Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 408-415

BINDING PROTEINS FOR INSULIN-LIKE GROWTH FACTORS IN ADULT RAT SERUM. COMPARISON WITH OTHER HUMAN AND RAT BINDING PROTEINS

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Received July 8, 1987

SUMMARY : Insulin-like growth factor (IGF) binding protein has been purified from adult rat serum by affinity chromatography on agarose-IGF-II and high performance reverse-phase chromatography. The final preparation contains two components, of apparent molecular mass 50 and 56 kDa nonreduced, or 44 and 48 kDa reduced, both of which specifically bind IGF-I and IGF-II. Competitive binding data indicate association constants of 5-10 x 10^{10} l/mol for both IGFs, with a slightly higher affinity for IGF-II than IGF-I. Amino-terminal sequence analysis yields a unique sequence, identical in 11 of the first 15 amino acids with that of a human plasma IGF binding protein (Martin, J. L., and Baxter, R. C. (1986) *J. Biol Chem.* 261, 8754-8760), and with slight homology to other human and rat IGF binding proteins characterized to date. By analogy with the binding protein from human plasma, it is likely that the rat protein is part of the growth-hormone dependent complex which appears to carry most or all of the circulating IGFs.

In recent years, binding proteins for peptides of the insulin-like growth factor (IGF) family have been purified from a variety of sources. A BP preparation isolated in this laboratory from human plasma was found to contain two protein species: a major component of molecular mass 53 kDa and a minor component of 47 kDa, nonreduced, or 43 kDa and 40 kDa respectively, reduced (1). Both species had IGF binding activity, and the mixture yielded a single amino-terminal sequence, suggesting identity in this region between the two components (2). An antibody raised against this preparation reacts predominantly with a GH-dependent species of 125-150 kDa in human plasma (3,4). A smaller BP, with an apparent molecular mass of about 34 kDa reduced and 28 kDa nonreduced, and an amino-terminal sequence different from that of the plasma protein, has been isolated from human amniotic fluid (5-7) and from culture medium conditioned by the human hepatoma line, HEP-G2 (8). Human placenta contains a similar protein, termed placental protein 12, with IGF binding activity and an identical amino-terminal decapeptide (9). RIAs developed against these BP types detect a protein of similar size in human plasma, which is present in much higher concentrations in fetal and cord blood than in the adult circulation (6, 10,11).

Abbreviations : IGF, insulin-like growth factor; BP, binding protein; GH, growth hormone; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

0006-291X/87 $1.50

The first non-human BP to be purified was a protein from the rat liver-derived cell line BRL-3A. Originally isolated by Knauer et al. (12), this protein has been characterized extensively in three laboratories (13-15). It has an apparent molecular mass of 33-36 kDa reduced and 31.5 kDa nonreduced (13-15), and approximately equal affinity for IGF-I and IGF-II (14). RIAs for this protein fail to detect activity in adult rat serum, whereas fetal and neonatal sera contain high concentrations (13,15). Thus this BP bears a strong resemblance to the human protein of about 30 kDa, both in its physical properties and in the age-dependence of its circulating levels. Although adult rat serum contains undetectable levels of the ~30 kDa BP, significant IGF binding activity is present in the adult rat circulation, in a variety of molecular forms (16-21). This study aimed to isolate and characterize the proteins responsible for this activity, and in this report we describe the physical and binding properties of a rat serum BP preparation, and compare its amino-terminal sequence with that of the other known BP species.

MATERIALS AND METHODS

Reagents — IGF-I and IGF-II were isolated from Cohn fraction IV of human plasma, as in our previous studies (22). Both peptides were iodinated using chloramine T and purified by gel chromatography at neutral pH. Iodinated IGF-I was further purified by hydrophobic interaction chromatography (23). The specific activities of the iodinated peptides were estimated to be 150 Ci/g for IGF-I and 100 Ci/g for IGF-II. The IGF-II affinity column was prepared using Affi-Gel 15 (Bio-Rad, Richmond, CA), as previously described (1). Bovine albumin (RIA grade) was from Sigma; most electrophoresis reagents and the reverse phase HPLC column from Bio-Rad. Molecular weight markers for electrophoresis were purchased from Pharmacia (Sydney, NSW), the Coomassie blue stain (Gradipure) from Gradipore, Sydney, NSW, amido black 10B from Corning, Palo Alto, CA, and nitrocellulose membrane (BA 85) from Schleicher and Schuell, Dassell, West Germany. Hyperfilm-MP for autoradiography was obtained from Amersham, Bucks, England.

Purification of Rat BP — The starting material for this preparation was a pool of 240 ml of normal adult rat serum, and 100 ml of serum from adult rats bearing the GH-secreting tumor MtT/W15. We have shown previously that the hepatic production rate of IGF BP in adult rats bearing this tumor is approximately double that seen in control rats (24). The pool was mixed with an equal volume of 2 M acetic acid, and the pH adjusted to 3.0. After standing for 2 h at 22 ° C, the acidified serum was pumped at 24 ml/h onto a 2.5 x 19 cm column of SP-Sephadex, previously equilibrated with 1 M acetic acid, 75 mM NaCl, pH 3.0. Serum remaining on the column was washed through with equilibration buffer, and the entire flow-through volume (690 ml) was adjusted to pH 6.5 with 7 M NaOH, and centrifuged to remove precipitated material. The supernatant was pumped at 18 ml/h onto a 1 x 12 cm column of agarose-IGF-II, the column was washed with 100 ml of 0.25 M NaCl at 75 ml/h, and BP was eluted with 0.5 M acetic acid, pH 3.0, at 60 ml/h. Fractions of 1 ml were collected and assayed for IGF-II binding activity, as previously described (25). Active fractions were pooled, lyophilized, and dissolved in 15% acetonitrile in 0.1% trifluoroacetic acid, then subjected to reverse-phase HPLC on a Bio-Rad Hi-Pore RP-318 column, exactly as previously described for the human protein (1). Active fractions were pooled and freeze-dried, and the dry powder weighed and dissolved in 1 M acetic acid at a nominal concentration of 1 mg/ml.

SDS-PAGE and Electroblotting — SDS-PAGE was performed according to the method of Laemmli (26) on 7-18% gradient slab gels. Standards and lyophilized samples were reconstituted in 10 mM Tris-Cl, 1% SDS, pH 8.0 before being applied to the gel. Electrophoresis at 50 V was continued until the sample had run into the stacking gel, then at 100 V until the dye front had reached the bottom of the gel. For protein staining, gels were fixed in methanol-acetic acid-water (40 : 10 : 50), then stained using Gradipure Coomassie blue. For blotting, gels were equilibrated after electrophoresis for 1 h in 25 mM Tris-Cl, 192 mM glycine, 20% methanol, pH 8.3. Proteins were transferred from the gel to

nitrocellulose membrane at 45 V for 6 h in the Bio-Rad Trans-Blot cell. After blotting, the lanes containing molecular weight markers were cut off , stained in 0.1% amido black in 45% methanol, 7% acetic acid, and destained in 7% methanol, 5% acetic acid. The nitrocellulose sheets containing binding proteins were incubated overnight at 37 °C in a 3% bovine albumin solution in 50 mM sodium phosphate, pH 6.5, then sealed in plastic bags with 35 ml of a solution containing 2 x 10^6 cpm of iodinated IGF-I (10 ng) or IGF-II (15 ng), with or without 10 μg of unlabeled IGF-I or IGF-II respectively, in 50 mM sodium phosphate, 3 % bovine albumin, pH 6.5. After incubation at 22 °C for 2 h, non-bound radioactivity was removed with three washes in cold phosphate buffer, the sheets were dried in air, and exposed to Hyperfilm-MP autoradiography film for 24 h at -80 °C.

Competitive Binding Studies — Binding was performed in 0.3 ml of 0.05 M sodium phosphate buffer, 0.25 % bovine albumin, pH 6.5, for 2 h at 22 °C. Incubations contained rat BP (routinely 0.5 ng, based on dry weight), iodinated IGF-I or IGF-II (30 pg), and various concentrations of unlabeled IGF-I or IGF-II ranging from 0.01 to 5 ng/tube. Nonspecific binding was measured in the presence of 100 ng of either peptide. At the end of the incubation period, bound radioactivity was separated from free by precipitation with 200 μg/tube concanavalin A, as previously described for the human binding protein (1). This method was chosen after preliminary experiments had established that precipitation of bound counts with concanavalin A gave slightly higher binding than adsorption of free tracer with charcoal.

Sequence Determination — Approximately 40 μg (0.8 nmol) of unreduced rat binding protein was sequenced by Dr. M. I. Tyler, School of Chemistry, Macquarie University, North Ryde, NSW, Australia, by automatic Edman degradation on an Applied Biosystems 470 A protein sequencer, as previously described (2). Sequences of human plasma and amniotic fluid proteins were determined as previously described (2).

RESULTS

When HPLC-purified rat serum IGF BP was subjected to SDS-PAGE and stained with Coomassie blue, two protein components were evident, of apparent molecular mass 50 kDa and 56 kDa. Figure 1(a) compares these two bands with those seen in preparations of human plasma BP, to which we have previously assigned molecular masses of 53 and 47 kDa (1). Unlike the human BP preparation, in which the higher molecular mass species was always the more heavily Coomassie stained, in the rat BP preparation the lower molecular mass (50 kDa) band generally appeared somewhat darker than the 56 kDa band. After reduction with 2-mercaptoethanol, the two bands corresponded to apparent molecular masses of 44 and 48 kDa (data not shown), showing a comparable decrease to that previously reported for the human BP preparation (1). Following electroblotting onto nitrocellulose after SDS-PAGE, and incubation with IGF-I or IGF-II tracer, both protein components were found to bind both IGFs. As shown in Figure 1(b), the 50 kDa component of rat BP showed greater binding of both tracers than the 56 kDa component, in contrast to the human BP preparation which showed higher binding to the higher molecular mass (53 kDa) band. Tracer binding was specific, as indicated by the elimination of bound radioactivity when incubations were performed in the presence of excess unlabeled IGF-I or IGF-II.

The specificity of IGF binding was examined in more detail by performing competitive binding experiments in liquid phase. As preliminary experiments had indicated that rat BP was glycosylated, we separated bound from free ligand in these experiments by precipitating the BP-IGF complex with concanavalin-A plus polyethylene glycol. As shown in Figure 2, rat BP bound IGF-II tracer slightly

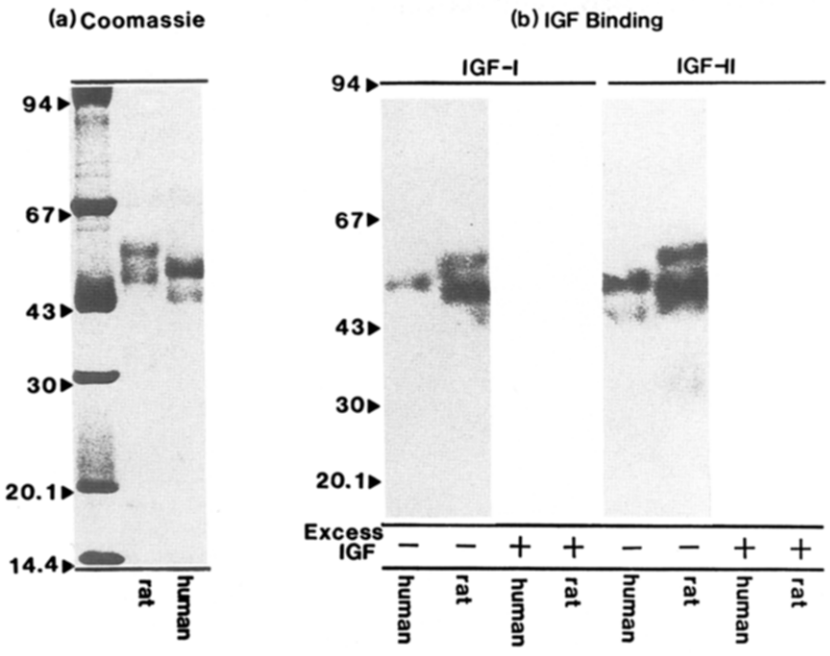

Figure 1. Nonreduced SDS-PAGE of human and rat serum IGF binding proteins. (a) 10 μg of nonreduced rat and human BP, as indicated, were electrophoresed on 7-18% gradient gels as described in the Methods. Binding proteins and molecular weight markers (*left lane*, values in kDa) were stained with Coomassie blue. (b) 1.25 μg per lane of nonreduced rat or human BP were electrophoresed as described above, blotted onto nitrocellulose, and probed with IGF-I tracer in the absence (-) or presence (+) of excess unlabeled IGF-I, or IGF-II tracer in the absence (-) or presence (+) of excess unlabeled IGF-II, as indicated in the figure. The nitrocellulose sheets were then autoradiographed as described in the Methods. IGF-I binding to the minor 47 kDa band of the human preparation, not visible in this experiment, has been demonstrated previously (2).

better than IGF-I tracer, and IGF-II was more potent than IGF-I in displacing either tracer. Scatchard analysis of binding data indicated a high affinity binding component (Ka = 5-10 x 10^{10} l/mol) plus a nonspecific binding component (Ka < 10^{7} l/mol) for each tracer (Figure 3). Based on a molecular mass of 50 kDa, the binding capacity for either ligand was approximately 0.5 mol/mol, suggesting a single binding site for either ligand per 50 kDa protein.

Amino-terminal sequence analysis on 40 μg of rat BP yielded a unique sequence, suggesting that the two protein components were identical in their amino-terminal region (or that one component was terminally blocked). Figure 4 compares this sequence to that obtained previously for the human plasma BP preparation (2), now extended to 15 residues, and to those reported by Mottola et al. (14) and Lyons and Smith (15) for the BP from rat BRL-3A cells. Six of the ten residues previously reported for the human BP (2) are identical in the rat protein, and positions 11 - 15 are entirely homologous, assuming residue 13 (not determined) to be cysteine. In contrast, the homology with the BRL-3A protein is much weaker in this region, although residues 5, 6 and 8 in the sequence of Lyons and Smith (corresponding

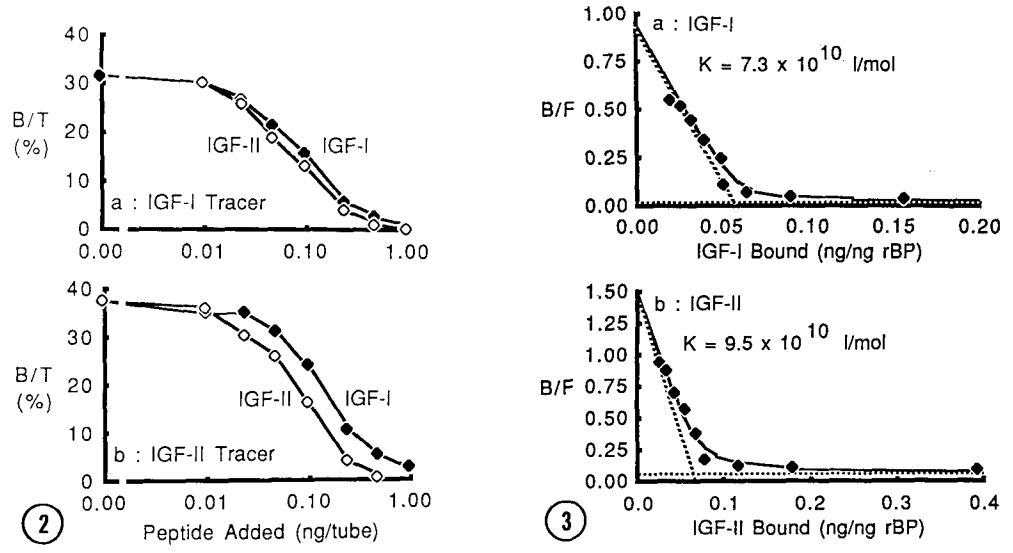

Figure 2. Competitive binding curves showing the displacement of (a) IGF-I tracer and (b) IGF-II tracer from purified rat IGF BP (0.5 ng) by increasing concentrations of unlabeled IGF-I (*solid symbols*) or IGF-II (*open symbols*). The reaction volume was 0.3 ml. Bound radioactivity was separated from free by precipitating the BP-IGF complex with concanavalin A plus polyethylene glycol (1). B/T is the ratio of protein-bound radioactivity to total radioactivity.

Figure 3. Scatchard plots of (a) IGF-I and (b) IGF-II binding to rat serum IGF BP (rBP). *Points* represent experimental data, *solid lines* are computer-assisted fits to a two-site model (high-affinity binding plus nonspecific binding), and *dotted lines* represent the resolved components. B/F is the ratio of bound to free ligand.

to residues 2,3 and 5 in the sequence of Mottola et al.) appear to correspond to residues 12, 13 and 15 of the human and rat serum proteins. Also shown for comparison are the first 15 residues of human amniotic fluid IGF BP isolated in this laboratory (2,7), initially reported up to residue 10 by Póvoa et al. (5). The sequence differs from that reported for the related or identical protein, placental protein 12, which has -Asp-Glu- at residues 11 and 12 (9). Amniotic fluid IGF BP, although only weakly homologous to the human and rat serum proteins at the amino-terminus, is identical in 6 of its first 15 residues with the rat BRL-3A protein.

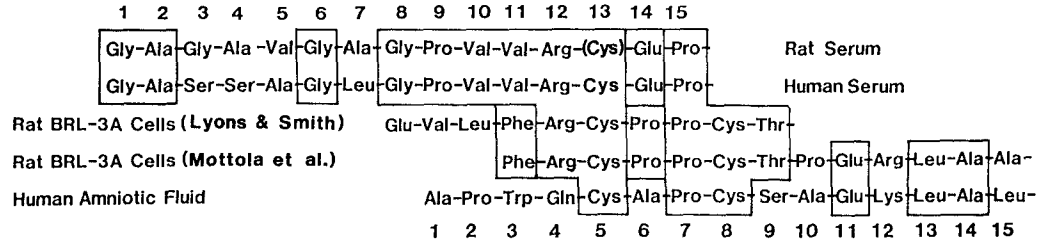

Figure 4. Amino-terminal sequence of rat serum IGF binding protein, compared to sequences of IGF BPs from human serum, rat BRL-3A cell culture medium, and human amniotic fluid. Residue 13 in the rat serum BP sequence, not identified, is assumed to be Cys. The BRL-3A BP sequences are those of Lyons and Smith (15) and Mottola et al. (14); the latter sequence was reported to 31 residues. *Enclosed areas* represent regions of homology between different sequences.

DISCUSSION

In this study we have demonstrated that adult rat serum contains a glycosylated binding protein for IGF-I and IGF-II which is different in size and amino-terminal sequence from that isolated from culture medium conditioned by rat BRL-3A cells, but homologous with a protein which we previously isolated from human plasma. The preparation contains two binding components, of apparent molecular mass 50 and 56 kDa nonreduced, or 44 and 48 kDa reduced. It thus resembles an IGF BP preparation isolated from human plasma which also contains two binding components, both of which decrease significantly in apparent molecular mass on reduction (1,2). In contrast, the rat BRL-3A binding protein, and the binding protein from human amniotic fluid, have nonreduced molecular masses around 30 kDa, which show a significant increase on reduction (5,7,14,15).

The rat BP preparation described in this report showed slightly higher affinity for IGF-II than for IGF-I. The relatively close affinity of this BP for the two IGFs resembles that of the BP in acid-chromatographed rat serum, and in medium conditioned by primary cultures of adult rat hepatocytes (25). IGF-I has been reported to show twice the potency of IGF-II in displacing IGF-I tracer from a BP preparation from cultured rat liver explants (27), but, given slight differences between the potencies of IGF preparations from different laboratories, this may not be inconsistent with our results. Binoux et al. (28) have also suggested that rat liver may produce a BP with selective affinity for IGF-II; however, this still remains to be conclusively demonstrated.

The exact relationship between the two IGF binding components of our BP preparation, and rat serum IGF BPs described in other studies, is not clear. In rat serum, much of the endogenous IGF activity and IGF binding sites have an apparent molecular mass, determined by gel chromatography at neutral pH, of about 150 kDa, whereas after acidification the binding activity corresponds to an acid-stable protein somewhat smaller than albumin (17). This observation parallels that seen in human plasma (29), where antibodies raised against the acid-stable 53 kDa BP have been shown to react predominantly with the plasma BP-IGF complex of approximately 150 kDa (3). This complex, which carries most of the endogenous IGFs (30,31), contains sufficient binding subunits (as determined by RIA) to account fully for the total circulating concentrations of IGF-I and IGF-II (4), assuming one binding site per 53 kDa protein, as previously demonstrated by direct binding studies (1).

By analogy with the binding proteins of human plasma, the purified rat binding species of 50 and 56 kDa described in this paper may be structurally related to the major circulating BP-IGF complex in

rat serum. The two proteins may also be related to some of the binding species demonstrated by D'Ercole and Wilkins using affinity labeling (20). In that study the major GH-dependent BP in rat serum appeared to form a complex with IGF-I of 95 kDa, with a fainter 49 kDa band also present. These might correspond to a monomer and dimer of one or both of the binding proteins we have isolated, which had reduced molecular masses of 44 and 48 kDa. Rat hepatocytes in primary culture also produce binding activity of 50 kDa after acidification (25), consistent with the size of the purified rat serum BP. Future structural and immunological studies may explain how these proteins aggregate to form complexes of 95 kDa or 150 kDa with the IGFs, and how they regulate the delivery of IGFs to target cell receptors.

ACKNOWLEDGEMENTS

This study was supported by the National Health and Medical Research Council, Australia. The assistance of Dr. M. I. Tyler, School of Chemistry, Macquarie University, NSW, who performed the amino acid sequence analyses, is gratefully acknowledged. Donor rats bearing the MtT/W15 tumor were originally obtained from the Mason Research Institute, Worcester, MA.

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