IDENTIFICATION OF A SOMATOMEDIN-BINDING PROTEIN PRODUCED BY A RAT HEPATOMA CELL LINE

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1. Introduction

Somatomedins, a class of 5000–10 000 molecular mass peptide hormones which function as growth factors in mediating the effects of pituitary growth hormone [1–3], circulate in human and rat serum complexed to specific high M_r binding proteins [4–7].

There appear to be at least two species of SmBPs*, a larger ($M_r \approx 150\,000$) which is growth hormone dependent and a smaller ($M_r \approx 60\,000$) which is not. Acid treatment converts the higher M_r species to the lower one and dissociates Sm from both [5–7].

Both Sm and SmBp are synthesized by the liver in vivo [8] and by primary hepatocyte cultures [9] and a line of transformed Buffalo rat liver cell, BRL-3A in vitro. The latter cells produce the Sm, multiplication-stimulating activity [10], and a single SmBP comparable to the smaller M_r species found in serum [6,11]. A clone of these cells, BRL-3A-2, produces the SmBP but, in contrast, no MSA [6].

The present study is the first to report the isolation and partial characterization of a SmBP ($M_r \approx 45\,000$) synthesized by a line of rat hepatoma cells, H-35 Rueber, which, like the BRL-3A-2 cells, produce no detectable Sm [15].

2. Materials and methods

2.1. *Cell culture*

Rat Rueber H-35 hepatoma cells were grown as monolayers in Richter's Imemzo medium containing insulin (4 μg/ml) (Associated Biomedic Systems, Inc., Buffalo) supplemented with $NaHCO_3$ (2.2 g/l), plus 10% tryptose phosphate broth (Difco) and 5% fetal calf serum (Gibco). At the time of cell confluence (5–6 days), the plating medium was replaced with 25 ml of serum-free medium which, after a period of 18 h, was discarded. Fresh SFM was added and harvested successively at 48-h intervals, and aliquots of this 'conditioned' medium frozen and lyophilized.

2.2. *Somatomedins*

Highly purified (approx. 200 μU/μg) Sm, insulin-like growth factor I (gift of Dr René Humble), was iodinated (spec. act. ≈ 50 μCi/μg) by the chloramine T method [12] as in [13]. Partially purified (approx. 1 μU/μg) MSA was obtained by gel chromatography (Sephadex G-50, 1 M acetic acid) of the conditioned medium from BRL-3A cells. Sm activity of 1 μU is defined in terms of its insulin-like action as being equivalent to 1 μU of porcine insulin standard in promoting glucose oxidation in isolated rat adipocytes.

2.3. *Chromatography*

A 1 g aliquot of the lyophilized powder (equivalent to approx. 80 ml) was reconstituted in 4.0 ml of 0.15 M ammonium acetate, pH 7.0, and centrifuged to remove particulate material. A 3.6 ml aliquot of the supernatant (approx. 8 mg protein) was subsequently incubated with 75 000 cpm ^{125}I-IFG-I (approx. 2.5 ng) for 18 h at 4°C. This sample was applied to a 2.5 × 134 cm Sephacryl S-200 (Pharmacia) column equilibrated in and eluted with 0.15 M ammonium acetate, pH 7.0, at 4°C.

2.4. *Competitive protein binding assay*

Estimation of SmBP activity was determined as in

* *Abbreviations:* Sm, somatomedin; SmBP, somatomedin-binding protein; ^{125}I-IGF-I, ^{125}I-labeled insulin-like growth factor I; SFM, serum-free medium; MSA, multiplication-stimulating activity; M_r, relative molecular mass

[13]. Briefly, the binding of ^{125}I-IGF-I by a sample of SmBP isolated from conditioned medium is quantitated by comparing it with that of known standards of partially purified human SmBP, 1 μg of which binds 30% of approx. 0.2 ng of ^{125}I-IGF-I.

2.5. *Analytical gel electrophoresis*

Electrophoresis was carried out for 2 h at 3 mA/tube in a 10% polyacrylamide gel system, pH 8.5, as in [14]. Subsequently, gels were either stained for protein with Coomassie blue or sliced in 1 mm sections using an electrophoresis gel slicer (Bio-Rad, Richmond, CA). Individual slices were minced, transferred to disposable glass culture tubes (12 X 75 mm) and suspended in 0.4 ml of 0.1 M sodium acetate buffer, pH 4.0. The tubes were then capped and gently shaken at room temperature overnight. Binding protein activity of the supernatants was determined by competitive protein binding assay.

3. Results

The results of Sephacryl S-200 filtration at neutral pH of a sample of conditioned medium incubated with ^{125}I-IGF-I are shown in fig.1A. The elution profile of radioactivity is confined to a single peak, fractions 90–115, with a K_{av} value of 0.33–0.34. In addition to containing approx. 95% of the radioactively labeled peptide added, this peak constituted about 0.04% of the total protein applied and eluted well before 'free' ^{125}I-IGF-I (K_{av} = 0.64–0.66) as determined in a separate run. Binding protein activity of individual fractions from a similar chromatographic separation (but without added ^{125}I-IGF-I) was determined by competitive protein binding assay (fig.1B). This band of BP activity includes, but is broader than, the ^{125}I-IGF-I complexed peak seen in fig.1A. The specificity of the BP activity in the K_{av} = 0.33–0.34 peak is demonstrated by the displacement of ^{125}I-IGF-I by increasing amounts of added unlabeled MSA (table 1).

An estimation of the apparent M_r of the K_{av} = 0.33–0.34 peak is presented in fig.2. From a plot of the K_{av} of various protein standards *vs* their known M_r, we calculate that the peak of SmBP activity has a M_r of 45 000 ± 4000 based on multiple determinations.

Polyacrylamide gel electrophoresis was utilized in an attempt to further analyze the S-200 peak of SmBP

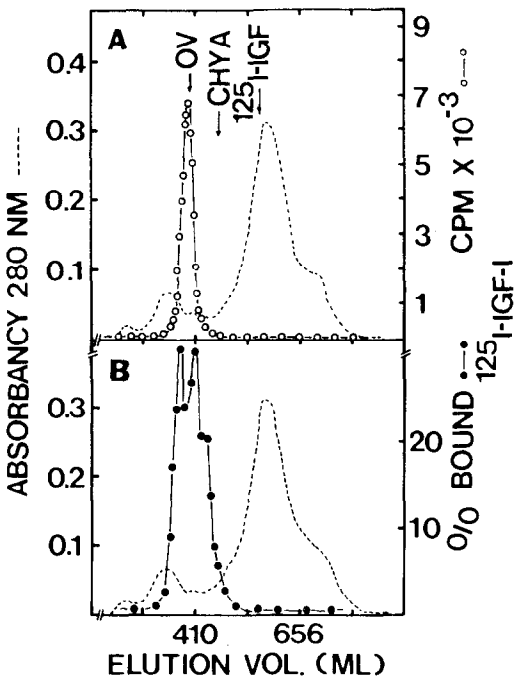

Fig.1. Chromatography of rat H-35 hepatoma conditioned medium on Sephacryl S-200 in 0.15 M ammonium acetate, pH 7.0. Samples were prepared and chromatographed as in section 2. Fraction size: 4.1 ml; flow rate: 35 ml/h. (A) Binding profile after prechromatography incubation with ^{125}I-IGF-I. Fractions were monitored individually for radioactivity. (B) Binding profile in the absence of added ^{125}I-IGF-I as determined by competitive protein binding assay. Column markers were ovalbumin (OV), α-chymotrypsinogen A (CHY A) and ^{125}I-labeled IGF-I (^{125}I-IGF-I).

activity (fractions 90–115). A single sharp peak of BP activity was detected in a region corresponding to a discrete protein band in a parallel stained gel (fig.3). The band of BP activity has an R_F value near to but slightly greater than rat serum albumin.

Table 1
Displacement of ^{125}I-IGF-I from H-35 hepatoma SmBP by added unlabeled MSA

MSA added[a] (μg)	^{125}I-IGF-I bound[b] (% of maximum)
0	100
20	40
50	23

[a] 1 μU/μg
[b] Determined by competitive protein binding assay

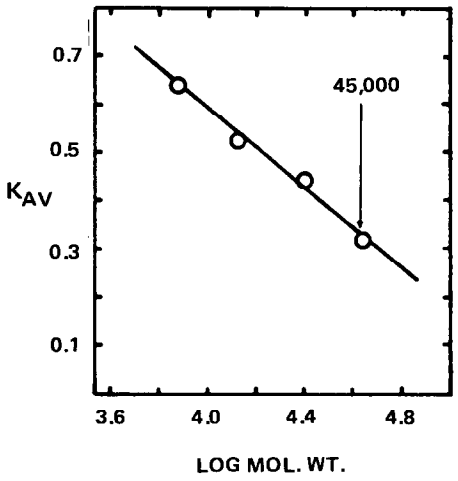

Fig.2. Estimation of the M_r-value of rat H-35 hepatoma binding protein by chromatography on Sephacryl S-200. Sample preparation and chromatography conditions are the same as given in fig.1A. Logarithm of the M_r-value of multiple protein standards *vs* their K_{av} ($K_{av} = (V_e - V_o)/(V_t - V_o)$; V_e, elution volume; V_o, void volume; V_t, total bed volume) is plotted and yielded a straight line which is used to estimate the M_r-value of the BP. In addition to bovine thyroglobulin (750 000) and tyrosine to determine the V_o and the V_t, respectively, the following peptide or protein standards from left to right were used: ^{125}I-IGF-I (7500), α-lactalbumin (14 400), α-chymotrypsinogen A (25 000) and ovalbumin (45 000). Arrow, elution position of the hepatome SmBP.

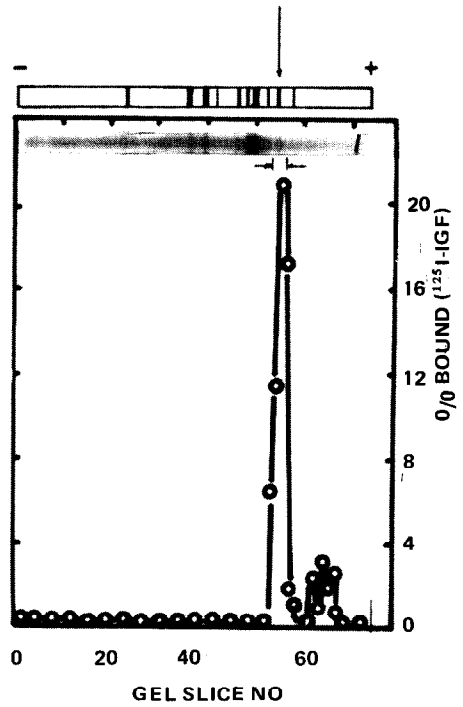

Fig.3. Distribution of H-35 hepatoma SmBP activity in a polyacrylamide disc gel electrophoresed at pH 8.5. Pooled and lyophilized Sephacryl S-200 fractions 90–115 (fig.1B) were reconstituted in 150 μl of pH 8.5 running buffer. Aliquots of 50 μl (100 μg protein) were electrophoresed for 2 h at 3 mA/tube. One of the gels was stained for protein (upper section); the other was sliced into 1 mm sections, extracted and then assayed for SmBP activity (lower section) as described in text. A drawing of the stained gel is at the top of the figure. Arrow, protein band associated with SmBP activity.

4. Discussion

A SmBP has been partially purified and characterized from SFM conditioned by cultured rat H-35 hepatoma cells. The H-35 hepatoma cell line reported here resembles the BRL-3A-2 subclone in that neither produce Sm, but both produce a SmBP eluting as a single peak of ^{125}I-Sm-complexed activity on gel filtration analysis [6,15]. However, these SmPBs appear to differ in their respective apparent M_r values, the H-35 hepatoma SmBP complex having an apparent M_r of approx. 45 000 on Sephacryl S-200 *vs* an apparent M_r of approx. 60 000 for both the BRL-3A-2 and BRL-3A SmBP complexes. The latter, consists of two distinct proteins with M_r values of 30 000 and 31 500 in sodium dodecyl sulfate-polyacrylamide gels [11]. Under non-denaturing conditions, both the BRL-3A-2 and BRL-3A SmBPs resemble in size the smaller SmBP ($M_r \approx 60\,000$) reported in growth hormone-deficient serum. In vivo growth hormone treatment shifts the size of the SmBP present in the latter to the larger form ($M_r \approx 150\,000$) found in normal serum [6,7]. While growth hormone stimulates Sm production in cultured primary hepatocytes [9], we have not yet tested its influence on either Sm or SmBP production by H-35 hepatoma cells.

Treatment of human and rat serum with acid shifts the SmBP from the larger to smaller M_r species, suggesting the larger form is acid labile [5–7]. Prechromatographic incubation of H-35 conditioned SFM with ^{125}I-IGF-I followed by Sephacryl S-200 chromatography under acid conditions (pH 4.0) similarly shifts the radioactive complex seen under pH 7.0 conditions to a smaller size ($M_r \approx 19\,000$) (data not shown). This suggests the $M_r \approx 45\,000$ species both is acid labile and therefore distinct from the low M_r

SmBP in serum, and subdividable into smaller components which retain binding activity.

Data from this and previous studies provide evidence that SmPBs, like the Sms themselves, constitute a family of forms which at this time are not fully understood with respect to their structure and function. We believe that the H-35 hepatoma cell line offers a unique model for the study of a previously undescribed, acid-labile, low M_r SmBP. The synthesis of this protein in the absence of Sm provides a significant advantage for its isolation without prior cleavage of its ligand.

Acknowledgements

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