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M_r 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor

(plasminogen activator/capillary endothelial cells/heparin affinity/basic fibroblast growth factor receptor/radioimmunoassay)

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ABSTRACT **A M_r 25,000 form of basic fibroblast growth factor (bFGF) has been isolated from guinea pig brain along with the typical M_r 18,000 form. Both forms were purified to homogeneity by a combination of heparin-affinity chromatography and ion-exchange chromatography on an FPLC Mono S column. The M_r 25,000 form, like the M_r 18,000 form, was not eluted from the heparin-affinity column with 0.95 M NaCl, but was eluted with 2 M NaCl. The M_r 25,000 guinea pig protein stimulated plasminogen activator production by cultured bovine capillary endothelial cells in a dose-dependent manner at concentrations of 0.1–10 ng/ml, the same range that was effective for guinea pig and human M_r 18,000 bFGFs. The binding of human ^{125}I-labeled bFGF to baby hamster kidney cells is inhibited equally by the M_r 25,000 guinea pig protein and the M_r 18,000 guinea pig and human bFGFs. Polyclonal antibodies raised against human bFGF recognize both the M_r 25,000 and 18,000 guinea pig proteins in an immunoblot analysis. In a radioimmunoassay, both the M_r 25,000 and M_r 18,000 guinea pig proteins compete equally well with iodinated human bFGF for binding to the anti-human bFGF antibodies. When treated with low concentrations of trypsin, the M_r 25,000 guinea pig bFGF was converted to a M_r 18,000 protein. These results show that the two molecules are closely related and suggest that the M_r 25,000 protein shares substantial homology with the M_r 18,000 bFGF.**

Basic fibroblast growth factor (bFGF) is an endothelial cell mitogen that binds strongly to heparin-Sepharose (1). bFGF isolated from bovine pituitary has a molecular weight of ≈16,500 and is composed of 146 amino acids (2). bFGFs of the same molecular weight have been purified from bovine and human brain (1, 3), human placenta (4), bovine retina (5), and bovine adrenal gland (6). Truncated forms of bFGF lacking the first 15 amino acids have been isolated from bovine kidney (2), adrenal gland (6), and corpus luteum (7). Heparin-binding endothelial cell mitogens with molecular weights of 16,000–18,000 have also been isolated from a variety of tissues (8–14). These molecules appear to be closely related or identical to bFGF.

A M_r 18,000 heparin-binding protein that stimulates plasminogen activator (PA) and collagenase production in cultured bovine capillary endothelial (BCE) cells has been purified from human placenta (15) and from cultured human hepatoma cells (16). This molecule also stimulated DNA synthesis and motility in BCE cells (15, 16). Amino acid sequence analysis of the placental protein has shown that it is human bFGF. The human bFGF is a 157-amino acid protein, having an 11-amino acid extension at the N-terminal end compared to bovine pituitary bFGF (17).

Here we describe a M_r 25,000 form of bFGF that is present in guinea pig brains in addition to the characteristic M_r 18,000 form described for a variety of tissues. The M_r 25,000 form is shown to be bFGF by its interaction with polyclonal antibodies to human placental bFGF, its competition with bFGF for binding to the bFGF receptor, and its ability to stimulate the production of PA in BCE cells, a known biological activity for bFGF isolated from a variety of cell types (18).

METHODS

Cell Culture. BCE cells were isolated from the adrenal cortex of recently slaughtered yearling cattle by the method of Folkman *et al.* (19). Initial isolates were grown in the α modification of minimal essential medium (α-MEM) containing 10% (vol/vol) calf serum and supplemented with medium conditioned by mouse sarcoma 180 cells as described (19). Once cultures were established, they were grown in α-MEM containing 5% (vol/vol) calf serum and no conditioning factors.

Baby hamster kidney (BHK) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) calf serum.

Assays for Induction of PA. The medium on confluent cultures of BCE cells was replaced by fresh α-MEM containing 5% (vol/vol) calf serum and the substance to be tested. After incubation in this medium for 18 hr at 37°C, the cells were washed twice with cold PBS1 (137 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.5) and were extracted with 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. The extracts were assayed for PA as described (20).

Purification of bFGF. Frozen guinea pig brains were obtained from Rockland Farms (Gilbertsville, PA) and from Bio-Trol (Indianapolis, IN). The brains were homogenized while still frozen in cold 20 mM Tris·HCl (pH 7.5) containing 3 mM EDTA and were sonicated for 10 min at 50 W. The sonicate was adjusted to pH 4.0, held at this pH for 2 min, and then neutralized. Sodium chloride was added to a final concentration of 0.5 M, and the sonicates were centrifuged at 10,000 × *g* for 45 min. The supernatant was loaded on a 85 × 40 mm column of heparin-Sepharose (Pharmacia), and the column was washed with 0.5 M NaCl in 20 mM Tris·HCl (pH 7.5) and 3 mM EDTA. Partially purified bFGF was then eluted with 2 M NaCl in the same buffer. The eluate was diluted with 20 mM Tris·HCl (pH 7.5) containing 3 mM EDTA until the conductance was 24 mS and loaded on a second heparin-Sepharose column (25 × 60 mm). This column was washed sequentially with 0.8 M NaCl and 0.95 M NaCl, each in 20 mM Tris·HCl (pH 7.5) and 3 mM EDTA. bFGF was eluted with 2 M NaCl in the same buffer. The eluate was dialyzed against 0.2 M NaCl in 20 mM Mes (pH 6.0) and centrifuged at 100,000 × *g* for 60 min. The supernatant was

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Abbreviations: bFGF, basic fibroblast growth factor; BCE, bovine capillary endothelial; BHK, baby hamster kidney; PA, plasminogen activator.

loaded on an FPLC (fast protein liquid chromatography) Mono S column (Pharmacia) equilibrated with the same buffer. The Mono S column was eluted with a 0.2–0.6 M NaCl gradient in 20 mM Mes (pH 6.0).

$NaDodSO_4$/PAGE. $NaDodSO_4$/polyacrylamide gels with 3% stacking gels and 12% or 15% resolving gels were prepared and electrophoresed according to the method of Laemmli (21). Proteins were detected with the silver stain procedure (22).

Protein Determination. Protein concentrations were determined from absorbance at 280 nm, using an extinction coefficient determined experimentally for human placental bFGF.

Competition with ^{125}I-Labeled bFGF for Binding. Competition experiments were done as described (23). Briefly, confluent cultures of BHK cells on 24-well plates were preincubated for 2 hr at 37°C in DMEM containing 0.15% gelatin. The cultures were washed twice with cold PBS1, changed to DMEM containing 0.15% gelatin, 25 mM Hepes (pH 7.5), ^{125}I-labeled human placental bFGF at 2 ng/ml, and various concentrations of unlabeled human placental bFGF, M_r 18,000 guinea pig bFGF, or M_r 25,000 guinea pig bFGF and were incubated for 2 hr at 4°C on a rotary shaker. The cells were washed three times with PBS1, and once with 2 M NaCl containing 20 mM Hepes (pH 7.5) to remove ^{125}I-labeled bFGF bound to low-affinity sites as described (23). ^{125}I-labeled bFGF bound to high-affinity sites was extracted with 0.5% Triton X-100 in 0.1 M sodium phosphate (pH 8.1).

Radioimmunoassay for bFGF. Polyclonal anti-human bFGF antibodies were raised in rabbits as described (16, 18) and were purified by affinity chromatography on a bFGF-Affigel 10 column. The affinity-purified bFGF antibodies were diluted to 4 μg/ml in carbonate coating buffer (0.05 M sodium carbonate, pH 9.6) and were passively adsorbed to the wells of Falcon Microtest III flexible assay plates during a 4-hr incubation at 37°C. Following washing of the wells with PBS2 (0.15 M NaCl in 50 mM sodium phosphate, pH 7.4) containing 0.05% Tween-20, 50 μl of human bFGF, guinea pig bFGF, or guinea pig M_r 25,000 protein were incubated for 1 hr at 37°C with 50 μl of PBS2 containing 0.05% Tween-20 plus bovine serum albumin at 1 mg/ml and 15,000 cpm per well of ^{125}I-labeled bFGF (specific activity, 25 μCi/μg; 1 Ci = 37 GBq). Following this incubation the wells were washed extensively with PBS2 containing 0.05% Tween-20, and radioactivity was measured using a γ counter. The percent of ^{125}I-labeled bFGF bound per well was calculated and was plotted versus ng of human bFGF, guinea pig bFGF, or M_r 25,000 protein per well. Serial dilutions of guinea pig proteins from 200 ng per well to 195 pg per well were used.

Immunoblots. Human placental bFGF, guinea pig bFGF, and guinea pig M_r 25,000 protein were electrophoresed on $NaDodSO_4$/polyacrylamide gels containing 15% polyacrylamide according to the method of Laemmli (21). Proteins were then transferred electrophoretically to nitrocellulose (24) and probed with a gamma-globulin fraction of polyclonal anti-human bFGF antibodies. Briefly, the nitrocellulose blot was incubated in blocking buffer (PBS2 containing 5% Carnation nonfat dry milk and 0.1% Nonidet P-40) for 1 hr followed by incubation for 2 hr in a 1:100 dilution of the anti-human bFGF antibodies in blocking buffer. Blots were washed over 1 hr in four changes of blocking buffer prior to incubation with peroxidase-conjugated goat anti-rabbit secondary antibody (Miles-Yeda, Rehovet, Israel). Following a 2-hr incubation with these antibodies diluted in blocking buffer, blots were again washed in blocking buffer as above, and finally washed once in PBS2 alone prior to development in 0.05% 4-chloro-1-naphthol/0.01% H_2O_2.

Trypsin Treatment of M_r 25,000 Guinea Pig bFGF. Purified M_r 25,000 guinea pig bFGF was iodinated with Iodo-Gen (Pierce) according to the procedure of Neufeld and Gospodarowicz (25). The iodinated M_r 25,000 bFGF was repurified by chromatography on heparin-Sepharose. The column was washed with 0.5 M NaCl in 20 mM Tris·HCl (pH 7.5), and bFGF was eluted with 2 M NaCl in the same buffer. Aliquots of the 2 M eluate were mixed with 0.1 vol of a trypsin solution to achieve final concentrations of 0.01, 0.1, 1, or 10 μg of trypsin per ml and were incubated at 37°C for 6 hr. The reaction was stopped by adding an equal volume of 2× electrophoresis reducing sample buffer (21) and heating in a boiling water bath for 2 min. The samples were electrophoresed on $NaDodSO_4$/PAGE with a 15% polyacrylamide resolving gel and a 3% polyacrylamide stacking gel. The gels were dried and exposed to Kodak X-Omat film for autoradiography.

RESULTS

Guinea pig brains were extracted at neutral pH in 3 mM EDTA by the procedure we have used for the isolation of bFGF from human placenta (15) and SK-HEP-1 human hepatoma cells (16). bFGF was isolated from the extract by chromatography on two consecutive heparin-Sepharose columns. The first heparin-Sepharose column was washed with 0.5 M NaCl, and bFGF was eluted with 2 M NaCl. The 2 M NaCl eluate was bound to a second heparin-Sepharose column, the column was washed with 0.95 M NaCl, and bFGF was eluted from the column with 2 M NaCl. Final purification was achieved by ion-exchange chromatography on an FPLC Mono S column. bFGF was eluted with a linear gradient of 0.2–0.6 M NaCl. A peak of protein eluted from the Mono S column with 0.45–0.50 M NaCl (Fig. 1), a characteristic of bFGF isolated from human placenta and SK-HEP-1 cells. $NaDodSO_4$/PAGE showed that this peak contained a single protein with a molecular weight of 18,000 (Fig. 2, lane a) and, thus, was similar to the human bFGFs. A second peak of protein was eluted from the Mono S column with 0.54–0.58 M NaCl (Fig. 1). This peak contained a single protein with a molecular weight of 25,000 (Fig. 2, lane b) as demonstrated by $NaDodSO_4$/PAGE under both reducing and nonreducing conditions (data not shown). One thousand guinea pig brains yielded about 45 μg of the M_r 18,000 bFGF-like protein and 15 μg of the M_r 25,000 protein. Since the M_r 25,000 protein was eluted from heparin-Sepharose under the same conditions used to purify bFGFs, it was further characterized.

The M_r 18,000 and the M_r 25,000 proteins were assayed for their ability to stimulate PA production in cultured BCE cells. The guinea pig bFGF-like protein (M_r 18,000) stimulated PA production by BCE cells in a dose-dependent manner be-

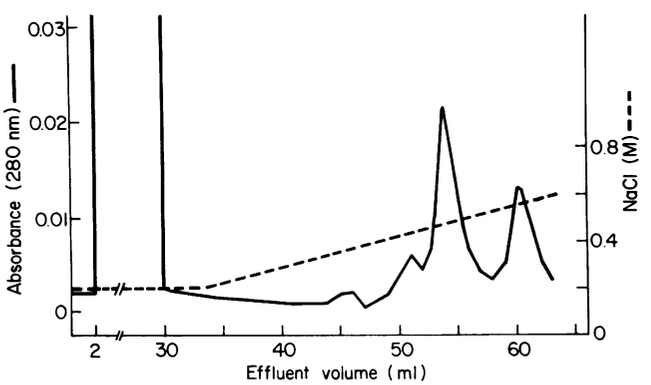

FIG. 1. Elution of guinea pig heparin-binding proteins from the Mono S column. The 2 M NaCl eluate from heparin-Sepharose chromatography was dialyzed against 0.2 M NaCl in 20 mM Mes (pH 6.0), was clarified by centrifugation, and was loaded on an FPLC Mono S column. The column was eluted with a 0.2–0.6 M linear NaCl gradient. Two protein peaks were detected.

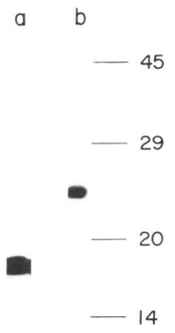

FIG. 2. $NaDodSO_4$/PAGE of guinea pig brain heparin-binding proteins. Samples (≈200 ng each) of the two protein peaks from Mono S chromatography were electrophoresed on $NaDodSO_4$/polyacrylamide gels under reducing conditions. A 12% polyacrylamide resolving gel with a 3% stacking gel was used. Proteins were visualized with silver stain. Lane a, 0.45–0.50 M NaCl eluate. Lane b, 0.54–0.58 M NaCl eluate. Molecular weight markers were aldolase (45,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), and lactalbumin (14,200); values shown are $M_r \times 10^{-3}$.

tween 0.1 and 10 ng/ml, the same concentrations that were effective for human bFGF (Fig. 3). In addition, the M_r 25,000 protein stimulated PA production over the same concentration range. Half-maximal stimulation of PA production occurred with a concentration of ≈1.5 ng/ml for both guinea pig proteins. Thus, both guinea pig proteins were as active as human bFGF for the stimulation of PA production in BCE cells, suggesting that the M_r 18,000 and the M_r 25,000 proteins were forms of bFGF.

The two guinea pig proteins were tested for their ability to compete with ^{125}I-labeled human bFGF for binding to high-affinity receptors on BHK cells. Medium containing ^{125}I-labeled human bFGF at 2 ng/ml and various concentrations of human bFGF, guinea pig M_r 18,000 bFGF, or the guinea pig M_r 25,000 protein were incubated with BHK cells for 2 hr at 4°C. At concentrations from 3 to 30 ng/ml, unlabeled human bFGF competed with ^{125}I-labeled human bFGF for binding to BHK cell receptors (Fig. 4). The ability of the guinea pig M_r 18,000 bFGF to compete with ^{125}I-labeled human bFGF for binding to BHK cell receptors was equal to that of the human bFGF (Fig. 4). Likewise, the guinea pig M_r

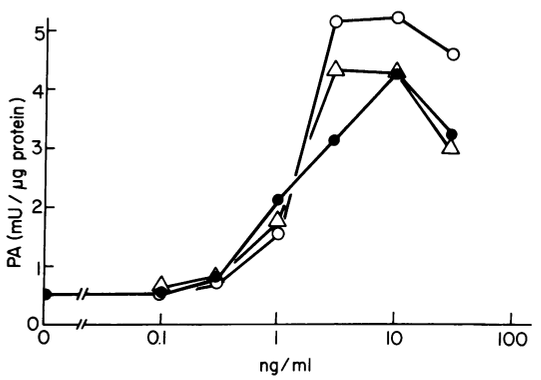

FIG. 3. Stimulation of endothelial cell PA production by guinea pig heparin-binding proteins. Confluent cultures of BCE cells were given fresh α-MEM containing 5% (vol/vol) calf serum and the indicated concentrations of human placental bFGF (●), guinea pig M_r 18,000 heparin-binding protein (△), or guinea pig M_r 25,000 heparin-binding protein (○) and were incubated at 37°C overnight. The cells were washed twice with PBS1 and were extracted with 0.5% Triton X-100. The extracts were assayed for PA. Each point is the result of duplicate assays performed on each of duplicate extracts. mU, milliunits.

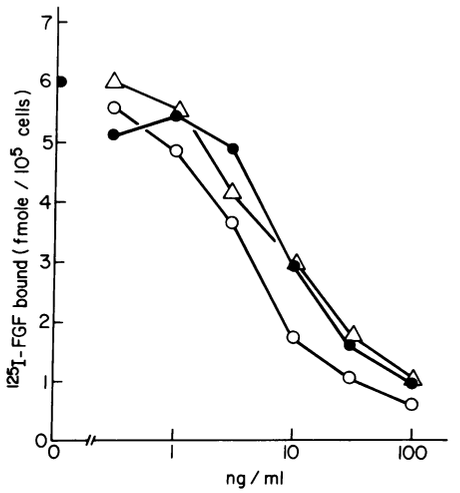

FIG. 4. Competition for binding of ^{125}I-labeled bFGF (^{125}I-FGF) to BHK cells by guinea pig heparin-binding proteins. BHK cells were incubated for 2 hr at 4°C with DMEM containing 0.15% gelatin, 25 mM Hepes (pH 7.5), ^{125}I-labeled human placental bFGF at 2 ng/ml, and the indicated concentrations of unlabeled human placental bFGF (●), guinea pig M_r 18,000 heparin-binding protein (△), or guinea pig M_r 25,000 heparin-binding protein (○). The cells were washed three times with PBS1 and once with 2 M NaCl in 20 mM Hepes (pH 7.5), to remove ^{125}I-labeled bFGF bound to low-affinity sites. The cells were extracted with 0.5% Triton X-100, and the radioactivity in the extracts was determined. Each point represents the average of triplicate samples.

25,000 protein also competed with ^{125}I-labeled human bFGF for binding to BHK cell receptors over this same concentration range (Fig. 4), but appeared to compete at slightly lower concentrations. Thus, both guinea pig proteins have approximately the same affinity for the BHK bFGF receptor as does human bFGF.

The relationship of these two proteins to bFGF was further tested immunologically. Guinea pig M_r 18,000 and M_r 25,000 proteins were probed by immunoblot analysis with polyclonal anti-human bFGF antibodies. Crossreactivity equal to that observed with human placental bFGF was seen for both guinea pig proteins (Fig. 5). When nonimmune gamma globulins were used to probe the transferred proteins, no immunoreactive bands were present.

In addition, when the M_r 18,000 and M_r 25,000 proteins were assayed in a radioimmunoassay for bFGF using the polyclonal anti-human bFGF antibodies, both proteins com-

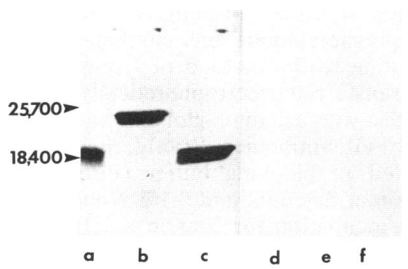

FIG. 5. Immunoblot analysis of guinea pig heparin-binding proteins using anti-human bFGF antibodies. Guinea pig proteins or human placental bFGF were electrophoresed on $NaDodSO_4$/polyacrylamide gels under reducing conditions, and the proteins were then electrophoretically transferred to nitrocellulose. The nitrocellulose was then probed with either anti-human bFGF antibodies (lanes a–c, gamma globulin fraction at a 1:100 dilution) or nonimmune gamma globulins (lanes d–f, 1:100 dilution). Lanes: a and d, human bFGF; b and e, M_r 25,000 guinea pig bFGF; c and f, M_r 18,000 guinea pig bFGF. Positions of molecular weight markers are indicated.

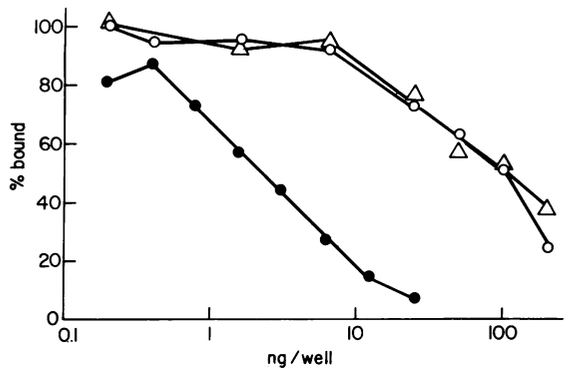

FIG. 6. Competition with ^{125}I-labeled human bFGF for binding to anti-human bFGF antibodies by the guinea pig M_r 18,000 and M_r 25,000 bFGFs. Decreasing concentrations of either the M_r 18,000 or M_r 25,000 form of guinea pig bFGF were assayed for their ability to compete with ^{125}I-labeled human bFGF for binding to solid-phase anti-human bFGF antibodies. The amount of ^{125}I-labeled bFGF binding to the antibody was measured using a γ counter, and the percent ^{125}I-labeled bFGF bound was plotted vs. the amount of unlabeled protein in the assay. Both proteins competed with ^{125}I-labeled bFGF for binding to the antibodies in a dose-dependent manner. ○, M_r 25,000 guinea pig bFGF; △, M_r 18,000 guinea pig bFGF; ●, human placental bFGF.

peted with radiolabeled human bFGF for binding to the antibodies in a dose-dependent manner (Fig. 6). The competition curve generated for the M_r 18,000 form of guinea pig bFGF was parallel to that generated for human placental bFGF; however, the guinea pig bFGF competed less strongly than human placental bFGF with ^{125}I-labeled human bFGF for binding to the antibodies (Fig. 6). This suggests that the guinea pig and human M_r 18,000 forms are similar but not identical. The competition curve generated for the M_r 25,000 form of the guinea pig protein was virtually identical to that obtained with the M_r 18,000 guinea pig bFGF (Fig. 6), indicating a high degree of relatedness between these two proteins.

The close immunological relationship of the two guinea pig bFGFs suggested that the M_r 18,000 form might be a product of proteolytic cleavage of the M_r 25,000 form. To test if the M_r 25,000 form could be converted to an M_r 18,000 form by proteolytic enzymes, the M_r 25,000 form was labeled with ^{125}I, and the ^{125}I-labeled M_r 25,000 bFGF was incubated for 6 hr at 37°C with trypsin. The ^{125}I-labeled M_r 25,000 bFGF was contaminated with a small amount of low molecular weight material (Fig. 7, lane b). Treatment of the ^{125}I-labeled M_r 25,000 dalton bFGF with trypsin at 0.1 μg/ml caused a

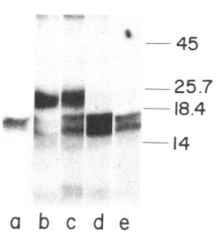

FIG. 7. Trypsin treatment of ^{125}I-labeled M_r 25,000 guinea pig bFGF. Guinea pig M_r 25,000 bFGF was labeled with ^{125}I and was repurified by heparin-Sepharose chromatography. Aliquots of the 2 M NaCl eluate of the column were incubated with trypsin at 37°C for 6 hr. The samples were electrophoresed on NaDodSO$_4$/polyacrylamide gels with 15% polyacrylamide resolving gels. The gels were dried and exposed for autoradiography. ^{125}I-labeled human bFGF was included as a marker (lane a); ^{125}I-labeled M_r 25,000 guinea pig bFGF was incubated with no trypsin (lane b); trypsin at 0.01 μg/ml (lane c), trypsin at 0.1 μg/ml (lane d); or trypsin at 1 μg/ml (lane e).

complete disappearance of the M_r 25,000 band. The trypsin treatment resulted in the formation of two products: one that comigrated with M_r 18,000 ^{125}I-labeled human bFGF, and a second that was slightly smaller, $M_r \approx 16{,}000$ (Fig. 7, lane d). In samples that were treated with 10-times less trypsin (0.01 μg/ml), a partial conversion of the M_r 25,000 bFGF to M_r 18,000 and M_r 16,000 forms was observed (Fig. 7, lane c). In samples that were treated with trypsin at 1 μg/ml, the M_r 18,000 and M_r 16,000 bands were still present, although slightly diminished (Fig. 7, lane e), demonstrating that the M_r 18,000 and M_r 16,000 products are more trypsin-resistant than the M_r 25,000 precursor. These results suggest that the M_r 18,000 bFGF may be derived from the M_r 25,000 dalton form by the action of trypsin-like enzymes in the tissue.

DISCUSSION

We have demonstrated here the presence of two forms of bFGF in guinea pig brain. The M_r 18,000 form is homologous to the M_r 16,000–18,000 bFGFs that have been isolated from a variety of tissues. The nature of the M_r 25,000 form is presently unclear. It may represent a post-translationally modified form of the M_r 18,000 bFGF. Alternatively, the trypsin treatment experiments suggest that the M_r 25,000 form contains a longer amino acid sequence than the M_r 18,000 form and is its precursor. Elucidation of the nature of the difference between these two bFGFs awaits further structural analysis.

The fact that the M_r 25,000 form of bFGF has the same biological activity as the M_r 18,000 form suggests either that the M_r 25,000 form can be processed into a biologically active M_r 18,000 form extracellularly or that both forms are intrinsically active. The high specific activity of the M_r 25,000 form raises the possibility that the M_r 18,000 form may be an artifact of proteolytic cleavage during isolation, and a larger form may be the principal active form *in vivo*. Conversely, it is also possible that two unique, active forms are produced in the brain.

Amino acid sequence analysis of bovine pituitary bFGF has demonstrated that it is a 146-amino acid protein (2). From the nucleotide sequence of a cDNA clone for bovine bFGF, a primary translation product of 155 amino acids has been predicted (26). A bFGF that appeared to contain all of the predicted primary sequence except the N-terminal methionine was obtained by purifying bFGF from bovine pituitary in the presence of protease inhibitors (27). The coding region of a genomic clone of human bFGF appears to be highly homologous to the bovine bFGF cDNA (28), suggesting that human bFGF has a structure similar to bovine bFGF. However, a human bFGF isolated from placenta contained 157 amino acids, including 2 amino acids on the N-terminal side of the proposed initiator methionine (17). These results suggested that in humans alternative splicing of the mRNA for bFGF may occur and/or that larger primary translation products might exist. The present results suggest that such a larger primary translation product may be present in guinea pig brain.

The predicted primary translation product of the bovine bFGF gene contained no classical signal sequence (26), leading to the proposal of alternative secretory mechanisms for bFGF (29, 30). The existence of larger forms of bFGF raises the possibility that a primary translation product containing a classical signal sequence may exist and suggests that discussion of alternative secretory mechanisms may be premature.

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