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Note

Method for purification of an insulin-like growth factor-binding protein produced by human HEP G2 hepatoma cells

DAVID R. POWELL*

Department of Pediatrics, Baylor College of Medicine, 8080 North Stadium Drive, Houston, TX 77054 (U.S.A.)

PHILLIP D.K. LEE

Division of Endocrinology, The Children's Hospital, 1056 E. 19th Street, Denver, CO 80218 (U.S.A.)

JOHN E. SHIVELY

Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA 91010 (U.S.A.)

and

MARINA ECKENHAUSEN and RAYMOND L. HINTZ

Department of Pediatrics, Divison of Pediatric Endocrinology, Stanford University Medical Center, Stanford, CA 94305 (U.S.A.)

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Insulin-like growth factors I (IGF-I) and II (IGF-II) are the major peptides in a family of hormones which appear to mediate the growth-promoting effects of growth hormone [1, 2]. These IGF peptides have significant structural homology with insulin and can evoke many of the same biological actions as insulin [3, 4]. Unlike insulin, however, the IGF peptides circulate in the bloodstream tightly bound to specific IGF-binding proteins [5]. IGF-binding proteins have been purified by affinity chromatography of conditioned medium from cultured rat BRL cells [6, 7], rat H35 hepatoma cells [7] and human HEP G2 hepatoma cells [8]. Affinity chromatogaphy has also been employed to purify an acid-stable IGF-binding protein from Cohn Fraction 4 of human plasma [9]. These methods can provide mg yields of binding protein but require the covalent attachment of mg amounts of the relatively scarce IGF peptides or anti-binding protein immunoglobulins to the affinity support. IGF-binding proteins have also been purified

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by non-affinity techniques from human amniotic fluid [10, 11], but for most investigators amniotic fluid is difficult to obtain, and yields can be quite low [11]. In the present study we report a simple three-step method which can purify 3–4 mg of IGF-binding protein from 1 l of medium conditioned by HEP G2 cells in culture, and which does not require high-affinity ligands in the purification scheme. This method also demonstrates that 100 m*M* ammonium acetate, pH 6.5, is valuable as an easily removed aqueous mobile phase for the reversed-phase high-performance liquid chromatography (HPLC) of proteins which are contaminated by acid proteases.

EXPERIMENTAL

Reagents and chemicals

Formic acid (88% solution), ammonium acetate and HPLC-grade acetonitrile and glacial acetic acid were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Trifluoroacetic acid (free acid stored under argon), ammonium bicarbonate and ammonium sulfate were obtained from Sigma (St. Louis, MO, U.S.A.).

Chromatography

Chromatography was performed with a Beckman HPLC system consisting of two Model 110B pumps, a 421 A system controller and an Altex 210A sample injector. Absorbance of column eluates was monitored at 280 nm with a Beckman Model 163 variable-wavelength UV detector. The Aquapore RP-300 C_8 analytical column (25 cm × 4.6 mm I.D., 7 μm particle size) was from Brownlee Labs. (Santa Clara, CA, U.S.A.). C_{18} Sep-Pak cartridges were from Waters Assoc. (Milford, MA, U.S.A.). In some experiments the aqueous phase consisted of 0.1% trifluoroacetic acid (TFA). In other experiments the aqueous phase was prepared by titrating 100 m*M* ammonium acetate to pH 6.5 with glacial acetic acid and then passing it through a bed of octadecylsilyl-silica (C_{18} Sep-Pak) to remove UV-absorbing contaminants. In all experiments the organic phase was HPLC-grade acetonitrile.

Cell culture

HEP G2 cells, originally isolated from a human hepatoma [12], were kindly provided by Dr. A.D.Cooper, Stanford University. Monolayers were maintained for continuous culture in 80-cm flasks in modified Eagle's medium supplemented with 10% fetal bovine serum, 20 m*M* L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. They were incubated at 37°C in carbon dioxide–air (5:95) and passaged 1:4 weekly at confluence after detachment with brief exposure to 0.25% trypsin–0.1% EDTA. For collection of conditioned medium, 5–20 million cells in 50–100 ml complete medium were transferred into 490-cm plastic roller bottles (Corning Glass Works, Corning, NY, U.S.A.) and rotated at 0.5 rpm at 37°C in room air. The cells formed discrete tumors on the sides of the roller bottles. After one month of weekly medium changes, the size of these tumors stabilized and no further enlargement was noted. At this point, the cells were washed with 100 ml saline and the medium then changed to 200 ml per bottle of

RPMI 1640 supplemented only with antibiotics and glutamine. After four days of exposure to the cells, this serum-free medium was collected, clarified by centrifugation at 2500 *g* and frozen at −20°C until use. The cells were then refed with 50–100 ml complete medium for three days. This cycle was repeated weekly. Cells in individual roller bottles remained viable for periods of up to ten months.

Preliminary purification

Proteins were precipitated by dissolving 390 g ammonium sulfate in each liter of HEP G2-conditioned medium (60% ammonium sulfate final concentration). After overnight incubation, this solution was centrifuged at 10 000 *g* for 30 min at 4°C and the supernatant was discarded. The precipitate from each liter of medium was solubilized in 25–30 ml of 50 m*M* ammonium bicarbonate, pH 7.8. Aliquots (40–50 ml) were introduced onto a 100×5 cm Pharmacia K 50/100 column packed with Sephacryl S-300. The column was eluted in the same ammonium bicarbonate buffer at 4°C at a flow-rate of 0.7 ml/min. Fractions were collected over 30 min and assayed for specific IGF-I binding activity. Peak binding fractions were pooled and then stored at −20°C.

HPLC purification

The initial experiments used 0.1% TFA as aqueous phase. The frozen aliquots of partially purified binding protein were lyophilized and 1–5 mg was then solubilized in 1–2 ml of 0.1% TFA. This was loaded onto the column at a flow-rate of 0.5 ml/min. After 10 min at 0% organic modifier, the acetonitrile concentration increased to 20% with a linear gradient over 5 min and then to 60% over the following 75 min.

Later experiments used 100 m*M* ammonium acetate, pH 6.5, as aqueous phase. In some experiments, ca. 2 mg of lyophilized and partially purified binding protein was solubilized in 10 ml of 100 m*M* ammonium acetate, pH 6.5 and loaded onto the column. The column was eluted with the same acetonitrile gradient as was used above with the 0.1% TFA aqueous phase. Yield was determined on binding protein which had been purified from 1850 ml of conditioned HEP G2 medium, requiring the pooling of binding protein peaks from three independent HPLC experiments. In other studies designed to maximize yield, the ammonium bicarbonate solution containing the partially purified binding protein was adjusted to pH 6.5 with HPLC-grade glacial acetic acid and then a 10–15 ml aliquot was loaded onto the column. The column was eluted with the same acetonitrile gradient as above. Yield was determined on an aliquot of HPLC-purified binding protein which derived from 200 ml of HEP G2 medium. After lyophilization and reconstitution of this binding protein aliquot in 50 m*M* ammonium bicarbonate, pH 7.8, absorbance was measured at 220 nm. A 268-μg amount of purified binding protein was weighed out on an electronic microbalance, solubilized in 50 m*M* ammonium bicarbonate and used as standard.

Microsequence analysis

HPLC performed with 100 m*M* ammonium acetate (pH 6.5) as aqueous phase yielded a binding protein peak which was subjected to microsequence analysis

before and after S-carboxymethylation (3 μg for each analysis, the first performed in duplicate). The gas phase microsequencer is similar to that described by Hawke et al. [13]. Phenyl/thiohydantoin-amino acid analysis was performed either on line or according to Hawke et al. [14].

Assays

In all experiments, samples were assayed for binding protein. Briefly, each sample was first incubated with [^{125}I]IGF-I for 18 h at 4°C and then combined with a solution of 1% activated charcoal for 15 min. Samples were then centrifuged. Radioligand not bound to binding protein was adsorbed onto the charcoal and counted in the pellet after centrifugation [15].

The partially purified binding protein preparation was evaluated for the presence of acid protease activity. A 2-mg amount of lyophilized, partially purified binding protein was reconstituted in 14 ml of 50 m*M* ammonium bicarbonate, pH 7.8, and divided into seven equal aliquots. A 50-μl sample of one aliquot was immediately lyophilized. The remainder of this aliquot was kept at pH 7.8 while the pH of the remaining six aliquots was lowered to either 7.1, 6.1, 4.8, 3.8, 2.7 or 1.8 by titration with 88% formic acid. All aliquots were then incubated at room temperature for 3 h after which a 50-μl sample was lyophilized. Samples were reconstituted in binding protein assay buffer and then tested for binding activity.

RESULTS

Preliminary studies demonstrated that virtually all of the binding protein activity present in HEP G2 medium was precipitated in 60% ammonium sulfate and that this precipitated activity was soluble in a much smaller volume of 50 m*M* ammonium bicarbonate, pH 7.8. The majority of UV-absorbing material in the ammonium bicarbonate aliquot was eluted from the column of Sephacryl S-300 at a molecular mass greater than that of bovine serum albumin. The peak binding protein activity eluted after albumin and before myoglobin at an estimated molecular mass of 30 000.

When partially purified binding protein was loaded onto the HPLC column in 0.1% TFA and then eluted with acetonitrile, the absorbance pattern at 280 nm demonstrated multiple broad and poorly resolved peaks. The pattern of peaks was not reproducible and the majority of binding protein activity was no longer detectable in the lyophilized fractions eluted from the column. To test whether an acid protease was responsible for the loss of binding activity, aliquots of ca. 140 μg of partially purified binding protein were titrated to various acid pH levels and then ca. 3.5 μg of each aliquot was evaluated for its ability to bind [^{125}I]IGF-I after a 3-h incubation. Both the non-incubated control sample and the sample incubated at pH 7.8 specifically bound 47% of added [^{125}I]IGF-I. However, binding activity was decreased at an incubation pH below 6.1 and above 1.8, with no specific binding of radioligand detectable at pH 2.7.

Since binding activity was more stable when the impure preparation was kept at neutral pH, HPLC of this preparation was performed using 100 m*M* ammonium acetate, pH 6.5 as aqueous phase. This approach resulted in one major UV-

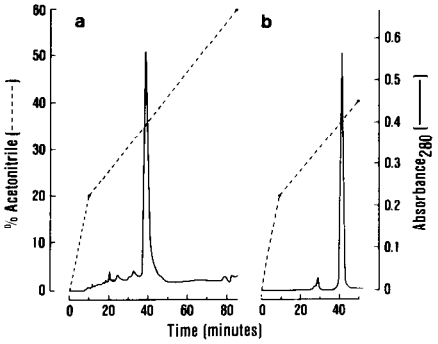

Fig. 1. C_8 reversed-phase HPLC of IGF-binding protein derived from the conditioned medium of HEP G2 cells in culture. (a) Chromatography of a partially purified preparation of IGF-binding protein; aqueous phase was 100 m*M* ammonium acetate, pH 6.5, and organic phase was acetonitrile; ca. 2 mg of purified IGF-binding protein was recovered. (b) Rechromatography of the purified IGF-binding protein demonstrated in Fig. 1a; aqueous phase was 0.1% TFA and organic phase was acetonitrile. See Experimental section for detailed description of the chromatographic conditions.

absorbing peak which eluted at ca. 35% acetonitrile and which coincided with IGF-I binding activity (Fig. 1a). This protein was found to be homogenous by amino terminal microsequence analysis, and the first 21 amino acids in this sequence were determined. As is demonstrated in Table I, the first ten amino acids in this sequence were homologous to that previously reported for IGF-binding protein isolated from both amniotic fluid and HEP G2 cells. When partially purified binding protein from 1.85 l of conditioned medium was lyophilized and then chromatographed by this method, 6 mg of pure binding protein were obtained. When the partially purified peparation from 200 ml of conditioned medium was not lyophilized but instead was loaded directly onto the column, 0.88 mg of binding protein were obtained.

In order to determine whether the binding protein purified by neutral pH HPLC in Fig. 1a was acid-stable, all fractions containing significant IGF binding activity were pooled and acidified to pH 2.5 with glacial acetic acid. This aliquot was loaded onto the column with 0.1% TFA serving as aqueous phase, and the column was eluted with the same acetonitrile gradient as before. As demonstrated in Fig. 1b, only one significant UV-absorbing peak was detected at 280 nm, which eluted at ca. 36% acetonitrile coincident with the binding protein activity. Serial dilutions of binding activity demonstrated > 75% recovery of activity after HPLC in 0.1% TFA.

DISCUSSION

IGF-I and IGF-II are mitogenic peptides which may play a major in vivo role in cellular growth by mediating the growth promoting effects of growth hormone [1]. In addition, abnormal mitogenic activity has recently been associated with the presence of the IGF-I peptide in the case of breast cancer [16] and with increased transcription of the gene encoding IGF-II in the case of Wilm's tumor

TABLE I

AMINO TERMINAL MICROSEQUENCE ANALYSIS OF HEP G2 AND AMNIOTIC FLUID BINDING PROTEINS

The sample (100 pmol) was sequenced before and after S-carboxymethylation, the first performed in duplicate. Cys was determined as the S-carboxymethyl derivative. Aliquots (20%) of the cycles were counted, confirming the presence of S-carboxymethylcysteine at these cycles (207 cpm, cycle 5; 275 cpm, cycle 8). For further details, see Experimental.

Cycle	Picomoles	HEP G2-binding protein, present report	HEP G2-binding protein, protein [8]	Amniotic fluid binding protein [10]
1	66	Ala	Ala	Ala
2	45	Pro	Pro	Pro
3	33	Trp	Trp	Trp
4	63	Gln	Gln	Gln
5	54	Cys-a	-	Cys
6	63	Ala	Ala	Ala
7	33	Pro	Pro	Pro
8	54	Cys-a	–	Cys
9	24	Ser	Ser	Ser
10	60	Ala	Ala	Ala
11	48	Glu		
12	60	Lys		
13	27	Leu		
14	54	Ala		
15	24	Leu		
16	24	Leu		
17	33	Pro		
18	36	Pro		
19	12	Val		
20	6	Ser		
21	24	Ala		
22	4	(Ser)		
23	8	(Leu)		

[17]. Clearly, the IGF peptides may play a key role in both normal and abnormal cell growth.

Both IGF peptides circulate in serum bound to specific binding proteins. It is estimated that less than 4% of free IGF-I circulates unbound [18]. At least two different binding proteins exist in humans. A binding protein purified from amniotic fluid has an amino terminal sequence identical to the IGF-binding protein produced by human HEP G2 hepatoma cells in culture (Table I) [8, 10]. In contrast, an acid-stable growth hormone-dependent binding protein has been purified by biospecific affinity chromatography and found to have a different amino terminal sequence from the HEP G2 form [9]. In addition, a third binding protein purified from the conditioned medium of rat BRL cells in culture has an amino terminal sequence different from either of the two human forms [6, 7]. The physiologic role of these binding proteins is not clear. Purified BRL-binding protein can inhibit the ability of purified IGF-II to stimulate glucose transport and DNA synthesis in chick embryo fibroblasts in vitro [19], but an IGF-binding protein complex is quite potent at stimulating sulfate incorporation in a hypophysectomized rat costal cartilage bioassay [20]. Another study has sug-

gested that binding protein may mediate the interaction between IGF-I and the IGF-I receptor on cultured human fibroblasts [21].

Most of the methods cited above used ligand-affinity techniques as the key step in the purification of the IGF-binding proteins. The binding proteins were bound to either IGF- or anti-IGF-binding protein antibodies covalently linked to Sepharose supports, and then eluted in a low pH buffer [6–9]. We initially purified HEP G2-binding protein in this way, using rat IGF-II as the affinity ligand. However, affinity column performance decreased steadily over five acid elutions and no binding protein was obtained after the sixth elution. Since IGFs and antibodies to binding proteins are relatively scarce, we sought high yield, non-affinity approaches to purify this protein.

The partially purified binding protein preparation which we had used to isolate binding protein by the biospecific affinity method was found to lose binding activity when it was either incubated at acid pH or loaded onto a reversed-phase C_8 HPLC column using 0.1% TFA as aqueous phase. Since other studies have demonstrated that the IGF-binding proteins are acid-stable, these results suggested that an acid protease was responsible for the loss of binding activity and might also account for the progressively poor yield provided by the affinity column after a number of acid elutions.

Recent experience has suggested that excellent separation and recovery of a number of polypeptide and protein hormones can be achieved on silica-based supports by using either 100 m*M* ammonium bicarbonate, pH 7.8, as the aqueous phase or by using an aqueous phase which changes from 100 m*M* ammonium acetate, pH 7.0, to 15 m*M* TFA, pH 2.0, as the chromatographic run progresses [22, 23]. We chose to use 100 m*M* ammonium acetate titrated down to pH 6.5 as the aqueous phase because our binding protein appeared to be stable at pH > 6.1 and because the risk of damage to silica-based supports increases above pH 7. Using this aqueous phase, the HEP G2-binding protein was eluted from the C_8 column as one sharp peak at ca. 35% acetonitrile and appeared to be the major protein present in the partially purified Sephacryl S-300 preparation that had been loaded onto this C_8 column. Amino terminal microsequence analysis demonstrated that the HPLC-purified binding protein was homogeneous and had an N-terminus identical with that reported for both the human HEP G2 and amniotic fluid-binding proteins. When this purified sample was rerun over the same column using 0.1% TFA as aqueous phase, HEP G2-binding protein was again eluted as one sharp peak of binding activity, demonstrating that the binding activity is truly acid stable. The acid protease was probably either inactivated by the first elution at pH 6.5 or separated from the peak of binding protein activity. In either case, 100 m*M* ammonium acetate, pH 6.5, proved useful as an aqueous phase since it protected the binding protein from acid proteolysis while offering ease of sample handling due to its volatility.

The final yield of HEP G2-binding protein ranged from 3.2 to 4.4 mg per l of conditioned medium. This is much greater than the yield of 200 μg HEP G2-binding protein per 900 ml conditioned medium obtained by biospecific affinity chromatography as reported by Povoa et al. [8]. In their experiments, conditioned medium was harvested from cells grown to confluent monolayers in 175-cm^2 flasks. With our method, medium was conditioned by HEP G2 cells which

were grown in large roller bottles and actually formed discrete tumors on the sides of the bottles. It is probable that these differences in plating and growth pattern allowed the HEP G2 cells in our experiments to produce larger quantities of binding protein per liter of conditioned medium. Confirmation of this hypothesis awaits development of a binding protein radioimmunoassay.

There are certain theoretical advantages to studying this binding protein over other purified IGF-binding proteins. First, it is produced by a human cell line. Second, this cell line is derived from the liver, which appears to be the major site for the production of IGF-binding proteins in vivo. Future studies can use the HEP G2 cell line as a model to examine the transcriptional and translational events involved in the processing of the IGF-binding protein, while our purification method will provide adequate quantities for further investigation of the structure and function of this important regulatory protein.

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