Cell Transformation by *kFGF* Requires Secretion But Not Glycosylation

Frances Fuller-Pace, Gordon Peters, and Clive Dickson

Imperial Cancer Research Fund Laboratories, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Abstract. The *Kfgf* gene, which encodes a member of the fibroblast growth factor family, was originally discovered by assaying human tumor DNA for dominantly transforming oncogenes. The 22-kD kFGF product contains a single site for asparagine-linked glycosylation and an amino-terminal signal peptide for vectorial synthesis into the endoplasmic reticulum and eventual secretion. To determine whether these features are necessary for transformation, we have constructed mutants of kFGF that are impaired for glycosylation or secretion. All mutants retained the ability to induce DNA synthesis when added to quiescent cells, and the absence of glycosylation had no appreciable effect on the transformation efficiency on NIH3T3 cells. In contrast, mutants of kFGF that remain in the cytoplasm or are retained in the secretory pathway, through addition of a KDEL motif, score negative in standard transformation assays. Since transformation by either the glycosylated or unglycosylated form of kFGF can be reversed by addition of suramin, the data imply that secretion of kFGF, or surface localization of the ligand/receptor complex, is a prerequisite for transformation.

THE *Kfgf* gene, referred to in the literature as *Hst*, *Hst-1*, *HSTF1*, *FGFK*, and *KS3*, was identified as a transforming gene after transfection of human tumor DNA into mouse NIH3T3 fibroblasts (10, 44). The profusion of names stems in part from its simultaneous discovery in different laboratories, using DNA from a variety of human tumors, including stomach cancer, Kaposi's sarcoma, melanoma, colon carcinoma, and hepatoma (3, 10, 29, 36, 44). However, there are no indications that *Kfgf* played any role in the genesis of these tumors since the same gene isolated from normal human DNA is also capable of eliciting morphological transformation of transfected NIH3T3 cells (45). The transforming activity is therefore assumed to reflect transcriptional activation of the gene during transfection. In contrast, the mouse homologue of the gene has been implicated in a naturally occurring malignancy, namely the mammary carcinomas associated with infection by mouse mammary tumor virus (4, 35, 39). Here again the effects appear to be transcriptional implying that it is the normal gene product that contributes to neoplasia.

Cloning and sequence analysis of these independently isolated genes showed that they encoded a product of ~22 kD that has significant homology to the fibroblast growth factor (FGF) family (11, 47, 55). The prototypes for this family, the so-called acidic and basic FGFs (aFGF and bFGF, respectively), are broad spectrum mitogens that have been isolated from a wide variety of tissues (8, 21, 41, 49). Recently, however, the classification of the FGFs has come to rely more on sequence comparisons than on functional equivalence. On this basis, the family also includes int-2, FGF-5, FGF-6, and keratinocyte growth factor (KGF),[1] as well as kFGF (9, 13, 14, 17, 31, 43, 56). As the family has grown, it is the two prototypic FGFs that now appear unusual in that they lack typical signal sequences for passage through the ER, whereas all the other FGF-related genes encode secreted proteins (1, 12, 15, 17, 26, 31, 55, 56). For example, the sequence of human *Kfgf* predicts a hydrophobic amino terminus from which the first 29 amino acids are removed during transit through the secretory pathway (12, 47). A similar situation prevails for mouse *Kfgf* although there is some sequence variation at the presumed signal peptide cleavage site (7, 25). Otherwise, the two proteins are 82% homologous and share a single consensus site for asparagine-linked glycosylation.

Not surprisingly, kFGF can be readily detected in the secretory pathway and conditioned medium of producing cells, either immunologically or functionally as a heparin binding mitogen (12, 32, 52). The protein is also reported to induce phenotypic changes, analogous to transformation, when added to cultured cells (11, 32). Such findings suggest that transformation requires interaction between the secreted protein and specific cell surface receptors (48, 52). Similar conclusions have been drawn from studies on bFGF where the provision of a signal sequence appears to enhance its transforming potential (6, 40, 54). However, these analyses did not exclude the possibility of intracellular interactions

Dr. Fuller-Pace's present address is CRC Cell Transformation Laboratory, Department of Biochemistry, The University, Dundee DD1 4HN, United Kingdom.

1. *Abbreviation used in this paper*: KGF, keratinocyte growth factor.

between the growth factor and high-affinity receptors, as previously suggested for the product of the v-*sis* oncogene (5, 18, 23, 28). We have addressed this issue more directly by constructing mutant forms of mouse *Kfgf* whose products are impaired for glycosylation, secretion or both. Although all the mutant proteins retained biological activity, as judged by their ability to induce DNA synthesis in quiescent cells, only the secreted forms of kFGF were able to induce morphological transformation.

Materials and Methods

Construction of Mutant kFGFs

All of the kFGF mutants were based on a cDNA clone of mouse *Kfgf* prepared using the polymerase chain reaction (PCR) with RNA from F9 teratocarcinoma cells (7). This clone contains one difference (valine to alanine at 127) from the sequence of Hebert et al. (25). The primers for the PCR reaction were designed so that the coding sequences are bounded by XbaI and EcoRI sites (Fig. 1), and the ATG that specifies the beginning of the open reading frame is contained within a unique NcoI site. The sequences immediately preceding the ATG conform to the consensus for translation initiation (30).

To construct the KDEL mutant, two complementary oligonucleotides (5'-GTAACCCACTTCCTTCCTAGACTGAGCGAGAAGGACGAGCTGT GATAAG-3' and 5'-AATTCTTATCACAGCTCGTCCTTCTCGCTCAGT-CTAGGAAGGAAGTGG-3') were used to replace the carboxy-terminal sequences, between unique BstEII and EcoRI sites. As well as the last eight amino acids of mouse kFGF, these oligonucleotides encoded the SEKDEL motif followed by two in frame stop codons (Fig. 1). For the NSP mutant, a primer was designed to place an in-frame initiation codon at the signal peptide cleavage site. The 28-nucleotide primer, 20 nucleotides of which were based on the mouse *Kfgf* sequence, was used as the 5' partner in a PCR reaction in which the 3' primer was centered on a unique SacI site in the *Kfgf* cDNA (Fig. 1). The 265-bp NcoI–SacI product was then used to replace the corresponding section of wild-type cDNA, thereby excluding the amino-terminal 77 bp that specify the signal peptide of kFGF. As a result, the sequences immediately preceding the new ATG were identical to those in the wild type construct, and optimized for initiation of translation. Finally, in the GLY$^-$ mutant, the asparagine codon (AAC) at residue 32 was changed to a glutamine codon (CAG) using a mutagenesis kit supplied by Amersham International (U.K.). The complementary oligonucleotide used to generate the mutation was 5'-GCGTGCCCTGGGGTGC-3' and the template was the coding strand of mouse *Kfgf* cDNA in M13mp19. The modified cDNA was then recloned as a double stranded XbaI–EcoRI fragment in the pGEM4 vector (Promega Biotec, Madison, WI). The relevant sections of each mutant were subjected to DNA sequence analysis using specific oligonucleotides to prime synthesis on the completed double-stranded plasmids.

The various mutant forms of *Kfgf* cDNA were initially constructed in pGEM4 and transferred into the pKC4 vector (kindly provided by D. Hanahan, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as XbaI–EcoRI fragments. The same fragments were ligated between the BamHI and EcoRI sites of the pDOBS retrovirus vector (33) by filling in the XbaI and BamHI cleavage sites with T4 DNA polymerase to create blunt ends.

Cell-free Transcription and Translation

The pGEM4-based plasmids, containing wild-type and mutant *Kfgf* cDNAs, were linearized with EcoRI and transcribed into sense RNA with T7 RNA polymerase (Promega Biotec) in the presence of m^7GpppG (New England Biolabs, Boston, MA), as recommended by Promega Biotec. Approximately 1 μg of each transcript was used to direct protein synthesis in a rabbit reticulocyte lysate (Promega Biotec). To produce ^{35}S-labeled products, the reactions were carried out in the presence of 5 μCi of [^{35}S]methionine (Amersham International). Otherwise, reaction conditions were as specified by the supplier.

Thymidine Incorporation Assays

C57MG mammary epithelial cells (50) were grown in DMEM containing 10% newborn calf serum (Gibco Laboratories, Grand Island, NY). For mitogenicity assays, 5×10^4 cells were transferred to each well of a 24-well tissue culture plate in 500 μl of medium. After 24 h, the medium was replaced with DMEM containing 0.1% serum, and after a further 72 h the cells were considered quiescent. The cells were then treated with samples (2 or 10 μl) of the various cell-free translation reactions in 500 μl of fresh DMEM and 0.1% serum, for 16 h. The induction of DNA synthesis was assessed by labeling the cells with 5 μCi per well of [^3H]thymidine (Amersham International) for 3 h and measuring the incorporation of label into acid-insoluble material. The cells were washed twice in PBS, fixed for 20 min in cold 5% (wt/vol) trichloroacetic acid, allowed to dry and finally lysed in 500 μl of a solution containing 1 M NaOH, 2% Na_2CO_3, and 1% SDS. The amount of ^3H-labeled material in the acid precipitate was determined by liquid scintillation counting. Each assay was performed in duplicate.

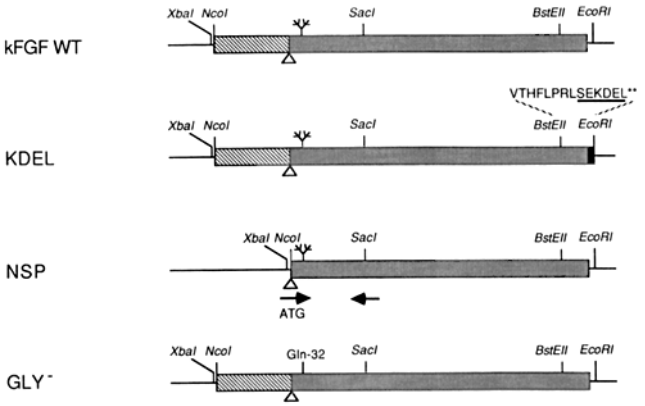

Figure 1. Structures of wild type and mutant kFGFs. The 609-nucleotide coding domain of mouse *Kfgf* is represented by the shaded box in which the lighter shading depicts the amino-terminal signal sequence. The signal peptide cleavage site (△) and the single consensus for asparagine linked glycosylation (Ψ) are indicated, but the diagram is not drawn to scale. The cDNA product generated by PCR is bounded by sites for XbaI and EcoRI, and there are unique internal sites for NcoI (spanning the AUG codon), SacI and BstEII. The essential features of the KDEL, NSP, and GLY$^-$ mutants are also illustrated. In the KDEL mutant, two complementary oligonucleotides encoding the amino acids -Ser-Glu-Lys-Asp-Glu-Leu- (SEKDEL) were used to replace the sequences between the BstEII and EcoRI sites of *Kfgf*. In the NSP mutant, synthetic oligonucleotide primers (bold arrows) were used to amplify sequences between the signal peptide cleavage site and the SacI site, enabling translational initiation at an ATG contained within a novel NcoI site. This fragment was used to replace the corresponding section of wild type *Kfgf* cDNA. In the GLY$^-$ mutant, the Asn-32 codon was changed to Gln, preventing carbohydrate addition.

Cell Transformation Assays

NIH3T3 cells, grown in DMEM plus 10% calf serum, were passaged 24 h prior to transfection with the various *Kfgf* cDNAs in the MuLV-based retrovirus vector, pDOBS (33). For each transfection, 10^6 cells in a 9-cm culture dish were exposed to 100 ng of plasmid DNA and 20 μg of human placental DNA as a calcium phosphate precipitate (53). After 24 h, the cells were passaged at ratios of 1:3 and 1:30, and duplicate pools established of each dilution. One group was placed in DMEM containing 10% calf serum and G418 (Gibco Laboratories) at 500 μg/ml. Since the retrovirus vector contained the neomycin resistance gene, all cells that take up and express the input DNA will acquire resistance to G418. These pools therefore provided a measure of transfection efficiency, and subsequently allowed the isolation of individual colonies of transfected cells. The second group was placed in DMEM containing 5% calf serum, to promote the appearance of transformed foci against a background of normal cells. The numbers of foci and of G418^R colonies were counted at 10–12 d after transfection.

Electroporation of COS-1 Cells

COS-1 monkey cells, constitutively expressing SV40 T-antigen (19), were

cultured in DMEM supplemented with 10% FCS. The various *Kfgf* cDNAs, in the SV40-based vector pKC4 (from D. Hanahan) were introduced by electroporation of 10^6 cells with 20 μg of purified plasmid DNA at 450 V/250 μF, using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA). Cells were harvested ~60 h later when transient expression of the input DNA had reached a maximum.

Analysis of kFGF Proteins in Transfected Cells

Transfected NIH3T3 and COS-1 cells were labeled by addition of 500 μCi of [^{35}S]methionine and [^{35}S]cysteine ("Express Labeling Mix" from New England Nuclear) in DMEM (depleted of methionine and cysteine) and 2% dialyzed calf serum. After labeling for 4 h, the medium (1.5 ml per 9-cm dish) was recovered for further analysis and the cells were washed twice in PBS. Total cell extracts were prepared by lysis in RIPA buffer, essentially as described in Harlow and Lane (24). The media and cell extracts were immunoprecipitated with a polyclonal rabbit serum raised against the carboxy-terminal 12 amino acids of mouse kFGF. Immune complexes were recovered by association with protein A–Sepharose (Pharmacia Fine Chemicals) and separated by electrophoresis in 12.5% polyacrylamide gels in the presence of SDS (SDS-PAGE) according to standard procedures (24). The ^{35}S-labeled products were detected by autoradiography.

Results

Construction of kFGF Mutants

Modified forms of mouse *Kfgf* cDNA (7) were constructed so that the encoded products would be impaired for secretion or glycosylation (Fig. 1). In the first of these, the carboxy-terminus of the protein was modified to include the Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) motif that has been shown to cause retention of proteins in the ER (34, 38). Since the signal sequence was left intact, this product would be expected to enter but effectively remain in the secretory pathway. In the NSP mutant, on the other hand, the hydrophobic amino terminus was removed so that the translation product would no longer pass into the ER (Fig. 1). The NSP form of kFGF would therefore be expected to reside in the cytoplasm. In the third mutant, designated GLY$^-$, the asparagine-32 codon of mouse kFGF was changed to a glutamine codon. This point mutation removed the Asn-X-Ser/Thr target for carbohydrate addition but left the remainder of the protein unchanged. Details of these modifications are presented in Materials and Methods.

Each of the mutated cDNAs was inserted into three different vectors to facilitate various functional assays. For cell-free translation, the cDNAs were cloned into the pGEM4 plasmid to permit transcription of sense RNA with T7 RNA polymerase; for transient expression in COS cells, the constructs were based on the pKC4 vector carrying the SV40 early promoter (15); and for the transformation assays in NIH3T3 cells, we used a retrovirus-based vector carrying an independently expressed neomycin-resistance gene (20).

Mitogenic Activity of kFGF Mutants

As we had previously shown that synthetic kFGF, produced by cell-free translation, is capable of inducing DNA synthesis in serum-deprived cells (15), we first wanted to confirm that the various mutant kFGFs had retained full activity in this assay. In vitro translation reactions were therefore performed in rabbit reticulocyte lysates programmed with 1 μg of sense transcript from each of the cDNAs. The incorporation of [^{35}S]methionine allowed both qualitative and quantitative comparison of the products by electrophoresis in SDS-polyacrylamide gels. As shown in Fig. 2, the mutant proteins

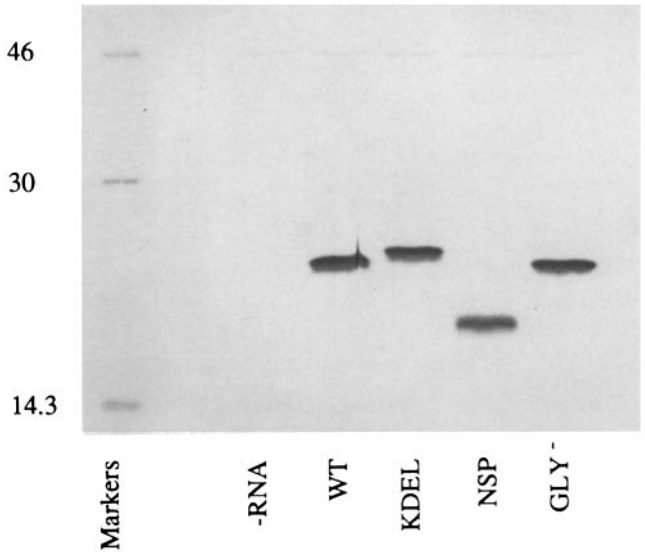

Figure 2. Cell-free translation of kFGF proteins. The wild type and mutant *Kfgf* sequences, in the pGEM4 vector, were transcribed into sense RNA with T7 polymerase, and 1 μg of each RNA was used to direct protein synthesis in a reticulocyte lysate. Products labeled by incorporation of [^{35}S]methionine were fractionated by electrophoresis in a 12.5% polyacrylamide gel and visualized by autoradiography. The various *Kfgf* constructs are as indicated, and -RNA signifies a control reaction in which no RNA was added. Numbers on the left refer to the sizes, in kilodaltons of the protein markers (Amersham International).

were of the predicted sizes and the translation efficiencies were similar for all the constructs. To assess their mitogenic properties, unlabeled products from parallel translation reactions were used in thymidine incorporation assays.

C57MG mouse mammary epithelial cells (50) were rendered quiescent by reducing the serum concentration to 0.1%. Samples of each translation reaction were then added to the cells, in medium containing 0.1% serum, and 16 h later the effects on DNA synthesis were assessed by measuring the incorporation of [^{3}H]thymidine into acid insoluble material (see Materials and Methods). As shown in Fig. 3, the mutant kFGFs were all capable of inducing DNA synthesis in these cells. With 10 μl samples of each lysate, the incorporation of [^{3}H]thymidine was close to the maximum achieved by addition of 10% serum. The assay was therefore repeated using an amount of lysate (2 μl) that gave an approximately half-maximal response with the wild-type kFGF. Within the limits of such an assay, each of the mutant kFGFs elicited comparable mitogenic responses (Fig. 3).

Transformation of NIH3T3 Cells by Mutant kFGFs

We next wished to determine whether the various mutants also had comparable transforming activities. To this end, the different cDNAs were ligated into an MuLV-based retrovirus vector to ensure efficient expression in mouse cells. The plasmid DNAs were then introduced into NIH3T3 cells as calcium phosphate precipitates and the transfected cells scored for focus formation after 10–12 d. Since the vector also encoded the neomycin-resistance gene, the efficiency of transfection could be monitored by counting the numbers of colonies that acquired resistance to the drug G418 (see Materials and Methods).

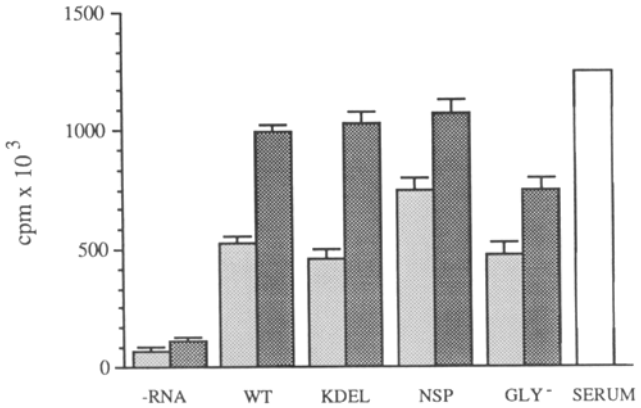

Figure 3. Induction of DNA synthesis by synthetic kFGFs. The products of cell-free translation reactions, performed as in Fig. 2 but without [^{35}S]methionine, were added to quiescent C57MG cells, and the effects on DNA synthesis assessed after 16 h. The histograms indicate the incorporation of [^{3}H]thymidine into acid-insoluble material in response to either 2 μl (*lighter shading*) or 10 μl (*darker shading*) of reticulocyte lysate in a total of 500 μl of medium with 0.1% serum. The different *Kfgf* constructs are as indicated, with -RNA referring to the background level of incorporation observed with the reticulocyte lysate alone. The addition of 10% serum was used as a measure of the maximum response.

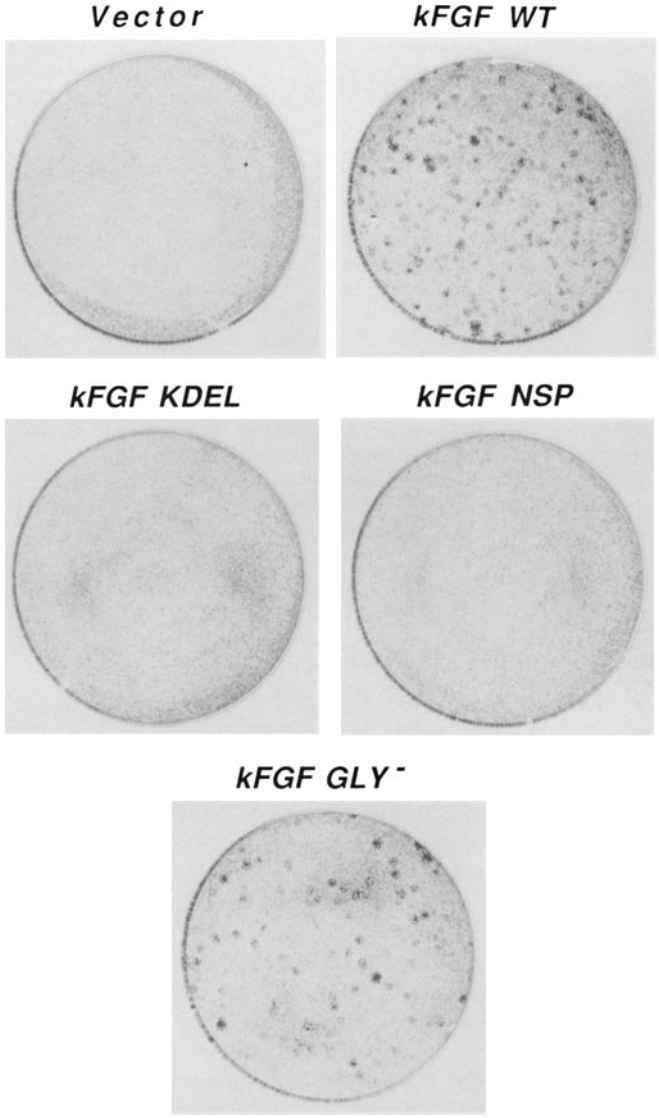

Figure 4. Transformation of NIH3T3 cells by *Kfgf* DNA. NIH3T3 cells were transfected with the wild-type and mutant *Kfgf* constructs in the pDOBS vector, as described in Materials and Methods, and stained with Giemsa after 12 d. Multiple foci were discernible with the wild type and GLY$^-$ mutant, as indicated, but not with the KDEL and NSP mutant cDNAs, or with the vector alone.

As illustrated in Fig. 4, and quantitated in Table I, both the wild-type and GLY$^-$ mutants induced numerous foci of transformed cells, whereas no foci were detected with the KDEL and NSP mutants, or with the vector alone. The transfection efficiencies, as measured by numbers of G418^R colonies, were roughly the same for each DNA, although in this experiment the GLY$^-$ yielded fewer colonies (Table I). Nevertheless, the transforming efficiency of the GLY$^-$ DNA, as reflected in the percentage of G418^R colonies that appeared morphologically transformed, was indistinguishable from that of wild-type kFGF. These transformed cells were also similar in their ability to grow as anchorage-independent colonies in soft agar (not shown). In contrast, the G418^R colonies obtained with the KDEL and NSP mutants appeared morphologically normal and did not grow in soft agar (not shown).

Expression of Mutant kFGFs in NIH3T3 Cells

As the mutant forms of kFGF were designed to have altered subcellular fates, it was essential to confirm that the differences in transforming efficiencies were attributable to differences in the processing and/or secretion of the products. Pools of G418^R NIH3T3 cells, transfected with the various constructs, were labeled with [^{35}S]methionine and cysteine and the culture media and total cell extracts recovered for analysis. After immunoprecipitation, using a rabbit polyclonal antiserum raised against a COOH-terminal peptide from mouse kFGF, the labeled proteins were fractionated by polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 5). We confirmed that this antibody recognized wild-type and mutant kFGFs with similar efficiency, as judged by immunoprecipitation of [^{35}S]methionine-labeled in vitro translation products (not shown). The wild-type and GLY$^-$ products were detectable in both the cells and medium, as expected for secreted proteins. The major difference noted for the GLY$^-$ mutant was in the smaller size of the protein, which reflected the absence of glycosylation. In contrast, the slightly larger KDEL mutant was apparently glycosylated, but could not be detected in the medium, despite relatively high intracellular levels (Fig. 5). This would be con-

Table I. Transformation Efficiency of Kfgf cDNAs

Plasmid	G418^R colonies per μg DNA	Foci per μg DNA	Transformation efficiency*
pDOBS	2,625	0	–
WT	2,170	2080	0.96
KDEL	2,580	0	–
NSP	2,280	0	–
GLY$^-$	1,430	1350	0.94

* Transformation efficiency was calculated as number of foci/number of G418^R colonies.

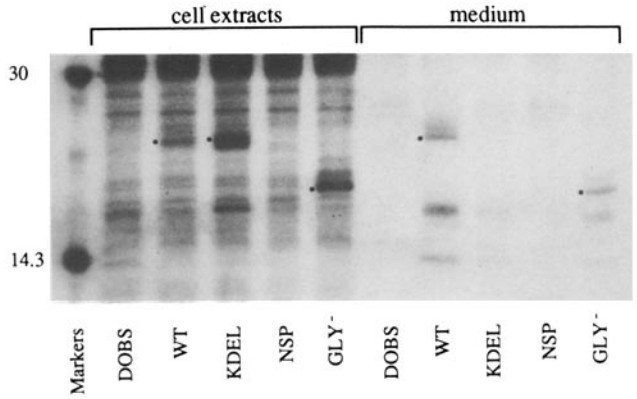

Figure 5. Expression of kFGF proteins in NIH3T3 cells. Pools of G418^R NIH3T3 cells transfected with the indicated cDNAs in the pDOBS vector were metabolically labeled with [^{35}S]methionine and cysteine and the cell extracts (*left*) and conditioned media (*right*) were immunoprecipitated with an antiserum against the carboxy terminus of mouse kFGF. The immunoprecipitates were fractionated by SDS-PAGE in a 12.5% gel and the labeled proteins were visualized by autoradiography. Equivalent amounts of the extracts and media were analyzed and the expected kFGF products are identified by •. The sizes (kilodaltons) of ^{14}C-labeled protein standards (Amersham International) are shown on the left.

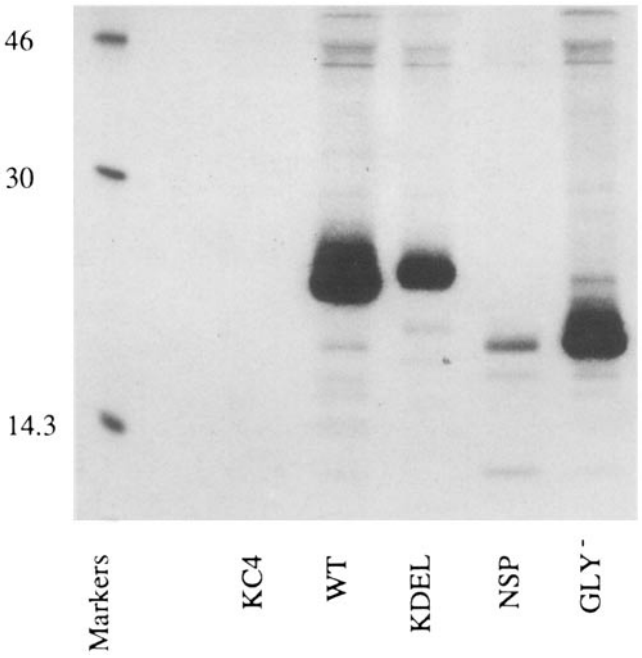

Figure 6. Expression of kFGF proteins in COS-1 cells. The various *Kfgf* cDNAs in the pKC4 vector, as well as the vector alone as a control, were introduced into COS-1 cells by electroporation. After 60 h, the cells were labeled with [^{35}S]methionine and cysteine and the kFGF-related proteins in the cell extracts were analyzed by immunoprecipitation and SDS-PAGE, as in Fig. 5. The positions and sizes (kilodaltons) of ^{14}C-labeled proteins markers (Amersham International) are indicated on the left.

sistent with retention within the ER. With the NSP mutant, which was expected to be entirely cytosolic, it proved difficult to detect the protein in either fraction, possibly reflecting a reduced stability in the cytoplasm as opposed to the ER (Fig. 5). In the wild-type and GLY$^-$ mutant, immunoreactive proteins of a smaller size were seen in the medium; these appear to be degradation products of kFGF.

Expression of Mutant kFGFs in COS-1 Cells

To compare the processing and stability of the mutant kFGFs, we chose to use the COS cell system where it is possible to achieve high levels of expression using an SV40-based vector (19). The various cDNAs, in the pKC4 vector (2, 15), were introduced into COS-1 cells by electroporation and ~60 h later, when levels of expression had reached their peak, the cells were labeled with a 2-h pulse of [^{35}S]methionine and [^{35}S]cysteine. As shown in Fig. 6, the wild-type kFGF, and the KDEL and GLY$^-$ mutant proteins were readily detectable, and at comparable levels. However, with the NSP mutant, where the block to secretion was likely to be much tighter, the intracellular levels were again low as compared to the other mutants. This was not a function of transfection efficiency since immunofluorescence assays confirmed that a similar proportion of transfected cells were expressing the kFGF products in each case (not shown). Thus, the cytoplasmic form encoded by the NSP mutant appeared inherently less stable or was less efficiently expressed in both COS-1 and NIH3T3. Pulse–chase experiments (not shown) indicated that the half-life of the NSP protein in COS-1 cells is ~40 min, compared to ~100 min for the wild-type kFGF, but it is not clear whether this twofold difference is enough to account for the low levels of NSP protein observed.

Transformation Can Occur via an Autocrine Loop

The data shown above imply that the ability of kFGF to transform NIH3T3 cells could be abrogated by mutations that impaired the secretion of the protein. However, we felt it necessary to confirm that the secreted forms of the protein were indeed active, and that no functional protein was being released from the KDEL and NSP expressing cells, at levels below the sensitivity of the immunological assay. Since the medium from normal NIH3T3 cells was found to contain significant mitogenic activity on the C57MG indicator cells, irrespective of transfection by the various kFGFs, the most direct functional test was whether the protein itself could elicit transformation. Conditioned medium taken from pools of transfected NIH3T3 cells, grown for 24 h in the absence of G418, was added to fresh cultures of NIH3T3 cells, and their morphology assessed after a further 16–20 h. As shown in Fig. 7, the media containing either wild-type kFGF or the GLY$^-$ mutant were able to cause dramatic changes in morphology, whereas media from untransfected NIH3T3 cells or from cells expressing the KDEL and NSP mutants did not. The phenotype was even more pronounced if heparin was included in the conditioned medium. These morphological responses to exogenous kFGF were transient in that removal of the conditioned medium allowed the cells to revert to a normal phenotype. In the converse experiment, we asked whether the polyanionic compound suramin, which has been shown to dissociate some growth factors from their cognate receptors, was able to block transformation. Addition of 1 mM suramin for 16 h was sufficient to revert the phenotype of transfected cells expressing either wild-type or GLY$^-$ kFGF (Fig. 8). Since these cells are constitutively expressing kFGF, morphological transformation was reestablished when the suramin was removed (not shown). Neither heparin nor

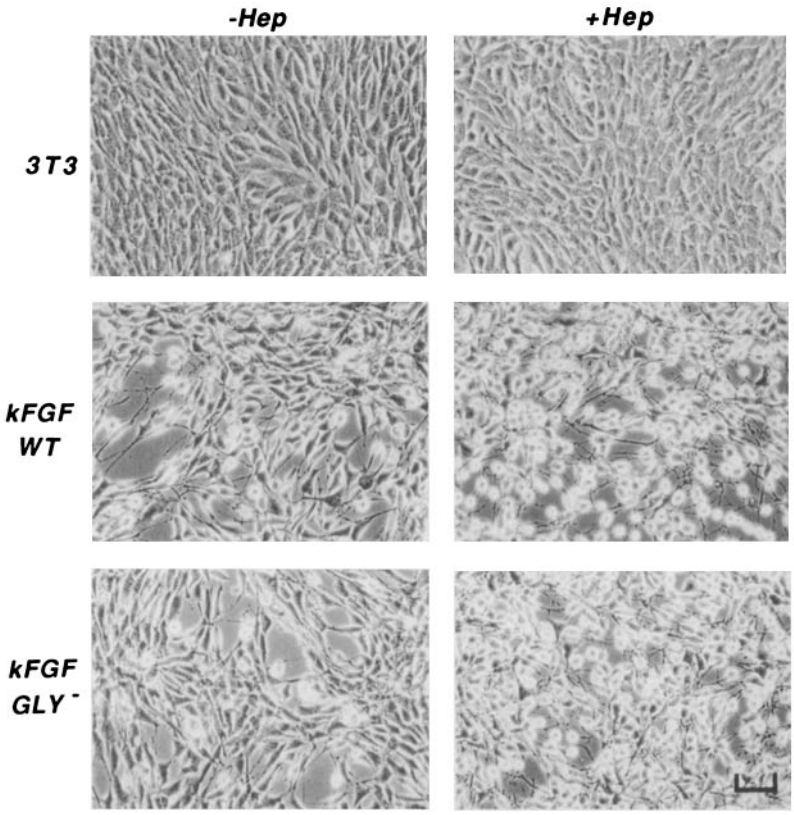

Figure 7. Transformation of NIH3T3 cells by conditioned medium from cells expressing kFGF. The media recovered from pools of NIH3T3 cells expressing wild type kFGF, the GLY^- mutant or from untransfected cells, were clarified by centrifugation and added to fresh cultures of NIH3T3 cells in the presence or absence of heparin at 50 μg/ml (Sigma Chemical Co.). The cells were photographed under phase contrast 16 h after addition of the medium. Bar, 25 μm.

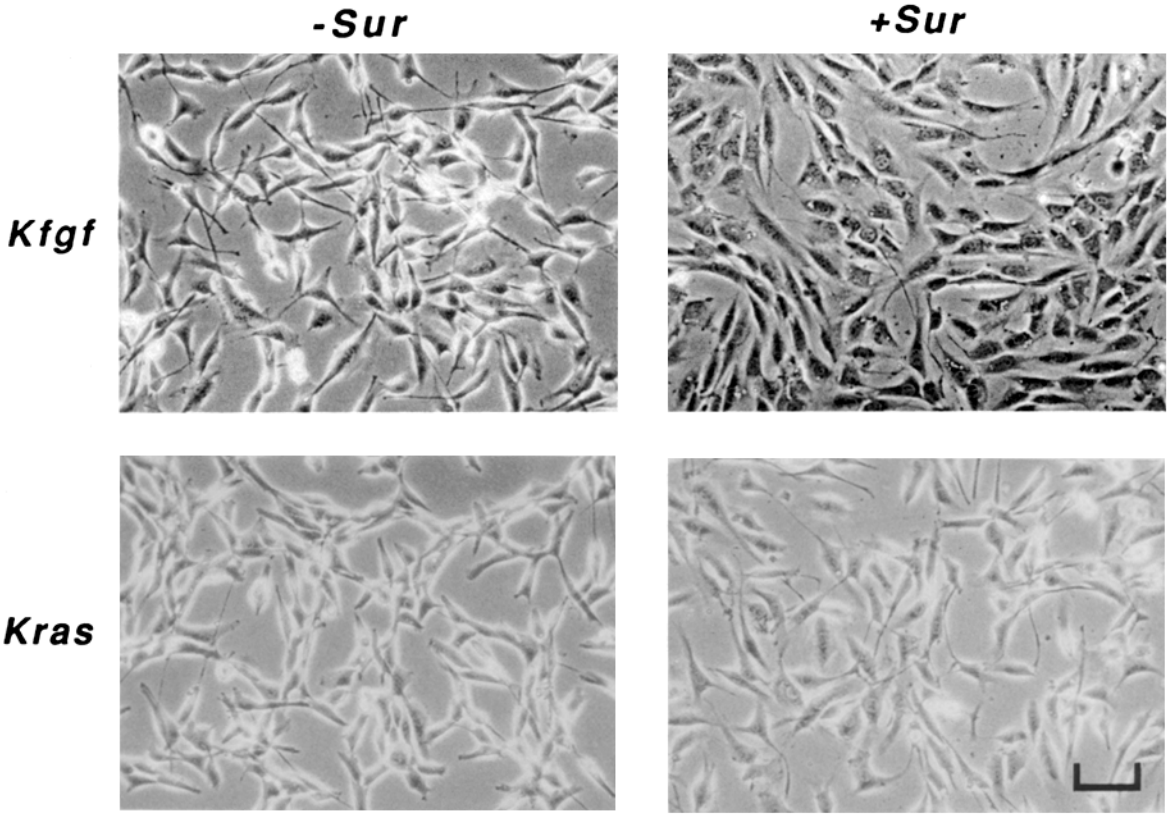

Figure 8. Reversal of *Kfgf*-induced transformation by suramin. NIH3T3 cells transformed by either wild-type *Kfgf* in the pDOBS vector or by human K-*ras* DNA were grown for 24 h in the presence or absence of 1 mM suramin. The cells were then photographed under phase contrast. Suramin caused morphological reversion of the kFGF-expressing cells but had no discernible effect on the K-*ras* control cells. Bar, 25 μm.

suramin had any effect on the normal appearance of NIH3T3 cells transfected with the KDEL or NSP mutants, or on the phenotype of cells transformed by K-*ras* DNA (Fig. 8).

Discussion

The notion that autocrine stimulation of cell growth can play a significant role in tumorigenesis has gained considerable support from the fact that certain oncogenes, implicated in experimental and naturally occurring cancers, encode growth factors or products related to growth factors. The classic example is the relationship between the v-*sis* oncogene and the B-chain of PDGF (16, 51), but subsequent examples have mostly involved members of the FGF family. Thus, the genes for int-2, kFGF, FGF-5, and FGF-6 can all be classed as oncogenes because they are capable of inducing morphological transformation of NIH3T3 cells (11, 20, 31, 47, 56), and both *int-2* and *Kfgf* have been implicated in virally induced mammary tumors in mice (14, 39). The point at issue is whether transformation as a result of autocrine stimulation is dependent on interactions between ligand and receptor at the cell surface, or whether intracellular encounters between the two can also complete the autocrine loop. Here we present evidence that for kFGF, the former situation prevails.

Our approach was to construct mutant forms of kFGF that differed in post-translational processing and subcellular fate but retained intrinsic mitogenic activity. The three mutants compared here were indistinguishable from the wild type kFGF in their ability to induce DNA synthesis in quiescent C57MG cells (Fig. 3), suggesting that the addition or loss of residues at the carboxy or amino termini had little influence on the function of the protein. However, the experiments presented in Figs. 2 and 3 relied on products that were neither cleaved nor glycosylated since they were translated in the absence of microsomes. It was therefore important to determine whether the wild type kFGF and the GLY$^-$ mutant expressed in cells were also functionally equivalent, particularly in view of reports that inhibition of glycosylation by tunicamycin can impair secretion of kFGF (12). In the pools of transfected NIH3T3 cells, we observed only marginal differences between the secretion of the wild type and GLY$^-$ forms (Fig. 5); otherwise the two products were functionally indistinguishable. For example, the two cDNAs were able to transform NIH3T3 cells with similar efficiency (Fig. 4 and Table I), the two pools of transformed cells behaved identically in colony assays in soft agar (not shown), and the conditioned media from these cells were equally capable of eliciting morphological transformation of untransfected NIH3T3 cells (Fig. 7). With COS-1 cells, on the other hand, a much smaller proportion of the GLY$^-$ protein appeared in the conditioned medium (not shown), suggesting that the impairment to secretion may be a feature of the COS cell system. We have previously noted striking differences between the secretion of the int-2 protein from NIH3T3 and COS-1 cells (unpublished results; 15, 20).

The ability of *Kfgf*-conditioned medium to directly influence cellular morphology (Fig. 7) provides compelling evidence that transformation of NIH3T3 cells can be mediated by extracellular kFGF (11, 48). That the transformed phenotype can be enhanced by low concentrations of heparin and essentially reversed by suramin (Fig. 8) lends further support, yet these data do not prove that an extracellular loop is essential for transformation. For example, secreted growth factors have to traverse the same intracellular compartments as their cognate transmembrane receptors, and it remains possible that interactions leading to autocrine stimulation might occur within the cell, without the need to externalize the receptor/ligand complex. This was first suggested for PDGF/*sis*, where retention in the ER through the addition of KDEL sequences had little effect on the transforming properties (5, 28). However, it now appears that surface localization of the receptor may be required to trigger the appropriate signal transduction pathways (18, 23).

With kFGF, addition of the KDEL motif to the carboxy terminus blocked both the release of immunologically detectable protein into the medium (Fig. 5) and the capacity to transform NIH3T3 cells (Fig. 4 and Table I). Otherwise, the rate of synthesis, intracellular stability and mitogenic activity of the protein appeared unimpaired, although it is conceivable that the presence of the KDEL motif had subtle effects that were not apparent in the experiments we describe. For example, the glycosylated protein expressed in transfected cells may be a less effective ligand than the unprocessed form synthesized in vitro. However, after extended periods in culture, the morphologies of some of the NIH3T3 colonies transfected with the KDEL mutant began to resemble those of transformed cells. We attribute this to the selection of clones expressing high levels of intracellular kFGF, allowing the release of enough extracellular protein to effect transformation. Immunoprecipitation analyses confirmed that these partially transformed colonies contained elevated levels of intracellular kFGF compared with their nontransformed counterparts (not shown). During the preparation of this manuscript, Talarico and Basilico reported similar observations using a modified form of human kFGF bearing the KDEL motif (48). In their studies, sporadic transformation also correlated with high expression of the protein and was partially reversible with neutralizing anti-kFGF serum, consistent with an extracellular interaction between the ligand and receptor.

As anticipated, we did not observe transformation with the NSP mutant of mouse kFGF (Fig. 4 and Table I) since the protein remains largely cytosolic and should not encounter receptors within the cell. In this regard, the NSP form resembles aFGF and bFGF. Although these factors are reputed to induce morphological changes when added to cells (22, 49), neither gene scores positive in a conventional focus assay unless provided with a signal sequence for secretion of the protein (6, 42). However, there are also indications that overexpression may elicit transformation, possibly through paracrine effects by factors released from damaged or dying cells (27, 37, 40, 46). In the experiments we report here, the intracellular levels of NSP mutant were relatively low (Figs. 5 and 6), so that leakage into the extracellular compartment may never have reached the threshold needed for transformation.

The studies reported here support the findings of Talarico and Basilico (48) and clearly imply that to initiate signal transduction, the interaction between kFGF and its receptor must occur at the cell surface. This does not rule out association between ligand and receptor in an intracellular compartment, but suggests that such interactions are only productive when localized at the plasma membrane. The threshold of extracellular ligand needed for autocrine stimulation and

transformation will naturally depend on its affinity for the receptor(s). Since it is now clear that there is a family of FGF receptors, and that each may be expressed as alternative isoforms, the phenotype of a cell expressing kFGF, or the mutant kFGFs described here, will depend on the integrated response to multiple receptor-ligand interactions. Dissection of these events will be an obvious focus of future research.

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References

1. Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes. 1986. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science (Wash. DC).* 233:545–548.
2. Acland, P., M. Dixon, G. Peters, and C. Dickson. 1990. Subcellular fate of the Int-2 oncoprotein is determined by choice of initiation codon. *Nature (Lond.).* 343:662–665.
3. Adelaide, J., M.-G. Mattei, I. Marics, F. Raybaud, J. Planche, O. De Lapeyriere, and D. Birnbaum. 1988. Chromosomal localization of the *hst* oncogene and its coamplification with the *int*.2 oncogene in a human melanoma. *Oncogene.* 2:413–416.
4. Akakura, K., Y. Furuya, N. Sato, T. Kodama, S. Teshima, Y. Shimosato, T. Yoshida, M. Terada, and J. Shimazaki. 1990. Acquired expression of *hst*-1 in an autonomous subline (Chiba subline 2) derived from androgen-responsive mouse mammary tumor (Shionogi carcinoma 115). *Jpn. J. Cancer Res.* 81:554–556.
5. Bejcek, B. E., D. Y. Li, and T. F. Deuel. 1989. Transformation by v-*sis* occurs by an internal autoactivation mechanism. *Science (Wash. DC).* 245:1496–1499.
6. Blam, S. B., R. Mitchell, E. Tischer, J. S. Rubin, M. Silva, S. Silver, J. C. Fiddes, J. A. Abraham, and S. A. Aaronson. 1988. Addition of growth hormone secretion signal to basic fibroblast growth factor results in cell transformation and secretion of aberrant forms of the protein. *Oncogene.* 3:129–136.
7. Brookes, S., R. Smith, J. Thurlow, C. Dickson, and G. Peters. 1989. The mouse homologue of *hst/k*-FGF: sequence organization and location relative to *int*-2. *Nucleic Acids Res.* 17:4037–4045.
8. Burgess, W. H., and T. Maciag. 1989. The heparin binding (fibroblast) growth factor family proteins. *Annu. Rev. Biochem.* 58:575–606.
9. De Lapeyriere, O., O Rosnet, D. Benharroch, F. Raybaud, S. Marchetto, J. Planche, F. Galland, M.-G. Mattei, N. G. Copeland, N. A. Jenkins, F. Coulier, and D. Birnbaum. 1990. Structure, chromosome mapping and expression of the murine *Fgf*-6 gene. *Oncogene.* 5:823–831.
10. Delli Bovi, P., and C. Basilico. 1987. Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. *Proc. Natl. Acad. Sci. USA.* 84:5660–5664.
11. Delli Bovi, P., A. M. Curatola, F. G. Kern, A. Greco, M. Ittmann, and C. Basilico. 1987. An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. *Cell.* 50:729–737.
12. Delli-Bovi, P., A. M. Curatola, K. M. Newman, Y. Sato, D. Moscatelli, R. M. Hewick, D. B. Rifkin, and C. Basilico. 1988. Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potential. *Mol. Cell. Biol.* 8:2475–2484.
13. Dickson, C., and G. Peters. 1987. Potential oncogene product related to growth factors. *Nature (Lond.).* 326:833.
14. Dickson, C., R. Deed, M. Dixon, and G. Peters. 1989. The structure and function of the *int*-2 oncogene. *Prog. Growth Factor Res.* 1:123–132.
15. Dixon, M., R. Deed, P. Acland, R. Moore, A. Whyte, G. Peters, and C. Dickson. 1989. Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. *Mol. Cell. Biol.* 9:4896–4902.
16. Doolittle, R. F., M. W. Hunkapiller, L. E. Hood, S. G. Devare, K. C. Robbins, S. A. Aaronson, and H. N. Antoniades. 1983. Simian sarcoma virus *onc* gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science (Wash. DC).* 221:275–277.
17. Finch, P. W., J. S. Rubin, T. Miki, D. Ron, and S. A. Aaronson. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science (Wash. DC).* 245:752–755.
18. Fleming, T. M., T. Matsui, C. J. Molloy, K. C. Robbins, and S. A. Aaronson. 1989. Autocrine mechanism for v-*sis* transformation requires cell surface localization of internally activated growth factor receptors. *Proc. Natl. Acad. Sci. USA.* 86:8063–8067.
19. Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell.* 23:175–182.
20. Goldfarb, M., R. Deed, D. MacAllan, W. Walther, C. Dickson, and G. Peters. 1991. Cell transformation by Int-2- a member of the fibroblast growth factor family. *Oncogene.* 6:65–71.
21. Gospodarowicz, D., G. Neufeld, and L. Schweigerer. 1986. Fibroblast growth factors. *Mol. Cell. Endocrinol.* 46:187–204.
22. Gospodarowicz, D., N. Ferrara, L. Schweigerer, and G. Neufeld. 1987. Structural characterization and biological functions of fibroblast growth factor. *Endocrine Rev.* 8:95–114.
23. Hannink, M., and D. J. Donoghue. 1988. Autocrine stimulation by the v-*sis* gene product requires a ligand-receptor interaction at the cell surface. *J. Cell Biol.* 107:287–298.
24. Harlow, E., and D. Lane. 1988. *In* Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 421–470.
25. Hebert, J. M., C. Basilico, M. Goldfarb, O. Haub, and G. R. Martin. 1990. Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev. Biol.* 138:453–463.
26. Jaye, M., R. Howk, W. Burgess, G. A. Ricca, I.-M. Chiu, M. W. Ravera, S. J. O'Brien, W. S. Modi, T. Maciag, and W. N. Drohan. 1986. Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. *Science (Wash. DC).* 233:541–545.
27. Jaye, M., R. M. Lyall, J. Schlessinger, and N. Sarver. 1988. Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:963–969.
28. Keating, M. T., and L. T. Williams. 1988. Autocrine stimulation of intracellular PDGF receptors in v-*sis*-transformed cells. *Science (Wash. DC).* 239:914–916.
29. Koda, T., A. Sasaki, S. Matsushima, and M. Kakinuma. 1987. A transforming gene, *hst*, found in NIH3T3 cells transformed with DNA from three stomach cancers and a colon cancer. *Jpn. J. Cancer Res.* 78:325–328.
30. Kozak, M. 1989. The scanning model for translation: an update. *J. Cell Biol.* 108:229–241.
31. Marics, I., J. Adelaide, F. Raybaud, M.-G. Mattei, F. Coulier, J. Planche, O. de Lapeyriere, and D. Birnbaum. 1989. Characterization of the *HST*-related *FGF*.6 gene, a new member of the fibroblast growth factor gene family. *Oncogene.* 4:335–340.
32. Miyagawa, K., H. Sakamoto, T. Yoshida, Y. Tamashita, Y. Mitsui, M. Furusawa, S. Maeda, F. Takaku, T. Sugimura, and M. Terada. 1988. *hst*-1 transforming protein: expression in silkworm cells and characterization as a novel heparin-binding growth factor. *Oncogene.* 3:383–389.
33. Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587–3596.
34. Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.
35. Murakami, A., H. Tanaka, and A. Matsuzawa. 1990. Association of *hst* gene expression with metastatic phenotype in mouse mammary tumors. *Cell Growth Differ.* 1:225–231.
36. Nakagama, H., S. Ohnishi, M. Imawari, H. Hirai, F. Takaku, H. Sakamoto, M. Terada, M. Nagao, and T. Sugimura. 1987. Identification of transforming genes as *hst* in DNA samples from two human hepatocellular carcinomas. *Jpn. J. Cancer Res.* 78:651–654.
37. Neufeld, G., R. Mitchell, P. Ponte, and D. Gospodarowicz. 1988. Expression of human basic fibroblast growth factor cDNA in baby hamster kidney-derived cells results in autonomous cell growth. *J. Cell Biol.* 106:1385–1394.
38. Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.
39. Peters, G., S. Brookes, R. Smith, M. Placzek, and C. Dickson. 1989. The mouse homolog of the *hst/k-FGF* gene is adjacent to *int*-2 and is activated by proviral insertion in some virally induced mammary tumors. *Proc. Natl. Acad. Sci. USA.* 86:5678–5682.
40. Quarto, N., D. Talarico, R. Florkiewicz, C. Basilico, and D. B. Rifkin. 1989. Transformation by basic fibroblast growth factor requires high levels of expression: comparison with transformation by *hst*/K-*fgf*. *Oncogene Res.* 5:101–110.
41. Rifkin, D. B., and D. Moscatelli. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* 109:1–6.
42. Rogelj, S., R. A. Weinberg, P. Fanning, and M. Klagsbrun. 1988. Basic fibroblast growth factor fused to a signal peptide transforms cells. *Nature (Lond.).* 331:173–175.
43. Rubin, J. S., H. Osada, P. W. Finch, W. G. Taylor, S. Rudikoff, and S. A. Aaronson. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl. Acad. Sci. USA.* 86:802–806.
44. Sakamoto, H., M. Mori, M. Taira, T. Yoshida, S. Matsukawa, K. Shimizu, M. Sekiguchi, M. Terada, and T. Sugimura. 1986. Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc. Natl. Acad. Sci. USA.* 83:3997–4001.
45. Sakamoto, H., T. Yoshida, M. Nakakuki, H. Odagiri, K. Miyagawa, T. Sugimura, and M. Terada. 1988. Cloned *hst* gene from normal human

leukocyte DNA transforms NIH3T3 cells. *Biochem. Biophys. Res. Commun.* 151:965–972.
46. Sasada, R., T. Kurokawa, M. Iwane, and K. Igarashi. 1988. Transformation of mouse BALB/c 3T3 cells with human basic fibroblast growth factor cDNA. *Mol. Cell Biol.* 8:588–594.
47. Taira, M., T. Yoshida, K. Miyagawa, H. Sakamoto, M. Terada, and T. Sugimura. 1987. cDNA sequence of human transforming gene *hst* and identification of the coding sequence required for transforming activity. *Proc. Natl. Acad. Sci. USA.* 84:2980–2984.
48. Talarico, D., and C. Basilico. 1991. The K-*fgf*/*hst* oncogene induces transformation through an autocrine mechanism that requires extracellular stimulation of the mitogenic pathway. *Mol. Cell. Biol.* 11:1138–1145.
49. Thomas, K. A. 1988. Transforming potential of fibroblast growth factor genes. *Trends Biochem. Sci.* 13:327–328.
50. Vaidya, A. B., E. Y. Lasfargues, J. B. Sheffield, and W. G. Coutinho. 1978. Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. *Virology.* 90:12–22.
51. Waterfield, M. D., G. T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.-H. Heldin, J. S. Huang, and T. F. Deuel. 1983. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. *Nature (Lond.).* 304:35–39.
52. Wellstein, A., R. Lupu, G. Zugmaier, S. L. Flamm, A. L. Cheville, P. Delli Bovi, C. Basilico, M. Lippman, and F. G. Kern. 1990. Autocrine growth stimulation by secreted Kaposi fibroblast growth factor but not by endogenous basic fibroblast growth factor. *Cell Growth Differ.* 1: 63–71.
53. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from prokaryotes and eucaryotes. *Cell.* 16:777–785.
54. Yayon, A., and M. Klagsbrun. 1990. Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: reversal by suramin. *Proc. Natl. Acad. Sci. USA.* 87:5346–5350.
55. Yoshida, T., K. Miyagawa, H. Odagiri, H. Sakamoto, P. F. R. Little, M. Terada, and T. Sugimura. 1987. Genomic sequence of *hst*, a transforming gene encoding a protein homologous to fibroblast growth factors and the *int*-2-encoded protein. *Proc. Natl. Acad. Sci. USA.* 84:7305–7309.
56. Zhan, X., B. Bates, X. Hu, and M. Goldfarb. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell Biol.* 8:3487–3495.