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EFFECT OF CYSTEINE SUBSTITUTIONS ON THE MITOGENIC ACTIVITY AND STABILITY OF RECOMBINANT HUMAN KERATINOCYTE GROWTH FACTOR

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Human Keratinocyte growth factor (hKGF), a member of the FGF family of growth factors, contains five cysteines at amino acid positions 1, 15, 40, 102, and 106. We expressed five cysteine mutants of hKGF in which the cysteines were cumulatively replaced with alanine or serine, starting with cysteine-1. Recombinant hKGF has an inherently higher mitogenic activity and stability to heat and acid than reported for glycosylated hKGF. Mitogenic activity is increased an additional 2.6 fold by substitution of cysteine-1 with alanine. Mutants with the conserved cysteine substituted at position 40 were more susceptible to heat inactivation than rhKGF, but showed no significant difference in acid inactivation. Cysteine-free rhKGF is mitogenic,demonstrating that neither cysteines nor disulfide bonds are required for mitogenic activity. However, cysteine-free rhKGF does not bind Heparin-Sepharose and is unstable to heat and acid compared to rhKGF, suggesting that the cysteines have a role in maintaining KGF's structure. This information will useful in the development of a more stable and more potent wound healing agent from hKGF.

Keratinocyte growth factor (KGF or FGF-7) is a recent member of the FGF-family of growth factors (1,2), It is a potent mitogen of epithelial cells, but lacks mitogenic activity toward fibroblast and endothelial cells. This unique target cell specificity distinguishes it from the other FGF family members, and suggests a potential use as a wound healing agent (18). Human KGF is a 163 amino acid protein that contains five cysteines at amino acid positions 1, 15, 40, 102 and 106 (2). The cysteines at positions 40 and 106 are conserved in all members of the FGF family

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The abbreviations used are: KGF, Keratinocyte growth factor; hKGF, human KGF; rhKGF, recombinant hKGF; FGF, Fibroblast growth factor ; aFGF, acidic FGF; bFGF, basic FGF; SA1, rhKGF [Cys-1 Ala]; SA2, rhKGF [Cys-1 to Ala, Cys-15 to Ser]; SA3, rhKGF [Cys-1 to Ala, Cys-15 to Ser, Cys-40 to Ser]; SA4, rhKGF [Cys-1 to Ala, Cys-15 to Ser, Cys-40 to Ser, Cys-102 to Ser]; SA5, rhKGF [Cys-1 to Ala, Cys-15 to Ser, Cys-40 to Ser, Cys-102 to Ser, Cys-106 to Ser]; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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(2). Although the cysteines in aFGF and bFGF are not involved in disulfides nor are they required for mitogenic activity (3,4,5,6,7,8), substitutions of cysteine in aFGF and bFGF do have effects on activity. Substitution of the two non-conserved cysteines of bFGF has been shown to reduce disulfide formation and to increase its acid stability (5), and substitution of cysteines in aFGF was also shown to affect mitogenic activity(4,6). To study the role of cysteines in hKGF, we constructed a synthetic hKGF gene with unique restriction sites. These restriction sites were used to construct cysteine mutants of hKGF, which were expressed at mg/liter[1] in *E.coli* using a T7 promoter (15). In this study, the effects of cysteine substitutions on mitogenic activity and on heat and acid stability were determined.

MATERIALS AND METHODS

DNA Methods. Restriction enzymes (New England Biolabs or Gibco/BRL) and T4 DNA ligase (Gibco/BRL) were used in the buffers provided. LB and M9 media (Gibco/BRL) contained 200ug/ml Ampicillin (Sigma). M9 media was supplemented with 20ml of 20% glucose and 1ml of 1M $MgSO_4$ per liter. Phosphorylation of oligos with T4 Polynucleotide Kinase (Pharmacia) was performed at 37°C for 1 hour in the provided reactions buffer containing 750 uM ATP and then stopped by heating at 80°C for 5'. Cloning and ligations were performed using standard protocols from Maniatis(10). Transformations of competent cells were performed according to the manufacturer's protocols for DH5α (Gibco/BRL) and BL21(DE3) (Novagen) *E.coli* cells. *E.coli* BL21(DE3) is a phage lysogen containing T7 RNA polymerase under the control of the lac UV5 promoter-operator sequence and is inducible with IPTG. The oligodeoxyribonucleotides were chemically synthesized using a Milligen Biosearch Cyclone Plus DNA Synthesizer and were purified and detritylated on NENSORB columns (Dupont).

Construction of rhKGF Mutants. Mutants were made by replacing sections of the synthetic hKGF gene in pET-11a, pSynKGF (Genbank U01670), or mutants thereof with chemically or PCR synthesized DNA fragments. Correct constructs were confirmed by DNA sequencing (11). Sense and antisense strands were synthesized for SA1, TATGGCTAACGATA-TGACTCCGGAACAGATGGCTACTAACGTTAACTGC and TCGAGCAGTTAACGTTAG-TAGCCATCTGTTCCGGAGTCATATCGTTAGCCA, and for SA2, TATGGCTAACGATAT-GACTCCGGAACAGATGGCTACTAACGTTAACAGC and TCGAGCTGTTAACGTTAGT-AGCCATCTGTTCCGGAGTCATATCGTTAGCCA. The oligonucleotides were annealed by heating at 80°C for 1 minute and allowed to cool to room temperature over 15 minutes. The double stranded fragment contained Nde1 and Xho1 compatible ends and were ligated in pSynKGF in which the Nde1/Xho1 fragment had been removed. The sense and antisense DNA strands were synthesized for SA3, GTACGTCGTCTGTTCTCTCGTACTCAGTGGTATC-TGCGTATCGACAAACGTGGTAAAGTTAAAGGTAC and CTTTAACTTTACCACG-TTTGTCGATACGCAGATACCACTGAGTACGAGAGAACAGACGAC, were annealed and ligated into SA2 in which the Spl1/Kpn1 fragment had been removed. To create SA4, the Kpn1-Sac1 fragment of SA3 was replaced with a 165 bp fragment constructed from four over-lapping oligonucleotides designated A-D. The oligos were **A**, CCAGGAAATGAAAAACAACTAC-AACATCATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCTATCAAAA; **B**, CAAC-ACCTTTGATAGCAACGATACCAACAGCAACAGTACGGATTTC-

[1] Manuscript in preparation.

CATGATGTTGTAGTTGTTTTTCATTTCCTGGGTAC; **C**, GGTGTTGAATCTGAATTCTA-CCTGGCTATGAACAAAGAAGGTAAACTGTACGCTAAAAAAGAATCTAACGAAGA-CTGCAACTTCAAAGAGCT; **D**, CTTTGAAGTTGCAGTCTTCGTTAGATTCTTTTTTAG-CGTACAGTTTACCTTCTTTGTTCATAGCCAGGTAGAATTCAGATT. Oligos B and C were phosphorylated at the 5' end with T4 polynucleotide kinase. Oligonucleotides A and B, and C and D were annealed as previously described and ligated together using T4 DNA Ligase overnight at 16°C. This 165 base-pair fragment contained Kpn1 and Sac1 compatible ends and was ligated into SA3 plasmid in which the Kpn1 and Sac1 fragment had been removed. The oligonucleotide required to construct SA5 was PCR amplified from 1ug of the 165 bp Kpn1/Sac1 fragment of SA4 using 1 ug each of primers **A**, GTTAAAGGTACCCAGGAAATGAAAAAC and **B**, CAGGATGAGCTCTTTGAAGTTAGAGTCTTCG. The reaction was performed in the buffer provided and contained 300 uM dNTP, 1 U of AmpliTaq (Perkin-Elmer) in a 100ul reaction. All PCR was initiated by a 5 minute incubation at 95°C followed by 35 cycles of 1.5 minutes at 94°C, 2 minutes at 30°C, and 3 minutes at 72°C. The amplified product was electrophoresed on a 1.5% agarose gel and electroeluted. The fragment was digested with Kpn1 and Sac1, and then ligated into the SA4 plasmid in which the Kpn1 and Sac1 fragment had been removed.

Inductions and Purifications of KGF Mutants SA1 through SA4. pET-11a SynKGF mutants were transformed into BL21(DE3) cells and single colonies was used to inoculate a 500 ml culture of M9-Amp. The cells were grown at 37°C until they reached an optical density between 0.8 and 1.0 at 600nm. Aliquots were removed for an induction test (15) and the remaining culture induced by adding IPTG to 1mM. The inductions were done at 28°C for 3.5 hours. Cell extract were prepare as describe by (9) and the hKGF mutant were purified basicaaly as decribed by Ron et al. (17). The supernatant was loaded onto a 5ml Heparin-Sepharose column equilibrated in 20mM Tris-HCl pH 7.5 and 0.2 M NaCl. The KGF protein was eluted by stepping to 0.3 M NaCl and running a gradient from 0.3 M NaCl to 1M NaCl in 30 ml, diluted with 15 ml of water and loaded onto a 1ml MonoS column equilibrated in 50mM NaH_2PO_4 pH 6.8 and 0.1M NaCl. The KGF protein was eluted using a 48ml linear gradient from 0.1M to 1M NaCl. The KGF protein eluted at 0.5M NaCl. The purified samples were concentrated 6 to 10 fold using a Centricons-10. N-terminal protein sequencing and amino acid analysis were kindly performed by Dr. Roy Harris at Delta Biotechnology, Nottingham, UK. The KGF mutants had molecular weights of 21.5 kDa, and were greater than 95% pure on 15% SDS gels.

Purification of SA5 KGF. SA5 is expressed in amounts similar to the other SynKGF mutant; however, less than 10% of it was expressed as soluble protein, and neither soluble nor resolublized inclusion bodies bound to Heparin-Sepharose. SA5 inclusion bodies were prepared and solubilized as described by Lin and Chang (12). 50 mg of 30% pure rhKGF SA5 as determined by SDS gel electrophoresis was obtained from 1 liter of culture.

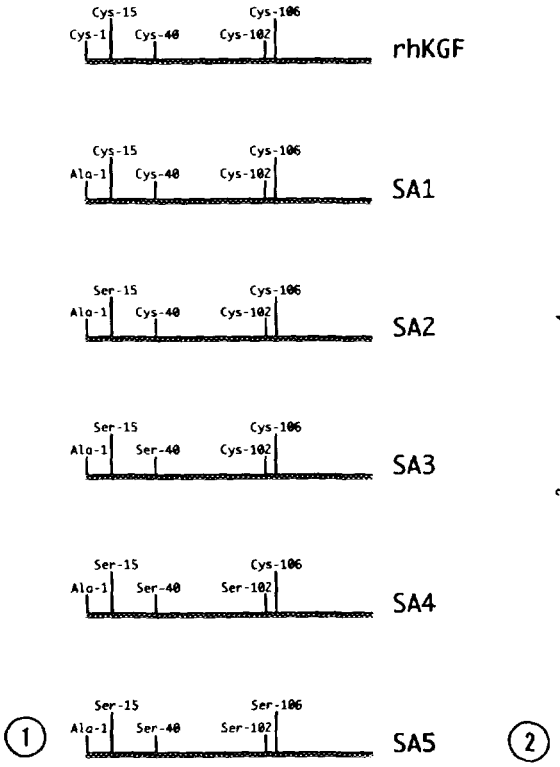
Cell Lines and Culture Conditions. BALB/MK, mouse keratinocytes (13) were cultured in EMEM (low Ca^{++} media, Biofluids) containing 10% dialyzed fetal bovine serum and 10 ng EGF. Cells were cultured at 37°C in 7% CO_2. The BALB/MK was a gift of Dr. Stuart Aaronson.

[^{3}H]Thymidine Assay. Mitogenic activity of the various growth factors was assayed using the method described by Falco et al. (14). Acid stability was determined by diluting the purified KGF constructs to 1µg/µl in 0.5 M acetic acid, and incubating for the desired length of time. The samples were neutralized and diluted to 500ng/ml in 10 mM Hepes pH 7.5 and 0.1% BSA. Heat stability assays were done by diluting the purified KGF constructs to 500ug/ml in 10 mM Hepes pH 7.5, and incubating the samples at 60°C for an appropriate length of time. The samples were immediately diluted with ice cold buffer containing 10 mM Hepes pH 7.5 and 0.1% BSA . For both assays, appropriate aliquots were added to the media of the mitogenic assay. All assays were done in triplicate; results are the average of three or more experiments.

RESULTS AND DISCUSSION

Using the unique restriction sites in a synthetic hKGF gene (SynKGF) to specifically mutate the cysteines in rhKGF, we systematically substituted the cysteines starting at the N-terminus until all five cysteines were replaced creating mutants, SA1 to SA5, where the number indicates the number of cysteines substituted (Figure 1). In all the mutants, the amino acid replacing the cysteine at position-1 was alanine whereas serine replaced cysteine in all subsequent substitutions. In the recombinant gene, a methionine was added to the N-terminus as the initiating amino acid, but was shown by N-terminal sequencing to be removed in SA1-4 (Data not shown).

Mitogenic activity of rhKGF and rhKGF cysteine mutants to Balb/MK. Substitution of the cysteines in rhKGF changes the protein's mitogenic activity. The bar graph in figure 2 compares the activities of rhKGF and the rhKGF cysteine mutants SA1 - SA5. The proteins were assayed at 2 ng/ml; error bars are shown on the graph. All the cysteine mutants of rhKGF except SA5 show a substantial increase in mitogenic activity over the rhKGF. Substitution of the N-



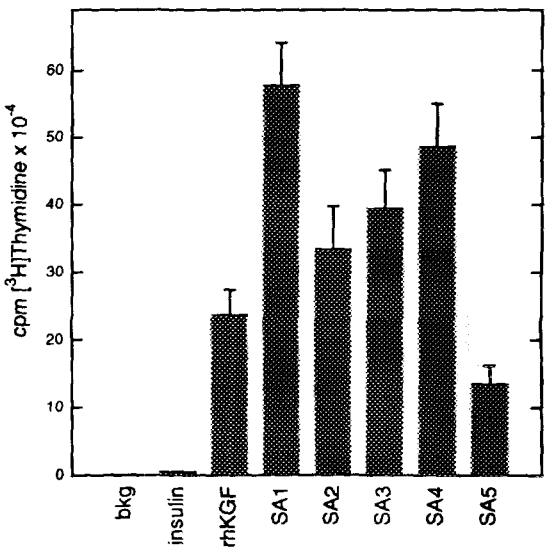


Figure 1. Diagrams of rhKGF Cysteine Mutants.
The location of the 5 cysteine residues at amino acids positions 1, 15, 40, 102, and 106 in rhKGF and the amino acid, alanine or serine, substituted are illustrated here.

Figure 2. Mitogenic Activity of rhKGF and the Cysteine Mutants of rhKGF at 2nM. The protein concentrations were determined spectrophotometrically and estimated by densitometry of SDS gels to be greater than 95% pure (except SA5, see METHODS).

terminal cysteine of rhKGF with alanine, SA1, produced the greatest increase in mitogenic activity, 2.6-fold. The additional substitution of the second cysteine at position 15 with serine in SA2 caused a 1.5-fold increase in the mitogenic activity compared to rhKGF, but a 40% decrease in activity compared to SA1. If the effects of the substitutions are assumed to be additive, substitution of Cys-15 may have negative effects on mitogenic activity of the protein. Further substitution of the third and fourth cysteine with serine in SA3 and SA4 caused stepwise increases in mitogenic activity compared to SA2, but the activity was still less than that found for SA1. SA3 and SA4 showed mitogenic activity which was 1.8- and 2.2-fold higher than rhKGF, respectively. Because SA5 did not bind Heparin-Sepharose, its concentration was estimated by densitometry of the SA5 preparation on an SDS-PAGE gel in which the SA5 band was compared to a known concentration of rhKGF protein. Even though the relative activity of SA5 to the other mutant could not be quantitatively assessed due to its impurity, the SA5 mutant has significant amounts of mitogenic activity. Thus, as has been shown for aFGF and bFGF, cysteines are not required for mitogenic activity of rhKGF.

Relative acid stability of rhKGF and the cysteine-substituted mutants. Human KGF mitogenic activity to BALB/MK was reported to be reduced by 86% after a 10' exposure to 0.5 M acetic acid (1). However, as shown in Figure 3A, rhKGF is inherently more stable, losing

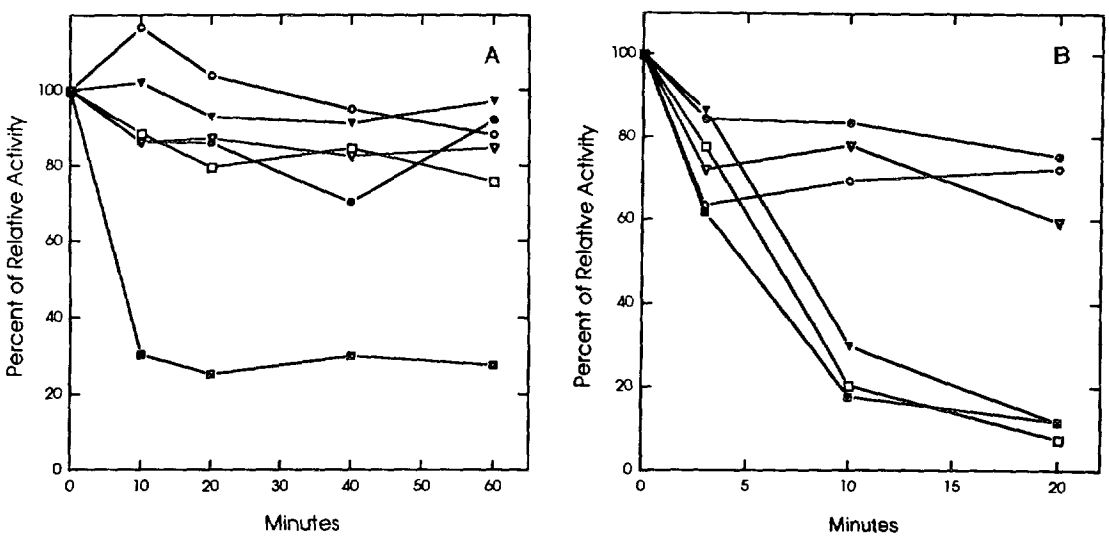


Figure 3. Stability of rhKGF and Cysteine Mutants of rhKGF.
Panel A. Stability in 0.5M acetic acid. Samples were diluted with 0.5M acetic acid for a specific time, neutralized, and assayed for mitogenic stimulation of BALB/MK cells.
Panel B. Stability at 60°C. Sample were heated to 60°C for a specified time, chilled on ice, and assayed for mitogenic stimulation of BALB/MK cells. The 100% relative activity was determined as the activity at time zero. The samples assayed were rhKGF, ●; SA1, ○; SA2, ▽; SA3, ▼; SA4, □; SA5, ■.

only 5 percent of its mitogenic activity when exposed to this acidic condition. The cysteine-substituted mutants SA1 through SA4 showed a 10 to 20% drop in mitogenic activity after a 60' exposure to this acidic condition. However, the 30% pure SA5 mutant showed significant inactivation by this acid treatment, dropping to 30% of its total activity in 10' and to 10% after 60' of the 0.5 M acetic acid treatment. The marked decrease in acid stability of SA5 compared to SA4 suggests that Cys-106 may play a role in rhKGF's stability. Alternatively, it could indicate that the partially pure SA5 preparation is contaminated with acid proteases. However, comparisons of SA5 on SDS PAGE before and after acid treatment were identical.

Relative heat stability of rhKGF and the cysteine-substituted mutants. The stability of rhKGF at 60°C is shown in figure 3B. Human KGF was reported to lose 68% of its mitogenic activity after a 10' incubation at 60°C (1). In comparison, rhKGF lost about 30% of its mitogenic activity. The cysteine mutants could be separated into two groups. Substitution of the first two cysteines of rhKGF produced mutants with similar or slightly greater stability at 60°C than rhKGF. Substituting the Cys-1 of rhKGF with alanine in SA1 produced the most stable mutant, which lost only 16% of its activity after 10' at 60°C. SA2 had stability similar to rhKGF, losing 24% of its mitogenic activity after 10' and 40% after 20' at 60°C. Additional cysteine-to-serine substitution in SA3, SA4 and SA5 caused a substantial decrease in stability at 60°C. SA3, SA4, and SA5 lost 70, 80, and 82% of their mitogenic activity respectively after 10' at 60°C, and 89, 92, and 90%, respectively after 20' at 60°C. Those mutants in which the conserved cysteines at position 40 were replaced have greatly reduced heat stability compared to rhKGF indicating that the conserved cysteine, Cys-40, is important for the stability of the hKGF protein.

Together, the acid and heat stability of the cysteine mutants suggest that the conserved cysteines play important roles in hKGF's stability. However, since the rapid inactivation of hKGF mutants occur at high temperture when Cys-40 is substituted and in acid when Cys-106 is substituted, their mechanisms of inactivation are different.

We were surprised at the increased stability of rhKGF to acid and heat, even though it is identical in amino acid sequence to hKGF. Based on units of activity, our rhKGF construct has about 2 times higher specific activity than reported for wild type hKGF by Rubin, et al. (1). A notable difference between hKGF and our rhKGF is the proposed carbohydrate modification on hKGF from fibroblast culture media (1,16,17). If glycosylation destabilizes or reduces the inherent mitogenic activity of hKGF, then unglycosylated hKGF should have greater mitogenic activity. Ron, et al. (17) reported a 10 fold higher activity for rhKGF over wild type hKGF compared to the two fold that we observed. The difference in activity is most likely to due to an

extended N-terminus (Met-Ala), which is absent in our rhKGF and the wild type hKGF. We observe an additional 2.6 fold increase in mitogenic activity when the N-terminal cysteine is substituted with alanine in SA1. This mutant, lacking both glycosylation and an N-terminal cysteine, shows about a 6 fold increase in mitogenic activity over glycosylated hKGF, which is similar to the increase observed by Ron, et.al.(17).

Heparin affinity is a characteristic of the FGF growth factor family. Salt concentrations greater than 1.2M are required to displace aFGF or bFGF from Heparin-Sepharose whereas hKGF, with its much lower affinity, is displaced by 0.6M NaCl (1). Substitution of the first four cysteines in hKGF had little effect on hKGF's affinity for heparin since they elute from Heparin-Sepharose at the same salt concentration. However, the additional substitution in SA5 of the conserved cysteine at position 106 caused the complete loss of heparin affinity. The loss of heparin affinity was not due to gross structural changes in SA5, since resolubilized SA5 was still mitogenic. It has been observed that mutations of the conserved cysteines of bFGF caused a small shift in the affinity of bFGF for heparin (5), and that mutation of the cysteines in aFGF affected heparin's stimulation in mitogenic activity (4). This suggests that mutation of Cys-106 in rhKGF by itself or in combination with other cysteine mutations may cause slight structural changes that interfere with the already-weak interactions between rhKGF and heparin, but does not significantly affect mitogenic activity.

These data demonstrate that the cysteines of rhKGFare important for the protein's stability and mitogenic activity. Reconbinant hKGF's mitogenic activity is stimulated 2.6 fold when the N-terminal cysteine is substituted with alanine, but further substitution of cysteines in addition to Cys-1 show less stimulation. Mutants in which Cys-40 was substituted reduced rhKGF's stability to heat treatment, and the substitution of Cys-106 in SA5 decreased acid stability and caused a structural change that resulted in loss of heparin affinity. Further studies to determine the structural changes caused by cysteine mutations will clarify the role of cysteines in protein stability and mitogenicity of hKGF. A better understanding of hKGF's structure-activity relationship may aid in the development of a wound healing agent with improved pharmaceutical characteristics.

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