Expression of Biologically Active Recombinant Keratinocyte Growth Factor

STRUCTURE/FUNCTION ANALYSIS OF AMINO-TERMINAL TRUNCATION MUTANTS*

(Dina Ron‡, Donald P. Bottaro, Paul W. Finch§, David Morris¶, Jeffrey S. Rubin, and Stuart A. Aaronson||

From the Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892)

Keratinocyte growth factor (KGF) is a newly identified member of the fibroblast growth factor (FGF) family (FGF-7). KGF is expressed by stromal fibroblasts and acts on epithelial cells in a paracrine mode. To facilitate structure/function studies, we utilized the T7 prokaryotic expression system to synthesize this growth factor. Recombinant KGF (rKGF) was mitogenic with a specific activity around 10-fold higher than native KGF. By in vitro mutagenesis, we generated a series of KGF mutants with sequential deletions of the amino-terminal domain, the most divergent region among different FGF members. Mutant proteins, produced in bacteria, were tested for their ability to bind heparin, bind and activate the KGF receptor, and induce DNA synthesis. Heparin binding properties were preserved with deletion of up to 28 amino-terminal residues of the mature KGF but lost by the deletion of an additional 10 residues. Biological activity of mutants with deletions of up to 10 residues was comparable to that of rKGF. However, deletion of 29 residues resulted in significantly reduced ability to stimulate KGF receptor tyrosine-kinase activity and DNA synthesis, although this mutant bound the receptor at high affinity. These characteristics of a partial agonist may be useful in the development of competitive antagonists of KGF action.

Keratinocyte growth factor (KGF)† is a recently identified member of the FGF family (FGF-7) (1). Unlike other members of this family with a whole spectrum of target cells, KGF appears to be restricted in its activity to epithelial cells (2). Its expression by stromal fibroblasts of many epithelial tissues suggests a role for KGF as a major paracrine mediator of epithelial cell proliferation. Recently, a high affinity receptor for KGF was cloned and found to be an alternatively spliced isoform of fibroblast growth factor receptor-2 (bek/FGFR2) (3, 29). KGF was initially purified from conditioned medium of human embryonic fibroblasts (2). To obtain large quantities of the growth factor required for structure/function studies, we sought to express recombinant KGF in bacteria. We report here a method that produces high yields of functional human recombinant KGF. We have utilized this expression system as a means to generate and characterize KGF mutants with the goal of localizing domains important for KGF biological function.

MATERIALS AND METHODS

Construction of Recombinant Plasmids—Plasmid pet8C (4) was initially used to clone the KGF coding sequence. This insert was adapted for cloning into the NcoI/BamHI sites of pet8C by means of the polymerase chain reaction (PCR) (5, 6), as described under "Results." The recombinant plasmid, propagated in HB101 cells, was used to transform BL21(DE3) plys E (4) for induction of KGF expression.

Amino-terminally truncated KGF mutants were generated by the PCR technique (5, 6). A pair of oligonucleotide primers was used to generate each of the mutants; one was derived from the carboxyl-terminal region of the KGF open reading frame common to each mutant: tgg gat cca tta agt tat tgc  cat agg aag. The second primer was derived from the sequence immediately distal to the region to be deleted (Table I). Ndel and BamHI sites were included in the primer to allow cloning of the amplified inserts into the pet3C plasmid. An Ndel site in the KGF coding sequence was first eliminated using two steps of site-directed mutagenesis which did not change the primary amino acid sequence.

DNA was amplified by PCR as described previously (6). Briefly, 1 ng of plasmid DNA containing KGF cDNA was used as a template. Amplification was performed for 25 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 3 min in a total volume of 100 µl. The amplified DNA was purified by Sephadex G-50 spin columns and subjected to restriction enzyme digestion to allow cloning into the appropriate vectors. Plasmid DNA was prepared from positive colonies, and the nucleotide sequence of each mutant was confirmed using the dideoxy sequencing method (7).

Production and Purification of Recombinant Human KGF—

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second primer generated by PCR</td>
</tr>
<tr>
<td>Mutant Deletion</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>K10 1-34 atc ata tga ctc cac gag cag cag aag</td>
</tr>
<tr>
<td>K11 1-38 atc ata tga aag gag ggg ata ta</td>
</tr>
<tr>
<td>K12 1-58 atc ata tga aag gag ggg ata ta</td>
</tr>
<tr>
<td>K13 1-69 atc ata tga aag gag ggg ata ta</td>
</tr>
<tr>
<td>K14 1-79 atc ata tga aag gag ggg ata ta</td>
</tr>
</tbody>
</table>

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Technion-Israeli Institute of Technology, Technion City, Haifa-32000, Israel.

§ Present address: Dept. of Neurosurgery, Rhode Island Hospital, Providence, RI 02903.

¶ Present address: Massachusetts General Hospital, Boston, MA 02114.

|| To whom correspondence should be addressed: Bldg. 37, Rm. 1E24, NIH, Bethesda, MD 20892. Tel.: 301-496-9885; Fax: 301-496-8479.

1 The abbreviations used are: KGF, keratinocyte growth factor; rKGF, recombinant KGF; FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
BL21(DE3) pLys E cells bearing recombinant plasmid were grown at 37 °C in terrific broth (8) containing 100 μg/ml ampicillin. When A660 reached 1.0, isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added, cultures were incubated for 3 h, and cells were collected by centrifugation. The cell pellet was resuspended in TENG buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 5 mM MgCl2, pH 7.5) containing protease inhibitors, and the cells were lysed by three successive cycles of freezing and thawing. The lysate was disrupted by sonication, cleared by centrifugation, and the supernatant was frozen prior to growth factor purification.

Purification of recombinant KGF was performed by applying the bacterially-synthesized material to a heparin-Sepharose column (4-ml bed volume). The column was washed with phosphate buffer (50 mM, pH 7.2, containing 0.2 M NaCl) until the absorbance fell to near base line and then subjected to a linear step gradient of increasing NaCl concentration. Aliquots from fractions were analyzed by SDS-PAGE and tested for mitogenic activity. Selected fractions were concentrated in 50 mM phosphate buffer, pH 6.8, and subjected to fast protein liquid chromatography/Mono S cation exchange chromatography using a gradient of increasing NaCl concentration. rKGF was eluted around 0.45 M NaCl. The amino acid composition and final concentrations of purified KGF and KGF mutants were determined by amino acid analysis.

Protein Detection, Binding Assays, and Mitogenic Activity—Polyacrylamide gels were prepared with SDS by the procedure of Laemmli (9). Samples were boiled for 3 min in the presence of 2.5% (v/v) 2-mercaptoethanol. The gels were fixed and silver stained (10) by using the reagents and protocol available from Bio-Rad, or they were transferred without fixation to polyvinylidene difluoride membranes for immunoblotting. Rabbit polyclonal antisera raised against full-length KGF or synthetic peptides corresponding to KGF residues 33-44 or 179-194 (where residue 1 is the initiation codon in the KGF open reading frame) were used for KGF detection. Immunoprecipitation and immunoblotting of cellular proteins phosphorylated on tyrosine in response to KGF were performed as described previously (11).

**RESULTS**

**Construction of a Prokaryotic KGF Expression Vector**—For prokaryotic expression of KGF, we utilized the pet& vector containing the promoter of the highly expressed φ10 gene of bacteriophage T7 (4). This vector contains the first 11 amino acids of φ10 as well as a Tp transcription termination signal recognized by the T7 RNA polymerase. The amino-terminal residues of the T7 φ10 gene product were excised, and one of the upstream cloning sites was used to express the KGF gene product. The sequence corresponding to KGF residues Ala31 to Thr184 was amplified by PCR. The 5’ and 3’ ends of the DNA were designed to include Ncol and BamHI recognition sites, respectively, to allow cloning of the insert into the Ncol/BamHI sites of the pet& vector. An amino-terminal methionine codon was included for initiation of translation. This construct was designated k4-9 (Fig. 1).

The amino acid sequence of mature KGF purified from human fibroblast culture fluids consists of Cys83-Thr184, numbered from the KGF initiation codon (1). We included Ala31 in our prokaryotic expression vector because the codon which follows the AUG can influence the translation efficiency of prokaryotic proteins (12). Cys in this position has been shown to reduce translation efficiency, and it rarely exists in this position in highly expressed proteins (12). In contrast, Phe, Ala, and Lys are commonly found in this position, and these amino acids do not decrease translation efficiency.

**Expression and Characterization of Recombinant KGF**—We initially attempted to express recombinant KGF (rKGF) in BL21(DE3) pLys E cells. However, KGF was highly toxic to these cells, and their growth was dramatically inhibited even by basal levels of KGF expression in the absence of isopropyl-1-thio-β-D-galactopyranoside induction. We next analyzed BL21(DE3) pLys E cells, which express a T7 RNA polymerase-specific inhibitor, the T7 lysozyme (13). This inhibitor reduces the basal level of target gene expression prior to induction and thereby minimizes potential toxic effects (4). Since expression of the T7 lysozyme is also driven by the T7 RNA polymerase promoter, its level of expression also increases upon induction, resulting in slower accumulation of the target protein (4). In this system, by 4 h postinduction, rKGF comprised 1–2% of the total cellular protein (data not shown).

rKGF was purified as described under "Materials and Methods" and eluted from heparin-Sepharose in 0.5 M NaCl as determined by mitogenic assay on Balb/MK cells. The elution profile from the Mono S cation exchange column revealed two protein peaks, both of which had mitogenic activity on Balb/MK cells (Fig. 2A). Silver staining of the Mono S peak fractions showed that rKGF was purified to homogeneity (Fig. 2B, fourth through seventh lanes). The apparent molecular mass of the polypeptide from the first peak was around 21 kDa, as expected for the intact rKGF product, whereas that of the polypeptide(s) eluted in the second peak was 16–17 kDa (Fig. 2B, eighth and ninth lanes).

Amino acid analysis of the 21-kDa protein observed by silver stain revealed that its composition was in good agreement with the predicted KGF sequence (data not shown). To prove that the 21-kDa protein was indeed rKGF and to investigate the smaller protein(s) further, we subjected the Mono S peak fractions to immunoblot analysis. As shown in Fig. 3A, antiserum directed against purified KGF readily recognized both the 21-kDa and smaller recombinant protein(s). In contrast, antiserum raised against an amino-ter-
The molecular masses observed for rKGF and native KGF were 21 and 26-28 kDa, respectively (Fig. 4B). The faster mobility of rKGF likely reflects the absence of glycosylation of the bacterially expressed protein. Whether this accounts for its greater specific mitogenic activity remains to be determined.

Expression and Characterization of KGF Amino-terminal Mutants—Comparison of the predicted amino acid sequence of KGF with that of other FGF family members revealed greatest divergence from KGF in their amino-terminal domains. Since amino-terminal proteolytic digestion resulted in rKGF products with mitogenic activity, we sought to study more thoroughly the effects of sequential amino-terminal deletions on KGF biological properties. Mutants were generated by PCR as described under “Materials and Methods.” We took advantage of the presence of Met codons within the KGF open reading frame at positions 35, 40, and 59 and deleted sequences 5' of each of these codons. Two additional mutants were engineered in which the first 70 or 80 residues were deleted and an AUG codon was added to initiate translation. A schematic diagram of each mutant is shown in Fig. 5A.

K10, K11, and K12 retained the ability to bind heparin and eluted at the same salt concentration as the parental molecule (data not shown). Whereas K13 and K14 mutants were expressed at levels similar to wild type rKGF (Fig. 5B), these mutants completely lost the ability to bind heparin. Attempts to purify K13 and K14 using Mono S or CM-Sephadex resins were also unsuccessful. Since K13 and K14 lacked detectable mitogenic activity, they were not characterized further. K10, K11, and K12 mutants were purified to homogeneity by Mono S chromatography (Fig. 5C) and their identities confirmed by immunoblot analysis using rabbit polyclonal antisera directed against a synthetic peptide derived from the KGF carboxy-terminal sequence (Fig. 5D).

We next investigated the abilities of K10, K11, and K12 mutants to displace radiolabeled KGF from its receptor, trigger receptor-kinase activity, and stimulate Balb/MK DNA synthesis. Each of the mutants effectively displaced 125I-KGF bound to NIH/3T3 cells overexpressing the KGF receptor aFGF and hst, the mitogenic activities of both naturally occurring and rKGF were significantly inhibited by heparin. When the concentration dependence of rKGF mitogenic activity on Balb/MK cells was compared with that of the native protein purified from M426 fibroblasts, we found that the dose required for half-maximal stimulation by rKGF was 1 ng/ml. This was in striking contrast to native KGF, which required a 10-fold higher concentration to achieve a similar level of activity (Fig. 4A). The molecular masses observed for rKGF and native KGF were 21 and 26-28 kDa, respectively (Fig. 4B). The faster mobility of rKGF likely reflects the absence of glycosylation of the bacterially expressed protein. Whether this accounts for its greater specific mitogenic activity remains to be determined.

A hallmark of FGF family members is their ability to bind heparin. For aFGF and hst, it has been reported that heparin can augment their biological activities (14, 15). Thus, we examined the effect of heparin on the mitogenic activity of native and recombinant KGF. Table II shows that, unlike native KGF, recombinant KGF was significantly inhibited by heparin. To determine whether this accounts for its greater specific mitogenic activity, recombinant and native KGF were compared with that of other FGF family members revealed greatest divergence from KGF in their amino-terminal domains. Since amino-terminal proteolytic digestion resulted in rKGF products with mitogenic activity, we sought to study more thoroughly the effects of sequential amino-terminal deletions on KGF biological properties. Mutants were generated by PCR as described under “Materials and Methods.” We took advantage of the presence of Met codons within the KGF open reading frame at positions 35, 40, and 59 and deleted sequences 5' of each of these codons. Two additional mutants were engineered in which the first 70 or 80 residues were deleted and an AUG codon was added to initiate translation. A schematic diagram of each mutant is shown in Fig. 5A.

K10, K11, and K12 retained the ability to bind heparin and eluted at the same salt concentration as the parental molecule (data not shown). Whereas K13 and K14 mutants were expressed at levels similar to wild type rKGF (Fig. 5B), these mutants completely lost the ability to bind heparin. Attempts to purify K13 and K14 using Mono S or CM-Sephadex resins were also unsuccessful. Since K13 and K14 lacked detectable mitogenic activity, they were not characterized further. K10, K11, and K12 mutants were purified to homogeneity by Mono S chromatography (Fig. 5C) and their identities confirmed by immunoblot analysis using rabbit polyclonal antisera directed against a synthetic peptide derived from the KGF carboxy-terminal sequence (Fig. 5D).

We next investigated the abilities of K10, K11, and K12 mutants to displace radiolabeled KGF from its receptor, trigger receptor-kinase activity, and stimulate Balb/MK DNA synthesis. Each of the mutants effectively displaced 125I-KGF bound to NIH/3T3 cells overexpressing the KGF receptor aFGF and hst, the mitogenic activities of both naturally occurring and rKGF were significantly inhibited by heparin. When the concentration dependence of rKGF mitogenic activity on Balb/MK cells was compared with that of the native protein purified from M426 fibroblasts, we found that the dose required for half-maximal stimulation by rKGF was 1 ng/ml. This was in striking contrast to native KGF, which required a 10-fold higher concentration to achieve a similar level of activity (Fig. 4A). The molecular masses observed for rKGF and native KGF were 21 and 26-28 kDa, respectively (Fig. 4B). The faster mobility of rKGF likely reflects the absence of glycosylation of the bacterially expressed protein. Whether this accounts for its greater specific mitogenic activity remains to be determined.

Expression and Characterization of KGF Amino-terminal Mutants—Comparison of the predicted amino acid sequence of KGF with that of other FGF family members revealed greatest divergence from KGF in their amino-terminal domains. Since amino-terminal proteolytic digestion resulted in rKGF products with mitogenic activity, we sought to study more thoroughly the effects of sequential amino-terminal deletions on KGF biological properties. Mutants were generated by PCR as described under “Materials and Methods.” We took advantage of the presence of Met codons within the KGF open reading frame at positions 35, 40, and 59 and deleted sequences 5' of each of these codons. Two additional mutants were engineered in which the first 70 or 80 residues were deleted and an AUG codon was added to initiate translation. A schematic diagram of each mutant is shown in Fig. 5A.

K10, K11, and K12 retained the ability to bind heparin and eluted at the same salt concentration as the parental molecule (data not shown). Whereas K13 and K14 mutants were expressed at levels similar to wild type rKGF (Fig. 5B), these mutants completely lost the ability to bind heparin. Attempts to purify K13 and K14 using Mono S or CM-Sephadex resins were also unsuccessful. Since K13 and K14 lacked detectable mitogenic activity, they were not characterized further. K10, K11, and K12 mutants were purified to homogeneity by Mono S chromatography (Fig. 5C) and their identities confirmed by immunoblot analysis using rabbit polyclonal antisera directed against a synthetic peptide derived from the KGF carboxy-terminal sequence (Fig. 5D).

We next investigated the abilities of K10, K11, and K12 mutants to displace radiolabeled KGF from its receptor, trigger receptor-kinase activity, and stimulate Balb/MK DNA synthesis. Each of the mutants effectively displaced 125I-KGF bound to NIH/3T3 cells overexpressing the KGF receptor aFGF and hst, the mitogenic activities of both naturally occurring and rKGF were significantly inhibited by heparin. When the concentration dependence of rKGF mitogenic activity on Balb/MK cells was compared with that of the native protein purified from M426 fibroblasts, we found that the dose required for half-maximal stimulation by rKGF was 1 ng/ml. This was in striking contrast to native KGF, which required a 10-fold higher concentration to achieve a similar level of activity (Fig. 4A). The molecular masses observed for rKGF and native KGF were 21 and 26-28 kDa, respectively (Fig. 4B). The faster mobility of rKGF likely reflects the absence of glycosylation of the bacterially expressed protein. Whether this accounts for its greater specific mitogenic activity remains to be determined.
we incubated equal amounts of KGF or K12 at 37 °C in Dulbecco's modified Eagle's medium in the presence or absence of Balb/MK cells for 48 h. The medium was then removed, clarified by centrifugation, and tested for mitogenic activity. We observed no evidence that K12 was relatively more labile by this type of analysis.

**DISCUSSION**

We demonstrate that recombinant expression of KGF in bacteria results in efficient production of biologically active growth factor. We estimate that in the inducible system utilized, rKGF represented as much as 1-2% of bacterial proteins. By means of heparin-Sepharose chromatography, it was possible to achieve a preparation that was approximately 90% pure. Subsequent Mono S cation exchange chromatography led to an essentially homogeneous rKGF preparation, making this system practical for producing large quantities of pure recombinant growth factor.

Recombinant KGF was substantially more active as a mitogen than the native molecule. Since both proteins were purified to homogeneity, the 10-fold greater specific activity of rKGF likely reflects differences in intrinsic properties. Consistent with the presence of a potential Asn-linked glycosylation site in its predicted amino acid sequence (1), KGF mitogenic activity appears to be cell type-dependent. Whether the absence of glycosylation is responsible for the greater specific mitogenic activity of rKGF remains to be elucidated.

The mitogenic activities of both rKGF and native KGF were found to be strongly inhibited by heparin. Yet, heparin has been shown to protect aFGF and bFGF from heat inactivation and proteolytic digestion (for review see Ref. 14) and to stabilize K-GFG/HST/FGF-4 (15). Recently, it was reported that interaction with heparin or heparin sulfate type proteoglycans is essential for bFGF binding to its high affinity receptor as well (16, 17). However, the effect of heparin on bFGF mitogenic activity appears to be cell type-dependent. Shipley et al. (18) showed that heparin concentrations as high as 10 μg/ml had no effect on bFGF mitogenic activity for human fibroblasts but markedly reduced its activity on human...**

\[^2\] D. Ron, unpublished data.
keratinocytes (18). Heparin inhibition of bFGF mitogenic activity has been observed with Balb/MK cells as well (19). Thus, it is possible that the inhibitory effect of heparin on KGF mitogenic activity also may be cell type-dependent.

By analysis of a series of genetically engineered or naturally occurring rKGF amino-terminal truncation mutants, it was possible to define the extent to which such residues contribute to KGF heparin binding properties and mitogenic activity. Deletion of as many as 28 residues from the amino terminus of the mature molecule did not affect heparin binding properties, but deletion of an additional 10 amino acids resulted in complete loss of heparin binding. This domain contains 3 positively charged amino acids, which might contribute directly to heparin binding. Efforts to localize heparin binding sites in aFGF and bFGF have led to evidence that their carboxyl-terminal halves are involved (20-24). Since these molecules share significant similarity to KGF, it is possible that the amino-terminal deletion resulting in loss of KGF heparin binding affects the tertiary structure in such a way as to affect heparin binding sites indirectly in the carboxyl terminus of the molecule. Thus, more subtle mutagenesis will be required to localize KGF heparin binding domains precisely.

Mitogenic activity was retained by K10 and K11 mutants as well as by amino-terminal proteolytic digestion products of rKGF, whose mobilities suggest loss of the first 18-19 amino-terminal residues. The K12 mutant, which lacked 28 residues, retained the capacity for high affinity receptor interaction but showed substantially decreased ability to activate the receptor kinase and stimulate cell DNA synthesis. A previously reported aFGF mutant with a Gly substitution for Lys182 (22) has been reported to exhibit substantially reduced mitogenic activity but no detectable loss of ability to stimulate early events including tyrosine phosphorylation of p90 and phospholipase Cγ as well as the transcriptional activation of fos and myc genes (22, 25). Thus, both mutants imply that high affinity receptor binding is not sufficient to initiate the cascade of biochemical events required for a mitogenic response.

Accumulating evidence indicates that a number of growth factors activate their receptors by inducing receptor dimer formation (for review see Ref. 26). This appears to be the case for FGF receptors as well (30). The most well studied ligand receptor interactions involve growth hormone and its receptor, in which crystallography of the ligand-receptor complex has revealed that two separate sites in the growth hormone molecule bind to identical sites on two receptors (27). Recently, a potent competitive antagonist of growth hormone action has been generated by mutagenesis which enhances site 1 and diminishes site 2 binding. By this approach, the antagonist competes effectively for receptor binding and blocks dimer formation required for receptor activation (28).

It will be of interest to determine whether the K12 mutant acts as a partial agonist through a similar mechanism. The ability to generate large quantities of rKGF should make it possible to explore KGF structure/function relationships further as well as to investigate possible applications of this growth factor to clinical conditions requiring enhanced epithelial cell renewal.

Acknowledgments—We thank W. Taylor and D. Hirschfield for technical assistance, J. Thompson for photography, W. G. Burgess for amino acid analysis, and N. Lichtenberg for secretarial assistance.

REFERENCES