Transformation of NIH 3T3 Cells with Basic Fibroblast Growth Factor or the hst/K-*fgf* Oncogene Causes Downregulation of the Fibroblast Growth Factor Receptor: Reversal of Morphological Transformation and Restoration of Receptor Number by Suramin

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Abstract. When NIH 3T3 cells were transfected with the cDNA for basic fibroblast growth factor (bFGF), most cells displayed a transformed phenotype. Acquisition of a transformed phenotype was correlated with the expression of high levels of bFGF (Quarto et al., 1989). Cells that had been transformed as a result of transfection with bFGF cDNA had a decreased capacity to bind ^{125}I-bFGF to high affinity receptors. NIH 3T3 cells transfected with bFGF cDNA that expressed lower levels of bFGF were not transformed and had a normal number of bFGF receptors. NIH 3T3 cells transfected with the hst/Kfgf oncogene, which encodes a secreted molecule with 45% homology to bFGF, also displayed a transformed phenotype and decreased numbers of bFGF receptors. However, NIH 3T3 cells transfected with the H-*ras* oncogene were transformed but had a normal number of bFGF receptors. Thus, transformation by bFGF-like molecules resulted in downregulation of bFGF receptors. Receptor number was not affected by cell density for both parental NIH 3T3 cells and transformed cells. In the cells transfected with bFGF cDNA that were not transformed, the receptors could be downregulated in response to exogenous bFGF. Conditioned medium from transformed transfected cells contained sufficient quantities of bFGF to downregulate bFGF receptors on parental NIH 3T3 cells. Thus, the downregulation of bFGF receptors seemed related to the presence of bFGF in an extracytoplasmic compartment. Treatment of the transformed transfected NIH 3T3 cells with suramin, which blocks the interaction of bFGF with its receptor, reversed the morphological transformation and restored receptors almost to normal numbers. These results demonstrate that in these cells bFGF transforms cells by interacting with its receptor and that bFGF and hst/K-*fgf* may use the same receptor.

Basic fibroblast growth factor (bFGF)[1] stimulates the growth and modulates the differentiated function of cells of mesenchymal, ectodermal, and endodermal origin (Gospodarowicz et al., 1987; Rifkin and Moscatelli, 1989). Acidic FGF (aFGF), a polypeptide mitogen with a 55% amino acid sequence homology to bFGF, shares many of the same biological properties (Gospodarowicz et al., 1987). The overlap in biological activities may be due to the fact that in several cell types these two mitogens seem to share the same receptors (Neufeld and Gospodarowicz, 1986; Huang et al., 1986; Olwin and Hauschka, 1986; Hoshi et al., 1988). Recently, three oncogenes have been identified that code for proteins with 40–45% sequence homology to bFGF (Taira et al., 1987; Yoshida et al., 1987; Delli-Bovi et al., 1987; Dickson and Peters, 1987; Zhan et al., 1988). The product of one of these oncogenes, hst/K-*fgf*, has been partially characterized and has many biological properties in common with bFGF (Delli-Bovi et al., 1988). The hst/K-*fgf* protein also seems to use the same receptors as bFGF (unpublished observations).

Receptors for bFGF have been identified on a variety of cell types (Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986; Moenner et al., 1986; Moscatelli, 1987; Neufeld et al., 1987; Neufeld and Gospodarowicz, 1988; Presta et al., 1988). In many cell types, two putative receptor proteins of 125,000 and 145,000 molecular weight can be cross-linked to radiolabeled bFGF. Some variation in size of the receptor among various cell types has been reported (Neufeld and Gospodarowicz, 1988). Occupation of the receptor leads to internalization of bFGF and a decrease in the number of cell surface receptors (Neufeld and Gospodarowicz, 1985; Moscatelli, 1988). For several cell types, the number of receptors per cell seems to be roughly inversely

1. *Abbreviations used in this paper*: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor.

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proportional to the cell content of bFGF (Moscatelli, 1987; Tsuboi, R., Y. Sato, and D. B. Rifkin, manuscript submitted for publication). This apparent downregulation of receptors in cells that produce bFGF may be related to the observation that bFGF acts in an autocrine manner in some cell types (Sato and Rifkin, 1988).

In addition to binding to receptors, bFGF also binds to heparan sulfate proteoglycans produced by the cell (Moscatelli, 1987; Saksela et al., 1988). It is unclear whether these heparan sulfate proteoglycans are cell-surface molecules or components of the extracellular matrix or both. For the purposes of this discussion, the term matrix will refer to all of these possibilities. The binding of bFGF to the cell-associated heparan sulfate proteoglycans can complicate the measurement of bFGF receptors. However, bFGF bound to matrix can be separated from bFGF bound to the receptor by washing cells with 2 M NaCl at neutral pH (Moscatelli, 1987). The 2 M NaCl wash removes bFGF bound to matrix while the bFGF bound to receptors remains cell associated. A subsequent wash with 2 M NaCl at pH 4.0 removes bFGF bound to receptors. These procedures can be used to quantitate bFGF receptors despite high levels of bFGF binding to matrix.

Both bFGF and aFGF lack a conventional signal sequence for secretion and appear to be localized primarily within the cell. It thus is unclear how these growth factors interact with cell surface receptors in autocrine systems. Cells with a transformed phenotype as a result of transfection with genes of the FGF family can be viewed as a model for an autocrine system using FGF. Cells transfected with bFGF to which the immunoglobulin signal sequence has been spliced have a transformed phenotype, suggesting that in these cells the bFGF is secreted and interacts with bFGF receptors in an extracytoplasmic compartment (Rogelj et al., 1988). However, in these experiments no bFGF could be detected in the conditioned medium of these cells (Rogelj et al., 1988). Cells producing a high level of unmodified bFGF or aFGF also appear to be transformed (Neufeld et al., 1988; Sasada et al., 1988; Jaye et al., 1988; Quarto et al., 1989). In the case of aFGF, no decrease in receptor level was detected in the transformed cells (Jaye et al., 1988). This finding raises the possibility that FGFs can transform without interacting with their receptors. It recently has been shown that in NIH 3T3 cells transfected with bFGF, transformation is related to the level of bFGF expression (Quarto et al., 1989). Here we demonstrate that in these cells transformation is also correlated with a decrease in receptor number, suggesting that bFGF transforms through an interaction with its receptor.

Materials and Methods

Reagents

bFGF was purified from human placentas as described previously (Moscatelli et al., 1986*b*; Presta et al., 1986). Recombinant bFGF was a gift from Synergen, Inc. (Boulder, CO). Natural and recombinant bFGF behaved similarly and were used interchangeably. bFGF was iodinated using the Iodo-Gen reagent (Pierce Chemical Co., Rockford, IL) as described previously (Neufeld and Gospodarowicz, 1985; Moscatelli, 1987). ^{125}I-bFGF had a specific activity of ~500 cpm/fmol.

Cells

Mouse NIH 3T3 cells were transfected with the retroviral vector pZipNeoSV(X) alone, pZipNeoSV(X) containing a human bFGF cDNA insert, or pZipNeoSV(X) containing an hst/K-*fgf* cDNA insert as described previously (Quarto et al., 1989). Briefly, 3 × 10^5 NIH 3T3 cells/100-mm plate were transfected with a calcium phosphate precipitate containing 0.5 μg of plasmid DNA and 20 μg of NIH 3T3 high-molecular weight DNA. After 24 h in culture, the cells were split 1:5 and cultured thereafter in DME containing 10% calf serum (Gibco Laboratories, Grand Island, NY) and 500 μg/ml of G418 (Gibco Laboratories). After 2 wk, the G418-resistant clones were isolated and expanded, and the cells were analyzed. In some experiments, NIH 3T3 cells transfected with pZipNeoSV(X) containing a mutated bFGF cDNA insert were used. Mutations were made as described by Florkiewicz and Sommer (1989). In the mutant 365 bFGF cDNA, the ATG (met) codon at nucleotide positions 364–366 initiating translation of 18 kD bFGF was changed to GCT (ala). In the mutant 43 bFGF cDNA, a 17-nucleotide sequence was inserted between nucleotides 352 and 353 causing a +1 frame shift for the bFGF species initiating translation 5′ to the start codon for 18 kD bFGF.

NIH 3T3 cells transfected with the H-*ras* oncogene were provided by Dr. Claudio Basilico of the New York University Medical Center (New York).

Determination of High-affinity Binding of ^{125}I-bFGF

Cells were plated at 1 × 10^6 per 60-mm dish. After 36 h at 37°C, the cultures were washed twice with cold PBS and incubated for 2 h at 4°C in DME containing 0.15% gelatin, 25 mM Hepes, pH 7.4, and 10 ng/ml ^{125}I-bFGF. This concentration of ^{125}I-bFGF was sufficient to saturate bFGF receptors. To control for nonspecific binding, parallel cultures were incubated under the same conditions with the addition of 200-fold excess unlabeled bFGF. At the end of the incubation period, the medium was removed, and the cells were washed once with PBS and twice with 2 M NaCl in 20 mM Hepes, pH 7.4, to remove ^{125}I-bFGF bound to the extracellular matrix (Moscatelli, 1987). ^{125}I-bFGF bound to receptors was released by two subsequent washes with 2 M NaCl in 20 mM sodium acetate, pH 4.0 (Moscatelli, 1987, 1988). The amount of radioactivity released in the washes was determined in a gamma scintillation counter (model 5210; Packard Instruments, Downers Grove, IL). Parallel cultures were trypsinized, and the number of cells per culture was determined with a particle counter (Coulter Electronics, Hialeah, FL).

In some experiments, cells were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with cold PBS before incubation with ^{125}I-bFGF to remove any bFGF already bound to the cells.

Cross-Linking of ^{125}I-bFGF to Cells

Cells were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with cold PBS and were incubated in DME containing 0.15% gelatin, 25 mM Hepes, pH 7.4, and 10 ng/ml ^{125}I-bFGF for 2 h at 4°C. The medium was removed and replaced with PBS containing 1 mM bis(sulfosuccinimidyl)suberate (Pierce Chemical Co.) and 10% DMSO. After a 15-min incubation at room temperature, the cells were extracted with electrophoresis sample buffer and the extract was heated for 2 min in a boiling water bath. The samples were subjected to SDS-PAGE on a 3% stacking gel and a 5–15% gradient resolving gel. The gels were dried and exposed to Kodak X-MAR (Eastman Kodak, Rochester, NY) autoradiography film.

Suramin Treatment of Cells

24 h after plating the cells, the medium of cultures was changed to fresh DME containing 10% calf serum with or without 1 mM suramin (Mobay Chemical Corp., New York, NY). After a 12-h incubation at 37°C, the cells were washed twice with PBS and incubated for a further 24 h in DME containing 10% calf serum with or without 1 mM suramin. The cells were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with cold PBS and used for binding studies. To compensate for the fact that the cells treated with suramin did not grow during the treatment, these cultures were plated at 1.0 × 10^6 cells per 60-mm dish, while untreated cultures were plated at 0.5 × 10^6 cells per 60-mm dish.

Western Blot Analysis of Suramin-treated Cells

Untreated clone B3 cells and cells treated with 1 mM suramin for 36 h as described above were washed twice with cold PBS, scraped from the plate in PBS, sonicated for 1 min at 20 W, and heated in a boiling water bath for 5 min. 40 μg of the cell sonicate was subjected to SDS-PAGE on a 3% stacking gel and a 12% resolving gel. The gel was electroblotted onto a nitrocel-

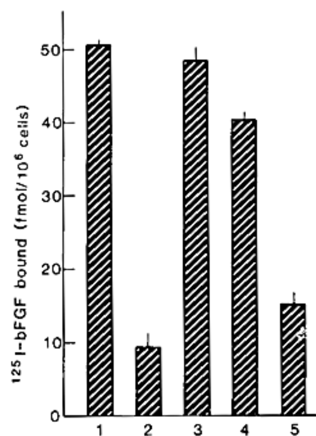

Figure 1. Binding of ^{125}I-bFGF to high-affinity receptors on transfected and nontransfected NIH 3T3 cells. Cultures containing ~1.5 × 10^6 cells/60-mm dish of nontransfected NIH 3T3 cells (column *1*), clone B3 cells (column *2*), clone B14 cells (column *3*), NIH 3T3 cells transfected with the Zip plasmid only (column *4*), or NIH 3T3 cells transfected with the hst/K-*fgf* oncogene (column *5*) were incubated for 2 h at 4°C with DME containing 0.15% gelatin and 10 ng/ml ^{125}I-bFGF. The cells were washed once with PBS and twice with 2 M NaCl in 20 mM Hepes, pH 7.4, to remove ^{125}I-bFGF bound to matrix. ^{125}I-bFGF bound to receptors was removed by two washes with 2 M NaCl in 20 mM sodium acetate, pH 4.0, and was quantitated in a gamma scintillation counter. Nonspecific binding was determined by incubating parallel cultures with the above solution containing a 200-fold excess of unlabeled bFGF.

lulose filter. The blot was probed for 30 min at room temperature with anti-bFGF antibodies diluted 1:200 in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, plus 5% nonfat dry milk, washed three times with the same buffer, and incubated with ^{125}I-protein A (New England Nuclear, Boston, MA) in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, plus 0.5% gelatin for 30 min at room temperature. After three washes in the same buffer, the filter was dried and exposed to Kodak X-MAR film. The intensity of the bands on the autoradiogram were compared after scanning with a soft laser densitometer.

Results

NIH 3T3 cells normally produce undetectable levels of bFGF. When these cells were transfected with bFGF cDNA, clones were isolated that express 0.66 pg/cell of bFGF (Quarto et al., 1989), 300 times as much bFGF as expressed by SK-HEP-1 human hepatoma cells, which are among the highest natural producers of bFGF described (Moscatelli et al., 1986*a*). Cells of clones expressing these high levels of bFGF have a transformed phenotype (Quarto et al., 1989). The number of receptors for bFGF on the transfected cells was compared to the number of receptors on the parental nontransfected cells. In these experiments, cells were incubated with 10 ng/ml ^{125}I-bFGF, a concentration of bFGF sufficient to saturate bFGF receptors (data not shown). ^{125}I-bFGF bound to matrix was removed with a wash with 2 M NaCl at pH 7.4. ^{125}I-bFGF bound to receptors was then removed with a wash with 2 M NaCl at pH 4.0 and quantitated in a gamma scintillation counter.

The cells of one transformed clone, B3, bound only 20% as much ^{125}I-bFGF to high-affinity receptors as control nontransfected cells (Fig. 1). Similar results were obtained with the cells from another independently isolated transfected clone (B1) that displayed a transformed phenotype (data not shown). Cells from a clone transfected with the same plasmid without the bFGF cDNA had the same number of receptors as nontransfected cells, demonstrating that transfection and the selection of the clones had no effect on receptor number (Fig. 1). Cells from another clone (B14) transfected with the bFGF cDNA, which expressed 10–20% of the amount of bFGF in clone B3 cells, did not exhibit a transformed phenotype. These cells bound as much ^{125}I-bFGF to high affinity receptors as the nontransfected cultures (Fig. 1). Cells transfected with the hst/K-*fgf* oncogene also displayed a lower number of receptors than nontransfected cultures (Fig. 1). In contrast, there was no difference among these clones in the amount of ^{125}I-bFGF bound to matrix (data not shown).

Similar results were obtained when the receptors were visualized by cross-linking them to radioactive ^{125}I-bFGF (Fig. 2). In these experiments, the cells were incubated for 2 h at 4°C with 10 ng/ml ^{125}I-bFGF, and then the cross-linking agent bis(sulfosuccinimidyl)suberate was added for 15 min at room temperature. When extracts of the cells were analyzed by electrophoresis followed by autoradiography, high molecular weight bands containing radioactive bFGF could be detected in nontransfected NIH 3T3 cells. These bFGF-receptor complexes had molecular weights of ~165,000 as described previously (Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986). The complexes detected in cells transfected with the vector without bFGF cDNA or cells transfected with bFGF that have a nontransformed phenotype were equal in intensity to the complexes detected in nontransfected cells. In cells transfected with bFGF that display a transformed phenotype and cells transfected with the hst/K-*fgf* oncogene, only low levels of ^{125}I-bFGF-receptor complex could be formed.

The low level of bFGF receptors measured in clone B3 was not due to the occupancy of receptors by bFGF produced by the cells. To show this, clone B3 cells were washed with cold 2 M NaCl at pH 4.0, which has been shown to remove bFGF from receptors and from the matrix (Moscatelli, 1987), and then were used for binding studies. The acid-washed clone B3 cells bound the same amount of ^{125}I-bFGF as PBS-washed cells (data not shown). The amount of bFGF bound by nontransfected NIH 3T3 cells was not affected by the acid wash (data not shown), demonstrating that the wash procedure did not alter the receptor (Moscatelli, 1988). Similarly, the low number of receptors measured in the transfected cells

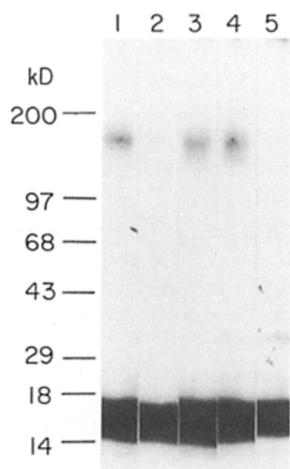

Figure 2. Cross-linking of ^{125}I-bFGF to high affinity receptors on transfected and nontransfected NIH 3T3 cells. Cultures of nontransfected NIH 3T3 cells (lane *1*), clone B3 cells (lane *2*), clone B14 cells (lanes *3*), NIH 3T3 cells transfected with the Zip plasmid only (lane *4*), or NIH 3T3 cells transfected with the hst/K-fgf oncogene (lane *5*) were incubated for 2 h at 4°C with DME containing 0.15% gelatin and 10 ng/ml ^{125}I-bFGF. The cells were washed with PBS and incubated in PBS containing 1 mM bis(sulfosuccinimidyl)suberate and 10% DMSO for 15 min at room temperature. The cells were extracted in electrophoresis sample buffer, and the extracts were analyzed on a 5–15% gradient SDS polyacrylamide gel, followed by autoradiography.

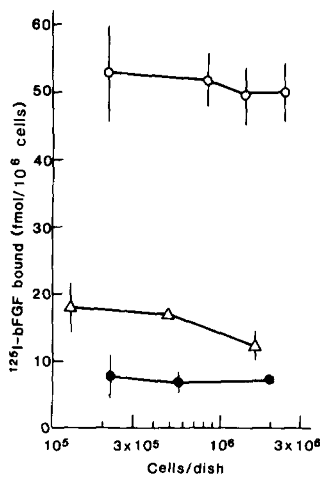

Figure 3. Effect of cell density on high-affinity bFGF receptors of transfected and nontransfected NIH 3T3 cells. Nontransfected NIH 3T3 cells (○), clone B3 cells (●), and NIH 3T3 cells transfected with the hst/K-*fgf* oncogene (△) were plated at various concentrations on 60-mm dishes in DME containing 10% calf serum. After 24 h at 37°C, the cells were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with PBS to remove any bound bFGF. The cells were incubated with 10 ng/ml ^{125}I-bFGF for 2 h at 4°C, and the amount of ^{125}I-bFGF bound to high-affinity receptors was determined as described in the legend to Fig. 1. Parallel cultures were trypsinized and the number of cells was determined on a particle counter (Coulter Electronics).

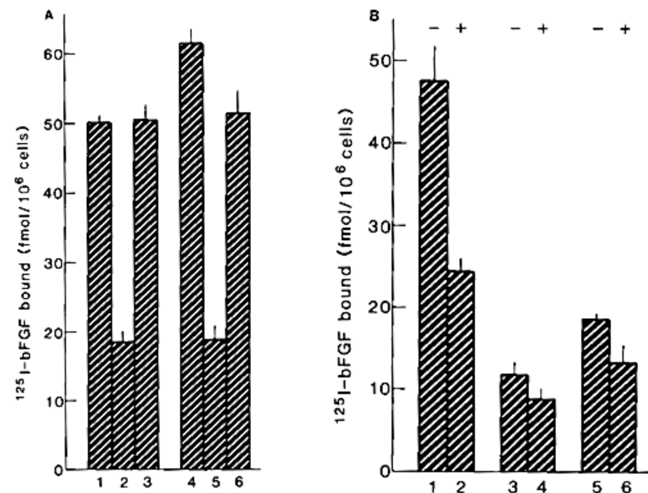

Figure 4. Downregulation of bFGF receptors in transfected and nontransfected NIH 3T3 cells. (*A*) Cultures containing ~1.5 × 10^6 cells/60-mm dish of nontransfected NIH 3T3 cells (columns *1–3*) or clone B14 cells (columns *4–6*) were changed to fresh DME containing 10% calf serum and 10 ng/ml bFGF (columns *2* and *5*) or no bFGF (columns *1*, *3*, *4*, and *6*) and incubated at 37°C for 2 h. Some of the cultures incubated without bFGF were then changed to cold DME containing 10% calf serum and 10 ng/ml bFGF and incubated at 4°C for 10 min (columns *3* and *6*). All cultures were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with PBS to remove bound bFGF. The cells were incubated with 10 ng/ml ^{125}I-bFGF for 2 h at 4°C, and the amount of ^{125}I-bFGF bound to high-affinity receptors was determined as described in the legend to Fig. 1. (*B*) Cultures containing ~1.5 × 10^6 cells/60-mm dish of nontransfected NIH 3T3 cells (columns *1* and *2*), clone B3 cells (columns *3* and *4*), or NIH 3T3 cells transfected with the hst/K-*fgf* oncogene (columns *5* and *6*) were changed to fresh DME containing 10% calf serum and 10 ng/ml bFGF (columns *2*, *4*, and *6*) or no bFGF (columns *1*, *3*, and *5*) and incubated at 37°C for 2 h. Binding of ^{125}I-bFGF to high-affinity receptors was determined as described above.

was not due to the release of bFGF from the cells during the binding assay, which then competed with ^{125}I-bFGF for binding to its receptor. To demonstrate this, clone B3 cells were washed with 2 M NaCl, pH 4.0, and incubated for 2 h at 4°C in binding buffer without ^{125}I-bFGF. This buffer was collected, ^{125}I-bFGF was added to it, and it was compared with fresh buffer in a binding assay on nontransfected NIH 3T3 cells. A similar number of receptors was calculated for cells incubated in either buffer (data not shown). Thus, insufficient amounts of bFGF are released by the transfected cells during the binding assay to compete with the ^{125}I-bFGF.

It has been reported that for some cell types bFGF receptors appear to be regulated by cell density (Neufeld and Gospodarowicz, 1985; Rizzino et al., 1988). Because of differences in cell shape, cell-to-cell contact occurs at lower densities for nontransformed NIH 3T3 cells than for transformed cells. To determine whether the differences in receptor number between nontransformed and transformed cells was related to cell contact, we investigated whether receptor number was modulated by cell density. There was virtually no change in receptor number in nontransfected NIH 3T3 cells, clone B3 cells, or cells transfected with the hst/K-*fgf* oncogene over a 10-fold range of cell densities from sparse to highly confluent (Fig. 3).

The normal number of receptors in clone B14 was not due to an inability of these cells to downregulate their receptors. When 10 ng/ml of bFGF was added to these cultures and the cells were incubated at 37°C for 2 h, the number of receptors was decreased by 50% (Fig. 4 *A*). A similar decrease was observed in nontransfected NIH 3T3 cells that had been incubated with 10 ng/ml bFGF at 37°C for 2 h. When the 10 ng/ml bFGF was added at 4°C for 10 min, there was no change in receptor number, showing that this effect is not due merely to the addition of unlabeled bFGF (Fig. 4 *A*). Addition of 10 ng/ml of exogenous bFGF for 2 h at 37°C caused no further decrease in the number of bFGF receptors in clone B3 cells or in cells transfected with the hst/K-*fgf* oncogene (Fig. 4 *B*). Thus, the difference in receptor number between clones B3 and B14 is not related to their ability to downregulate receptors and is likely to be a result of the different levels of bFGF expression in these cells.

The amount of bFGF necessary to downregulate bFGF receptors on NIH 3T3 cells was investigated. Subconfluent cultures of NIH 3T3 cells were incubated with various concentrations of bFGF for 5 h at 37°C. These cells were washed with 2 M NaCl at pH 4.0 to remove bound bFGF and were used for binding studies with ^{125}I-bFGF at 4°C. Incubation with 0.5 ng/ml bFGF resulted in a half-maximal downregulation of bFGF receptors (Fig. 5). Maximal downregulation of bFGF receptors was obtained with 3 ng/ml bFGF. In contrast, incubation of the cells with bFGF had no effect on ^{125}I-bFGF binding to the matrix (data not shown). The transformed clone B3 cells have been demonstrated to release bFGF into their medium (Quarto et al., 1989). Medium collected after a 24-h incubation on clone B3 cells and transferred to nontransformed NIH 3T3 cells for 5 h at 37°C caused a downregulation of bFGF receptors on the NIH 3T3 cells to a level equivalent to that caused by 0.5 ng/ml bFGF (Fig. 5, ○), demonstrating that the bFGF released by these cells is capable of downregulating cellular receptors.

Clone B1, B3, and B14 cells were transfected with bFGF cDNA that encodes four species of bFGF with molecular

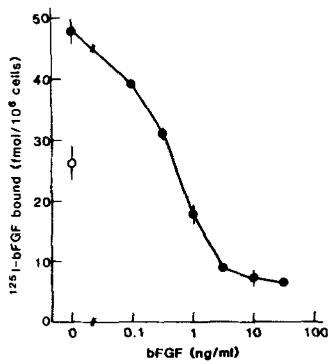

Figure 5. Dose-dependent downregulation of high-affinity bFGF receptors in NIH 3T3 cells. Nontransfected NIH 3T3 cells (1.5×10^6/60 mm dish) were changed to fresh DME containing 10% calf serum and the indicated concentrations of bFGF and incubated at 37°C for 5 h. In addition, some cultures were incubated with DME containing 10% calf serum that had been conditioned by clone B3 cells for 24 h (o). All cultures were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with PBS to remove bound bFGF. The cells were incubated with 10 ng/ml ^{125}I-bFGF for 2 h at 4°C, and the amount of ^{125}I-bFGF bound to high-affinity receptors was determined as described in the legend to Fig. 1.

masses of 18, 22, 22.5, and 24 kD (Quarto et al., 1989; Florkiewicz and Sommer, 1989). These species arise from initiation of translation at separate codons (Florkiewicz and Sommer, 1989) and differ from each other in the length of NH_2-terminal extensions (Florkiewicz and Sommer, 1989; Sommer et al., 1989). All four forms are present in extracts of these cells in approximately equal amounts (Quarto et al., 1989). Western blot analysis of medium conditioned for 24 h by clone B3 cells demonstrated that all four forms were also present in the medium (data not shown). To determine whether the downregulation of bFGF receptors could be attributed to expression of either 18-kD bFGF or the higher molecular weight bFGFs, receptor number on cells transfected with mutated bFGF cDNAs was investigated. NIH 3T3 cells transfected with bFGF cDNA in which the codon initiating 18-kD bFGF was mutated (365 bFGF cells) express only 22-, 22.5- and 24-kD bFGFs (Quarto, N., D. Talarico, R. Florkiewicz, C. Basilico, and D. B. Rifkin, manuscript in preparation). Clone 365 bFGF C7 cells, which express high levels of the higher molecular weight bFGFs and display a transformed morphology, had low levels of bFGF receptor (Table I). Clone 365 bFGF C6 cells, which expressed lower levels of 22-, 22.5-, and 24-kD bFGFs, did not exhibit a transformed morphology and bound as much ^{125}I-bFGF on high-affinity receptors as nontransfected cells (Table I). Thus, in cells that express only higher molecular weight bFGFs, transformation is also correlated with downregulation of the bFGF receptor, suggesting that the higher molecular weight bFGFs are able to reach an extracytoplasmic compartment and interact with the receptor. NIH 3T3 cells transfected with a bFGF cDNA carrying a frame-shift insertion 5′ to the initiation codon for 18-kD bFGF (43 bFGF cells) synthesize only 18-kD bFGF (Quarto, N., D. Talarico, R. Florkiewicz, C. Basilico, and D. B. Rifkin, manuscript in preparation). Clone 43 bFGF C31 cells that express high levels of 18-kD bFGF exhibit a transformed morphology and have down-regulated bFGF receptors (Table I). Thus, the high level expression of either high molecular weight bFGFs or 18 kD bFGF results in transformation of cells and downregulation of bFGF receptors.

It appears that the decreased number of bFGF receptors

Table I. Receptor Content of NIH 3T3 Cells Transfected with Mutant bFGF cDNAs

Cell line	^{125}I-bFGF bound
	fmol/10⁶ cells
NIH 3T3 parental	49.8 ± 0.5
365 bFGF C7	10.6 ± 2.0
365 bFGF C6	46.6 ± 3.0
43 bFGF C31	3.4 ± 0.8

Cultures containing ~1.5×10^6 cells/60-mm dish of nontransfected NIH 3T3 cells or NIH 3T3 cells transfected with mutant bFGF cDNAs were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with PBS. The cells were incubated with 10 ng/ml ^{125}I-bFGF for 2 h at 4°C, and the amount of ^{125}I-bFGF bound to high-affinity receptors was determined as described in the legend to Fig. 1.

in clone B3 cells is related to their high level expression of bFGF and is due to bFGF produced by the cells causing a downregulation of bFGF receptors. We investigated whether receptor number in clone B3 cells could be restored to the level seen in nontransfected cells with suramin or protamine sulfate, two agents that block bFGF interaction with its receptor (Coffey et al., 1987; Neufeld and Gospodarowicz, 1987). Suramin, which also prevents the interaction of platelet-derived growth factor (PDGF) with its receptor, reverses the morphological transformation of cells expressing v-*sis* (PDGF B chain) and decreases the rate of turnover of PDGF receptors in these cells (Garrett et al. 1984; Betsholtz et al., 1986; Huang and Huang, 1988).

Cell morphology and receptor number of clone B3 cells were not affected by protamine sulfate at concentrations up to 0.3 mg/ml (data not shown). However, when clone B3 cells were incubated with 1 mM suramin for 36 h, their transformed morphology was lost (Fig. 6, *C* and *D*). Suramin had a less dramatic effect on the morphology of NIH 3T3 cells transfected with the plasmid alone (Fig. 6, *A* and *B*), and had little effect on the morphology of NIH 3T3 cells transformed with the H-ras oncogene (Fig. 6, *E* and *F*). Suramin had no effect on the amount of bFGF produced by clone B3 cells. This was demonstrated by Western blot analysis of extracts of suramin-treated and untreated clone B3 cells (Fig. 7 *A*). Extracts of treated cells contained all four forms of bFGF in amounts equivalent to untreated cells. (In these gels, the 22- and 22.5-kD forms run together.) Dot blots of dilutions of the extracts confirmed that the total amount of bFGF in suramin-treated clone B3 cells was equal to the amount in nontreated cells (data not shown). However, suramin treatment resulted in an increase in the number of cell surface bFGF receptors, restoring them almost to control levels (Fig. 7 *B*). Suramin had little effect on receptor number in cells transfected with the plasmid alone or with the H-*ras* oncogene. A similar reversal of the transformed morphology was observed when cells transfected with the hst/K-*fgf* oncogene were treated with suramin (data not shown).

Discussion

The decreased number of bFGF receptors in some clones of transfected NIH 3T3 cells can be correlated with their transformed phenotype. Clones that expressed high levels of bFGF exhibited a transformed phenotype and had a de-

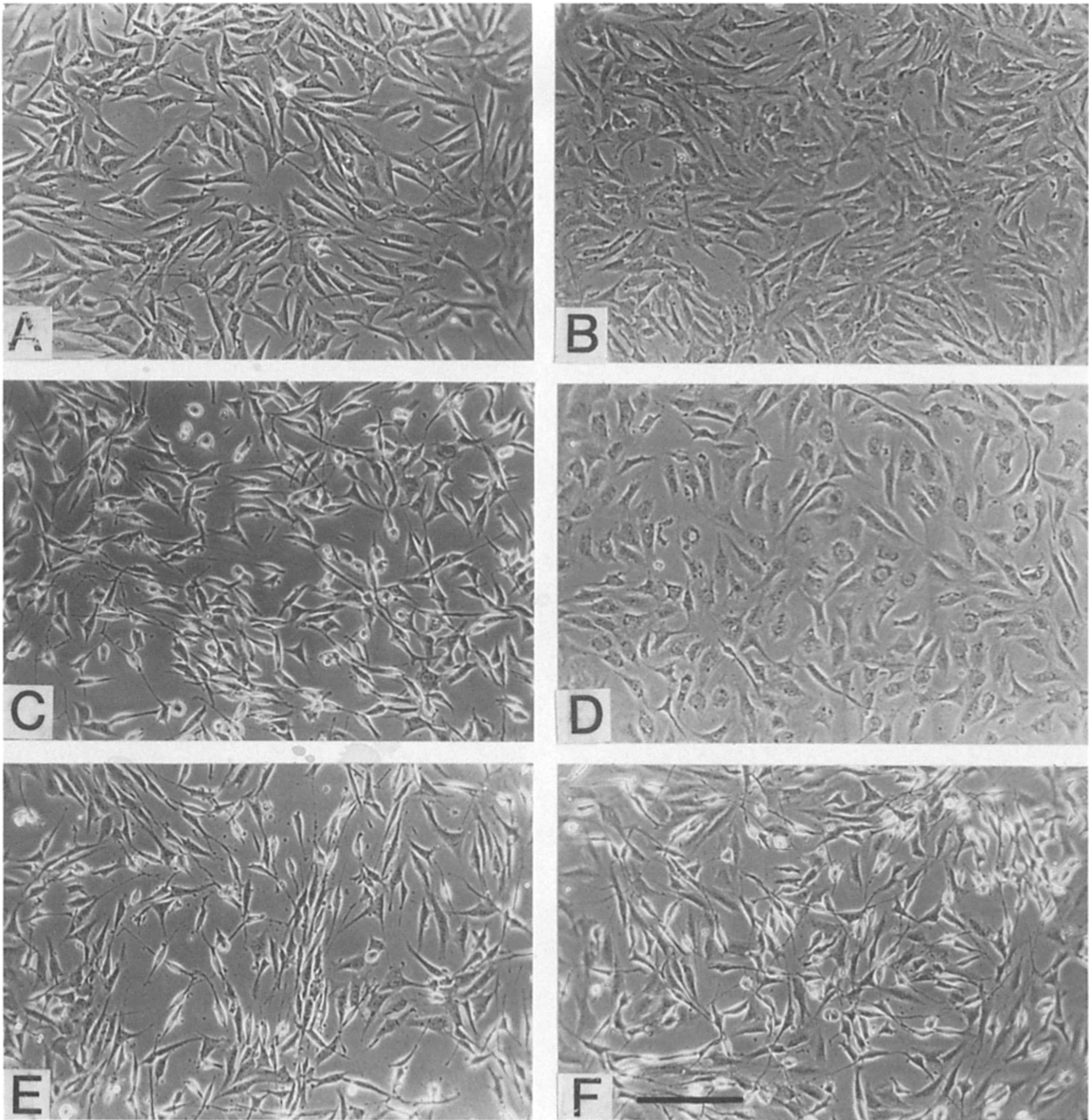

Figure 6. Effect of suramin on the morphology of transfected NIH 3T3 cells. NIH 3T3 cells transfected with the Zip plasmid alone (*A* and *B*), clone B3 cells (*C* and *D*), and NIH 3T3 cells transfected with the H-*ras* oncogene (*E* and *F*) were incubated with DME containing 10% calf serum and 1 mM suramin (*B*, *D*, and *F*) or no suramin (*A*, *C*, and *E*) as described in Materials and Methods. After a 36-h treatment at 37°C, the cells were photographed. Bar in *F*, 200 μm.

creased number of bFGF receptors. Likewise, cells that expressed the hst/K-*fgf* oncogene, a bFGF-like molecule that contains a signal sequence and is secreted, also exhibited a transformed phenotype and decreased bFGF receptors. In contrast, clones that expressed lower levels of bFGF were not transformed and had the same number of bFGF receptors as nontransfected cells. Suramin, which has previously been shown to block the interaction of bFGF with its receptor (Huang et al., 1986; Coffey et al., 1987), restored the clones that expressed high levels of bFGF or the hst/K-*fgf* oncogene to a morphologically nontransformed phenotype and increased receptor number to control levels. These results suggest that, in NIH 3T3 cells, bFGF transforms by interaction with its receptor.

Expression of PDGF, transforming growth factor-α, granulocyte-macrophage colony-stimulating factor, or colony-stimulating factor 1 in cells that have functional receptors for the appropriate growth factor also leads to transformation of the

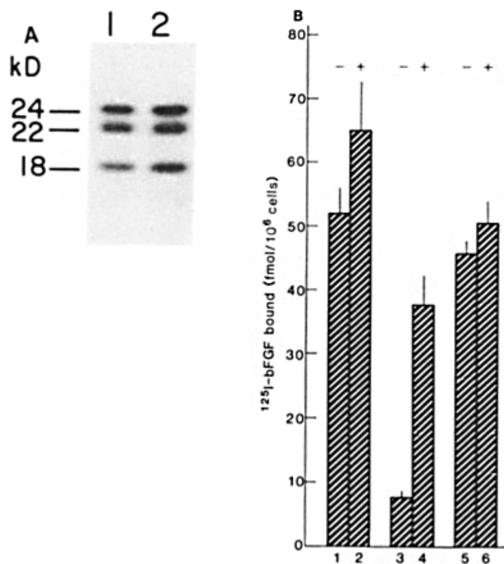

Figure 7. Effect of suramin on bFGF expression and bFGF receptors in transfected NIH 3T3 cells. (*A*) Cultures of clone B3 cells incubated with no suramin (lane *1*) or 1 mM suramin (lane *2*) for 36 h were extracted, and the extracts were assayed for bFGF content by Western blot as described in Materials and Methods. (*B*) Parallel cultures of NIH 3T3 cells transfected with the Zip plasmid alone (columns *1* and *2*), clone B3 cells (columns *3* and *4*), and NIH 3T3 cells transfected with the H-*ras* oncogene (columns *5* and *6*) were incubated with DME containing 10% calf serum and 1 mM suramin (columns *2*, *4*, and *6*) or no suramin (columns *1*, *3*, and *5*) as described in Materials and Methods. After a 36-h treatment at 37°C, the cells were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with PBS. The cells were incubated with 10 ng/ml ^{125}I-bFGF for 2 h at 4°C, and the amount of ^{125}I-bFGF bound to high-affinity receptors was determined as described in the legend to Fig. 1.

cell (Betsholtz et al., 1984; Garrett et al., 1984; Huang et al., 1984; Lang et al., 1985; Rosenthal et al., 1986; Rettenmier et al., 1987). These growth factors have signal sequences and the newly synthesized proteins are rapidly released into the endoplasmic reticulum. In the vesicular compartment of the cell, these growth factors may interact with newly synthesized receptors or are efficiently secreted and bind to cell-surface receptors. In contrast, bFGF lacks a classical signal sequence and appears to be primarily retained in an intracellular compartment. That bFGF interacts with its receptor in clone B3 cells indicates that despite the absence of a signal sequence some bFGF reaches an extracytoplasmic compartment where it can bind to its receptor.

Because clone B3 cells synthesize four forms of bFGF, the form of bFGF that stimulates receptor downregulation in these cells is not certain. All four bFGFs were found in the conditioned medium, and therefore any of the forms may be responsible. In cells transfected with mutated bFGF cDNA that express only 18 kd bFGF, bFGF receptors were downregulated, demonstrating that the information included in the NH_2-terminal extensions of the higher molecular weight bFGFs is not required for the release of bFGF and its modulation of its receptor in these cells. Cells transformed by transfection with bFGF cDNA mutated so that only the 22-, 22.5-, and 24-kD bFGFs are expressed also had downregulated bFGF receptors, suggesting that the higher molecular weight bFGFs are also able to modulate receptor levels. However, the medium from these cultures contained, in addition to 22-, 22.5-, and 24-kD bFGFs, small amounts of 18-kD bFGF that most likely arose by proteolysis of the higher molecular weight forms (Quarto, N., D. Talarico, R. Florkiewicz, C. Basilico, and D. B. Rifkin, manuscript in preparation). This raises the possibility that receptor downregulation in these cells was also caused by 18-kD bFGF. Nevertheless, other evidence suggests that each of the forms of bFGF is able to interact with the bFGF receptor. The 25- and 18-kD bFGFs isolated from guinea pig brain had equivalent biological activities and competed equally with ^{125}I-human bFGF for binding to the bFGF receptor (Moscatelli et al., 1987). In addition, high molecular weight human bFGFs were shown to possess mitogenic activity for 3T3 cells equivalent to that of 18-kD human bFGF, suggesting that all forms of human bFGF interact equally with the receptor (Florkiewicz and Sommer, 1989). Finally, when 25-kD guinea pig bFGF was added to cultures of bovine capillary endothelial cells at 37°C, it was internalized into the cells in an intact form (unpublished observations), suggesting that the 25-kD bFGF has an intrinsic ability to activate the receptor and does not need to be converted to the 18-kD form. Thus, the modulation of bFGF receptors on clone B3 cells is most likely due to the combined action of all four forms of bFGF.

Although bFGF was found in the conditioned medium of clone B3 cells, it was present at <1% of the levels found in cell extracts (Quarto et al., 1989). The mechanism by which bFGF is released by these cells is unclear. A low-efficiency system for bFGF release may exist. In transfected NIH 3T3 cells that express high levels of bFGF, enough bFGF may be released by this system to stimulate the cells. This system may be more efficient in nontransfected cells that normally produce bFGF, allowing bFGF to be released in adequate amounts for autocrine activation even though these cells synthesize much lower levels of bFGF than clone B3 cells. Alternatively, bFGF may be released from clone B3 cells by cell lysis. Since it has been estimated that clone B3 cells contain 0.66 pg of bFGF per cell, the death of only 0.4% of the cells on a semiconfluent 60-mm dish would result in the release of enough bFGF to give a 1 ng/ml solution in the medium. Addition of 1 ng/ml bFGF to nontransfected NIH 3T3 cells is sufficient to cause downregulation of bFGF receptors. However, in sparse cultures a greater proportion of the cells would have to lyse to release this concentration of bFGF. For clone B3 cells, the number of receptors per cell was not altered with changes in cell density over a 10-fold range, suggesting that cell lysis may not be a primary mechanism of release.

The absence of any alteration in the number of bFGF receptors per cell with changes in cell density for NIH 3T3 cells is in contrast to what has been reported for bFGF receptors in baby hamster kidney, normal rat kidney, and 3T3 cells (Neufeld and Gospodarowicz, 1985; Rizzino et al., 1988). This difference may be explained by the fact that the earlier investigators did not separate receptor-bound bFGF from matrix-bound bFGF in their studies. When matrix-bound bFGF is eliminated, no difference in the number of receptors per cell can be observed over a range of cell densities in NIH 3T3 cells (Fig. 3) or BHK cells (unpublished observations).

That transfection of NIH 3T3 cells with the hst/K-*fgf* oncogene also results in the downregulation of bFGF receptors suggests that these homologous molecules may share the same receptors. This possibility is supported by the finding that crude preparations of the hst/K-*fgf* oncogene product compete with ^{125}I-bFGF for binding to its receptor (unpublished observations). In this respect, the hst/K-*fgf* oncogene product is similar to aFGF, which has also been shown to share receptors with bFGF. Thus, specific receptors for each member of the FGF family may not exist, and the FGF receptors may have overlapping affinities for different FGF-like molecules.

The authors thank Pierre Devesly for expert technical help, Dr. Daniel B. Rifkin for helpful advice, and Dr. Claudio Basilico for providing the human hst/K-*fgf* cDNA.

This work was supported by grants 1811 from the Council for Tobacco Research U.S.A., Inc., CA-42229 and CA-34282 from the National Institutes of Health, and BC-554C from the American Cancer Society.

Received for publication 15 May 1989 and in revised form 10 July 1989.

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