Bovine Fibroblast Growth Factor: 
Comparison of Brain and Pituitary Preparations

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ABSTRACT Bovine brain and pituitary fibroblast growth factors (FGF) have been compared 
with regard to their chemical and biological properties. Pituitary and one preparation of brain 
FGF (Prep A) contain a basic mitogenic activity, which migrates to the same position on 
electrophoresis in acid pH gels as detected by incorporation of [methyl-3H]-thymidine into 
BALB/c 3T3 cells. In contrast, another preparation of brain FGF (Prep B) contains two mitogens, 
one (20–30%) indistinguishable from the basic components in pituitary and brain (Prep A) FGF 
preparations and an acidic activity (70–80%), pl 5–6, that migrates more slowly on acid gels, 
corresponding to the acidic component of brain FGF described previously (Thomas, K. A., M. 
C. Riley, S. K. Lemmon, N. C. Baglan, and R. A. Bradshaw. 1980. 
J. Biol. Chem. 255:5517–5520.) 
In agreement with that report, 
none of the mitogens comigrates with fragments of myelin basic 
protein. Pituitary FGF was virtually inactive, brain (Prep A) FGF had a small amount of activity, 
and brain (Prep B) FGF was highly potent (50% maximal stimulation at 15–30 ng/ml) in 
stimulating the growth of human umbilical vein endothelial (HUVE) cells. The acidic compo-
nent of brain FGF, which is much more unstable at pH 8.5 than the basic one, can be protected 
by reducing agents, whereas the basic constituent of brain FGF as well as pituitary FGF is 
unaffected by reducing conditions. Thus, brain FGF preparations may contain two distinct 
mitogenic activities, one that is acidic and contains HUVE cell activity, and a basic mitogen 
that is similar to and may be identical with pituitary FGF.

In 1974, Gospodarowicz described the presence of a potent 
mitogen for mouse 3T3 fibroblasts in extracts of brain and 
pituitary (5). This activity was named fibroblast growth factor 
(FGF) and was subsequently purified from each of these 
bovine tissues (6, 7). Both were characterized as basic molecules 
of ~13,000 M, that were acid and heat labile and as having 
common biological activities for a variety of cultured cells 
derived from embryonic mesoderm (9, 12). It was suggested 
that they might be either related or identical (12) until it was 
shown that brain FGF could be separated into three forms, 
(FGF-1, 2, and 3) (7, 24), corresponding to different peptide 
fragments of myelin basic protein (MBP), a structural protein 
of the central nervous system. Because brain and pituitary 
FGF preparations had different amino acid compositions and 
antigenic properties (preparations of brain, but not pituitary, 
FGF caused autoimmune experimental encephalitis in guinea 
pigs presumably because of the presence of the MBP fragments 
(24)), it was concluded that they were distinct entities. 
Subsequently Thomas et al. (22) reported that, by three 
different criteria, the fragments of MBP did not possess the 
brain FGF activity. First, preparations of fragments produced 
from native bovine MBP, nearly identical to those reported to 
contain the brain FGF activity, were not mitogenic for BALB/ 
c 3T3 cells (22) or human dermal fibroblasts (1). Secondly, the 
mitogenic activity in brain FGF preparations could be sepa-
rated from the MBP fragments on an affinity column of 
insolubilized anti-MBP. Finally, the biological activity ap-
ppeared to be associated with an acidic molecule, pl 5–6, as
determined by isoelectric focusing.

Since brain FGF was found not to be associated with the MBP fragments, it again raised the possibility that the two mitogens could be the same, i.e. pituitary FGF might also be acidic, contrary to its reported properties (6). In this report, we confirm the basic character of pituitary FGF. We also show that brain FGF contains at least two different mitogens, one acidic and one basic, the latter of which has properties similar to and hence may be identical with pituitary FGF. In agreement with our previous report (22), neither the basic nor the acidic component of brain FGF corresponds to fragments of MBP.

**MATERIALS AND METHODS**

**Anti-MBP Affinity Column**

A chicken anti-bovine MBP affinity column was prepared and application and elution of samples was performed as described previously (21). Briefly, bovine brain FGF samples were applied in phosphate buffered saline (PBS) (2) at room temperature to a 1.0 ml column and allowed to bind for about 10 min. The column was washed with PBS until absorption at A280 and A320 was negligible and then was further washed with 1 M NaCl in PBS and 2 M guanidine-HCl. Aliquots from the three protein pools were taken for bioassay, diluted with 2.0 mg/ml bovine serum albumin (BSA) (#4378, Sigma Chemical Co., St. Louis, MO) in PBS, and dialyzed against PBS. The remainder of each sample was dialyzed with 1 mM acetic acid and lyophilized for application to gels.

**SDS and Acid Polyacrylamide Gel Electrophoresis**

15% SDS polyacrylamide gels were prepared and developed according to the method of Laemmli (14). Silver staining was performed as described previously (21). Acid-gel-disc electrophoresis was performed by the type C' modified Renfled method as described by Rodbard and Chrambach (20). 10%, 7.5% polyacrylamide resolving gels and 3.0% stacking gels were polymerized with light in the presence of riboflavin-5'-phosphate. Samples were dissolved in 10% glyceral and 1:10 dilution of upper running buffer. Gels were electrophoresed at approximately 4°C and 100 amp for 2 h. This was the amount of time it took for a cytochrome c marker, which ran near the buffer front, to migrate close to the bottom of the gel. Sample lanes to be tested for bioactivity were cut into 0.25-mm segments and eluted by shaking overnight at 4°C in 1.0 ml of PBS and 2 mg/ml BSA. The remainder of the gel was stained with Coomassie Blue R 250.

**Isoelectric Focusing in Polyacrylamide Slab Gels**

Isoelectric focusing slab gels, pH 3–10, 7.5% acrylamide, and 10-cm long were prepared as follows: 7.5 ml of 30% acrylamide/0.8% bisacrylamide, 7.5 ml of 50% glycerol, 0.75 ml each of Pharmalytes pH 3–10 and pH 6.5–9 (Pharmacia Fine Chemicals, Uppsala, Sweden), and 12.5 ml H2O were combined and degassed. After the mixture had reached room temperature, utilizing ice-cold buffers and refrigerated (4°C) conditions, 5% dithiothreitol was added, mixed, poured into an 8.0-cm diameter and the wattage and amperage had stabilized, ~1.5-~2 h total electrofocusing time. Gel lanes to be tested for biological activity were cut into 0.5-mm segments and eluted as described for acid gel samples. Gels were stained by the method of Reiner et al. (19).

**BALB/c 3T3 Cell Bioassay**

The assay of mitogenic activity for BALB/c 3T3 fibroblasts was performed by measuring incorporation of [3H]-thyminde into cells grown at 37°C in a humidified 5% CO2 atmosphere. Cells were plated onto Costar 12 well-dishes (4 cm) at 8,000 cells/well in 1.0 ml of medium (designated DME) which consisted of Dulbecco's modified Eagle's medium (high glucose) (DM 326 with 3.7 g/l of sodium bicarbonate added, KC Biologicals, Inc., Lenexa, KS) containing 100 units penicillin, 0.1 mg of streptomycin (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), and 0.1 mg of glutamine (DME). This was supplemented with 10% calf serum (CS) (KC Biologicals, Inc.). ~17 h later, the cells were washed two times with DME without serum and then 1.0 ml of the same medium containing 0.4% calf serum was added to wells. Cells were allowed to become quiescent for 30–54 h, after which samples to be tested were introduced in an additional 100 ml or less of DME containing 2 mg/ml BSA along with 1.12 mg/ml [3H]-thyminde (~70 Ci/mmol. ICN Pharmaceuticals, Inc., Irvine, CA) and 3 mg of unlabeled thymidine in 50 µl of DME were added. After a further 25 h, the assay was harvested. The cells were washed three times with 1.0 ml 2.0 mg/ml BSA in HBSS and then precipitated with 1.0 ml of 5% TCA for 30 min on ice. After two more washes with cold 5% TCA, precipitable material was solubilized with 1.0 ml of 2% NaOH, in 0.1 M NaOH for 1 h. A sample of 0.5 ml of the incorporated label was neutralized with 70 µl of 50% TCA and 1.0 ml scintillation cocktail (Aq70, Research Products International Corp., Mt. Prospect, IL) was added for counting. Results were obtained from triplicate determinations and standard deviations were generally <10% of reported values.

**Human Umbilical Vein Endothelial Cell Growth Assays**

Human umbilical vein endothelial (HUVE) cells were maintained as previously described (16). For biological assay, cells between passage 3 and 11 were seeded into 35-mm culture dishes coated with 10 µg human fibronectin/cm² at a density of 1 x 10⁵ cells/dish in medium 199 and 20% fetal bovine serum (PBS) (Gibco Laboratories). Endothelial-cell growth factor (ECGF) (an extract as prepared in (15)) or FGF samples were added at this time, and cell cultures were fed every 2–3 d with the appropriate supplements. The addition of FGF samples was performed after 10 d. The cells were harvested by treatment with 0.05% trypsin-0.02% EDTA (Gibco Laboratories). trypsin inactivated by the addition of soybean trypsin inhibitor (Sigma Chemical Co.) and duplicate hemocytometer counts obtained. Results are reported directly as the number of viable endothelial cells per dish.

**Preparation of Brain FGF**

Bovine pituitary FGF was prepared by the method of Gospodarowicz et al (6, 7) with minor modifications. Frozen pituitaries (Pel-Freeze Biologicals, Rogers, AR, or the generous gift of Dr. D. A. K. Roncan, University of Toronto, Toronto, Canada) were homogenized in 0.15 M (w/v) SO₄ in the presence of 2.5 mg/l leupeptin, 2.5 mg/l pepstatin (Sigma Chemical Co.), 0.25 mM phenyl methyl sulfonyl fluoride (PMSF), and 1 mM EDTA. Acid extraction, subsequent ammonium sulfate precipitation, dialysis, and carboxymethyl-Sephadex (CM-Sephadex) C-50 stepwise elutions were performed as previously described (6, 7) with the inclusion of 0.25 mM PMSF and 1 mM EDTA in all solutions. The Sephadex G-75 gel filtration column (2.5 x 90 cm) was run with 10–40 ml bed resin in 0.1 M ammonium bicarbonate. The active G-75 pool was loaded to the final CM-Sephadex C-50 column in 0.1 M sodium phosphate, pH 6.0, containing 0.1 M NaCl. The column was washed extensively with 0.1 M NaCl and 0.15 M NaCl in the phosphate buffer, followed by a gradient from 0.15 to 0.6 M NaCl in the same buffer. The final active fractions were pooled, dialyzed versus H₂O and stored desalted at ~20°C for ~6 month. Final amounts of protein were estimated by amino acid analysis on a Durrum D-500 amino acid analyzer (Dionex Corp., Sunnyvale, CA).

**Preparations of Brain FGF**

FGF from bovine brain was isolated by the method of Gospodarowicz et al (7, 24) with the following modifications. In one purification from 11 kg of tissue (Prep A), the active pool from the CM-Sephadex C-50 column was dialyzed overnight against 0.1 M ammonium bicarbonate, pH 8.5, in tubing with a Mₐ = 3,500 cutoff. This pool (1,400 ml) was then concentrated at 4°C by pressure filtration over a 4-d period using a PM 10 membrane to 50 ml, followed by further concentration in a smaller diameter UM 10 membrane to 30 ml. This step replaced the more rapid lyophilization normally used. This material was loaded directly onto the Sephadex G-75 column and the active fraction purified through the last CM cellulose step. The pooled FGF fractions (Prep A) were stored in 30 mM ammonium formate, pH 6.0, at ~20°C for 2–3 yr.

In a second purification (Prep B), initial homogenization, extraction, and ammonium sulfate precipitation of 16 kg of brains were performed in the laboratory at room temperature, utilizing ice-cold buffers and refrigerated (4°C) centrifugation. All subsequent steps were done entirely in the cold. The following modifications were made to accelerate the first chromatography step. The material from the second ammonium sulfate precipitation was dialyzed against water, lyophilized, and dissolved in 500 ml of 0.1 M sodium phosphate, pH 6.0. This was mixed with 40 g of CM-Sephadex resin, equilibrated in the same buffer, stirred for 15 min, poured into a sintered glass filter and the excess liquid drawn out. The resin was mixed with starting buffer, poured into an 8.0-cm diameter
RESULTS

Isoelectric Focusing of Brain and Pituitary FGF

In initial studies to compare the properties of the two brain FGFs and pituitary FGF, appropriate preparations were analyzed by isoelectric focusing. Pituitary (10 μg), brain Prep A (25 μg), and Prep B (90 μg) FGF were focused in 7.5% polyacrylamide slab gels, pH range 3–10, after which slices were cut, eluted, and tested for stimulation of incorporation of [methyl-3H]-thymidine into BALB/c 3T3 cells. Fig. 1 shows the activity profiles from these experiments. As seen, pituitary FGF (Fig. 1A, closed circles) focused in the basic region of the gel with a pI of 8–9, confirming earlier reports of the basic nature of this mitogenic activity (6). Unexpectedly, the older preparation of brain FGF (Prep A) (Fig. 1B) showed a similar broad activity-peak in this region of the focusing profile, with possibly a very small amount of activity (<5%) at a pI of ~6.0. This was in apparent contrast to our previous results in which we found all of the brain FGF activity was associated with an acidic molecule (22). However, when a second preparation of brain FGF (Prep B) was analyzed by isoelectric focusing (Fig. 1C), two active components were detected, a basic mitogenic activity with a pI similar to that in brain Prep A and pituitary FGF, and a more prominent species that focused with a pI of about 6.0 in this system. Myelin basic protein fragments, which were identified by staining of the gels, appeared to separate from the activities and migrated to the cathode, pI ≥10.0. In control experiments, in which blank lanes with no applied protein were sliced, eluted, and tested in the 3T3-cell bioassay, no activity was seen (Fig. 1A, open triangles).

Anti-MBP Affinity Column Chromatography of Brain FGF

In view of the fact that the Prep A of brain FGF contained primarily a basic mitogenic activity, we reexamined this material after passage through an anti-MBP affinity column (22). 300 μg of brain Prep A FGF were loaded in PBS and the column was washed sequentially with PBS, 1 M NaCl, and 2 M guanidine-HCl. As reported previously (22), almost all of the biological activity recovered, as determined in the BALB/c 3T3 assay, was not retained by the column and came through the breakthrough PBS pool (87%) even though this sample consisted largely of a basic mitogen. 11% was found in the NaCl pool and <2% was contained in the guanidine–HCl fraction, which included the MBP fragments, the bulk of the protein loaded on the column (data not shown).

The introduction of the highly sensitive silver-staining method for SDS polyacrylamide gels made it possible to identify the components in all of the fractions eluted from the anti-MBP column (Fig. 2). ~1–2 μg of protein, estimated by absorption at A280, were analyzed from each fraction. As can be seen, the PBS pool (lane 1) contained three major staining bands that did not comigrate with any of the MBP fragments eluted by 2 M guanidine-HCl (lane 3). The material eluted by 1 M NaCl (lane 2) contained some components found in both the guanidine–HCl and PBS pools. Thus, the biological activity did not adhere to the anti-MBP affinity column and was not associated with fragments of MBP, confirming our previous report (22).

Acid Gel Electrophoresis of Brain and Pituitary FGF

Preparations of pituitary, brain Prep A FGF, containing primarily a basic mitogenic component, and brain Prep B FGF, with two separable mitogenic activities, one acidic and one basic, were further examined by acid gel electrophoresis. 10 μg of pituitary and 10–20 μg of brain Prep A FGF were electrophoresed on 10% polyacrylamide acid pH gels, slices cut, and samples eluted for the 3T3-cell assay. As seen in Fig. 3, pituitary (panel A) and brain Prep A FGF (panel B) had a single mitogenic activity that migrated to the same apparent position in the gel, very close to the chicken egg-white lysozyme marker. Although the brain FGF preparation used in this experiment had been partially purified by passage through the anti-MBP affinity resin, unfraccionated samples of brain Prep A FGF gave identical activity profiles in the acid gels.

FIGURE 1 Isoelectric focusing profiles of FGF activity. The 7.5% polyacrylamide gels with ampholytes of pH 3–10 were focused for 1½–2 h as described in the text. Gels were sliced into 0.5-cm slices and eluted in 1.0 ml PBS, 2.0 mg/ml BSA for bioassay. Results are expressed as percent of maximal stimulation for each FGF, with two separable mitogenic activities, one acidic and one basic, were further examined by acid gel electrophoresis. 10 μg of pituitary and 10–20 μg of brain Prep A FGF were electrophoresed on 10% polyacrylamide acid pH gels, slices cut, and samples eluted for the 3T3-cell assay. As seen in Fig. 3, pituitary (panel A) and brain Prep A FGF (panel B) had a single mitogenic activity that migrated to the same apparent position in the gel, very close to the chicken egg-white lysozyme marker. Although the brain FGF preparation used in this experiment had been partially purified by passage through the anti-MBP affinity resin, unfraccionated samples of brain Prep A FGF gave identical activity profiles in the acid gels.
FIGURE 2 Silver-stained pattern of SDS polyacrylamide (15%) gel electrophoresis of fractions eluted from the anti-MBP Sepharose chromatography. Gels were developed for approximately 3 h at 20 mAmp and processed for silver staining as described previously (21). Samples, 1–2 μg, of each of the protein pools eluted from the anti-MBP Sepharose chromatography of brain Prep A FGF as described in the text were applied to lanes 1–3: lane 1, PBS pool; lane 2, 1 M NaCl pool; lane 3, 2 M guanidine-HCl pool. Lane 4, 500 ng each of molecular weight markers: ovalbumin, 43,000 Mr [43]; chymotrypsinogen, 25,000 Mr [25]; cytochrome c, 12,500 Mr [12.5].

FIGURE 3 Activity profiles of acid gel electrophoresis of FGF. Samples applied to 10% polyacrylamide type ‘G’ acid gels (19) were electrophoresed as described in the text. Gels were sliced into 0.25-cm fragments and eluted as in Fig. 1 for the 3T3-cell bioassay. Results are expressed as described in Fig. 1 with each FGF preparation examined in a separate experiment. Electrophoresis of samples was from right to left and migration was analyzed relative to the cytochrome c marker. Arrows indicate chymotrypsinogen (chy), lysozyme (lys), and cytochrome c (cyt). (A) Pituitary FGF, 10 μg loaded, 0.1 μl assayed; maximal stimulation by FGF, 31,600 ± 701 cpm; 10% CS, 26,219 ± 2546 cpm. (B) Brain-Prep A FGF, partially purified on an anti-MBP affinity column, 10–20 μg loaded, 1.0 μl assayed; maximal stimulation by FGF, 5397 ± 416 cpm; 10% CS, 8505 ± 726 cpm. (C) Brain-Prep B FGF, 90 μg loaded, 1.0 μl (○) or 5 μl (■) assayed; maximal stimulation by FGF, 8551 ± 767 cpm; 10% CS 11,344 ± 829 cpm.

The Coomassie Blue staining-patterns from the acid gel electrophoresis of pituitary and this further purified brain Prep A FGF are shown in Fig. 4. As can be seen, the brain FGF (lane 3) still contained residual MBP fragments when it was compared to the material eluted by 2 M guanidine–HCl from the anti-MBP affinity column (lane 2). However, these peptides served as a visual marker, because the biological activity, which migrated near lysozyme (lane 1, B), and the MBP fragments, which migrated more slowly, were clearly separated in the acid gels. Both the pituitary (lane 4) and this brain FGF sample had staining bands in the region of biological activity.

In contrast, when brain Prep B FGF (90 μg) was analyzed by acid gel electrophoresis, two separate mitogenic entities were seen (Fig. 3 C). There was a smaller peak of activity (20–30%) that migrated to the same position as pituitary and brain Prep A FGF and a slower migrating component (70–80%) that had a mobility in this system similar to chymotrypsinogen. This distribution of the two active components correlated well with the results of the isoelectric focusing gels. The MBP fragments migrated approximately between the two peaks.

In addition, it was found that virtually 100% recovery of activity of material loaded on the acid gels was obtained when slices were eluted into buffer containing carrier BSA. 10 μl from samples eluted from sliced-blank lanes run without applied protein showed no stimulation of [methyl-3H]-thymidine into 3T3 cells.

Effects of FGF on HUVE cells

FGF preparations were tested for their ability to stimulate the growth of HUVE cells (Fig. 5). In most experiments,
FIGURE 4 Acid gel electrophoresis of pituitary-and brain-Prep A FGF. Gels were developed as in Fig. 3 and then stained with Coomassie Blue R. Lane 1: chymotrypsinogen, 10 µg, (A); lysozyme, 10 µg, (B); cytochrome c, 20 µg, (C). Lane 2: 2 M guanidine-HCl fraction from anti-MBP affinity chromatography, 20 µg. Lane 3: brain-Prep A FGF, partially purified on the anti-MBP affinity column as in Fig. 3 B, 10–20 µg. Lane 4: pituitary FGF, 15 µg.

pituitary FGF showed no activity, although occasionally, as shown in Fig. 5B, a small amount was detectable when 100 ng/ml or greater were added. By comparison, this material showed half-maximal stimulation of activity in the BALB/c 3T3 cell assay at a dose of 0.1–0.5 ng/ml. Brain Prep A FGF, which produced a half-maximal response in the 3T3 cell bioassay at 5–10 ng/ml, displayed activity in the HUVE cell assay, but even at 250 ng/ml, it had not reached 50% of the ECGF level. In contrast, brain Prep B FGF was equipotent in both assays with half-maximal stimulation between 15 and 30 ng/ml. In addition, it was as active in the HUVE cell assay at 4 ng/ml as brain Prep A FGF was at 250 ng/ml, a 60-fold difference.

Stability Studies

These brain and pituitary FGF preparations were also analyzed for their stability when subjected to reducing conditions. Samples were treated with or without β-mercaptoethanol at 37°C, pH 8.5, for 30 min and then dialyzed to remove reducing agents. As shown in Table I, pituitary and brain Prep A FGF were not sensitive to these reducing conditions, as there was no significant difference in activities in the presence or absence of β-mercaptoethanol when tested in the BALB/c 3T3 bioassay. In contrast, preparation B of brain FGF was markedly stabilized by β-mercaptoethanol when subjected to pH 8.5 and 37°C and then dialyzed. In this experiment, there was a 40–50% reduction in biological activity in the absence of the thiol reagent, as compared with samples treated in its presence.

This stabilizing effect of β-mercaptoethanol was further analyzed by isoelectric focusing of brain Prep B FGF samples that had been incubated with or without this agent. Fig. 6
The biological activity focused in the basic region of the isoelectric focusing slab gels and experiments on focusing columns of pituitary FGF. Our experiments have confirmed that the present studies have reexamined the relationship of brain and pituitary FGF. All of the studies presented in our earlier paper (22) were performed with brain FGF purified on a column of CM-52 ion exchange column. However, this cannot account for the virtual absence of the acidic component in the further purified brain FGF. Although both the acidic and basic components were stimulatory for BALB/c 3T3 fibroblasts, only preparations that contained the acidic factor showed significant activity in the HUVE-cell assay. The unexpected result was that this preparation of brain FGF contained little of the factor with the acidic pl that we originally described (22). However, analyses, by the same methods, of a second preparation (Prep B), clarified this apparent discrepancy. We found this sample contained both an acidic component with mitogenic activity, corresponding to the activity described in our earlier report (22), and a basic constituent resembling pituitary and brain Prep A FGF.

TABLE I

| Treatment          | ng/ml | -βMEcpm | +βMEcpm |
|--------------------|-------|---------|---------|
| Pituitary FGF      | 0.5   | 5872 ± 329 | 5715 ± 90 |
|                    | 5     | 12590 ± 387 | 11771 ± 545 |
| Brain Prep A FGF   | 5     | 8413 ± 190  | 7431 ± 312 |
|                    | 50    | 11391 ± 704 | 13236 ± 514 |
| Brain Prep B FGF   | 10    | 364 ± 18    | 785 ± 72  |
|                    | 100   | 3261 ± 125  | 5083 ± 355 |

* Samples were treated with or without β-mercaptoethanol (βME), as described in the text, and tested in the 3T3-cell bioassay. Results are expressed as cpm ([3H]thymidine) incorporated into TCA-precipitable material. Pituitary FGF and brain Prep A were tested in the same experiment with 10% calf serum controls incorporating 16,999 ± 887 cpm; maximal stimulation was 13,236 ± 514 cpm. In a separate experiment, brain Prep B FGF gave a maximal stimulation of 5,083 ± 355 cpm with 10% calf serum controls incorporating 5640 ± 375 cpm.

shows that in the absence of reductant (panel B) the acidic component of this brain FGF was nearly undetectable; however, protection from inactivation was provided by the sulphydryl compound (panel A), although there was still some decrease in the acidic constituent. In contrast, there was no loss of activity of the basic component of brain Prep B FGF with or without reductant, revealing another similar property of this activity to the FGF of pituitary and the Prep A of brain. Samples prepared without growth factors but in the presence of β-mercaptoethanol were inactive when tested for biological activity.

**DISCUSSION**

The present studies have reexamined the relationship of brain and pituitary FGF. Our experiments have confirmed that pituitary FGF is indeed basic as originally described (6). All of the biological activity focused in the basic region of the isoelectric focusing slab gels and experiments on focusing columns in sucrose density-gradients with cruder preparations of pituitary FGF also showed that the major mitogenic activity is basic (data not shown).

When we compared pituitary FGF to two different preparations of brain FGF by isoelectric focusing, we found that the brain samples gave markedly different activity-profiles. One preparation (Prep A) contained primarily a basic mitogen that was similar to pituitary FGF in that it migrated to the same position in the acid pH gels and was insensitive to sulphydryl reagents at 37°C. It was also shown not to be related to the MBP fragments. This was established in both electrophoresis systems utilized in these studies and by the affinity chromatography and its subsequent analysis in SDS gels. Other investigators are in agreement with this finding (1, 13).

The unexpected result was that this preparation of brain FGF contained little of the factor with the acidic pI that we originally described (22). However, analyses, by the same methods, of a second preparation (Prep B), clarified this apparent discrepancy. We found this sample contained both an acidic component with mitogenic activity, corresponding to the activity described in our earlier report (22), and a basic constituent resembling pituitary and brain Prep A FGF.

Although both the acidic and basic components were stimulatory for BALB/c 3T3 fibroblasts, only preparations that contained the acidic factor showed significant activity in the HUVE-cell assay. The amount of activity for HUVE cells was reflected in the quantity of the acidic component in a given sample; that is, pituitary FGF had virtually no activity, brain Prep A FGF had very small amounts of activity, and brain Prep B FGF was a highly potent mitogen for these cells. In addition, only in the case of brain Prep B FGF was the growth-factor preparation as active in the HUVE-cell assay as it was in the 3T3-cell assay. That the acidic factor contains the HUVE-cell activity, though, is only inferred as we have been unable to test samples derived from polyacrylamide gels directly in these cultures without unacceptable losses in cell survival.

It is possible that the two components in preparation B of brain FGF are related in some way. For example, one activity could be produced from the other constituent by a latent proteolytic event. However, the fact that only brain FGF with the acidic material is active for HUVE cells, which is stabilized by β-mercaptoethanol, whereas the basic components from brain and pituitary FGF do not show these properties, suggests that the two mitogenic activities are distinguishable entities.

The fact that the acidic and basic mitogens are apparently distinct proteins suggests that the variable amounts of these constituents in different brain FGF preparations are probably due to other reasons. There were several differences in the purification and storage of the two brain FGF samples used in these studies. The most significant was that Prep B was only purified through the Sephadex G-75 column, whereas Prep A was purified through the final CM-52 ion exchange column. However, this cannot account for the virtual absence of the acidic component in the further purified brain FGF. First, all of the studies presented in our earlier paper (22) were performed with brain FGF purified on a column of CM-52 with gradient elution and only the acidic component was observed on isoelectric focusing. Second, both the acidic and basic mitogenic components were observed with isoelectric focusing and acid gel electrophoresis when brain Prep B FGF was further purified through a final CM-52 column (data not shown). However, it should be noted that the bound protein fraction was eluted in a single step because biological activity...
extended over the entire gradient profile in most of our prior purification of brain FGF. In these experiments, the acid gel electrophoresis showed identical amounts of the two activities, 20–30% basic and 70–80% acidic, as had been seen with the G-75 purified material.

Aging of brain FGF preparations may account, in part, for the loss of one constituent relative to the other. We have noted that brain FGF is generally less stable than pituitary FGF and this could be due to the presence of the more labile acidic component in brain FGF preparations. However, the difference in age in the brain-FGF samples used in these studies is not so great as to suggest that this is the main factor in the differences in content of the acidic mitogen. Rather, it is more likely that during manipulation of the samples or at other steps in the purification of brain FGF, recoveries of the two mitogens are altered. Slight variations in pH and ionic strength of buffers, temperature, length of dialysis or concentration, pooling of column fractions, storage conditions, and the age of the animals from which the bovine brains were obtained could determine the final ratio of the acidic to basic components. For example, it is possible that the loss of the acidic constituent in Prep A brain-FGF could be accounted for by the prolonged concentration step at pH 8.5 prior to the Sephadex G-75 purification, since it has been shown that the acidic component is labile to treatment at this pH at 37°C. Alternatively, storage of samples in solution (Prep A) versus lyophilized (Prep B) could be a contributing factor. At the present time, it is not clear what factors lead to the variable amounts of the acidic and basic constituents of brain FGF.

We are not certain why we did not observe the basic component of brain FGF in our earlier studies (22). This form was probably contained in the brain FGF we originally analyzed, but because of insensitivity of the biological assay and the isoelectric focusing methods utilized at that time, which was 20-fold less sensitive, it was not detected. However, we do not feel that loss of the basic constituent in the earlier experiments was due to dialysis of samples with 10 mM acetic acid, as has been suggested by Gospodarowicz and Mescher (10). In the initial reports of the characterization of brain FGF, substantial biological activity (80%) was retained when samples were treated for 24 h with as great as 100 mM acetic acid (7).

In view of the conflicting results that have been obtained from isoelectric focusing of brain-FGF preparations, this may not be an adequate method with which solely to analyze these growth factors, especially when identification depends upon a mitogenic bioassay. In particular, there are difficulties in interpreting the basic region of isoelectric focusing-profiles. It has been noted that basic ampholytes can stimulate fibroblasts to divide (4). Also, alkaline pH can result in mitosis of 3T3 cells (25). Although assaying a blank run without added protein is a useful control, there exists the possibility that some of the mitogenic activity in a test sample is due to ampholytes that may act differently when associated with proteins. In addition, we cannot eliminate the possibility that some artifactual mitogenic activity in focusing experiments was observed when larger sample volumes, 5–10 µl, were assayed, as we have sometimes seen >100% recovery, and the ratio of acidic to basic material in brain (Prep B) FGF did not always correlate with the analysis of the same preparation in acid gels. Therefore, this latter method that does not have unusual buffers, appears to give a more reliable indication of the amounts of the two constituents found in brain FGF preparations.

The presence of two distinct mitogens in preparations of brain FGF may explain the conflicting results with human endothelial-cell growth in culture. While Gospodarowicz and colleagues (8) have shown that high concentrations of brain and pituitary FGF can promote the growth of normal human-endothelial cells, these results have been difficult to confirm independently (15, 16). It is also possible that bovine pituitary contains the acidic growth activity. In our studies on pituitary FGF, we have noted the presence of other components mitogenic for 3T3 cells, especially as seen by gel filtration, and Maciag et al. (15) have shown that pituitary extracts contain significant levels of activity for HUVE cells. Because we have only studied normal human endothelial-cell growth with these reagents, it is presently not possible to extend our findings to normal bovine-endothelial cells (11, 18, 23). However, it should be noted that pituitary FGF, prepared in our laboratory, is highly active for an established line of bovine vascular-endothelial cells (P. R. Kelley, Collaborative Research Inc., Wallingford, MA, personal communication), an observation consistent with other laboratories (3, 11).

In conclusion, we feel that the experiments presented here have helped to clarify the contradictory findings surrounding studies with bovine fibroblast growth factors. We have definitively shown that brain FGF activity is not associated with fragments of MBP, but that these preparations do contain a basic component with properties very similar to pituitary FGF. At present, they are indistinguishable. The acidic constituent of brain FGF, present in some preparations, resembles closely and may be related to endothelial-cell growth factor, pf 5.0–6.0, that has been characterized by Maciag et al. (15, 17). If these biological assignments prove to be correct, it would be reasonable to refer to the basic mitogens of brain and pituitary as “fibroblast growth factor” and retain the designation “endothelial-cell growth factor” for the acidic component.

K. A. Thomas acknowledges the valuable assistance of Mari Candellore during the purification of one of the brain FGF samples.

This work was supported by a grant from the American Cancer Society, BC-273C to R. A. Brashaw and by a National Cancer Institute Award, DHHS FHS grant, CA-16217, to Washington University. It was also supported by a National Institutes of Health research grant, AG-00599, to T. Maciag.

Received for publication 11 February 1982, and in revised form 21 June 1982.

REFERENCES
1. Chiang, T. M., J. N. Whitsaker, J. M. Sayer, and A. H. King. 1980. Effect of peptides of bovine myelin basic protein on dermal fibroblasts. J. Neurosci. Res. 5:439–449.
2. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with polioviruses. J. Exp. Med. 99:147–199.
3. Duthe, G. S., and J. R. Smith. 1980. In vitro proliferation and life-span of bovine aortic endothelial cells: effects of culture conditions and fibroblast growth factor. J. Cell. Physiol. 103:385–392.
4. Gospodarowicz, D., H. Bialecki, and G. Greenberg. 1978. Purification of fibroblast growth factor activity from bovine brain. J. Biol. Chem. 253:3736–3743.
5. Gospodarowicz, D., H. Bialecki, and G. Greenberg. 1979. Mitogenic and inhibitory effects of carrier ampholytes on quiescent and stimulated human diploid lung fibroblasts. Adv. Biochem. 19:27–35.
6. Gospodarowicz, D. 1974. Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature (Lond.). 248:123–127.
7. Gospodarowicz, D. 1975. Purification of fibroblast growth factor from bovine pituitary. J. Biol. Chem. 250:2515–2520.
8. Gospodarowicz, D., H. Bialecki, and G. Greenberg. 1978. Purification of fibroblast growth factor activity from bovine brain. J. Biol. Chem. 253:3736–3743.
9. Gospodarowicz, D., K. D. Brown, C. R. Birdwell, and B. R. Zetter. 1978. Control of proliferation of human vascular endothelial cell characterization of the response of human umbilical vein endothelial cells to fibroblast growth factor, epidermal growth factor, and thrombin. J. Cell. Biol. 77:774–788.
10. Gospodarowicz, D., G. Greenberg, H. Bialecki, and B. R. Zetter. 1978. Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. In Vitro (Rockville). 14:85–118.
11. Gospodarowicz, D., A. L. Mescher. 1978. Fibroblast growth factor and vertebrate regeneration. Adv. Neurol. 29:149–171.
12. Gospodarowicz, D., J. Morais, D. Braun, and C. Birdwell. 1978. Clonal growth of bovine vascular endothelial cells: fibroblast growth factor as a survival agent. Proc. Natl. Acad. Sci. U. S. A. 75:4120–4124.
12. Gospodarowicz, D., P. Rudland, J. Lindstrom, and K. Benirschke. 1975. Fibroblast growth factor: its localization, purification, mode of action and physiological significance. Adv. Metab. Diord. 8:201–215.

13. Kellett, J. G., T. Tanaka, J. Rowe, R. P. C. Shiu, and H. G. Friessen. 1981. The characterization of growth factor activity in human brain. J. Biol. Chem. 256:54–58.

14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond). 227:680–685.

15. Maciag, T., J. Cerundolo, S. Boye, P. R. Kelley, and R. Forand. 1979. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc. Natl. Acad. Sci. U.S.A. 76:5674–5678.

16. Maciag, T., G. A. Hoover, M. B. Stemerman, and R. Weinstein. 1981. Serial propagation of human endothelial cells in vitro. J. Cell. Biol. 91:420–426.

17. Maciag, T., G. A. Hoover, and R. Weinstein. 1982. High and low molecular weight forms of endothelial cell growth factor. J. Biol. Chem. 257:5333–5336.

18. Mueller, S. N., E. M. Rosen, and E. M. Levine. 1980. Cellular senescence in a cloned strain of bovine fetal aortic endothelial cells. Science (Wash. D.C.). 207:889–891.

19. Reisner, A. H., P. Nemes, and C. Bucholtz. 1975. The use of Coomassie brilliant blue R-250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. Anal. Biochem. 64:509–516.

20. Rodbard, D., and A. Chrambach. 1971. Estimation of molecular radius, free mobility and valence using polyacrylamide gel electrophoresis. Anal. Biochem. 40:95–134.

21. Rubin, J. S., E. M. Jacob, W. H. Daughaday, and R. A. Bradshaw. 1982. Isolation and partial sequence analysis of rat basic somatomedin. Endocrinology. 110:734–740.

22. Thomas, K., A. M. C. Riley, S. K. Lawmon, N. C. Baglan, and R. A. Bradshaw. 1980. Brain fibroblast growth factor: nonidentity with myelin basic protein fragments. J. Biol. Chem. 255:5517–5520.

23. Wall, R. T., L. A. Harker, L. J. Quadracci, and G. E. Striker. 1978. Factors influencing endothelial cell proliferation in vitro. J. Cell. Physiol. 96:203–214.

24. Westall, F. C., V. A. Lentz, and D. Gospodarowicz. 1978. Brain-derived fibroblast growth factor: identity with a fragment of the basic protein of myelin. Proc. Natl. Acad. Sci. U.S.A. 75:4675–4678.

25. Zetterberg, A., and W. Engstrom. 1981. Mitogenic effect of alkaline pH on quiescent, serum-starved cells. Proc. Natl. Acad. Sci. U.S.A. 78:4334–4338.