Differential Modulation of Cell Phenotype by Different Molecular Weight Forms of Basic Fibroblast Growth Factor: Possible Intracellular Signaling by the High Molecular Weight Forms

Andreas Bikfalvi, Sharon Klein, Giuseppe Pintucci, Natalina Quarto, Paolo Mignatti,* and Daniel B. Rifkin

Department of Cell Biology and the Raymond and Beverly Sackler Foundation Laboratory, and the Kaplan Cancer Center, New York University Medical Center, New York 10016; and * Dipartimento di Genetica e Microbiologia, Università di Pavia, 27100 Pavia, Italy

Abstract. To study possible functional differences of the 18-kD and high molecular weight forms of basic fibroblast growth factor (bFGF), we have examined the effect of endogenous production of different bFGF forms on the phenotype of NIH 3T3 cells. Cells transfected with cDNAs coding for either 18-kD bFGF (18-kD bFGF) or all four molecular forms (18, 22, 22.5, 24 kD; wild type [WT] bFGF) exhibit increased migration and decreased FGF receptor number compared to parental cells. However, migration and FGF receptor number of cells transfected with a cDNA coding only for high molecular weight bFGF (22, 22.5, and 24 kD; HMW bFGF) were similar to that of parental cells transfected with vector alone. Cells expressing HMW, 18 kD, or WT bFGF grew to high saturation densities in 10% serum. However, only cells expressing HMW or WT bFGF grew in low serum. Cell surface or metabolic labeling of the different cell types followed by immunoprecipitation with anti-bFGF antibody showed primarily cell surface-associated 18-kD bFGF. In addition, when cells expressing exclusively HMW bFGF were transfected with a cDNA coding for 18-kD bFGF, migration was increased, bFGF receptors were down-regulated, and 18-kD bFGF was found on the cell surface. Cells expressing 18-kD bFGF transfected with a cDNA encoding FGF receptor-2 lacking the COOH-terminal domain (dominant negative bFGF receptor) exhibited a flat morphology and decreases in migration and saturation density. Cells expressing HMW bFGF transfected with the dominant negative bFGF receptor continued to grow to a high saturation density, proliferated in low serum, and exhibited no morphological changes. These results indicate that increased cell migration and FGF receptor down-regulation are mediated by the extracellular interaction of 18-kD bFGF with its cell surface receptor. Growth in low serum may be stimulated by the intracellular action of HMW bFGF through mechanisms independent of the presence of a cell surface receptor. Thus, the different molecular forms of bFGF may act through distinct but convergent pathways.

Basic fibroblast growth factor (bFGF) is the prototype member of the heparin-binding growth factor family that currently includes eight other proteins (Basilico and Moscatelli, 1992; Miyamoto et al., 1993). bFGF is a potent mitogen for a variety of cell types, including endothelial cells and fibroblasts, and affects a number of additional cellular functions such as protease production, surface integrin levels, and cell migration (Basilico and Moscatelli, 1992; Klein et al., 1993). Many cells produce bFGF both in vivo and in vitro. Four different tyrosine kinase plasma membrane receptors for FGF have been described and are found on many cell types (Jaye et al., 1992; Basilico and Moscatelli, 1992). Basic FGF, like acidic FGF (aFGF), does not contain a signal sequence (Abraham et al., 1986) and is not released via the classical secretory pathway (McNeil et al., 1989; Muthukrishnan et al., 1991; Mignatti et al., 1992). Moreover, the mechanism(s) for release of bFGF and aFGF remain(s) unknown. Different molecular forms of bFGF representing alternative translation products from a single mRNA have been described (Moscatelli et al, 1987b; Sommer, 1987; Florkiewicz and Sommer, 1989; Prats et al., 1989). The translation of the smallest molecular weight form (18 kD) of bFGF is initiated at an internal AUG codon, whereas the translation of the higher molecular
weight bFGF (HMW bFGF; 22-24 kD) forms is initiated at CUG codons 5' to the AUG codon. In vitro most cells produce all forms of bFGF. HMW bFGF is preferentially localized in the nucleus, whereas 18-kD bFGF is found predominantly in the cytosol (Quarto et al., 1999a,b; Renko et al., 1991; Bugler et al., 1991; Florkowski et al., 1991). The nuclear localization of HMW bFGF is determined by the additional amino-terminal sequences not found in 18-kD bFGF as proteins containing the unique amino-terminal sequences of HMW bFGF fused to β-galactosidase or chloramphenicol acetyltransferase accumulate in the nucleus (Quarto et al., 1999a; Bugler et al., 1991). Fusion of 18-kD bFGF to pyruvate kinase does not direct the chimeric protein to the nucleus (Quarto, N., unpublished observation).

The existence of multiple forms of bFGF with different subcellular localization raises the question of whether these forms of bFGF have different mechanisms of action and specialized functions. We have addressed this question by creating transfected NIH 3T3 cell lines that express exclusively 18-kD bFGF, HMW bFGF, or all forms of bFGF (wild type [WT] bFGF) (Quarto et al., 1989, 1991b). Cells expressing increased levels of 18-kD or WT bFGF had growth properties of transformed cells, including increased saturation density and anchorage-independent growth. Cells expressing HMW bFGF were of two types; many clones were small and had impaired growth as though HMW bFGF expression inhibited cell division. These cells contained low levels of HMW bFGF and had only one copy of the integrated HMW bFGF cDNA. Clones that expressed higher levels of HMW bFGF and had multiple copies of integrated HMW bFGF cDNA grew rapidly and had the properties of transformed cells (Quarto et al., 1991b).

To characterize potential functional differences between 18-kD and HMW bFGF, we extended our analysis of cells expressing specific forms of bFGF. In this paper, we provide evidence for a differential modulation of the cell phenotype by endogenous production of either 18-kD or HMW bFGF and for different mechanisms of action. Cells transfected with cDNAs for either 18-kD or WT bFGF display enhanced migration, whereas cells transfected with HMW bFGF cDNA exhibit no changes in migration. Although cells expressing HMW or WT bFGF grow in low serum, cells expressing 18-kD bFGF do not. Expression of a cDNA encoding a dominant negative bFGF receptor reverses those phenotypic properties associated with 18-kD bFGF expression but not those associated with HMW bFGF. Thus, high levels of expression of HMW bFGF may affect cell proliferation by a mechanism independent of the presence of a cell surface receptor. These data provide evidence for potentially different functional mechanisms for HMW and 18-kD bFGF action.

Materials and Methods

Reagents

Recombinant human bFGF (18 kD) was a gift from Synergen, Inc. (Boulder, CO). HMW bFGF was purified from guinea pig brains as described by Moscatelli et al. (1987). Na÷¹ (16.93 mCi/µg) was purchased from American Corp. (Arlington Heights, IL), and 35S-L-cysteine (1,220 Ci/mmol) and 32P-orthophosphate (8,300-9,120 Ci/mmol) from Dupont Radiochemicals (Boston, MA). All other reagents were research grade.

Cells

NIH 3T3 cells were transfected with the Zip-neo vectors containing either a 1.1-kb insert of a cDNA coding all bFGF forms (24, 22.5, 18, 12 kD; WTbFGFcl, WTbFGFc3, WTbFGFc5, WTbFGFc22), a cDNA coding only for the 24-22-kD bFGF (clones 365FGFIc1, 365FGFc2, 365FGFc9, 365FGFec11, 365FGFe5), or a cDNA coding only for 18-kD bFGF (clones 43FGFc8, 43FGFc21, 43FGFc31) as described (Quarto et al., 1989). The cells were grown in DME containing 10% FCS plus 500 µg/ml Genetecin (G418; Gibco BRL, Gaithersburg, MD) and were subcultured at a ratio of 1:10.

Secondary transfection of cells with 18-kD bFGF cDNA: NIH 3T3 cells transfected with the Zip-neo vector containing HMW bFGF cDNA were subsequently transfected with 18-kD bFGF cDNA inserted in the Zip-neo vector plus the pCEP4 vector containing a hygromycin resistance gene (kindly provided by Dr. C. Basilico, New York University Medical Center, New York, NY) at a ratio of 8:1. Hygromycin-resistant clones were selected in DME containing 10% FCS, 200 µg/ml of hygromycin (B Calbiochem-Behring Corp., La Jolla, CA) and 250 µg/ml of G418. Secondary transfec-
tions were identified by Western blotting of cell extracts with anti-bFGF antibodies (clones: 365/43 NCI, 365/43 NC2, 365/43 NC12, 365/43 NC33).

Transfection with a dominant negative bFGF receptor cDNA: NIH 3T3 cells transfected with the Zip-neo vector containing either HMW, 18 kD, or WT bFGF cDNAs were cotransfected with pRKS containing a 1.3-kb insert of a human bek (FGF receptor 2) cDNA that lacks the COOH-terminal tyrosine kinase domain (dominant negative bFGF receptor; kindly provided by Dr. J. Schlessinger, N.Y.U. Medical Center) and the pCEP4 vector. Hygromycin-resistant clones were selected in DME containing 10% FCS, 200 µg/ml of hygromycin, and 250 µg/ml of G418. Resistant clones were tested for high affinity bFGF receptors according to Moscatelli (1987) and by cross-linking to cell surface receptors with 125I-bFGF. HMW bFGF clones: 365DNc2, 365DNc4, 365DNc6, 365DNc7, 365DNc12, 365DNc14, 365DNc24, 365DNc28, 365DNc32, 365DNc38, 365DNc39, 365DNc42, 365DNc43, 365DNc46, 365DNc47, 365DNc51, 365DNc63, 365DNc67, 365DNc80; control clones transfected with the cDNA encoding the hygromycin-resistant gene alone: 365FGFHc3, 365FGFHc5, 365FGFHc8; 18-kD bFGF clones: 43DNc8, 43DNc10, 43DNc11, 43DNc30, 43DNc31, 43DNc33, 43DNc38, 43DNc62, 43DNc72, 43DNc73; control clones transfected with the cDNA encoding the hygromycin-resistant gene alone: 43FGFHc3, 43FGFHc8, 43FGFHc10; WT bFGF clones: WTbDNc1, WTbDNc2, WTbDNc3, WTbDNc4, WTbDNc7; control clones transfected with the cDNA encoding the hygromycin-resistant gene alone: WTbFGFHc3, WTbFGFHc5, WTbFGFHc10.

These clones were analyzed with comparable results in the experiments shown in the figures and were obtained from at least two independent transfections.

Cell Migration Assay

Cell migration was assayed by the phagokinetic track assay (Albrecht-Buehler, 1977) as modified by Mignatti et al. (1991, 1992). Briefly, 20 cells/well were seeded on coverslips coated with 1% BSA (Sigma Chem. Co., St. Louis, MO) and collodial gold and incubated at 37°C in 24-well plates.

The medium was replaced with fresh medium after 2 h. After overnight incubation at 37°C, the cells were fixed with 3.7% formaldehyde and measured at 37°C in 24-well plates.

The area units obtained with the image analyzer (pixel) were converted into µm² by interpolation with a calibration curve obtained by measuring the size of different squares of a hemocytometer. The data were plotted as a probability plot using Kaleidograph software run on an Apple Macintosh Ii computer.

Cell Proliferation Assay

20,000 cells were seeded in 3.5 cm culture dishes. After overnight incubation at 37°C, the cells were refed with DME containing either 10% or 1% FCS. The cells were counted with a Coulter counter (Coulter Electronics, Hialeah, FL) every day for 8 to 10 days.

Growth in Soft Agar

10,000-100,000 cells were seeded in 6 cm dishes in DME containing 10% FCS plus 0.3% agar (2 ml) on a 0.6% (3 ml) agar underlay. The cells were refed twice a week with DME containing 10% FCS and 0.3% (1.5 ml) agar. Colonies were counted after a 3-wk incubation at 37°C.
Metabolic Labeling and Immunoprecipitation

Cells were labeled with $^{35}$S-L-cysteine (250 μCi/ml) for 30 min in cysteine-free minimal Eagle's medium (MEM) and chased with MEM containing 7% FCS and 25 μCi/100 ml of L-cysteine. At the indicated times, the medium was collected, and the cells were washed twice with 20 mM Tris-HCl, 2 M NaCl, pH 4.0, to remove cell surface-associated bFGF. The cells were harvested with 20 mM Tris-HCl, pH 7.4, containing 2 M NaCl, 3 mM EDTA, 20 U/ml of Trasylol (Sigma Chem. Co.), 1 mM DTT (Sigma Chem. Co.), and 0.5 μg/ml of leupeptin (Sigma Chem. Co.) (buffer 1), sonicated and kept on ice for 30 min. The NaCl concentration was adjusted to 0.5 M with lysis buffer (20 mM Tris-HCl, pH 7.4, 3 mM EDTA, 0.3% SDS, 1% Triton X-100, 1 mM DTT, 0.5 μg/ml leupeptin, 20 U/ml of Trasylol, and 0.1% BSA). Conditioned media, 2 M NaCl cell surface washes, and cell extracts were precleared with 100 μl of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and immunoprecipitated with anti-bFGF antiserum (1/200 dilution) in buffer 1, sonicated and kept on ice for 30 min. The NaCl concentration was adjusted to below 0.5 M and immunoprecipitation was performed as indicated above. Sample volumes were normalized to protein concentration. After overnight incubation at 4°C, the beads were washed five times with lysis buffer, and the entire sample loaded in reducing sample buffer on 10 or 15% SDS-polyacrylamide gels. After electrophoresis the gels were dried and analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or by autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY).

$^{125}$I-bFGF Binding Experiments

Binding experiments for high or low affinity bFGF receptors were performed as described (Moscatelli, 1987). Cells in 35 or 60 mm dishes were incubated in duplicate with the indicated concentrations of $^{125}$I-bFGF at 4°C with or without recombinant bFGF. After a 2-h incubation, the cells were washed three times with ice-cold PBS and binding to high or low affinity receptors analyzed as described (Moscatelli, 1987). Cross-linking of $^{125}$I-bFGF receptors was performed as follows: After a 2-h incubation with $^{125}$I-bFGF, cells were washed three times with ice-cold PBS and incubated for 20 min at room temperature with 1 mM bis (sulfosuccinimidyl) suberate (BS3; Pierce Chem. Co., Rockford, IL). At the end of the incubation the cells were washed twice with PBS, lysed with sample buffer (0.1% bromphenol blue, 1% SDS, and 20% glycerol in 50 mM Tris, pH 6.8). The proteins were separated on either 6 or 8% SDS-polyacrylamide gels under reducing conditions, and the iodinated proteins visualized by autoradiography. The samples were analyzed in duplicate.

Tyrosine Phosphorylation

Cells incubated for 10 min with or without 100 ng/ml of bFGF were labeled for 3 h with $^{32}$P-orthophosphate (125 μCi/60 mm dish) in labeling medium (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl₂, 1.2 M MgSO₄, 5.6 mM glucose, and 0.1% BSA, pH 7.4). The cells were washed once with cold 125 μCi/ml of L-cysteine, 1% Triton X-100, and 1 mM PMFSF, pH 7.4). Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies coupled to Agarose (R & D Systems, Minneapolis, MN) or affinity-purified anti-phosphotyrosine antibodies bound to protein A-Sepharose (kindly donated by Dr. W. Burgess, Holland Laboratories, Holland, PA) were washed with PBS and detached with 5 mM EDTA. The suspended cells were exposed to XAR-5 film or analyzed by PhosphorImager scanning.

Results

Migration Is Stimulated in NIH 3T3 Cells Expressing 18-kD bFGF but not in Cells Expressing HMW bFGF

Because endogenous production of bFGF affects cell migration (Mignatti et al., 1991), we examined the migration of NIH 3T3 cells transfected with different species of bFGF. For these studies, we used the phagokinetic gold track assay as NIH 3T3 cells migrate in an FGF-dependent manner in this assay (Mignatti et al., 1991, 1992). As shown in Fig. 1a, NIH 3T3 cells transfected with a cDNA encoding 18-kD bFGF have an increased capacity to migrate when compared with control cells transfected with vector alone. This result is consistent with data obtained previously with cells overexpressing WT bFGF (Mignatti et al., 1991). The cells produce approximately 84 ng 18-kD bFGF or 190 ng WT bFGF per mg of total cell protein as determined by ELISA (Brunner et al., 1993). In contrast, NIH 3T3 cells from two clones transfected with the HMW bFGF cDNA migrated to the same extent as control cells. These cells produce ~85 ng of HMW bFGF per mg of cell protein.

The migration of WT bFGF expressing cells has been shown to depend upon the extracellular concentration of bFGF (Mignatti et al., 1991). Therefore, we investigated whether addition of exogenous recombinant bFGF also increased the migration of NIH 3T3 cells transfected with HMW bFGF cDNA (Fig. 1b). Addition of recombinant 18-kD bFGF increased the migration of cells transfected with the HMW bFGF cDNA (clone 365FGFc2) as well as that of control cells (Fig. 1b). Thus, the lack of migration of cells expressing high amounts of HMW bFGF is not the result of the cells being refractory to the stimulation of exogenous bFGF. In contrast, the migration of cells transfected with the cDNA encoding 18-kD bFGF was not enhanced by exposure to exogenous bFGF (Fig. 1c), probably because the migration of these cells is maximal under normal conditions (Mignatti et al., 1991, 1992).

HMW bFGF purified from guinea pig brain stimulated the migration of both control cells and cells expressing only HMW bFGF as efficiently as recombinant 18-kD bFGF (Table 1). Therefore, the low level of migration in NIH 3T3 cells expressing only HMW bFGF does not result from an inability to respond to extracellular bFGF or from HMW bFGF lacking migration-inducing activity on NIH 3T3 cells.

Low Serum Is Permissive for The Growth of Cells Expressing HMW bFGF but not for Cells Expressing 18-kD bFGF

In the presence of 10% serum, cells expressing high levels of 18-kD, HMW, or WT bFGF grow to high saturation den-
Figure 1. Phagokinetic track areas formed by NIH 3T3 cells transfected with different bFGF cDNAs or with the vector alone. Cells were seeded onto colloidal gold-coated coverslips at a density of 20 cells/coverslip. Following 16-h incubation at 37°C, measurement of the phagokinetic track areas and elaboration of the results were performed as described in Materials and Methods. Each point shows the probability of finding a phagokinetic track with an area equal to or lower than the value indicated on the abscissa. The data were plotted using a probability scale on the ordinate. (a) Migration of cells expressing HMW bFGF. (•) Cells expressing HMW bFGF, clone 365 FGFc2, mean track area ± SEM: 481.1 ± 28.8 μm²; (X) clone 365 FGF/e: 609.4 ± 64 μm²; (●) cells expressing 18-kD bFGF, clone 43 FGFc31: 1481.9 ± 90.1 μm²; (●) control cells, Zipneo: 716.2 ± 45.8 μm². (b) Migration of cells expressing HMW bFGF in the presence of exogenous bFGF. The migration of cells expressing HMW bFGF (clone 365 FGFc2) or control cells (Zipneo) was measured either in the absence (•, 365 FGFc2; ●, Zipneo) or the presence (△, 365 FGFc2; Zipneo) of 20 ng/ml of bFGF. In the presence of bFGF, the mean track area of control cells was 1339 ± 1287 μm² (unstimulated 679.6 ± 61.8 μm²) and that of 365 FGFc2 was 1153 ± 92 μm² (unstimulated 505.1 ± 35.5 μm²). (c) Migration of cells expressing 18-kD bFGF in the presence of exogenous bFGF or anti-bFGF antibodies. The migration of cells expressing only 18-kD bFGF (clone 43 FGFc31) was measured either in the presence of (△, 1717.1 ± 2050 μm²) or absence (●, 1802.3 ± 198.4 μm²) of 20 ng/ml bFGF with (●; mean track area ± SEM: 837.8 ± 608 μm²) or without (●) affinity-purified anti-bFGF antibodies (20 μg/ml). Control cells (○, Zipneo, 679.6 ± 61.8 μm²).

| Addition | Control cells | HMW bFGF expressing cells |
|----------|---------------|---------------------------|
| None     | 721 ± 64      | 460 ± 52.6                |
| HMW bFGF | 1236.9 ± 70.9 | 1005 ± 80.9               |

Cells were plated on colloidal gold-coated coverslips and their migration was measured as described in Materials and Methods. The track areas (μm²) of at least 15 cells were measured for each sample.

FGF Receptors Are Down-modulated in Cells Expressing 18-kD bFGF but not in Cells Expressing HMW bFGF

To investigate whether FGF receptors are modulated differently in NIH 3T3 cells expressing the different species of bFGF, the binding of 125I-bFGF to cells was examined. When the cells were incubated with saturating concentrations of 125I-bFGF, bFGF binding to high affinity receptors was decreased in cells expressing 18-kD bFGF compared to control cells (Fig. 3a). In contrast, the binding to high affinity receptors in cells transfected with HMW bFGF was only slightly decreased. These differences were maintained for at least six days after plating. Cells expressing all forms of bFGF also displayed strongly reduced binding of 125I-bFGF to high affinity receptors as described previously (Moscatelli and Quarto, 1989). These results suggest that high affinity receptors for bFGF were down modulated in cells expressing 18-kD bFGF but not in cells expressing HMW bFGF.
Figure 2. Growth of cells expressing HMW, 18 kD, or WT bFGF in 1% serum. Cell growth was monitored over the indicated time intervals in the presence of 1% FCS as described in Materials and Methods. Cells expressing HMW (•, clone 365FGFc2), 18-kD (•, clone 43FGFc31), or WT bFGF (○, clone WTFGFc3). Control (○, Zipneo). This experiment was repeated three times with similar results. Each point represents the mean ± SEM of duplicate samples.

incubating the cells with a 100-200-fold excess of unlabeled bFGF before cross-linking.

FGF receptor activation by exogenous bFGF was also investigated by immunoprecipitating 32P-orthophosphate-labeled cell extracts with antiphosphotyrosine antibodies. These results revealed that exogenous bFGF stimulated the phosphorylation of several proteins, including proteins at 130-150 and 90 kD, in both control and in cells expressing HMW bFGF, but not in cells expressing 18-kD bFGF (manuscript in preparation). In cells expressing 18-kD bFGF, however, enhanced phosphorylation of the 130-150-kD band was observed without the addition of bFGF. On some occasions addition of recombinant bFGF to these cells decreased the level of protein phosphorylation. The reasons for this decrease are not understood.

Transfection of Cells Expressing Only HMW bFGF with 18-kD bFGF cDNA Results in Increased Migration and FGF Receptor Down-regulation

To test the hypothesis that increased migration and receptor down-regulation were mediated by 18-kD bFGF, cells expressing only HMW bFGF were transfected with a cDNA encoding 18-kD bFGF and a cDNA containing a hygromycin resistance gene. The resultant cells that expressed both HMW and 18-kD bFGF were more refractile and spindle shaped than the parental cells that expressed only HMW bFGF (Fig. 4). These cells that expressed all forms of bFGF also resembled cells treated with recombinant 18-kD bFGF (data not shown).

The doubly transfected cells displayed an increase in migration compared to control cells (Fig. 5). In addition, when cells expressing HMW bFGF transfected with the 18-kD bFGF cDNA were assayed for bFGF binding to high affinity receptors, a decrease in receptor number was observed (data not shown). However, the ability of cells expressing HMW bFGF to grow in the presence of 1% FCS was essentially unaffected by transfection with the 18-kD cDNA (Fig. 6).

Cell Migration and Receptor Down-regulation Correlate With 18-kD bFGF Release

The previous results indicating differences between cells expressing HMW or 18-kD bFGF are most easily explained by postulating the release of 18-kD bFGF but not HMW bFGF.

Figure 3. High affinity binding and cross-linking of 125I-bFGF to NIH 3T3 cells expressing HMW or 18-kD bFGF. (a) Binding of 125I-bFGF to cells. Binding experiments were performed as described in Materials and Methods. Black bars, control cells (Zipneo); open bars, cells expressing 18-kD bFGF (clone 43 FGFc31); grey bars, cells expressing HMW bFGF (clone 365FGFc2). The values represent the mean ± SEM of duplicate samples. (b) Cross-linking of 125I-bFGF to cells. Control cells (Zipneo), cells expressing 18-kD (clone 43FGFc31), or HMW bFGF (clone 365FGFc2) were incubated with 125I-bFGF either in the absence (−) or presence (+) of unlabeled bFGF. Bound 125I-bFGF was cross-linked to cells as described in Materials and Methods and the samples analyzed by SDS-PAGE and autoradiography. The arrowhead indicates the position of the FGF receptor.
However, bFGF has no secretion signal and is released inefficiently. In order to establish whether the phenotypic changes observed with cells expressing 18-kD bFGF were due to extracellular release of the growth factor, we tested the effects of protein G-purified or affinity-purified anti-bFGF IgG on migration and FGF receptor levels in those cells. In the presence of anti-bFGF antibodies, the migration of cells transfected with the 18-kD bFGF cDNA was reduced to control levels (Fig. 1c). Previous experiments have shown that these antibodies also reduce the migration of cells expressing WT forms of bFGF (Mignatti et al., 1991). Because in this assay cell migration is mediated by the bFGF produced by the cells (Mignatti et al., 1991), this result demonstrated that 18-kD bFGF must be released in order to stimulate their migration. Treatment of the cells with protein G-purified or affinity-purified antibodies also increased the cell surface binding of bFGF to high affinity receptors (Fig. 7). This increase was 3.5-fold for cells expressing only 18-kD bFGF and sixfold for cells expressing WT bFGF. Similar results were obtained when receptor levels were measured by cross-linking of $^{125}$I-bFGF to cells treated with affinity-purified antibody to bFGF (data not shown). Thus, down-modulation of bFGF receptors also may be due, at least in part, to the release of 18-kD bFGF and its interaction with the cell surface receptor.

We next assayed for cell surface-associated bFGF by immunoprecipitation of extracts from surface-labeled cells with anti-bFGF antibodies. As shown in Fig. 8, bFGF was only found on the surface of cells expressing either 18-kD bFGF or all forms of bFGF. Cells that expressed exclusively HMW bFGF exhibited only trace amounts of HMW bFGF and a degradation product migrating slightly slower than 18-kD bFGF. However, the total amount of HMW bFGF released was less than found in 18-kD bFGF producing cells. Immunoprecipitation of surface-labeled extracts of cells expressing HMW bFGF that were subsequently transfected with the 18-kD bFGF cDNA also revealed a band of 18-kD bFGF (Fig. 8).

These results were confirmed by the analysis of the distribution of metabolically labeled bFGF. Cell extracts, culture media, and 2 M NaCl cell surface washes were immunoprecipitated with a specific anti-bFGF serum, and the immunoprecipitates were subjected to SDS-PAGE. bFGF was detected in the immunoprecipitates from the cell extracts of all transfected clones but not from the conditioned media (data not shown). In addition, 18-kD bFGF was present in the 2 M NaCl cell surface wash from cells transfected with 18-kD bFGF cDNA (clone 43FGFc3) or WT bFGF cDNA (clone WT FGFc3). No bFGF (either 18-kD or HMW bFGF) could be immunoprecipitated from the cell surface wash or from the medium of cells transfected with HMW bFGF cDNA (clone 365FGFc2) (data not shown). The difference in sensitivity between iodination (Fig. 8) and the metabolic labeling probably accounts for the inability to detect any surface-bound HMW bFGF by the latter approach. Thus, the changes in migration and bFGF receptor level of NIH 3T3 cells expressing different species of bFGF correlate with the release of 18-kD but not HMW bFGF on the cell surface.

Expression of a Dominant Negative bFGF Receptor Reverts the Phenotype of Cells Expressing 18-kD bFGF but not That of Cells Expressing HMW bFGF

To determine whether the interaction of bFGF with its high affinity receptor was responsible for the phenotypic changes
Figure 6. Proliferation of cells expressing HMW bFGF transfected with 18-kD bFGF cDNA. The experiment was carried out as described in Materials and Methods. (a) Growth in 10% FCS; (b) growth in 1% FCS. Cells expressing HMW bFGF (Δ, clone 365 FGFHe3); 365FGFHc3 cells transfected with 18-kD bFGF cDNA (A, 365/43nc33), or Zipneo (○). The data represent mean ± SEM of duplicate samples.

Figure 7. Effect of anti-bFGF antibodies on FGF receptor expression. Cells expressing 18-kD or WT bFGF were incubated for 24 h with or without 100 μg/ml of protein-G-purified anti-bFGF antibody (AB) or 100 μg/ml of non-immune antibody. Binding of 125I-bFGF to its receptor was determined as described in Materials and Methods. Open bars, non-immune antibody; filled bars, anti-bFGF antibody.

Figure 8. 125I-labeling of cell surface-associated bFGF. 125I-labeling of cells, immunoprecipitation, SDS-PAGE, and PhosphorImager analysis were carried out as described in Materials and Methods. Cells expressing 18-kD bFGF (clone 43FGFHc8), HMW bFGF (clone 365FGFHc3), WT bFGF (clone WT FGFc3), or cells expressing HMW bFGF transfected with a cDNA encoding 18-kD bFGF and the dominant negative receptor eDNA (HMW/18-kD bFGF; clone 365/43NC33) were immunoprecipitated with anti-bFGF antibodies. Control, cells transfected with the vector alone (Zipneo).

exhibited by cells expressing the different forms of bFGF, NIH 3T3 cells expressing 18-kD, HMW, or WT bFGF were transfected with a cDNA encoding a dominant negative type 2 FGF receptor. Ueno et al. (1992) have shown that expression of a dominant negative receptor of this type inhibits signaling initiated by all FGF receptor types. Stable transfectants were isolated and were characterized by binding and cross-linking of 125I-bFGF to cell surface receptors. By quantitating the amount of 125I-bFGF bound to cells, as illustrated in Fig. 3 a, clones were obtained that bound up to 17 times more bFGF than did the control Zipneo cells (data not shown). The presence of the dominant negative FGF receptor was verified by the demonstration of a major band of 90 kD and multimeric complexes of 150-200 kD seen after incubation of the cells with 125I-bFGF and cross-linking with BS3 (Fig. 9, insets).

Cells expressing 18-kD or WT bFGF and high levels of the dominant negative receptor cDNA lost their spindle shape and displayed a flat morphology (Fig. 9, compare images a to d and c to f). In contrast, cells co-expressing HMW bFGF and the dominant negative receptor exhibited no morphological change despite their high expression of the dominant negative bFGF receptor (Fig. 9, compare image b to e).

Cells expressing 18-kD or WT bFGF plus the dominant negative bFGF receptor were also characterized for migration. These cells showed decreased motility in phagokinetic assays (Fig. 10) compared to cells expressing only 18 kD or WT bFGF. Thus, expression of the dominant negative receptor prevented the signaling necessary for the stimulation of cell migration induced by 18-kD bFGF.
HMW bFGF Modulates Cell Growth by a Mechanism Independent of Its Cell Surface Receptor

In the presence of 10% FCS cells expressing the dominant negative FGF receptor and 18-kD bFGF grew to saturation densities that were lower than those of cells that did not express the dominant negative receptor (Fig. 11 a). These saturation densities were comparable to that of control cells transfected with the vector alone. In contrast, cells that expressed both HMW bFGF and the dominant negative FGF receptor grew to a saturation density comparable to that of cells that did not express the dominant negative receptor (Fig. 11 b).

In the presence of 1% FCS, cells transfected with 18-kD bFGF and the dominant negative FGF receptor, as well as cells transfected with the vector alone, grew slowly (Fig. 11 c). In contrast, cells expressing HMW bFGF and the dominant negative FGF receptor grew rapidly (Fig. 11 d, and data not shown). As shown above, transfection of cells expressing HMW bFGF with 18-kD bFGF cDNA did not affect their growth rate or saturation density in the presence of either 10 or 1% FCS (Fig. 6).

In addition, expression of the dominant negative receptor significantly inhibited the soft agar growth only of cells expressing exclusively 18-kD bFGF (Table II). The cloning efficiency of cells expressing only 18-kD bFGF was reduced...
Figure 10. Migration of cells expressing 18-kD bFGF or WT bFGF plus the dominant negative FGF receptor. Cells were isolated and their migration measured as described in Materials and Methods. (a and b) Cells expressing 18-kD bFGF and the dominant negative FGF receptor (open symbols; clone 43DNc11; closed symbols, clone 43DNc38); cells expressing only 18-kD bFGF (open symbols; clone 43FGFHc8). Cells transfected with the vector alone (closed symbols, Zipneo; mean track area + SEM = 666.4 ± 42.5 μm²).

Figure 11. Growth curves of cells expressing 18-kD or HMW bFGF and a dominant negative FGF receptor. Cells were isolated and grown experiments performed as described in Materials and Methods. (a and c) Cells expressing 18-kD bFGF transfected with the dominant negative FGF receptor (•, clone 43DNc11; ○, clone 43DNc31); cells transfected with 18-kD bFGF cDNA (•, 43FGFHc3). Control cells (○, Zipneo). (b and d) Cells expressing HMW bFGF transfected with the dominant negative FGF receptor (•, clone 365DNc5; ▲, 365DNc63); non-transfected cells (△, 365FGFHc3). Control cells (○, Zipneo). (a and b) 10% FCS; (c and d) 1% FCS. The data presented represent the mean ± SEM of duplicate samples and are representative of three experiments.

Discussion

The data reported in this paper show that the 18-kD and HMW forms of bFGF may mediate certain cell functions through separate mechanisms of action. This conclusion is based on the following observations: (a) Cells expressing 18-kD bFGF have high motility and surface-associated bFGF. In contrast, cells expressing HMW bFGF have low motility and virtually no surface-associated bFGF. In contrast, cells expressing HMW bFGF have low motility and virtually no surface-associated bFGF.

Table II. Growth in Soft Agar

| Cells                      | Control       | DN receptor transfected |
|----------------------------|---------------|-------------------------|
|                            | % ± SEM       |                         |
| 18 kD                      | 2.06 ± 0.05   | 0.21 ± 0.03             |
| WT bFGF                    | 2.85 ± 0.15   | 0.87 ± 0.10             |
| HMW bFGF                   | 2.04 ± 0.22   | 2.03 ± 0.26             |

Cells expressing 18-kD bFGF, WT bFGF, or HMW bFGF transfected with the dominant negative bFGF receptor (18-kD bFGF: clone 43 DNc11; WT bFGF: clone WTbFGHc2; HMW bFGF: clone 365DNc2) or control cells (18-kD bFGF: clone 43FGFHc10; WT bFGF: WTbFGFHc3; HMW bFGF: 365FGFHc3) were plated at a density of 100,000 cells in 60 mm dishes in 2 ml of 0.3% plating agar on a 0.6% agar underlay. After three weeks, the number of colonies in each plate was counted. The data are expressed as a percent ± SEM of the total cells plated.

These results indicate that HMW bFGF, but not 18-kD bFGF, stimulates cell growth by a mechanism independent of its cell surface receptor.
receptors are down-regulated in cells expressing 18-kD bFGF, but not in cells expressing HMW bFGF. (c) Cells expressing HMW bFGF have a reduced serum requirement for growth, whereas cells expressing 18-kD bFGF proliferate poorly in low serum. (d) Transfection of cells expressing HMW bFGF with 18-kD bFGF cDNA results in increased motility but has no effect on cell growth in low serum. (e) Expression of a dominant negative FGF receptor inhibits the migration and suppresses the growth in soft agar as well as the saturation density of cells expressing 18-kD bFGF, but has no effect on the growth of cells expressing HMW bFGF.

Measurement of the extracellular levels of 18 kD and HMW bFGF revealed significantly more 18-kD bFGF than HMW bFGF. We interpret these results to indicate that the extracellular release of 18-kD bFGF down-regulates receptors, increases motility, and stimulates growth via interaction with plasma membrane receptors. We propose that in the cells we have examined, HMW bFGF is not released in amounts sufficient to affect migration or bFGF receptor levels, but enhances growth by an intracellular mechanism. It is important to note that the responses we have observed are probably dependent upon the absolute expression levels of each of the individual forms of bFGF. As reported by Quarto et al. (1991b), transfection by the HMW bFGF cDNA we have used results in many clones of cells whose growth is inhibited and in a few clones of cells whose growth is rapid.

Among the transfected clones, those clones that grow rapidly have multiple copies of integrated HMW bFGF cDNA, whereas the poorly growing cells usually have only one copy. The mechanism of growth inhibition induced by low levels of HMW bFGF expression is not understood at this time. Our studies have characterized those cells which grow rapidly. No inhibition of cell growth was observed in cells expressing low levels of 18-kD bFGF (Quarto et al., 1991b). These cells grew in a manner similar to cells expressing high levels of 18-kD bFGF. However, the observation that even low expression of HMW bFGF results in impaired growth, whereas low expression of 18-kD bFGF does not, implies that HMW bFGF yields a phenotype different than that of 18-kD bFGF.

The absolute level of expression of 18-kD bFGF may also be critical for the results we have observed. Contrary to our results, others have reported the growth in low or no serum of cells transformed by 18-kD bFGF (Sasada et al., 1988; Neufeld et al., 1988). However, the cDNA used in those experiments was at or close to the AUG initiation codon specifying 18-kD bFGF and did not contain the 5' sequences required for HMW bFGF expression. Prats et al. (1992) have reported that these additional 5' sequences attenuate 18-kD bFGF translation. Therefore, the expression levels of 18-kD bFGF in the cells described by Sasada et al. (1988) and Neufeld et al. (1988) were probably higher than those achieved by our cells. At these higher levels of expression, the serum requirement we observed might be masked.

Cells expressing HMW bFGF are less motile than cells expressing 18-kD bFGF. However, exposure of these cells to exogenous HMW bFGF stimulates cell migration as efficiently as exposure to 18-kD bFGF. This indicates that the low motility of cells expressing HMW bFGF is not because HMW bFGF lacks migration-stimulating activity. Rather, the poor motility and normal levels of receptor in cells expressing HMW bFGF result from the extracellular concentra-

trations of bFGF insufficient to trigger receptor signaling. In addition, expression of a dominant negative FGF receptor reduces the motility of cells expressing 18-kD bFGF, indicating that increased cell migration requires the externalization of 18-kD bFGF and its interaction with cell surface receptors.

Expression of a dominant negative FGF receptor affects neither the growth in 1% FCS nor the anchorage-dependent growth of cells producing HMW bFGF. These data indicate that HMW bFGF can modulate cell proliferation by an intracellular mechanism independent of cell surface receptors. In the light of our present knowledge, the mechanism(s) by which intracellular HMW bFGF modulates cell proliferation is not clear. A number of hypotheses can be considered. First, several data suggest that HMW bFGF can be translocated into the nucleus to exert its mitogenic activity as has been described for the Schwannoma-derived growth factor (Kimura, 1993). The transport of exogenous 18-kD bFGF and aFGF to the nucleus, and their interaction with nuclear receptors or binding molecules have been described in several systems (Baldin et al., 1990; Bouche et al., 1987; Imamura et al., 1991; Zhan et al., 1994). Other proteins and cytokines, including interleukin-1, are also translocated into the nucleus and may interact with nuclear receptors (Curtis et al., 1990; Weitzmann and Savage, 1992). However, a receptor for bFGF localized in the nuclear membrane and processed by the pathway used by the plasma membrane receptors would not have its ligand-binding domain exposed to the nucleoplasm. Thus, the mechanism by which HMW bFGF might interact with this receptor is not obvious. Other molecules involved in signal transduction, including phosphoinositides and protein kinase C, are present in the nuclear membrane, suggesting the possibility of additional signaling systems in the nucleus (Divecha et al., 1991, 1993). Second, HMW bFGF may act as a transcription factor. This hypothesis is supported by the finding that 18-kD bFGF can modulate gene transcription in a cell-free system (Nakanishi et al., 1992), although the concentrations at which bFGF exhibits this activity are high. Also, bFGF lacks a consensus sequence for a classical transcription factor, and it is, therefore, unlikely that HMW bFGF directly promotes gene transcription. HMW bFGF might act indirectly by forming complexes with either transcription factors or growth suppressor genes, or HMW bFGF may interfere with the activity of molecules involved in growth control, such as cyclins or phosphatases. Third, HMW bFGF may interact with a cytoplasmic form of its membrane receptor. Overexpression of a cytoplasmic truncated form of type 1 FGF receptor results in cell transformation (Yan et al., 1993). Therefore, soluble cytoplasmic receptors may be functional, although the mechanism(s) by which these receptors transduce a signal is(are) unknown. The identification of proteins that specifically interact with HMW bFGF may help to elucidate the mechanism of transformation by HMW bFGF.

In conclusion, our results show that 18-kD and HMW bFGF can have distinct mechanisms of action. The former modulates cell motility and proliferation through the interaction with its cell surface receptors. The latter can act as a mitogen and an inducer of anchorage-independent growth through an intracellular mechanism of action but has essentially no effect on cell migration. Although additional roles
for the different molecular weight forms of bFGF cannot be ruled out, these conclusions indicate a spatial and functional bipolarity in the action of bFGFs; the cell membranes as the site of action for 18-kD bFGF, and an intracellular, perhaps nuclear, site for HMW bFGF.

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