Conversion of Cysteine to Serine Residues Alters the Activity, Stability, and Heparin Dependence of Acidic Fibroblast Growth Factor*

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Acidic fibroblast growth factor (aFGF) is a broad spectrum mitogen that is stabilized by complexation with heparin and heparan proteoglycans. The monoclonal human protein contains 3 reduced cysteine residues of unknown function, the first 2 of which are conserved among all seven known fibroblast growth factors. The influence of these free sulfhydryl groups on the level, stability, and heparin dependence of the mitogenic activity at physiological temperature and pH is characterized using a complete set of site-directed mutants in which either any 1, 2, or all 3 of the cysteine residues are converted to serines. Mutants of aFGF in which either any 2 or all 3 cysteine residues are substituted by serines are more active, have longer activity half-lives, and are less heparin dependent than wild-type aFGF. In contrast, wild-type aFGF and the three mutants that each retain 2 cysteine residues inactivate more rapidly in the absence of heparin by a nonproteolytic mechanism but are markedly stabilized by heparin. This cysteine-mediated destabilization of aFGF not only diminishes its activity in the absence of heparin in tissue culture but also could functionally restrict its activity in vivo to the vicinity of mast cell-derived heparins and heparan proteoglycans associated with cell surfaces and basement membranes.

The abbreviations used are: FGF(s), fibroblast growth factor(s); aFGF, acidic FGF; WT, wild-type; kb, kilobase(s); SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.

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EXPERIMENTAL PROCEDURES

Construction of the Mutants—Human aFGF contains 3 cysteine residues at amino acid positions 16, 83, and 117 in the 140-amino acid numbering system (24). The site-directed mutants S16, S83, and S117 that code for serine instead of cysteine in positions 16, 83, and 117, respectively, were generated previously from a gene encoding the human aFGF that in turn was derived by mutagenesis of a synthetic bovine gene (23).

Site-directed mutants of human aFGF with 2 of 3 cysteine residues converted to serines were generated by combining restriction fragments of wild-type (WT) and the S16, S83, and S117 mutant synthetic genes, cloned in a pKK-2.7 plasmid and subcloned in M13mp19 (Bethesda Research Laboratories). The S16S83 and S16S117 recombinants were constructed by first ligating a 0.24-kb EcoRI-BamHI restriction fragment of M13mp19-S16 coding Ser16, followed by ligation of the 0.20-kb BamHI-SalI restriction fragments from either M13mp19-S83 or M13mp19-S117, into the EcoRI-SalI sites of a modified pKK-2.7 vector in which a second BamHI site upstream of the tac promoter was eliminated. The S16S83S117 mutant was generated by ligating the gel-purified larger 3.0-kb SphII-SalI fragment of pKK-K5S83K117 encoding serines at 83 and 117. Transformants of this construct were selected for EcoRI-SalI digestion to select for constructs with the desired orientation of ligated fragments. All aFGF mutant genes were sequenced by the dideoxy method using the Sequenase kit (U. S. Biochemical Corp.).

Expression and Purification—WT and mutant aFGFs were expressed in the DH5 (Bethesda Research Laboratories) and the AB1899 protease-deficient lon strain of Escherichia coli (E. coli Genetic Stock Center, New Haven, CT). Cells containing the pKK recombinants were grown at 30 °C in Luria broth supplemented with
50 μg/ml ampicillin. At 0.1 A600, 1 mM isopropyl β-D-thiogalactopyranoside (Sigma) was added, and on reaching an A600 of 2, the cells were collected at 4°C by centrifugation at 4,000 x g for 25 min. Expression of aFGF mutants was monitored by Western gel blotting whole E. coli lysates after electrophoresis in a reducing SDS-polyacrylamide gel (15%) as described (22, 25).

WT and mutant aFGFs were each purified from 2-4-liter cultures of induced AB1899 lon" cells by sequential chromatography on CM-Sephadex C-50, heparin-Sepharose, and C16 reversed-phase HPLC columns (22, 25). Separate columns were used for WT aFGF and each of the mutants to avoid cross-contamination. Purity was monitored by reducing SDS-polyacrylamide electrophoretic gels (15%) followed by silver staining (26).

Mitogen Assay—All aFGF samples were prediluted to 50 μg/ml in 7 mM trifluoroacetic acid, 33% acetonitrile (v/v) and stored at −20°C. The purified mitogens were assayed in 96-well plates (Costar) for induction of incorporation of [methyl-3H]thyidine into the DNA of confluent Balb/c 3T3 fibroblasts maintained in serum-free media (3:1 ratio of Dulbecco’s modified Eagle’s medium to Ham’s F-12 (GIBCO)) supplemented with insulin-transferrin-selenium (Collaborative Research), histidine, ethanolamine, and linoleic acid as described (22). Assays were done in either the absence or the presence of 50 μg/ml heparin (porcine intestinal mucosa, grade I, Sigma), a control showing potentiation of the mitogenic activity of aFGF optimally for both vascular endothelial (15) and Balb/c 3T3 cells. One mitogenic unit is the amount of aFGF/ml that generates half-maximal stimulation as determined from full dose-response assays.

The specific mitogenic activity is the number of mitogenic units/ml of 50 pg/ml heparin (porcine intestinal mucosa, grade I, Sigma) was C02-buffered to pH 7.4 at 37°C. Mitogen samples were incubated in 7 mM trifluoroacetic acid, 33% acetonitrile (v/v) and stored at −20°C. Mitogen samples were incubated at 0.5 μg/ml, supplemented with 1 mg/ml human serum albumin to prevent nonspecific adsorption, with 0.5 mg/ml heparin, equivalent to the 10-fold concentrates from which the highest concentration point in the dose-response mitogenic assay is diluted. Recovery of activity and full-length aFGF were determined by dose-response assays and electrophoresis in SDS under reducing conditions in 15% polyacrylamide gels (22, 26), respectively. The aFGF gel bands were quantitated by densitometry using an LKB Ultrascan XL laser scanner interfaced to a Nelson Analytical 4400 recording integrator.

RESULTS

Expression and Purification of Wild-type and Mutant Human aFGFs in E. coli—A schematic representation and nomenclature of WT aFGF and all seven permutations of either one, two, or all three Cys → Ser mutations are shown in Fig. 1. WT aFGF and all seven mutants, including the four newly created forms in which either 2 (S16S33, S16S17, S33S17) or all 3 (S16S33S17) cysteine residues were converted to serines, were expressed from the pPK 2.7 vector in an E. coli AB1899 (lon") host, a strain mutationally inactivated in the gene coding for the La protease that degrades intracellular proteins. An anti-aFGF Western blot of lysates of these cells (Fig. 2A) demonstrates that although WT aFGF is present in the highest concentration, all four of the new mutants accumulate to significant levels. The lon" strain was used to express these mutants since all but the S33S17 mutant, the form containing only the first cysteine residue at position 16, were present in much lower amounts when expressed in the La protease-containing DH5α strain (Fig. 2B). The WT and mutant r-aFGFs were purified by sequential chromatography using CM-Sephadex C-50, heparin-Sepharose, and C16 reversed-phase HPLC. The recombinant mutants were highly purified since, as shown previously for WT aFGF (24) and the S16, S33, and S17 mutants (22), only single 16-kDa silver-stained bands were present after reduction and electrophoresis in SDS-polyacrylamide gels (15%) at loads 100-fold above the threshold of detection.

Relative Mitogenic Activities—The specific mitogenic activities of the wild-type and mutant proteins in the presence and absence of heparin were evaluated by full dose-response DNA synthesis assays using Balb/c 3T3 fibroblasts (Fig. 3).

The WT mitogen is 20-fold more active in the presence than in the absence of heparin. As described previously (22), two of the three mutants containing any 2 of the cysteine residues (S16, S17) are as active as WT aFGF in the presence of heparin whereas the third, S33, is somewhat less potent. This mutant is also the least active in the absence of heparin whereas S33 is approximately equally potent, and S17, the mutant containing the 2 evolutionarily conserved cysteine residues, is nearly 3-fold more active than wild-type aFGF.

The mutants retaining either only 1 or no cysteine residues are all more active than WT aFGF. The two most active forms, S16S33 and S33S17, are approximately 2.5-fold more potent than the WT mitogen in the presence of heparin. The activities of the forms in which either all, or any 2, cysteine residues are converted to serines are also less heparin dependent, being potentiated from only 3-5-fold by heparin. In fact, the most active mutant in the absence of heparin, S33S17, is
of Cys elicit a half-maximal response (ED50). The fold stimulation by heparin and without heparin is denoted between the bars).

The time (in h) required for loss of one-half of the mitogenic activity in the presence or absence of heparin were determined as a function of incubation time in serum-free media in the absence (Fig. 4) and the ratio of this inactivation time in the presence to the absence (Fig. 5A). In the absence of heparin, WT aFGF and the three mutant proteins containing any 2 cysteine residues are all more stable than their corresponding mitogenic activities (Fig. 5B). For example, WT aFGF inactivates (t1/2 = 0.26 h) 20 times more rapidly than the disappearance from solution of its corresponding protein band (t1/2 = 5.3 h). These data demonstrate that rapid inactivation can proceed by a nonproteolytic mechanism that is remarkably dependent on the number and location of the cysteine residues.

### DISCUSSION

The intrinsic specific mitogenic activity of aFGF is potentiated 20-fold by heparin in the serum-free assay of confluent Balb/c 3T3 cells. All of the Cys → Ser mutants except S16 are at least as active in both the presence and absence of heparin as WT aFGF. The mutants in which either all, or any 2, cysteine residues are converted to serines are more active and less heparin-dependent than WT aFGF and the three single Cys → Ser mutants. The activities of these double and triple Cys → Ser mutants not only confirm our previous conclusion approximately as active as WT aFGF in the presence of heparin.

**Relative Stabilities**—The relative stabilities of WT and mutant aFGFs in either the presence or absence of heparin were determined as a function of incubation time at 37 °C in Dulbecco’s modified Eagle’s tissue culture medium containing 1 mg/ml bovine serum albumin. The time (in h) required for loss of one-half of the mitogenic activity (Fig. 4) and the ratio of this inactivation time in the presence of heparin are listed.

<table>
<thead>
<tr>
<th>aFGF Form</th>
<th>Specific Activity (units/mg)</th>
<th>WT</th>
<th>S16</th>
<th>S17</th>
<th>S16S17</th>
<th>S16S83</th>
<th>S17S83</th>
<th>S16S17S83</th>
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<tr>
<td>+Heparin</td>
<td>100</td>
<td>26</td>
<td>12</td>
<td>17</td>
<td>240</td>
<td>92</td>
<td>79</td>
<td>73</td>
</tr>
<tr>
<td>−Heparin</td>
<td></td>
<td>0.26</td>
<td>&lt;0.08</td>
<td>0.62</td>
<td>1.4</td>
<td>13</td>
<td>5.6</td>
<td>7.0</td>
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The half-life was not determined because activity is lost in less than 5 min of incubation in the absence of heparin.

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**Fig. 3.** Mitogenic activities of WT and mutant aFGFs with and without heparin. The activities on confluent Balb/c 3T3 cells in serum-free media in the absence (open bars) and presence (solid bars) of 50 μg/ml of heparin were determined using a DNA synthesis mitogenic assay. Specific activity is expressed as units/mg of pure mitogenic assay. Specific activity is plotted as a function of incubation time in serum-free media in the absence of heparin. The time scale in the absence of heparin. The fold stimulation by heparin and without heparin.

**Fig. 4.** Inactivation rates of WT aFGF and all permutations of Cys → Ser mutants. The log of the percent initial mitogenic activity is plotted as a function of incubation time in serum-free medium, pH 7.4, 37 °C in the presence (A) and absence (B) of heparin. The most stable form in the absence of heparin, all but one form inactivated and disappeared at equivalent rates. The single exception was S16, the most rapidly inactivating form, which lost activity approximately 5 times faster than it disappeared from solution (Fig. 5A). In the absence of heparin, WT aFGF and the three mutant proteins containing any 2 cysteine residues are all more stable than their corresponding mitogenic activities (Fig. 5B). For example, WT aFGF inactivates (t1/2 = 0.26 h) 20 times more rapidly than the disappearance from solution of its corresponding protein band (t1/2 = 5.3 h). These data demonstrate that rapid inactivation can proceed by a nonproteolytic mechanism that is remarkably dependent on the number and location of the cysteine residues.

### TABLE I

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>% Initial Activity</th>
<th>% Initial Activity</th>
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<tr>
<td></td>
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<td>0</td>
<td>100</td>
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**Fig. 5.** Inactivation rates compared with protein loss for rapidly inactivating WT and mutant aFGFs. The log of the percent initial mitogenic activity (closed symbols, solid lines) and the corresponding 16-kDa protein band in reducing SDS-polyacrylamide electrophoretic gels (open symbols, dashed lines) are plotted as a function of incubation time in serum-free medium, pH 7.4, 37 °C in the presence (A) and absence (B) of heparin. Only the forms that inactivate faster than they disappear, WT (circles), S17 (squares), S83 (triangles), and S16 (inverted triangles) are shown. Other forms inactivate and disappear with similar kinetics.
that aFGF does not require an intramolecular disulfide bond (22) but also demonstrate that the inability to form such a bond correlates with increased activity, especially in the absence of heparin. Either full (28) or diminished (29) activities have been reported for a form of basic FGF in which all 4 cysteine residues were converted to serines.

The effective activity of aFGF both in vitro and in vivo might be a function not only of its intrinsic activity but also of its stability. In the absence of heparin, WT aFGF is remarkably unstable with a half-life for inactivation of only 15 min. Heparin dramatically extends the half-life 100-fold to over 1 day. The three mutants that retain any one of the three possible pairs of cysteine residues are also very labile in the absence of heparin, with inactivation half-lives ranging from less than 5 min for S16 to 1.4 h for S17. Heparin also significantly increases the activity half-lives of these three mutants by from 27 to 170-fold.

In contrast, all aFGF mutants retaining only 1 or 0 cysteine residues have longer half-lives in the absence of heparin and are stabilized to a lesser degree in its presence. The most stable mutant, which is devoid of all cysteine residues, has a half-life approximately 280 times longer than WT aFGF. The S6S17 mutant, which only contains the cysteine residue at position 16, is the most active, least heparin-dependent (at time 0), and one of the more stable forms. It is also the only double Cys → Ser mutant that is resistant to proteolytic degradation within the DH5 strain of E. coli. In contrast, S16 is the only mutant that appears to be less stable than WT aFGF in the absence of heparin, having little detectable activity at the earliest measurable time. The low activity and inefficient production in E. coli (22) could reflect the instability of this mutant. Therefore a cysteine residue at position 16 appears to contribute to increased activity, heparin independence, and stability of aFGF.

The mechanism of rapid inactivation of WT aFGF and the three double cysteine-containing mutants is not known. Acidic FGF has been shown previously to be protected by heparin from degradation by serum-derived proteases (17, 18). In addition, inactivation of basic FGF has been attributed to heparin-independent binding by α2-macroglobulin, an abundant plasma-derived protease inhibitor and growth factor sequesterant (30). Acidic FGF appears to be trapped similarly since it competes with basic FGF for binding to α2-macroglobulin. Yet despite the absence of both serum-derived proteases and α2-macroglobulin, WT aFGF and these three mutants containing different pairs of cysteine residues inactivate rapidly in the absence of heparin.

This inability is not the result of either digestion by protease contaminant or adsorption to the incubation tube surface as established by high recovery of the inactivated but undegraded protein on reducing SDS-polyacrylamide electrophoretic gels. Although rapid inactivation in the absence of heparin of WT aFGF and the three containing any 2 cysteine residues might be attributable to formation of intramolecular disulfide bonds (22), other mechanisms are feasible including either disulfide cross-linking to bovine serum albumin or formation of mixed disulfides with low mass thiols present in tissue culture medium.

In general, the rank order of activity half-lives of WT and all mutant aFGFs are similar with and without heparin except for S17, the mutant containing only the 2 cysteine residues conserved among not only the four sequenced aFGFs (2, 24, 31, 32) but also all known FGFs (1–8). In the absence of heparin this mutant is among the most rapidly inactivating forms. In contrast, in the presence of heparin its activity is very stable, equaled only by S6S16S17. The conserved cysteine residues at positions 16 and 83, therefore, appear to be correlated with the selective rapid inactivation of mitogenic activity in the absence of heparin while affording a substantially longer half-life in its presence. Therefore, by a combination of partitioning aFGF onto basement membranes and selected cell surfaces and of stabilizing the bound mitogen, heparin proteoglycans would both direct and promote the biological activity of this growth factor in vivo. Moreover, the selective evolutionary retention of these 2 conserved cysteine residues might, in part, be a consequence of the physiological advantage afforded by their compatibility with heparan-mediated stabilization while promoting rapid inactivation of aFGF not bound to these biological targets.

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REFERENCES

Wild-type and Mutant aFGF Activity and Stability