Acidic fibroblast growth factor (aFGF) contains a phosphorylation site recognized by protein kinase C. A non-mitogenic mutant growth factor is devoid of this phosphorylation site. We have changed amino acids in and close to the phosphorylation site and studied the consequences of this for binding of the growth factor to high affinity receptors as well as to heparin. We have also studied the ability of the mutants to stimulate DNA synthesis and cell proliferation as well as phosphorylation of mitogen-activated protein kinase and the ability of the growth factor mutants to be transported to the nucleus. The results indicate that while the mutations strongly affect the ability of the growth factor to bind to heparin, they do not affect much the binding to the specific FGF receptors, activation of mitogen-activated protein kinase or transport of the growth factor to the nucleus. The mutations affect to various extents the ability of the growth factor to stimulate DNA synthesis and to induce cell multiplication. We find that phosphorylation of aFGF is not required for mitogenic activity. The data suggest that altered interaction of the growth factor with a cellular component different from the receptor, possibly a component in the nucleus, is the reason for the different mitogenicity of the different growth factor mutants.

Acidic fibroblast growth factor (aFGF or FGF-1) belongs to large family of growth factors that bind to four closely related receptors, FGFRs 1–4 (1). The receptors contain, in their cytoplasmic part, a split tyrosine kinase domain that is activated upon binding of FGF to the extracellular receptor domain (2). Signaling through this tyrosine kinase with the activation of downstream signaling molecules is an important part in the signal transduction from FGF (3–7). However, there is also evidence that some growth factors of this family, in particular aFGF and basic FGF (bFGF), enter the cytosol and the nucleus and act directly on intracellular targets (8–18).

aFGF contains an exposed loop that is involved in binding to heparin (19). Mutation in this region of lysine 132 to glutamic acid (K132E) reduces heparin affinity and essentially abolishes the ability of the growth factor to stimulate DNA synthesis in cells (15, 20). aFGF(K132E) binds to FGFR with similar affinity as wild-type aFGF (15) and activates the tyrosine kinase of the receptor (15, 20) and stimulates MAP kinase. Furthermore, the K132E mutant stimulates expression of several immediate-early genes (15, 20, 21), and it is as potent as the wild-type aFGF in inducing mesodermal formation in Xenopus animal caps (22). The mutant was found to be transported to the nuclear fraction similarly as the wild-type (15). We recently cloned a novel, intracellular protein, FIBP, which binds the wild-type aFGF, but not aFGF(K132E) (23).

The K132E mutation destroys a consensus phosphorylation site for protein kinase C (PKC) in aFGF, Ser130-Cys-Lys. aFGF was previously shown to be phosphorylated by PKC on serine in vitro, but not by protein kinase A or casein kinase I and only negligibly by casein kinase II (24). We found that wild-type aFGF was phosphorylated in vitro in a cell lysate while aFGF(K132E) was not. In vitro phosphorylation in cell lysate was augmented in the presence of diacylglycerol and phosphatidylserine and inhibited by staurosporine (15). Furthermore, externally added wild-type aFGF, but not aFGF(K132E), was phosphorylated in intact cells (15). Phosphorylation in vivo depended on translocation of aFGF to the cytosol or nucleus, because phosphorylation was not observed when aFGF was incubated with cells transfected with FGFR4 constructs incapable of mediating translocation to these locations. Taken together, these data indicate that after translocation to the cytosol or nucleus aFGF becomes phosphorylated on serine 130 by PKC. In attempts to elucidate the role of the phosphorylation site in the exposed loop, we made several mutations in this region and measured the ability of the mutated growth factors to bind to heparin and FGFR and to stimulate MAP kinase, DNA synthesis, and cell proliferation.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Purification—Constructs for in vitro transcription and translation encoding aFGF with different mutations (Fig. 1A) were generated by polymerase chain reaction-directed mutagenesis with the plasmid paFGF(K132E) (15) as template. Polymerase chain reaction products were cut with Stu I and Eco RI and ligated into paFGF(K132E) between the same sites. Each construct contained a new restriction site for colony screening, and the sequence of each construct was verified by DNA sequencing. Constructs encoding maltose-binding protein (MBP) fusion proteins were generated by inserting the SplI-EcoRI fragment from each of these plasmids between the same sites in pMal-cN-aFGFCAAX, which had been previously constructed by inserting the NcoI-EcoRI fragment of pHBGFC-x1 (13) into the polylinker of pMal-cN (25). In some of the constructs a stop-codon had not been generated by polymerase chain reaction and was instead made by cutting the plasmids with EcoRI, filling in with T4 DNA polymerase and religating the plasmids. This procedure introduced an in-frame stop codon.

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1 The abbreviations used are: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; MAP kinase, mitogen-activated protein kinase; PKC; protein kinase C; MBP, maltose-binding protein; FIBP, aFGF intracellular binding protein; PAGE, polyacrylamide gel electrophoresis.
stop-codon and an AseI restriction site. MBP fusion proteins were affinity-purified on an amylose resin column as described by the manufacturer (New England Biolabs, Beverly, MA).

**Cell Culture and Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS-PAGE)**—Cells were propagated in Dulbecco's modified essential medium supplemented with 7.5% (v/v) serum, 1% (v/v) nonessential amino acids, and antibiotics. After 24 h, cells were washed twice with HEPES medium and subjected to immunoprecipitation with an anti-MAP kinase (ERK1 and ERK2) antibody (Zymed Laboratories Inc.), and the immunoprecipitates were subsequently incubated for 15 min at 37 °C in 25 μl of kinase buffer (20 mM Tris, pH 7.4, 10 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol) with 1 μM of γ-32P-ATP and 20 μg of myelin basic protein. SDS-PAGE sample buffer was added, and the samples were analyzed by SDS-PAGE and autoradiography (28).

**RESULTS**

**Generation of Mutants in the Exposed Loop of aFGF**—Since aFGF(K132E) exhibits strongly reduced ability to stimulate DNA synthesis in cells (15, 20, 22) and is incapable of being phosphorylated in vitro and in vivo (15), we carried out a number of additional mutations in this region of the molecule (Fig. 1A). Serine 130 in the consensus sequence for phosphorylation by PKC was mutated to glutamic acid (S130E) or to alanine (S130A) to mimic phosphorylated and unphosphorylated growth factor, respectively. Similar mutations have been made previously in other proteins to study the role of phosphorylation (29–32). To study if lysine 132 as such is required, we mutated it to alanine (K132A). Since this mutation destroys the PKC phosphorylation site, we also made the double mutant S130E/K132A as a “phosphorylated” control. We further made the conservative mutation of lysine 132 into arginine (K132R). Burgess et al. (22) made the double mutant cysteine 131 into serine and lysine 132 into glutamic acid (C131S/K132E) and reported that it was mitogenic to the same extent as the bovine wild-type aFGF. In this double mutant a new, potential PKC phosphorylation site is created (Ser131-Glu-Arg), and therefore we also made this double mutant to investigate if it would become phosphorylated.

**In Vitro Phosphorylation and Heparin Affinity of the Mutants**—Some of the mutants were expressed as fusion proteins with maltose-binding protein in bacteria and affinity-purified. These fusion proteins were then cleaved with the protease factor Xa, to separate the MBP part from the growth factor part, and analyzed by in vitro kinase assay with purified PKC. As shown in Fig. 1B, lower panel, lane 4, the wild-type growth factor was strongly phosphorylated. The mutants where serine 130 had been mutated to alanine (lane 1) or glutamic acid (lane 2) were not or negligibly phosphorylated, consistent with the notion that serine 130 is the major phosphorylation site in aFGF. The mutant C131S/K132E, where we had introduced a possible PKC site one amino acid away from the normal site, was also not phosphorylated (lane 3). In lanes 5 and 6 we used growth factor expressed as such in bacteria and purified as described previously (14, 15) as controls. The pure, wild-type growth factor was phosphorylated to a similar extent as wild-type growth factor expressed as MBP fusion protein (compare lanes 4 and 6). With pure aFGF(K132E) a weak phosphorylated band was detectable (lane 5) similarly as with the other mutants. Since aFGF(K132E) is not phosphorylated in vitro in a cell lysate or in vivo in intact cells (15), the weak phosphorylation observed in lanes 1–3 and 5 probably represents background phosphorylation in the in vitro assay with pure PKC. In aFGF(K132R) the PKC consensus site is intact, since both lysine and arginine are tolerated at this position, and we found that aFGF(K132R) was indeed substrate for PKC (not demonstrated).

aFGF(K132E) exhibits strongly reduced heparin affinity (20) as compared with wild-type aFGF. To measure heparin affinity of the different aFGF mutants, we bound in vitro translated, [35S]methionine-labeled growth factors to heparin-Sepharose columns, washed, and eluted with stepwise increasing concentra-
Concentrations of NaCl. The results, shown in Fig. 1C, demonstrate that the different mutants varied considerably in their affinity for heparin. The S130E mutant exhibited virtually identical heparin affinity as the wild-type, while the S130A mutant was eluted in a broad peak. This result was reproducible in four different experiments, but the reason for the unusual broadness of the peak is unclear. The mutants where lysine 132 had been changed all exhibited reduced heparin affinity. The most conservative mutation, K132R, reduced the affinity least, while the K132A mutation reduced heparin affinity considerably. We found that aFGF(K132E) eluted at 0.3M NaCl (not demonstrated). It appears that the heparin affinity of aFGF is very sensitive to mutations of lysine 132 and that more drastic mutations have a more pronounced effect than conservative ones.

Ability of Mutant Growth Factors to Bind to Cells—To test the ability of the different mutants to bind to cells, in vitro translated, [35S]methionine-labeled mutant and wild-type growth factors were incubated with calf pulmonary artery endothelial cells at 4 °C in the presence of heparin and in the absence or presence of excess unlabeled wild-type aFGF. The cells were then washed, lysed, and the postnuclear supernatant was analyzed by SDS-PAGE and fluorography. B, NIH 3T3 cells were incubated with [125I]labeled wild-type aFGF (5 ng/ml) for 3 h at 4 °C in medium containing 10 units/ml heparin in the absence or presence of increasing concentrations of aFGF constructs expressed and purified as MBP fusion proteins. The cells were washed three times, lysed in 0.1 M KOH, and the cell-bound radioactivity was measured. The data shown are mean of three experiments ± S.D.
mRNA had been added did not stimulate [3H]thymidine incorporation. aFGF(K132E) could not elicit a full mitogenic response on mutant, which was at least 100-fold less active than wild-type aFGF. For comparison, we also tested the K132E mutant and the S130E mutant was 3-fold less potent than wild-type to stimulate DNA-synthesis. The concentration of unlabeled growth factor was for each mutant estimated from parallel translation reactions containing [35S]methionine, which were analyzed by SDS-PAGE and densitometric scanning. The data in Fig. 3A are corrected for differences in translation efficiency. The different mutants varied strongly in their ability to stimulate DNA synthesis. Wild-type aFGF and the S130E and S130A mutants were equally potent in this matter. The K132A and the S130E/K132A mutants were both approximately 10-fold less potent, while the K132R mutant was 3-fold less potent than wild-type to stimulate DNA-synthesis. For comparison, we also tested the K132E mutant, which was at least 100-fold less active than wild-type aFGF. aFGF(K132E) could not elicit a full mitogenic response within the limits of this assay using in vitro translated growth factors. As control, addition of translation mixture to which no mRNA had been added did not stimulate [3H]thymidine incorporation at all (not demonstrated).

To study the ability of the different mutants to support proliferation, we incubated serum-starved NIH 3T3 cells for 48 h with different concentrations of wild-type and mutant growth factors that were made in vitro and then counted the cells in a cell counter (Fig. 3B). In this assay, one μl/ml of translation mixture gave optimal stimulation of proliferation for all constructs tested, and increasing the concentration to 3 μl/ml did not increase the effect reproducibly. Therefore, only results with the concentration of 1 μl/ml is shown in Fig. 3C. Wild-type aFGF and the S130E mutant stimulated proliferation most effectively, while the S130A and the K132R mutants were somewhat less effective. The K132A and the S130E/K132A mutants gave the weakest stimulation of proliferation. Together, these data indicate that phosphorylation of aFGF is not required for stimulation of DNA synthesis. It appears that lysine 132 is important for the mitogenic effect of aFGF, independent of phosphorylation of serine 130.

Stimulation of MAP Kinase by the Mutant Growth Factors—Upon binding of aFGF to the FGFR, the tyrosine kinase of the receptor is activated and several signal transduction cascades are initiated (33–35). Activation of the Ras/Raf/Mek/MAP kinase cascade has been suggested to be important for stimulation of cell proliferation by FGF (34, 36, 37). Therefore, we measured the ability of the different mutants to stimulate MAP kinase. Serum-starved NIH 3T3 cells were stimulated with different concentrations of in vitro translated wild-type or mutant growth factor for 5, 30, or 60 min, lysed, and MAP kinase (ERK1 and ERK2) was immunoprecipitated. MAP kinase activity in the immunoprecipitates was measured in an in vitro kinase assay with myelin basic protein as substrate.

There was virtually no MAP kinase activity immunoprecipitated from cells treated with a translation mixture to which no mRNA had been added (Fig. 4, lane 1). Ten percent fetal calf serum (lane 2) and the higher concentration used of wild-type aFGF (lane 4) stimulated MAP kinase activity to a similar extent. The aFGF induced MAP kinase activation was completely prevented when the cells were treated with genistein, a tyrosine kinase inhibitor (lanes 15 and 16, upper panel). Importantly, no detectable differences in MAP kinase activity could be measured when the cells were stimulated with the

**Fig. 3. Ability of the different mutants of aFGF to stimulate DNA synthesis and cell proliferation.** A, serum-starved NIH 3T3 cells were stimulated with different amounts of in vitro translated, unlabeled growth factors for 24 h in the presence of heparin (10 units/ml) and [3H]thymidine. The cell-associated, trichloroacetic acid-precipitable radioactivity was then measured. The experiments in A were repeated four to six times with similar results. The data shown are from a representative experiment. B, serum-starved NIH 3T3 cells were treated with 1 μl of in vitro translated, unlabeled growth factors for 48 h in the presence of heparin (10 units/ml), trypsinized, and counted in a cell counter. The data are plotted as percent of the cell number in the culture treated with wild-type growth factor. Data shown are mean (±S.E.) of four experiments with two or three parallels in each.
which we have found recently, is able to inhibit transport of aFGF by the phosphoinositide 3-kinase inhibitor LY294002 (Fig. 5). Transport to the nuclear fraction could be completely inhibited in cells expressing wild-type aFGF were found in the nuclear fraction (Fig. 5). Treatment with heparin and incubated at 37 °C for different periods of time. The cells were washed, treated with Pronase to digest growth factor at the cell surface or bound to the plastic, then the cells were lysed and fractionated into cytosol/membrane and nuclear fractions as described under “Experimental Procedures.” In all cases, the nuclear fraction was collected by centrifugation. After sonication, the trichloroacetic acid-precipitable material in the nuclear fraction was analyzed by SDS-PAGE and autoradiography.

The Ras/Raf/Mek/MAP kinase cascade is considered to be important for mitogenic activity of several growth factors, FGF included (41). Microinjection of an antibody against FRS2, a protein located in a part of bFGF reported to be involved in both heparin binding and high affinity receptor binding (40). Possibly, phosphorylation of aFGF may modulate its binding to FGFR.

In this paper we report the generation and characterization of several mutations in an exposed loop of aFGF, known to be of importance for heparin affinity and mitogenic activity of the growth factor. The mutants varied 10-fold in mitogenic activity, but were equally potent in stimulating MAP kinase activation. A correlation, however, could be observed between mitogenic activity and affinity to heparin.

High affinity receptor binding varied little among the constructs, with the exception of the S130E mutant, which apparently bound with higher affinity. Interestingly, bFGF phosphorylated by protein kinase A on threonine 112 (corresponding to Thr120 according to the numbering used in Ref. 1) was found to be three to eight times more potent at displacing 125I-labeled FGF from the high affinity receptors (24). This threonine is located in a part of bFGF reported to be involved in both heparin binding and high affinity receptor binding (40). Possibly, phosphorylation of aFGF may modulate its binding to FGFR.

**Fig. 4. Ability of aFGF mutants to stimulate MAP kinase activity.** Serum-starved NIH 3T3 cells were stimulated with 1 and 5 µl of in vitro translated, unlabeled growth factors (lanes 3–14), with 5 µl/ml of a translation mixture without added mRNA (lane 1) or with 10% fetal calf serum (lane 2) for 5 min (upper panel), 30 min (middle panel), or 60 min (lower panel). To be able to compare the intensity of the bands after 5 min of stimulation with the intensity of the bands after longer stimulation times, in lanes 15 and 16, middle and lower panels, the cells were treated for 5 min with in vitro translated wild-type aFGF, the samples were processed as described below and then run on the same gel as the other samples in the same panel. In lanes 15 and 16, upper panel, 50 µg/ml genistein was present during 15 min of preincubation and 5 min of stimulation. In all cases heparin (5 units/ml) was present. The cells were lysed, and the nuclear supernatant was subjected to immunoprecipitation with anti-ERK1 and anti-ERK2 antibodies and kinase activity in the immunoprecipitates was measured with myelin basic protein as substrate for 15 min at 37 °C in kinase buffer containing 132P[ATP. SDS-PAGE sample buffer was added, and the samples were analyzed by SDS-PAGE and autoradiography.

**Fig. 5. Transport of the different mutants to the nuclear fraction.** A, serum-starved NIH 3T3 cells were incubated at 37 °C for 9 h in the presence of 10 units/ml heparin and 2 µl/ml [35S]methionine-labeled growth factors, washed, and treated with 5 mg/ml Pronase at 37 °C for 5 min. The cells were washed in the presence of protease inhibitors, lysed, and the nuclear fraction was collected by centrifugation. After sonication, the trichloroacetic acid-precipitable material in the nuclear fraction was analyzed by SDS-PAGE and autoradiography. B, serum-starved NIH 3T3 cells were treated with wild-type aFGF as in A for 10 h or 28 h. In lanes 1 and 2, 50 µM LY294002 was present. The cells were further processed and analyzed as in A.

**DISCUSSION**

In this paper we report the generation and characterization of several mutations in an exposed loop of aFGF, known to be of importance for heparin affinity and mitogenic activity of the growth factor. The mutants varied 10-fold in mitogenic activity, but were equally potent in stimulating MAP kinase activity. A correlation, however, could be observed between mitogenic activity and affinity to heparin.

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The Ras/Raf/Mek/MAP kinase cascade is considered to be important for mitogenic activity of several growth factors, FGF included (41). Microinjection of an antibody against FRS2, a tyrosine-phosphorylated protein that links FGFR activation to activation of the Ras/Raf/Mek/MAP kinase cascade, inhibited stimulation of DNA synthesis by aFGF (34). A cell-permeable peptide derived from the Grb2-binding sequence of epidermal

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4. O. Klingenberg, A. Wiedlocha, and S. Olness, submitted for publication.
growth factor receptor was shown to inhibit MAP kinase activation in cells stimulated with epidermal growth factor or with platelet-derived growth factor, but not in cells stimulated with bFGF. The peptide also inhibited the mitogenic response of the cells to epidermal growth factor and to platelet derived growth factor, but not to bFGF (42). However, even though the different aFGF mutants described in the present paper differed 10-fold in mitogenic activity, they were equally potent in stimulation of MAP kinase. We have also found that the K132E mutant, which exhibits at least a 100-fold lower mitogenic activity than wild-type aFGF, shows a similar potency as the wild-type to induce MAP kinase activation. It therefore appears that activation of the Ras/Raf/Mek/MAP kinase cascade is not sufficient for stimulation of DNA synthesis by aFGF.

We previously provided data suggesting that the strongly reduced mitogenic activity of the K132E mutant is due to a defect in its intracellular activity (15). We also found that the K132E mutation rendered the growth factor incapable of being phosphorylated in vivo (15). One of the aims of the present study was to investigate whether there could be a cause-effect relationship between these observations. To use phosphorylated wild-type growth factor in experiments that last for 24 h or more, may not be optimal (24). Therefore, we took the strategy of mutating the serine in the phosphorylation site to the negatively charged (parts of) molecules. Activation of the Ras/Raf/Mek/MAP kinase cascade is not proportional reduction in activation of FGFR and its downstream effectors should be expected from this hypothesis. This is not the case. One possible explanation could be that interaction with intracellular targets of aFGF also depends on the ability of aFGF to interact with negatively charged (parts of) molecules. In this context, it is interesting that glypican, a heparan sulfate proteoglycan, and biglycan, a chondroitin sulfate proteoglycan, have been found to contain functional nuclear localization sequences and to exist in the nucleus (46).

In conclusion, phosphorylation of aFGF is not required for its mitogenic activity. Lysine 132 of aFGF is important both for mitogenic effect and for heparin affinity. We cannot detect a correlation between mitogenic activity and ability to activate MAP kinase.

Acknowledgements—The expert work with the cell cultures by Jorunn Jacobsen and the skilful technical assistance of Mette Sværen are gratefully acknowledged.