Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2628–2632, April 1991
Cell Biology

A six-amino acid deletion in basic fibroblast growth factor dissociates its mitogenic activity from its plasminogen activator-inducing capacity

(cell proliferation/endothelial cells/urokinase/basic fibroblast growth factor receptor/tyrosine phosphorylation)

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Communicated by David D. Sabatini, December 17, 1990 (received for review August 23, 1990)

ABSTRACT **A recombinant deletion mutant of the 155-amino acid form of human basic fibroblast growth factor (bFGF), lacking amino acid residues 27–32 (Lys-Asp-Pro-Lys-Arg-Leu), was expressed in *Escherichia coli* and purified to homogeneity by heparin-Sepharose affinity chromatography. When maintained in the presence of an equimolar concentration of soluble heparin, the bFGF mutant (M1-bFGF) is as potent as bFGF in stimulating cell proliferation in normal and transformed fetal bovine aortic endothelial cells, in adult bovine aortic endothelial cells, and in NIH 3T3 fibroblasts. However, under the same experimental conditions, M1-bFGF is at least 100 times less efficient than bFGF in stimulating plasminogen activator (PA) production in endothelial cells, as assayed by chromogenic PA assay, SDS/PAGE zymography, and Northern blot analysis of urokinase-type PA mRNA. In the presence of heparin, M1-bFGF binds to bFGF plasma membrane receptors present on endothelial cells in a manner undistinguishable from bFGF. It also induces the same tyrosine phosphorylation pattern when added to NIH 3T3 cells. The data suggest that the PA-inducing activity of bFGF may depend upon a functional domain that differs from those involved in the mitogenic activity of the growth factor and that the binding of bFGF to its plasma membrane receptor may not be sufficient to induce urokinase-type PA production in endothelial cells.**

Basic fibroblast growth factor (bFGF) is a heparin-binding mitogen endowed with angiogenic activity *in vivo* (1). *In vitro* bFGF induces different responses in endothelial cells, including cell proliferation and plasminogen activator (PA) production (2, 3). When added to cultured endothelial cells, bFGF binds to low- and to high-affinity binding sites (4). Low-affinity binding sites represent cell-associated heparin-like molecules (4), whereas high-affinity binding sites represent tyrosine kinase plasma membrane receptors (5). Interaction of bFGF with high-affinity binding sites, but not with low-affinity sites, is thought to be responsible for the biological activity of bFGF (4).

Two regions in the primary structure of the 155-amino acid bFGF molecule have been proposed to be involved in the receptor binding and mitogenic activity of this growth factor (6). These regions correspond to amino acid residues 33–77 and 102–129. In addition, we have recently observed that two synthetic peptides overlapping amino acid residues 38–61 and 82–101 exert a partial bFGF agonist and antagonist activity in mitogenic assays on endothelial cells (M.P., M.R., C. Urbinati, A. Sommer, and G.R., unpublished data). However, these peptides do not affect PA production in bFGF-treated and untreated endothelial cells. Also, antibodies raised against these peptides completely quench the mitogenic activity of bFGF, but they only partially inhibit its PA-inducing capacity. These data suggest that the PA-inducing activity of bFGF may depend upon a functional domain that differs from those involved in the mitogenic activity of this growth factor. In this respect, it is interesting to note that two different intracellular transduction signals mediate the capacity of bFGF to induce cell proliferation and PA production in fetal bovine aortic endothelial cells (FBAE cells): activation of protein kinase C is responsible for the mitogenic activity exerted by bFGF, whereas induction of PA activity is independent of protein kinase C activation and requires calcium uptake from the extracellular environment (7).

Here we describe a recombinant deletion mutant of the 155-amino acid form of human bFGF (M1-bFGF) lacking amino acid residues 27–32 (Lys-Asp-Pro-Lys-Arg-Leu). In the presence of an equimolar concentration of soluble heparin, this mutant retains the capacity to induce cell proliferation in cultured endothelial cells and NIH 3T3 fibroblasts. In these experimental conditions, M1-bFGF interacts with plasma membrane bFGF receptors in a manner indistinguishable from wild-type bFGF. However, M1-bFGF is 100 times less potent that bFGF in inducing the production of urokinase-type PA (uPA) in endothelial cells, indicating that different biological activities of bFGF may be dissociated at the structural level.

MATERIALS AND METHODS

Plasmid Construction. A synthetic gene for the 155-amino acid form of human bFGF (8) was obtained by assembling synthetic oligonucleotides. The sequence coding for M1-bFGF, a bFGF mutant lacking amino acid residues 27–32 (Lys-Asp-Pro-Lys-Arg-Leu), was obtained by deleting the corresponding codons from the bFGF gene. For this purpose, the native sequence was subcloned into M13mp9am16 and modified by site-directed mutagenesis, using the oligonucleotide 5′-AAGCCCCCGTTTTTGCAGTAGAAGTGGC-CGGGCGGGAAGG-3′ as a mutagenesis primer, yielding M13mp9-M1. The expression plasmid for bFGF, named pFC80, was obtained by assembling (*i*) a synthetic *Sal* I–*Nar* I fragment harboring the λ *cII* ribosome binding site (10) followed by the first 55 nucleotides of the bFGF sequence; (*ii*) a synthetic *Nar* I–*Bam*HI fragment encoding the bFGF sequence from nucleotides 56 to 405; (*iii*) the large *Sal* I–*Bam*HI fragment from plasmid pFC7 (10), which carries the

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Abbreviations: bFGF and aFGF, basic and acidic fibroblast growth factors; M1-bFGF, deletion mutant of bFGF; PA, plasminogen activator; uPA, urokinase-type PA; ABAE and FBAE cells, adult and fetal bovine aortic endothelial cells; FCS, fetal calf serum.

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ampicillan resistance gene, the P_{trp} promoter, and the origin of replication. The remaining nucleotides of the bFGF sequence were inserted into the unique *Bam*HI site of this new plasmid as a synthetic *Xho* II restriction fragment, yielding pFC80. Plasmid pFC86, encoding the sequence for M1-bFGF, was obtained by replacing a *Nar* I–*Bam*HI fragment in pFC80 with the corresponding fragment from the mutagenized M13mp9-M1. Plasmids pFC80 and pFC86 were used to transform an *Escherichia coli* type B strain (11).

Purification of bFGF and M1-bFGF. After induction (10), recombinant bFGF and M1-bFGF were purified from the soluble fraction of *E. coli* cell extract by heparin-Sepharose affinity chromatography (3). Proteins were probed in a Western blot with anti-human bFGF antibodies (provided by D. B. Rifkin, New York University Medical Center) as described (12). Purity of recombinant bFGF and M1-bFGF was determined by SDS/PAGE followed by silver staining (13).

Cell Cultures. Normal FBAE AG 7680 cells and transformed FBAE GM 7373 cells, corresponding to the described BFA-1c 1BPT multilayered transformed clone (14), were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). Adult bovine aortic endothelial cells (ABAE cells) were obtained from Istituto Scienze Farmacologiche Mario Negri (Milan). Cells were grown in Eagle's minimal essential medium (MEM) containing 10% or 20% fetal calf serum (FCS), vitamins, and essential and nonessential amino acids. NIH 3T3 fibroblasts were grown in Dulbecco's modification of MEM (DMEM) with 2 mM glutamine and 10% calf serum.

Cell Proliferation. *Short-term assay.* GM 7373 and AG 7680 cells (70,000 cells per cm^2) were incubated in fresh medium containing 0.4% FCS and different concentrations of bFGF or M1-bFGF. After 24 hr, cells were trypsinized and counted. In a typical experiment, cultures incubated in 0.4% FCS, in 0.4% FCS plus 30 ng of bFGF per ml, or in 10–20% FCS underwent 0.1–0.2, 0.7–0.8, and 1.0 cell population doubling, respectively.

Long-term assay. ABAE cells were seeded in 35-mm dishes at 2000 cells per dish. After 16 hr, cells were incubated in fresh medium with 10% FCS in the absence or in the presence of different concentrations of bFGF or M1-bFGF. Then, medium with or without the growth factor was changed every other day. After 6 days, cells were trypsinized and counted.

DNA Synthesis. bFGF and M1-bFGF were tested for their ability to stimulate [^{3}H]thymidine incorporation into the DNA of quiescent NIH 3T3 cells as described (15).

PA Assay and SDS/PAGE Zymography. Confluent cultures of GM 7373, AG 7680, and ABAE cells were incubated in fresh medium containing 0.4% serum and different concentrations of bFGF or M1-bFGF. After 24 hr, PA activity was measured in the cell extracts as described (7), by using the plasmin chromogenic substrate D-norleucylhexahydrotyrosyllysine *p*-nitroanilide acetate (American Diagnostica, Greenwich, CT). Human uPA (Calbiochem) was used as a standard. One international unit corresponds to 0.7 Ploug unit. Also, 20-μg samples of GM 7373 cell extracts were run on 10% SDS/polyacrylamide gel under nonreducing conditions. Then, zymography for the detection of PA activity was carried out on a fibrin/agarose gel as described (16).

Northern Blot Analysis. Northern blot analysis of total RNA from untreated, bFGF-treated, and M1-bFGF-treated GM 7373 cells was performed according to standard procedures (17). A *Sma* I–*Bam*HI fragment from pcUK176 (18), harboring the human uPA sequence, was used as a probe. After hybridization, the blot was washed and rehybridized with a probe for the dehydrofolate reductase gene (provided by L. Monaco, Farmitalia Carlo Erba) to determine the relative amount of RNA loaded per lane.

^{125}I-Labeled bFGF (^{125}I-bFGF) Binding Assay. bFGF was iodinated as described (19) (specific activity = 870 cpm/fmol). GM 7373 cells were incubated at 4°C in serum-free medium containing 10 ng of ^{125}I-bFGF per ml, 0.15% gelatin, 20 mM Hepes buffer (pH 7.5), and the indicated concentrations of unlabeled bFGF or M1-bFGF. After 2 hr, the binding of ^{125}I-bFGF to high-affinity binding sites was assayed as described (4).

Cross-Linking to the Receptor. GM 7373 cells were incubated at 4°C in serum-free medium containing 0.15% gelatin, 20 mM Hepes buffer (pH 7.5), and 30 ng of ^{125}I-bFGF or ^{125}I-labeled M1-bFGF (^{125}I-M1-bFGF specific activity = 978 cpm/fmol) per ml in the absence or in the presence of 1 μg of unlabeled bFGF or M1-bFGF per ml. After 2 hr, cross-linking of the iodinated ligand to its receptor and SDS/PAGE analysis of the ligand/receptor complex were carried out as described (12).

Down-Regulation of the bFGF Receptor. GM 7373 cells were incubated at 37°C in serum-free medium containing 0.15% gelatin and the indicated concentrations of bFGF or M1-bFGF. At different times, cells were washed with ice-cold 2 M NaCl in 20 mM sodium acetate (pH 4.0), to remove bound bFGF (4), and with ice-cold PBS. Then, cells were incubated for 2 hr at 4°C with 10 ng of ^{125}I-bFGF per ml and assayed for high-affinity binding sites as described (4).

Tyrosine Phosphorylation. Subconfluent cultures of NIH 3T3 cells were incubated in fresh medium containing 0.5% calf serum. After 24 hr, cells were exposed for 15 min to 20 ng of bFGF or M1-bFGF per ml in serum-free medium. Then, cells were washed with PBS, lysed with 2-fold concentrated SDS/PAGE reducing sample buffer, scraped from the plate with a rubber policeman, sonicated with two bursts of 10 sec each at 50 W, and incubated at 100°C for 3 min. Lysates were run on a 5–12% SDS/polyacrylamide gel and proteins were electrophoretically transferred to a nitrocellulose membrane. Membranes were probed with 20 μg of affinity-purified anti-phosphotyrosine antibodies per ml (provided by T. Maciag, American Red Cross, Rockville, MD) and immunocomplexes were visualized by incubation with ^{125}I-labeled protein A (^{125}I-protein A) (Amersham).

RESULTS

Biological Activity of M1-bFGF. The recombinant 155-amino acid form of human bFGF and the recombinant deletion mutant M1-bFGF were purified from *E. coli* cell extracts by heparin-Sepharose affinity chromatography. Both molecules were 95% pure or better, as evaluated by SDS/PAGE analysis, and cross-reacted with polyclonal anti-human bFGF antibodies (Fig. 1). The deletion of the amino acid residues 27–32 within M1-bFGF was confirmed by amino acid sequencing of the purified molecule (Table 1).

bFGF induces cell proliferation and PA production in different endothelial cell types (2, 3, 7). On this basis, we have compared the mitogenic and the PA-inducing activity of bFGF and of M1-bFGF in normal and transformed FBAE cells and in normal ABAE cells. In preliminary experiments we noticed some variability in the potency of the different preparations of the bFGF mutant when they were tested in cell proliferation assays. This variability could be overcome by storing M1-bFGF in the presence of an equimolar amount of soluble heparin. We interpret these data as an indication that the 6-amino acid deletion may cause a reduction of the stability of M1-bFGF that is prevented by maintaining M1-bFGF in the presence of soluble heparin, a molecule that is known to stabilize the tertiary structure of bFGF (20). Thus, all biological and receptor-binding assays described in the present paper were performed with wild-type bFGF and with M1-bFGF to which soluble heparin (1:1.5, wt/wt) was added immediately after their purification, before storage at −80°C. Under these experimental conditions, M1-bFGF and bFGF

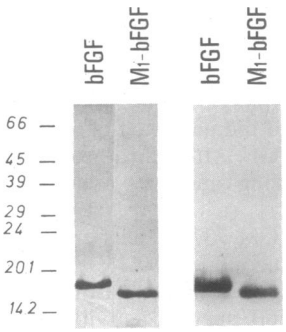

FIG. 1. SDS/PAGE and immunoblot analysis of purified recombinant bFGF and M1-bFGF. bFGF and M1-bFGF were run on an SDS/12% polyacrylamide gel under reducing conditions. Proteins were visualized by silver staining (*Left*) or probed with an anti-human bFGF antiserum (*Right*). Molecular weights are expressed as $M_r \times 10^{-3}$.

induce endothelial cells to proliferate with a similar potency in a short-term mitogenic assay performed on FBAE cells (Fig. 2) and in a long-term mitogenic assay performed on ABAE cells (Fig. 3). However, M1-bFGF is at least 100 times less potent than bFGF in stimulating PA production in all of the endothelial cell types tested (Figs. 2 and 3). Similar results were obtained when M1-bFGF and bFGF were assayed in the presence of an excess (10 μg/ml) of heparin (data not shown).

The inability of M1-bFGF to stimulate PA production was confirmed by SDS/PAGE zymography (Fig. 4*A*). Indeed, 30 ng of bFGF per ml caused an increase of cell-associated PA activity with an apparent M_r of ≈50,000 in GM 7373 cells. No significant increase in this activity was detectable in M1-bFGF-treated cells. Similar results were obtained when SDS/PAGE zymography was performed on the conditioned media from the same cultures (data not shown). The M_r 50,000 PA activity was identified as bovine uPA on the basis of its molecular weight (21) and of its complete inhibition after incubation with polyclonal anti-uPA antibodies (data not shown). Accordingly, a 5- to 10-fold increase of the levels of uPA mRNA was observed by Northern blot analysis of bFGF-treated cells but not of M1-bFGF-treated cells (Fig. 4*B*).

Binding of M1-bFGF to bFGF Plasma Membrane Receptor. Interaction of bFGF with its cell membrane receptor causes down-regulation of bFGF high-affinity binding sites (22). Accordingly, a 2-hr incubation of GM 7373 cells with bFGF or M1-bFGF causes a dose-dependent disappearance of ^{125}I-bFGF high-affinity binding sites, with similar dose–response curves for bFGF and for M1-bFGF (Fig. 5*A*). Moreover, the kinetics of down-regulation of ^{125}I-bFGF high-affinity binding sites is very similar for bFGF-treated and M1-bFGF-treated cells (Fig. 5*B*). These data suggest that bFGF and M1-bFGF interact with the bFGF plasma membrane receptor in the same manner.

Table 1. Amino-terminal sequence analysis of M1-bFGF

Cycle	Amino acid	pmol	Cycle	Amino acid	pmol
1	Ala	85.9	16	Gly	18.3
2	Ala	66.8	17	Ser	6.0
3	Gly	36.6	18	Gly	11.0
4	Ser	29.2	19	Ala	11.2
5	Ile	31.8	20	Phe	10.5
6	Thr	22.4	21	Pro	11.0
7	Thr	34.2	22	Pro	13.4
8	Leu	31.0	23	Gly	8.1
9	Pro	29.0	24	His	3.6
10	Ala	26.1	25	Phe*	7.4
11	Leu	28.8	26	Tyr	5.6
12	Pro	22.5	27	(Cys)	3.6
13	Glu	18.5	28	Lys	3.9
14	Asp	14.3	29	Asn	3.5
15	Gly	15.5	30	Gly	4.4

Note that in recombinant M1-bFGF, as for wild-type bFGF, the first methionine residue is removed *in vivo* by *E. coli*.
*Last residue before the 6-amino acid deletion.

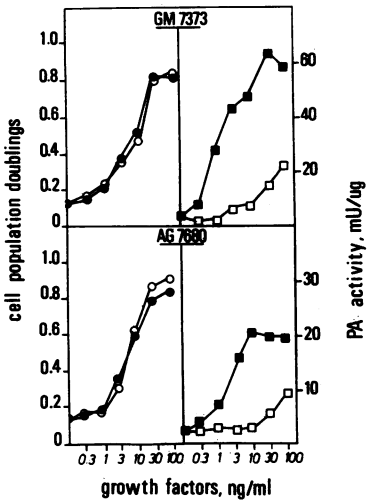

FIG. 2. Mitogenic and PA-inducing activity of bFGF and M1-bFGF in FBAE cells. GM 7373 cells and AG 7680 cells were incubated in the presence of bFGF (closed symbols) or M1-bFGF (open symbols). After 24 hr, cells were trypsinized and counted (●, ○) or extracted and assayed for cell-associated PA activity (■, □). PA activity is expressed as international milliunits per microgram of cell extract.

To confirm this hypothesis, we have compared the capacity of bFGF and M1-bFGF to compete with ^{125}I-bFGF for the binding to its high-affinity binding sites in GM 7373 cells. For this purpose, cells were incubated for 2 hr at 4°C with a constant amount of ^{125}I-bFGF in the presence of increasing concentrations of either unlabeled bFGF or unlabeled M1-bFGF, both maintained in the presence of an equimolar concentration of soluble heparin. Under these conditions, M1-bFGF was as effective as bFGF in competing with ^{125}I-bFGF for its high-affinity binding sites (Fig. 6*A*, closed symbols). Moreover, chemical cross-linking studies confirmed that ^{125}I-bFGF and ^{125}I-M1-bFGF bind to the same M_r 140,000 plasma membrane receptor. The binding of ^{125}I-bFGF and of ^{125}I-M1-bFGF to this receptor was fully inhibited competitively by a 66-fold excess of unlabeled M1-bFGF or of unlabeled bFGF (Fig. 6*B*). It must be noticed that, in the absence of an equimolar concentration of soluble heparin, M1-bFGF was less efficient by a factor of 100 than bFGF in displacing ^{125}I-bFGF from its receptor (Fig. 6*A*, open symbols). These data are in keeping with the reduced mitogenic activity exerted by some preparations of M1-bFGF when maintained in the absence of soluble heparin (data not shown) and they support the hypothesis that M1-bFGF undergoes

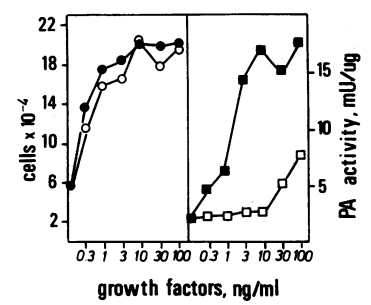

FIG. 3. Mitogenic and PA-inducing activity of bFGF and M1-bFGF in ABAE cells. ABAE cells were seeded in 35-mm dishes at 2000 cells per dish. After 16 hr, cells were added with bFGF (●) or M1-bFGF (○) in the presence of 10% FCS. Then, medium was changed every other day. Cells were trypsinized and counted after 6 days. For PA activity determination, confluent cultures were incubated with bFGF (■) or M1-bFGF (□). After 24 hr, cultures were extracted and assayed for cell-associated PA activity.

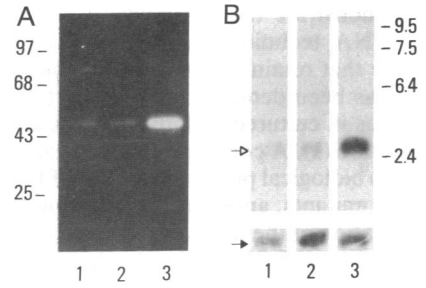

FIG. 4. uPA production in GM 7373 cells. (*A*) SDS/PAGE zymography. Cultures were incubated in serum-free medium with no addition (lane 1), 30 ng of M1-bFGF per ml (lane 2), or 30 ng of bFGF per ml (lane 3). After 24 hr, cell-associated PA activity was analyzed by SDS/PAGE zymography. Molecular weights are expressed as $M_r \times 10^{-3}$. (*B*) Northern blot analysis. Twenty-five micrograms of total RNA from untreated cells (lane 1), cells treated for 6 hr with 30 ng of M1-bFGF per ml (lane 2), or cells treated with 30 ng of bFGF per ml (lane 3) was sequentially hybridized with a uPA probe (open arrow) and with a dehydrofolate reductase probe (closed arrow). RNA markers of 2.4–9.5 kilobases were used.

structural modifications that are prevented by its incubation with soluble heparin.

M1-bFGF Induces Tyrosine Phosphorylation in NIH 3T3 Cells. The bFGF cell membrane receptor belongs to the tyrosine kinase receptor family (5). Exposure of 3T3 cells to bFGF causes tyrosine phosphorylation of the bFGF receptor and of other cellular proteins (20). Thus, to better characterize the interaction of M1-bFGF with bFGF cell membrane receptor, we have evaluated the effect of M1-bFGF on cell proliferation and tyrosine phosphorylation in NIH 3T3 cells. As shown in Fig. 7*A*, M1-bFGF exerted a mitogenic activity in NIH 3T3 cells. The effect was dose-dependent, with an ED_{50} equal to 0.3 ng/ml, very similar to that observed for bFGF. Accordingly, M1-bFGF and bFGF induced the same pattern of tyrosine phosphorylation when added for 15 min at 37°C to NIH 3T3 cells in serum-free conditions (Fig. 7*B*). Tyrosine phosphorylated proteins included a M_r 140,000 molecule, likely to correspond to the activated bFGF receptor, and three other major proteins, including the typical M_r 90,000 substrate (23). Thus, these data indicate that M1-bFGF activates bFGF receptor in NIH 3T3 cells in a manner indistinguishable from wild-type bFGF.

DISCUSSION

In the present study we report the characterization of M1-bFGF, a heparin-binding recombinant deletion mutant of the

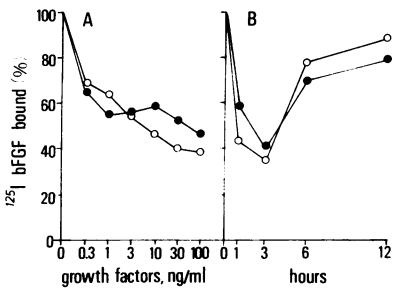

FIG. 5. Down-regulation of bFGF plasma membrane receptor. (*A*) Dose–response. GM 7373 cells were incubated at 37°C with increasing concentrations of bFGF (●) or of M1-bFGF (○). After 2 hr, cells were washed with 2 M NaCl (buffered at pH 4.0 to remove bound bFGF), incubated for 2 hr at 4°C with ^{125}I-bFGF (8 ng/ml), and assayed for bFGF high-affinity binding sites. (*B*) Kinetics of down-regulation. GM 7373 cells were incubated with 30 ng of bFGF per ml (●) or of M1-bFGF (○) for different times. After incubation, cells were assayed for bFGF high-affinity binding sites as described in *A*.

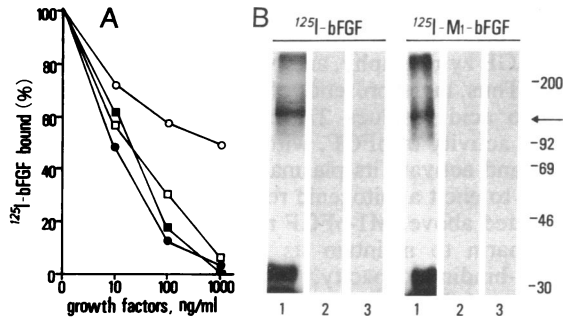

FIG. 6. Competition assay. (*A*) Competition for the binding of ^{125}I-bFGF to GM 7373 cells by bFGF and M1-bFGF. GM 7373 cells were incubated for 2 hr at 4°C with ^{125}I-bFGF (8 ng/ml) and increasing concentrations of unlabeled bFGF (■, □) or M1-bFGF (●, ○). The incubation was performed with unlabeled growth factors that were maintained either in the presence (closed symbols) or in the absence (open symbols) of an equimolar amount of soluble heparin. Then, binding of ^{125}I-bFGF to bFGF plasma membrane receptor was quantitated and expressed as percent of the binding in the absence of unlabeled molecules. (*B*) Cross-linking of ^{125}I-bFGF and ^{125}I-M1-bFGF to GM 7373 cells. GM 7373 cells were incubated for 2 hr at 4°C with 30 ng of ^{125}I-bFGF or ^{125}I-M1-bFGF per ml with no addition (lanes 1), 1 μg of unlabeled bFGF per ml (lanes 2), or 1 μg of unlabeled M1-bFGF per ml (lanes 3). Then cells were incubated with the cross-linking reagent bis[2-(succinimidoxycarbonyloxy)-ethyl]sulfone (1 mM) for 20 min at room temperature and lysed in SDS/PAGE reducing sample buffer. Extracts were analyzed on an SDS/3.5–12% polyacrylamide gel, followed by autoradiography. The arrow marks the bFGF receptor. Molecular weights are expressed as $M_r \times 10^{-3}$.

155-amino acid form of human bFGF lacking the amino acid sequence 27–32 (Lys-Asp-Pro-Lys-Arg-Leu). In the presence of an equimolar amount of soluble heparin, M1-bFGF exerts a mitogenic activity in different endothelial cell types and in NIH 3T3 fibroblasts, interacts with bFGF plasma membrane receptor, and induces tyrosine phosphorylation in NIH 3T3 cells in a manner indistinguishable from wild-type bFGF. However, M1-bFGF is at least 100 times less potent

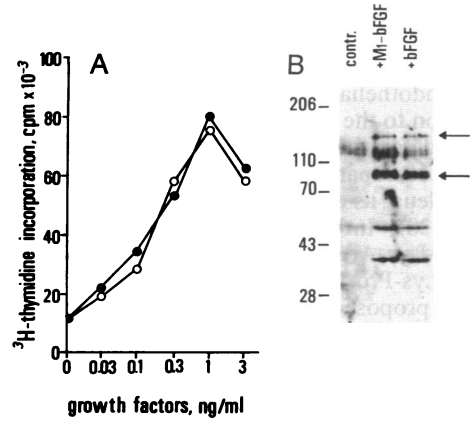

FIG. 7. Effect of bFGF and M1-bFGF on NIH 3T3 cells. (*A*) DNA synthesis. Quiescent cultures of NIH 3T3 cells were incubated in fresh medium containing [^{3}H]thymidine and increasing concentrations of bFGF (●) or M1-bFGF (○). After 24 hr, the amount of radioactivity incorporated into the trichloroacetic acid-precipitable material was measured. (*B*) Tyrosine phosphorylation in intact cells. NIH 3T3 cells were incubated for 15 min in serum-free medium with no addition (contr.), 20 ng of M1-bFGF per ml, or 20 ng of bFGF per ml. Then, cells were lysed in reducing sample buffer, run on an SDS/5–12% polyacrylamide gel, and immunoblotted with anti-phosphotyrosine antibodies, and immunocomplexes were visualized by incubation with ^{125}I-protein A. Arrows mark the M_r 140,000 putative bFGF receptor and the M_r 90,000 substrate. Molecular weights are expressed as $M_r \times 10^{-3}$.

than bFGF in stimulating PA production in endothelial cells. This has been demonstrated by a chromogenic PA assay, SDS/PAGE zymography, and Northern blot analysis of uPA mRNA. Thus, in the presence of soluble heparin, the deletion of amino acid residues 27–32 strongly reduces the PA-inducing activity of bFGF, without affecting its capacity to bind to and activate its plasma membrane receptor and its capacity to elicit a mitogenic response.

As stated above, M1-bFGF requires the presence of soluble heparin to maintain its full mitogenic activity and receptor-binding capacity. This would suggest that the 6-amino acid deletion present in M1-bFGF may affect the tertiary structure of the growth factor, whereas heparin is capable of preventing these structural modifications. Nevertheless, the capacity of M1-bFGF to induce PA production in endothelial cells is dramatically impaired also in the presence of heparin.

bFGF has to remain active in the extracellular environment for several hours to induce endothelial cells to proliferate and to produce PA (M.P., L. Tiberio, M.R., P. Dell'Era, and G.R., unpublished data). Thus, cell proliferation and PA production represent two late responses of the endothelial cell to stimulation by bFGF. While this work was in progress, the characterization of the biological activity of mutants of acidic fibroblast growth factor (aFGF), a heparin-binding mitogen strictly related to bFGF (20), has been reported (24, 25). These mutants show the capacity to induce early events in endothelial and/or 3T3 cells (i.e., receptor binding, tyrosine phosphorylation, and protooncogene activation), but they do not stimulate cell proliferation. Here, we demonstrate the possibility to dissociate at a structural level not only a late event, like PA production, from the early events of receptor binding and tyrosine phosphorylation but also two late events induced by bFGF—that is, cell proliferation from PA production. Accordingly, previous observations obtained in our laboratory have shown that the mitogenic activity and the PA-inducing activity of bFGF can be pharmacologically dissociated and that two different signal transduction pathways mediate the two responses in endothelial cells (7).

The capacity of M1-bFGF to bind to and to activate the bFGF receptor without stimulating PA production indicates that the interaction of bFGF with its receptor may not be sufficient to induce uPA gene expression. bFGF translocates to and accumulates into the cell nucleus when administered to cultured endothelial cells (26, 27). These findings suggest that in addition to the classical pathways of signal transduction proposed for the action of this growth factor, bFGF might exert at least part of its biological activity by interacting with cell nucleus, as already hypothesized for other growth factors (24, 28). Interestingly, the amino acid sequence Lys-Asp-Pro-Lys-Arg-Leu, deleted in M1-bFGF, is similar to the Lys-Lys-Pro-Lys-Leu-Leu aFGF-(23–28) sequence that has been proposed to be involved in the nuclear targeting and biological activity of aFGF (24). Thus, it is tempting to hypothesize that amino acid residues 27–32 represent a region involved in the interaction of bFGF with cell nucleus and that this interaction is required for the induction of the expression of the uPA gene in endothelial cells. However, it must be pointed out that a complex multigene family of bFGF receptors exists (5, 9, 29–31). Even though the physiological function of these receptors is still unknown, it is possible to hypothesize that different receptors may mediate different biological responses, elicited by bFGF. On this basis, M1-bFGF may have retained the capacity to interact with one or more receptors responsible for the mitogenic activity of bFGF, but it may have partially lost its capacity to interact with a hypothetical receptor responsible for the PA-inducing activity of bFGF.

The data demonstrate the possibility of synthesizing by recombinant DNA techniques a bioactive mutant of the bFGF molecule that retains only part of its biological properties. bFGF has been demonstrated to exert different biological responses in cultured endothelial cells and to induce neovascularization (1). A complete characterization of the *in vitro* and *in vivo* biological properties of M1-bFGF, as well as of other related mutants, appears to be of pivotal importance for the identification of the mechanism(s) responsible for the angiogenic activity of bFGF and for the development of new bFGF agonists and antagonists.

We thank Dr. G. Nitti for the amino acid sequencing of M1-bFGF, Drs. D. B. Rifkin, P. Dell'Era, and J. A. M. Maier for helpful discussion and criticism, and Miss M. Belleri for her expert technical assistance. This work was supported by grants from Consiglio Nazionale delle Ricerche (Progetto Finalizzato Biotecnologie e Biostrumentazioni, Sottoprogetto Biofarmaci) and from Associazione Italiana per la Ricerca sul Cancro to M.P. and by grants from Consiglio Nazionale delle Ricerche (Progetto Finalizzato Oncologia and Grant 89.02759.04) to G.R.

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