Acidic fibroblast growth factor (aFGF) is a potent mitogen for many cells. Exogenous aFGF is able to enter the cytosol and nucleus of sensitive cells. There are indications that both activation of the receptor tyrosine kinase and translocation of aFGF to the nucleus are of importance for mitogenesis. However, the mechanism of transport of aFGF from the cell surface to the nucleus is poorly understood. In this work we demonstrate that inhibition of phosphatidylinositol (PI) 3-kinase by chemical inhibitors and by expression of a dominant negative mutant of PI 3-kinase blocks translocation of aFGF to the cytosol and nucleus. Translocation to the cytosol and nucleus was monitored by cell fractionation, by farnesylation of aFGF modified to contain a farnesyl signal, and by phosphorylation by protein kinase C of aFGF added externally to cells. If aFGF is fused to diptheria toxin A-fragment, it can be artificially translocated from the cell surface to the cytoplasm by the diptheria toxin pathway. Upon further incubation, the fusion protein enters the nucleus due to a nuclear localization sequence in aFGF. We demonstrate here that upon inhibition of PI 3-kinase the fusion protein remains in the cytosol. We also provide evidence that the phosphorylation status of the fusion protein does not regulate its nucleocytoplasmic distribution.

Acidic fibroblast growth factor (aFGF) is considered to be involved in several important physiological and pathological processes, such as angiogenesis, wound healing (1), and atheromatoses (2–6). In cell cultures it can stimulate growth, cell migration, and differentiation. As the growth factor lacks a classical signal sequence, its mechanism of secretion is still unclear, but it appears to involve a pathway different from the classical secretion pathway through the endoplasmic reticulum and Golgi apparatus (7–9). At the cell surface, aFGF binds with high affinity to transmembrane FGF-receptors (FGFR) containing a cytoplasmic split tyrosine kinase domain. There are four different genes encoding FGFR (FGFR1–4) with several different splicing variants. aFGF also binds to heparan sulfate proteoglycans at the cell surface, although with lower affinity. Upon activation, the FGFR is phosphorylated on tyrosine residues, and it activates downstream effectors such as phospholipase Cγ and the mitogen-activated protein kinase pathway. There are some conflicting data as to whether phosphatidylinositol (PI) 3-kinase is also activated (10–13). After binding to high affinity cell surface receptors, aFGF is translocated across cellular membranes and transported to the nucleus (14–22). The mechanism of this process is not well defined. It depends on FGFR and, at least in the case of FGFR4, also on the intactness of the C terminus of its cytosolic part.2

Human PI 3-kinases phosphorylate PI in the 3′ position of the inositol ring and can be divided into three main classes on the basis of in vitro substrate specificity (23). Class I phosphates PI, PI 4-phosphate and PI 4,5-diphosphate and includes the p85/p110 heterodimeric PI 3-kinases and the G-protein βγ subunit-activated PI 3-kinases. Class II phospholipates PI and PI 4-phosphate, whereas class III phospholipates only PI and includes the human homologue of the yeast vps34 protein (23–27). PI 3-kinase has been reported to be involved in the regulation of diverse cellular processes, such as intracellular vesicle transport (26, 28–35), mitogenesis (36), cell motility and invasiveness (37), insulin-regulated glucose uptake (27, 38–40), and apoptosis (41–43), to mention some.

Best studied is the heterodimeric PI 3-kinase consisting of a 110-kDa catalytic subunit noncovalently bound to an 85-kDa regulatory subunit (25, 36). PI 4,5-diphosphate is likely its preferred physiological substrate (23, 26). The regulatory 85-kDa subunit contains several domains, among which are two SH2 (src homology 2) domains and one SH3 domain. The SH2 domains can bind specifically to phosphorylated tyrosine residues in the consensus sequence pYXXM (25, 36), contained in transmembrane receptors such as the platelet-derived growth factor receptor and the colony stimulating factor-1 receptor. Binding of PI 3-kinase by its SH2 domains to such phosphorylated tyrosine residues activates the enzyme. The same consensus sequence is conserved in all four FGF receptors (44), and the tyrosine in this sequence of FGFR1 was recently shown to be phosphorylated in vivo upon receptor activation (45). At high concentrations, a tyrosine-phosphorylated pentapeptide derived from this FGFR1 sequence was demonstrated to compete with platelet-derived growth factor receptor and v-fms for binding to PI 3-kinase in vitro (46). However, a direct interaction of FGFR and PI 3-kinase has been difficult to demonstrate (47, 48). There is one recent report that in extracts from Xenopus blastulae, p85 of PI 3-kinase co-precipitates with FGFR1 (49).

PI 3-kinase is also regulated by other means. Ras can bind to the catalytic subunit and thereby activate it (50–53). CDC42

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and Rac are reported to activate the enzyme by binding to the regulatory subunit (37, 54, 55), and tyrosine phosphorylation of this subunit also has a stimulatory effect (36, 56). Serine autophosphorylation down-regulates the enzyme (36).

Diphtheria toxin (DT) is synthesized as a single polypeptide chain that can be cleaved at a protease-sensitive site into two fragments, A and B (or DT-A and DT-B), held together by a disulfide bond. DT-B binds to diphtheria toxin receptors, and the complex is endocytosed. Triggered by the low pH in the endosomes, DT-A translocates across the endosomal membrane and reaches the cytosol, where it exerts its toxic effect by ADP-ribosylating elongation factor 2, thereby inhibiting protein synthesis (57). Experimentally, the translocation can be rapidly induced at the level of the surface membrane if cells with receptor-bound toxin are exposed to low pH, resembling the conditions in the endosomes (58, 59).

In this work we demonstrate that PI 3-kinase activity is required both for transport of exogenously added aFGF to the cytosol and to the nuclear fraction and for accumulation in the nucleus of aFGF that has been translocated to the cytosol as a fusion protein with diphtheria toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wortmannin and rapamycin were obtained from Sigma; LY294002 was from Calbiochem; rabbit reticulocyte lysate and RNAsin were from Promega, Madison, WI; T3 RNA polymerase was from Life Technologies, Inc.; [3H]methionine, [14C]mevalonolactone, and [3H]mevalonolactone were from NEN Life Science Products; protein A-Sepha-

rose and heparin-Sepharose were from Amersham Pharmacia Biotech; T4 DNA polymerase and restriction endonucleases were from New England Biolabs, Beverly, MA. Plasmids encoding dominant negative (Ap85α) and constitutively active (PI10-K227E) PI 3-kinase mutants were kindly provided by J. Downward, Signal Transduction Laboratory, Imperial Cancer Research Fund, London, UK.

**Cell Culture, Polyacrylamide Gel Electrophoresis (PAGE) in the Presence of SDS 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% CO2 atmosphere at 37 °C. SDS-PAGE was carried out with 7.5 or 15% gels as described by Laemmli (64) and reveal is shown for 1–2 h at 37 °C in the presence of protease inhibitors, either lysed or further incubated for an additional 2 h at 37 °C in the presence of wortmannin. Bands corresponding to phosphorylated Akt and 4E-BP1 were visualized by chemiluminescence with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). 

**Fractionation of Cells into Cytosol/Membrane and Nuclear Fractions**—After lysis in lysis buffer (0.1 M NaCl, 10 mM NaHPO4, 1% Triton X-100, 1 mM EDTA, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin), cells were centrifuged for 15 min at 15,800 × g and designated the cytosol/membrane fraction. The pellet was washed once by resuspension in lysis buffer and once 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 × g for 15 min at 4 °C, then sonicated in lysis buffer containing an additional 0.5 M NaCl and centrifuged for 5 min at 15,800 × g. The supernatant of this centrifugation was designated the nuclear fraction (6).

**Fractionation of Cells into Cytosol, Membrane, and Nuclear Fractions**—Cells were resuspended in homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPEs, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin), homogenized with 15 strokes in a 1-ml syringe with a 22 × 1.5 gauge needle and centrifuged for 15 min at 720 × g. The supernatant was further washed in lysis buffer containing sucrose as above. The supernatant was centrifuged for 60 min at 228,000 × g to separate the membrane fraction (pellet) from the cytosol fraction (supernatant). The membrane fraction was finally dissolved in lysis buffer.

In Vivo Farnesylation—NIH 3T3 cells were serum-starved for 1–2 days in the presence of 2.5–5 μg/ml insulin and 2.5–5 μg/ml transferrin. The cells were then preincubated for 30–60 min in serum-free DMEM containing 5 μg/ml lovastatin, 10 units/ml heparin, 3–5 μ Ci/ml [3H]mevalonic acid, and 1 μCi/ml [14C]mevalonic acid in the presence of wortmannin, LY294002, or carrier. 10–100 ng/ml recombinant protein was added, and the incubation was continued for 9–18 h. In experiments where wortmannin was used, the medium was replaced every third hour. Subsequently, the cells were washed twice in HEPES medium containing 10 units/ml heparin and twice in HEPES medium without heparin (17). The cells were lysed and 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 lysis buffer containing 0.5 M NaCl and analyzed by SDS-PAGE.

**Internalization of aFGF—NIH 3T3 cells were incubated at 37 °C in HEPES medium containing 10 units/ml heparin and 10 ng/ml [125I]aFGF in the absence or presence of 50 μM LY294002. At different time points the medium was removed, and the cells were washed four times in PBS. Surface-bound [125I]-aFGF was then obtained by washing the cells twice in acid-salt buffer. The cells were solubilized in 0.1 M KOH, and the radioactivity in the acid-salt wash and in the solubilized cells was measured. The incubation medium was assayed for degraded [125I]-aFGF as trichloroacetic acid-soluble radioactivity.

**Translation of aFGF-DT-A Fusion Protein in Cells**—[3H]Methionine-labeled aFGF-DT-A fusion proteins and DT-B were synthesized in vitro and dialyzed together, allowing disulfide bonds to be formed. The translated fusion proteins were incubated with cells for 1 h at 4 °C. The cells were washed and treated with buffer, pH 4.5, for 5 min at 37 °C, treated for 10 min at 37 °C with 5 μg/ml Pronase, washed, and the presence of protease inhibitors, and either lysed or further incubated for an additional 2 h at 37 °C in the presence of wortmannin. Bands corresponding to phosphorylated Akt and 4E-BP1 were visualized by chemiluminescence with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). 

**In Vivo Phosphorylation of aFGF—Transiently transfected, serum-starved COS cells were preincubated at 37 °C for 3 h in phosphate-free DMEM containing 100 μCi/ml [32P]Pi and 10 units/ml heparin. aFGF (100 ng/ml) was added, and incubation was continued for 12 h more. The cells were washed twice in HEPES medium with hargin and three times in HEPES medium without heparin, lysed in P-lysis buffer (10 mM Tris/HC1 (pH 7.4), 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) with 16 μg/ml protease inhibitors added, and fractionated into nuclear and cytosol/membrane fractions, which were subsequently adsorbed to heparin-Sepharose for 2 h at 4 °C. The heparin-Sepharose was washed once in P-lysis buffer and then treated for 45 min at 20 °C with 0.1 mg/ml trypsin. Due to the tight conformation imposed on aFGF by heparin, the growth factor was resistant to this treatment (61). The heparin-Sepharose was then

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washed once in HEPES medium containing 1 mM phenylmethylsulfonyl fluoride and 4 μg/ml aprotinin and once in PBS with 0.1% Triton X-100 containing additional 0.7 M NaCl, and bound proteins were eluted with 2 M NaCl in PBS with 0.1% Triton X-100 on ice for 15 min. After dilution 10 times with PBS with 0.1% Triton X-100, aFGF was immunoprecipitated with a rabbit antihuman aFGF antibody (Sigma) and analyzed by SDS-PAGE and autoradiography.

RESULTS

Effect of Inhibition of PI 3-Kinase on Binding and Internalization of aFGF—We and others found earlier that exogenous aFGF is transported from the cell surface to the cytosol and nucleus (14-22). In attempts to characterize this process, we have tested compounds that interfere with intracellular transport to see if such compounds would interfere with the internalization of the growth factor. PI 3-kinase has been reported to be involved in the regulation of intracellular trafficking processes (26), and we therefore tested the effect of two inhibitors of this enzyme, wortmannin and LY294002, on transport of aFGF to the cytosol and nucleus. We first tested if the drug inhibited binding of the growth factor to the cells. The data in Fig. 1A demonstrate that wortmannin did not reduce the binding of [35S]methionine-labeled aFGF (5 ng/ml) and heparin (10 units/ml) were added with or without excess unlabeled aFGF (1 μg/ml). After 1 h, the cells were washed and lysed, and the postnuclear supernatant was analyzed by SDS-PAGE and autoradiography. B, U2OSDr1-R4 cells were preincubated for 30 min at 37 °C with heparin and bovine serum albumin in the presence or absence of 50 μM LY294002. The temperature was shifted to 4 °C, increasing concentrations of [125I]aFGF were added, and incubation was continued for 3 h more. The cells were washed, and cell-associated radioactivity was measured.

C and D, NIH 3T3 cells (C) or U2OSDr1-R4 cells (D) were incubated at 37 °C for 30 min with or without 50 μM LY294002 in the presence of heparin and bovine serum albumin. Ten ng/ml [125I]aFGF was added, and incubation was continued for different periods of time. The cells were then washed twice with acid-salt buffer and lysed in KOH. Radioactivity in the acid-salt wash and in the lysed cells was measured. E, NIH 3T3 cells were incubated at 37 °C with 10 units/ml heparin and 100 ng/ml [125I]aFGF in the absence or presence of 50 μM LY294002. The cells were preincubated with LY294002 for 1 h or 18 h as indicated. At different time points, the medium was removed and assayed for degraded [125I]aFGF as trichloroacetic acid-soluble radioactivity. Degraded growth factor is expressed as percent of the radioactivity added. F, NIH 3T3 cells were preincubated with or without 50 μM LY294002, fixed, and stained with rhodamin-coupled phallolidin to label actin or with anti-tubulin or anti-vimentin antibodies and then with CY3-coupled secondary antibodies as described (101). The cells were examined by confocal microscopy.

Fig. 1. Effect of PI-3 kinase inhibitors on binding (A and B), endocytosis (C and D), and degradation (E) of aFGF and on cytoskeletal proteins (F). A, NIH 3T3 cells were preincubated for 15 min at 37 °C with increasing concentrations of wortmannin. The temperature was shifted to 4 °C, and [35S]methionine-labeled aFGF (5 ng/ml) and heparin (10 units/ml) were added with or without excess unlabeled aFGF (1 μg/ml). After 1 h, the cells were washed and lysed, and the postnuclear supernatant was analyzed by SDS-PAGE and autoradiography. B, U2OSDr1-R4 cells were preincubated for 30 min at 37 °C with heparin and bovine serum albumin in the presence or absence of 50 μM LY294002. The temperature was shifted to 4 °C, increasing concentrations of [125I]aFGF were added, and incubation was continued for 3 h more. The cells were washed, and cell-associated radioactivity was measured. C and D, NIH 3T3 cells (C) or U2OSDr1-R4 cells (D) were incubated at 37 °C for 30 min with or without 50 μM LY294002 in the presence of heparin and bovine serum albumin. Ten ng/ml [125I]aFGF was added, and incubation was continued for different periods of time. The cells were then washed twice with acid-salt buffer and lysed in KOH. Radioactivity in the acid-salt wash and in the lysed cells was measured. E, NIH 3T3 cells were incubated at 37 °C with 10 units/ml heparin and 100 ng/ml [125I]aFGF in the absence or presence of 50 μM LY294002. The cells were preincubated with LY294002 for 1 h or 18 h as indicated. At different time points, the medium was removed and assayed for degraded [125I]aFGF as trichloroacetic acid-soluble radioactivity. Degraded growth factor is expressed as percent of the radioactivity added. F, NIH 3T3 cells were preincubated with or without 50 μM LY294002, fixed, and stained with rhodamin-coupled phallolidin to label actin or with anti-tubulin or anti-vimentin antibodies and then with CY3-coupled secondary antibodies as described (101). The cells were examined by confocal microscopy.
3T3 cells were incubated for 8 h at 37 °C in the presence of heparin, the same whether or not 50 μM LY294002 was present (Fig. 1, C and D). The amount of cell surface bound 

\[ ^{125}\text{I}-\text{aFGF} \] was also not affected by LY294002 (Fig. 1, C and D). Furthermore, prolonged preincubation (18 h) with the inhibitor had no inhibitory effect (not demonstrated). Also, 100 nM wortmannin had no effect on endocytosis in experiments where 

\[ ^{125}\text{I}-\text{aFGF} \] was prebound to cells at 4 °C, the cells were washed, and endocytosis was then measured after 10 and 30 min (not demonstrated). Degradation of 

\[ ^{125}\text{I}-\text{aFGF} \] in NIH3T3 cells, as measured by trichloroacetic acid-soluble radioactivity, was slightly decreased by LY294002 (Fig. 1E).

To investigate the possible effects PI 3-kinase inhibition might have on cytoskeletal proteins, we incubated NIH 3T3 cells for 3 h with or without 50 μM LY294002, fixed the cells, and stained for actin, tubulin, or vimentin. By confocal microscopy we could not detect any difference between treated and untreated cells for any of the proteins examined (Fig. 1F).

Similar data were obtained for 100 nM wortmannin (not demonstrated).

**Effect of Inhibition of PI 3-Kinase on Transport of aFGF to Cytosol and Nucleus**—Externally added aFGF accumulated slowly in the nucleus over a time-span of several hours (22). To test if PI 3-kinase inhibition interferes with transport of the growth factor to the nucleus, NIH 3T3 cells were incubated with 

\[ ^{35}\text{S}\text{-methionine-labeled aFGF} \] and different concentrations of LY294002. The cells were then washed three times with acid-salt buffer to remove aFGF at the cell surface, homogenized, and fractionated into nuclear (N), cytosolic (C), and membrane (M) fractions as described under “Experimental Procedures” and analyzed by SDS-PAGE and fluorography.

10 ng/ml 

\[ ^{125}\text{I}-\text{aFGF} \] and heparin, the rate of endocytosis was the same whether or not 50 μM LY294002 was present (Fig. 1, C and D). The amount of cell surface bound 

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Similar data were obtained for 100 nM wortmannin (not demonstrated).

In similar experiments where the cells were dissolved with Triton X-100 instead of being homogenized and then fractionated into a cytosol/membrane fraction and a nuclear fraction, we obtained labeled aFGF in the nuclear fraction in the absence of wortmannin. The amount of labeled aFGF in the nuclear fraction decreased with increasing concentration of the drug and was absent at 75 nM wortmannin (not demonstrated). Also, LY294002 inhibited accumulation of 

\[ ^{35}\text{S}\text{-methionine-labeled aFGF} \] in the nuclear fraction in NIH 3T3 cells assessed by this method (not demonstrated).

To test if exogenous aFGF is translocated to the cytosol and nuclear fraction, we took advantage of the fact that farnesyl transferase is an enzyme of the cytosol and possibly the nucleus (67–71) and that farnesylation of a protein added externally to the intact cells must therefore mean that the protein has been translocated across cellular membranes to reach the compartments where the farnesyl transferase is located. The requirement for farnesylation of a protein is that the C-terminal end of the protein contains a CAAX motif appropriate for farnesylation. We therefore modified the growth factor to contain the C-terminal extension CVIM (Cys-Val-Ile-Met). When unlabeled, modified aFGF (aFGF-CAAX) is incubated with FGFR-positive cells in the presence of radiolabeled mevalonic acid, a precursor of the farnesyl group, and lovastatin, an inhibitor of mevalonic acid biosynthesis included to increase labeling efficiency, the growth factor becomes labeled (14, 17, 18).

We incubated NIH 3T3 cells in the presence of aFGF-CAAX, radiolabeled mevalonic acid, and increasing concentrations of the PI 3-kinase inhibitors for 15–20 h at 37 °C. Then the cells were washed, lysed, fractionated, and analyzed by SDS-PAGE and fluorography. In the absence of inhibitor, labeled aFGF-CAAX was obtained from both the cytoplasmic and nuclear fractions (Fig. 3A, lanes 1 and 2). With increasing concentrations of LY294002 (lanes 3 to 8) the amount of farnesylated aFGF-CAAX decreased, and at 50 μM LY294002, we obtained no labeled aFGF-CAAX. Similar results were obtained with wortmannin, with full inhibition of farnesylation of aFGF-CAAX at 100 nM (not demonstrated).

In control experiments, 50 μM LY294002 and 100 nM wortmannin had no detectable effect on in vitro farnesylation of aFGF-CAAX (17) in a rabbit reticulocyte lysate, whereas 50 μM B581, a farnesyl transferase inhibitor (17, 72), clearly inhibited the farnesylation (not demonstrated). Therefore, the data strengthen the notion that transport of aFGF-CAAX to the cytosol and nucleus in NIH 3T3 cells is inhibited by the PI 3-kinase inhibitors.

U2OSDr1-R4 cells, a human osteosarcoma cell line stably transfected with FGFR4, bind aFGF and transport the growth factor to the nuclear fraction (14, 18, 63). The results in Fig. 3B demonstrate that also in these cells 50 μM LY294002 completely inhibited accumulation in the nuclear fraction of 

\[ ^{35}\text{S}\text{-methionine-labeled aFGF} \] (upper panel) as well as farnesylation of aFGF-CAAX (lower panel).

We recently demonstrated that aFGF contains a functional phosphorylation site for protein kinase C (18, 73) and that the growth factor can be phosphorylated at this site upon incubation with COS cells transfected with functional FGFR4.2 In this paper we use phosphorylation of aFGF as a measure of translocation to the cytosol and nucleus, since these are the only places in the cell where protein kinase C is known to exist. In untransfected COS cells, which express little or no FGFR, aFGF is not translocated to the cytosol and nucleus, and hence, it is also not phosphorylated. This allowed us to do co-transfection experiments with FGFR4 and PI 3-kinase mutants as described below.

We found that phosphorylation of externally added aFGF is

![Graph showing the effect of PI 3-kinase inhibition on transport of aFGF to cytosol and nucleus of NIH 3T3 cells.](image-url)
fractionated into cytosol/membrane (C) fractions. Each incubation was continued for 16 h more. The cells were washed, lysed, and fractionated into cytosol/membrane (C) and nuclear (N) fractions. Each fraction was subjected to heparin-Sepharose precipitation and analyzed by SDS-PAGE and autoradiography.

The cells were fractionated, and phosphorylated aFGF was analyzed by SDS-PAGE and fluorography. Migration of aFGF into different cells.

When the cells were transfected with FGFR4, aFGF was phosphorylated (Fig. 3C). Also in this assay 50 μM LY294002 (lanes 1 and 8) inhibited labeling of the growth factor. We also used a well-characterized dominant negative mutant of PI-3 kinase, Δp85 (53). This mutated regulatory subunit of the heterodimeric PI-3 kinase lacks the binding site for the catalytic subunit (74), and upon overexpression, it is thought to block upstream stimulatory signaling by titrating out stimulatory molecules. When Δp85 was co-transfected with FGFR4, it completely inhibited labeling of aFGF (lanes 4 and 9), whereas co-transfection with an activated point mutant of PI-3 kinase, p110α(K227E) (52), had no inhibitory effect (lanes 5 and 10). It therefore appears that the activity of the heterodimeric PI-3 kinase is necessary for transport of aFGF into the cytosol and nucleus.

Effect of PI-3-Kinase Inhibitors on Tyrosine Phosphorylation of FGFR—NIH 3T3 cells express endogenous FGFR1 (14). To test if the PI-3-kinase inhibitors inhibit activation of FGFR, serum-starved NIH 3T3 cells were preincubated for 15 min without or with the inhibitors, treated with aFGF for 10 min or left untreated, lysed, and analyzed by Western blotting with an antiphosphotyrosine antibody. The membrane was stripped and reprobed with an antibody that recognizes Akt independent of its phosphorylation status (Anti-Akt, lower panel).

**FIG. 3.** Effect of PI-3 kinase inhibition on transport of aFGF into different cells. A, serum-starved NIH 3T3 cells were preincubated for 2 h at 37°C with radiolabeled mevalonate in the presence of lovastatin and heparin and for 30 min with different concentrations of LY294002. Unlabeled aFGF-CAAX (10 ng/ml) was added, and the incubation was continued for 16 h more. The cells were washed, lysed, and fractionated into cytosol/membrane (C) and nuclear (N) fractions. Each fraction was subjected to heparin-Sepharose precipitation and analyzed by SDS-PAGE and fluorography. Migration of aFGF-CAAX and molecular weight markers are indicated. B, upper panel, serum-starved U2OSDR1-R4 cells were incubated for 16 h at 37°C with [3H]methionine-labeled aFGF (5 ng/ml) and heparin (10 units/ml) in the presence or absence of LY294002. The cells were then washed twice in acid-salt buffer, lysed, fractionated into cytosol/membrane (C) and nuclear (N) fractions, subjected to heparin-Sepharose precipitation, and analyzed by SDS-PAGE and fluorography. Lower panel, serum-starved U2OSDR1-R4 cells were treated and analyzed as described in A. C, COS-1 cells were transiently transfected with a plasmid without insert (Mock) or with a plasmid encoding p85Δ (Δp85) (dominant negative PI-3 kinase mutant) or p110αK227E (p110Δ) (constitutively active PI-3 kinase mutant). The cells were serum-starved and preincubated at 37°C for 3 h in phosphate-free DMEM containing 100 μM unlabeled aFGF (100 ng/ml) and 10 units/ml heparin. aFGF (100 ng/ml) was added, and the incubation was continued for 12 h more. Fifty μM LY294002 (LY) was also present where indicated. The cells were fractionated, and phosphorylated aFGF was collected as described under “Experimental Procedures” and analyzed by SDS-PAGE and autoradiography.

**FIG. 4.** Effect of inhibitors on tyrosine phosphorylation of FGFR1 (A) and on phosphorylation of Ser473 of Akt (B) in NIH 3T3 cells. Serum-starved NIH 3T3 cells were preincubated for 15 min in the presence of heparin (10 units/ml) and 50 μM LY294002 (LY), 100 nM wortmannin (W), 50 μg/ml genistein (GEN) or carrier and then stimulated for 10 min with 100 ng/ml recombinant aFGF or left untreated. The cells were washed once, lysed, and analyzed by Western blotting. A, membrane was first probed with an antiphosphotyrosine antibody (Anti-PY, upper panel), stripped, and reprobed with an anti-FGFR1 antibody (Anti-FGFR1, lower panel). In B, the membrane was first probed with an antibody that recognizes Akt only when phosphorylated on Ser473 (Anti-P-Akt, upper panel), stripped, and reprobed with an antibody that recognizes Akt independent of its phosphorylation status (Anti-Akt, lower panel).
loaded (lower panel). aFGF-induced phosphorylation of Akt (compare lanes 1 and 2) and both LY294002 (lane 3) and wortmannin (lane 4) inhibited Akt phosphorylation. Also genistein (50 μg/ml) (lane 5) inhibited Akt phosphorylation. It may be concluded that although wortmannin and LY294002 do not interfere with autophosphorylation of the receptor, they inhibit downstream signaling from the FGF receptor depending on PI 3-kinase.

**Effect of PI 3-Kinase Inhibition on the Accumulation in the Nucleus of a Fusion Protein of aFGF and Diphtheria Toxin A-fragment Translocated to the Cytosol by the Diphtheria Toxin Pathway**—The observations that in the presence of the PI 3-kinase inhibitors, aFGF-CaAX was not farnesylated (Figs. 3, A and B, lower panel), that[^35S]methionine-labeled aFGF was recovered from the membrane fraction but not from the cytosol or nuclear fractions (Fig. 2), and that aFGF was not phosphorylated by protein kinase C (Fig. 3C) suggested that these drugs prevented transport of aFGF to the nucleus by inhibiting translocation across cellular membranes. However, these data could not exclude the possibility that PI 3-kinase inhibition could also affect the distribution of aFGF between the cytosol and nucleus. To investigate this, we used a fusion protein of aFGF and diphtheria toxin to artificially translocate the growth factor to the cytosol. We could not use NIH 3T3 cells for this purpose as they lack receptors for diphtheria toxin. We therefore used U2OSDr1 (16) and COS 110 (derived from COS 1 cells) cells, both of which are rich in diphtheria toxin receptors but lack FGF receptors. Importantly, both cell lines are resistant to the intracellular action of the toxin.

We demonstrated earlier that when aFGF is fused to the N terminus of diphtheria toxin A-fragment and the fusion protein is reconstituted with diphtheria toxin B-fragment, the fusion protein can be translocated into the cytosol by the diphtheria toxin pathway (16, 18, 61). The translocation can be induced by low pH treatment of cells with surface-bound toxin. Under these conditions the fusion protein was rapidly translocated to the cytosol (Fig. 5A, lane 1). Upon subsequent incubation of the cells, the translocated fusion protein accumulated in the nucleus (lane 4). Accumulation of the fusion protein in the nucleus was slow, as it took about 8 h to be complete (not demonstrated). Nevertheless, it appeared to be due to a nuclear localization sequence (NLS) in the growth factor (16, 20). When this sequence, which is located in the N terminus of the growth factor, was removed, the translocated fusion protein stayed in the cytosol (lanes 5–8). Fusion protein containing a mutant growth factor that is unable to stimulate DNA synthesis in cells (18, 78) but that has an intact NLS was translocated to the nucleus (lanes 9–12) like the wild-type aFGF.

Increasing concentrations of wortmannin inhibited accumulation of the fusion protein in the nucleus in U2OSDr1 cells (which are resistant to the intracellular action of diphtheria toxin) (Fig. 5B) with an IC₅₀ between 15 and 25 nM, and at 50 nM the fusion protein was only recovered from the cytosol fraction. Also, increasing concentrations of LY294002 inhibited accumulation of the fusion protein in the nucleus of these cells, with an IC₅₀ between 5 and 10 μM, and at 20 μM, all was retained in the cytosol (Fig. 5C). Similar results were obtained in COS 110 cells (not demonstrated). The PI 3-kinase inhibitors have also been reported to inhibit other enzymes than PI 3-kinase, although often only at higher concentrations than required for PI 3-kinase inhibition (25, 79). The mammalian target of rapamycin, mTOR, which shares homology with PI 3-kinase, has been reported to be directly inhibited by both LY294002 and wortmannin (79). We therefore tested the effect of rapamycin, which inhibits mTOR as well as p70 S6 kinase downstream of mTOR, on accumulation of aFGF-DT-A in the nucleus. At 100 ng/ml rapamycin, which is a fairly high concentration, the fusion protein was recovered only from the nuclear fraction (Fig. 5C, lanes 9 and 10) as in the control (lanes 1 and 2).

To further investigate whether inhibition of PI 3-kinase was responsible for the effect of wortmannin and LY294002 on nuclear accumulation of aFGF-DT-A, we transfected COS 110 cells transiently with the dominant negative mutant of PI 3-kinase, Δp85α. The transfection efficiency was only 20–30%, as assayed by co-transfection with a plasmid encoding green fluorescent protein and analysis by flow cytometry (not demonstrated), whereas all cells contained endogenous diphtheria toxin receptors. Therefore, even if Δp85α would inhibit completely accumulation of the fusion protein in the nucleus, we could only expect a 20–30% reduction in intensity of the band representing aFGF-DT-A in the nuclear fraction, with a con-
comitant appearance of a weak band in the cytosol fraction. As shown in Fig. 5D, this was what we observed (compare lanes 1 and 2 with lanes 5 and 6).

We recently found that the mutation K132E (78) in aFGF (aFGF(K132E)) renders the growth factor incapable of being phosphorylated in vitro and in vivo (18), apparently because the mutation destroys a consensus site for phosphorylation by protein kinase C. Also the fusion protein aFGF(K132E)-DT-A does not become phosphorylated in vitro in a cell lysate, whereas aFGF-DT-A does (not demonstrated). aFGF(K132E)-DT-A accumulated in the nuclear fraction in a similar manner as aFGF-DT-A (Fig. 5D, lanes 3 and 4), and the accumulation was inhibited by Δp85α (lanes 7 and 8) and LY294002 (not demonstrated).

Together, these data indicate that accumulation in the nuclear fraction of aFGF-DT-A translocated into the cytosol by the diphtheria toxin pathway is dependent on the activity of the heterodimeric PI 3-kinase. Furthermore, the findings argue against the possibility that phosphorylation of the fusion protein itself regulates its accumulation in the nucleus.

**DISCUSSION**

The data here presented demonstrate that two inhibitors of PI3-kinase, wortmannin and LY294002, inhibited transport to the cytosol and nuclear fraction of aFGF added externally to cells. Also, the drugs inhibited accumulation in the nucleus of the fusion protein aFGF-DTA that had been translocated into the cytosol by the diphtheria toxin pathway. The concentrations required to inhibit these processes suggest that in both cases, inhibition of PI 3-kinase was responsible for the effect, since most other targets for these inhibitors require higher concentrations of the drugs (25, 79–81). This notion is also substantiated by the findings that the dominant negative PI 3-kinase mutant, Δp85α, inhibited transport to the cytosol and nucleus of aFGF, as measured by phosphorylation as well as the nuclear accumulation of aFGF-DT-A.

When cells with bound aFGF-DT-A fusion protein reconstituted with DT-B are exposed to low pH, the fusion protein is immediately located in the cytosol (61, 82). Complete accumulation of the fusion protein in the nuclear fraction takes, however, several hours. The reason for the slow kinetics of nuclear accumulation is not clear, but the possibility that aFGF-DT-A is actually shuttling between the cytosol and nucleus is presently under investigation. If this is the case, the slow nuclear accumulation could be the result of competition between nuclear export and binding to nuclear structures retaining the fusion protein in the nucleus. In any event, nuclear accumulation of aFGF-DT-A appears to depend on the NLS of aFGF since ΔaFGF-DT-A, where this sequence has been deleted, remained in the cytosol. The NLS of aFGF has been shown to be able to direct nuclear translocation of a chimeric protein when fused to the N terminus of β-galactosidase and expressed in NIH 3T3 cells (19). However, when full-length aFGF was fused to β-galactosidase, the construct remained in the cytosol, as was the case when aFGF alone was expressed (19). Exogenously added aFGF, on the other hand, was transported to the nucleus (14–22).

With aFGF as such, the transport pathway from the cell surface into the nucleus is not clear. It is not known whether aFGF is first translocated to the cytosol and then to the nucleus or vice versa. For the NLS of aFGF to be functional (20), interaction with cytoplasmic transport proteins appears to be required, which favors the first alternative. The NLS could also function to counteract diffusion from the nucleus into the cytosol. We incubated calf pulmonary artery endothelial cells in the presence of 125I-labeled aFGF for different periods of time, fractionated the cells in membrane, cytosol, and nuclear fractions, and analyzed the fractions by SDS-PAGE. At the earliest time points, aFGF was contained in the membrane fraction. Later, it was also obtained from the nuclear fraction, and finally aFGF was found also in the cytosol fraction. These data argue for a transport mechanism in which aFGF is first translocated into the nucleus and then later exported to the cytosol. However, the data can also be explained by a model in which aFGF is first translocated to the cytosol and then rapidly transported from the cytosol to the nucleus, so that the concentration in the cytosol is below the detection limit in our assay. This issue requires further work.

PI 3-kinase inhibition seems to interfere with nuclear localization at different steps for aFGF and aFGF-DT-A. In the case of aFGF, it appears that a transport step prior to or involved in membrane translocation is blocked by PI 3-kinase inhibition since labeled aFGF was only found in the membrane fraction in the presence of PI 3-kinase inhibitors and since, upon PI 3-kinase inhibition, aFGF-CAAX was not farnesylated, and aFGF was not phosphorylated. It could therefore not have reached even the cytosol.

PI 3-kinase has a well established role in intracellular vesicle transport, from yeast to man. In Saccharomyces cerevisiae the VPS34 gene product, which is the only PI 3-kinase in yeast, is involved in protein sorting to the vacuole (30). In mammals, PI 3-kinase is not required for the initial endocytic process but for later sorting events (26). A mutant platelet-derived growth factor receptor that is deficient in PI 3-kinase binding and activation did not traffic normally to the juxtanuclear region but stayed at the cell periphery (29). It was recently shown that the Rab5 binding human early-endosomal autoantigen, EEA1, binds directly to PI 3-phosphate (34, 35), providing a molecular link between PI 3-kinase and endosome fusion (83). It is therefore quite possible that PI 3-kinase inhibition interferes with intracellular transport of aFGF at a sorting step after endocytosis. If this is the case, it can be invoked that endocytosis is required for membrane translocation of aFGF to take place. For other proteins that translocate across membranes, this is often the case as for e.g. diphtheria toxin (58, 59) and ricin (84). There are, however, also examples of proteins that most likely are able to translocate across the cell surface membrane such as the invasive adenylate cyclase from Bordetella pertussis (85), the VP22 protein of herpesvirus (86, 87) and the Antennapedia homeodomain from Drosophila (88). It should be kept in mind that the data here presented do not rule out the possibility that the effect of PI 3-kinase inhibition is not on intracellular transport but rather on the membrane translocation process as such.

The cytoplasmic retention of aFGF-DT-A observed upon PI 3-kinase inhibition could not be caused by a block in vesicle sorting since the fusion protein was translocated into the cytosol during a brief low pH treatment in the absence of wortmannin and LY294002. In the case of the fusion protein, either transport from the cytosol to the nucleus (89–91), intranuclear binding (92, 93), or exit from the nucleus must have been affected by PI 3-kinase inhibition. Wortmannin blocked the nuclear translocation of protein kinase Cζ (PKCζ) during ischemia, but this result was interpreted as inhibition of protein kinase Cζ activation, since this isotype of protein kinase C is stimulated by PI 3-kinase lipid products (94). Wortmannin also prevented nuclear translocation of mitogen-activated protein kinase in polyomavirus middle-T-transformed cells, but also in this case inhibition of nuclear localization of mitogen-activated protein kinase was coupled with inhibition of its activation (95). Protein kinase B, a signal transducer downstream of PI

3 A. Wiedlocha, unpublished results.
3-kinase, was reported to undergo nuclear translocation within 20–30 min after stimulation (96). Even PI 3-kinase itself was reported to translocate to the nucleus after treatment of PC 12 cells with nerve growth factor (97). However, in all these cases nuclear translocation was coupled to activation of the proteins.

Protein kinase activity has been involved in regulation of transport of proteins to the nucleus. In many cases a phosphorylation site close to a nuclear localization sequence regulates nucleocytoplasmic distribution (90, 98, 99). aFGF is phosphorylated in living cells (18, 100), and both aFGF and aFGF-DT-A are phosphorylated in vitro in a cell lysate (18). However, the nucleocytoplasmic distribution of aFGF(K132E)-DT-A was apparently regulated by PI 3-kinase in the same manner as aFGF-DT-A. aFGF(K132E)-DT-A was not phosphorylated in vitro because the Lys-132Gl answer mutation destroys a consensus site for phosphorylation by protein kinase C, which appears to be the major phosphorylation site in the growth factor (18, 73). Therefore, it is unlikely that the effect of PI 3-kinase inhibition on nucleocytoplasmic distribution is mediated by phosphorylation of aFGF-DT-A.

In conclusion, we have demonstrated the requirement of PI 3-kinase activity for transport of externally added aFGF to the cytosol and nucleus. We have also demonstrated that nuclear accumulation of aFGF-DT-A, translocated to the cytosol by the diphtheria toxin pathway, depends on PI 3-kinase activity. The molecular mechanisms of these observations remain to be elucidated.

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