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Purification and characterization of acidic fibroblast growth factor from bovine brain

(mitogen/brain protein/reversed-phase HPLC)

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ABSTRACT **Acidic brain fibroblast growth factor has been purified a minimum of 35,000-fold to apparent homogeneity by a combination of differential salt precipitation, ion exchange chromatography, gel filtration, isoelectric focusing, and hydrophobic chromatography on a C_4 reversed-phase HPLC column. Two microheterogeneous forms of the molecule are obtained with apparent molecular masses of 16,600 and 16,800 daltons. The mitogen is highly active with half-maximal stimulation of BALB/c 3T3 fibroblasts at about 40 pg/ml in an assay using incorporation of [*methyl*-^{3}H]thymidine into DNA.**

Protein growth factors are important signals for both the maintenance of viability and the induction of growth of their target cells (1). Although serum is a common source of mitogenic activities used to maintain a wide variety of types of cells in culture, growth factors are also found in media conditioned by transformed cells and both embryonic and adult animal tissue extracts.

Mitogenic activity for fibroblasts was recognized in brain extracts more than 40 years ago (2, 3). The first claim of purification to homogeneity of a brain-derived growth factor was made by Gospodarowicz *et al.* (4, 5), who described a variety of activities and target cells of the mitogen (6). After an approximately 1,000-fold purification from a crude bovine brain homogenate the active protein, fibroblast growth factor (FGF), was reported to be a family of three proteolytic fragments of myelin basic protein (7), a constituent of the myelin sheath surrounding many brain and peripheral neurons.

The identification of the mitogens as degradation products of myelin basic protein subsequently was disputed (8, 9). This controversy was based, in part, on inability to demonstrate mitogenic activity of polypeptides, similar to those claimed to be active, that were generated from pure myelin basic protein by limited proteolysis *in vitro* with either pepsin or lysosomal cathepsin D. Furthermore, although both these inactive myelin basic protein fragments generated *in vitro* and almost all of the protein from the FGF purification bound to an anti-myelin basic protein immunoaffinity column, the mitogenic activity was unretarded. Finally, both acidic (8) and basic (9) activities were separated by isoelectric focusing of the putatively pure FGF, neither of which was bound by the antibody column. Therefore, the fragments of myelin basic protein were confirmed to be the major protein species in these preparations but not the active mitogens. The generation of inactive fragments of myelin basic protein may occur early in purification during incubation of the brain homogenate at pH 4.5 for 1–2 hr. The acidic pH would favor degradation of the protein by lysosomal proteases released by homogenization.

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Although both acidic and basic FGFs stimulate cell division of BALB/c 3T3 fibroblasts, the similarity in their ranges and target cells remains to be determined. The structural relatedness, if any, of the two mitogens must be characterized by using the purified proteins. We report the purification to homogeneity and initial characterization of the acidic FGF from bovine brain.

METHODS AND MATERIALS

Extraction and Salt Precipitation. Bovine brains were obtained from a local slaughterhouse and transported on ice. Visible blood clots and the outer membranes of the meninges with their constituent blood vessels were removed. The brains were sliced into cubes approximately 2 cm on an edge, quick frozen in liquid N_2, and stored at −70°C. Distilled water was used to make all solutions and pH values were adjusted against standards at the temperature of use. All steps were carried out at 4°C unless otherwise noted.

A 4-kg mass of tissue (about 12 whole adult bovine brains) was thawed in 4 liters of 0.15 M $(NH_4)_2SO_4$ and homogenized in a Waring blender. The homogenate was adjusted to pH 4.5 using 6 M HCl while vigorously mixing with a 6-in-diameter (1 in = 2.54 cm) propeller stirrer and, after 1 hr, it was centrifuged at 13,800 × *g* for 40 min, the supernatant was adjusted to pH 6.75 with 1 M NaOH, and 200 g of $(NH_4)_2SO_4$ per liter (1.52 M) was slowly added while stirring. After centrifugation of the mixture at 13,800 × *g* for 30 min, 250 g of $(NH_4)_2SO_4$ was added per liter of supernatant (3.41 M). The mixture was recentrifuged, and the resulting pellet was dissolved in 200 ml of water, dialyzed for 18 hr in M_r 6,000–8,000 cutoff bags (Spectrum Medical Industries, Los Angeles) against two 14-liter volumes of water, and lyophilized.

CM-Sephadex Chromatography. Lyophilized protein from the salt precipitate of 16 kg of brain was dissolved in 900 ml of 0.05 M sodium phosphate (pH 6.0), and the mixture was readjusted to pH 6.0 with 1 M NaOH and clarified by centrifugation at 23,300 × *g* for 30 min. The supernatant was stirred for 15 min with 800 ml of hydrated CM-Sephadex C-50 (Pharmacia) equilibrated with 0.1 M phosphate buffer, the unadsorbed protein was sucked out on a coarse sintered glass filter, and the resin was washed with 3 liters of 0.1 M buffer and packed into a column. The column was sequentially eluted with 0.1 M buffer containing 0.15 M and 0.60 M NaCl to remove the protein. The approximately 500-ml pool of the protein peak eluted by 0.6 M NaCl was dialyzed in M_r 6,000–8,000 bags for 18 hr against two 14-liter volumes of water and lyophilized.

Sephadex G-75 Chromatography. One-quarter of the lyophilized protein from the C-50 column was dissolved in 20 ml of 0.1 M ammonium bicarbonate (pH 8.5), and the solution was clarified by a 15-min centrifugation at 27,000 × *g*

Abbreviation: FGF, fibroblast growth factor.

and fractionated on a Sephadex G-75 (Pharmacia) column. Pooled fractions were directly lyophilized.

CM-Cellulose Chromatography. Protein from the Sephadex G-75 column was dissolved in 10 ml of 0.1 M ammonium formate (pH 6.0), the pH was readjusted to 6.0 with 0.1 M formic acid, the solution was clarified by centrifugation at 27,000 × *g* for 15 min, and the supernatant was loaded on a CM-cellulose (CM52) (Whatman) column. The protein was removed by elution with 0.2 M followed by 0.6 M ammonium formate (pH 6.0). The active pool was lyophilized directly.

Isoelectric Focusing. Protein samples were isoelectric focused in Ultrodex (LKB) using a modified LKB Multiphor flatbed focusing plate with miniaturized focusing lanes. Focusing was typically carried out on a plate containing three lanes of 0.5 × 10 cm, each with 75 mg of Ultrodex in 1.9 ml of water containing 126 μl of pH 3–10 Pharmalyte (Pharmacia) and 47 μl of 9–11 Ampholine (LKB). The liquid was evaporated 32% by weight. Either the FGF sample or 1 mg each of cytochrome *c* and hemoglobin (Sigma) was loaded in 100 μl of the diluted ampholyte solution. The pH gradient reached equilibrium as monitored by the stability of the current, the positions of the cytochrome *c* and hemoglobin standards, and the final pH profile. The gel was divided into ten 1-cm slices and each segment was eluted by three 5-min centrifugations with 333 μl of 0.6 M NaCl at 200 × *g* in a MF-1 microfiltration tube containing a 1-μm pore size regenerated cellulose RC-60 filter (Bioanalytic Systems, West Lafayette, IN). The pH of each 1-ml eluate was measured against 0–5°C standards.

Reversed-Phase HPLC. Final purification was achieved on a Vydac C_4 silica-based HPLC column (The Separations Group, Hesperia, CA) equilibrated with a 10-mM trifluoroacetic acid solution that had been passed through a C_{18} preparative reversed-phase HPLC column to remove UV-absorbing contaminants. The mitogenically active acidic fractions that eluted from the focusing resin were injected directly on the column.

Mitogenic Assay. Incorporation of [^{3}H]thymidine by BALB/c 3T3 A31 fibroblasts (American Type Culture Collection) was used to monitor mitogenic activity (8). Each dose–response point was the average of triplicate determinations. The amount of mitogenic activity in each chromatographic pool was determined from dose–response curves. One unit of mitogenic activity is defined as the amount of protein that elicits half-maximal increase in activity from which the total number of activity units per pool was calculated.

Polyacrylamide Gel Electrophoresis. The procedure of O'Farrell (10) was used to prepare $NaDodSO_4$ gels. Samples were heated (100°C, 2 min) with or without 5% (vol/vol) 2-mercaptoethanol. Gels were glutaraldehyde fixed and silver stained (11, 12).

Amino Acid Analysis. Protein samples eluted from the HPLC column were evaporated to dryness and hydrolyzed in 6 M HCl (Ultrex, Baker) containing 2% phenol for 24, 48, and 72 hr. Cysteine content was determined as cysteic acid after performic acid oxidation (13). Tryptophan was measured after hydrolysis in 4 M methanesulfonic acid (Pierce) for 24 hr (14). All analyses were carried out with a Beckman 121 MB amino acid analyzer.

RESULTS

Protein Purification. The differential salt precipitation, CM-Sephadex C-50, Sephadex G-75, and CM-cellulose (CM52) steps were based on the protocol developed by Gospodarowicz and colleagues so that the FGF activity that they described would be generated. Using their procedure, we found both acidic (8) and basic (9) mitogenic activities in the final product by isoelectric focusing. Subsequent minor modifications of decreasing by one-third the initial volume of homogenization, decreasing the incubation time from 2 to 1 hr at pH 4.5, and batch adsorption of the sample to the CM-

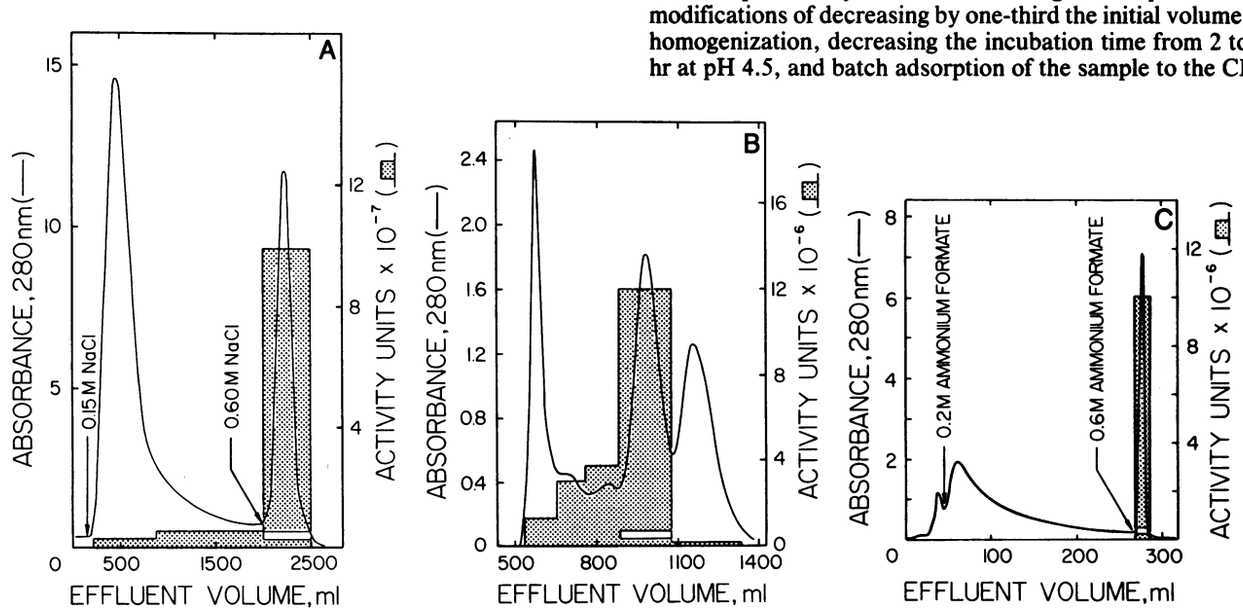

FIG. 1. (*A*) CM-Sephadex C-50 chromatography. Lyophilized protein (72 g) from the dialyzed product of 3.41 M $(NH_4)_2SO_4$-precipitated crude extract from 16 kg of bovine brains was batch adsorbed to 40 g of Sephadex C-50 equilibrated with 0.1 M sodium phosphate (pH 6.0). The resin was rinsed with the same buffer, packed into an 8.3-cm-diameter column, washed with 0.1 M sodium phosphate, pH 6.0/0.15 M NaCl, and eluted with buffer containing 0.6 M NaCl. The flow rate was 30 ml/min and 22.5-ml fractions were collected. The material pooled for subsequent purification is indicated by the open horizontal bar. (*B*) Sephadex G-75 chromatography. One-quarter of the lyophilized protein (590 mg) in the dialyzed 0.6 M NaCl-eluted pool from the CM-Sephadex C-50 column was loaded on a 5 × 90 cm Sephadex G-75 (particle size, 40–120 μm) column equilibrated with the same buffer. The column was eluted at a flow rate of 74 ml/hr and 17.5-ml fractions were collected. The material pooled for subsequent purification is indicated by the open horizontal bar. (*C*) CM-cellulose chromatography. The lyophilized protein (210 mg) from the highest activity pool (885 to 1,075-ml elution volume) of the G-75 column was loaded on a 1.5 × 6.5 cm CM52 column equilibrated with 0.2 M ammonium formate (pH 6.0). The resin was washed with 0.2 M, and eluted with 0.6 M, ammonium formate (pH 6.0). The column was eluted at a flow rate of 60 ml/hr and 4.25-ml fractions were collected. The material pooled for subsequent purification is indicated by the open horizontal bar.

Table 1. Purification of acidic brain FGF

Purification step	Protein recovery, mg	Activity recovery Units	Activity recovery %	Specific activity, units/mg	Purification factor
Brain homogenization	1.1×10^5*	7.9×10^7	100	7.2×10^2	1.0
Salt fractionation	1.8×10^4*	5.2×10^7	66	2.9×10^3	4.0
Chromatography					
Sephadex C-50	5.9×10^2†	2.5×10^7	32	4.2×10^4	58.0
Sephadex G-75	2.1×10^2†	1.2×10^7	15	5.7×10^4	79.0
CM52	54‡	1.0×10^7	13	1.85×10^5	2.6×10^2
Isoelectric focusing	NQ§	3.6×10^6	4.6	—	—
C_4 HPLC	0.144¶	3.2×10^6	4.1	2.5×10^7	3.5×10^4

Values are based on 4 kg of bovine brain. Except for the value for the homogenate, purification factors are minimum values estimated by assuming that all of the initial mitogenic activity is acidic brain FGF.
*Protein was estimated by the A_{260}/A_{280} ratio method (15).
†Protein was estimated by using $A_{280}^{1\%} = 10$.
‡Protein was estimated by using $A_{280}^{1\%} = 7.9$ (9).
§Not quantitated.
¶Protein was quantitated by amino acid analysis.

Sephadex C-50 column prior to pouring the column were made. These changes decreased the time required to prepare the acidic mitogen without a sacrifice in final yield.

Lyophilized protein from the salt precipitation of the homogenized brains was chromatographed on CM-Sephadex C-50. As shown in Fig. 1*A*, the bulk of the bound mitogenic activity was eluted by 0.6 M NaCl. A 14-fold purification was achieved by this step (Table 1). The mitogen was fractionated next on Sephadex G-75. The profile of activity (Fig. 1*B*) was similar to that described previously (4). The highest activity pool was applied to a CM52 carboxymethyl-cellulose column. After first washing with 0.2 M ammonium formate, we originally eluted the activity with a 0.2–0.6 M gradient of the same buffer. However, because both (*i*) peaks of protein eluted by the gradient were poorly resolved (5) and (*ii*) we observed the mitogenic activity to span multiple peaks, the active protein was subsequently eluted with a single step of 0.6 M ammonium formate (Fig. 1*C*).

After the above purification, both acidic and basic mitogenic activities were observed by isoelectric focusing. The flatbed focusing apparatus was miniaturized to increase recovery of small amounts of protein. In a typical activity profile (Fig. 2), 71% of the applied activity of a sample of protein eluted from the CM52 column was recovered, with slightly more than one-half in the acidic pool. Because silver-stained one-dimensional isoelectric focusing and two-dimensional isoelectric focusing–NaDodSO$_4$ polyacrylamide gels (data not shown) of the same CM52-derived sample showed that almost all of the protein focused in the basic range, no attempt was made to read the A_{280} profile of the focused protein to avoid losses of the acidic mitogen due to surface adsorption to the glass spectrophotometer cuvettes. Control experiments showed that 0.6 M NaCl, used to elute the protein from the Ultrodex resin, increased the measured pH by about 0.1 unit (16) over that previously observed in the absence of NaCl (8). The corrected pH-values for the two active pools centered at 5.8 ± 0.5. The slight increase in pH of the acidic mitogen over that previously reported, pH 5.3 ± 0.5 (8), may be correlated with the 50% decrease in incuba-

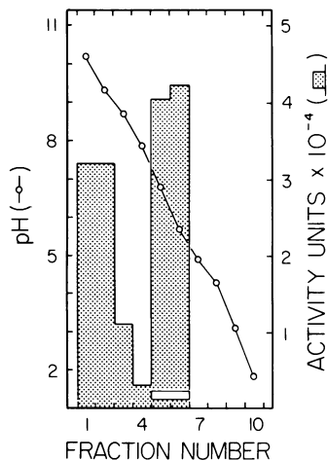

FIG. 2. Isoelectric focusing in Ultrodex. A 1.25-mg lyophilized sample from the 0.6 M ammonium formate-eluted pool of the CM52 column was dissolved in the diluted pH 3–11 ampholyte mixture used to hydrate the Ultrodex resin and loaded dropwise over the length of a 0.5 × 10 cm flatbed miniaturized focusing lane. Cellulose paper electrode wicks were hydrated with 1 M NaOH (cathode) or 1 M H_3PO_4 (anode). The sample was focused at a maximum of 0.12 W per focusing lane for 2,800 V·hr over a 24-hr period at 7°C in a N_2 atmosphere. After focusing, the resin was sliced into 1-cm segments and these were eluted with 0.6 M NaCl. The acidic fractions typically pooled for subsequent purification are indicated by the open horizontal bar.

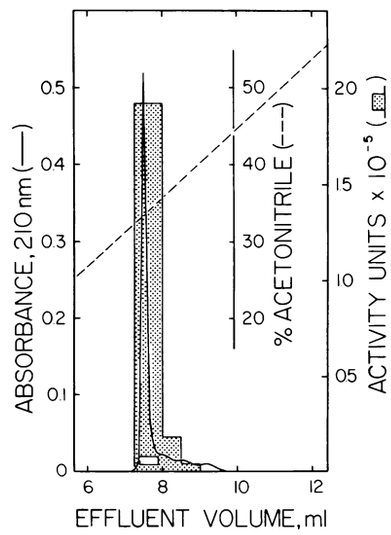

FIG. 3. Reversed-phase C_4 HPLC. The acidic pool (pH 4.9–6.6) from the isoelectric focusing of 4 mg of partially purified FGF from the CM52 column was loaded on a 4.6 mm × 5 cm C_4 Vydac HPLC column (particle size, 5 μm; pore size, 330 Å) equilibrated with 10 mM trifluoroacetic acid and eluted with a 0–67% linear gradient of acetonitrile over 30 min at 20–22°C. Elution solvents were vacuum deaerated immediately before use and maintained under argon during chromatography. The flow rate was 0.5 ml/min and 0.25-ml samples were collected in polypropylene tubes. The material pooled for subsequent characterization is indicated by the open horizontal bar.

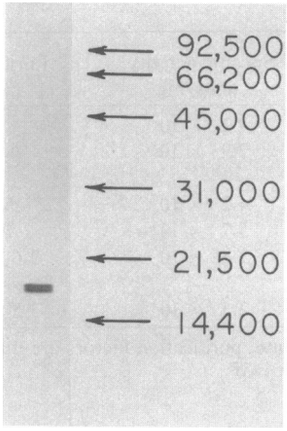

FIG. 4. Polyacrylamide/NaDodSO$_4$ gel of reduced acidic brain FGF. About 100 ng of HPLC-purified acidic FGF was heat denatured and reduced with NaDodSO$_4$/2-mercaptoethanol, electrophoresed at 10 mA through a 0.75-mm-thick 15% polyacrylamide gel with a 4.5% stacking gel, and silver stained. The protein molecular weight standards were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). The lower limit of contaminant detection is about 1 ng.

tion time of the acidified brain homogenate.

The inability to quantitatively remove the carrier ampholytes by the conventional means of dialysis, gel filtration, or electroelution resulted in difficulty in characterizing the focused acidic mitogen. The polar ampholytes did not bind to a C_4 reversed-phase HPLC column as monitored by absorbance at 210 nm. Therefore, the acidic pool, typically from about pH 5 to pH 7, was chromatographed on the C_4 column (Fig. 3). The activity coelutes with a single large A_{210} peak followed by a few smaller peaks. Minor contaminants, occasionally observed at the extreme basic end of the mitogenically active acidic pool, are also removed by this step. The recovery of active mitogen from the column is almost 90%. The combined steps of isoelectric focusing and reversed-phase HPLC result in an estimated 135-fold increase in specific activity of acidic FGF. Final recovery of mitogenic activity is 4.1% with an estimated 35,000-fold purification (Table 1).

Table 2. Amino acid composition of acidic brain FGF

Amino acid	Observed	Integer
Aspartic acid	14.1	14
Threonine*	8.6	9
Serine*	9.8	10
Glutamic acid	16.4	16
Proline	6.8	7
Glycine	14.2	14
Alanine	4.6	5
Half-cystine†	4.1	4
Valine	4.7	5
Methionine	0.9	1
Isoleucine	5.6	6
Leucine	18.8	19
Tyrosine	7.4	7
Phenylalanine	7.0	7
Histidine	5.4	5
Lysine	13.0	13
Arginine	5.8	6
Tryptophan‡	1.2	1
No. of residues		149
Calculated M_r		16,811

*Determined from 24-, 48-, and 72-hr extrapolation.
†Determined after performic acid oxidation.
‡Determined after methanesulfonic acid hydrolysis.

Characterization of Acidic FGF. Polyacrylamide/NaDodSO$_4$ gels of the purified acidic FGF with (Fig. 4) or without (data not shown) disulfide reduction by 2-mercaptoethanol show a pair of very close bands at 16,600 and 16,800 daltons. Because the molecular mass is not altered by reduction, these chains are not constituent polypeptides in a larger disulfide-linked protein. These two polypeptides have been identified as microheterogeneous forms of the same protein (see *Discussion*).

The amino acid composition of acidic brain FGF is given in Table 2. The analysis confirms previous results (8) that this FGF is not derived from myelin basic protein. The dose–response profile of the purified growth factor (Fig. 5) shows that 1 unit of mitogenic activity for BALB/c 3T3 cells corresponds to 40 pg of protein per ml (2.4 pM) as quantitated by amino acid analysis.

DISCUSSION

Acidic FGF, having a very high specific mitogenic activity, has been purified from bovine brain to apparent homogeneity. The purification resulted in an apparent increase in specific activity of 35,000-fold with a 4.1% recovery of mitogenic activity. The net recovery and degree of purification of acidic FGF may actually be greater because other mitogens, including basic FGF and somatomedins (17), occur in brain and would be expected to contribute to the total mitogenic activity for BALB/c 3T3 cells in the crude brain homogenate.

The molecular basis for the difference between the two microheterogeneous forms remains to be determined. Both the lack of binding of the mitogen to either concanavalin A or wheat germ lectin-Sepharose and the inability to detect amino sugars by amino acid analysis indicate that variations in the degree of glycosylation seem to be an unlikely explanation for differences in apparent molecular weight. After fractionation by shallow gradient elution from the C_4 HPLC column, both molecules are observed to have nearly identical amino acid compositions (unpublished data).

The inability of Gospodarowicz *et al.* (18) to identify the

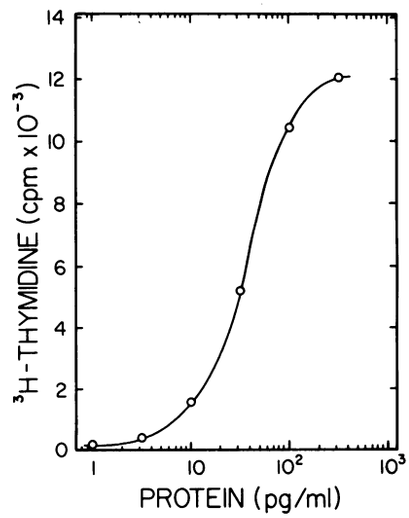

FIG. 5. DNA synthesis dose–response assay of purified acidic brain FGF on BALB/c 3T3 cells. Incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble DNA of quiescent BALB/c 3T3 cells was measured as a function of the concentration of HPLC-purified acidic brain FGF. Background values with no sample added were 125 cpm. Full stimulation by 10% heat-inactivated calf serum was 13,750 cpm.

acidic mitogen could arise from modifications that they made to their original published protocol (4). These include elution of the CM-Sephadex C-50 column with 0.5 M instead of 0.6 M NaCl, concentration of the active pool by ultrafiltration versus dialysis and lyophilization, and an increase in the pH of the Sephadex G-75 column from 8.5 to 9.2. As evidence for the absence of the acidic mitogen from their putatively pure FGF preparations, assays of isoelectric-focused FGF were presented. Unfortunately, only ampholytes from pH 9 to pH 11 were used to generate the pH gradient. The resulting steep acidic region presumably was formed from the mixing of the 10 mM acetic acid anode solution with trace amounts of ampholytes and, therefore, would be expected to be of low buffering strength and poor focusing potential. Finally, the mitogenic activity measurements of the focused pools were not made with the BALB/c 3T3 cell line originally used to describe the activity.

Acidic brain FGF appears to be unlike any of the few purified and well-characterized mitogenic growth factors. The isoelectric point (pI) and mass distinguish it from others as follows: murine epidermal growth factor, M_r = 6,045, pI = 4.6 (19); human insulin-like growth factors I (somatomedin C), M_r = 7,649, pI = 7.8–8.6 (20, 21), and II (multiplication stimulating activity), M_r = 7,471, pI = 7.8–8.6 (21, 22); murine sarcoma growth factor, $M_r \cong$ 13,000 (23); human melanoma growth factor, $M_r \cong$ 7,400 (24); human platelet-derived transforming growth factor, $M_r \cong$ 25,000 per dimer (25); human platelet-derived growth factor, M_r = 30,000, pI $\cong$ 10 (26–28); and basic pituitary fibroblast growth factor, $M_r \cong$ 14,500, pI $\geq$ 8.5 (29). Partially purified acidic fibroblast mitogens from human brain (30), bovine hypothalamus (31), retina (32), and pituitary (33) may be related or identical to bovine brain acidic FGF. Unambiguous determination of either the uniqueness or degree of similarity of acidic FGF to other growth factors will require comparison of their complete amino acid sequences and spectrum of target cells.

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