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 26 residues. Neither protein had detectable carbohydrate side chains and each had a similar affinity for radiolabeled IGF-I (1.7-2.2 x 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 [3H]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 inhibited IGF-I binding to its receptor and to adherent peak B. We conclude that human amniotic fluid contains two forms of insulin-like proteins that have very similar amino acid and chemical 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), 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 (6). 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 two different types of IGF 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-dextran, ammonium persulfate, sodium thiocyanate, Sephadex G-100, polyethylene glycol (M, 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 Co 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, ServaIyt ioelectric 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 x g for 20 min. The pellet...
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 X 15 cm) that had been previously equilibrated with 0.05 M Tris, pH 7.4.

Following sample loading, the column was washed with the equilibration buffer until the absorbance (280 nm) returned to baseline. 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 to 0.02 M Tris, pH 9.0; and 4) 1.0 M NaCl. 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 mixture applied to a C-4 Vydac reverse-phase HPLC column (0.46 X 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 applied directly to the reverse-phase C-4 column. The elution conditions that were identical to those stated previously were used.

[125I]-IGF-I Binding Capacity—IGF-I binding activity of the column fractions was determined as follows using a polyethylene glycol precipitation method (18). 10 ml of each fraction was incubated with [125I]-IGF-I (340 Ci/mg) (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 Na-HCO₃, 0.02% NaN₃, pH 6.0 (250 ml total volume). The IGF-I was iodinated by a published method (19). Bound and free [125I]-IGF-I were separated by adding 250 μl of 1% human γ-globulin and 500 μl of 25% polyethylene glycol (M, 8000) (final concentration of 12.5%). The mixture was centrifuged at 10,000 X g for 15 min. The pellet was washed with 1 ml of 6.25% polyethylene glycol and the final pellet counted in an Applied Biosystems model 470A gas phase sequenator and the mixture applied to a reverse-phase HPLC column (20). The reducing agent was 0.05 M dithiothreitol. Alkylation of the 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. Phenylthiocarbonyl derivatives were identified by comparing their HPLC elution profiles using a Waters Associates 540A liquid chromatograph and a NOVA-PACK C-18 reverse phase column with the elution profiles of known mixtures of phenylthiocarbonyl derivatives.

Determination of Carbohydrate Content—To determine whether either peak B or peak 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% isopropanol. 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) Schiffs reagent (overnight), and 6) 0.5% sodium metabisulfite/0.1% 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 CaCl₂, and 2 mM MgCl₂. 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 reequilibrated with the same buffer. The fractions were tested for IGF binding activity as described previously.

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 cultured 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% fetal calf serum. [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 (Servaipyte Precote). 20 μg of known standards was run in a parallel lane. The proteins were electrofocused for 1 h at 1000 volts. The gel was divided into two sections and one half was fixed in 10% trichloroacetic acid and 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 [125I]-IGF-I and [125I]-IGF Binding Protein Binding
to Cell Monolayers and Affinity Labeling—Prior to conducting the binding experiments, pig smooth muscle cells were grown to confluence 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 125I-IGF-I (0.27 nCi/0.25 ml) in 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 125I-IGF-I was determined as previously described (18). Nonspecific binding was determined in the presence of 900 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 125I-IGF-I (19). Nonspecific binding was estimated 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 125I-IGF-I (19). Nonspecific binding was determined in the presence of 500 ng/ml unlabeled IGF-I and that value was subtracted for all points.

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 125I-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 ml 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 X 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 33% ammonium sulfate pellet and this was chosen for further purification. During phenyl-Sepharose chromatography, the majority of contaminating protein eluted with 0.3 M sodium thioacetate 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 56% 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 nonequilibrium 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 contaminating proteins. Comparison of peaks B and C on SDS-PAGE showed that they both had identical Rf 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 artificial. 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-glycosidase 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
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<table>
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<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
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<td>19</td>
<td>373</td>
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</table>

* 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.
* Based on binding capacity assay protein determination.
* Based on absorbance at 280 nm.
* 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 (NH₄)₂CO₃, 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 (NH₄)₂CO₃, pH 7.2, as indicated. Absorbance was measured at 280 nm and percent maximum IGF-I binding activity is indicated. Fractions pooled 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 (NH₄)₂CO₃, 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 few tetanus 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 [³H]-IGF-I 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 × 10⁵ liters/mol, respectively (Fig. 7).

In spite of their physiochemical similarity, the peak B and C materials were found to have markedly 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 IGF-I binding (Fig. 9). In contrast, addition of peak C protein resulted in a 36% decrease in the amount of IGF-I that was specifically bound.

To further characterize potential differences in the cellular response to the peak B and peak C proteins, [³H]-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 72% 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). [³H]-IGF-I binding to this band was specific since it was
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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 dH2O) followed by a linear gradient to 100% solvent B (0.04% trifluoroacetic acid 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 H2O) 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.

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 radiolabeled 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.

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."

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
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 \[^{3}H\] thymidine incorporation by a mechanism similar to the effect of transforming growth factor-\(\beta\) 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 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 stain. 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 radiolabeled 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 \(^{125}\text{I}\)-IGF-I that was bound. In contrast, non-radio-

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**TABLE II**

**Amino acid composition of the IGF-I binding protein**

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<th>Amino acid</th>
<th>Peak B</th>
<th>Peak C</th>
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<tr>
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<td>Lysine</td>
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<td>3.8</td>
</tr>
</tbody>
</table>

*Based on recovery of cysteine standard following hydrolysis.

*Tryptophan is not detectable following hydrolysis.
Purification of a Human Amniotic Fluid Protein

TABLE III

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>N-terminal Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF binding protein</td>
<td>Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-Glu-Lys-Leu-Ala-Leu-Cys-Pro-Pro-Val-Ser-Ala-Ser-Cys-Ser-Glu-Val-</td>
</tr>
<tr>
<td>Placental protein 12</td>
<td>Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-Asp-Glu-Leu-Ala-Leu-</td>
</tr>
</tbody>
</table>

Fig. 7. Scatchard plots of IGF-I binding to the peaks B and C proteins. 125I-IGF-I (0.27 ng/ml) and increasing concentrations of unlabeled IGF-I were incubated with 14 ng/ml of either peak B (A) or peak C (●) binding protein. Following a 48-h incubation at 4 °C the bound and free 125I-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. 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 [3H]thymidine incorporation into DNA was determined. The values plotted are the means of triplicate determinations.

Fig. 9. Alteration in 125I-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 125I-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 125I-IGF-I was determined as described under “Experimental Procedures.”

Fig. 10. Affinity labeling of cell-associated binding protein with 125I-IGF-I. Pure IGF binding protein, peaks B and C, were incubated with smooth muscle cells. The monolayers were then washed and 125I-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).
potentiation of the DNA synthesis response. Since at present type I receptor interactions that might be modified by retardation of the rate of type I receptor internalization, block that results in enhanced affinity for IGF-I or direct binding of IGF binding protein. The results are expressed as the mean ± 1 S.D. of triplicate determinations.

Identification of this specific difference would be of major importance in understanding the control of IGF-I action at the cell surface.

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REFERENCES