Acidic fibroblast growth factor (aFGF) added externally to cells has been proposed to enter the nucleus and stimulate DNA synthesis, but it has remained controversial whether or not exogenous aFGF has the capability to enter the nucleus. When incubated with NIH3T3 cells overnight, about one-third of the cell-associated, aFGF-tagged growth factor was farnesylated, indicating that efficient translocation had taken place. Binding to specific FGF receptors was required for translocation to occur. Part of the farnesylated growth factor was found in the nuclear fraction. The data indicate that aFGF-tagged aFGF added externally to cells is able to cross cellular membranes and enter the cytosol and the nucleus.

Acidic fibroblast growth factor (aFGF), which promotes cell differentiation and proliferation (Burgess and Maciag, 1989; Basilio and Moscatelli, 1992), binds to transmembrane receptors containing a cytoplasmic tyrosine kinase domain that is activated upon binding (Johnson and Williams, 1993; Ruta et al., 1989; Mason, 1994). It has remained controversial if aFGF transmits signals only through the receptor or if the growth factor is also translocated into the cells and to the nucleus to stimulate DNA synthesis (Imamura et al., 1990; Cao et al., 1993). We recently demonstrated that aFGF fused to diptheria toxin A fragment could be translocated (microinjected) by the diphtheria toxin pathway to the cytosol and to the nucleus and stimulate DNA synthesis in cells lacking functional aFGF receptors (Wiedlocha et al., 1994). Clearly, therefore, intracellular growth factor can convey a biological signal.

Morphological and biochemical evidence for nuclear targeting of externally added aFGF and certain other growth factors has been provided by several groups (Imamura et al., 1990; Baldin et al., 1990; Kimura, 1994; Wiedlocha et al., 1994; Moroianu and Riordan, 1994; Imamura et al., 1994). A major problem in such studies is to demonstrate that the protein has really crossed cellular membranes to gain access to the cytosol or to the nucleus. Alternatively, it could be present in intracellular vesicles or cytoskeletal compartments that may have a juxtanuclear location (Prudovsky et al., 1994), or it could be trapped in cytoskeletal material adhering to the nucleus upon disrupting or dissolving the cells.

To test for translocation in a more rigorous manner, we have developed a novel principle to study whether or not a protein is exposed to the cytosol. We added to aFGF a C-terminal CAAX (C = Cys, A = an aliphatic amino acid, and X = any amino acid) motif, which, upon exposure to the cytosol, will be modified by attachment of a prenyl group followed by proteolytic removal of the last three amino acids and carboxyl methylation of the appearing C-terminal cysteine (Clarke, 1992; Cox and Der, 1992). Prenylating enzymes have been observed only in the cytosol (Reiss et al., 1990; Schaber et al., 1990; Casey et al., 1991) and possibly in the nucleus (Lutz et al., 1992; Sinensky et al., 1994), and there is no indication that prenylation occurs at the cell surface or inside compartments of the endocytic pathway. In the present study, we have found that externally added aFGF is efficiently farnesylated by cells, indicating that translocation of the growth factor to the cytosol and to the nucleus does indeed take place.

**EXPERIMENTAL PROCEDURES**

Buffers and Media—HEPES medium consisted of bicarbonate- and serum-free Eagle’s minimal essential medium buffered with HEPES to pH 7.4. Lysis buffer consisted of 0.1 M NaCl, 20 mM NaH2PO4, 10 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, pH 7.4. PBS consisted of 140 mM NaCl, 10 mM NaH2PO4, pH 7.4. Cell Cultures—NIH3T3 cells and the human osteosarcoma cell line U2OS Dr1 were propagated as earlier described (Wiedlocha et al., 1994). Cells were seeded into Costar (Cambridge, MA) microtiter plates or 25-cm² Falcon flasks the day preceding the experiments.

Transfection of Cells—The expression plasmid LTRFGFR4 encoding FGF receptor 4 (Partanen et al., 1993) was a kind gift from Dr. Aaltala. pMamNeo encoding the neomycin phosphotransferase gene was obtained from Clontech. U2OS Dr1 cells were seeded out in 10-cm Petri dishes (10 cells per dish) in Dulbecco’s modified minimal essential medium (DMEM) containing 5% fetal calf serum (FCS). The next day the cells were washed in serum-free medium, and then 2.5 ml of serum-free DMEM, containing 5 μg of LTRCFGFR4 and 5 μg of pMamNeo DNA, and 2.5 ml of a 1 mg/ml aqueous solution of DOTAP (Boehringer Mannheim), which had previously been vortexed and left for 5 min at room temperature, was added. The cells were incubated for 5 h at 37 °C with occasional careful shaking. Then 250 μl of FCS and 2.5 ml of medium containing 5% FCS was added, and the cells were incubated overnight. The next day, the medium was changed, the cells were trypsinized, diluted 1:3, and seeded out into new Petri dishes. After 4 days the medium was removed, and DMEM containing 5% FCS and 1 mg/ml...
gentamycin was added. Small colonies developed after 2 weeks. The cells were then incubated further in DMEM containing 0.5% FCS and 10 ng/ml aFGF. Colonies that grew under these conditions were tested for aFGF-stimulated incorporation of \([^{3}H]H_{3}P_{3}H_{3}\) as described (Wiedlocha et al., 1994). One transfected (U2OS DR1 R4) was selected.

Plasmid Construction—E. coli strain DH5\(_{a}\) was used in the cloning procedures. DNA for aFGF (Immura et al., 1992; Wiedlocha et al., 1994) was cloned into pTrc-99A (Pharmacia Biotech Inc.) for expression in bacteria (pTrc-aFGF). To introduce a C-terminal CAAX motif, an extension encoding Cys-Val-Ile-Met (TGGGTAACTCATGTAATGGA) was generated by polymerase chain reaction, cloned into the aFGF DNA by standard techniques, cloned into pBluescribe (Stratagene) for expression in a cell-free system (pHBGF-cax), and cloned into pTrc-99A for expression in bacteria (pTrc-aFGF-cax). The plasmid encoding dتا-CAAX was constructed as described by Faînes et al. (1995).

Expression and Purification of Recombinant Proteins—pTrc-aFGF and pTrc-aFGF-cax in E. coli DH5\(_{a}\) were induced with 5 mM isopropyl-\(\beta\)-D-thiogalactopyranoside. The bacterial pellet was suspended in 20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA and sonicated. The supernatant was applied to a heparin cartridge (Bio-Rad), and the bound material was eluted with an NaCl gradient (0.2–2 M) in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA.

Microsomes

| aFGF-CaX | aFGF |
|---------|------|
| B 581   | +    |

**RESULTS**

Acidic Fibroblast Growth Factor with a CAAX Tag Is Biologically Active—The farnesylation signal (Cys-Val-Ile-Met) of the K-Ras-4B protein has been shown to be sufficient for farnesylation both in vitro (Goldstein et al., 1991) and in vivo (Hancock et al., 1991a), and we added this CAAX motif to the C terminus of aFGF. When recombinant aFGF-CAAX and \([^{3}H]H_{3}P_{3}H_{3}\)farnesylphosphate were incubated in a reticulocyte lysate in the presence of \([^{3}H]farnesyl\) pyrophosphate in the absence and presence of microsomes and 50 \(\mu\)M B581. The samples were then immunoprecipitated with immobilized anti-aFGF antibodies and analyzed by SDS-PAGE and fluorography. In lane 1 is non-farnesylated \([^{3}S]methylone-labeled aFGF-CAAX for comparison.**

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During the last 6 h, the cells were incubated with 1 unit/ml heparin, and the incubation was continued for 24 h at 37°C. Amounts of aFGF or aFGF-C AAX incubated for 48 h in serum-free medium at 37°C; then, increasing amounts of aFGF or aFGF-C AAX were added with and without 10 units/ml heparin, and the incubation was continued for 24 h at 4°C with cells growing as monolayers in 24-well microtiter plates at a density of 5 × 10^4 cells/well. The cells were washed, and the bound radioactivity was measured. We and others (Zhan et al., 1992; Wiedlocha et al., 1994) have earlier found that when NIH3T3 cells are incubated with labeled aFGF, part of the growth factor is recovered in the nuclear fraction. In the experiments in Fig. 3B, we therefore analyzed both the cytoplasmic and the nuclear fractions for material adsorbing to heparin-Sepharose and to immobilized anti-aFGF. When cells were incubated with aFGF in the presence of labeled mevalonate, as expected no labeled growth factor was, like aFGF (Imamura et al., 1990), adsorbed to heparin-Sepharose at high ionic strength (lane 4). In the case of aFGF, no labeled material was adsorbed to heparin-Sepharose (lane 5).

To compare the labeling of aFGF-C AAX with that of endogenous prenylated proteins, we analyzed the total trichloroacetic acid-precipitable material in the cytoplasmic fraction. The results (lane 6) showed a labeled band migrating as expected for prenylated aFGF-C AAX (position indicated with an asterisk). There was no band at the same position in cells treated with aFGF (lane 7). The labeling extent of externally added aFGF-C AAX was comparable to that of prenylated endogenous proteins in the cells, such as the small G proteins (lane 6). There was no labeling when aFGF-C AAX was added to lysed cells on ice, excluding the possibility that the labeling seen in Fig. 3A could have occurred after lysis of the cells (data not shown).

Fig. 2. Ability of aFGF-C AAX to bind to and stimulate DNA synthesis in NIH3T3 cells. A, to measure binding, 5 ng/ml 125I-aFGF and increasing concentrations of unlabeled aFGF or aFGF-C AAX in the presence of 10 units/ml heparin were incubated for 2 h at 4°C with cells growing as monolayers in 24-well microtiter plates at a density of 5 × 10^4 cells/well. The cells were washed, and the bound radioactivity was measured. B, to measure [3H]thymidine incorporation, cells were preincubated for 48 h in serum-free medium at 37°C; then, increasing amounts of aFGF or aFGF-C AAX were added with and without 10 units/ml heparin, and the incubation was continued for 24 h at 37°C. During the last 6 h, the cells were incubated with 1 μCi/ml [3H]thymidine, and the incorporated radioactivity was measured.

Fig. 3. Farnesylation of aFGF-C AAX in NIH3T3 cells. A, serum-starved cells were incubated with 10 units/ml heparin and 100 ng/ml aFGF or aFGF-C AAX overnight in the presence of lovastatin and labeled mevalonate. The cells were lysed, and the cytoplasmic fraction was divided into three parts. One part was treated with anti-aFGF adsorbed to protein A-Sepharose, and one part was treated with heparin-Sepharose, which was subsequently washed with 0.7 M NaCl. The second part was treated with 5% trichloroacetic acid. The precipitated material was analyzed by SDS-PAGE and fluorography. The position of small GTP-binding proteins (Small G) and of farnesylated aFGF-C AAX (asterisk) are indicated. B, increasing amounts of aFGF or aFGF-C AAX were added to 2 × 10^6 serum-starved cells in the absence and presence of 50 μM B581. The cells were incubated overnight and lysed. Growth factor in the cytoplasmic fraction and in material extracted with 0.5 M NaCl from the sonicated nuclei was precipitated with heparin-Sepharose (upper panel) or with anti-aFGF-protein A-Sepharose (lower panel). In all cases, the material was analyzed by SDS-PAGE and fluorography.
factor was precipitated with heparin-Sepharose (upper panel) or with immobilized anti-aFGF (lower panel) either from the cytoplasmic (lanes 2 and 3) or from the nuclear fractions (lanes 8 and 9). When aFGF-CAAX was used instead, labeled growth factor was precipitated from both fractions, both with immobilized heparin and immobilized anti-aFGF (lanes 4, 5, and 10, 11). The relative amount of growth factor in cytoplasm and nucleus varied somewhat between experiments. In the presence of the farnesylation inhibitor B581, no labeling was observed (lanes 6 and 12). The results indicate that externally added aFGF-CAAX is indeed farnesylated by the cells and that part of the modified protein migrates to the nucleus.

Requirement of FGF Receptors for in Vivo Farnesylation of aFGF-CAAX—To test if binding to FGF receptors is required for farnesylation, NIH3T3 cells were incubated with aFGF-CAAX in the absence and presence of excess aFGF, which competes out binding of aFGF-CAAX to the specific receptors but not to surface heparans. Fig. 4A, lane 2, shows that aFGF-CAAX was not labeled in the presence of excess aFGF. Binding of aFGF-CAAX to specific receptors therefore appears to be required for translocation of the growth factor to the cytosol.

The possibility existed that the labeling obtained was derived from a subpopulation of cells that, although gently treated, could have been damaged but not killed, and that the growth factor could have entered the cells by a mechanism similar to that in scrape loading (Frankel and Pabo, 1988; Leeners and Marshall, 1992; Morris et al., 1993). To test this, we first measured the ability of NIH3T3 cells to modify a protein that does not bind to the cells, viz. diphtheria toxin A fragment with a C-terminal CAAX motif (dtA-CAAX). This protein is efficiently farnesylated in vitro (data not shown). The cells were incubated with increasing concentrations of dtA-CAAX and subjected to the process of scrape loading. A faint 20-kDa band was observed when 100 μg/ml of the construct had been present in the medium (Fig. 4B, lane 8). This is 10^4 times the concentration required to detect labeled aFGF-CAAX in these cells (lane 2).

U2OS Dr1 cells lack specific aFGF receptors, but the growth factor binds extensively and with high affinity to surface heparans on the cells (Sakaguchi et al., 1991; Wieełocha et al., 1994). In these cells farnesylation of the growth factor was observed only after exposure to 100 μg/ml aFGF-CAAX and scrape loading (Fig. 4B, lanes 10–13). These data indicate that the farnesylation of aFGF-CAAX in lanes 2–4 was not due to “leakage” of the growth factor into wounded cells, and they support the finding above that specific FGF receptors are required for translocation.

When the U2OS Dr1 cells were transfected with FGF receptor 4 (FGFR4), they bound aFGF specifically in the sense that excess unlabeled aFGF in the presence of heparin prevented the binding (Fig. 5A). The untransfected cells bound very little aFGF in the presence of heparin. When aFGF-CAAX was added to these cells in the presence of labeled mevalonic acid, a strong band corresponding to the growth factor appeared (Fig. 5B, lane 2), which was absent in the untransfected cells (lane 1). In the presence of excess unlabeled growth factor, only a weak band appeared (lane 3). Clearly, farnesylation of externally added aFGF-CAAX was dependent upon binding to the specific receptor in U2OS Dr1 R4 cells.

Partitioning into Triton X-114 of Farnesylated aFGF-CAAX—To estimate how much of the cell-associated aFGF-CAAX becomes farnesylated, we used Triton X-114 partitioning. Farnesylated Ras has been shown to enter the detergent phase when submitted to Triton X-114 partitioning (Gutierrez et al., 1989). This was also the case with ^35S)methionine-labeled aFGF-CAAX, which had been farnesylated in vitro (Fig. 6A, lanes 1 and 2), but not when the farnesylation had been prevented by B581 (lanes 3 and 4).

When NIH3T3 cells were incubated with ^35S)methionine-labeled aFGF and then treated with Triton X-114, all the labeled protein partitioned into the water phase (Fig. 6B, lanes 1 and 2). On the other hand, when aFGF-CAAX was used, part of the protein partitioned into the detergent phase (lanes 3 and 4). This was not the case when the cells had been incubated with labeled aFGF-CAAX in the presence of the farnesylation inhibitor B581 (lanes 5 and 6). Densitometric analysis indicated that approximately one-third of the cell-associated aFGF-CAAX was present in the detergent phase and therefore farnesylated.

Experiments with ^125I)-aFGF-CAAX indicated that ~200,000 molecules were associated with each cell after overnight incubation under conditions as in Fig. 6B. Since not all aFGF-CAAX molecules that enter the cytosol may be farnesylated (see “Discussion”), this indicates that at least 70,000 molecules of aFGF-
The main conclusion from the present work is that externally added aFGF-CAAAX (and therefore also aFGF) is able to cross cellular membranes to gain access to the cytosol and/or the nucleus with 0.5 M NaCl from the sonicated nuclei was mixed and submitted to immunoprecipitation with anti-aFGF-protein A-Sepharose.

AAX per cell entered the cytosol.

Kinetics of Prenylation of Externally Added aFGF-CAAX—When cells were incubated with aFGF-C-CAAAX and labeled mevalonate acid for increasing periods of time, the extent of labeling increased considerably between 8 and 12 h, but further incubation for an additional 12 h did not increase the labeling detectably (Fig. 7). This is consistent with previous observations that the entry into the cell occurs mainly in the late G1 stage (Friedman et al., 1994; Imamura et al., 1994).

DISCUSSION

The main conclusion from the present work is that externally added aFGF-CAAX (and therefore also aFGF) is able to cross cellular membranes to gain access to the cytosol and/or the nucleus. FGFR receptors appear to be required for this, as aFGF-CAAX was not farnesylated when added to U2OS Dr1, a human osteosarcoma cell line, which does not express measurable amounts of functional FGFR receptors and which does not respond to addition of aFGF with increased growth or with increased thymidine incorporation (Wie ¸dłocha et al., 1994). While these cells bind aFGF extensively in the absence of heparin, no binding was detected in the presence of heparin (Wie ¸dłocha et al., 1994), indicating that binding occurs only to surface heparans. The data therefore indicate that binding to specific FGFR receptors is required for translocation to the cytosol.

Scrape loading has been used to introduce exogenous proteins into the cytosol, such as the Tat-encoded protein from human immunodeficiency virus (Franke and Pabo, 1988), human papillomavirus E7 protein (Morris et al., 1993), and p21ras (Leevers and Marshall, 1992). In these cases, extracellular protein concentrations in the range of 0.1–1 ng/ml were required to obtain measurable biological effects. This is similar to the concentration of aFGF-CAAX here found to be required to detect farnesylation in cells lacking FGFR receptors, as well as of dTAAAX, which does not bind to cells. We therefore consider it unlikely that the farnesylation detectable in NIH3T3 cells at 10 ng/ml of extracellular aFGF-CAAX is due to growth factor that enters through occasional and transient membrane defects in the cells.

The Triton X-114 partitioning experiments indicated that about one-third of the total cell-associated aFGF-CAAX is farnesylated, which may amount to ~70,000 molecules per cell. The rest may either still be bound to cell surface receptors or it could be located in endocytic vesicles and other membrane-bound compartments. Also, some of the CAAX-tagged growth factor present in the cytosol or in the nucleus may for some reason not be farnesylated. If farnesylation of aFGF-CAAX can...
only occur in the cytosol, transport of the unmodified growth factor to the nucleus could prevent farnesylation from reaching completion. Our recent data demonstrate that farnesylation of CAAX-modified diphtheria toxin A fragment is completed only 1 h after translocation to the cytosol (Falnes et al., 1995).

Although farnesylation can only be taken as evidence for transport to the cytosol, once present in the cytosol the growth factor can migrate into the nucleus (Imamura et al., 1992; Cao and Pettersson, 1993). Also, the farnesylated growth factor is partly found in the nucleus. In the experiment shown in Fig. 3B, the major part is in the cytoplasmic fraction, whereas in other experiments more was found in the nuclear fraction. The reason for this variation is not clear. The ability of aFGF to locate to the nucleus has been found to depend upon the presence of a positively charged sequence near the N terminus, and removal of this sequence rendered the growth factor unable to stimulate RNA synthesis but not to bind to and activate the specific FGFR receptors (Imamura et al., 1990; Wiedlocha et al., 1994).

Unmodified aFGF could migrate more efficiently into the nucleus than the farnesylated growth factor, which may occur at the level of the cell surface membrane or across the membranes of intracellular vesicles. It has recently been demonstrated that the translocation of the growth factor takes place. Certain protein transport mechanisms developed by the cells for other purposes, such as transport of certain growth factors.

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