Isolation and characterization of two different molecular forms of basic fibroblast growth factor extracted from human placental tissue

SYLVIE UHLRICH*, JÉRÔME TIOLLIER, MICHEL TARDY and JEAN-LOUIS TAYOT

IMEDEX, Z.I. Les Troques, B.P. 38, 69630 Chaponost (France)

ABSTRACT

Basic fibroblast growth factor (bFGF) was purified to homogeneity from human placental tissue on a semi-large scale. Placental bFGF consists of two proteins of apparent molecular masses 16 000 and 18 000 dalton, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis under non-reducing conditions. Microsequence analysis showed that both proteins have the same N-terminal sequence Pro–Ala–Leu–Pro–Glu–Asp–Gly–Gly Ser–Gly–Ala–Phe..., which is identical with that of (1–146) bFGF extracted from human brain. After reduction by dithiothreitol or mercaptoethanol, placental bFGF appears as a single protein of 16 000 dalton. The reduced protein displays the same ability to stimulate the proliferation of CCL39 fibroblasts as the non-reduced doublet. These data indicate that bFGF extracted from placental tissue consists of two proteins with different apparent molecular masses which do not differ in their N-terminal sequence but in their oxidation state.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a heparin-binding polypeptide mitogen for a wide variety of mesodermal and neuroectodermal cells [1,2]. It was first isolated from bovine pituitary as a single-chain 16 500-dalton protein composed of 146 amino acids [3]. A number of N-terminal modified forms of bFGF have been identified, depending on the source and the method of isolation: bFGF extracted from bovine brain, retina and human brain under acidic conditions had 146 amino acids [4–6] whereas extraction of bovine kidney, adrenal, corpus luteum and testis [7–10] under acidic conditions yielded a truncated form of bFGF lacking the first fifteen amino acids. N-Terminal extended forms of bFGF have been described: an eight amino acid extension on extraction of human prostate under basic conditions [11] and a thirteen amino acid extension on extraction of human placenta after brief acidification [12]. A 25 000-dalton form has also been purified from guinea pig brain [13] and a 22 000-dalton immunoreactive form has been isolated from rat brain [14]. These observations indicate that bFGF consists of different molecular forms. These forms may be naturally occurring processed forms or may be generated by proteases released during the purification procedure.

Elucidation of the molecular heterogeneity of bFGF was also done by studying

the role of the four cysteines contained in bFGF. By using site-directed mutagenesis, Arakawa *et al.* [15] demonstrated that the analogue protein deprived of all four cysteines exhibited mitogenic activity on NIH 3T3 cells which was indistinguishable from natural sequence protein. In a same manner, Seno *et al.* [16] showed that selective substitution of cysteine residue 69 or 87 by serine did not affect the heparin-binding ability of the protein but reduced its heterogeneity, recognized as several peaks of bFGF eluted from a heparin affinity column.

We report here for the first time the molecular heterogeneity of naturally occurring bFGF due to different oxidation states. Whereas all variations in apparent molecular mass of extracted bFGF which have been described up to now could be attributed to N-terminal variations, we show here that bFGF extracted from placental tissue under acidic conditions consists of a doublet of proteins which display the same N-terminal sequence but differ from each other in their oxidation state.

EXPERIMENTAL

Purification of placental bFGF

Placentas were collected at delivery and immediately frozen at $-20°C$. Pools of 500–600 placentas were mechanically ground into small pieces and then stirred, until completely thawed, in an 8% (v/v) ethanol water solution. Placental blood was separated from the tissue on a press. The placental tissue pulp underwent further acid extraction at pH 3.2 at 4°C for 30 min. The acid extract was separated from the residual placental pulp on a press, neutralized and precipitated with 15% ethanol. The supernatant was recovered by centrifugation, concentrated by ultrafiltration and finally diafiltered against 0.1 M sodium phosphate (pH 6.0) with 10 000-dalton cut-off membranes. One litre of final placental tissue crude extract corresponded to *ca.* 15 kg of placental tissue pulp. The extract (198 g of proteins) was then applied to a 30 $\times$ 10 cm I.D. column of CM-Trisacryl (IBF Biotechnics, Villeneuve la Garenne, France) and eluted with the above-mentioned buffer until the absorbance at 280 nm dropped to the baseline at 2.0 a.u.f.s. The unretained fraction was discarded. The CM-Trisacryl column was eluted with 0.1 M sodium phosphate (pH 6.0) containing 0.15 M NaCl and then with 0.6 M NaCl buffer. The 0.15 M NaCl eluate showed of three successive absorbance peaks, denoted CM-1, CM-2 and CM-3. Fractions CM-2 and CM-3 contained most bFGF and were further purified on heparin–Sepharose (Pharmacia, Uppsala, Sweden). Each fraction was adjusted to 0.1 M sodium phosphate–0.65 M NaCl (pH 7.2) and pumped onto a heparin–Sepharose column (11 $\times$ 9 mm I.D.) that had previously been equilibrated with 6.5 mM Na$_2$HPO$_4$–1.5 mM KH$_2$PO$_4$–2.7 mM KCl 0.65 M NaCl (pH 7.2). The heparin–Sepharose column was washed with the same buffer until the absorbance at 206 nm dropped to the baseline at 0.5 a.u.f.s. and then eluted with the same buffer containing successively 1 and 2 M NaCl. Both 2 M NaCl eluates obtained from the CM-2 and CM-3 fractions were analysed and further stored at $-70°C$.

Protein determination

Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Labs., Richmond, CA, U.S.A.) using human albumin (Pasteur Mérieux Serums et Vaccins, Lyon, France) as a standard.

Analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE following standard techniques [17] in the presence or absence of 2% (v/v) mercaptoethanol in 1-mm thick 20% acrylamide gels stained with silver nitrate [18]. About 1 μg of each sample was desalted on a PD10 column (Pharmacia) and lyophilized before SDS-PAGE. Molecular mass standards (Boehringer, Mannheim, Germany) were α_2-macroglobulin (170 000), phosphorylase B (97 400), glutamate dehydrogenase (55 400), lactate dehydrogenase (36 500), trypsin inhibitor (21 500), cytochrome c (12 500) and aprotinin (6500 dalton).

Preparative SDS-PAGE

Proteins (typically 5–10 μg per lane) were loaded on the same SDS-polyacrylamide gels as for analytical purposes (see above). After electrophoresis, the gels were dipped for a few seconds in water and then reversibly stained by gentle rocking in 250 ml of 0.3 M CuCl$_2$ solution for 5 min at room temperature [19]. The gels were then washed in water to remove excess of reagent. The protein bands appeared as negatively stained areas. Each band of the bFGF doublet (namely 16K and 18K, where K = kilodalton) was excised from the gel. Copper stain was eliminated by incubation in three changes (10 min each) of 0.25 M EDTA 0.25 M Tris HCl (pH 9.0). Proteins were then eluted from the gels by vigorous rocking for 72 h at 4°C in 20 mM Tris–HCl–0.15 M glycine–0.01% SDS. The recovery was between 10 and 20%.

Reversed-phase high-performance liquid chromatography (HPLC)

A Waters Model 600 HPLC system was used, coupled with a Waters 490 programmable multi-wavelength detector (Millipore–Waters, Milford, MA, U.S.A.). Samples were loaded onto a C$_4$ reversed-phase HPLC column (Vydac 214TP54) (250 × 4.6 mm I.D.) equilibrated with 10% acetonitrile 0.05% trifluoroacetic acid at a flow-rate of 1.0 ml/min. The column was washed with 30 ml of this solvent and bound proteins were eluted with a 60-ml gradient of 10–50% acetonitrile–0.05% trifluoroacetic acid. Proteins were detected by monitoring the absorbance at 215 nm. Fractions of 1 ml were collected. For 16K and 18K chromatography after elution from SDS-polyacrylamide gels, samples were submitted to a first run as described. The main peak was collected, lyophilized, resuspended in 20 mM Tris–0.15 M glycine–0.01% SDS and rechromatographed under the same conditions before sequencing.

Protein sequence analysis

Protein sequence analysis was carried out at the Laboratoire Central d'Analyses (CNRS, Solaise, France) on a Model 470A gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.) connected "on-line" with a Model 120A phenylthiohydantoin amino acid analyser (Applied Biosystems).

Growth factor assays

The biological characterization of growth factor activities was performed by proliferation assays on bovine corneal endothelial cells and CCL39 fibroblasts. Corneal endothelial cells (EC) were isolated from bovine eye corneal endothelium by trypsinization and scraping. Once cultures had been established, EC were grown and maintained in culture flasks using Minimum Essential Medium (MEM) containing 6% foetal calf serum (FCS) and 100 μg/ml of crude placental extract. CCL39

fibroblasts (ATCC, Rockville, MD, U.S.A.) were subcultivated in MacCoy's medium supplemented with 20% FCS.

For both types of cells, proliferation assays were performed in 24-well plates (Falcon), in serum-free medium (DMEM/F_{12}) supplemented with insulin, transferrin, albumin and selenite and with increasing amounts of bFGF fractions to be tested. For EC, 10 000 cells were seeded at day 0 (D0) and stimulation with bFGF fractions were performed at D0, D1, D2, D3 and D4. Medium changes occurred at D2 and D4 and the total number of cells was determined after trypsinisation at D7. For CCL39 fibroblasts, 50 000 cells per well were seeded at D0. Stimulation with bFGF fractions was performed at D0, D1, D2 and D3. The medium was changed at D2 and the final total number of cells was determined at D4.

Reduction of bFGF

A 30-μg sample of bFGF was adjusted in 0.1 M Tris (pH 8)–1.5 mM EDTA–10 mM dithiothreitol and incubated for 1.5 h at 37°C. Reduced sulphydryl groups were then blocked with 20 mM iodoacetamide for 30 min at 37°C. Samples were then either desalted against water for SDS-PAGE or desalted against PBS–0.02% Tween 20 and assayed for biological activity.

RESULTS

Purification of placental bFGF

Placental tissue was processed after separation of the placental blood on a press. The extraction of placental tissue at pH 3.2 yielded a final placental extract which contained *ca.* 2.6 g of proteins per kilogram of tissue. When applied to a CM-Trisacryl in 0.1 M phosphate at pH 6.0, 90–95% of the proteins were recovered in the unretained fraction. bFGF was bound to the gel and eluted with 0.15 M NaCl. The 0.15 M NaCl eluate showed of three successive absorbance peaks (namely CM-1–3) (Fig. 1). After a second chromatography on heparin–Sepharose, only fractions CM-2 and CM-3 appeared to contain bFGF, which was eluted from heparin–Sepharose by stepwise elution at 2 M NaCl. The final bFGF fractions obtained from either CM-2 or CM-3 (namely HS-CM-2 and HS-CM-3), were analysed by SDS-PAGE (Fig. 2) under non-reducing conditions and consisted of two proteins of *ca.* 16K and 18K. The recovery was quantitative with a yield of purification of 15 μg of bFGF per kilogram of placental tissue.

The biological activity of placental bFGF was characterized on endothelial cells (Fig. 3): both bFGF fractions HS-CM-2 and HS-CM-3 stimulated the proliferation of bovine corneal endothelial cells. The ED_{50} (concentration of proteins necessary to induce half-maximum stimulation) was equivalent for both fractions and *ca.* 0.5 ng/ml. Cell growth depended on the concentration of bFGF in the medium and reached a plateau at 20 ng/ml.

Further characterization of placental bFGF

Separation of 16K and 18K proteins. We tried to separate the 16K and 18K proteins contained in the bFGF fraction by reversed-phase HPLC with an acetonitrile gradient, but both proteins coeluted in the same peak at 35% acetonitrile. However, both proteins could be separated by SDS-PAGE. The bFGF fraction was applied to an
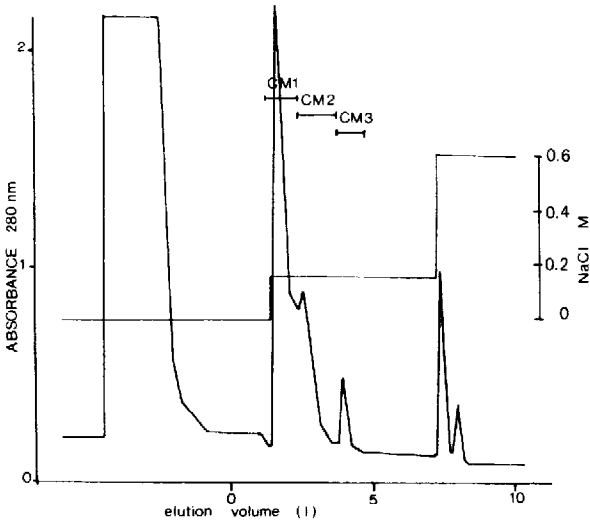
Fig. 1. Purification of crude acid placental extract by cation-exchange chromatography on CM-Trisacryl (30 × 10 cm I.D.) in 0.1 M sodium phosphate (pH 6.0). Stepwise elution with NaCl (0.15 and 0.6 M); flow-rate, 1.4 l/h.
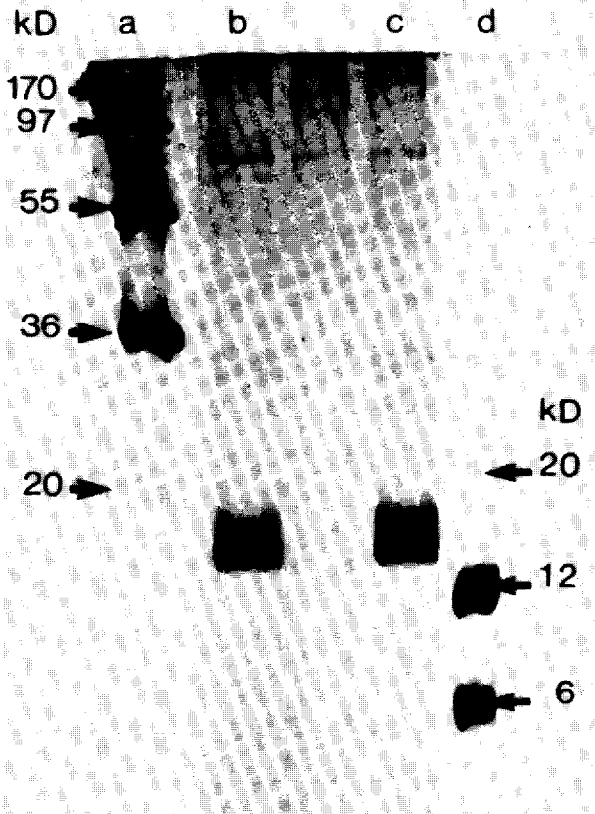

Fig. 2. SDS-PAGE (20%) under non-reducing conditions. Lanes a–d = molecular mass standards; b = placental bFGF purified from CM-2 eluate (HS-CM-2); c = placental bFGF purified from CM-3 eluate (HS-CM-3). kD = Kilodalton.
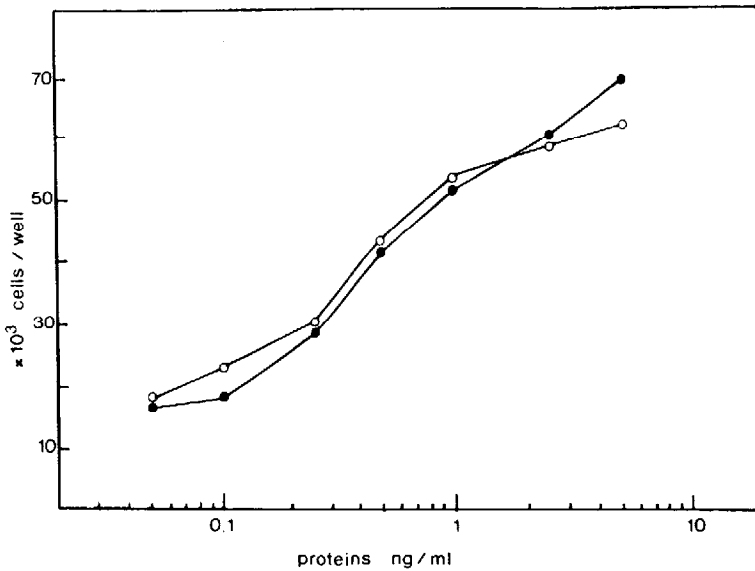
Fig. 3. Biological activity of placental bFGF HS-CM-2 (●) and HS-CM-3 (○) eluate on proliferation of bovine corneal endothelial cells. 10 000 cells at day 0; stimulation with bFGF fractions at day 1, 2, 3 and 4; medium changes at day 2 and 4; determination of total number of cells at day 7.

SDS-PAGE (20%) gel. The bands were reversibly stained with copper chloride, cut out from the gel and eluted by incubation in Tris glycine buffer. The two proteins were further chromatographed by reversed-phase HPLC. Each protein eluted as a sharp peak at 44% acetonitrile (Fig. 4). SDS-PAGE of each HPLC eluate (Fig. 5) showed that the main peak consisted of the homogeneous protein, but the presence of dimeric (Fig. 5, lanes d and j) and oligomeric structures (Fig. 5, lanes e–f and k–l) was detected in the tail of the peak. This observation could be made for both 16K and 18K, indicating that both proteins are able to make aggregates (either by covalent or non-covalent bonds) under the physico-chemical conditions of reversed-phase HPLC elution. The amino-terminal sequence analysis of 16K and 18K showed that both proteins have the same sequence, Pro Ala Leu–Pro Glu–Asp–Gly–Gly–Ser–Gly–Ala–Phe–Pro–Pro–Gly–His–Phe–Lys–, which is identical with that of (1–146) bFGF extracted from human brain (Table I). Moreover, for both proteins, microsequence analysis showed the presence of degraded polypeptides lacking the last amino acid (Pro) and the last four amino acids (Pro–Ala–Leu–Pro).

Effect of reducing agents on 16K and 18K proteins. The placental bFGF fraction, which consists of a 16K–18K doublet, was submitted to reduction by dithiothreitol followed by iodoacetamide alkylation. SDS-PAGE of the reduced fraction (Fig. 6, lane d) showed a single band at 16 000 dalton. Moreover, the direct analysis of the bFGF fraction in the presence of mercaptoethanol confirmed that the 18K bFGF form is transformed into the 16K bFGF form by reduction (Fig. 6, lane c). The biological activity of placental bFGF was also determined after reductive alkylation on CCL39 fibroblasts. As shown in Fig. 7, the bFGF fraction was equally active after
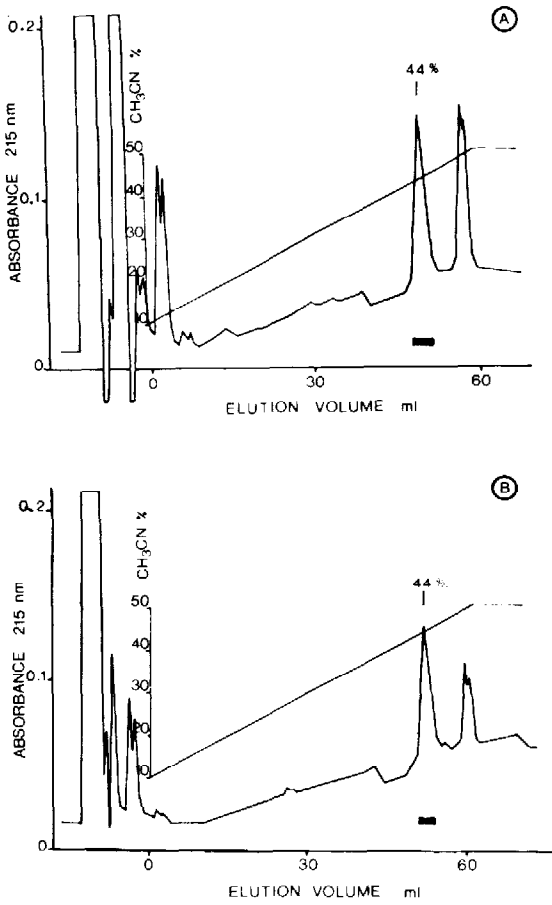
Fig. 4. Purification of 16K and 18K placental bFGF by reversed-phase HPLC on a Vydac C₄ column (250 × 4.6 mm I.D.). Flow-rate, 1.0 ml/min; 60-min gradient elution from 10 to 50% acetonitrile in water–0.05% trifluoroacetic acid. Elution profiles of (A) 16K and (B) 18K protein.

and before reduction, ED_{50} values ranging between 0.7 and 1 ng/ml as determined from four different experiments.

DISCUSSION

After extracting placental tissue by brief exposure to an acidic pH, we purified placental bFGF with two chromatographic steps involving cation-exchange chromatography followed by affinity chromatography on immobilized heparin. The resulting bFGF fraction consists of two proteins of apparent molecular masses 16K and 18K, as determined by SDS-PAGE under non-reducing conditions. Two molecular forms of bFGF have already been described in human placenta: the classical (1–146) bFGF identical with human brain bFGF [20] with a molecular mass of 16 400 dalton and a thirteen amino acid N-terminally extended form [12] with a molecular mass of
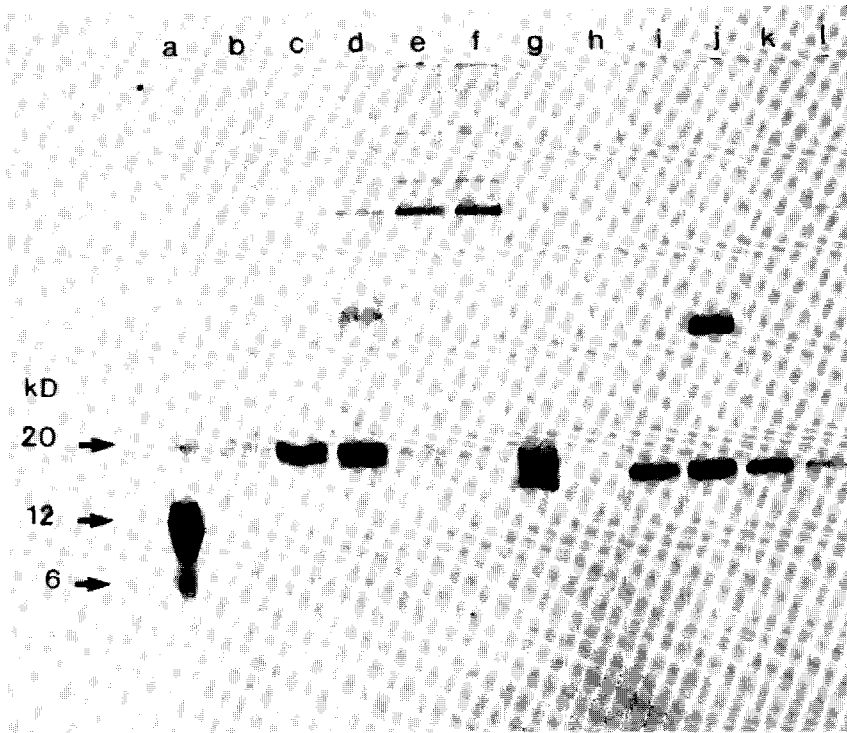
Fig. 5. SDS-PAGE (20%) under non-reducing conditions. Lanes: a = molecular mass standards; b = beginning of 18K peak on HPLC; c = 18K HPLC peak; d,e,f = tail of 18K HPLC peak; g = placental bFGF; i = 16K HPLC peak; j,k,l = tail of 16K HPLC peak. kD = Kilodalton.

17 500 dalton. However, in our case, N-terminal amino acid sequence analysis of the 16K and 18K proteins revealed that both proteins have the same N-terminal sequence, which is identical with that of brain (1–146) bFGF. Moreover, we showed that the 16K–18K doublet appears as a single 16K protein after reductive alkylation or by SDS-PAGE under reducing conditions. (1–146) bFGF contains four cysteines at positions 25, 69, 87 and 92, among which only residues 25 and 92 are conserved through the whole FGF family [21]. By site-directed mutagenesis and replacement of cysteine with serine residues, it was shown that substitution of cysteine residues at positions 69 and 87 did not affect the biological activity and heparin-binding ability of bFGF [16]. Moreover, in contrast to the natural sequence form, SDS-PAGE of the serine-69, 87 analogue under non-reducing conditions revealed little dimer formation [22]. Our data show that placental bFGF is equally active before and after reductive alkylation, indicating that the 18K protein displays the same biological activity as the 16K reduced form. Reversed-phase HPLC of each protein revealed the presence of dimers and oligomers in both instances, which disappeared on analysis in the presence of reducing agent (data not shown). These observations suggest that the 16K and 18K proteins differ from each other only in their oxidation state. The observation that they are both able to form intermolecular bonds supports the hypothesis that both proteins

TABLE I

AMINO-TERMINAL SEQUENCE ANALYSIS OF BOTH PLACENTAL bFGF MOLECULAR FORMS

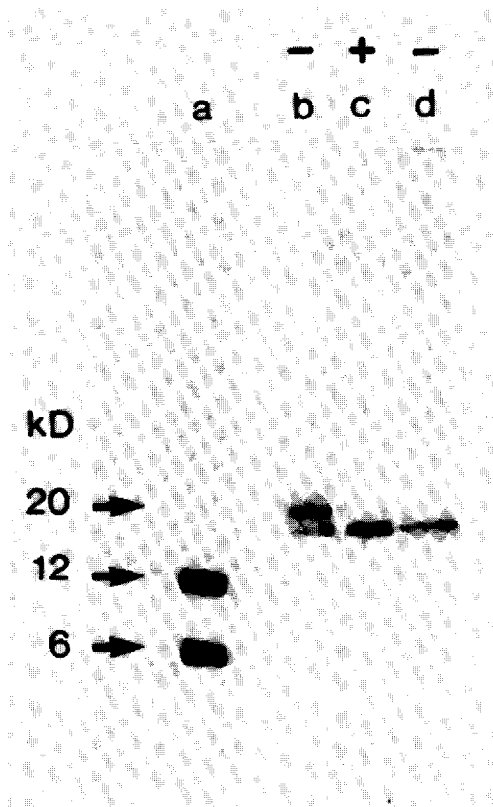| Cycle	PTH-amino acid	bFGF 16K yield (pmol)	bFGF 18K yield (pmol)	Cycle	PTH-amino acid	bFGF 16K yield (pmol)	bFGF 18K yield (pmol)
1	Pro	42	22	11	Ala	10	4
2	Ala	18	7	12	Phe	10	4
3	Leu	16	6	13	Pro	6	4
4	Pro	15	8	14	Pro	5	1
5	Glu	15	5	15	Gly	4	?
6	Asp	10	4	16	His	3	2
7	Gly	3	4	17	Phe	6	2
8	Gly	7	0. 5	18	Lys	4	2
9	Ser	n.d.[a]	n.d.[a]	19	Asp	3	
10	Gly	7	2	20	Pro	2	

[a] n.d. = Not determined.

Fig. 6. SDS-PAGE (20%) in the presence (+) or absence (−) of mercaptoethanol. Lanes: a = molecular mass standards; b,c = native placental bFGF; d = placental bFGF after reductive alkylation (dithiothreitol + iodoacetamide). kD = Kilodalton.
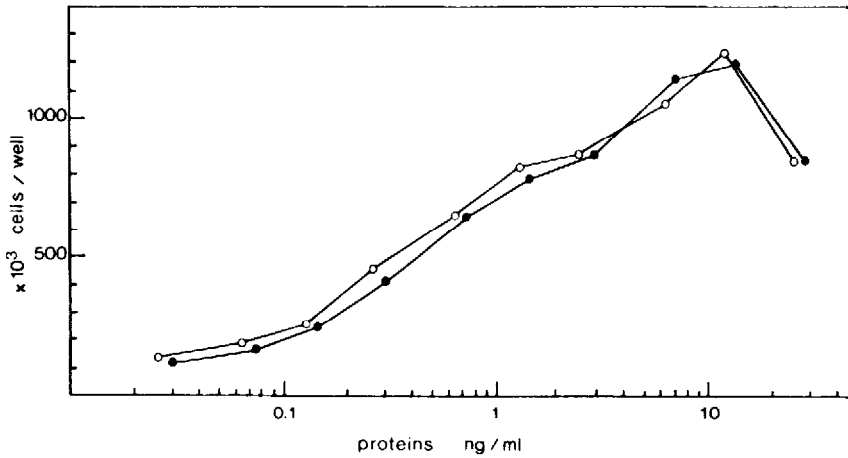
Fig. 7. Biological activity of placental bFGF native (●) or after reduction by dithiothreitol (○) on proliferation of CCL39 fibroblasts. 50 000 cells at day 0; stimulation with bFGF fractions at day 0, 1, 2 and 3; medium changes at day 2 and 4; determination of total number of cells at day 7.

contain free sulphydryl groups, the 16K protein containing all four cysteines free whereas the 18K protein contains at least one disulphide bridge. Moreover, the full biological potential of the 16K reduced form is consistent with the findings of Arakawa *et al.* [15], who showed that human bFGF analogue in which all four cysteines have been replaced with serine exhibited mitogenic activity on NIH 3T3 cell which was indistinguishable from the natural sequence molecule.

ACKNOWLEDGEMENTS

REFERENCES

1 D. Gospodarowicz and J. S. Moran, *Annu. Rev. Biochem.*, 45 (1976) 531.
2 A. Baird, F. Esch, P. Normede, N. Ueno. N. Ling, P. Bohlen, S. Y. Ying, W. B. Wehrenberg and R. Guillemin, *Recent Prog. Horm. Res.*, 42 (1986) 143.
3 F. Esch. A. Baird, N. Ling, N. Ueno, F. Hill, L. Denoroy. R. Klepper. D. Gospodarowicz, P. Bohlen and R. Guillemin, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 6507.
4 D. Gospodarowicz. J. Cheng, G. M. Lui, A. Baird and P. Bohlen, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 6963.
5 A. Baird, F. Esch, D. Gospodarowicz and R. Guillemin, *Biochemistry*, 24 (1985) 7856.
6 P. Bohlen, F. Esch. A. Baird, K. L. Jones and D. Gospodarowicz *FEBS Lett.*, 185 (1985) 177.
7 A. Baird, F. Esch, P. Bohlen. N. Ling and D. Gospodarowicz, *Regul. Pept.*, 12 (1985) 201.
8 D. Gospodarowicz, A. Baird, J. Cheng, G. M. Lui, F. Esch and P. Bohlen, *Endocrinology*, 118 (1986) 82.
9 D. Gospodarowicz, J. Cheng, G. M. Lui, A. Baird, F. Esch and P. Bohlen, *Endocrinology*, 117 (1985) 2383.

10 N. Ueno, A. Baird, F. Esch, N. Ling and R. Guillemin, *Mol. Cell. Endocrinol.*, 49 (1987) 189.
11 M. T. Story, F. Esch, S. Shimasaki, J. Sasse, S. C. Jacobs and R. K. Lawson, *Biochem. Biophys. Res. Commun.*, 142 (1987) 702.
12 A. Sommer, M. T. Brewer, R. C. Thompson, D. Moscatelli, M. Presta and D. B. Rifkin, *Biochem. Biophys. Res. Commun.*, 144 (1987) 543.
13 A. Sommer, D. Moscatelli and D. B. Rifkin, *Biochem. Biophys. Res. Commun.*, 160 (1989) 1267.
14 M. Presta, M. Rusnati, J. A. M. Maier and G. Ragnotti, *Biochem. Biophys. Res. Commun.*, 155 (1988) 1161.
15 T. Arakawa, Y. R. Hsu, S. G. Schiffer, L. B. Tsai, C. Curless and G. M. Fox, *Biochem. Biophys. Res. Commun.*, 161 (1989) 335.
16 M. Seno, R. Sasada, M. Iwane, K. Sudo, T. Kurokawa, K. Ito and K. Igarashi, *Biochem. Biophys. Res. Commun.*, 151 (1988) 701.
17 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
18 W. Wray, T. Boulikas, V. P. Wray and R. Hancock, *Anal. Biochem.*, 118 (1981) 197.
19 C. Lee, A. Levin and D. Branton, *Anal. Biochem.*, 166 (1987) 308.
20 D. Gospodarowicz, J. Cheng, G. M. Lui, D. K. Fujii, A. Baird and P. Bohlen, *Biochem. Biophys. Res. Commun.*, 12 (1985) 554.
21 T. Yoshida, K. Miyagawa, H. Odagiri, H. Sakamoto, P. F. R. Little, M. Terada and T. Sugimura, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 7305.
22 G. M. Fox, S. G. Schiffer, M. F. Rohde, L. B. Tsai, A. R. Banks and T. Arakawa, *J. Biol. Chem.*, 263 (1988) 18452.