Cell. Physiol. 107*, 345–358.

Sirbasku, D. A., Ikeda, T., & Danielpour, D. (1985) in *Growth and Differentiation of Cells in a Defined Environment* (Murakami, H., Yamane, I., Barnes, D. W., Mather, J. P., Hayashi, I., & Sato, G. H., Eds.) pp 325–330, Springer-Verlag, New York.

Smith, E. P., Svoboda, M. E., Van Wyk, J. J., Kierszenbaum, A. L., & Tres, L. L. (1987) *Endocrinology 120*, 186–193.

Soule, H., Vazquez, J., Long, A., Albert, J., & Brennan, M. (1973) *J. Natl. Cancer Inst. 51*, 1409–1413.

Steck, G., Leuthard, P., & Burk, R. R. (1980) *Anal. Biochem. 107*, 21–24.

Szabo, L., Mottershead, D., Ballard, F. C., & Wallace, J. C. (1988) *Biochem. Biophys. Res. Commun. 151*, 207–214.

Zapf, J., Walter, H., & Froesch, E. R. (1981) *J. Clin. Invest. 68*, 1321–1330.