Purification of two astroglial growth factors from bovine brain

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The astroglial growth factor (AGF), which induces a characteristic morphological change in cultured rat astroglial cells and stimulates their proliferation, was purified to homogeneity from bovine brain. Two different methods were used, the second one including heparin-Sepharose affinity chromatography. AGF is actually composed of two factors, AGF1 and AGF2, which both modify the morphology and stimulate the proliferation of the astroglial cells. Several data suggest that the AGFs are similar or possibly identical to the fibroblast growth factors (FGFs) isolated from brain [(1984) Proc. Natl. Acad. Sci. USA 81, 357–361; and 6963–6967]. A specific antiserum against AGFs was raised in mouse.

Growth factor *Cell culture* *Glial cell*

1. INTRODUCTION

The morphological modification of cultured astroglial cells under the influence of brain soluble extracts was first reported in 1972 [3]. Later it was found that these extracts also elicited an increase in the level of the S-100 protein [4] as well as the glutamine synthetase activity [5] and stimulated the proliferation of the cultured astroblasts [6,7].

Partial purification of factors from brain active on the morphology or the proliferation of astroglial cells in culture has been reported by us [7] and by Barritault et al. ([8], our observations), Lim and Miller [9] and Lemke and Brockes [10]. Different denominations were given to these factors: glial maturation factor and subsequently astroglial growth factor (AGF), brain derived growth factor (BDGF), glial maturation factor (GMF) and glial growth factor (GGF), respectively. The first three factors could be identical since they are acidic and their M_r values are not very different: 20000 for AGF [7], 14000 for BDGF [8], 23000 and 13000 for GMF [9,11]. GGF is a basic molecule with an M_r of 31000 [10].

Here we describe two methods for large scale purification of AGF to apparent homogeneity and we bring evidence that there are actually two different factors, AGF1 and AGF2.

2. MATERIALS AND METHODS

2.1. *Test of the AGF on the astroblast morphology*

Cultures of astroblasts were prepared from brain hemispheres of newborn Wistar rats [7]. Cells from one brain were seeded in 30 tissue culture Falcon petri dishes (35 mm diameter), in 2 ml of culture medium. Chromatographic fractions to be tested were added to the medium on day 5 or 6 after seeding.

2.2. *^{125}I-iododeoxyuridine (IdU) incorporation in astroblasts*

Chromatographic fractions found to be active on astroblast morphology were pooled. Aliquots and 1 μCi IdU (5 Ci/mg) were then added to 6-day-old cultures. After 24 h the dishes were rinsed 3 times with 0.9% NaCl. The cells were scraped with a rubber policeman and sedimented at 2000 × g for 10 min; the cell pellets were

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counted in an LKB 1260 gamma counter. Preliminary experiments performed using either cell pellets prepared as described or cells lysed in water and precipitated with 10% trichloroacetic acid showed that these two preparations contained the same radioactivity.

2.3. *Production of mouse polyclonal antibodies*

1 ml of the Pro RPC active fractions (see section 3) was mixed with 10 μl of a control mouse serum and lyophilized. The dried material was dissolved in 100 μl water and mixed with 100 μl of complete Freund's adjuvant. This preparation was injected intraperitoneally into a 2-month-old Balb/c mouse. Three injections were performed every third week.

2.4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining*

SDS-PAGE was performed according to Laemmli [12]. Gels (140 × 160 × 0.75 mm) were composed of a separating gel containing 15% acrylamide and of a stacking gel containing 4% acrylamide. Gels were fixed for at least 60 min in 12% trichloroacetic acid, 24% methanol and 0.01% Coomassie blue R250. They were rinsed twice in 30% methanol for 30 min, and treated with 0.005% dithiothreitol 3 times for 15 min. Staining was achieved by treatment with 0.1% $AgNO_3$ for 25 min, followed by washing with 3% Na_2CO_3 twice for 1 min, and development was performed with 3% Na_2CO_3, 0.03% formaldehyde for 5 min. Stained gels were kept in 3% Na_2CO_3.

2.5. *Electrophoretic transfer and immunoblotting*

To obtain very thin protein bands on the nitrocellulose paper after electrophoretic transfer, the SDS-PAGE was modified: the acrylamide concentration was lowered to 10% and the electrophoresis was performed for 2 h at 400 V instead of 12 h at 120 V.

The procedure of Towbin et al. [13] was used for the transfer of proteins from polyacrylamide gels to nitrocellulose paper. After transfer the nitrocellulose blots were cut into vertical strips and incubated overnight at 4°C in PBS containing 3% bovine serum albumin (BSA). The blot strips were then incubated for 2 h at room temperature with the mouse-antiserum to AGF diluted 1:200 in PBS containing 3% BSA and 10% normal goat serum.

The strips were then washed for 30 min with 5 changes in PBS and incubated at room temperature for 2 h with horseradish-peroxidase coupled goat antimouse immunoglobulins diluted 1:1000. After 30 min washing in PBS (with 5 changes), bound peroxidase was detected using 180 μg 4-chloro-1-naphthol per ml PBS containing 0.01% H_2O_2.

2.6. *Amino acids determination*

Protein samples were hydrolyzed in 6 M HCl containing 2% phenol and 1% β-mercaptoethanol for 24 h at 110°C in nitrogen atmosphere. Cysteine content was determined as cysteic acid after performic acid oxidation [14]. Tryptophan was determined after hydrolysis in 3 M methanesulfonic acid containing 0.2% tryptamine [15].

3. RESULTS

3.1. *First method of purification*

3.1.1. Acidic extraction

Bovine brains (120) obtained fresh from the slaughterhouse were transported on ice to the laboratory. All subsequent steps were carried out at 4°C. Blood clots and bloody meninges were removed. Brains were homogenized through a continuous homogenizer. Then 2 vols of 35 mM citric acid containing 0.5 mM PMSF were added and thoroughly mixed to give a homogenous suspension. This homogenate was centrifuged at 5000 × *g* for 12 min. Supernatants were pooled. Solutions of 2 M Tris and of 0.5 M EDTA in 2 M Tris were added to give a final concentration of 60 mM Tris and 10 mM EDTA. The pH, which was 4.3 in the initial supernatant, was brought to 7.8 with 10 N NaOH.

3.1.2. Blue-Trisacryl M affinity chromatography (IBF, France)

The extract (about 100 l) was applied to a 3.5 l column of Blue-Trisacryl (11 × 36 cm) previously equilibrated in 20 mM Tris-HCl, pH 7.5 (Tris-buffer). The column was washed with 10 l Tris-buffer. Stepwise elution was performed successively with 10 l of 0.5 M NaCl in Tris-buffer and 10 l of 2 M NaCl in Tris-buffer. Activity on the morphology of astroblasts was found in about 6 l of the last eluate.

3.1.3. Acidic precipitation

The solution was acidified to pH 2.5 by addition of 1 N HCl and incubated at 4°C for 2 h. The precipitate was removed by sedimentation at 5000 × *g* for 30 min. The supernatant was diluted with one volume of water and its pH was brought to 6.4 with 1 N NaOH.

3.1.4. Hydroxyapatite-Ultrogel (HA) chromatography (IBF, France)

The solution was applied to an HA column (3.5 × 20 cm) equilibrated with 5 mM sodium phosphate, pH 6.5. The column was washed with 50 mM sodium phosphate pH 6.5, then with 25 mM sodium phosphate, pH 9.0, and the activity was eluted with 200 mM sodium phosphate, pH 9.0.

3.1.5. Mono S chromatography (FPLC Pharmacia)

The active fraction from the HA column (300 ml) was diluted with 900 ml water and the pH was adjusted to 4.5. The solution was applied to a Mono S HR 10/10 column at 6 ml/min. Stepwise elution was performed at 0.36 M and 0.46 M NaCl; the activity was eluted at 0.46 M NaCl in about 40 ml.

3.1.6. Pro RPC reversed phase chromatography (FPLC Pharmacia)

10 ml aliquots of the active fraction from the Mono S column were applied to a Pro RPC HR 5/10 column at 0.25 ml/min. Column was washed with 0.05% trifluoroacetic acid and eluted with a gradient of acetonitrile. Activity was found in 2 areas (fig.1).

3.2. *Second method of purification*

In preliminary experiments we tried a heparin-affinity column which had been used for the purification of an endothelial cell derived growth factor [16]. We found that the heparin column also binds the AGFs and therefore used this for our second method of purification.

This method differs from the first method only by the omission of HA and Mono S columns and the use of a heparin-affinity column instead. It was found also that skipping the 0.5 M NaCl elution of the Blue-Trisacryl column and the acidification of the 2 M NaCl eluate from that column had no effect on the final purification.

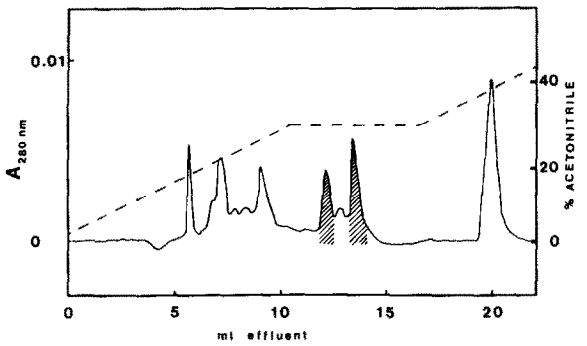

Fig.1. Reversed phase chromatography on Pro RPC Pharmacia column of 10 ml from the Mono S active fraction. Activity on astroglial morphology was found in hatched areas.

3.2.1. Heparin-Sepharose CL-6B affinity chromatography (Pharmacia)

The 2 M NaCl eluate from the Blue-Trisacryl column was diluted with 3 vols of water, its pH was adjusted to 7.0. The preparation was applied to a 25 ml heparin-Sepharose column (1.6 × 12 cm) previously equilibrated with 0.6 M NaCl in 10 mM Tris-HCl, pH 7.0. Stepwise elution was performed in the same buffer but at 1 M NaCl and 2 M NaCl. Activity was found in both eluates (fig.2A). The factors were called AGF1 and AGF2, respectively.

3.2.2. Pro RPC reversed phase chromatography

7 ml aliquots of the 2 active peaks were applied successively to the Pro RPC column. AGF1 was eluted at 25% acetonitrile (fig.2C), and AGF2 at 20% acetonitrile (fig.2B). AGF2 was almost pure after the heparin-Sepharose chromatography but AGF1 was still contaminated. However, after Pro RPC chromatography, in the area of the activity, the AGF1 was also pure.

Analysis by SDS-PAGE (fig.3) shows that AGF1 is composed of two very close bands with an M_r of 17500 and AGF2 is homogenous with an M_r of 18500.

Purification of AGFs is summarized in table 1. The higher amount of AGF1 necessary for half-maximal proliferation stimulation after the Pro RPC chromatography indicates that some inactivation of this factor occurs during the purification step.

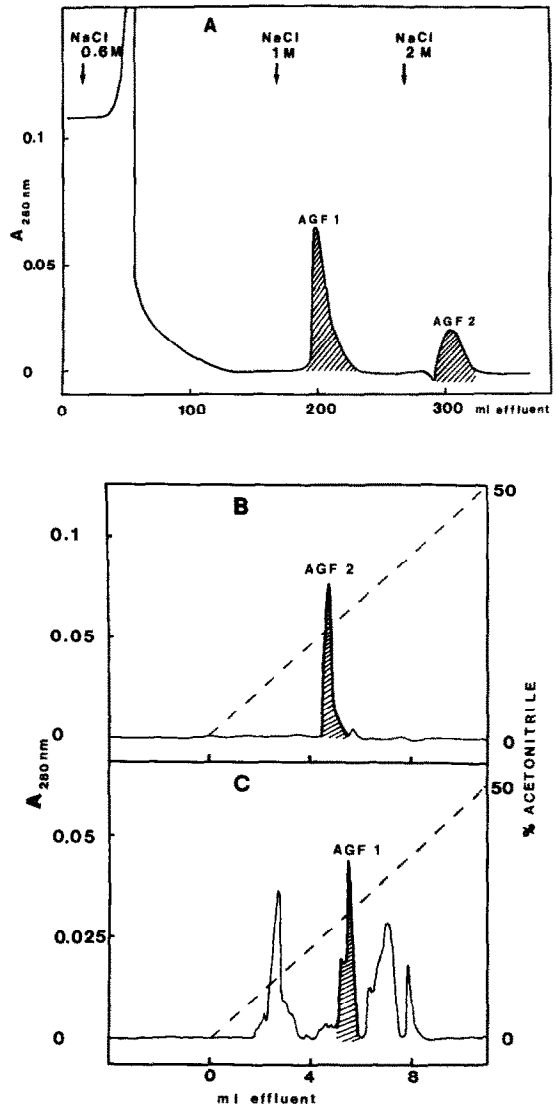

Fig.2. Heparin-Sepharose affinity chromatography of the 2 M NaCl eluate from the Blue-Trisacryl column (A). Reversed phase chromatography on Pro RPC column of the 2 M NaCl eluate (B) and of the 1 M NaCl eluate (C) from heparin-Sepharose. Activity was found in hatched areas.

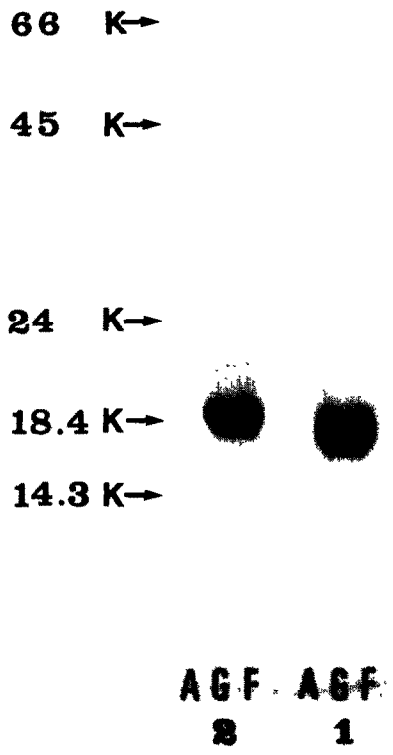

Fig.3. SDS-PAGE of AGFs after reversed phase chromatography.

The amino acid compositions of AGF1 and AGF2 are distinct (table 2).

Antiserum was raised in mouse by immunization with a mixture of AGF1 and AGF2 obtained with the first purification method. The mixture was used because at that time we thought that the two factors were microheterogenous forms of the same protein. The specificity of this antiserum was tested by immunoblotting: AGF1 and AGF2, isolated by the second method, were submitted separately to SDS-PAGE and then transferred to nitrocellulose. The antiserum was found to react with both factors (fig.4). It is specific since it did not react with a blot of the eluate from the Blue-Trisacryl column; however, with this fraction no reaction was seen at the level of the two factors probably because of their very low amount (not shown).

4. DISCUSSION AND CONCLUSION

Since our previous method of purification of the AGF from bovine brain published in 1980 [7], we have tried to improve it and developed various pro-

Table 1

Purification of AGF1 and AGF2 (second method)

Purification step	Protein recovery (mg)	1/2 maximal activity (ng/ml)	Total[a] activity units	Activity recovery (%)	Fold purification
Acidic extract	3.5×10^5	23400	1.50×10^7	100	–
Blue-Trisacryl	1.8×10^4	1920	9.4×10^6	63	12
Heparin peak 1	2.30	3.1	7.4×10^5	4.9	7500
Heparin peak 2 AGF2	1.49	0.47	3.2×10^6	21	49800
Pro RPC AGF1	0.77	5.7	1.4×10^5	1	4100

[a] One unit is defined as the amount of protein necessary to stimulate half-maximal IdU incorporation

Table 2

Amino acid composition of the AGFs

Amino acid	AGF1[a]	Brain[b] HGFα	Acidic[c] brain FGF	AGF2[d]	Brain[b] HGFβ	Basic[e] brain FGF
Asx	14.8 (15)	15	14	13.9 (14)	14	12
Thr	9.7 (10)	9	9	5.5 (6)	5	4
Ser	8.7 (9)	9	10	10.6 (11)	10	9
Glx	15 (15)	18	16	12.2 (12)	14	11
Pro	6.9 (7)	7	7	10.2 (10)	12	9
Gly	13.7 (14)	14	14	16.7 (17)	18	16
Ala	4.8 (5)	5	5	10 (10)	9	9
Cys	4.4 (4)		4	6.2 (6)		5
Val	4.4 (4)	4	5	6.6 (7)	6	5
Met	0.9 (1)	1	1	1.8 (2)	3	2
Ile	6.3 (6)	6	6	4.4 (4)	3	3
Leu	20.8 (21)	19	19	14.8 (15)	13	12
Tyr	7.5 (8)	7	7	7.7 (8)	7	6
Phe	7.2 (7)	7	7	8.6 (9)	8	7
His	5.5 (6)	6	5	3.3 (3)	3	2
Lys	14.2 (14)	13	13	15.6 (16)	14	14
Arg	7.5 (8)	5	6	14.4 (14)	11	10
Trp	0.8 (1)		1	1.08 (1)		1
Total	155			165		
Mean deviation		6.0%	7.1%		14.9%	17.3%

[a] Based on an M_r of 17500

[b] Heparin-binding growth factors purified from brain by Lobb and Fett [18]

[c] From Thomas et al. [1]

[d] Based on an M_r of 18500

[e] Isolated by heparin-Sepharose chromatography by Gospodarowicz e al. [2]

Mean deviation is the mean of the differences between our values and those from other authors, for every amino acid

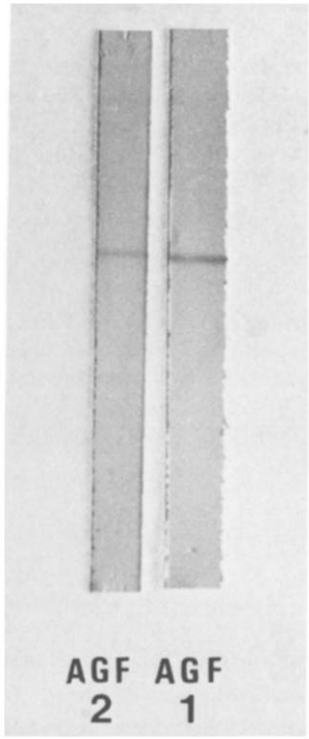

Fig.4. Immunoblot of the mouse AGFs antiserum after reaction with the two isolated factors.

cedures. The two last procedures which allow purification to homogeneity are reported here. The first one gave a very low yield (1–2%) mainly due to the HA column which attached only about 10% of the activity. No more activity was attached in a second passage through a new HA column. This behaviour is not explained. The second method is a combination of the first one and of a method described by Shing et al. [16] using heparin-affinity chromatography for the purification of an endothelial cell growth factor. Since then, the heparin-affinity step has also been used by several other authors to purify growth factors from nervous tissues [2,17,18] or from other sources [18,19].

We determined that AGF1 is acidic and has an isoelectric point of 5.5 and AGF2 is basic with an isoelectric point around 9.5. This observation added to the quasi-identity of their molecular masses suggest that the other acidic factors active on glial cells previously described [7,8,9,11] are of the same family or identical to AGF1. The GGF [10] which is basic could be related to AGF2, however, the molecular masses are different.

Comparison of the amino acid composition of AGFs with that of other growth factors isolated

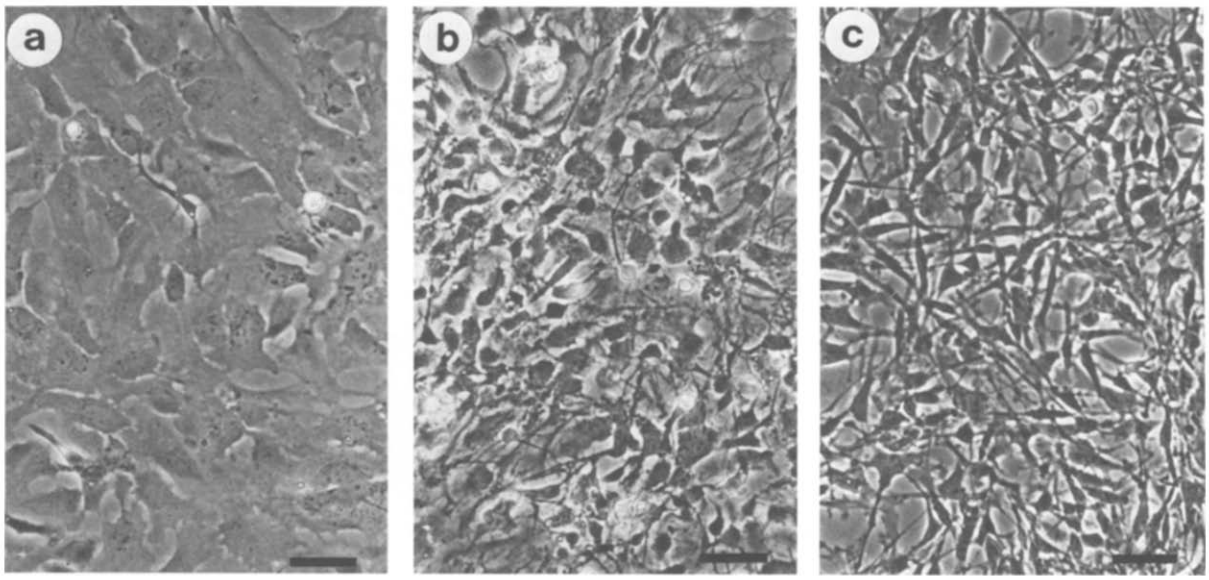

Fig.5. Astroglial cultures from cerebral hemispheres of newborn rat grown in nutrient medium alone for 8 days (a), in the presence of 10 ng/ml AGF1 from day 6 to 8 (b), in the presence of 10 ng/ml AGF2 from day 6 to 8 (c). Scale bar: 50 μm.

from brain and affecting proliferation of various cell types (table 2) shows a high homology between AGF1, the acidic FGF purified by Thomas et al. [1], and the heparin-binding growth factor α (HGFα) from Lobb and Fett [18] which was also eluted at 1 M NaCl from heparin-Sepharose. Good homology is also found between AGF2 and the basic FGF from Gospodarowicz et al. [2] or the HGFβ from Lobb and Fett [18], both eluted at high salt molarity from heparin-Sepharose. Molecular masses of all these factors are very close. These different data suggest that there are two families of growth factors, or possibly only two growth factors in the brain which are able to stimulate the proliferation of a variety of cell types.

The two AGFs modify the morphology of the astroglial cells but the effect is not identical (fig.5). The effects on the proliferation are similar, although AGF2 seems to be more active than AGF1. More extensive comparison of the effects elicited by the two factors on the astroglial cells has now to be considered.

The antiserum raised against AGFs might allow us to gain information about the possible physiological role of these growth factors.

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