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

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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 fg 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; Mansukhani 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; Klagsbrun 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-aminoc 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 EcoRl-SacI fragment, corresponding to the 5' half of the K-fgf cDNA, (Delli 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 Kunzel 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, to reconstruct the full length eDNA. The 1.1-kb EcoRI fragment was then cloned in the EcoRI site of the K1403B expression vector (Wong et al., 1985).

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% FCS, 0.1 mM hypoxanthine, 0.02 mM thymidine, and 10 mM proline. CHO-4-1 clone (Mansukhani et al., 1990) and CHO 3.7-5 clone (Mansukhani et al., 1992) were grown in medium lacking hypoxanthine and thymidine. 32D bk clone B3 cells (Mansukhani et al., 1992) were maintained in Isco's medium with 10% heat-inactivated FCS, interleukin-3 (10 U/ml) and gentamicin (400 mg/ml). COS-7 cells were transfected by the DEAE-dextran chloroquine procedure (Lutman and Magnuson 1983) using 3 µg of plasmid per 100-mm dish.

Immunoprecipitation

COS-7 cells were labeled for 8 h with 200 µCi/ml of $[^{35}]$S methionine and then lysed in RIPA buffer (Delli-Bovi et al., 1988). The cell lysate was centrifuged at 12,500 rpm for 30 min at 4°C. The medium was mixed with 1/4 vol of 5× RIPA buffer. Cell lysate and media were immunoprecipitated overnight at 4°C with rabbit anti-K-FGF antiserum. Immunoprecipitates were washed with RIPA buffer and analyzed on a 12% SDS-PAGE. After electrophoresis, the dried gels were fluorographed at $\sim$80°C.

Induction of Transformed Morphology by K-FGF

NIH3T3 cells were seeded at 3× $10^{4}$/well in DME supplemented with 10% calf serum. The next day the cells were washed and starved in DME with 0.5% calf serum. 24 h later different concentrations of the supernatants from COS-7 cells, containing the secreted growth factors, were added. 12 h later the cells were analyzed for their transformed phenotype.

Sequencing the NH$_2$ Terminus of the Truncated K-FGF Forms

Tritiated leucine-labeled proteins from the medium of COS-7 cells transfected with the plasmid expressing the glycine K-FGF were purified by precipitation with a polyclonal antibody raised against full length K-FGF. The amino terminus of the purified proteins was sequenced using a protein sequencer (model 470A, Applied Biosystems, Foster City, CA) with the antibody acting as a carrier protein. The samples from each round of Edman degradation were tested for radioactivity and tritium was found in several cycles allowing them to be assigned as leucine residues in the K-FGF sequence. There was a major sequence and a minor sequence. In both cases there was only one possible start site that would generate the Leucine residue pattern identified by the sequencer. The major sequence started at residue 67 of the K-FGF full length sequence and the minor at residue 110.

Expression and Purification of Recombinant K140

The full length cDNA for K-FGF was mutated using site-directed mutagenesis to remove the nucleotides coding for the first 66 amino acids and replace them with the sequence for a methionine codon. For high level expression, it was found necessary to change the codon usage pattern of the first seven amino acids to decrease the GC bias of the sequence. The new sequence reads 5'-ATG GCA GCA GTT CAA TCA GGA GCA-3' and does not change the amino acid sequence of the protein. This mutated sequence was inserted into a vector PA/L7-81 which allowed expression under the control of the pL promoter and the cII ribosome binding site (Shimakata and Rosenberg 1981). The GI-595 Escherichia coli strain used to express K-FGF had a temperature sensitive lambda repressor. After growth at the permissive temperature, the temperature of the culture was raised allowing expression of K140. Cells were broken in a Gaulin homogenizer and the insoluble K140 was extracted using a high salt buffer (0.75 M NaCl, 50 mM Hepes, pH 7.5), and purified by Heparin/Toyopearl column chromatography. Protein concentration of recombinant growth factor preparations 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.

Mitogenic Assays

BALB/c3T3 cells were plated at 3× $10^5$/cells/well in 24-well plates in DME supplemented with 10% calf serum. The cells were starved in 0.5% calf serum and after 4 h serial dilutions of recombinant growth factors were added. 18 h later the cells were labeled with 1 µCi/ml of [methyl-$^{3}H$]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× $10^5$/cells/well, were seeded in 96-well plates in Isonic’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 [methyln-$^{3}H$]thymidine and the incorporation 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$^5$ cpm/ng) were iodinated with $[^{125}]$I-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×$10^5$/cells/35-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 concentrations of $[^{125}]$I-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 released and extracted in 0.5% SDS, 50 mM TRIS/HCl pH 7.4, 0.15 M NaCl, 5 mM EDTA. Nonspecific binding was determined using the same amount of growth factor on parental CHO DG-44 cells that do not express FGF receptors.

Results

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 (Fig. 1). 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, and the cell lysates or the conditioned medium of transfected COS cells. Cells were labeled with [35S]methionine and the cell extracts or 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 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.

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   201 HFLPRL 206

**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.

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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.

Figure 4. Elution of truncated K-FGF forms from heparin affinity columns. Conditioned medium labeled with [35S]methionine produced from COS-7 cells transfected with the wt or glyc- K-fgf cDNA was absorbed to heparin-Sepharose and eluted with the indicated molar NaCl concentrations. Fractions were immunoprecipitated with anti-K-FGF antibodies and run on 12.5% SDS-PAGE. The molecular weights of the K-FGF forms are indicated by the arrows. The molecular weight markers are on the right.

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 acids polypeptide (K140) 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 KI40 Protein**

To study the biological activity of the recombinant KI40 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 KI40. 18 h later the cells were labeled with [\(^{3}\)H]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, KI40 was about five times more efficient than K-FGF in inducing DNA synthesis. At low doses, about five times more K-FGF than KI40 is required to achieve the same stimulation of DNA synthesis, and maximal stimulation was observed at 1 ng/ml with KI40 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 KI40 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 KI40 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 KI40 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 KI40 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 KI40 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 KI40. BALB/c-3T3 cells were incubated for 2 d in medium containing 0.5% serum, cells were treated with the indicated concentration of KI40 (●) or K-FGF (○). 18 h later the cells were labeled with [\(^{3}\)H]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 KI40 to CHO cells expressing FGFR-1 or CHO cells expressing FGFR-2. CHO cells were incubated at 4°C with [\(^{125}\)I]-labeled bFGF (4 ng/ml) and the indicated concentrations of unlabeled bFGF (△), K-FGF (●), or KI40 (○), 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 [\(^{125}\)I]-labeled bFGF bound to high affinity receptors was determined.
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 $\sim 90$ pM for K140 and 280 pM for K-FGF. Similar results were obtained for cells expressing FGFR-2, where the $K_d$ was $\sim 60$ pM for K140 and 140 pM for K-FGF (not shown).

We also measured binding of K140 and K-FGF to cells expressing 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.

Figure 9. Stimulation of DNA synthesis in 32D bek B3 cells by K-FGF or K140. Cells were incubated in Iscove's medium containing 10% FCS and different concentrations of heparin. After 2 h the medium was removed, and the radioactivity bound to the high affinity receptors was determined as described in Materials and Methods.

**Discussion**

The studies presented in this manuscript were initiated to determine precisely whether glycosylation of the K-FGF molecule, a growth factor of the FGF family that is normally efficiently secreted, was important for processing and secretion. We thus eliminated the unique glycosylation signal which is present in the K-FGF molecule near its NH₂ terminus 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 glycosylation 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 externalization 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...
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 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, flg 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 NH2-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 NH2-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.

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