Purification of a 31,000-Dalton Insulin-like Growth Factor Binding Protein from Human Amniotic Fluid

ISOLATION OF TWO FORMS WITH DIFFERENT BIOLOGIC ACTIONS*

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Human amniotic fluid has been shown to contain a protein that binds insulin-like growth factor I and II (IGF-I and IGF-II). Partially purified preparations of this protein have been reported to inhibit the biologic actions of the IGFs. In these studies our laboratory has used a modified purification procedure to obtain a homogeneous preparation of this protein as determined by polyacrylamide gel electrophoresis and amino acid sequence analysis. During purification the ion exchange chromatography step resulted in two peaks of material with IGF binding activity termed peaks B and C. Each peak was purified separately to homogeneity. Both peaks were estimated to be 31,000 daltons by polyacrylamide gel electrophoresis and their amino acid compositions were nearly identical. Amino acid sequence analysis showed that both peaks had identical N-terminal sequences through the first 28 residues. Neither protein had detectable carbohydrate side chains and each had a similar affinity for radiolabeled IGF-I (1.7–2.2×10^{10} liters/mol). In contrast, these two forms had marked differences in bioactivity. Concentrations of peak C material between 2 and 20 ng/ml inhibited IGF-I stimulation of [^{3}H]thymidine incorporation into smooth muscle cell DNA. In contrast, when peak B (100 ng/ml) was incubated with IGF-I there was a 4.4-fold enhancement of stimulation of DNA synthesis. Additionally, pure peak B was shown to adhere to cell surfaces, whereas peak C was not adherent. The non-adherent peak C inhibited IGF-I binding to its receptor and to adherent peak B. We conclude that human amniotic fluid contains two forms of IGF binding protein that have very similar physiochemical characteristics but markedly different biologic actions. Since both have similar if not identical amino acid compositions, N-terminal sequences, and do not contain carbohydrate, we conclude that they differ in some other as yet undefined post-translational modification.

Somatomedin C, also termed insulin-like growth factor I (IGF-I),[1] is a peptide growth factor that stimulates growth in many types of cultured cells (1, 2). Although many cell types and tissues secrete IGF-I (3, 4), it is uncertain whether this locally produced IGF-I stimulates growth in the regional microenvironment or is transported through blood to stimulate growth at sites distant from its site of synthesis (5). Compounding this difficulty in understanding the mechanisms by which the IGFs stimulate growth is the observation that IGF-I and IGF-II circulate in blood bound to binding proteins (6, 7). Extracellular fluids (8) and cell culture supernatants (9) also contain IGF binding proteins, suggesting that the IGFs are present in a bound form in the extracellular microenvironment. Since these proteins bind IGF-I and IGF-II they are believed to inactivate these substances (10). There are two major classes of IGF binding proteins. One is a glycoprotein (~53 kDa) that is synthesized by hepatocytes (11) and in plasma forms a stable 150-kDa complex with IGF-I (12). This protein is growth hormone-dependent (13). In contrast, extracellular fluids such as ascites (14), spinal (15), follicular (16), and amniotic (17) contain a protein whose molecular size has been estimated to be between 30 and 38 kDa and is not growth hormone-dependent. Recently our laboratory has shown that human fibroblasts secrete this protein and that it adheres to the fibroblast surface (18). The surface-adherent protein directly alters IGF-I binding such that the binding of radiolabeled IGF-I is paradoxically increased when low concentrations of unlabeled IGF-I are added (18). Since this form of binding protein is present in many types of extracellular fluids it has the potential to alter the cellular responses to IGF-I. These studies were undertaken to determine the physiochemical properties of a pure preparation of the human amniotic fluid derived IGF binding protein and to determine whether it could alter the biologic effects of IGF-I.

EXPERIMENTAL PROCEDURES

Materials—Human amniotic fluid was obtained from Dr. A. J. D'Ercole (University of North Carolina) from discarded amniocentesis samples. Ammonium sulfate and sodium chloride were purchased from EM Sciences, Cherry Hill, NJ. Rabbit γ-globulin, DEAE-cellulose, ammonium persulfate, sodium thiocyanate, Sephadex G-100, polyethylene glycol (M_r 8,000) and ammonium carbonate were purchased from Sigma. Phenyl-Sepharose CL-4B was purchased from Pharmacia LKB Biotechnology Inc., and the C-4 reverse-phase HPLC column from Vydac, Hesperia, CA. Acetonitrile, Gel Code silver stain kit, and trifluoroacetic acid were purchased from Pierce Chemical Co. Tris, SDS, and TEMED were obtained from Bethesda Research Laboratories. Glycine, bromphenol blue, Servalyt isoelectric focusing precotes, and glycerol were obtained from Serva, Heidelberg, West Germany. Tissue culture plates were purchased from Falcon Labware Division, Becton Dickinson, Oxnard, CA.

Protein Purification—Crude amniotic fluid (230 ml) was equilibrated with 45 g of ammonium sulfate (33% saturation), stirred for 30 min at 4 °C and centrifuged at 27,000 × g for 20 min. The pellet

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[1] The abbreviations used are: IGF-I, insulin-like growth factor; PPP, platelet-poor plasma; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEMED, N,N,N',N'-tetramethylethylendiamine; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

was discarded and the supernatant was adjusted to 50% saturation with ammonium sulfate and stirred for 30 min and the centrifugation step repeated. This pellet (33–50%) was resuspended in 50 ml of 0.05 M Tris, pH 7.4, and 1.2 ml of saturated ammonium sulfate added to achieve a final concentration of 0.14 M. This solution was applied to a phenyl-Sepharose column (2.2 × 15.0 cm) that had been previously equilibrated with 0.05 M Tris, pH 7.4, in 10% ammonium sulfate. Following sample loading, the column was washed with the loading buffer until the absorbance (280 nm) returned to base line. The column was eluted with step gradients of the following composition: 1) 0.05 M Tris, 0.5 M sodium thiocyanate, pH 7.4; 2) 0.05 M Tris, pH 7.4; 3) 0.02 M Tris, pH 9.0; and 4) H_2O. Each fraction was assayed for IGF-I binding activity (see below). The active fractions were pooled, the pH was adjusted to 7.2 with 1.0 M acetic acid, and the solution was applied directly to a DEAE-cellulose column that had been equilibrated with 0.01 M $(NH_4)_2CO_3$, 0.01 M NaCl, pH 7.2. After sample application the column was washed extensively with the equilibration buffer until the absorbance (280 nm) returned to base line. The column was eluted with step salt gradients containing 0.1, 0.25, and 1.0 M NaCl in the equilibrium buffer. The fractions were assayed for IGF binding as described. Greater than 80% of the activity eluted with 100 or 250 mM NaCl. These two peaks, termed B and C, were purified separately. 1.5 ml of the peak B pool was applied to a C-4 Vydac reverse-phase HPLC column (0.46 × 25 cm) that had been equilibrated with 0.04% trifluoroacetic acid. The mobile phase was run isocratically for 5 min and then a linear gradient from 0 to 100% acetonitrile plus 0.04% trifluoroacetic acid was run over 25 min. The IGF binding protein activity of each fraction was determined and the active fractions were pooled and stored at −20 °C.

Pool C from the ion exchange column was first purified by Sephadex G-100 column chromatography. 10 ml of pool C was applied to a 2.2 × 90 cm column that had been equilibrated with 0.01 $(NH_4)_2CO_3$, 0.05 M NaCl, pH 7.2. The column was eluted using a flow rate of 30 ml/h and approximately 9-ml fractions were collected. The IGF-I binding activity was determined (as described below). The active fractions were pooled and applied directly to the reverse-phase C-4 column. The elution conditions that were identical to those stated previously were used.

^{125}I-IGF-I Binding Capacity—IGF-I binding activity of the column fractions was determined as follows using a polyethylene glycol precipitation method (18). 10 μl of each fraction was incubated with ^{125}I-IGF-I (340 μCi/μg) (final concentration of 0.27 ng/ml) for 60 min at 22 °C in 0.1 HEPES, 0.1% BSA, 0.01% Triton X-100, 44 mM NaHCO$_3$, 0.02% NaN$_3$, pH 6.0 (250 μl total volume). The IGF-I was iodinated by a published method (19). Bound and free ^{125}I-IGF-I were separated by adding 250 μl of 1% human γ-globulin and 500 μl of 25% polyethylene glycol (M_r 8000) (final concentration of 12.5%). The mixture was centrifuged at 1000 × g for 15 min. The pellet was washed with 1 ml of 6.25% polyethylene glycol and the final pellet counted in a γ spectrometer. Nonspecific binding was determined by measuring the amount of ^{125}I-IGF-I that could be precipitated in the presence of 1.0 μg/ml unlabeled IGF-I. It was consistently <5% and was subtracted from the total radioactivity that was precipitated. To determine overall recovery, each pool of active fractions was reassayed at several concentrations and the binding capacity of the pools was compared to a human amniotic fluid standard. The data were used to assign a unit value to each pool. One unit was the quantity of the binding protein in human amniotic fluid necessary to achieve half-maximal IGF-I binding in that assay. This corresponds to 250–300 pg of pure IGF binding protein which binds approximately 83–100 pg of IGF-I.

To determine the binding capacity and affinity of the pure IGF binding protein for IGF-I, radiolabeled IGF-I (340 μCi/μg; 0.27 ng/ml) was incubated with 14 ng/ml each binding protein and increasing concentrations of unlabeled IGF-I in 0.25 ml of 0.03 M NaH$_2$PO$_4$, 0.01 M EDTA, 0.05% Tween 20, pH 7.4. After 48 h at 4 °C the bound and free ^{125}I-IGF-I were separated by adding a 1:250 dilution of a rabbit anti-binding protein antibody which had been prepared using a mixture of peaks B and C binding proteins and the incubation continued for 24 h. At that time 8 μl of goat anti-rabbit serum was added and the mixture incubated for 1 h at 22 °C. 2 μl of normal rabbit serum was added and the mixture incubated for an additional 1 h. The bound and free growth factors were separated by centrifugation at 8000 × g for 10 min.

Physiochemical Analysis—The purity of both peaks B and C was determined by SDS-polyacrylamide gel electrophoresis. The running gel was 12% acrylamide containing 0.375 M Tris, 0.1% SDS, pH 8.8, and the stacking gel was 4% acrylamide in 0.125 M Tris, 0.1% SDS,

pH 6.8. 0.1–10 μg of sample was diluted to 75 μl in 0.1 M Tris, pH 6.8, 10% glycerol, 5% SDS, and 0.02% bromphenol blue and the samples were heated to 100 °C for 5 min. The supernatants were clarified, the gel lanes loaded, and the proteins separated for 14 h at 65 V. Silver staining was performed using Gel code silver staining kit. The lower limit of detection of the technique was 25 ng as determined using known protein standards.

Amino Acid Composition and Sequence Analysis—Amino acid analysis was performed by the PICO-TAG method (19), described briefly as follows. 500 ng of each protein was hydrolyzed in an evacuated, sealed vessel containing fumes of 6 N HCl and 0.1% phenol at 150 °C for 1 h. The hydrolysate was derivatized with phenylisothiocyanate to generate the phenylthiocarbamyl derivative of each amino acid and the mixture applied to a reverse-phase HPLC column (20).

To determine the amino acid sequence of peak B, an aliquot (12 μg) was extensively reduced and alkylated to modify cysteine residues to their more stable carboxymethyl derivatives. Briefly, the aliquot was reduced in the presence of 5 M guanidine HCl at pH 8.6 for 1 h at 37 °C. The reducing agent was 0.05 M dithiothreitol. Alkylation was performed at room temperature by adding iodoacetic acid to 0.11 M and incubating the mixture in the dark for 1 h. The alkylated protein was separated from reagents by rechromatography using HPLC (C-4 column) as previously described. Guanidine and other reagents are not retained by this reverse-phase column. 6 μg of eluted, modified protein was placed on a polybrene (21)-treated glass fiber filter in an Applied Biosystems model 470A gas phase sequenator and subjected to repetitive Edman degradation. Phenylthiohydantoin derivatives were identified by comparing their HPLC elution profiles obtained using a Waters gradient HPLC and a NOVA-PAK C-18 reverse phase column with the elution profiles of known mixtures of phenylthiohydantoin derivatives.

Determination of Carbohydrate Content—To determine whether either peak B or C contained carbohydrate, 20.0 μg of each peak was loaded on a 12% SDS-polyacrylamide gel and separated for 14 h as described previously. Fetuin was run in parallel as a standard. The gel was fixed with 10% acetic acid/25% isopropyl alcohol. The gel was then washed sequentially with 1) 0.5% periodic acid, 2) 0.5% sodium arsenite/5% acetic acid, 3) 0.1% sodium arsenite/5% acetic acid, 4) 5% acetic acid, 5) Schiff's reagent (overnight), and 6) 0.6% sodium metabisulfite/0.01 M HCl.

To further determine whether either peak B or peak C contained carbohydrate, 1.5 μg of each protein was applied to a concanavalin A-Sepharose column that had been equilibrated in 0.02 Tris, pH 7.5, 2 mM CaCl$_2$, and 2 mM MgCl$_2$. The column was slowly loaded over 2 h and allowed to stand for 45 min at 22 °C. The column was further washed with 20 ml of starting buffer and then eluted with 10 ml of 0.02 M Tris, pH 7.5, containing 0.5 M α-methyl-D-mannoside and 0.1 M NaCl. After standing for 1 h, the column was reeluted with the same buffer. The fractions were tested for IGF binding activity as described previously.

Determination of [³H]Thymidine Incorporation into DNA—The biologic activity of pure peak B and C material was assessed by determining the capacity of each to stimulate DNA synthesis in porcine aortic smooth muscle cells. The smooth muscle cells were isolated and maintained in stock cultures using previously described methods (22). The cells from stock cultures were subcultured in microtest 96-well plates (Falcon 3004) by plating at 8000 cells/well in DMEM (GIBCO) containing 10% fetal bovine serum. 5 days after plating, the wells were washed once with serum-free DMEM, and then test factors were added to each well in 0.2 ml of DMEM supplemented with 1% platelet-poor plasma (PPP) and 0.5 μCi of [³H]thymidine. PPP was prepared by a previously described method (23). After 36 h of incubation the wells were washed twice with Ringer's bicarbonate and twice with 5% trichloroacetic acid (4 °C), and the DNA was extracted twice with 0.4 ml of 0.3 N NaOH. [³H] Thymidine incorporation was determined by liquid scintillation counting.

Isoelectric Focusing—To determine their isoelectric points, 2.5 μg of peak B and C proteins were loaded onto precast isoelectric focusing plates, pH 3–10 (Servalyt Precotes). 20 μg of known standards was run in a parallel lane. The proteins were electrofocused for 1.5 h at 200 V, and then for 1.5 h at 1000 volts. The gel was divided into two sections and one half was fixed in 10% trichloroacetic acid and then stained with Serva Blue according to directions. The other half was cut into 0.5-cm sections and eluted with 0.04% trifluoroacetic acid. The eluates were analyzed for IGF binding activity as described previously.

Determination of ^{125}I-IGF-I and ^{125}I-IGF Binding Protein Binding

to Cell Monolayers and Affinity Labeling—Prior to conducting the binding experiments, pig smooth muscle cells were grown to confluency in 24-well plates (Falcon 3003) and washed three times in PBS. The cultures were then incubated for 14 h at 37 °C with varying concentrations of pure peak B or C in 0.5 ml of minimum essential medium. The plates were washed twice with PBS and fresh peak B or C was added with ^{125}I-IGF-I (0.27 ng) to 0.25 ml of minimum essential medium containing 20 mM HEPES and 0.1% BSA. After 2 h at 4 °C the media was aspirated, the monolayers were washed four times in PBS, and the cell-associated ^{125}I-IGF-I was determined as previously described (18). Nonspecific binding was determined in the presence of 500 ng/ml unlabeled IGF-I and that value was subtracted for all points. The IGF binding protein was iodinated using a modification of the method that was used to prepare ^{125}I-IGF-I (19). 0.5 μCi of Na-^{125}I was added to 0.1 ml of 0.5 M NaPO$_4$, pH 7.5, containing 2.0–4.0 μg of protein. Chloramine T (50 μM) was added. After 3 min, the percentage precipitability in 20% trichloroacetic acid was determined and further chloramine T was added until the iodinated protein was 70% precipitable. The mixture was purified by Sephadex G-100 chromatography. The relative specific activity of peaks B and C was 81 and 152 μCi/μg, respectively. Direct measurements of ^{125}I-binding protein binding were determined as described for ^{125}I-IGF-I except that the concentration of radiolabeled protein in the incubation mixture was 2.0 ng/ml.

Affinity labeling was performed using a previously described method (18). The cells were grown to confluency in 35-mm dishes (Falcon 3002). The preincubation step with peaks B and C and the binding reaction were carried out as described above except that a 1.0 ml incubation volume and 2.0 ng/ml of ^{125}I-IGF-I was used. Following the binding experiment, the monolayers were washed and disuccinimidyl suberate was added in a final concentration of 0.1 mM in 1.0 ml of binding buffer without BSA (18). After 10 min at 22 °C, the reaction was quenched with 10 mM Tris, pH 7.0. The cell monolayers were extracted with 1% SDS and boiled for 5 min, and the supernatant was clarified by centrifugation at 10,000 × g for 3 min. The supernatants were loaded on to 10% SDS-polyacrylamide gels and the proteins separated as described previously (18). The gels were fixed with 10% acetic acid and 30% methanol, washed, dried, and exposed to Kodak X-O-mat film.

RESULTS

Ammonium sulfate precipitation of 230 ml of amniotic fluid resulted in recovery of IGF binding activity in both the 33 and 50% pellets. The majority of the activity was present in the 50% pellet and this was chosen for further purification. During phenyl-Sepharose chromatography, the majority of contaminating protein eluted with 0.5 M sodium thiocyanate as described previously (24) (Fig. 1). The peak containing the IGF binding protein eluted with 0.02 M Tris, pH 9.0, and had been purified 9.5-fold (Table I). Further purification by ion exchange chromatography resulted in separation of two major peaks of binding activity which eluted at 100 and 250 mM salt (Fig. 2). These peaks (termed peaks B and C) were pooled and further purified separately. Peak C material was purified by Sephadex G-100 chromatography. The binding protein activity eluted over a broad peak but was separated from larger molecular weight contaminants (Fig. 3). 60 μg of G-100 purified material was further purified by reverse-phase HPLC using a C-4 column. The active material was eluted as a single peak at 50% acetonitrile and was stable during storage at −20 °C for periods of up to 3 months (Fig. 4A). Peak B was purified by reverse-phase HPLC and this step resulted in a 9.4-fold purification (Fig. 4B).

To determine the purity, estimate the molecular size of each protein, and determine the efficacy of each separation method in removing contaminants, the protein at each stage of purification of peak C was subjected to polyacrylamide gel electrophoresis under nonreducing conditions followed by silver staining (Fig. 5). The pure product has a molecular mass estimate of 31 kDa and appears as a single band after the final purification step (*panel F*). The phenyl-Sepharose step appeared to be the most effective procedure for removing the
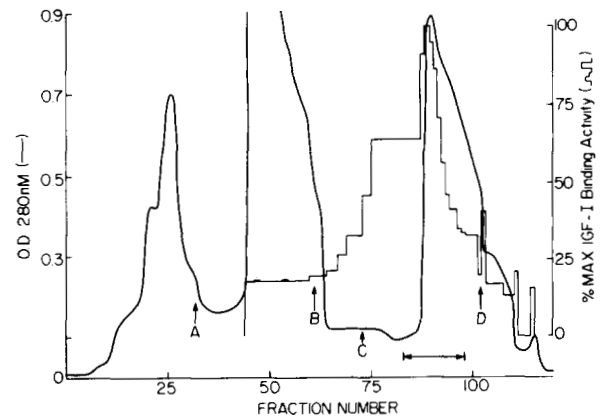
FIG. 1. **Phenyl-Sepharose fractionation of the 33–50% ammonium sulfate pellet obtained from human amniotic fluid.** The pellet was dissolved in 0.05 M Tris, 0.14 M (NH$_4$)$_2$SO$_4$, pH 7.4, and applied to the column that had been preequilibrated with 0.05 M Tris, pH 7.4, containing 10% w/v ammonium sulfate. Following sample loading, the column was rinsed with equilibrating buffer and eluted with the following buffers as indicated by *arrows*: A, 0.05 M Tris, 0.5 M sodium thiocyanate, pH 7.4; B, 0.05 M Tris, pH 7.4; C, 0.02 M Tris, pH 9.0; D, deionized water. *Heavy dark line* represents the absorbance at 280 mM, *bars* depict the percent maximum IGF-I binding activity. Fractions (83–98), pooled for further purification, are indicated (↔).

contaminating proteins. Comparison of pure peaks B and C on SDS-PAGE showed that they both had identical R_F values (Fig. 6). The molecular mass estimates were 31 kDa under nonreducing conditions but the estimate of each increased to 36 kDa if the proteins were reduced prior to electrophoresis (data not shown). This gel was deliberately overloaded (10 μg of each protein) to detect contaminants. The 69-kDa band is a dimer. Isoelectric focusing of each protein showed peak B had a PI value of 5.4, whereas peak C was 5.3 (Fig. 6).

When the amino acid compositions of peaks B and C were determined nearly identical amino acid ratios were obtained (Table II). The actual composition is in close agreement with previously published data (24). Reduction and alkylation of peak B and peak C followed by N-terminal sequence determination is shown in Table III. The result for amino acids 1–10 agrees with that published by two groups (24, 25) and further confirms that the protein that was purified was the IGF binding protein. Positions 11 and 12 differ from the published sequence for placental protein 12 (25). However, these differences are only one base substitution in the codons coding for each amino acid. This suggests that the placental protein 12 sequence may be distinct and that the difference is not artifactual. When compared with the sequence of the rat IGF binding protein, the cysteine positions at 5, 8, and 16 appear to be conserved. Both proteins were stable after heating to 100 °C for 10 min and were stable to pH 2.5.

Further physiochemical analysis was performed to determine whether carbohydrate side chains were present. Although one group had reported that the protein contained no carbohydrate (24) we noted that pure preparations of peak B or C adhered to concanavalin A. When 2 μg of each protein was applied to the concanavalin A column, 51% of peak C adhered and was eluted with 0.5 M α-methyl-D-mannoside, whereas only 24% of peak B was adherent. When each peak was treated with N-glycanase prior to concanavalin A chromatography, no change in the elution pattern was noted (data not shown). This suggested that the binding to concanavalin A was nonspecific. This result was confirmed by SDS gel electrophoresis of 20 μg of either peak B or C followed by staining with Schiff's base, which showed that neither protein

TABLE I

Recovery of IGF binding protein during purification

Purification steps	Total protein		Total activity[a]		Specific activity		Recovery		Purification	
	B	C	B	C	B	C	B	C	B	C
	mg		*units*		*units/mg*		*%*		*-fold*	
Amniotic fluid	1,242[b]		92,000		7.4×10^1		100		1	
Ammonium sulfate fractionation (33–50% saturation)	186.5[b]		62,500		3.35×10^2		68		4.5	
Phenyl-Sepharose	18.12[b]		55,000		3.04×10^3		60		41.0	
DEAE-Cellulose	5.15[c]	7.13[c]	17,600	29,300	3.4×10^3	4.1×10^3	19	32	46	35
Sephadex G-100		3.92[c]		27,800		7.1×10^3		30		96
HPLC C-4	0.545[d]	0.992[d]	17,600	27,800	3.2×10^4	2.8×10^4	19	30	432	378

[a] One unit of activity is the quantity of fluid necessary to stimulate one-half of maximal binding activity in the IGF-I binding capacity assay.

[b] Based on binding capacity assay protein determination.

[c] Based on absorbance at 280 mM.
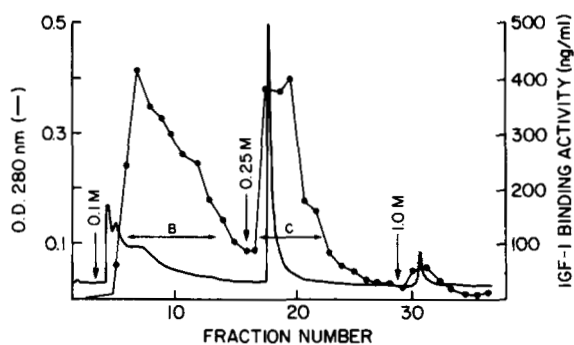
[d] Based on amino acid composition.

FIG. 2. **DEAE-cellulose chromatography of partially purified IGF-I binding activity.** Fifty-five ml of the IGF-I binding protein activity obtained from the phenyl-Sepharose chromatography (fractions 83–98) was acidified to pH 7.2 with acetic acid and applied to a DEAE-cellulose column previously equilibrated with 0.01 M $(NH_4)_2CO_3$, 0.01 M NaCl, pH 7.2. After sample loading, the column was rinsed with equilibrating buffer and eluted stepwise with 0.1, 0.25, and 1.0 M NaCl, all containing 0.01 M $(NH_4)_2CO_3$, pH 7.2, as indicated. Absorbance at 280 mM (——) and percent maximum IGF-I binding activity (●——●) are indicated. Fractions pool for further purification are depicted as pools B and C (↔); fractions 6–14 and 18–22, respectively.
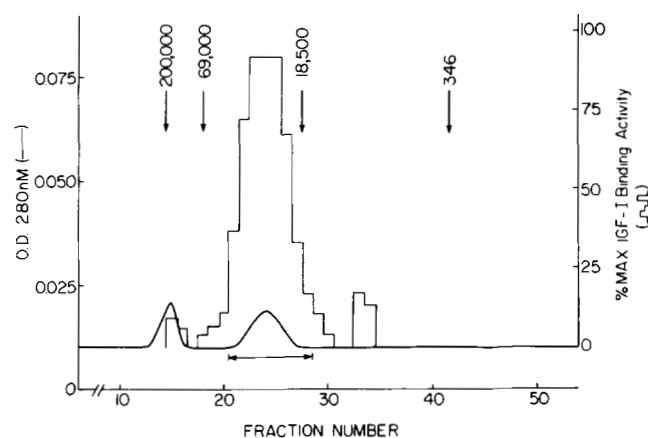

FIG. 3. **Sephadex G-100 chromatography of DEAE-pool C IGF-I binding activity.** Ten ml of DEAE-pool C was fractionated on a Sephadex G-100 column (22 mm × 90 cm). The column was equilibrated and eluted with 0.01 M $(NH_4)_2CO_3$, pH 7.1, containing 0.05 M NaCl. Fractions of approximately 9 ml were collected. IGF-I binding activity is indicated (⊓) as is absorbance at 280 nm (——). ↔ indicates region of pooling for further purification. Catalase (200,000), BSA (69,000), myoglobin (18,500), and phenol red (346) were used as molecular weight standards.

contained detectable carbohydrate. Based on the staining intensity of a fetuin standard it could be determined that each protein contained less than 0.5% of its weight as carbohydrate.

To determine the affinity of each protein for IGF-I, increasing concentrations of unlabeled IGF-I and ^{125}I-IGF were incubated with peak B or C and the bound complexes immunoprecipitated. The data were analyzed using Scatchard plots. Both proteins have binding characteristics that are consistent with either a two-site model with high and low affinity binding sites or a one-site model with negative cooperativity. The relative affinities of the high affinity sites of the peak B and C proteins are very similar: 1.7 and 2.2 × 10^10 liters/mol, respectively (Fig. 7).

In spite of their physiochemical similarity, the peak B and C materials were found to have markedly distinct biologic properties. Pure peak B material greatly potentiated the smooth muscle cell DNA synthesis response to IGF-I, but had no effect alone or with an equivalent concentration of human insulin (Fig. 8). In contrast, peak C material inhibited basal and IGF-I-stimulated [³H]thymidine incorporation and markedly inhibited the response to peak B plus IGF-I (Fig. 8). This effect was detectable at concentrations as low as 2.0 ng/ml peak C and was maximal at 20 ng/ml. To exclude the possibility that these changes in [³H]thymidine incorporation were

due to changes in the cell cycle kinetics, the rate of entry into DNA synthesis of smooth muscle cells was determined after the addition of peaks B and C. Peak B induced a significant increase in the amount of [³H]thymidine incorporation into DNA at all time points that were tested between 14 and 36 h (data not shown). Peak C inhibited [³H]thymidine incorporation between 14 and 36 h and did not appear to merely cause a delay in [³H]thymidine incorporation.

To further characterize potential differences in the cellular response to the peak B and peak C proteins, ^{125}I-IGF-I binding was determined in the presence of both forms of the binding protein. Addition of 100 ng/ml peak B to the cultures for 14 h prior to and during the binding reaction resulted in a 72% increase in IGF-I binding (Fig. 9). In contrast, addition of 25 ng/ml peak C protein resulted in a 36% decrease in the amount of IGF-I that was specifically bound.

To determine whether the differences were due to differences in the capacity of each form of the binding protein to adhere to cell surfaces, smooth muscle cell cultures were exposed to 50 ng/ml peak B binding protein for 14 h at 37 °C and during the binding reaction. Following binding and affinity labeling, a band was detected at 42 kDa (Fig. 10, *lane B*). ^{125}I-IGF-I binding to this band was specific since it was
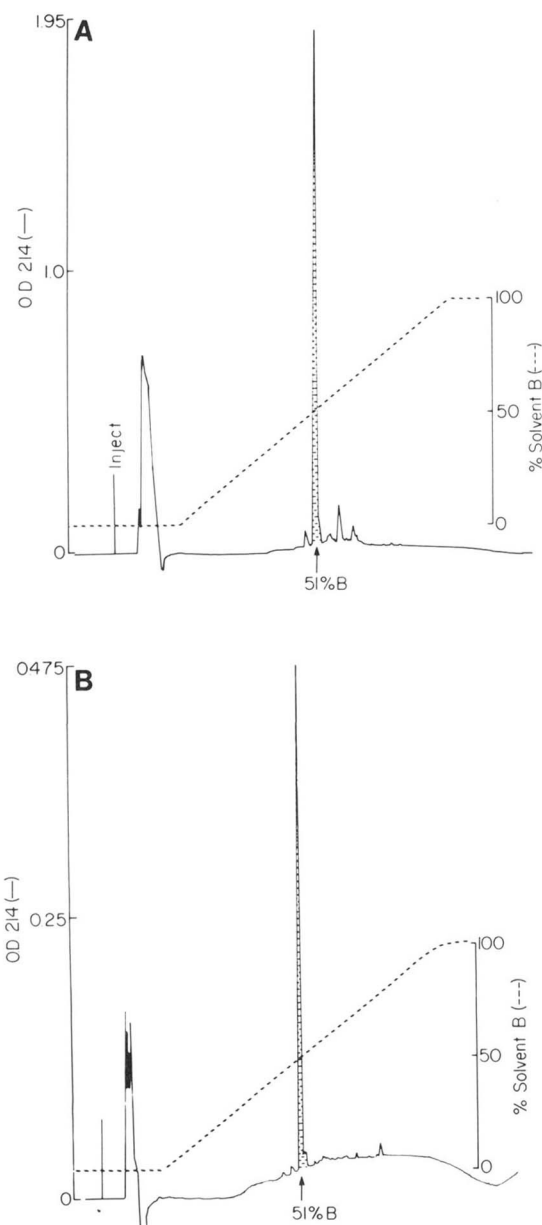
FIG. 4. *A*, HPLC of G-100 pool of peak C IGF-I binding activity. One ml was injected on to a Vydac C-4, reverse-phase column (4.6 mm × 25 cm). Sample was eluted isocratically for 5 min with 100% solvent A (0.04% trifluoroacetic acid in dH$_2$O) followed by a linear gradient to 100% solvent B (0.04% trifluoroacetic in acetonitrile) for 25 minutes. The flow rate was 1.5 ml/min and absorbance was monitored at 214 nm. IGF-I binding activity (indicated by shaded area) eluted at 51% solvent B. *B*, HPLC of DEAE-pool B IGF-I binding activity. 1.5 ml of DEAE-pool B was injected on to a Vydac C-4, reverse-phase column (4.6 mm × 25 cm). Sample was eluted isocratically for 5 min with 100% solvent A, (0.04% trifluoroacetic acid and H$_2$O) followed by a linear gradient to 100% solvent B (0.04% trifluoroacetic acid in acetonitrile) for 25 min. The flow rate was 1.5 ml/min and absorbance was monitored at 214 nm. IGF-I binding activity (indicated by shaded area) eluted at 51% B.

FIG. 5. **Analysis of purity of peak C IGF-I binding protein at each step of purification.** Samples were analyzed for purity on a 12% SDS-polyacrylamide gel. The experimental conditions are described under "Experimental Procedures." Ten µg of total protein that was present after each chromatographic step was loaded per lane. *Lane A*, crude amniotic fluid; *lane B*, phenyl-Sepharose chromatography; *lane C*, peak B after DEAE-cellulose chromatography; *lane D*, peak C after DEAE-cellulose chromatography; *lane E*, peak C further purified by reverse-phase HPLC C-4 column; *lane F*, peak C further purified by G-100 and HPLC. BSA (69,000), ovalbumin (43,000), and myoglobin (18,500) were run as standards as indicated. Silver staining was performed as described under "Experimental Procedures."

inhibited by excess unlabeled IGF-I but not by insulin. When peak C was added, no labeled band was detected in the 42-kDa region of the gel (*lane E*) and binding to the type I receptor appeared to be reduced. To determine whether the differences in the 42-kDa band intensity were due to differences in the adherence properties of peaks B and C, we determined the capacity of radiolabeled forms of each protein to bind to smooth muscle cell cultures. The addition of radi-
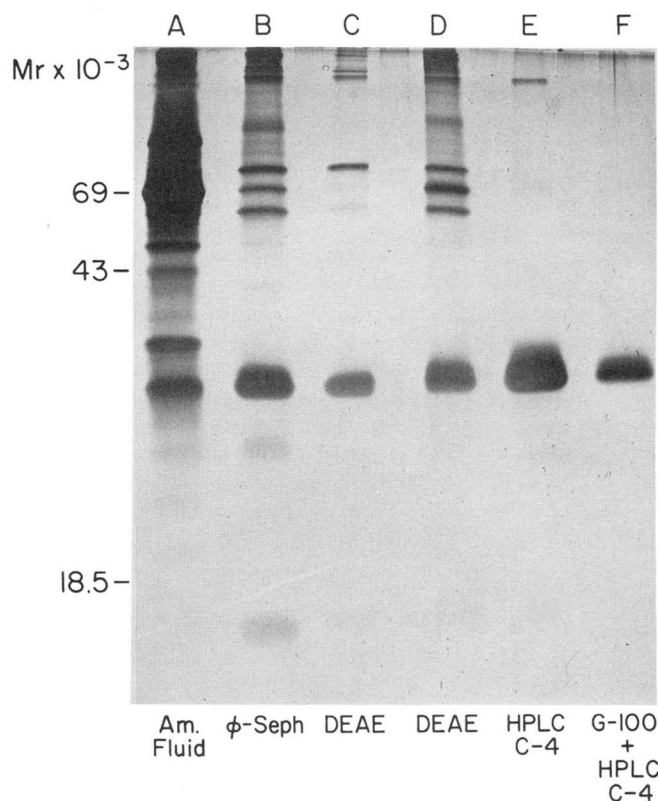
olabeled peak B resulted in 8% of the total counts per minute added being specifically bound to the cell surface, whereas incubation with an equal amount of peak C showed no specific binding (Fig. 11). Addition of 50 ng/ml non-radiolabeled peak B resulted in significant competition, whereas an equal amount of peak C showed no competition.

DISCUSSION

The insulin-like growth factor binding proteins are known to circulate in blood and to be present in extracellular fluids. The extracellular fluid form(s) of the protein are usually unsaturated; therefore, they have the potential to bind free IGF-I and IGF-II. It has been assumed that this large pool of carrier protein can act as a storage reservoir for IGF-I and that bound IGF-I is in an inactive form. These studies demonstrate that this model is too simplistic. The results show that human amniotic fluid contains two forms of the IGF binding protein that have similar physiochemical properties but differ in their capacity to bind to cell surfaces and in their capacity to enhance the cellular DNA synthesis response to IGF-I. Following separation on DEAE-cellulose the two proteins were purified to homogeneity and the homogeneous preparations had markedly different biologic actions. The peak B form of the binding protein potentiates smooth muscle cell DNA synthesis 4.4-fold above the rate that can be
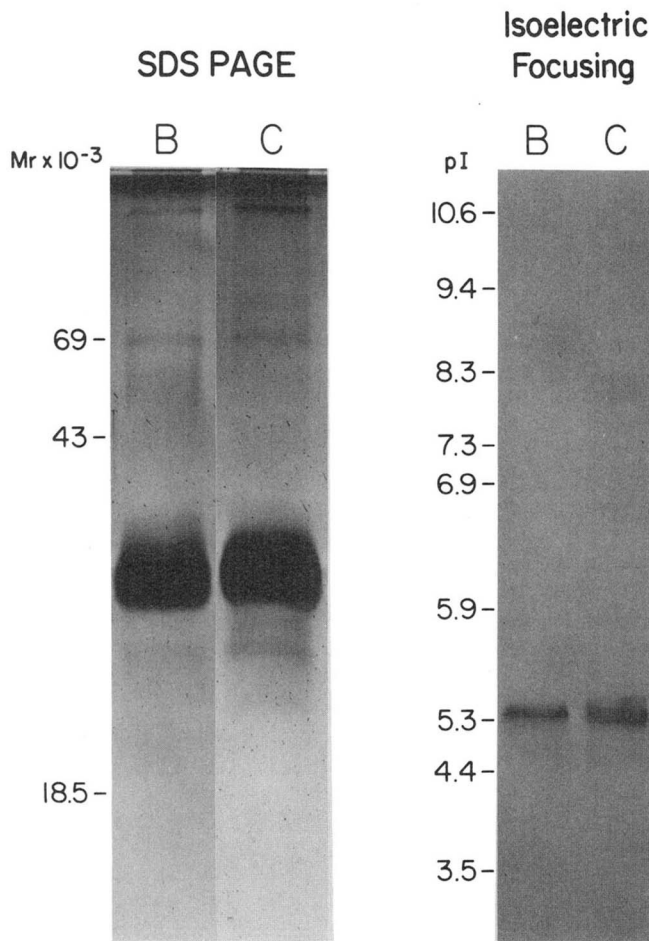
SDS PAGE

Isoelectric Focusing

FIG. 6. **Molecular weight estimate of peaks B and C by SDS-PAGE.** Ten μg of peaks B and C were loaded on to a 12% SDS-polyacrylamide gel and the proteins separated under nonreducing conditions as described. The gel was stained as described previously. For isoelectric focusing of peaks B and C, 2.5 μg of each protein were separated on precast isoelectric focusing plates as described under "Experimental Procedures." The gel was stained with Serva Blue or cut into 0.5-cm slices and eluted with 0.04% trifluoroacetic acid. IGF binding activity of the eluates was determined and was detected in the slice corresponding to the stained protein.

TABLE II

Amino acid composition of the IGF-I binding protein

Amino acid	Peak B	Peak C
	mol %	
Aspartic acid and asparagine	7.3	7.6
Glutamic acid and glutamine	15.1	14.9
Serine	8.5	8.7
Glycine	9.0	7.7
Histidine	2.1	2.1
Arginine	4.9	4.6
Threonine	3.8	4.5
Alanine	12.4	11.1
Proline	8.0	8.8
Tyrosine	2.8	2.5
Valine	3.8	3.8
Methionine	0.7	0.6
Cysteine	4.4[a]	5.6[a]
Isoleucine	3.6	3.9
Leucine	8.1	8.2
Phenylalanine	2.0	1.7
Lysine	3.7	3.8
Tryptophan[b]		

[a] Based on recovery of cysteine standard following hydrolysis.
[b] Tryptophan is not detectable following hydrolysis.

showed that it blocked the DNA synthesis response of chick embryo fibroblasts to MSA (10). Since many of the purification schemes that were used to purify these proteins did not include DEAE-cellulose chromatography it is possible that these partially purified preparations contained both the peak B and C forms of the binding protein. Since peak C is capable of inhibiting the cellular response to peak B plus IGF-I, failure to separate these two forms during purification could lead to these results. It is also possible that species differences could account for these discrepancies since the rat homologue of the extracellular binding protein has a different N-terminal sequence (30) and therefore it might not be capable of eliciting the same biologic response.

The exact molecular property that accounts for the differences in the cellular response to peaks B and C was not identified. Although we found that the two components had slightly different elution profiles from DEAE-cellulose, they had nearly identical isoelectric point determinations. This discrepancy could be due to preferential association of the peak B form of the protein with other proteins that elute at lower salt concentrations. A second possibility is that peak B aggregates into multimeric forms during the ion exchange step as a result of concentration and that such aggregation alters the exposed charge groups but that aggregation does not occur during isoelectric focusing. In addition, both forms had identical molecular weight estimates, very similar amino acid compositions, and identical N-terminal sequences, and both had no detectable carbohydrate content by Schiff's staining. The binding affinity estimates of each form of the protein for IGF-I showed complex kinetics that were consistent with a two-site model of competition for each form, but the affinity estimates for each form were not substantially different. The affinity of these proteins for IGF-II was not determined and, therefore, we cannot directly compare our binding results to those of Binoux *et al.* (15) who found two binding proteins with different affinities for IGF-I and IGF-II in a crude preparation of human spinal fluid.

A major difference in the membrane adherence properties of these two proteins was noted. Direct measurements of the binding of radiodinated forms of each protein showed that peak B attached to the cell surfaces, whereas peak C did not. Likewise, non-radiolabeled peak B was shown to both adhere to smooth muscle cell surfaces and to increase the total amount of ^{125}I-IGF-I that was bound. In contrast, non-radio-

achieved with IGF-I and PPP alone (26). This result is not accounted for by a contaminant since contaminants composed less than 0.5% of the sample and the addition of the peak B protein without IGF-I or in the presence of insulin has no stimulatory activity (26). In contrast, the peak C form of the protein inhibited the effect of IGF-I alone or the combined stimulatory effect of peak B plus IGF-I. Therefore, the peak C form appears to be able to negate the effect that the peak B form exerts on IGF-I action. These effects are not due to changes in cell cycle kinetics since addition of peak B did not alter the time course of DNA synthesis by smooth muscle cells. Likewise, peak C did not simply delay the onset of [³H] thymidine incorporation by a mechanism similar to the effect of transforming growth factor-β on ARK-2B cells (27). Since the net effect of the two proteins appears to determine the cellular response to IGF-I, it will be important to determine the relative abundance of these two forms in extracellular fluids.

In contrast to these findings, several investigators have reported that partially purified preparations of IGF binding protein inhibit either the insulin-like (28) or growth-promoting actions (29) of IGF-I. Furthermore, one group used a homogeneous preparation of the rat MSA binding protein and

TABLE III

N-terminal amino acid sequence of IGF binding protein and placental protein 12

IGF binding protein

Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-<u>Glu</u>-<u>Lys</u>-Leu-Ala-Leu-Cys-Pro-Pro-Val-Ser-Ala-Ser-Cys-Ser-Glu-Val-
10 20 Thr-Arg

Placental protein 12

Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-<u>Asp</u>-Glu-Leu-Ala-Leu
10

FIG. 7. **Scatchard plots of IGF-I binding to the peaks B and C proteins.** ^{125}I-IGF-I (0.27 ng/ml) and increasing concentrations of unlabeled IGF-I were incubated with 14 ng/ml of either peak B (▲) or peak C (●) binding protein. Following a 48-h incubation at 4 °C the bound and free ^{125}I-IGF-I were separated by immunoprecipitation as described under "Experimental Procedures." 83% of the total bound IGF-I was precipitated under the conditions used.

FIG. 9. **Alteration in ^{125}I-IGF binding induced by cell exposure to the IGF binding proteins.** Porcine aortic smooth muscle cell cultures were incubated with peak B or peak C for 14 h at 37 °C. At that time the cultures were washed and fresh peak B or C added with ^{125}I-IGF-I (1.0 ng/ml) and the binding reaction carried out for 2 h at 8 °C. The cultures were washed four times with PBS and the cell-associated ^{125}I-IGF-I was determined as described under "Experimental Procedures."

FIG. 8. **Effects of peak B and C IGF-binding proteins on IGF-I-stimulated DNA synthesis.** Quiescent porcine smooth muscle cell cultures were exposed to a basal medium containing 0.2 ml DMEM and 1% PPP. Additional cultures received 20 ng/ml IGF-I or 10 μg/ml insulin. Other cultures were exposed to pure peak B or C with or without IGF-I. After a 36-h incubation [^{3}H]thymidine incorporation into DNA was determined. The values plotted are the means of triplicate determinations.

labeled peak C did not attach to cell surfaces and exposure of cells to peak C did not result in an enhancement of ^{125}I-IGF-I binding. Since peak C inhibits the DNA synthesis response to IGF-I, it is possible that the presence of peak C in the incubation medium competitively inhibits ^{125}I-IGF binding, and thereby reduces the amount of IGF-I that is available to attach to the type I IGF receptor. These findings also suggest that attachment of peak B to the cell surface and the subsequent increase in IGF-I binding that is detected are linked to

FIG. 10. **Affinity labeling of cell-associated binding protein with ^{125}I-IGF-I.** Pure IGF binding protein, peaks B and C, were incubated with smooth muscle cells. The monolayers were then washed and ^{125}I-IGF-I (2.0 ng/ml) added. After a 2-h incubation at 8 °C, affinity labeling was performed as described under "Experimental Procedures." The labeled proteins were solubilized and separated by 10% SDS-PAGE. The autoradiographs show the following: *lane A*, control, no addition; *lane B*, peak B (50 ng/ml); *lane C*, peak B + unlabeled IGF-I (50 ng/ml); *lane D*, peak B + insulin (10 μg/ml); *lane E*, peak C (50 ng/ml).
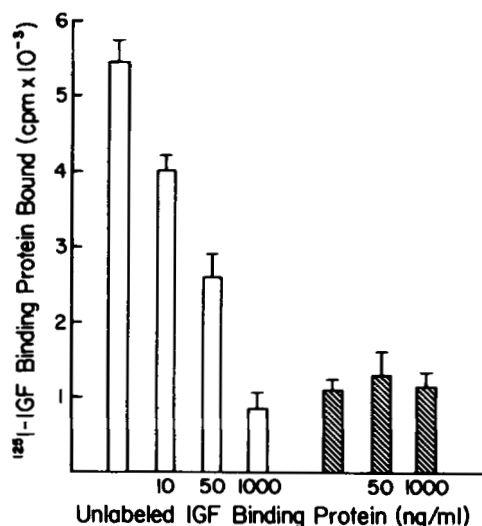
FIG. 11. **Binding of** ^{125}**I-IGF binding proteins to smooth muscle cell monolayers.** Confluent smooth muscle cell monolayers (76,000 cells/cm^2) were exposed to ^{125}I-IGF binding protein peak B (*open boxes*) or peak C (*hatched boxes*), 80,000 cpm in 0.5 ml of binding medium (as described under "Experimental Procedures"). After a 2-h incubation at 4 °C the cells were washed three times with PBS and the cell-associated radioactivity was determined. Duplicate wells contained increasing concentrations of each form of unlabeled IGF binding protein. The results are expressed as the mean ± 1 S.D. of triplicate determinations.

potentiation of the DNA synthesis response. Since at present this difference in the membrane adherence of these two forms of IGF binding protein is the only identifiable distinctive feature that has been linked to the differences in the cellular DNA synthesis response, it is critical to determine how this increase in the amount of IGF-I that is bound affects the type I receptor signaling mechanism. Potential binding protein-type I receptor interactions that might be modified by adherence of the IGF-I-binding protein complex to cell surfaces and subsequently enhance the transmembrane mitogenic signaling would include acceleration of receptor clustering, retardation of the rate of type I receptor internalization, blocking IGF-I degradation, and a change in receptor confirmation that results in enhanced affinity for IGF-I or direct binding of the IGF-I-binding protein complex to the receptor to a site that is distinct from the IGF-I binding site. Direct evidence supporting one of these mechanisms is not available, but since the IGF-I mitogenic signal is believed to be type I receptor-mediated each of these potential mechanisms is worthy of consideration.

The structural difference between peak B and C that accounts for the differences in membrane adherence and biologic response was not identified. Although the amino acid compositions were very similar and the first 28 residues of each protein are identical, it is possible that there are other as yet unidentified minor sequence differences. Likewise, other post-translational modifications such as fatty acid addition (31), carboxylation (32), phosphorylation, or internal disulfide bond rearrangements have not been excluded. It is likely that one of these modifications exists and that it accounts for the observed differences in biologic activity. Addi-

tion of fatty acids such as palmitate can account for the membrane adherence properties of proteins. Specifically, the P-21 RAS protein will not adhere to the cytoplasmic surface of the plasma membrane unless palmitate has been added (33). Since the membrane adherence properties of the peak B binding protein correlate with its capacity to simulate DNA synthesis, it is possible that such a modification could explain both of the observed differences between peaks B and C. Identification of this specific difference would be of major importance in understanding the control of IGF-I action at the cell surface.

REFERENCES

1. Van Wyk, J. J. (1985) in *Hormonal Proteins and Peptides*, Vol XXII (Li, C. H., ed) pp. 82–125, Academic Press, New York
2. Nissley, S. P., and Rechler, M. M. (1985) in *Hormonal Proteins and Peptides*, Vol. XII (Li, C. H., ed) pp. 128–203, Academic Press, New York
3. D'Ercole, A. J., Applewhite, G. T., and Underwood, L. E. (1980) *Dev. Biol.* **75**, 315–328
4. Clemmons, D. R., Underwood, L. E., and Van Wyk, J. J. (1981) *J. Clin. Invest.* **67**, 10–19
5. D'Ercole, A. J., Stiles, A. D., and Underwood, L. E. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 935–940
6. Zapf, J., Waldvogel, M., and Froesch, E. R. (1975) *Arch. Biochem. Biophys.* **168**, 638–645
7. Hintz, R. L., and Liu, F. (1977) *J. Clin. Endocrinol. Metab.* **45**, 988–995
8. Drop, S. L. S., Valiquette, G., Guyda, H. J., Corvol, M. T., and Posner, B. I. (1979) *Acta Endocrinol.* **90**, 505–519
9. Moses, A. C., Nissley, S. P., Passamani, J., and White, R. M. (1979) *Endocrinology* **104**, 536–546
10. Knauer, D. J., and Smith, G. L. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 7252–7254
11. Scott, C. D., Martin, J. L., and Baxter, R. C. (1985) *Endocrinology* **116**, 1094–1101
12. Copeland, K. C., Underwood, L. E., and Van Wyk, J. J. (1980) *J. Clin. Endocrinol. Metab.* **50**, 690–697
13. Martin, J. L., and Baxter, R. C. (1985) *J. Clin. Endocrinol. Metab.* **61**, 799–801
14. Moses, A. C., Nissley, S. P., Passamani, J., White, R. M., and Rechler, M. M. (1979) *Endocrinology* **104**, 536–546
15. Binoux, M., Hardouin, S., Lassarre, C., and Hossenlopp, P. (1982) *J. Clin. Endocrinol. Metab.* **55**, 600–602
16. Seppälä, M., Wahlström, T., Koskimies, A. I., Tenhunen, A., Rutanen, E.-M., Koistinen, R., Huhtaniemi, I., Bohn, H., and Stenman, U.-H. (1984) *J. Clin. Endocrinol. Metab.* **58**, 505–510
17. Chochinov, R. H., Mariz, I. K., Hajek, A. S., and Daughaday, W. H. (1977) *J. Clin. Endocrinol. Metab.* **44**, 902–908
18. Clemmons, D. R., Elgin, R. G., Han, V. K. M., Casella, S. J., D'Ercole, A. J., and Van Wyk, J. J. (1986) *J. Clin. Invest.* **77**, 1548–1556
19. D'Ercole, A. J., Underwood, L. E., Van Wyk, J. J., Decedue, C. J., and Foushee, D. B. (1976) in *Growth Hormone and Related Peptides* (Pecili, A., and Mueller, E. E., eds) pp. 190–201, Excerpta Medica, Amsterdam
20. Cohen, S. A., Tarvin, T. L., and Bidingmeyer, B. A. (1984) *Am. Lab.* **34**, 48–59
21. Tarr, G. E., Beecher, J. F., Bell, M., and McKean, D. J. (1978) *Anal. Biochem.* **84**, 622–627
22. Ross, R. (1971) *J. Cell Biol.* **50**, 172–186
23. Clemmons, D. R. (1983) *J. Cell. Physiol.* **114**, 61–67
24. Povoa, G., Enberg, G., Jornvall, H., and Hall, K. (1984) *Eur. J. Biochem.* **144**, 199–204
25. Kositinen, R., Kalkkinen, N., Huhtala, M. L., Seppala, M., Bohn, M., and Rutanen, E. M. (1986) *Endocrinology* **118**, 1375–1379
26. Elgin, R. G., Busby, W. H., and Clemmons, D. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3254–3258
27. Shipley, G. D., Childs, C. B., Volkenant, M. E., and Moses, H. L. (1984) *Cancer Res.* **44**, 710–716
28. Zapf, J., Schoenle, E., Jagars, G., Sand, I., Grunwald, J., and Froesch, E. R. (1979) *J. Clin. Invest.* **63**, 1077–1084
29. Massague, J., Kelley, B., and Mottola, C. (1985) *J. Biol. Chem.* **260**, 4551–4554
30. Mottola, C., MacDonald, R. G., Brackett, J. L., Mole, J. E., Anderson, J. K., and Czech, M. (1986) *J. Biol. Chem.* **261**, 11180–11186
31. Olson, E. N., Towler, D. A., and Glaser, L. (1985) *J. Biol. Chem.* **260**, 3784–3790
32. Gallop, P., Lian, J. B., and Hauschka, P. V. (1980) *N. Engl. J. Med.* **302**, 1460–1466
33. Chen, Z. Q., Ulsh, L. S., DuBois, G., and Shih, T. Y. (1985) *Virology* **56**, 607–612