Cleavage of K-FGF Produces a Truncated Molecule with Increased Biological Activity and Receptor Binding Affinity

Paola Bellosta, Daniela Talarico, David Rogers,* and Claudio Basilico

Department of Microbiology and Kaplan Cancer Center, New York University School of Medicine, New York 10016; and * Genetics Institute, Cambridge, Massachusetts 02140

Abstract. The K-FGF/HST (FGF-4) growth factor is a member of the FGF family which is efficiently secreted and contains a single N-linked glycosylation signal. To study the role of glycosylation in the secretion of K-FGF, we mutated the human K-fgf cDNA to eliminate the glycosylation signal and the mutated cDNA was cloned into a mammalian expression vector. Studies of immunoprecipitation from the conditioned medium of cells expressing this plasmid revealed that the lack of glycosylation did not impair secretion, however the unglycosylated protein was immediately cleaved into two NH2-terminally truncated peptides of 13 and 15 kD, which appeared to be more biologically active than the wild-type protein. These two proteins also showed higher heparin binding affinity than that of wt K-FGF. We have expressed in bacteria the larger of these two proteins (K140), in which the NH2-terminal 36 amino acids present in the mature form of K-FGF have been deleted. Mitogenicity assays on several cell lines showed that purified recombinant K140 had approximately five times higher biological activity than wild-type recombinant K-FGF. Studies of receptor binding showed that K140 had higher affinity than wt K-FGF for two of the four members of FGF receptor's family, specifically for FGFR-1 (fig) and FGFR-2 (bek). K140 also had increased heparin binding ability, but this property does not appear to be responsible for the increased affinity for FGF receptors. Thus removal of the NH2-terminal 36 amino acids from the mature K-FGF produces growth factor molecules with an altered conformation, resulting in higher heparin affinity, and more efficient binding to FGF receptors. Although it is not clear whether cleavage of K-FGF to generate K140 occurs in vivo, this could represent a novel mechanism of modulation of growth factor activity.

The K-fgf/hst oncogene (Delli-Bovi et al., 1987; Taira et al., 1987) encodes a heparin-binding protein (FGF-4) of the FGF family, whose members share a varying degree of homology at the amino acid sequence, and are mitogenic for a variety of cell types of epithelial, mesodermal, and neuroectodermal origin. They can also stimulate neurite outgrowth and inhibit the differentiation of myoblasts. In vivo FGFs accelerate wound repair, stimulate angiogenesis, and can induce the formation of mesoderm in Xenopus oocytes (for review see Basilico and Moscatelli, 1992).

The response of cells to FGF is mediated through binding of two types of cell surface receptors. High affinity receptors are tyrosine kinases and include the fig receptor or FGFR-1 (Lee et al., 1989; Dionne et al., 1990), the bek receptor or FGFR-2 (Kornbluth et al., 1988; Dionne et al., 1990; Manskhan et al., 1992), FGFR-3 (Keegan et al., 1991), and FGFR-4 (Partanen et al., 1991), while low affinity receptors comprise a heterogeneous population of heparan sulfate proteoglycans (HSPG) (Moscatelli, 1987). Although it is clear that all or most FGF have to interact with HSPG or heparin to bind and activate the tyrosine kinase receptors (Yayon et al., 1991; Klagesbrun and Baird, 1991), little is known about the FGF domains that are important for HSPG binding and receptor specificity.

The human K-fgf cDNA encodes a 206-amino acid polypeptide which contains an NH2-terminal hydrophobic leader sequence and a single N-linked glycosylation signal. The mature protein is efficiently secreted as a glycosylated 22-kD polypeptide (Delli-Bovi et al., 1988). N-linked glycosylation has been shown to play a role in protein transport to the cell surface, and it is known that suppression of glycosylation by site-directed mutagenesis or tunicamycin treatment may lead to reduced levels of secretion (Guan et al., 1985; Dorner et al., 1987).

To study the role of glycosylation in the secretion of K-FGF, we eliminated the N-linked glycosylation signal in the K-FGF protein by site-directed mutagenesis of K-fgf cDNA. Cells transfected with a plasmid encoding the unglycosylated (glyc−) K-FGF appeared to secrete the growth factor in the medium, indicating that lack of glycosylation does not impair K-FGF secretion. The absence of glycosylation however leads to cleavage of K-FGF at the cell surface or immediately prior
to secretion, producing two NH$_2$-terminally truncated polypeptides of $\sim$15 and 13 kD. These proteins exhibit higher affinity for heparin than mature K-FGF. Bacterial expression of a recombinant K-FGF form corresponding to the 15-kD polypeptide (K140) reveals that this protein has higher mitogenic activity than wt K-FGF and an increased affinity for at least two of the FGF tyrosine kinase receptors.

Materials and Methods

Construction of a Plasmid Expressing Unglycosylated K-FGF

A 595-bp EcoRI-SacI fragment, corresponding to the 5' half of the K-fgf cDNA, (Delili Bovi et al., 1987) was cloned into the M13mp18 vector. Preparation of the single-stranded template and site-directed mutagenesis were performed as described by Kunkel et al. (1987). A 25-nucleotide (nt) oligonucleotide complementary to nt 341 to 366 of the K-fgf cDNA and carrying a T-->C substitution in position 354 to change the threonine codon to alanine was used as a primer. DNA from single plaques was purified and sequenced by the dideoxynucleotide method (Sanger et al., 1977). Double-stranded DNA was extracted from a clone carrying the desired mutation. The insert was purified after digestion with SacI, and cloned into the SacI site of a pGEM3 derivative that carries the 3' SacI-EcoRI fragment of the human K-fgf cDNA, (Delli Bovi et al., 1987) to reconstruct the full length cDNA. The 1.1-kb EcoRI fragment was then cloned in the EcoRI site of the 91023B expression vector (Wong et al., 1983).

Cell Culture and Transfection

Mouse NIH3T3 and monkey COS-7 cells were grown in DME supplemented with 10% calf serum. CHO-DG44 cells, which are dihydrofolate reductase-negative (DHFR-), were maintained in DME containing 10% fetal calf serum. 32D bek clone B3 cells (Mansukhani et al., 1992) were maintained in Iscove's medium with 10% heat-inactivated FCS, interleukin-3 (10 U/ml), and 0.5% calf serum. 24 h later different concentrations of the supernatants containing the secreted growth factors were added. 18 h later the cells were labeled with 1 μCi/ml of [methyl-3H]thymidine for 6 h, washed with PBS and the incorporation into acid-precipitable material was determined. Each assay was performed in triplicate.

32D cells at 2.5×10$^4$ cells/well, were seeded in 96-well plates in Iscove's medium in the presence of 10% heat-inactivated FCS and different concentrations of heparin and growth factors. After 18 h the cells were labeled with [methyl-3H]thymidine and the incorporated radioactivity was determined as described above.

Radioiodination and Binding of FGFs

Human recombinant bFGF (specific activity 5×10$^4$ cpm/ng) was radioiodinated using the mild chloramine-T method (McConahey and Dixon, 1980) and purified over a Sephadex G-25 column in the presence of 1% BSA. Recombinant K-FGF (specific activity 9×10$^4$ cpm/ng) and K140 (specific activity 1.5×10$^4$ cpm/ng) were iodinated with 125I-labeled Bolton-Hunter reagent for 4 h on ice and purified on Sephadex G-25 column using as buffer PBS/2 M NaCl/0.25% gelatin. For competition binding assays, CHO cells were plated at 1×10$^6$ cells/25-mm dish and analysis was performed as described (Mansukhani et al., 1990). For Scatchard analysis cells were incubated at 4°C with DME containing 0.15% gelatin, 25 mM Hepes, pH 7.4, heparin (10 μg/ml), and various concentration of 125I-labeled K-FGF or K140. After 2 h the medium was removed, the cells were washed with ice-cold TRIS and the radioactivity bound to high affinity receptors was measured spectrophotometrically by its absorbance at 280 nm and by the Bradford procedure (Bio Rad Labs. Hercules, CA). Both assays were calibrated using K-FGF protein, whose concentration had been determined by amino acid composition analysis.

Effect of the Lack of Glycosylation on K-FGF Processing

The human K-FGF precursor protein is a 206-amino acid polypeptide with a hydrophobic leader sequence and an Asn-Gly-Thr consensus sequence for N-linked glycosylation. Processing of the immature form in the ER cleaves the signal peptide at residues 30 or 31, and the protein is glycosylated and secreted in the culture medium as a mature
protein of 175 or 176 amino acids (Delli-Bovi et al., 1988). To study the role of glycosylation on secretion of K-FGF, we eliminated the N-linked glycosylation signal sequence by substitution of threonine 38 with alanine, using oligonucleotide-directed mutagenesis (Fig. 1). The modified glyc- cDNA was cloned into the 9102303 mammalian expression vector, which contains the adenovirus major late promoter and the SV-40 replication origin (Wong et al., 1985), and the plasmid was introduced into simian COS cells, that constitutively express SV-40 large T antigen. 48 h later the conditioned medium from COS cells transfected with the glyc- mutant was tested for its ability to induce transformed morphology or DNA synthesis in serum starved NIH3T3 cells. This activity was found comparable with or even higher than that of the conditioned medium from COS cells transfected with the unmodified K-fgf cDNA, indicating that in the absence of glycosylation the protein is efficiently secreted. Transfection of plasmids expressing the glyc- K-FGF into NIH3T3 cells showed that their transforming ability was comparable to that of plasmids expressing wild-type (wt) K-FGF. Since transformation by K-FGF requires secretion (Talarico and Basilico, 1991; Fuller-Pace et al., 1991), this also indicated that lack of glycosylation did not significantly impair secretion.

Surprisingly, however, when the medium of COS cells transfected with the plasmid expressing the glyc- K-FGF was subjected to immunoprecipitation with anti-K-FGF antibodies, no K-FGF species of 18 kD (the expected molecular weight of unglycosylated, mature K-FGF) was observed (Fig. 2). While this form was easily detected in the cell lysate, the conditioned medium only contained two K-FGF forms of 15–13 kD, suggesting that the absence of glycosylation had exposed sites sensitive to proteases, active at the cell surface or just before secretion, leading to immediate cleavage of the protein upon release. To confirm this conclusion, COS cells transfected with the wt or the glyc- K-fgf plasmids were grown in the presence or the absence of tunicamycin, an 18-kD protein is detected in the cell lysates, as in cells transfected with the glyc- mutant. Tunicamycin causes a somewhat reduced secretion, but lower molecular weight K-FGF forms comigrating with those produced by the glyc- cDNA can be detected in the culture medium. Thus in both cases the absence of glycosylation results in extracellular cleavage of K-FGF to generate two lower molecular weight forms. Similar results were obtained by the analysis of NIH3T3 cells stably transfected with the glyc- K-fgf plasmid (data not shown).

**Heparin Binding Properties of the Truncated Forms of K-FGF**

To determine whether heparin interacted with the truncated K-FGF forms and stabilized them as it does with mature K-FGF (Delli Bovi et al., 1988), we tested the effect of heparin on the recovery of these polypeptides from the conditioned medium of transfected COS cells. Cells were labeled for 8 h with [35S]methionine in the presence or in the absence of heparin (10 µg/ml, H). Culture medium and cell lysates were immunoprecipitated and analyzed. In some cases (+ h) equal aliquots of the medium or lysates collected in the absence of heparin were incubated with 10 µg/ml of heparin for 1 h before immunoprecipitation (Fig. 3). As previously described (Delli Bovi et al., 1988), the presence of heparin in the medium during culture increases the recovery of K-FGF, although in this experiment the effect of heparin does not appear striking because of the labeling conditions. Addition of heparin to the culture medium before immunoprecipitation had no significant effect. On the other hand, in the case of the proteins produced by the glyc- mutant, the amounts of the truncated proteins detected in the medium are maximal.

![Figure 2](image-url)

**Figure 2.** Immunoprecipitation of the K-FGF species produced in COS-7 cells transfected with the glyc- K-fgf cDNA. Autoradiography of an SDS-PAGE (12.5% acrylamide) of immunoprecipitates of the conditioned medium (M) and lysates (L) from [35S]methionine-labeled COS-7 cells transfected with the glyc- K-fgf or the wt K-fgf cDNA plasmids incubated in the presence (+) or absence (−) of tunicamycin (10 µg/ml). The arrows indicated the K-FGF forms immunoprecipitated by anti-K-FGF antibodies. The molecular weight markers are indicated on the right.

![Figure 1](image-url)

**Figure 1.** Amino acid sequence of the human K-FGF precursor protein. The hydrophobic signal peptide is underlined. The arrows indicate the sites of cleavage of the mature, secreted form of K-FGF. Asterisks indicate the glycosylation signal. The mutation introduced in the cDNA to eliminate glycosylation is indicated above (Thr→Ala). The brackets indicate the sites of cleavage which generates the 15-kD K140 and the 13-kD K97 protein.
Heparin affects the recognition by antibodies of the truncated forms of K-FGF produced by the glyc- mutant. COS-7 cells transfected with the wt or glyc-K-fgf cDNA plasmids were labeled with [35S]methionine for 8 h in the presence (H) or absence of heparin in the culture medium (10 µg/ml). Media and cell lysates were immunoprecipitated with anti-K-FGF antibodies. Equal aliquots of the medium or lysate collected in the absence of heparin were also incubated with 10 µg/ml of heparin for 1 h before immunoprecipitation (+h). The immunoprecipitates were run on 12.5% SDS-PAGE followed by autoradiography. The K-FGF forms are indicated by arrows on the left. The migration of molecular weight standards are indicated on the right.

in the absence of heparin. When the medium is preincubated with heparin before immunoprecipitation (+h), there is a considerable decrease in the amount of recovered proteins (Fig. 3). Western analysis showed that the amounts of truncated proteins present in these media were not affected by the addition of heparin after harvesting, and that the presence of heparin during culture actually increased somewhat the recovery of the truncated K-FGF forms (data not shown). Thus these data suggest that the binding of heparin to the truncated K-FGF molecules inhibits or competes for antibody recognition.

To investigate further the interaction of heparin with the truncated K-FGF species, the conditioned medium labeled with [35S]methionine collected from COS cells transfected with either the K-fgf cDNA or the glyc- mutant was absorbed to heparin-Sepharose column and eluted with increasing NaCl concentration. Fractions were immunoprecipitated with anti-K-FGF antibodies, and run on SDS-PAGE. It can be seen in Fig. 4 that K-FGF eluted at 1.1 M NaCl, while the 13- and 15-kD proteins eluted with a peak at 1.3–1.5 M NaCl, indicating that the affinity for heparin of these proteins is higher than that of K-FGF. Thus the truncated forms of K-FGF also bind heparin with higher affinity than the wild type.

**Expression of a Truncated Form of K-fgf**

Titration of the mitogenic activity present in the medium of cells transfected with the glyc- K-fgf cDNA, relative to the amounts of truncated proteins estimated by Western blot, indicated that these truncated K-FGF forms had a higher specific activity than that of wt K-FGF. To verify this finding, we decided to characterize the cleavage sites to genetically engineer recombinant versions of the truncated K-FGF, purify the expressed protein to homogeneity and compare its properties with that of full length recombinant K-FGF. Immunoprecipitation analysis with antibodies raised against peptides that recognize the COOH-terminus of K-FGF, showed that the two truncated species were deleted in the NH2-terminal part of the molecule (data not shown).

To sequence the two polypeptides of 13 and 15 kD, COS cells, transfected with glyc- K-fgf cDNA, were metabolically labeled with [3H]leucine. The labeled proteins were purified by immunoprecipitation with anti-K-FGF antibodies. The amino terminus of the purified proteins was sequenced as described in Materials and Methods. The sequence of the largest polypeptide was identified as starting at residue 67 of the full length K-FGF protein, and that of the shorter and less abundant protein at residue 110 (Fig. 1). The 140–amino acid polypeptide (K140) has lost most of the amino-terminal region unique to K-FGF within the FGF family, but retained the central homologous core region. The 97-amino acid polypeptide has also lost part of the central homologous core region and has not been further studied.

The K-fgf cDNA sequence was altered using site-directed mutagenesis to delete the first 66 amino acids and place an initiator methionine in front of residue 67. The 140-amino acid polypeptide was cloned in the bacterial expression vector PA/L-781 under the control of the pL promoter and the cII ribosome binding site and expressed in E. coli. To increase the amount of K140 produced by the bacteria, the cDNA was modified, to reduce its GC content, without changing the amino acid sequence of the protein (see Materials and Methods). The protein was purified and its biological activity compared with that of recombinant K-FGF. As
expected it had a higher heparin affinity than that of similarly produced, full length K-FGF (data not shown).

**Mitogenic Activity of the K140 Protein**

To study the biological activity of the recombinant K140 molecule, its ability to stimulate cell proliferation was compared with that of recombinant K-FGF using a DNA synthesis assay on several fibroblast and endothelial cell lines. Cells were incubated for 2 d in medium containing 0.5% serum, and then treated with different concentration of K-FGF or K140. 18 h later the cells were labeled with [3H]thymidine for 6 h and the effects on DNA synthesis were calculated by measuring the incorporation of radioactivity into acid-insoluble material. A representative experiment using the BALB/c 3T3 cell line is shown in Fig. 5. As can be seen, K140 was about five times more efficient than K-FGF in inducing DNA synthesis. At low doses, about five times more K-FGF than K140 is required to achieve the same stimulation of DNA synthesis, and maximal stimulation was observed at 1 ng/ml with K140 and 5 ng/ml with K-FGF. Similar results were obtained using NIH 3T3 cells and human umbilical vein endothelial cells (data not shown). Surprisingly, the stimulation of DNA synthesis obtained with K140 was always somewhat higher than that produced by K-FGF, even at growth factor concentrations producing maximal stimulation. This small but reproducible difference could be due to the fact that K140 can recruit into DNA synthesis cells that are not induced by K-FGF, perhaps because they express a low number of receptors.

The lower mitogenic activity of K-FGF with respect to K140 does not appear to be the result of higher susceptibility of the former to proteolytic digestion by factors present in the conditioned medium. Western blotting analysis, using anti-K-FGF antibodies, showed that incubation of K-FGF and K140 in the conditioned medium of NIH 3T3 at 37°C for 24 h did not induce significant proteolytic degradation (data not shown). It is worth mentioning here that the biological activity of K140 can be neutralized as well as that of K-FGF by neutralizing antibodies raised in rabbits against the mature recombinant K-FGF protein.

**Figure 5.** Mitogenic activity in quiescent BALB/c-3T3 cells by human recombinant K-FGF or by recombinant K140. BALB/c-3T3 cells were incubated for 2 d in medium containing 0.5% serum, cells were treated with the indicated concentration of K140 (■) or K-FGF (□). 18 h later the cells were labeled with [3H]thymidine for 6 h and radioactivity incorporated into cellular DNA was counted after TCA precipitation. Stimulation by 10% serum (○).

**Figure 6.** Competition binding of bFGF, K-FGF, and K140 to CHO cells expressing FGFR-1 or CHO cells expressing FGFR-2. CHO cells were incubated at 4°C with [125I]-labeled bFGF (4 ng/ml) and the indicated concentrations of unlabeled bFGF (△), K-FGF (■), or K140 (○), in the presence of heparin (10 μg/ml) as described in Materials and Methods. After 2 h the cells were washed with 2 M NaCl buffered at pH 7.4 to remove the growth factor bound to low affinity receptors, and with 2 M NaCl buffered at pH 4.0 to remove the ligand bound to high affinity receptors. The amount of [125I]-labeled bFGF bound to high affinity receptors was determined.

Bellosta et al. *Truncated K-FGF with Higher Biological Activity* 709
Receptor Binding Analysis on CHO Cells Expressing FGFR-1 and FGFR-2

The higher mitogenic activity of K140 could have been related to a higher affinity for FGF receptors. Since 3T3 cells express multiple species of FGF receptors (Partanen et al., 1991; our own unpublished results), and that would have complicated the analysis, we performed ligand binding analysis on CHO cells expressing the murine FGFR-1 (flg) (Mansukhani et al., 1990) or FGFR-2 (bek) receptors (Mansukhani et al., 1992). In the first series of experiments, we performed competition experiments for binding to FGFR-1 or -2, using iodinated bFGF as the ligand (Fig. 6). As previously described, bFGF binds FGFR-1 with ~10 times higher affinity than K-FGF, while both growth factors bind FGFR-2 with the same high affinity (Mansukhani et al., 1990; 1992). The K140 protein competes for bFGF binding to FGFR-1 as effectively as bFGF itself, and significantly more efficiently than wild-type K-FGF. Binding to FGFR-2 is also more efficient with about two or three times higher affinity than that of bFGF and K-FGF.

To confirm this conclusion, CHO cells expressing the flg or bek receptor were incubated with iodinated K-FGF or K140, and binding was competed with various concentrations of unlabeled ligands (Fig. 7). In all cases, and irrespective of whether the labeled ligand was K-FGF or K140, about five times more K-FGF than K140 was required to compete for binding to receptor 1, while in the case of receptor 2 the affinity of K140 was about twice that of K-FGF. To confirm that K140 has higher affinity than K-FGF for FGFR-1 and FGFR-2, the relative dissociation constants were determined by saturation binding analysis. As shown in Fig. 8 Scatchard analysis for FGFR-1 gave a straight line, indicating a single...
class of binding sites. From these data the K_d for FGFR-1 is
~90 pM for K140 and 280 pM for K-FGF. Similar results
were obtained for cells expressing FGFR-2, where the K_d
was ~60 pM for K140 and 140 pM for K-FGF (not shown).

We also measured binding of K140 and K-FGF to cells ex-
pressing FGFR-4. This receptor has been shown to have
maximal affinity for aFGF, with bFGF and K-FGF binding
about ten times less efficiently (Vainikka et al., 1992). K140
did not appear to bind FGFR-4 more efficiently than K-FGF
(data not shown), suggesting that the increased affinity of
K140 for FGFR-1 and 2 is receptor-specific.

DNA Synthesis in Transfected 32D Cell Expressing
FGF Receptors

FGFs require an interaction with heparin or HSPGs to be
capable of binding and activating their tyrosine kinase recep-
tors (Yayon et al., 1991). It was therefore conceivable that
the higher affinity of the K140 protein for two of the FGF recep-
tors could have been due, at least in part, to its higher affinity
for heparin with respect to K-FGF. To test this hypothesis,
we measured the mitogenic effect of K140 and K-FGF on
32D cells expressing the bek receptor. 32D cells are IL-3 de-
pendent murine hematopoietic cells, that do not express de-
tectable levels of HSPGs or high affinity FGF receptors.
When these cells are transfected to express FGF receptors,
they can be induced to proliferate by FGFs, but this response
requires the presence of heparin in the culture medium (Man-
sukhani et al., 1992). 32D cells expressing the bek receptor
were seeded at 25,000 cells/well in 96-well plates in 10% 
FCS and different concentrations of growth factors or heparin
were added. After 18 h the cells were labeled with [3H]-
thymidine and the effects on DNA synthesis were calculated
by measuring the incorporation of radioactivity into acid in-
soluble material (Fig. 9). The data show that in the absence
of heparin both K140 or K-FGF failed to stimulate DNA syn-
thesis. At high growth factor concentrations (10 ng/ml),
K140 does not appear to exhibit lower heparin requirements
than K-FGF, as both factors begin to be mitogenic at a con-
centration of heparin of ~50 ng/ml and produce maximal
stimulation in the presence of ~1 µg/ml of heparin (Fig.
9 A). At low concentrations of growth factor (0.3 ng/ml),
K-FGF appears to be slightly more heparin dependent for its
mitogenic activity than K140, but this difference is not strik-
ing (Fig. 9 B). We interpret these data as indicating that
binding of K140 to its receptors also requires heparin (and
this was also confirmed by direct binding experiments, not
shown), but that the higher mitogenic and receptor binding
activity of K140 with respect to wild-type K-FGF cannot be
simply explained by its higher affinity for heparin, although
this property could be a contributing factor. Similar results
were obtained with 32D cells expressing the fig (FGFR-1)
receptor.

Discussion

The studies presented in this manuscript were initiated to de-
termine precisely whether glycosylation of the K-FGF mole-
cule, a growth factor of the FGF family that is normally
efficiently secreted, was important for processing and secre-
tion. We thus eliminated the unique glycosylation signal
which is present in the K-FGF molecule near its NH2 ter-
minus by site-directed mutagenesis of the human K-fgfcDNA.
Expression of this mutated form of K-FGF in mammalian
COS and NIH3T3 cells showed that the absence of glycosyla-
tion does not impair processing and secretion of K-FGF. The
cleaved, mature form of unglycosylated K-FGF is however
only detected intracellularly. All extracellular K-FGF is
shortened by cleavage at residues 66 or 109 to generate two
NH2-terminally truncated molecules.

Cleavage must predominantly occur during the external-
ization process or at the cell surface, as we were never able
to detect any full length unglycosylated protein in the culture
medium. The nature of the proteases involved in the cleavage
is unknown. There is a potential Staphylococcus aureus protease cleavage site at amino acid 66, and thus these proteases could be similar. Thus, the absence of glycosylation leads to exposure of protease sensitive sites that are normally protected in the K-FGF molecule. These sites are not immediately located within the region of attachment of the sugar chains, but are distal to it. It appears therefore that the exposure of these protease-sensitive sites results from changes in the structure or folding of the K-FGF molecule, resulting in the uncovering of sites which are normally protected by steric interference of sugar residues or by the folding of the molecule.

The unexpected result of this proteolytic cleavage is the generation of two lower molecular weight K-FGF species which have a higher affinity for heparin and of which one at least, has higher biological activity. These findings suggest that the truncated K-FGF forms must undergo some changes in tertiary structure, which differentiate them from mature K-FGF. It is particularly worth mentioning here that they do not only exhibit higher affinity for heparin, but that heparin appears to bind these molecules in a different way from that of mature K-FGF. In fact, while heparin binding does not generally affect K-FGF recognition by antibodies, the binding of heparin to the truncated molecules appears to inhibit antibody recognition (Fig. 3), and this effect is particularly evident with antipeptide sera raised against the COOH terminus of the protein (data not shown). This finding suggests that heparin binding is not only a function of primary amino acid sequence but is also influenced by the configuration of the protein molecule, although alternative explanations cannot be ruled out. It is interesting to note that an earlier study (Burgess et al., 1985) had suggested that a truncated form of acidic FGF had increased heparin affinity.

Another finding supporting the hypothesis that these truncated molecules must assume a novel spatial configuration is that fact that the longest of them, K140, has higher mitogenic activity than that of wild-type K-FGF. This higher biological activity seems to result, for the most part, from the increased affinity for two of the FGF receptors, fgl and bek, which are the most widely expressed members of this receptor family. It is not immediately apparent why a truncated form of this growth factor would have higher receptor binding. The higher affinity for heparin, with its consequent faster “activation” of the growth factor to a receptor binding form does not seem to be responsible (although it may contribute to its higher biological activity), as our binding experiments were performed using growth factor concentrations (2–4 ng/ml) at which we cannot detect any differential effect of heparin on K140 with respect to K-FGF (not shown).

It cannot be excluded however that binding of K140 to heparin results in conformational changes which are more favorable to receptor binding than those undergone by full length K-FGF. It has been suggested that heparin facilitates FGF dimerization, a property that may be important for receptor activation (Ornitz et al., 1992). It is possible that the interaction of K140 with heparin or HSPG leads to the formation of dimers that are more stable than those formed by K-FGF.

Analysis of the crystal structure of basic and acidic FGF shows that the NH$_2$-terminal region of these molecules is in a flexible and disordered configuration, and does not contribute to the folding of the rest of the molecule (Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991). If the K-FGF crystal structure is analogous, it could be speculated that removal of its NH$_2$-terminal region leads to the formation of a molecule which is more “compact” and organized, and thus could form a more stable association with the ligand binding domain(s) of the receptor. It is likely however that the explanation of this phenomenon is more complex, as K140 does not bind more efficiently than wt K-FGF to a third FGF receptor, FGFR-4. Thus a component of specificity must be involved in the increased receptor binding of K140. The answer to these questions will probably have to wait for the elucidation of the crystal structure of the K-FGF and K140 molecules.

The expression of wild-type K-FGF by both COS and CHO cells generates a low level of a truncated form of growth factor, suggesting that cleavage of glycosylated K-FGF can occur (our own unpublished results). The fact that the response of target cells in vitro is altered in both the maximum response (cells recruited) and sensitivity, hints at the intriguing possibility that truncated K-FGF has a specific function in vivo. The cellular location, distribution, and regulation of the proteases that cleave K-FGF are unknown but may play a role in producing novel forms of K-FGF. Currently few tissues have been shown to express K-FGF and those that do, do so during early stages of development (Niswander and Martin, 1992) making the natural synthesis of this growth factor difficult to characterize. An understanding of the biological role of K140 will require further study.
Kunkel, T. A., J. D. Roberts, and R. A. Lakowe. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367–382.

Lee, P. L., D. E. Johnson, L. S. Cousens, V. A. Fried, and L. T. Williams. 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science (Wash. DC).* 245:57–60.

Luthman, H., and G. Magnusson. 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* 11:1295–1308.

Mansukhani, A., P. Dell’Era, D. Moscatelli, S. Kornbluth, H. Hanafusa, and C. Basilico. 1992. Characterization of the murine BEK fibroblast growth factor (FGF) receptor: activation by three members of the FGF family and requirement for heparin. *Proc. Natl. Acad. Sci. USA.* 89:3305–3309.

Mansukhani, A., D. Moscatelli, D. Talarico, V. Levytska, and C. Basilico. 1990. A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF. *Proc. Natl. Acad. Sci. USA.* 87:4378–4382.

McConahey, P. J., and F. J. Dixon. 1980. Radioldination of proteins by the use of the chloramine-T method. *Methods Enzymol.* 70:210–213.

Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell Physiol.* 131:123–130.

Niswander, L., and G. R. Martin. 1992. FGF-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development (Camb.).* 114:755–768.

Ornitz, D. M., A. Yayon, J. G. Flanagan, C. M. Svahn, E. Levi, and P. Leder. 1992. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.* 12:240–247.

Partanen, J., T. P. Mäkelä, E. Eerola, J. Korhonen, H. Hirvonen, L. Claesson-Welsh, and K. Alitalo. 1991. FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1347–1354.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Shimatake, H., and M. Rosenberg. 1981. Purified X regulatory protein cII positively activates promoters for lysogenic development. *Nature (Lond.).* 292:128–132.

Tanaka, 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.

Talarico, D., and C. Basilico. 1991. The K-fgflhst oncogene induces transformation through an autocrine mechanism that requires extracellular stimulation of the mitogenic pathway. *Mol. Cell Biol.* 11:1138–1145.

Vainikka, S., J. Partanen, P. Bellotta, F. Coulier, C. Basilico, M. Jaye, and K. Alitalo. 1992. Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction via phospholipase C-γ. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4237–4240.

Wong, G. G., J. S. Witek, P. A. Temple, K. M. Wilkens, A. C. Leary, D. P. Luxenberg, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. A. Wang, and S. C. Clark. 1985. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science (Wash. DC).* 228:810–815.

Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 64:841–848.

Zhang, J., L. S. Cousens, P. J. Barr, and S. R. Sprang. 1991. Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1β. *Proc. Natl. Acad. Sci. USA.* 88:3446–3450.

Zhu, X., H. Komiya, A. Chirino, S. Faham, G. M. Fox, T. Arakawa, B. T. Hsu, and D. C. Ress. 1991. Three-dimensional structures of acidic and basic fibroblast growth factors. *Science (Wash. DC).* 251:90–93.