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Inability of the Acidic Fibroblast Growth Factor Mutant K132E to Stimulate DNA Synthesis after Translocation into Cells*

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Acidic fibroblast growth factor (aFGF) is a potent mitogen. It acts through activation of specific cell surface receptors leading to intracellular tyrosine phosphorylation cascades, but several reports also indicate that aFGF enters cells and that it has an intracellular function as well. The aFGF(K132E) mutant binds to and activates fibroblast growth factor receptors equally strongly as the wild-type, but it is a poor mitogen. We demonstrate that aFGF(K132E) enters NIH 3T3 cells and is transported to the nuclear fraction like wild-type aFGF. A fusion protein of aFGF(K132E) and diphtheria toxin A-fragment (aFGF(K132E)-DT-A) and a similar fusion protein containing wild-type aFGF (aFGF-DT-A) were reconstituted with diphtheria toxin B-fragment. Both fusion proteins were translocated to the cytosol by the diphtheria toxin pathway and subsequently recovered from the nuclear fraction. Whereas translocation of aFGF-DT-A stimulated DNA synthesis in U2OSDR1 cells lacking functional fibroblast growth factor receptors, aFGF(K132E)-DT-A did not. The mutation disrupts a protein kinase C phosphorylation site in the growth factor making it unable to be phosphorylated. The data indicate that a defect in the intracellular action of aFGF(K132E) is the reason for its strongly reduced mitogenicity, possibly due to inability to be phosphorylated.

In cell culture acidic fibroblast growth factor (aFGF or FGF-1)[1] stimulates DNA synthesis, cell migration, and cell differentiation. *In vivo* it is thought to be involved in mesoderm induction, angiogenesis, and in several other processes (1). There is increasing evidence that aFGF as well as basic FGF (bFGF or FGF-2) do not fit into the paradigm that protein growth factors act only through cell surface receptors inducing phosphorylation cascades and other second messengers. Thus, there are several indications that after binding to FGF receptors, aFGF and bFGF enter the nucleus, and that this is required for the mitogenic response, at least in certain cells. Baldin *et al.* (2) obtained evidence for G_1 phase-specific nuclear translocation of externally added bFGF. Imamura *et al.* (3) deleted a putative nuclear localization sequence from aFGF. This abolished mitogenicity, while the addition of a nuclear localization sequence from yeast histone 2B restored this function. Wiedlocha *et al.* (4) found that when aFGF was translocated into the cytosol as a fusion protein with diphtheria toxin (DT) in cells lacking functional FGF receptors, the fusion protein was transported to the nuclear fraction and stimulated DNA synthesis.

Evidence that exogenous aFGF as such is able to enter the cytosol was obtained by fusing a signal for farnesylation, a CAAX-tag, onto the C terminus of aFGF. When aFGF-CAAX was incubated with cells expressing FGF receptors, farnesylated growth factor was obtained (5). Since the enzymes catalyzing farnesylation are only found in the cytosol and possibly in the nucleus, this indicates that aFGF-CAAX had traversed cellular membranes.

Harper and Lobb (6) found that reductive methylation of aFGF reduced its affinity both for heparin and surface FGF receptors as well as its mitogenic activity. They found that Lys-132 (using numbering 1–154 for full-length aFGF, in their paper denoted Lys-118 due to different numbering) was the primary site of modification. Burgess *et al.* (7) mutated Lys-132 to Glu (K132E), and found that the affinity for heparin as well as mitogenicity was reduced, but in this case the high affinity binding to the specific receptors was not reduced. The mutant and wild-type aFGF-stimulated protein tyrosine phosphorylation and induction of proto-oncogenes to the same extent, and aFGF(K132E) was equally efficient as wild-type aFGF in induction of mesoderm formation in *Xenopus* (8). The reason for the strongly reduced mitogenicity of aFGF(K132E) remains unknown.

In the present paper we have studied whether the lack of mitogenicity of aFGF(K132E) is due to impaired entry into cells. First, we have investigated whether exogenous aFGF(K132E) enters the nuclear fraction in a similar manner as wild-type aFGF. We have also introduced the mutant artificially into the cytosol as a fusion protein with DT-A and studied the ability of this fusion protein to stimulate DNA synthesis and proliferation in cells with and without functional FGF receptors. Finally, we have studied phosphorylation of aFGF and aFGF(K132E) *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

Rabbit reticulocyte lysate and rRNasin were from Promega, Madison, WI; T3 RNA-polymerase from Life Technologies, Inc., Gaithersburg, MD; [^{35}S]methionine from NEN Research Products, Wilmington, DE; protein A-Sepharose and heparin-Sepharose from Pharmacia, Up-

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[1] The abbreviations used are: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; DT-A, diphtheria toxin A-fragment; DT-B, diphtheria toxin B-fragment; BrdUrd, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

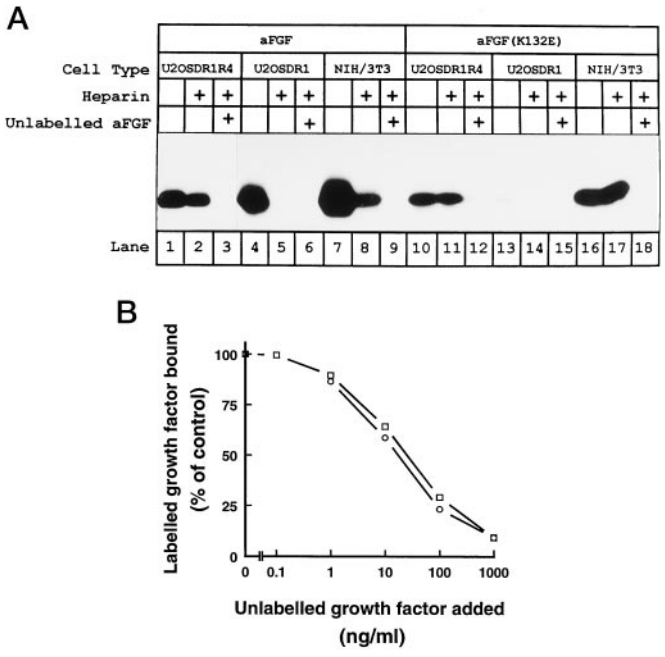

FIG. 1. **Binding of aFGF and aFGF(K132E) to different cell types.** *A,* cells as indicated were kept on ice for 2 h with [^{35}S]methionine-labeled proteins in the presence or absence of heparin (10 units/ml) and excess unlabeled aFGF (5 μg/ml), subsequently washed, and analyzed by SDS-PAGE and fluorography. *B,* NIH 3T3 cells were kept on ice for 4 h in the presence of ^{125}I-aFGF (5 ng/ml), heparin, and different concentrations of unlabeled aFGF (*circles*) or aFGF(K132E) (*squares*). After washing, cell associated radioactivity was measured.

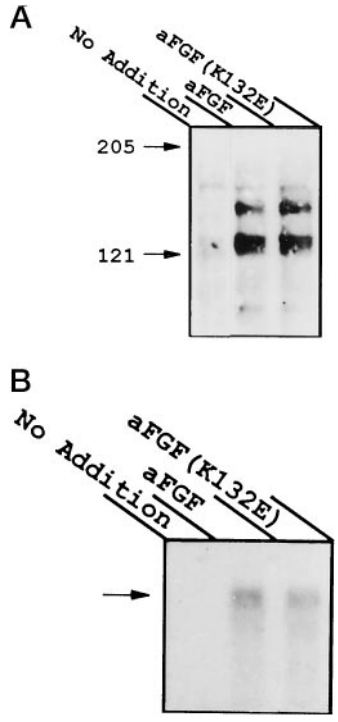

FIG. 2. **Stimulation of tyrosine phosphorylation (*A*) and c-*fos* induction (*B*).** *A,* serum-starved NIH 3T3 cells were left untreated or stimulated for 8 min with aFGF or aFGF(K132E) (100 ng/ml) in the presence of 10 units/ml heparin. The cells were subsequently lysed and FGF receptor 1 was immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine antibody. Migration of molecular mass standards is indicated. *B*, serum-starved NIH 3T3 cells were left untreated or stimulated with aFGF or aFGF(K132E) (10 ng/ml). Total RNA was isolated and analyzed by Northern blotting with a c-*fos* probe. The position of c-*fos* is indicated by an *arrow*.

psala, Sweden; restriction endonucleases from New England Biolabs, Beverly, MA.

Plasmid Construction and Protein Purification

paFGF(K132E)—The cDNA sequence of aFGF (9) in the plasmid pHBGF-1α (3) was changed by polymerase chain reaction-directed mutagenesis introducing a Glu codon instead of Lys-132 and a silent mutation giving a *Stu*I restriction site.

paFGF(K132E)-DT-A—The plasmids paFGF(K132E) and pHBGF-DT1 (10) were cut with *Eco*RI or *Nco*I, respectively, digested with mung bean nuclease and cut with *Nde*I. Fragments from paFGF(K132E) and vector from pHBGF-DT1 were ligated, creating a plasmid containing the sequence encoding aFGF(K132E) in front of DTA. pBD-23 encodes the B-fragment of DT (11).

pTrc-aFGF(K132E) and pTrc-aFGF(K132E)CAAX were obtained by cloning the cDNA for the mutant growth factor with and without the terminal CAAX motif between the *Nco*I site and the *Sal*I or *Pst*I sites, respectively, in pTrc99a (Pharmacia).

Recombinant proteins were expressed and purified as described previously (12) except that for aFGF(K132E) and aFGF(K132E)-CAAX the clarified bacterial lysates were applied to heparin-Sepharose in a buffer containing 0.1 M NaCl instead of 0.5 M and were further purified using an Econo-Pac Q cartridge (Bio-Rad).

Cell Culture, Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS-PAGE), and in Vitro Transcription and Translation

Cells were propagated in Dulbecco's modified essential medium (DMEM) with 7.5% (v/v) fetal calf serum in a 5% CO_2 atmosphere at 37 °C. SDS-PAGE was carried out with 7.5 or 15% gels as described by Laemmli (13) and processed as described (4). The plasmids were linearized, transcribed *in vitro*, and translated in a rabbit reticulocyte lysate system in the presence of unlabeled methionine or [^{35}S]methionine as described (4). After translation, when appropriate, lysates containing fusion protein were mixed with an equal volume of lysate containing unlabeled DT-B. The lysates were dialyzed against dialysis buffer (20 mM HEPES, pH 7.0, 140 mM NaCl, 2 mM $CaCl_2$) to remove free [^{35}S]methionine and reducing agent, allowing disulfide bridges to be formed (11).

Fractionation of Cells

After lysis in lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, pH 7.4) cells were centrifuged for 15 min at 720 $\times$ *g*. The supernatant was centrifuged again for 5 min at 15,800 $\times$ *g* and the second supernatant was designated the cytosol/membrane fraction. The pellet was washed twice by resuspension in lysis buffer containing 0.3 M sucrose that was layered on lysis buffer containing 0.8 M sucrose and centrifuged at 720 $\times$ *g* for 15 min at 4 °C then sonicated and centrifuged for 5 min at 15,800 $\times$ *g*. The supernatant of this centrifugation was designated the nuclear fraction (4).

In Vivo Farnesylation

NIH 3T3 cells were seeded in 6-well plates at a density of 2–4 $\times$ 10^5 cells/well. After 24 h the cells were serum starved for 1–3 days in DMEM containing 2.5 μg/ml insulin and 2.5 μg/ml transferrin, then incubated in the same medium containing 5 μg/ml lovastatin, 5–20 units/ml heparin, 3–5 μCi/ml [^{3}H]mevalonic acid, and 1 μCi/ml [^{14}C]mevalonic acid together with 10–100 ng/ml of the recombinant proteins for 18 h. Subsequently, the cells were washed twice in HEPES medium containing 20 units/ml heparin and twice in HEPES medium without heparin (5). The cells were lysed, fractionated, and both the nuclear and the cytosol/membrane fractions were incubated with heparin-Sepharose for 2 h at 4 °C. The beads were washed with phosphate-buffered saline (140 mM NaCl, 10 mM Na_2HPO_4, pH 7.2) and analyzed by SDS-PAGE.

Measurements of DNA Synthesis and Proliferation

To measure [^{3}H]thymidine incorporation, cells growing in 24-well microtiter plates were kept for 1–3 days in serum-free medium; in the case of NIH 3T3 cells, 2.5 μg/ml insulin and 2.5 μg/ml transferrin were present. Then medium with or without heparin and different concentrations of either purified recombinant proteins or dialyzed translation mixtures was added. Incubation was continued for 48 h when the fusion proteins of diphtheria toxin were used, and for 24 h when aFGF (or its mutant) as such was used. During the last 6 h of incubation, 1 μCi/ml

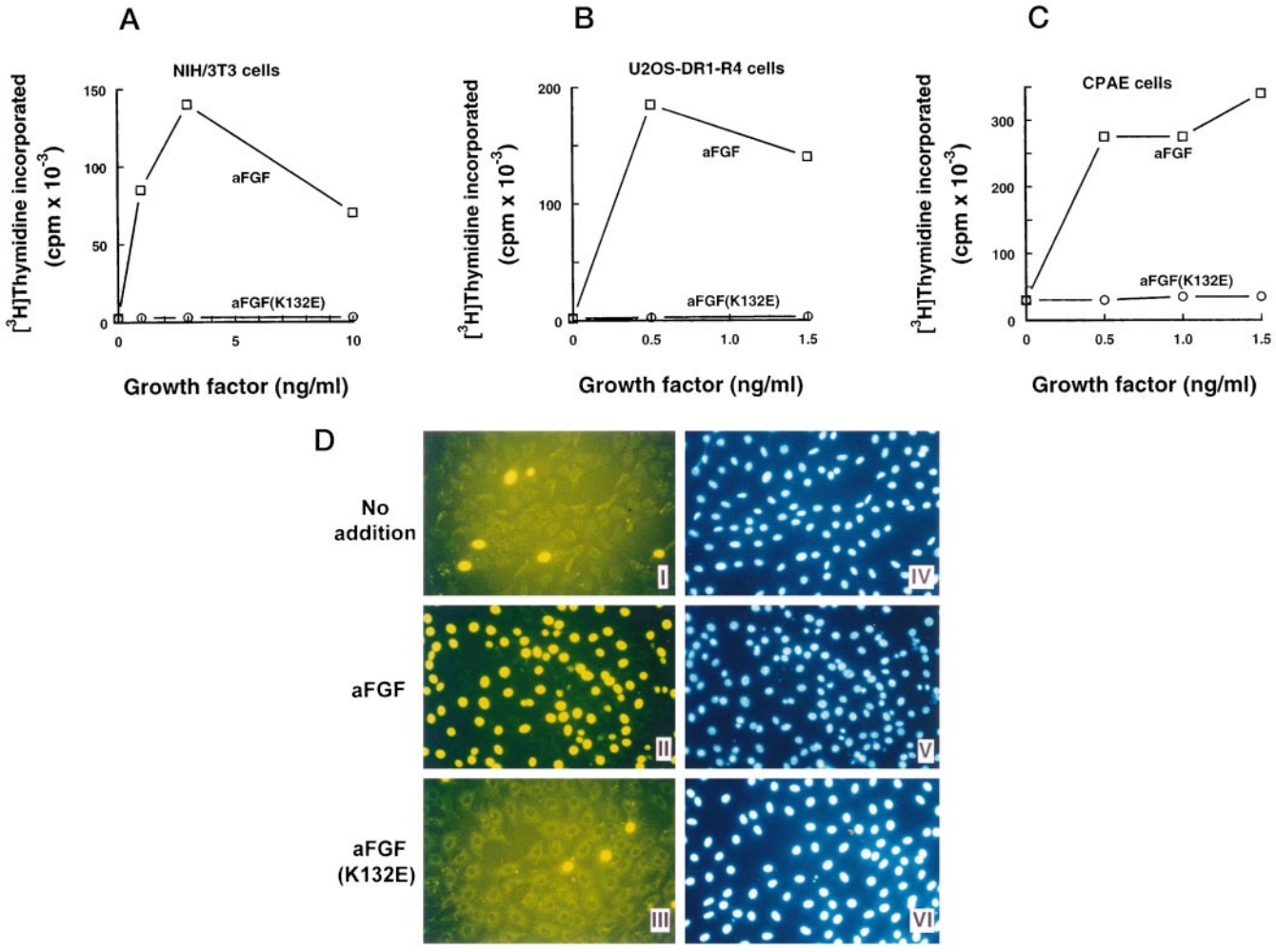

FIG. 3. **DNA synthesis stimulation in various cell types.** *A-C,* cells as indicated were rendered quiescent by serum deprivation and subsequently incubated for 24 h in the presence of different concentrations of aFGF or aFGF(K132E), for the last 6 h in the presence of [^{3}H]thymidine. Incorporated radioactivity was measured. *D*, NIH 3T3 cells were treated with or without 10 ng/ml growth factor as indicated. Incubation for the last 6 h was in the presence of BrdUrd. Incorporated BrdUrd was then visualized as described under "Experimental Procedures" (I–III). Hoechst staining was used to visualize all nuclei (IV–VI).

[^{3}H]thymidine was present. Finally, the incorporated radioactivity was measured (3). To measure cell proliferation, incubation with fusion proteins was continued for 96 h, cells were trypsinized, and cell number counted in a cell counter. To measure bromodeoxyuridine (BrdUrd) incorporation, NIH 3T3 cells were grown on glass coverslips and rendered quiescent by incubation in serum-free medium in the presence of 5 μg/ml insulin and 5 μg/ml transferrin for 72 h. The cells were incubated for 24 h with 10 ng/ml aFGF or aFGF(K132E). During the last 6 h of this period, 100 μM BrdUrd (Amersham) was present. After labeling, the cells were fixed, immunostained with a monoclonal mouse anti-BrdUrd antibody (Amersham) and a biotinylated horse anti-mouse antibody, followed by fluorescein isothiocyanate-streptavidin and then analyzed by fluorescence microscopy as described (14).

Binding and Translocation of Diphtheria Toxin Fusion Proteins

Cells growing in 6-well plates were incubated for 2 h on ice with dialyzed translation mixtures with fusion protein with or without DT-B in the presence of 1 mM unlabeled methionine, unlabeled aFGF, and in the absence or presence of 10 units/ml heparin, and then washed five times. To measure binding, cells were lysed and analyzed by SDS-PAGE. To measure translocation, cells were treated with medium, pH 4.5, for 5 min at 37 °C, washed and treated for 10 min at 37 °C with 3 mg/ml Pronase in the presence of 10 μM monensin (15), washed again, lysed, and centrifuged for 2 min at 15,800 × *g*. The supernatant was analyzed by SDS-PAGE. When appropriate, after Pronase treatment the cells were washed and further incubated at 37 °C for 24 or 48 h in DMEM. Then the cells were fractionated and analyzed by SDS-PAGE (4, 15).

Transport of Externally Added Protein to the Nuclear Fraction

Cells were incubated for 2–24 h in medium containing [^{35}S]methionine-labeled growth factor in the presence of 0.1–1 mM unlabeled methionine, washed and, when appropriate, incubated further for 24 h (4). The cells were then treated with 3 mg/ml Pronase at 37 °C for 5 min, transferred to an Eppendorf tube, and 1 mM phenylmethylsulfonyl fluoride was added. Cells were washed once in HEPES medium with 1 mM phenylmethylsulfonyl fluoride, lysed, and subjected to fractionation and heparin-Sepharose precipitation and analyzed by SDS-PAGE.

Cytotoxicity

Dialyzed unlabeled translation mixtures were added to U2OS cells growing as monolayers in 24-well plates in DMEM with 7.5% fetal calf serum in the presence or absence of heparin. The cells were incubated overnight and the rate of protein synthesis was measured by incorporation of [^{3}H]leucine into trichloroacetic acid precipitable material for 30 min (10, 16).

Tyrosine Phosphorylation of FGF Receptor 1

NIH 3T3 cells were serum-starved for 3 days in the presence of 2.5 μg/ml insulin and 2.5 μg/ml transferrin, then treated for 8 min with purified, recombinant aFGF or aFGF(K132E). The cells were then washed and lysed in the presence of phosphatase and protease inhibitors (4), centrifuged for 5 min at 15,800 × *g* at 4 °C. The supernatant was centrifuged again, and incubated with a rabbit anti-FGF receptor 1 antibody (Santa Cruz Biotechnology, CA). Immunecomplexes were collected with protein A-Sepharose, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The membrane was

probed with a mouse anti-phosphotyrosine antibody and visualized with a horseradish peroxidase-conjugated second antibody and enhanced chemiluminescence.

Induction of c-fos mRNA

NIH 3T3 cells were seeded out in 25-cm^2 tissue culture flasks at a density of 1×10^6 cells/flask in DMEM containing 10% fetal calf serum. On the following day the medium was changed to DMEM without fetal calf serum and the cells were grown for 48 h. The cells were subsequently incubated for 30 min at 37 °C in HEPES medium with additions as indicated, and total RNA was isolated from the cells by the LiCl/urea/SDS method (17). Samples of 5 μg of total RNA were separated on 1% agarose-formaldehyde gels (18) and blotted onto Hybond-N membranes (Amersham, United Kingdom), according to the manufacturer's instructions. The membranes were baked at 80 °C for 2 h and UV cross-linked. The blots were hybridized (19) with a v-*fos* probe (1.0-kilobase *Pst*I fragment) (20) labeled with ^{32}P by the random primer technique (21). Kodak (Rochester, NY) X-Omat AR films were exposed to the filters at −80 °C in the presence of intensifying screens.

In Vitro Phosphorylation

Cells were lysed in Tris lysis buffer (25 mM Tris, pH 7.5, 20 mM NaCl, 2 mM dithiothreitol, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.5% Triton X-100), diluted 5 times in kinase buffer (20 mM Tris, pH 7.6, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM EGTA, 0.1 mM Na_3VO_4, 1 μg/ml aprotinin, 0.1 mM ATP, 40 μCi/ml [γ-^{32}P]ATP), and then incubated at 37 °C for 1 h with 0.1 mg/ml (final concentration) aFGF-CAAX or aFGF(K132E)-CAAX. The reaction mixtures were analyzed by SDS-PAGE and autoradiography.

In Vivo Phosphorylation

Serum-starved U2OSDR1-R4 cells were preincubated at 37 °C for 3 h in phosphate-free DMEM containing 100 μCi/ml $^{32}PO_4^{3-}$. aFGF or aFGF(K132E) (50 ng/ml) and heparin (5 units/ml) was added and incubation was continued for 12 h. The cells were washed twice in phosphate-buffered saline with heparin and once without heparin and lysed in the presence of phosphatase and protease inhibitors. The postnuclear supernatant was subjected to immunoprecipitation with a rabbit anti-aFGF antibody (Sigma) and analyzed by SDS-PAGE and autoradiography.

RESULTS

aFGF(K132E) Binds to and Activates FGF Receptors, but Does Not Stimulate DNA Synthesis—cDNA encoding aFGF (9) was mutated at codon 132 by polymerase chain reaction-directed mutagenesis creating a glutamic acid codon instead of lysine. This mutation has been described to strongly reduce the mitogenic activity of the growth factor (7). cDNA for wild-type and mutant growth factor were transcribed in a cell-free system and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. To check that the mutated aFGF had retained its ability to bind to FGF receptors, we added the labeled growth factor to different cell lines and kept them on ice for 2 h, then the cells were washed and analyzed by SDS-PAGE. In the absence of heparin, wild-type growth factor bound extensively to untransfected U2OSDR1 cells as well as to the same cells transfected with FGF receptor 4 (U2OSDR1-R4) (12) and to NIH 3T3 cells expressing FGF receptor 1 (22) (Fig. 1*A, lanes 1, 4,* and *7*), while aFGF(K132E) bound to a lesser extent under the same conditions (*lanes 10* and *16*). In the presence of heparin, there was no binding in U2OSDR1 cells lacking FGF receptors (*lanes 5* and *14*), whereas NIH 3T3 cells and U2OSDR1-R4 cells bound wild-type and mutant aFGF to a similar extent (*lanes 2, 8, 11,* and *17*). This binding could be competed out with excess unlabeled aFGF (*lanes 3, 9, 12,* and *18*). The observation that aFGF(K132E) was bound to a similar extent in the presence and absence of heparin, while wild-type growth factor bound considerably more extensively when heparin was absent indicates that, under the conditions used, aFGF(K132E) does not bind well to surface heparans in accordance with earlier observations (7).

To compare in more detail the affinities of wild-type and mutant aFGF for the FGF receptor, we performed a binding competition experiment. The results (Fig. 1*B*) showed that the ability of wild-type and mutant aFGF to compete out the binding of ^{125}I-labeled wild-type growth factor was essentially the same.

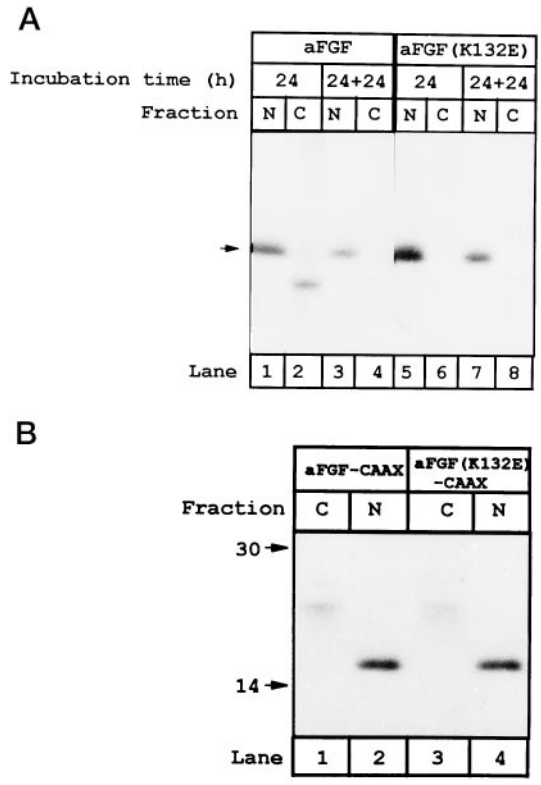

FIG. 4. **Transport of [^{35}S]methionine-labeled growth factor to the nuclear fraction (*A*) and farnesylation of CAAX-tagged growth factor *in vivo* (*B*).** *A,* serum-starved NIH 3T3 cells were incubated for 24 h in the presence of [^{35}S]methionine-labeled growth factor as indicated, washed, and either Pronase-treated and lysed (24 h) or incubated further for 24 h in the absence of growth factor and then Pronase-treated and lysed (24 + 24 h). The cells were then subjected to cell fractionation and analyzed by SDS-PAGE and fluorography. *Arrow* indicates migration of aFGF. *N*, nuclear fraction; *C*, cytoplasmic fraction. *B,* serum-starved NIH 3T3 cells were incubated for 24 h in the presence of unlabeled aFGF-CAAX or aFGF(K132E)-CAAX together with radiolabeled mevalonate (a farnesyl precursor) and lovastatin (a mevalonate biosynthesis inhibitor). The cells were subsequently subjected to cell fractionation and analyzed by SDS-PAGE and fluorography. Migration of molecular mass standards is indicated. *N*, nuclear fraction; *C*, cytoplasmic fraction.

Burgess *et al.* (7) demonstrated that both mutant and wild-type aFGF stimulate protein tyrosine phosphorylation and phospholipase C-γ phosphorylation, and that both induce immediate early gene mRNA expression. By immunoprecipitation with anti-FGF receptor 1 antibodies and Western blotting with anti-phosphotyrosine we found that both the mutant and the wild-type growth factor induced tyrosine phosphorylation of FGF receptor 1 in serum-starved NIH 3T3 cells (Fig. 2*A*). Approximately the same concentration of the two proteins were required to obtain tyrosine phosphorylation of FGF receptor 1 (data not shown). In the same cells we also detected induction of c-*fos* mRNA in response to both mutant and wild-type aFGF (Fig. 2*B*).

It was previously reported that aFGF(K132E) has strongly reduced mitogenic activity as compared with wild-type aFGF (7). We confirmed this in NIH 3T3 cells (Fig. 3*A*), U2OSDR1-R4 cells (Fig. 3*B*), and calf pulmonary artery endothelial cells (Fig. 3*C*) assayed by [^{3}H]thymidine incorporation, and in NIH 3T3 cells also measured by BrdUrd incorporation (Fig. 3*D*). In un-

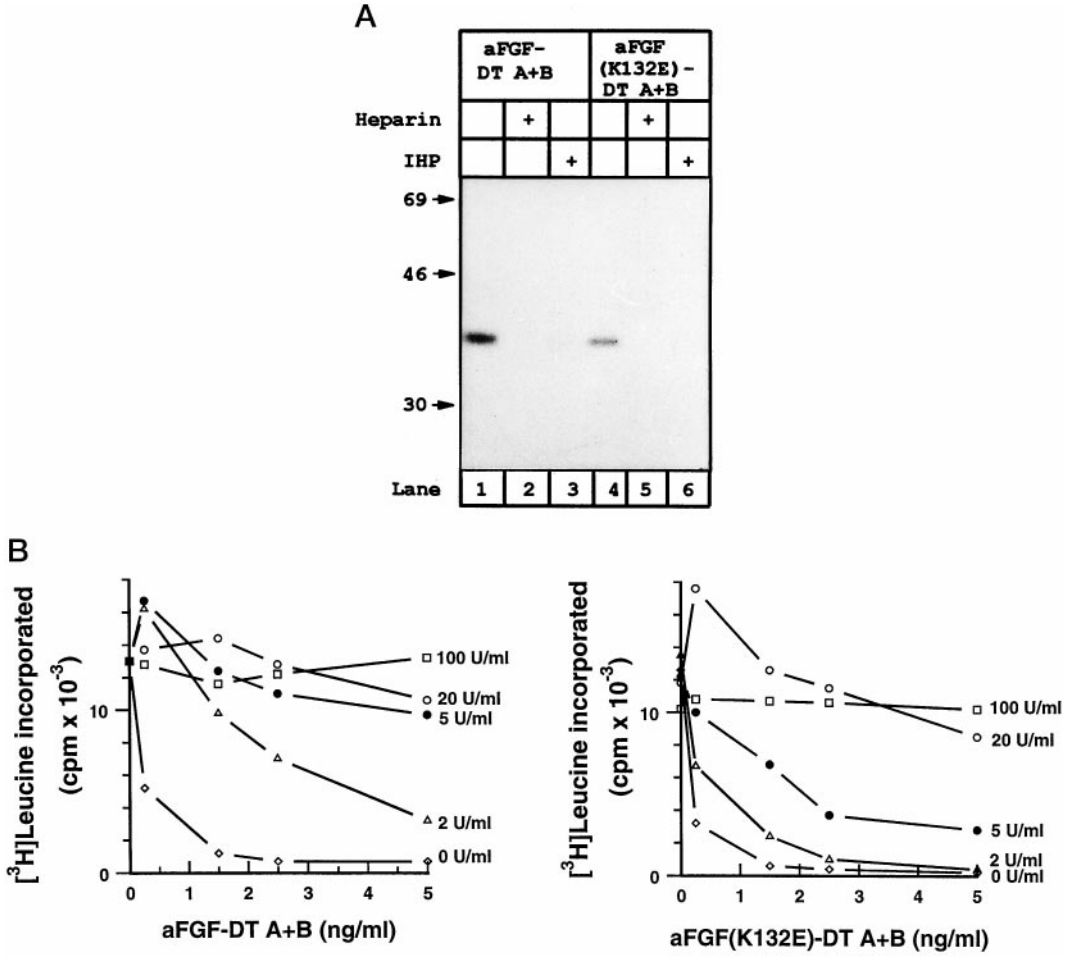

FIG. 5. **Translocation to cytosol (*A*) and cytotoxicity (*B*) of DT-fusion proteins.** *A,* [^{35}S]methionine-labeled aFGF-DT-A or aFGF(K132E)-DT-A fusion proteins reconstituted with unlabeled DT-B, were bound to Vero cells that were subsequently treated briefly with pH 4.5 and Pronase. The cells were then analyzed by SDS-PAGE and fluorography. Heparin (10 units/ml) or inositol hexaphosphate (*IHP*) (1 mM) was present as indicated. Migration of molecular mass standards is indicated. *B,* DT-sensitive U2OS cells were incubated for 16 h in the presence of different concentrations of heparin (none, *diamonds*; 2 units/ml, *triangles*; 5 units/ml, *closed circles*; 20 units/ml, *open circles*; 100 units/ml, *squares*) and increasing concentrations of aFGF-DT-A+B or aFGF(K132E)-DT-A+B. During the last 30 min, [^{3}H]leucine was present and radioactivity incorporated into trichloroacetic acid-precipitable material was measured.

transfected U2OSDR1 cells there was no mitogenic activity of aFGF in accordance with earlier findings (4). Altogether, the data confirm and extend previous findings that although aFGF(K132E) binds to and activates FGF receptors equally well as wild-type aFGF, it is a poor inducer of DNA synthesis.

aFGF(K132E) Is Translocated to the Nuclear Fraction—There are several reports that externally added aFGF is translocated to the nuclear fraction in cells containing FGF receptors (4, 5, 12, 23, 24). This transport appears to be required for stimulation of DNA synthesis, at least in certain cells (3, 4, 12). After transfection of NIH 3T3 cells with the growth factor, the distribution between the nuclear and the cytosolic fractions was similar for the aFGF(K132E) and wild-type aFGF (25). Nevertheless, the possibility existed that exogenously added mutant growth factor might not be transported to the nuclear fraction.

To investigate this, we incubated *in vitro* translated, [^{35}S]methionine-labeled growth factor with serum-starved NIH 3T3 cells at 37 °C for 24 h. The cells were washed and in some cases incubated further for 24 h. Finally, the cells were treated with Pronase, washed, lysed, and fractionated into a nuclear and a cytosol/membrane fraction which were analyzed by SDS-PAGE and fluorography. There was no appreciable difference between the wild-type and the mutant growth factor in their capability of being transported to the nuclear fraction within 24 h, and in both cases the growth factor could still be detected in the nuclear fraction after an additional incubation for 24 h in several experiments (Fig. 4*A*). It should be noted, however, that in some experiments the growth factor (both wild-type and mutant) was not stable in the nuclear fraction after the additional 24-h incubation (data not shown). The reason for this variation between experiments is not known. In U2OSDR1 cells lacking FGF receptors no translocation of growth factor to the nuclear fraction was seen (data not shown) in accordance with earlier data (12).

When bacterially expressed growth factor with a farnesylation signal (a CAAX motif) at the C terminus (aFGF-CAAX) is added to cells in the presence of lovastatin and radiolabeled mevalonate, part of the growth factor is labeled indicating that it is exposed to the cytosol (5). Farnesylation is only known to take place in the cytosol (26–28) and possibly in the nucleus (29, 30), but not extracellularly or in vesicular compartments (5). Mevalonate is a precursor of the farnesyl moiety and lovastatin is an inhibitor of mevalonate biosynthesis (31), included to increase the labeling efficiency.

To study in more detail translocation of aFGF(K132E) into cells, we incubated serum-starved cells for 24 h at 37 °C with unlabeled aFGF-CAAX and aFGF(K132E)-CAAX and labeled mevalonate. Cells were then washed, lysed, and fractionated into nuclear and cytosol/membrane fractions, protein was col-

lected on heparin-Sepharose and analyzed by SDS-PAGE. In FGF receptor-positive cells, both the wild-type and the mutant form of aFGF-CAAX gave bands of similar intensity, mainly present in the nuclear fraction (Fig. 4*B*). The distribution between the cytosol/membrane and nuclear fractions of the mevalonate-labeled aFGF-CAAX varied somewhat from experiment to experiment and up to 50% could sometimes be detected in the cytosol/membrane fraction. There was no difference between the wild-type and mutant growth factor in this respect. The data indicate that there is no difference between wild-type and mutant growth factor in its ability to penetrate into cells and enter the nuclear fraction.

Translocation into Cells of aFGF(K132E) with Diphtheria Toxin as Carrier—We have earlier shown that when aFGF is fused to the N terminus of diphtheria toxin A-fragment (aFGF-DT-A) and this fusion protein is reconstituted with diphtheria toxin B-fragment (DT-B) to form aFGF-DT-A+B, aFGF-DT-A can be translocated via the diphtheria toxin pathway into diphtheria toxin receptor-positive cells. Furthermore, translocation of aFGF-DT-A was found to stimulate DNA synthesis in FGF receptor-negative cells resistant to the intracellular action of diphtheria toxin (4). However, aFGF-DT-A+B does not stimulate cell proliferation unless both FGF receptors and diphtheria toxin receptors are present on the cells (12).

In further attempts to elucidate the mechanisms for the reduced mitogenicity of aFGF(K132E), we fused the mutant with DT-A and reconstituted it with DT-B. Radiolabeled aFGF(K132E)-DT-A+B bound to the same extent as aFGF-DT-A+B to Vero cells and U2OS cells which are both rich in diphtheria toxin receptors (12, 32), and in both cases the binding was competed out with excess unlabeled DT, indicating that the fusion proteins were bound in a specific manner (data not shown). Diphtheria toxin is normally endocytosed and, upon exposure to the low pH of the endosomes, a conformational change takes place and DT-A is translocated to the cytosol (33).

To mimic this process experimentally, we bound reconstituted fusion proteins to Vero cells on ice and exposed them briefly to a low pH buffer at 37 °C, removed non-translocated material with Pronase, and analyzed the cellular pellet by SDS-PAGE (4, 15). Labeled fusion proteins of wild-type and mutant aFGF were translocated to a Pronase inaccessible location, presumably the cytosol, with similar efficiencies (Fig. 5*A, lanes 1* and *4*) and it was in both cases inhibited by heparin (*lanes 2* and *5*) and by inositol hexaphosphate (*lanes 3* and *6*). Earlier work has demonstrated that heparin and inositol hexaphosphate inhibit translocation in this system (10), probably because they inhibit unfolding of the aFGF moiety. Unfolding of DT-A as well as of a passenger protein appears to be a prerequisite for translocation by the diphtheria toxin pathway (10, 34–36).

To compare the translocation competence of the two fusion proteins in a more sensitive system, we incubated increasing concentrations of each reconstituted fusion protein with diphtheria toxin-sensitive U2OS cells at 37 °C for 16 h and then measured the rate of protein synthesis as [^{3}H]leucine incorporation during 30 min. DT-A exerts its action on sensitive cells by inactivating elongation factor 2 and thereby blocking protein synthesis (37). The two fusion proteins were found to be equally toxic and the toxicity could be inhibited by heparin (Fig. 5*B*). At low heparin concentrations the toxicity of fusion protein containing the mutant was less reduced than in the case of fusion protein with wild-type aFGF. At higher concentrations heparin reduced the toxicity of the two to the same extent. This is in good accordance with the reduced, but not abolished heparin affinity of the mutant.

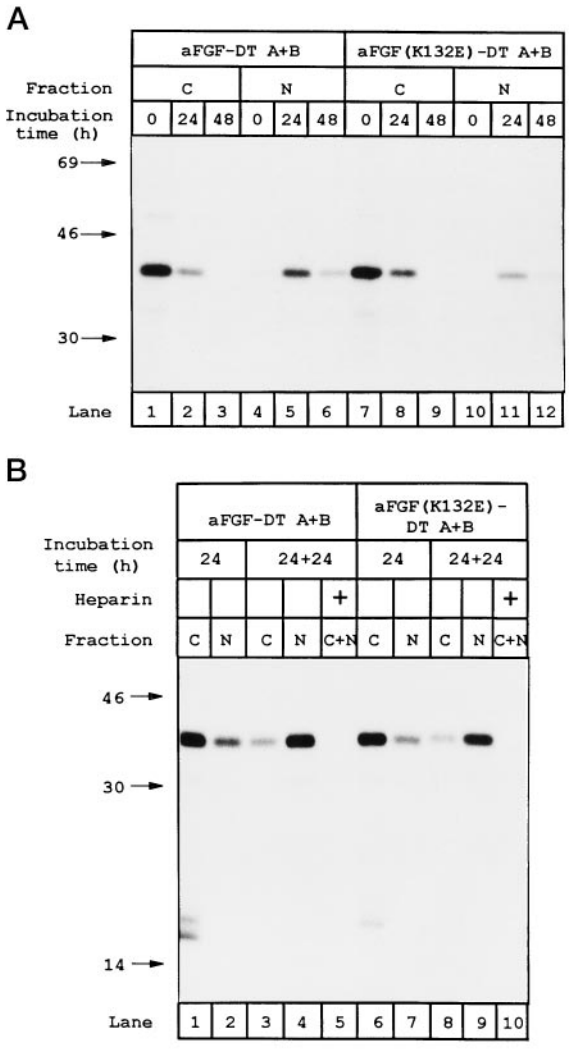

FIG. 6. **Presence in the nuclear fraction of translocated fusion protein.** *A,* reconstituted [^{35}S]methionine-labeled fusion proteins as indicated were bound to Vero-DR22 cells (containing DT receptors, but resistant to the intracellular action of DT), exposed briefly to pH 4.5, Pronase treated and either lysed (0 h) or further incubated for 24 or 48 h before lysis. Cells were subjected to cell fractionation and analyzed by SDS-PAGE and fluorography. Migration of molecular mass standards is indicated. *B,* Vero-DR1 cells were incubated for 24 h in the presence of reconstituted [^{35}S]methionine-labeled fusion proteins as indicated, washed, and either lysed (24 h) or incubated further for 24 h (24+24 h) in the absence of fusion proteins and then lysed. The cells were then fractionated into a cytosol/membrane (*C*) and a nuclear (*N*) fraction and analyzed by SDS-PAGE and fluorography. In *lanes 5* and *10*, heparin was present throughout the experiment. Migration of molecular mass standards is indicated.

We have earlier demonstrated that when aFGF-DT-A is translocated to the cytosol by the diphtheria toxin pathway, it is subsequently transported to the nuclear fraction (4, 12). To investigate if this were also the case with aFGF(K132E)-DT-A, Vero cells expressing diphtheria toxin receptor, but resistant to the toxin due to inability of their elongation factor 2 to be modified by the toxin (38, 39), were first kept on ice with radiolabeled fusion protein reconstituted with DT-B, then exposed to low pH in order to translocate the fusion protein, subsequently treated with Pronase, washed, and further incubated for 0, 24, or 48 h. Then the cells were lysed, fractionated, and analyzed by SDS-PAGE. When the cells were harvested immediately, fusion protein was only found in the cytosol/membrane fraction (Fig. 6*A, lanes 1* and *7*) and not in the nuclear fraction (*lanes 4* and *10*). After 24 h incubation, the

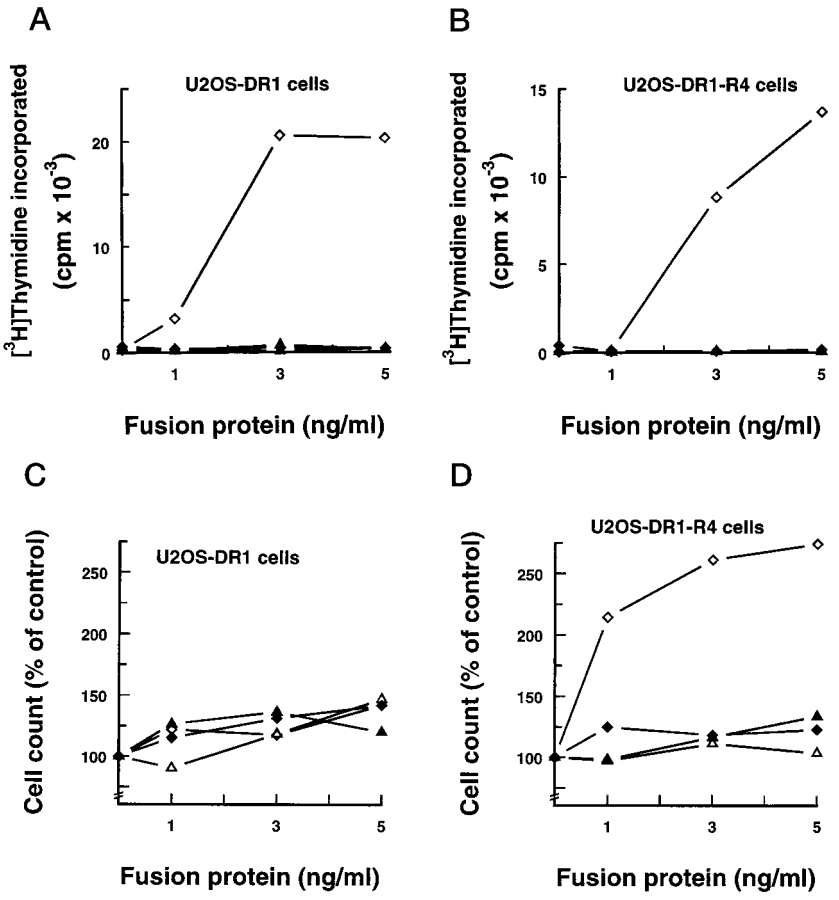

FIG. 7. **Effect of translocated fusion protein on DNA synthesis and proliferation.** U2OSDR1 cells (containing DT receptors, but lacking functional FGF receptors) (*A* and *C*) or U2OSDR1-R4 cells (U2OSDR1 cells stably transfected with FGF receptor 4) (*B* and *D*) were incubated at 37 °C for 48 h (*A* and *B*) or 96 h (*C* and *D*) with aFGF-DT-A+B (*diamonds*) or aFGF(K132E)-DT-A+B (*triangles*) in the presence (*closed symbols*) or absence (*open symbols*) of heparin. In *A* and *B* the last 6 h of incubation was in the presence of [^{3}H]thymidine and the trichloroacetic acid precipitable radioactivity was measured. In *C* and *D* the cell number was measured by a cell counter at the beginning and end of the experiment.

amount of translocated fusion protein in the cytosol/membrane fraction was reduced (*lanes 2* and *8*), while some fusion protein now could be detected in the nuclear fraction (*lanes 5* and *11*). When the incubation was continued for 48 h after the Pronase treatment, there was no detectable fusion protein in the cytosol/membrane fraction (*lanes 3* and *9*) while it was still detectable in the nuclear fraction (*lanes 6* and *12*).

To study conditions more similar to the DNA synthesis stimulation experiments described below, instead of inducing translocation of the fusion proteins from the cell surface by low pH treatment, we allowed DT-resistant Vero cells to endocytose the reconstituted fusion proteins during 24 h at 37 °C. We then either lysed the cells immediately or continued the incubation for 24 h in the presence of heparin to prevent further translocation followed by lysis, fractionation, and SDS-PAGE. After the first 24 h the fusion proteins were mainly present in the cytosol/membrane fraction, but also easily detectable in the nuclear fraction (Fig. 6*B, lanes 1, 2, 6,* and *7*). Following further incubation for 24 h, most of the translocated fusion protein was now present in the nuclear fraction (*lanes 3, 4, 8,* and *9*). As a control, cells were treated identically except that heparin was present throughout the experiment. No translocated fusion protein was detectable under these conditions (*lanes 5* and *10*). It may therefore be concluded that there is no appreciable difference between aFGF(K132E)-DT-A and aFGF-DT-A in their ability to be translocated into cells by the diphtheria toxin pathway and subsequently to be transported to the nuclear fraction.

Translocation into Cells of aFGF(K132E)-DT-A Does Not Stimulate DNA Synthesis—To study if the K132E mutant posses mitogenic activity when bypassing the FGF receptor, we incubated unlabeled aFGF(K132E)-DT-A+B at 37 °C for 48 h with serum-starved U2OSDR1 or U2OSDR1-R4 cells. aFGF-DT-A+B was included as a control. The last 6 h, [^{3}H]thymidine was present to measure DNA synthesis. U2OSDR1 cells express diphtheria toxin receptors but are resistant to the toxin and do not express functional FGF receptors. U2OSDR1-R4 cells are the same cells stably transfected with FGF receptor 4 (12).

Whereas aFGF-DT-A+B stimulated DNA synthesis in both cell types in a dose-dependent and heparin-inhibitable manner, aFGF(K132E)-DT-A+B did not stimulate DNA synthesis in any of the cell types (Fig. 7, *A* and *B*). When aFGF-DT-A was not reconstituted with DT-B there was no stimulation of DNA synthesis (data not shown).

To measure the proliferative response, a similar experiment was carried out, except that the incubation time was increased to 96 h and instead of measuring DNA synthesis the cell number was measured in a cell counter. In accordance with previous results (12), aFGF-DT-A+B stimulated cell proliferation in U2OSDR1-R4 cells, but not in U2OSDR1 cells. By contrast, aFGF(K132E)-DT-A+B did not stimulate cell proliferation in any of the two cell types (Fig. 7, *C* and *D*), and this was also the case with aFGF-DT-A as such (data not shown). Altogether, the data indicate that the inability of aFGF(K132E) to induce DNA synthesis is not due to inability of being transported into the cells and to the nucleus, but rather to inability of the mutated growth factor to interact with the appropriate intracellular target.

aFGF, but Not aFGF(K132E), Is Phosphorylated in a Cell Lysate and in Living Cells—Mascarelli *et al.* (40) obtained evidence that endogenous aFGF was phosphorylated in rod outer segments, and that this phosphorylation enhanced aFGF release. Phosphorylation was stimulated by phorbol ester, indicating that protein kinase C was responsible. The K132E mutation disrupts a consensus phosphorylation site for protein

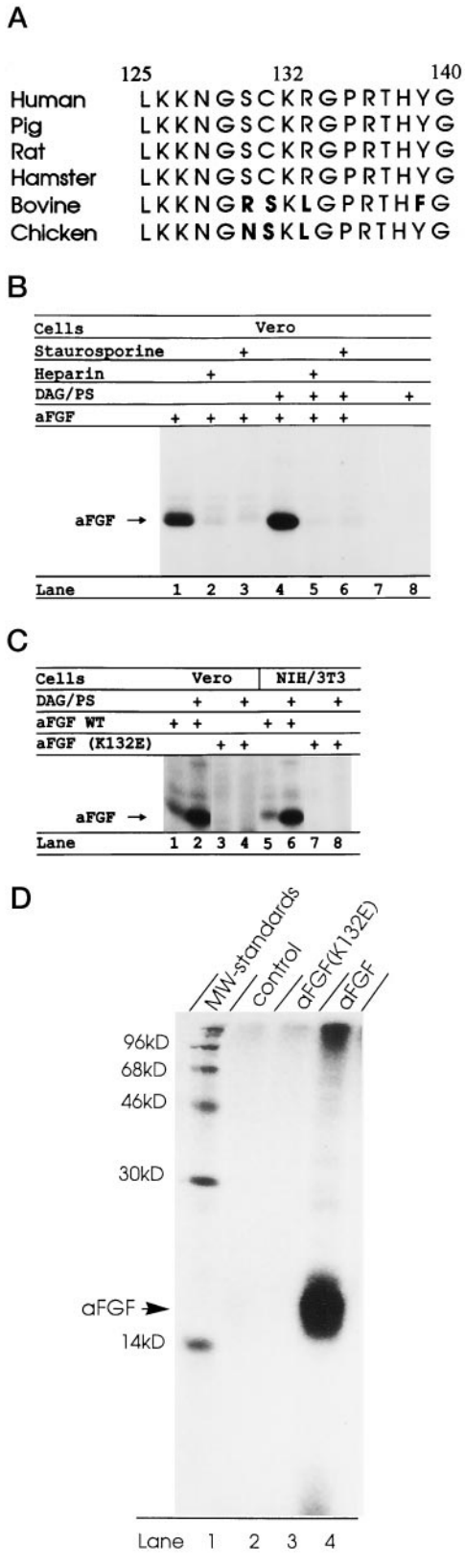

FIG. 8. **Phosphorylation of aFGF.** *A,* schematic overview of amino acids 125–140 of aFGF from different species. Amino acids that differ from the human sequence are in *bold. B* and *C*, aFGF or aFGF(K132E) were incubated with cell lysates at 37 °C in phosphorylation buffer containing [γ-^{32}P]ATP and additions as indicated and analyzed by SDS-PAGE and radiography. *D*, U2OSDR1-R4 cells were incubated without (control) or with aFGF or aFGF(K132E) for 12 h in the presence of $^{32}PO_4^{3-}$. The cells were lysed, centrifuged, and the growth factor was immunoprecipitated from the supernatant. The precipitated material was analyzed by SDS-PAGE and autoradiography.

kinase C ((S/T)*X*(R/K)) in human aFGF (Fig. 8*A*). Therefore, we compared phosphorylation of aFGF(K132E) and wild-type aFGF in cell lysates and in living cells. NIH 3T3 cells and Vero cells were lysed, and the lysates were incubated at 37 °C for 1 h with aFGF or aFGF(K132E) in phosphorylation buffer containing [γ-^{32}P]ATP. Proteins were then analyzed by SDS-PAGE and autoradiography. When wild-type aFGF was used a phosphorylated band corresponding to aFGF was obtained both with NIH 3T3 cells and Vero cells (Fig. 8, *B, lane 1* and *C, lanes 1* and *5*). Diacylglycerol and phosphatidylserine increased the intensity of the band (Fig. 8, *B, lane 4* and *C, lanes 2* and *6*). Phosphorylation was inhibited by heparin (10 μg/ml) (Fig. 8*B, lanes 2* and *5*) and by staurosporine (20 nM) (Fig. 8, *B lanes 3* and *6*). When no aFGF was added, the phosphorylated band was not obtained (Fig. 8*B, lanes 7* and *8*). In the case of aFGF(K132E) no phosphorylated protein was obtained in any of the tested cell lines, neither in the absence (Fig. 8*C, lanes 3* and *7*) nor in the presence (Fig. 8*C, lanes 4* and *8*) of diacylglycerol and phosphatidylserine. When we used fusion proteins of aFGF or aFGF(K132E) and DT-A as substrates in the phosphorylation reactions, we obtained phosphorylation of aFGF-DT-A, but not of aFGF(K132E)-DT-A (data not shown).

We then studied the ability of the two proteins to become phosphorylated *in vivo*. U2OSDR1-R4 cells were incubated with unlabeled aFGF or aFGF(K132E) in the presence of $^{32}PO_4^{3-}$. Then the cells were lysed and the growth factor was immunoprecipitated from the lysates. As shown in Fig. 8*D*, only the wild-type aFGF was labeled under these conditions.

DISCUSSION

The main findings in this paper are that aFGF(K132E) appears to enter cells in a similar manner as wild-type growth factor and that after artificial translocation of the mutant as a fusion protein with DT, it is not able to stimulate DNA synthesis. Wild-type aFGF fused to DT stimulated DNA synthesis under the same conditions. Whereas wild-type aFGF is phosphorylated *in vitro* and *in vivo*, apparently by protein kinase C, the mutant aFGF(K132E) is not.

Several recent reports have suggested an intracellular, probably intranuclear, function of the fibroblast growth factor prototypes aFGF and bFGF and that the externally added growth factors are able to enter the nucleus (2–5, 12, 24, 41). We here studied if the reason for the reduced mitogenicity of aFGF(K132E) is a defect in transport of the growth factor into cells. To test this we used two approaches. In the first approach we allowed FGF receptor-positive cells to take up radiolabeled mutant or wild-type growth factor, then removed with Pronase growth factor that remained bound to the cell surface or to the plastic, and finally lysed the cells and analyzed for radiolabeled growth factor in cytosol/membrane and nuclear fractions. In the second approach we tagged both mutant and wild-type aFGF with a CAAX box, incubated the modified growth factors with cells, and assayed for farnesylated aFGF in nuclear and cytosol/membrane fractions. The advantage of this method is that the growth factor must have reached the cytosol or the nucleus in order to be labeled (5). We could not detect differences between mutant and wild-type aFGF in transport to the cytosol or to the nucleus in either of these assays.

We also used diphtheria toxin as a carrier to translocate the mutant growth factor into the cytosol. For this we constructed a fusion protein of aFGF(K132E) and DT-A and reconstituted it with DT-B. Previously, we showed that when the wild-type aFGF is translocated into cells by this method, it induces DNA synthesis in cells that lack functional FGF receptors, but contain diphtheria toxin receptors (4). aFGF(K132E) fused to DT-A did not induce DNA synthesis even though it was translocated to the cytosol and transported to the nuclear fraction in

a similar way and with a similar efficiency as the fusion protein with wild-type aFGF. Since the difference in mitogenicity was still present when mutant and wild-type aFGF were translocated as DT fusion proteins to the cytosol of U2OSDR1 cells lacking functional FGF receptors (4, 12), thereby bypassing the FGF receptor, the possibility that a defect in receptor stimulation could be the reason for reduced mitogenicity of aFGF(K132E) is unlikely. Rather, the data suggest that an intracellular function of aFGF is affected by the mutation. Also the possibility that the reduced heparin affinity of aFGF(K132E) is the reason for its reduced mitogenicity (7) is disfavored, since it is not probable that heparin-aFGF interaction was of importance after the fusion protein was translocated to the cytosol. Heparin in fact blocked translocation of the fusion proteins, which makes it unlikely that surface heparans were translocated into the cytosol together with the fusion proteins.

The fact that the fusion protein of aFGF(K132E) and DT-A did not stimulate DNA synthesis while the wild-type aFGF fusion protein did, supports the idea that translocation of aFGF with DT as vector can be used as a method to study aFGF signal transduction, and that results obtained with this method reflect physiological phenomena. It rules out the possibility that the stimulation of DNA synthesis observed is due to some artifact caused by DT-A in the nucleus or by the translocation process as such.

In the *in vitro* and *in vivo* phosphorylation experiments there was a clear difference between wild-type and K132E mutant aFGF, the wild-type was phosphorylated while the mutant was not. We have provided evidence that the kinase responsible for phosphorylating aFGF was protein kinase C. If the difference in ability to become phosphorylated is the reason for the difference in activity between wild-type and mutant aFGF, the phosphorylated aFGF could be the active form that interacts with components in the cytosol or in the nucleus. If this is the case, it is surprising that bovine and chicken aFGF do not have a phosphorylation site at this position. In these cases there are three amino acid changes around the conserved Lys-132 which could compensate for the lacking phosphorylation. Experiments are in progress to study the potential functional role of phosphorylation of aFGF transported into target cells.

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Inability of the Acidic Fibroblast Growth Factor Mutant K132E to Stimulate DNA Synthesis after Translocation into Cells

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