Purification and Amino-terminal Sequence of an Insulin-like Growth Factor-binding Protein Secreted by Rat Liver BRL-3A Cells*

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A protein preparation that specifically binds insulin-like growth factors (IGFs) I and II was purified from medium conditioned by rat liver BRL-3A cells using molecular sieve chromatography in 1 M acetic acid followed by affinity chromatography on IGF-II-agarose. The affinity-purified IGF-binding protein exhibits a single major band with apparent M, = 36,300 under reducing conditions on sodium dodecyl sulfate-polyacrylamide gels. The IGF-binding protein is efficiently and specifically cross-linked to either 125I-IGF-I (human) or 125I-IGF-II (rat) using disuccinimidyl suberate. An IGF-binding protein of similar apparent molecular weight was also affinity purified from rat hepatoma H-35 cell conditioned medium and found to differ from the BRL-3A protein such that potent polyclonal antisera prepared in rabbits against the purified BRL-3A IGF-binding protein exhibited a much lower titer for the H-35 protein in an enzyme-linked immunosorbent assay and upon immunoblotting. In order to determine whether a single BRL-3A IGF-binding protein is present in the affinity-purified preparation, the protein was prepared for sequencing on a Sephadryl S-300 column in 6 M guanidine HCl after reduction and alkylation. The amino acid composition (expressed in percentages) of this IGF-binding protein was determined to be: Cys = 5.5, Lys = 4.8, His = 2.8, Arg = 7.0, Asp = 10.2, Thr = 5.1, Ser = 3.9, Glx = 15.7, Gly = 17.4, Ala = 7.3, Val = 4.6, Met = 1.4, Ile = 2.4, Leu = 8.3, Tyr = 1.0, Phe = 1.9. Sequencing of the NH2-terminal portion of this protein led to the identification of 31 amino acids in the following order: Phe-Arg-Cys-Pro-Pro-Cys-Thr-Pro-Glu-Arg-Leu-Ala-Ala-Cys-Gly-Pro-Pro-Pro-Asp-Ala-Pro-Cys-Ala-Glu-Leu-Val-Arg-Glu-Pro-Gly-Cys. We conclude that rat liver BRL-3A cells secrete a single major IGF-binding protein capable of binding both IGF-I and IGF-II.

Insulin-like growth factor (IGF) I (or somatomedin C) and IGF-II (or multiplication-stimulating activity) are small serum peptides that resemble insulin in their structures (1, 2) and exert a variety of both rapid and slow biological actions (3–7). The metabolic effects of these peptides are similar to those stimulated by insulin, although the IGFs are more potent in stimulating cell proliferation in most cell types (5, 8–11). The amount of circulating IGFs is rather high (12), but their concentrations in the free, biologically active form are extremely low (13, 14). Thus, unlike insulin, they are found in the serum as a high molecular weight complex, due to their association with specific carrier or binding proteins.

The precise number of circulating binding proteins and their respective affinities for IGF-I and/or IGF-II are still unknown, but an IGF-binding protein complex with the size of γ-globulins seems to predominate at physiological pH (15). Exposure to acid dissociates the binding protein from the IGFs and considerably reduces the molecular weight of the IGF-binding unit to a size smaller than bovine serum albumin (15).

Several investigators have reported the secretion of IGF-binding proteins by the liver and a number of human and rat cultured cell lines, mainly of hepatic origin (15–19, reviewed in Ref. 20) but including fibroblasts (21, 22). The size of such proteins consistently appears to be in the range of 30,000–50,000 daltons, suggesting similarity to the acid-treated form of the serum IGF-binding protein. Cell lines shown to produce IGF-binding protein usually also release IGF-I or -II into the medium, but studies performed with the mutant line BRL-3A2, which secretes binding protein but not the IGFs, have demonstrated that the synthesis of the two products is independent (15).

The binding of IGFs to their carrier proteins increases their half-life (20) and prevents their biological effects (24, 25). A precise regulatory function of these binding proteins in controlling the concentration of free IGFs, however, is not fully understood. Our goal in the present study, therefore, has been the development of a purification procedure that would yield completely purified IGF-binding proteins from conditioned medium of cultured cells. Using immobilized IGF-II, we have prepared IGF-binding protein from rat liver BRL-3A and rat hepatoma H-35 cells. We have determined a single NH2-terminal sequence of the BRL-3A IGF-binding protein preparation, indicating that the IGF-binding activity secreted by these cells is due to a single major IGF-binding protein of apparent M, = 36,300.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human IGF-I was a gift of Professor R. E. Humbel (Biochemisches Institut der Universität, Zurich, Switzerland). Rat multiplication-stimulating activity was purified from conditioned medium of BRL-3A liver cells by the procedure of Marquardt et al. (26) as previously described (27). We refer to this preparation as rat IGF-II. Porcine insulin was a gift of Dr. Ronald Chance (Eli Lilly). The peptides were iodinated by the immobilized lactoperoxidase method (Enzymochromes, Bio-Rad).

**IGF-Binding Protein Purification from BRL-3A and H-35 Cell Con-
diluted Media—Serum-free Waymouth's medium (75 ml/bottle) was conditioned by Buffalo rat liver BRL-3A cells in 850-cm² roller bottles and collected every other day according to the procedure described by Marquardt et al. (26). Rat hepatoma H-35 cells (gift of Dr. Gerald Litwack, Temple University) were grown under analogous conditions. Serum-free Dulbecco's modified Eagle's medium was collected from the cell culture every 2 days. The whole cell line was seeded to avoid overgrowth of the cells and a new culture was transferred to the containers. Batches of media (typically 20 liters from BRL-3A cells and 0.7 to 3 liters from H-35 cells) were clarified by centrifugation for 20 min at 10,400 × g and concentrated to one-tenth of the original volume using a Pellicon (Millipore) or a crude preparation of mouse liver cellulosic PSAC filter, M, cutoff 1,000.

The concentrated fluid was then dialyzed against 8 volumes of 0.1 M acetic acid at 4 °C. The dialysis buffer was replaced after 1 day and, after an additional 24-h period, the media were clarified by centrifugation and lyophilized. The dry material derived from 600 ml of concentrated medium was resuspended in 6–7 ml of 1 M acetic acid and the insoluble residue sedimented by centrifugation at 146,000 × g for 30 min. The supernatant was chromatographed on a Bio-Gel P-10 column (2.5 × 85 cm, 200–400 mesh, Bio-Rad) equilibrated in 1 M acetic acid, at a flow rate of 20 ml/h. Four-milliliter fractions were collected and an aliquot of this fraction was used to assay for the ability to compete with 125I-IGF-I for binding to IGF receptors. The fractions containing the greatest amount of this activity, recovered in the void volume between 120 and 150 ml of the effluent, were pooled and lyophilized or used as a starting material for analogous procedures. A low molecular weight calibration kit containing bovine serum albumin, ovalbumin, and insulin was used as standard. When 125I-labeled ligand was accompanied by other peptides, the low molecular weight calibration kit was supplemented with microgram amounts of porcine insulin and crystalline bovine insulin.

Preparation and Sequencing of an IGF-binding Protein

Preparation of Anti-IGF-binding Protein Ig—Two 3-month-old female New Zealand rabbits (designated as P and F) were injected subcutaneously in the scapular region with BRL-3A IGF-binding protein eluted from IGF-II-agarose. The antigen (45 µg/rabbit) was resuspended in 0.55 ml of Krebs-Ringer phosphate buffer, pH 7.4, and emulsified with an equal volume of Freund's complete adjuvant (Cohn's). Four weeks after this initial injection, the rabbits were boosted with 25 µg each of similarly prepared IGF-binding protein in Krebs-Ringer phosphate buffer, thoroughly mixed with an equal volume of Freund's incomplete adjuvant (Cohn's). The rabbits were bled from the ear 2 weeks later and then regularly every 14 days. The serum obtained was frozen or sequentially stored in liquid nitrogen for 40, 37, and 33% saturated ammonium sulfate to partially purify the Ig fraction (31). Control serum and Ig were prepared from a preimmune rabbit following the same procedure.

Enzyme-linked Immunosorbent Assay—The affinity of anti-binding protein serum toward its antigen was titrated by enzyme-linked immunosorbent assay (32) following the procedure reported by Oka et al. (34). Unless otherwise indicated, 50 µg of BRL-3A IGF-binding protein in 50 µl of phosphate-buffered saline were used to cover each well and the antigen was allowed to react with anti-serum dilutions from 1:2,000 to 1:10,000 in the same buffer.

Preparation of Peptide—The IGF-binding protein originally described by Burnette (35) was used with some modifications. At the end of the electrophoretic separation on an 8–14% polyacrylamide gradient gel, as reported above, proteins (0.5–3 µg of binding protein when purified) were transferred onto nitrocellulose paper (Schleicher and Schuell) by electroblotting for 4 h at 200 mA (constant current, approximately 10 V/A). The elution buffer, extensively degassed before use, contained 20% Tris, 150 mM glycine, 0.1% NaDodSO4, and 20% methanol. Subsequently, the paper was saturated with 2% bovine serum albumin in a solution of 150 mM Tris, 150 mM NaCl, pH 7.5, by incubation on a shaker at 15 °C. The nitrocellulose paper was then dried with a stream of air. The nitrocellulose was then placed on an end over end mixer for 4 h at 22 °C or for 18 h at 4 °C. The paper was washed three times with 15 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40 for a period of 10 min at 22 °C with shaking. A final wash was carried out in the absence of the detergent. The nitrocellulose was again placed in a plastic bag and incubated in the same Tris-NaCl buffer containing 5% bovine serum albumin and 2 × 10^6 cpm of 125I-protein A (New England Nuclear) for 40 min at 22 °C with end over end mixing. The washing cycle was repeated with three changes of 30 mM Tris, pH 7.4, 300 mM Ac, 0.05% Nonidet P-40 for 40 min at 22 °C with shaking. The paper was dried and exposed to Kodak XAR-5 film.

Determination of Amino Acid Composition—In order to undergo sequencing, BRL-3A IGF-binding protein was reduced, alkylated, and further purified. Approximately 4 µg of protein eluted from IGF-II-agarose were dried, resuspended in 2 mM Tris, pH 8.3, and extensively dialyzed at 4 °C against the same buffer. The sample was then lyophilized and redissolved in 4 ml of 6 M guanidine hydrochloride, 0.5 M Tris, pH 8.8, 10 mM dithiothreitol. The reduction was carried out for 90 min at 22 °C, then [2-3H]iodoacetic acid was added at a final concentration of 50 mM (specific activity 10 mCi/ml, New England Nuclear). After 30 min at 22 °C, the reduced sample was applied onto a 2.5 × 85 cm column of Sephacryl S-300 (Pharmacia) equilibrated in 6 M guanidine hydrochloride, 0.1 M NaHCO3, pH 8.2. The chromatographic separation was performed at a flow rate of 12 ml/h, collecting 2-mlliter fractions. The elution profile was monitored by counting 1% of each fraction for radioactivity; fractions containing the main peak of label were pooled and desalted by passage over a 1.5 × 50-cm column of Bio-Gel P-6 (Bio-Rad) in the presence of 0.1 M NH4HCO3, pH 8.2. The radioactive fractions eluting at the void volume of this column were combined and lyophilized. The lyophilized sample was then dissolved in 2 ml of 24% acetonitrile and 10-µl aliquots were transferred into microfuge tubes. A blank was prepared utilizing effluent buffer from the Bio-Gel P-6 column. After lyophilization, the samples were hydrolyzed for 24, 48, and 72 h in vacuo at 110 °C, hydrolyzed, and derivatized with phenylisothiocyanate according to the procedure of Slaunwhite et al. (36). The fluorometric amino acid analyses were conducted on a Waters HPLC using an UltraspHERE ODS reverse-phase column and a series of
two cell types was tested for its inhibitory activity on \( ^{125}\text{I}-\text{IGF-II} \) binding to H-35 cells. Similar results were obtained when the binding assay was performed on plasma membrane fractions from tissues rich in IGF-II receptors (data not shown). This inhibitory effect on labeled ligand binding to cells or plasma membranes exerted by the conditioned medium appeared to be specific against \( ^{125}\text{I}-\text{IGF-II} \) binding. Under the same conditions, the binding of \( ^{125}\text{I}-\text{insulin} \) or \( ^{125}\text{I}-\text{EGF} \) to their respective receptors was unaffected by addition of conditioned medium (data not shown).

Following acid treatment of the conditioned medium and its fractionation on a Bio-Gel P-10 column, the IGF-II-competting activity could be recovered in association with two main peaks, as shown in Fig. 2 for BRL-3A cells medium. This graph illustrates results obtained from the chromatography in 1 M acetic acid of medium conditioned by BRL-3A cells. The per cent inhibition of either \( ^{125}\text{I}-\text{IGF-II} \) (solid line) or \( ^{125}\text{I}-\text{insulin} \) binding (broken line) produced by the material eluting in each fraction was measured using a crude preparation of mouse liver plasma membrane. The major peak of \( ^{125}\text{I}-\text{IGF-II} \) competing activity eluted in the void volume of the column, indicating it contains the IGF-binding protein. The binding of \( ^{125}\text{I}-\text{insulin} \) to the membrane was not altered by conditioned medium.
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incubation with these fractions. A second peak that exhibited competition for \(^{125}\text{I}\)-IGF-II binding appeared in the effluent between 280 and 300 ml and co-eluted with a standard preparation of labeled \(^{125}\text{I}\)-IGF-II. A similar profile of distribution of \(^{125}\text{I}\)-IGF-II competing activity was obtained from Bio-Gel P-10 gel chromatography of medium conditioned by H-35 cells (not shown).

IGF-binding protein was separated from the bulk of other proteins present in the conditioned medium by affinity chromatography on IGF-II linked to Sepharose 4B. After this step, the binding protein preparation was enriched to approximately 60% homogeneity. The silver-stained pattern of the proteins eluted from IGF-II-agarose, after electrophoretic separation on polyacrylamide gradient gels (Fig. 3), exhibited a very evident major band (lane a) migrating with apparent \(M_r\) of 36,300 (average of 12 determinations) under reducing conditions. In the absence of reductants, this band showed a shift in apparent \(M_r\), to 33,000 (data not shown). Under either condition, the reproducible presence of other major components was not detectable, although several minor bands were consistently observed. Analogous preparations of medium conditioned by H-35 cells yielded a protein band indistinguishable from that produced by BRL-3A cells. This band is also a specific IGF-binding protein, as suggested by its absence from column eluates in experiments in which the IGF-II-agarose had been replaced with insulin-agarose. Incubation with neuraminidase (40-100 milliunits/\(\mu\)g protein) or endoglycosidase H (25–100 ng/\(\mu\)g protein) under standard conditions failed to influence the electrophoretic mobility of the IGF-binding protein purified from either one of the two cell sources employed, indicating that the presence of oligosaccharide side chains linked to this protein is unlikely (data not shown).

In order to provide a rough quantitative estimate of the IGF-binding protein recovered in each preparation known amounts of ovalbumin were run in parallel with an aliquot of the protein eluted from IGF-II-agarose (lanes b, c, and d in Fig. 3). The average yield of a typical preparation was estimated to be 1.5–2 mg of binding protein, starting from 6 liters of conditioned medium. In the representative experiment of Fig. 3, 0.2% of one such preparation was loaded on the gel (lane a). Due to the larger availability of the medium conditioned by BRL-3A cells (see "Experimental Procedures"), IGF-binding protein from these cells was selected for procedures requiring a significant amount of protein, such as the rabbit immunizations and sequencing of the binding protein amino terminus.

Chemical cross-linking of the binding protein at different stages of purification revealed its ability to specifically bind both \(^{125}\text{I}\)-IGF-I or \(^{125}\text{I}\)-IGF-II. Fig. 4 shows the results of an experiment in which H-35 binding protein following affinity chromatography was cross-linked to either \(^{125}\text{I}\)-IGF-I or \(^{125}\text{I}\)-IGF-II. In the autoradiograph of the representative gel depicted in Fig. 4, only one band appears to be diminished in intensity by the presence of unlabeled IGFs during the incubation. The molecular weight of this band, calculated from several determinations, is 43,000 which includes both the binding protein and ligand. The other less intense bands in the autoradiograph, not specifically diminished in intensity by the presence of unlabeled IGFs, are likely to be derived from the bovine serum albumin present in the assay. In this experiment, IGF-I and IGF-II seem to exhibit similar potency in competing for the binding of either one of the \(^{125}\text{I}\)-IGFs to the binding protein. The different specific activities of the \(^{125}\text{I}\)-iodinated ligands does not allow a direct comparison of the intensities of the labeled bands as true reflections of the affinities of the binding protein for \(^{125}\text{I}\)-IGF-I versus \(^{125}\text{I}\)-IGF-II. Several other experiments indicated that these affinities are similar, however (see also Fig. 9).

The binding protein secreted from BRL-3A cells exhibited IGF binding characteristics similar to those of the binding protein derived from H-35 cells. Fig. 5 shows the results of affinity cross-linking of \(^{125}\text{I}\)-IGF-II to the BRL-3A IGF-binding protein. When the cross-linking reaction was carried out with acid-treated material from the void volume of the Bio-Gel P-10 column or following purification by IGF-II-agarose chromatography, a specifically labeled band was easily detectable, exhibiting a molecular weight corresponding to that of the cross-linked H-35 binding protein. In both the partially purified and affinity-purified states, unlabeled IGF-1 or IGF-II effectively displaced the labeled ligand from this \(M_r\) \(=\) 43,000 major component. Again, labeled IGF-II binding to several minor bands, probably deriving from the serum albumin used in the assay, could not be displaced by unlabeled IGFs.

Potency and Specificity of Anti-BRL-3A IGF-binding Protein Polyclonal Antibodies—Two populations of polyclonal antibodies directed against purified BRL-3A IGF-binding protein were raised in two rabbits, designated as F and P. The
The affinity cross-linking reaction was performed in Krebs-Ringer phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin and 0.5 μg of purified IGF-binding protein in a final volume of 60 μl. Unlabeled 1 μM IGF-I or IGF-II was added to the indicated samples followed by incubation for 1 h at 22 °C with gentle mixing. After this period, the incubation was extended for an additional hour under the same conditions in the presence of either 5 nM 125I-IGF-I or 125I-IGF-II. The tubes were then transferred to a 10 °C bath and 0.4 mM disuccinimidyl suberate was added. After 15 min the cross-linking reaction was ended with an excess of Tris buffer, pH 7.4. The samples were solubilized and analyzed electrophoretically on an 8–14% polyacrylamide gradient slab gel, as described in the legend to Fig. 3. An autoradiograph of the Coomassie Blue-stained frozen gel is shown. Lanes a and d, samples containing 1 μM IGF-II; lanes b and e, samples containing 1 μM IGF-I; lanes c and f, controls, in the absence of unlabeled hormone. Specific activity of 125I-IGF-II = 65 Ci/g, specific activity of 125I-IGF-I = 127 Ci/g. The relative molecular mass of standard proteins is indicated; K = 1000.

The specificity of antisera F toward IGF-binding protein in the presence of other components in the conditioned medium was analyzed performing an immunoblot. In the results of the experiment depicted in Fig. 7 the sample used in lane A is an aliquot of total dialyzed serum before acid treatment, while samples B and C are different amounts of acidic material derived from the bulk of proteins eluting at the void volume of the Bio-Gel P-10 column. In both cases, the antisera react with high specificity exclusively with the IGF-binding protein, confirming the notion that the antigen used was sufficiently purified prior to immunization of rabbits. In addition, these data provide evidence that the binding protein can be efficiently recognized by this Ig population at every stage of the purification procedure.

In spite of the apparent similarity of the binding proteins from BRL-3A and H-35 cells, the anti-BRL-3A binding protein antibody exhibits a considerably lower sensitivity in reacting with binding protein secreted by H-35 cells (Fig. 8). When tested by enzyme-linked immunosorbent assay (upper panel) a 1:2000 dilution of antiserum F yielded a very low reaction with 250 ng of H-35 binding protein, while producing a strong signal (10-fold higher) in the presence of 50 ng of BRL-3A binding protein. Similar results were obtained from the immunoblot of the two proteins with antiserum F (dilution 1:250), as illustrated in the lower panel of Fig. 8. After reaction with the antibodies, a lower amount of 125I-protein A was bound to 3 μg of H-35 binding protein (lane c) than to 0.5 μg of BRL-3A binding protein (lane a). Although exhibiting a decreased sensitivity in recognizing H-35 binding protein versus BRL-3A binding protein in the above assays, both the polyclonal antibodies were shown to efficiently compete for the binding of 125I-IGFs to purified H-35 binding protein, when tested by affinity cross-linking (Fig. 9). In this experiment, 100 μg of protein from the Ig fraction of either serum P or F could almost completely prevent the labeling by 125I-IGF-I or 125I-IGF-II of 0.4 μg of binding protein from H-35 cells.

Amino Acid Composition and Sequence of the Amino Terminal of the BRL-3A Binding Protein—Two preparations of BRL-3A binding protein (amounting to about 4 mg of total protein) were combined and further purified prior to amino acid composition analysis and sequencing of the protein from its NH2-terminus. The affinity-purified sample was reduced and alkylated in the presence of [2-14C]iodoacetic acid, as described under "Experimental Procedures." Chromatography of this material on a Sephacyr S-300 column in 6 M guanidine hydrochloride buffered at pH 8.2 resulted in the resolution of a major sharp peak of labeled protein and a minor peak. The elution profile, as recorded in terms of radioactivity, is shown in Fig. 10. The second, smaller component is likely to be derived from proteolysis, as a minor band with lower molec-
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**Fig. 6. Titration of anti-IGF-binding protein antiserum by enzyme-linked immunosorbent assay.** Polyclonal antibodies were raised in two rabbits (F and P) by injection with BRL-3A IGF-binding protein eluted from IGF-II-agarose, as specified under "Experimental Procedures." The potency of each antiserum was tested by enzyme-linked immunosorbent assay. Purified binding protein (50 ng) was adsorbed to microtiter wells in 50 μl of 5 mM NaP, 0.9% NaCl, pH 7.4, for 2 h at 37°C. The excess protein still in suspension was discarded and the wells were covered with 1% bovine serum albumin in 100 μl of phosphate-buffered saline. After extensive washing with the same buffer containing either 0.5% Triton X-100 or 2% γ-globulin-free horse serum, 50 μl of anti-binding protein serum at the dilutions indicated were layered on each well and the reaction allowed to proceed for 17 h at 4°C. This first antibody was removed and the washing procedure repeated. A second antibody (10 pg/ml of 10 μg/ml of IGF-binding protein) was added to the incubation wells. After 2 h at 37°C, the adsorbed material was washed again as above and visualized by addition of 100 μl of phosphate-buffered saline. After extensive washings, 20 μg/ml of 1% Triton X-100 or 2% γ-globulin-free horse serum, 50 μl of anti-binding protein serum at the dilutions indicated were layered on each well and the reaction allowed to proceed for 17 h at 4°C. This first antibody was removed and the washing procedure repeated. A second antibody (10 pg/ml of 10 μg/ml of IGF-binding protein) was added to the incubation wells. After 2 h at 37°C, the adsorbed material was washed again as above and visualized by addition of 100 μl of phosphate-buffered saline. After extensive washings.

**Fig. 7. Specificity of the anti-IGF-binding protein antiserum.** Immunoblot of BRL-3A binding protein from conditioned medium before acid treatment and after elution from the Bio-Gel P-10 column in 1 M acetic acid is shown. The immunoblot was performed according to the method described under "Experimental Procedures." The proteins were electroeluted from an 8–14% polyacrylamide gradient gel onto nitrocellulose paper for 4 h at 200 mA (constant current). After saturation with bovine serum albumin, the paper was incubated with anti-BRL-3A IGF-binding protein serum (rabbit F) at 1:250 dilution for 18 h at 4°C. The presence of bound immunoglobulin, following extensive washing, was detected by adding 125I-protein A (2 × 10^6 cpm) for 40 min at 22°C and finally washing the nitrocellulose. The figure shows the autoradiograph pattern of the immunoblot. A, 1.25 μl of medium conditioned by BRL-3A cells was dialyzed against 2000 volumes of 2 mM Tris, pH 7.4, for 2 h at 4°C (M cutoff of the tubing 5000). This material was then concentrated by evaporation under vacuum to one-tenth of the original volume and 125 μl of Laemmli sample buffer (29) containing 100 mM thiodithioletritol and 1% NaDodSO₄ were added. The sample was solubilized for 2 min at 100°C and applied to the gel. The fractions eluting at the void volume of the Bio-Gel P-10 column in 1 M acetic acid in a typical preparation were combined and lyophilized (see "Experimental Procedures" for details). The total dry material was reconstituted in 200 μl of 10 mM Tris buffer, pH 7.4, and 60 μl (B) or 120 μl (C) of this suspension were solubilized and analyzed as above.

3H-S-carboxymethylated binding protein, confirmed those 22 residues and extended the identifications to 31 amino acids, as follows: Phe-Arg-Cys-Pro-Pro-Cys-Thr-Pro-Glu-Arg-Leu-Ala-Ala-Ala-Cys-Gly-Pro-Pro-Pro-Asp-Ala-Pro-Cys-Ala-Glu-Leu-Val-Agl-Glu-Pro-Gly-Cys. It is of interest that this region of the protein is particularly rich in proline (26%) and cysteine (16%).

The amount of each determined amino acid (expressed as logarithm) measured at each cycle of the sequence is reported in Fig. 11. This graph indicates an overall satisfactory efficiency of the sequencing process. Only the values for arginine are low, as expected from its instability under sequencing conditions. A best-fit line was calculated by linear regression analysis, excluding the values of arginine. According to this plot, at the starting point the sequencing yield relative to the total amount of sample employed for the analysis was 43%. The repetitive yields calculated on a standard protein (sperm whale myoglobin) were between 94 and 95%. The repetitive yield value calculated on the 2 leucine residues at positions 11 and 25 of the BRL-3A IGF-binding protein, after subtracting the background from the previous cycles, was 92%.

**DISCUSSION**

The presence in the serum of proteins that bind IGF-I and IGF-II specifically and with high affinity provides a means of greatly decreasing the effective circulating concentrations of these peptides in free form (13, 14). The high affinity binding constants that characterize interactions (15) between IGFs and binding proteins assures that most of the IGFs circulate in bound form. Both degradation and biological activity of the IGFs are inhibited when the peptides are associated with the

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binding proteins (24, 25). Thus, the binding proteins might be considered a protection mechanism because most of insulin's biological effects can be mimicked by the IGFs and vice versa, whenever the concentration of one of these three peptides is increased to a level sufficiently high to allow their interaction with insulin or IGF receptors (3, 7, 8, 13, 28).

Recent data indicate that there may be proteolytic or other mechanisms that mediate the rapid release of IGFs from their binding proteins (Figs. 1 and 2). Subsequently, the binding proteins and should be readily applicable to other purification has allowed us to establish that IGF-binding proteins are the major products released by BRL-3A and H-35 hepatoma cells. The first separation step, as described by a number of laboratories, is the chromatography on IGF-1 agarose (Fig. 3), using modifications of a technique previously successfully employed in our laboratory for the isolation of IGF-II receptor (27) and similar but faster than that used by Knauer et al. (44). Electrophoretic analysis of the IGF-binding protein at different stages of purification has allowed us to establish that IGF-binding proteins are the major products released by BRL-3A and H-35 cells into the medium and that the purification procedure does not lead to major changes in the mass of the proteins. This second observation was confirmed by immunoblotting the conditioned media with antisera prepared against the purified IGF-binding protein (Fig. 7). The protocol developed in this study is therefore a convenient procedure for the rapid isolation of milligram amounts of BRL-3A or H-35 cell IGF-binding proteins and should be readily applicable to other cellular systems or sera.

A key finding in this present study is that the purified BRL-3A cell IGF-binding protein preparation represents a single protein. This point is documented by the detection of a single amino-terminal amino acid sequence upon analysis of the

**FIG. 8.** Cross-reactivity of anti-BRL-3A binding protein serum toward H-35 binding protein. Upper panel, enzyme-linked immunosorbent assay was performed as described in the legend to Fig. 6. The indicated amount of IGF-binding protein prepared from medium conditioned by BRL-3A cells (●) or H-35 cells (▲) was incubated with antisera (dilution 1:2000) raised in rabbit P by injection with BRL-3A binding protein purified on IGF-II-agarose. Blanks were run in the presence of 1% bovine serum albumin and their value subtracted from the data plotted in the graph. Lower panel, autoradiograph of an immunoblot carried out as described in the legend to Fig. 7. After elution from IGF-II-agarose, 0.5 μg (a) and 1 μg (b) of BRL-3A IGF-binding protein and 3 μg (c) of H-35 IGF-binding protein were run on an 8-14% polyacrylamide gradient slab gel, and subsequently transferred by electrophoresis for 4 h at 200 mA to nitrocellulose paper. The paper was soaked in buffer containing 2% bovine serum albumin and anti-BRL-3A IGF-binding protein serum (P, dilution 1:250) for 4 h at 22°C, on an end over end mixer. After extensive washing, 125I-labeled protein A (2 × 10^6 cpm) was allowed to react with the bound antibody for 40 min at 22°C. The excess 125I-protein A was discarded and the paper repeatedly washed.

**FIG. 9.** Inhibitory effect of the anti-IGF-binding protein Ig on affinity cross-linking of purified H-35 binding protein to 125I-IGFs. H-35 cell binding protein (0.4 μg) obtained by affinity chromatography was used for each assay that was performed in a final volume of 200 μl. The incubation mixture contained Krebs-Ringer phosphate buffer, pH 7.4, 0.5% bovine serum albumin, and 100 μg of the indicated Ig fractions, prepared as described under "Experimental Procedures." The incubation mixture was kept for 40 min at 22°C with constant stirring. Subsequently, 1 μM unlabeled IGF-II was added to two of the samples (lane D in the figure) and all the tubes received either 125I-IGF-I or 125I-IGF-II at 5 nM. The binding of the IGFs was carried out for 4 h at 4°C with constant mixing and visualized by affinity cross-linking followed by electrophoresis on an 8-14% polyacrylamide gradient slab gel, exactly as described previously (Figs. 4 and 5). The autoradiographic pattern of the gel is shown. Specific activity of 125I-IGF-I = 72 Ci/g; specific activity of 125I-IGF-I = 127 Ci/g. The relative molecular mass of standard proteins is indicated.

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Purification and Sequencing of an IGF-binding Protein

The purified protein by both a gas-phase microsequenator and a liquid phase sequenator. The validity of this strategy becomes evident from the sequencing yields in this second run. The best-fit line plotted in Fig. 11 yields a constant slope, indicating the nature of each residue is indicated. Ordinate, residue yield expressed as logarithm of the number of picomole. "Cys (Cm) confirmed by the presence of radioactivity; amount calculated from the specific activity of the label (see "Experimental Procedures"). The plotted line was obtained by the linear regression method; Arg (○) values were not included.

binding proteins of physiological significance is supported by several pieces of evidence. (a) Strongly acidic conditions are necessary to separate the competing activity for 125I-IGF-II binding present in the conditioned medium from IGF-II itself. (b) After this step, only one major protein from the medium selectively binds to IGF-II-agarose. (c) Binding proteins from both H-35 and BRL-3A cells can be specifically affinity-labeled with 125I-IGF-I or 125I-IGF-II. The representative data in Figs. 4 and 5 indicate that unlabelled IGF-I and IGF-II compete with similar potency for the binding of either labeled IGFs to the binding protein. The conclusion that these binding proteins exhibit comparable affinity for IGF-I and IGF-II is in agreement with data reported by Moses et al. (15) and D'Ercole and Wilkins (17). (d) Purified H-35 binding protein is capable of blocking the biological action of IGFs (45). (e) The general characteristics of the proteins described in this

![Fig. 10. Purification of the BRL-3A cell IGF-binding protein by Sephacryl S-300 chromatography.](image)

![Fig. 11. Sequencing yields of the amino-terminal portion of IGF-binding protein. A preparation (4.67 nmol) of [3H]-carboxymethylated binding protein was sequenced on a Beckman liquid-phase instrument, as described under "Experimental Procedures." Abscissa, cycle number. The nature of each residue is indicated. Ordinate, residue yield expressed as logarithm of the number of picomole. "Cys (Cm) confirmed by the presence of radioactivity; amount calculated from the specific activity of the label (see "Experimental Procedures"). The plotted line was obtained by the linear regression method; Arg (○) values were not included.](image)

### Table I

<table>
<thead>
<tr>
<th>Amino acid composition of the BRL-3A IGF-binding protein</th>
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<tbody>
<tr>
<td>Amino acid</td>
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<td>Cysteine (Cm)</td>
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<td>Lysine</td>
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<td>Histidine</td>
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<td>Arginine</td>
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<td>Aspartic acid and asparagine</td>
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<td>Threonine</td>
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<td>Serine</td>
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<td>Alanine</td>
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<td>Tyrosine</td>
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<td>Phenylalanine</td>
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<sup>a</sup>Three sets of samples (1 µg each) were hydrolyzed in 5.7 N HCl at 108 °C under vacuum for 24, 48, and 72 h. These values were plotted as a function of hydrolysis time for each amino acid. Values listed were calculated as the y intercepts (t = 0) of regression lines for each plot.

<sup>b</sup>Calculations are based on an estimated molecular weight of 36,000 assuming the protein contains no carbohydrate, proline, or tryptophan.

...
study, such as their molecular weight range, agree with those reported for several analogous IGF-binding proteins (15, 18-22).

The purified BRL-3A binding protein is a good immunogen, having elicited the production of specific antibodies in both rabbits injected. The two immunoglobulin populations recognize with considerable sensitivity and specificity the BRL-3A IGF-binding protein before and after the purification process (Figs. 6 and 7). These anti-IGF-binding protein antisera should be valuable tools in future studies on this protein. It is interesting that the H-35 binding protein was detected with considerably lower sensitivity by these two antisera (Fig. 8), suggesting that it may differ in molecular structure from the BRL-3A protein. Further studies will be required to establish the degree of homology existing between these two proteins, as well as their relationship to IGF-binding proteins in sera. Such studies are currently in progress.

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REFERENCES