Purification of a Chick Brain-Derived Growth Factor by Reversed-Phase High-Performance Liquid Chromatography

Robert Carlone,¹ J. K. Kim,² and Michel Rathbone²

¹Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada, and ²Department of Neurosciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

A chick brain-derived growth factor (CBGF) has been purified to apparent homogeneity by a combination of ultrafiltration, DEAE ion-exchange, and reversed-phase high-performance liquid chromatography on a C₁₈ column. CBGF has an apparent molecular mass of approximately 1500 Da. The amino acid composition of CBGF has been determined and reveals a peptide containing predominantly glycine, glutamic acid/glutamine, and aspartic acid/asparagine. This mitogen is highly active, with half-maximal stimulation of chick brain astrocytes at 5 ng/ml in an assay using incorporation of methyl-³H-thymidine into DNA. In addition to its effects on chick astrocytes, purified CBGF from the 18 d chick embryo is mitogenic for amphibian limb regeneration blastema cells *in vitro*. A possible role for this mitogen in the nerve-dependent regeneration of amphibian appendages is discussed.

The nervous system is a rich source of several mitogenic factors for cells derived from the embryonic mesoderm, as well as for neurons and glia (Brockes and Lemke, 1981; Brockes, 1984; Bunge and Waksman, 1985; Carlone and Mescher, 1985). Many of these factors are proteins and peptides with a broad range of target specificities, some of which have been purified to homogeneity and sequenced (James and Bradshaw, 1984). We have previously isolated and partially purified a low-molecular-weight peptide growth factor from adult chick brain, chick brain-derived growth factor (CBGF) (Carlone and Rathbone, 1985). This factor is a mitogen for cultured regeneration blastemas from amputated regenerating newt limbs. It also promoted proliferation of serum-starved Swiss 3T3 cells. A similar mitogenic activity was detected in the brains of adult newts and of 18 d chick embryos.

We now report the purification to homogeneity, chemical characteristics, and amino acid composition of the CBGF.

Materials and Methods

Materials. Fertile white Leghorn eggs were obtained from Fleming Chicks Ltd. (Beamsville, Ontario). Adult newts (*N. viridescens viridescens*) were obtained from Charles Sullivan (Nashville, TN). Insulin, sodium hydroselenite, progesterone, putrescine, human transferrin, phenylmethylsulfonyl fluoride, and crystalline BSA were purchased from Sigma

Chemical Company. Eagle's modified essential medium (MEM), Leibovitz's L-15, bovine achilles tendon collagen, and fetal bovine serum were from GIBCO. Tissue culture dishes and 24 well plates were from Falcon. DEAE 52 ion-exchange medium was obtained from Whatman. The C₁₈ μ Bondapak reversed-phase column was from Waters. Methyl-³H-thymidine (specific activity, 40–60 Ci/mmol) was obtained from Amersham Canada Ltd. All reagents were analytical grade.

Cell and tissue culture bioassays. Early to midcone stage (Iten and Bryant, 1973) regeneration blastemas from bilaterally denervated forelimbs of the newt were removed aseptically and cultured in 70% Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) and 0.14 units/ml insulin (basal medium) for 48 hr in 24 well test plates. Fractions to be tested for CBGF activity were then added to wells for 24 hr, followed by a 12 hr labeling period with 0.5 μ Ci/well of methyl-³H-thymidine. Contralateral control blastemas received fresh medium and label. Blastemas were washed, the wound epithelia removed, and mesenchymal tissues fixed in acid-alcohol. The tissues were then Feulgen-stained, squash preparations prepared, and slides processed for autoradiography.

Astroblasts used in bioassays for CBGF activity were prepared from 10 d chick embryo brains. Whole brains were dissected from decapitated 10–11 d chick embryos and the meninges removed. The brain tissue was dissociated by a combination of trypsinization and mechanical sieving through 63 μ m Nitex mesh and the non-neuronal cells selected by differential adhesion to collagen-coated petri dishes according to the methods of Hanson et al. (1982). The immunohistochemically defined astroblasts were maintained in Eagle's MEM supplemented with 33.3 mM D-glucose, 5 μ g/ml insulin, 5 μ g/ml human transferrin, 2×10^{-8} M progesterone, 100 μ M putrescine, and 3×10^{-8} M sodium selenite (basal medium) supplemented with 10% FBS. The cells for assay were harvested from stock dishes by trypsinization and seeded at a density of 3×10^4 cells/well in 24 well test plates. The cells were established in basal medium with 10% FBS for at least 48 hr, after which the medium was aspirated and replaced with basal medium without serum. After an additional 48 hr, the medium was replaced by basal medium + 1% FBS and varying concentrations of fractions to be tested. Control wells received fresh medium supplemented with either 1 or 10% FBS. The stimulation period lasted for 48 hr, with methyl-³H-thymidine (0.5 μ Ci/well) included in the assay wells for the last 24 hr. The cells were then washed, fixed in cold 10% TCA, air-dried, and solubilized in 0.5 ml of 1% SDS in 0.3 N NaOH. TCA precipitable material was then filtered (Whatman, Type GF/A), dried, and counted in Aqueous Counting Solution (Amersham) in a Beckman scintillation counter (model LS 1800). CBGF activity was determined on at least 6 replicate wells in repeated assays and expressed as a percentage of control cultures receiving only basal medium + 10% FBS.

Purification of CBGF. The initial steps involved in the purification of CBGF from 18 d chick embryo brain include homogenization of the tissue in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) followed by ultracentrifugation and pressure ultrafiltration of the 100,000 $\times$ g supernate through a series of Amicon Diaflo membranes of decreasing porosity (Types PM-30, YM-2, and UM-05) and are described in detail elsewhere (Carlone and Rathbone, 1985). The active material is retained and concentrated on the type UM-05 membrane (nominal molecular-weight cut-off, ~1000), dialyzed against 0.15 M ammonium acetate on the same membrane, lyophilized, and stored at -70°C .

DEAE ion-exchange chromatography. Whatman DE-52 was prepared

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Correspondence should be addressed to Dr. Robert Carlone at the above address.

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Figure 1. Fractionation of CBGF activity on DEAE ion-exchange chromatography. Approximately 5 mg UM-05 retentate protein was applied to a DEAE 52 column and eluted with a linear gradient of 0.05–0.65 M ammonium acetate (pH 7.2) as described in Materials and Methods. CBGF activity eluted in a pooled series of fractions, 35 ml (histogram) with 0.4–0.45 M ammonium acetate (dashed line). ^3H -thymidine incorporation values represent a percentage of control values obtained with similar astrocyte cultures treated with 10% serum.

according to the manufacturer's instructions and equilibrated against 0.05 M ammonium acetate. A 1.5×30 cm column, containing approximately 40 ml bed volume and eluted by gravity at a rate of 25 ml/hr was prepared. The lyophilized UM-05 retentate material was reconstituted in equilibration buffer (5 mg/ml) and applied to the column. The loaded column was eluted with a linear gradient (400 ml) of ammonium acetate (0.05–0.65 M) at a flow rate of approximately 10 ml/hr. Fractions, 3.5 ml, were collected, pooled, and lyophilized; CBGF activity was determined by bioassay.

Reversed-phase (RP) HPLC. Final purification was achieved on a Waters μ Bondapak C_{18} column (3.9 mm $\times$ 30 cm). Fractionation of the active material from the DEAE column was carried out with a shallow gradient of 0–10% acetonitrile in 0.05% TFA during a 10 min period. The column eluant was monitored at either 212 or 280 nm (no differences were detected in the elution profile of equal portions of the same sample), and peaks were collected manually, evaporated to dryness, lyophilized, and stored frozen until assayed for CBGF activity.

Amino acid analysis. Active fractions from the HPLC column were dried under vacuum and placed in a 40 ml vial containing 1 ml of a mixture of HCl : TFA : H_2O (6:2:3, vol/vol) and 100 μl of mercaptoacetic acid. The vial was purged with argon, sealed, and placed in a 165°C oven for 30 min. After cooling, the tubes were dried under vacuum and the hydrolysate reconstituted with 50 μl H_2O . Following derivatization, the PTH-amino acids were analyzed on an automated amino acid analyzer (Applied Biosystems model 120A).

Results

DEAE ion-exchange chromatography of the UM-05 retentate fraction

The elution profile and biological activity distribution after DEAE ion-exchange chromatography of the UM-05 retentate fraction is shown in Figure 1. Most of the biologically active material elutes in one peak (fractions 38–47) at approximately 0.45 M ammonium acetate. This active fraction at 50 ng/ml was roughly 10% more stimulatory to quiescent chick embryo astroblasts than was the addition of fresh basal medium containing 10% FBS and resulted in a 6- to 7-fold increase in ^3H -thymidine incorporation compared with the addition of basal medium supplied with 1% FBS (Table 1). As can be seen in Table 2, an approximately 18-fold purification was achieved by this chro-

matographic step. The material in tubes 38–47 was pooled, lyophilized after concentration, and further fractionated on an RP-HPLC column.

Reversed-phase HPLC separation of the DEAE fraction with CBGF activity

The protein distribution and growth-promoting activity of the fraction obtained by DEAE ion-exchange chromatography was further analyzed by submitting it to reversed-phase HPLC on a C_{18} μ Bondapak column. As shown in Figure 2, when 5–10 μg of protein was processed in this way, significant heterogeneity was apparent in the DEAE fraction. At least 4 UV-absorbing peaks could be distinguished and separated. UV-absorbing peaks were collected manually, evaporated, lyophilized, and assayed for activity on the chick astroblasts. The major peak, eluting at approximately 5% acetonitrile, contained all of the recoverable CBGF activity (Table 1) and represented a 12.5-fold further purification of the mitogenic activity from the DEAE cellulose-52 fraction (Table 2). Repeated chromatography of this active material on the same column under different elution conditions resulted in an apparently homogenous single peak with biological activity.

A typical dose response of HPLC-purified CBGF on ^3H -thymidine incorporation in cultured astrocytes is shown in Figure 3. Half-maximal and maximal stimulation of division in these cells occurred at CBGF concentrations of approximately 5 ng/ml (~ 3 nM) and 10 ng/ml (~ 6 nM), respectively.

Amino acid analysis of HPLC purified CBGF

The amino acid composition of CBGF eluted from the RP-HPLC column and rechromatographed on the same column is given in Table 3. Based on an estimated molecular weight of approximately 1500–2000 Da (Carlone and Rathbone, 1985) and extrapolating to the nearest integer for each determination, CBGF appears to be a small polar peptide, containing predominantly Gly, Glu/Gln, and Asp/Asn.

Figure 2. RP-HPLC fractionation of CBGF activity. CBGF activity recovered from the DEAE 52 column was concentrated, lyophilized, and chromatographed on a C_{18} μ Bondapak column as described under Materials and Methods. The majority of the recoverable CBGF activity elutes as a single, prominent peak (histogram).

Effect of RP-HPLC purified CBGF on cultured blastemas from the regenerating limb of the newt

In a previous study (Carlone and Rathbone, 1985), partially purified CBGF from adult chicken brain was shown to be mitogenic for cultured limb blastemas explanted from the regenerating forelimbs of the adult newt, *Notophthalmus viridescens*. It was of interest to determine whether the more highly purified factor from chick embryo brain was mitogenic for cultured regenerates from the amphibian limb. As can be seen from Table 4, CBGF activity purified by RP-HPLC is mitogenic for cultured newt limb blastemas when tested at 10 ng/ml. Treatment resulted in an average 3.6-fold increase in the percentage of labeled blastema nuclei.

Discussion

A low-molecular-weight peptide growth factor, CBGF, from embryonic chick brain has been purified to apparent homogeneity using DEAE ion-exchange chromatography and RP-HPLC. CBGF has a high specific mitogenic activity on cultured astrocytes from the 10 d chick embryo brain, as well as on cultured regeneration blastemas from the forelimbs of adult newts. Half-maximal stimulation (ED_{50}) of 3H -thymidine incorporation into cultured chick embryo astrocytes occurs at a concentration of approximately 3 nM CBGF. This value is comparable to values exhibited by other purified growth factors and neural peptides such as platelet-derived growth factor (ED_{50} = 1 ng/ml; Raines and Ross, 1985) and bombesin (ED_{50} = 1 nM; Rozengurt and Sinnet-Smith, 1983) and indicates that CBGF acts in a "physiologically relevant" concentration range.

A number of other cell types have been tested for their response to CBGF—including chick embryo myoblasts, meningeal fibroblasts, and neuroblasts and mouse embryo fibroblasts (both primary cultures and established lines)—and have been found to be unresponsive at all concentrations tested (Carlone

Table 1. Fractionation of CBGF activity

Fraction tested	3H -thymidine incorporation ($\times$ cpm/well) $\pm$ SD
Defined medium (DM)	5270 $\pm$ 621
DM + 1% FBS	10,000 $\pm$ 3045
DM + 10% FBS	26,060 $\pm$ 5365
DEAE 52 (pooled, fraction 4) ^a	36,645 $\pm$ 2487
HPLC (fraction 3) ^b	30,782 $\pm$ 3121

Effects of chromatographic fractions on 3H -thymidine incorporation in astrocyte cultures from the 10 d chick embryo. Values represent the means $\pm$ SD for at least 6 replicate cultures for each treatment. Fractions were added to the defined medium supplemented with 1% fetal bovine serum (FBS) in all cases. Concentrations tested were DEAE 52, 100 ng/ml; HPLC, 10 ng/ml.

^a See Figure 1.

^b See Figure 2.

et al., 1987). The spectrum of cell types affected by CBGF *in vitro* is somewhat limited and parallels (with one significant exception) the spectrum of cells stimulated by glial growth factor (GGF), a 31,000 Da protein isolated from bovine brain and pituitary (Lemke and Brookes, 1984). As well as being mitogenic for astrocytes (Brookes et al., 1980) and fibroblasts (Brookes and Lemke, 1981), the distinguishing feature of GGF is its mitogenicity on the cultured rat Schwann cell, a cell type normally unresponsive to a variety of purified growth factors (Salzer et al., 1980). We have not yet determined whether CBGF is mitogenic to cultured Schwann cells of the rat.

CBGF appears to be unlike any of the limited number of purified and well-characterized peptide growth factors. The mass and amino acid composition distinguish it from GGF, M_r = 31,000 (Lemke and Brookes, 1984); murine epidermal growth factor, 6045 (Taylor et al., 1972); human insulin-like growth factors I and II, 7649 and 7471, respectively (Rinderknecht and Humbel, 1978a, b); platelet-derived growth factor, 28–32,000 (Deuel et al., 1981; Raines and Ross, 1982); both acidic and basic fibroblast growth factors from brain, 16,600 and 16,000, respectively (Gospodarowicz et al., 1978; Thomas et al., 1984); and a human T-lymphocyte-derived glial growth promoting activity (GGPF), 30,000 (Benveniste et al., 1985).

Recently, interest has arisen in the concept of neuropeptides as mitogens, a previously unsuspected role for these neural factors (Rozengurt et al., 1979; Globus et al., 1983; Rozengurt and Sinnet-Smith, 1983; Nilsson et al., 1985). Because of its small size, it is possible that CBGF is more closely related to neuro-

Table 2. Summary of the purification of CBGF from chick embryo brain

Step	Total protein (mg)	ED_{50} ^a (ng)	Purification factor (fold)	Yield (% initial)
105,000 $\times$ g supernatant	23,800	125,000	—	100.0
UM-05 filter retentate	4.6	450	278	43.0
DEAE cellulose 52	0.18	25	5000	30.3
C_{18} HPLC	0.02	5	62,500	16.8

The activity was purified from 240 fresh 18 d chick embryo brains as described in the text.

^a ED_{50} is the amount of a given fraction required to give half-maximal stimulation of 3H -thymidine incorporation in the astrocyte cell assay described in the text and as illustrated in Figure 3.

Figure 3. Dose response of HPLC-purified CBGF on ³H-thymidine incorporation in cultured chick embryo astrocytes. Data points are means ± SD for 3 determinations. Bioassays are performed in 24 well culture plates as described under Materials and Methods. Control cultures (0 ng/ml CBGF) received only fresh medium.

peptides such as bombesin, vasopressin, substance P, and Hydra Head Activator, all demonstrated mitogens *in vitro* (Brockes, 1984). Unambiguous determination of the degree of similarity of CBGF to other growth-promoting proteins and peptides found in the nervous system awaits sequence analysis and the rigorous determination of the complete spectrum of cells stimulated by this mitogen *in vitro*.

Table 3. Amino acid composition of CBGF

Amino acid	Residues/mol ^a	
	Observed	Nearest integer
Asx	2.4	2
Thr	0.4	0
Ser	1.2	1
Glx	3.7	4
Pro	0.2	0
Gly	4.3	4
Ala	0.7	1
Cys	0.8	1
Val	0.7	1
Met	0.1	0
Ile	0.1	0
Leu	0.7	1
Tyr	0.2	0
Phe	0.3	0
Trp	0.0	0
His	0.1	0
Lys	0.4	0
Arg	0.0	0
Nle	0.0	0
Total residues		15

^a Based on an estimated molecular weight of 1500. The nearest integer for each determination is shown.

Despite the increasing number of growth factors purified from a variety of tissue sources, it has been difficult in most cases to determine their precise role *in vivo* in specific developmental and regenerative processes. The epimorphic regeneration of the amphibian limb, however, represents a system whereby a more rigorous assessment of the mechanisms underlying the control of cell division by growth factors *in vivo* is possible. In this system, the proliferation of a well-defined population of undifferentiated cells, the blastema, has been shown to be dependent upon a growth factor (or factors) produced by a single, identified heterologous tissue source, the peripheral nerves in the limb stump (Singer, 1978; Carlone and Mescher, 1985; Brockes and Kintner, 1986). Although a number of candidates have been proposed—including fibroblast growth factor (Gospodarowicz and Mescher, 1980), substance P (Globus et al., 1983), glial growth factor (Brockes and Kintner, 1986), and transferrin (Mescher and Munaim, 1984)—the identity of this neurotrophic growth factor remains unknown. The present results would sup-

Table 4. Effect of CBGF on proliferation of newt limb blastema cells *in vitro*

Animal	% Labeled mesenchymal nuclei ^a		
	Control	CBGF-treated ^b	Fold increase
1	1.48	5.18	3.5
2	0.76	3.61	4.8
3	1.56	4.64	3.0
4	0.74	5.51	7.5
5	3.28	5.34	1.6
6	3.89	5.27	1.4

^a Bud stage contralateral blastemas from each animal were excised 72 hr after bilateral surgical resection of the brachial plexi of each animal and cultured as described under Materials and Methods. The values represent percentages of labeled blastema cell nuclei in autoradiographs of mesenchymal squash preparations. More than 1000 nuclei were counted for each determination.

^b CBGF was added to cultures at 10 ng/ml.

port a role for CBGF (or a CBGF-like factor from amphibian neural tissue) in the nerve-dependent stimulation of blastema cell proliferation. Purified CBGF was shown to be mitogenic for blastema cells at physiologically relevant concentrations (10 ng/ml). In addition, in preliminary studies, fractionation of crude adult newt brain homogenates by ultrafiltration, and DEAE cellulose ion-exchange chromatography have revealed a blastema mitogen with biochemical characteristics identical to embryonic chick CBGF (data not shown). Studies are presently underway to determine whether CBGF stimulates proliferation of an identifiable subpopulation of blastema cells whose division has been shown to depend upon the action of peripheral nerve (Kintner and Brookes, 1985).

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