Purification of a pituitary polypeptide that stimulates the replication of adipocyte precursors in culture

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A bovine anterior pituitary polypeptide that stimulates the replication of rat and human adipocyte precursors has been purified. Its M_r is 44 000–53 000 and its isoelectric point is 9.8–10.3. While pituitary basic fibroblast growth factor is equally mitogenic on adipocyte precursors and skin fibroblasts, the polypeptide described here is selectively more active on the precursors. We postulate that this adipocyte growth factor plays a physiological role by modulating the number of adipocyte precursors.

Pituitary polypeptide *Growth factor* *Adipocyte precursor*

1. INTRODUCTION

The recent elaboration of a propagating cell culture system of rat and human adipocyte precursors has facilitated our studies on factors regulating the replication of developing adipocytes [1–4]. The purification and partial characterization of an anterior pituitary polypeptide that stimulates the replication of adipocyte precursors is the subject of this report.

2. MATERIALS AND METHODS

2.1. *Preparation of pituitary factors*

The initial steps of purification were similar to those reported for brain and pituitary fibroblast growth factors (FGF) [5–8]. Bovine anterior pituitaries (800) (Pel-Freez Biologicals, AR) were homogenized at 0–4°C in 1 *l* 0.15 M ammonium sulfate containing 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_2EDTA, and 2.5 mg each of leupeptin and pepstatin A (Sigma, St Louis MO). The homogenate was adjusted to pH 4.5, stirred for 2 h, and centrifuged at 11 000 × *g* for 1 h. The supernatant, after adjustment to pH 6.8, was subjected to ammonium sulfate fractionation (250 g/*l* followed by the addition of 290 g/*l* to the resultant supernatant). The precipitate was redissolved in distilled water containing PMSF and Na_2EDTA as above, and dialyzed against the same solution overnight in Spectrapor 1 tubing (Spectrum Medical Industries, CA) with a M_r 6000–8000 cutoff.

2.2. *Carboxymethyl-Sephadex C-50 and Sephacryl S-200 chromatography*

The crude extract was adjusted to pH 6.0 and 2 mg protein/ml packed resin were loaded on a carboxymethyl (CM)-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala) column and washed with 0.1 M sodium phosphate (pH 6.0) containing 0.25 mM PMSF and 1 mM Na_2EDTA. This was followed by a series of batch elutions with the same column buffer containing 0.1 M NaCl, 0.15 M NaCl and 0.6 M NaCl, respectively. (The exact concentration was obtained reproducibly by determining the conductivity of each solution.) The active fraction eluted with 0.6 M NaCl was concentrated by ultrafiltration using Amicon UM-2 filters (Amicon Corp., MA) with an M_r 1000 cutoff, before applying to a 2 × 90 cm Sephacryl S-200

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(Pharmacia) column developed in 0.2 M ammonium bicarbonate (pH 7.9), with PMSF and Na_2EDTA as described.

The active fractions were pooled, concentrated by ultrafiltration, and futher purified by a second CM-Sephadex C-50 step. The column was first washed with 0.1 M NaCl in 0.1 M phosphate buffer (pH 6.0) then with 0.15 M NaCl in the same buffer, and finally eluted with a linear 0.15–0.6 M NaCl gradient developed in column buffer.

2.3. *Chromatofocusing*

Each active fraction was purified further by chromatofocusing (Pharmacia). Both acid and alkaline pH gradient elutions were used. In the case of pH 4.0–7.0 gradient, the 'polybuffer' exchanger resin (PBE 94) was equilibrated with the start buffer, 25 mM imidazole–HCl (pH 7.4). The self-forming pH gradient was initiated using 'Polybuffer 74' elution buffer (pH 4.0). Different 'polybuffer' exchanger (PBE 118), start buffer (25 mM triethylamine–HCl, pH 11.0) and elution 'polybuffer' (Pharmalyte 8.0–10.5) were used for the alkaline pH gradient elution. The pH of each eluted fraction was measured and adjusted to 7.4 with either 2 M NH_4OH or 2 M HCl prior to assay for mitogenic activity.

2.4. *Sephadex G-75 filtration*

Following chromatofocusing, the elution 'polybuffer' was removed from the fractions to be assayed, with a 0.9 × 110 cm column of Sephadex G-75 Superfine (Pharmacia) equilibrated and eluted with 5 mM ammonium acetate. Fractions of 1 ml were collected and assayed.

2.5. *SDS–Polyacrylamide gel electrophoresis (SDS–PAGE)*

Disc gel electrophoresis of each active fraction was done with 15% polyacrylamide as in [9]. Following Sephadex G-75 chromatography, aliquots of each active fraction were run in parallel so that different gels were stained with Coomassie blue R or eluted for assay of any mitogenic activity. Elution was carried out overnight at 0–4°C in 1 ml phosphate-buffered saline (pH 7.4) containing 2 mg/ml of bovine serum albumin (BSA). Aliquots were then tested for their mitogenic properties using our adipocyte precursor culture system.

Protein concentrations were determined as in [10], or estimated by absorbance at 220 nm and 280 nm, based on BSA standards.

2.6. *Assay of biological activity*

The mitogenicity of the various fractions was based on stimulation of replication of rat and human adipocyte precursors in culture. The propagating cell culture system of adipocyte precursors has been detailed [1–4,11]. Cells in secondary culture were grown in alpha medium supplemented with 20% fetal calf serum (FCS) for 18 h. The concentration of FCS was reduced to 0.5% for an additional 32 h to allow the cells to enter a stage of quiescence. The cells were further incubated in the presence or absence of the putative mitogenic substance for 32–48 h. Cell replication was quantified by direct cell counting and by incorporation of [^{3}H]thymidine into DNA, as in [2,11]. Briefly, 10 μCi [*methyl*-^{3}H]thymidine (spec. act. ~80 Ci/mmol) were added to 5 ml growth medium in 25-cm^2 tissue culture flasks. The radioactivity retained on glass microfibre filters in a manifold Millipore filtering unit, was then determined [11].

3. RESULTS AND DISCUSSION

Following CM-Sephadex fractionation, Sephacryl S-200 gel filtration revealed 3 major peaks of mitogenic activity. The one containing the compound(s) of highest app. M_r revealed one major active fraction upon repeated CM-Sephadex chromatography (fig. 1). Further purification was achieved by chromatofocusing, which indicated that this compound had an isoelectric point (pI) of 9.8–10.3. (The 'polybuffer' did not affect the mitogenic effect.) Gel filtration of the active fraction on Sephadex G-75, revealed a single activity peak. The fractions constituting this peak gave rise to one major protein band upon SDS–PAGE (fig. 2). By comparison with several monomeric standard proteins, its M_r was 44 000–53 000. Elution of unstained gels indicated correspondence of mitogenic activity with the protein band. (About 30% of the mitogenic activity was retained after SDS–PAGE, reflecting remarkable stability.)

Four additional pituitary polypeptides that enhanced the replication of cultured adipocyte precursors, were purified using similar methods. (In some cases, SDS–PAGE and elution had to be repeated.) These polypeptides ranged from M_r

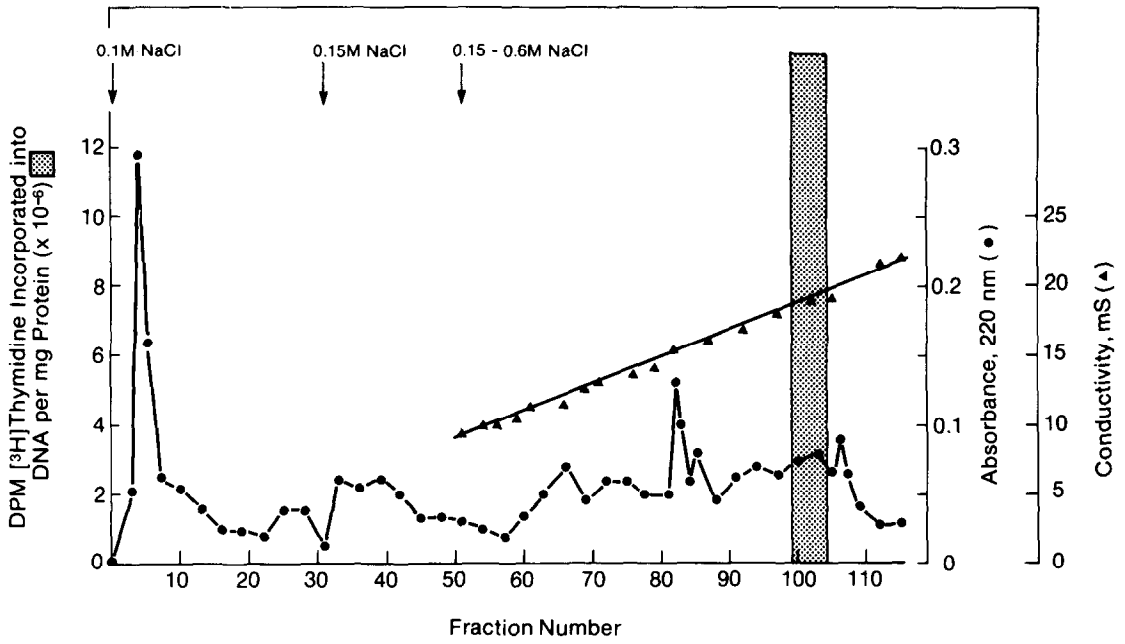

Fig. 1. Carboxymethyl-Sephadex C-50 chromatography of anterior pituitary factors that stimulate the replication of cultured adipocyte precursors. The pooled active fractions of highest M_r on Sephacryl S-200, was loaded (10 mg at 2 mg/ml packed resin) on a 1.6 × 10 cm column and washed with 0.1 M NaCl in 0.1 M sodium phosphate (pH 6.0) and then with 0.15 M NaCl. A linear 0.1–0.6 M NaCl gradient (conductivity (ms) ▲) was developed in column buffer at 6.6 ml/h, and 2.2 ml fractions were collected. The protein profile was monitored by absorbance at 220 nm (●). The region of the active fraction is indicated by the bar. Mitogenic activity of the active fraction was assayed by incorporation of [^{3}H]thymidine into DNA and by direct cell counting.

~8200–~38 000, and their isoelectric point from 4.7–7.4. Details of their purification and characterization will be described separately. In each case, activity as determined by quantifying radioactive thymidine incorporation into DNA, was confirmed by direct cell counting of adipocyte precursors. These polypeptides, moreover, stimulate the replication of both rat and human precursors in culture at a concentration of 10 ng/ml growth medium (lowest level tested). The polypeptide (M_r 44 000–53 000) described in this report may be a relatively active precursor of the smaller stimulatory factors.

From the crude extract to the early purification steps including the Sephacryl S-200 eluate, the ratio of adipocyte-precursor–growth-stimulatory activity to fibroblast–growth-stimulatory activity was ~1. Beginning with the active fraction of the second CM-Sephadex gradient eluate, the ratio increased to 1.6, and remained at about this value up to completion of purification. In contrast, pituitary basic fibroblast growth factor promotes the replication of both adipocyte precursors and skin fibroblasts to a similar extent [4]. The relative selectivity found for the polypeptides described in this communication is notable in view of the similar properties and the probable existence of a common progenitor of adipocyte precursors and fibroblasts [1,2]. On a molar basis, the novel pituitary polypeptide reported here is about twice as active as pituitary basic FGF in terms of mitogenic stimulation of cultured adipocyte precursors (at 3×10^{-10} M).

Other disparities exist between FGF and the pituitary polypeptides described here. The M_r-values or isoelectric points of the latter are distinct from the values for pituitary fibroblast growth factors [5,12]. The polypeptides stimulating adipocyte precursor replication, moreover, have different M_r or pI from those of a series of basic bovine pituitary polypeptides reported to have a relatively low mitogenic effect on cultured fibroblasts [13]. It

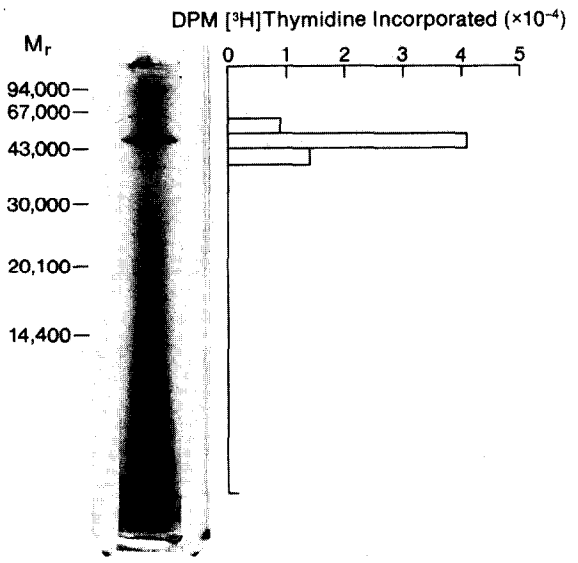

Fig. 2. SDS–polyacrylamide disc gel electrophoresis of an anterior pituitary factor that stimulates the replication of cultured adipocyte precursors. Following chromatofocusing and Sephadex G-75 filtration, the active fraction (~5 μg) was run in 15% polyacrylamide gels, which were either stained with Coomassie blue R or eluted with phosphate-buffered saline (pH 7.4) containing 2 mg BSA/ml. The mitogenic activity corresponds to the major protein band which has M_r 44 000–53 000. The M_r volumes of standard monomeric proteins are indicated on the left.

is not yet known whether the pituitary polypeptides described here are related to basic and acidic fibroblast growth factors isolated from bovine and human brain [6–8]. It is conceivable that the polypeptide (M_r 44 000–53 000) reported here may be a precursor of one or more fibroblast growth factors.

Our findings indicate that the anterior pituitary contains, in addition to FGF, a series of distinct polypeptides that stimulate the replication of cultured adipocyte precursors. In contrast to the polypeptides reported here and pituitary FGF, classical pituitary hormones such as somatotropin, prolactin, and corticotropin, do not enhance adipocyte precursor replication [4].

In view of the more selective mitogenic activity of the novel polypeptides on adipocyte precursor replication, we propose that they may function in vivo as adipocyte growth factors by increasing the number of adipocyte precursors.

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REFERENCES

[1] Van, R.L.R., Bayliss, C.E. and Roncari, D.A.K. (1976) J. Clin. Invest. 58, 699–704.
[2] Van, R.L.R. and Roncari, D.A.K. (1978) Cell Tiss. Res. 195, 317–329.
[3] Roncari, D.A.K. and Van, R.L.R. (1978) J. Clin. Invest. 62, 503–508.
[4] Roncari, D.A.K. (1981) Int. J. Obesity 5, 547–552.
[5] Gospodarowicz, D. (1975) J. Biol. Chem. 250, 2515–2520.
[6] Gospodarowicz, D., Bialecki, H. and Greenburg, G. (1978) J. Biol. Chem. 253, 3736–3743.
[7] Thomas, K.A., Riley, M.C., Lemmon, S.K., Baglan, N.C. and Bradshaw, R.A. (1980) J. Biol. Chem. 255, 5517–5520.
[8] Kellett, J.G., Tanaka, T., Rowe, J.M., Shiu, R.P.C. and Friesen, H.G. (1981) J. Biol. Chem. 256, 54–58.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Roncari, D.A.K., Lau, D.C.W. and Kindler, S. (1981) Metabolism 30, 425–427.
[12] Gambarini, A.G. and Armelin, H.A. (1982) J. Biol. Chem. 257, 9692–9697.
[13] Kelly, L. and Puett, D. (1980) Biochim. Biophys. Acta 626, 397–411.