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Effect of mutation of cytoplasmic receptor domain and of genistein on transport of acidic fibroblast growth factor into cells

Raquel Muñoz, Olav Klingenberg, Antoni Więdłocha, Andrzej Rapak, Pål Ø Falnes and Sjur Olsnes

Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

Acidic fibroblast growth factor (aFGF) binds to specific transmembrane receptors and is partly transported to a nuclear location. To study this transport we made a kinase-negative mutant of FGF receptor 4 as well as one where the major part of the cytoplasmic receptor domain was deleted, and expressed them in U2OSDr1 cells that lack functional FGF receptors. All receptors mediated endocytic uptake of aFGF. Translocation of the growth factor across cellular membranes was assayed using aFGF with a C-terminal CAAX-motif, which signals addition of a farnesyl group onto the protein once in the cytosol. CAAX-tagged aFGF was farnesylated when incubated with cells containing wild-type or kinase-negative receptors. It was not farnesylated in cells expressing the deleted receptor, or when the incubation was in the presence of genistein. aFGF incubated with cells transfected with wild-type or kinase-negative receptors, but not with the deleted receptor, was partly recovered from the nuclear fraction in the absence, but not in the presence of genistein. The data indicate that the cytoplasmic receptor domain, but not the active kinase, is required for transport of the growth factor into cells, and that genistein inhibits the process.

Keywords: acidic fibroblast growth factor; receptor mutant; genistein; farnesylation

Introduction

Acidic fibroblast growth factor (aFGF) belongs to a family of nine related growth factors (Burgess and Maciag, 1989; Mason, 1994). It induces morphological changes or mitosis in a variety of cells (Burgess and Maciag, 1989; Basilico and Moscatelli, 1992; Crumley *et al*., 1991). The growth factor binds to specific FGF receptors carrying a split tyrosine kinase domain in their cytoplasmic part. There are four known FGF receptor genes and a number of splicing variants of three of them (Hou *et al*., 1991; Johnson *et al*., 1990; Johnson *et al*., 1991; Chellaiah *et al*., 1994). Binding of the growth factor induces tyrosine phosphorylation of the receptor with subsequent downstream signalling by phospholipase Cγ and by activation of MAP-kinase and early genes (Mason, 1994). aFGF does not have a typical leader sequence for secretion through the endoplasmic reticulum and Golgi apparatus. After synthesis as a cytosolic protein, the growth factor is exported from the cells by an unknown mechanism (Jackson *et al*., 1992). aFGF and some other members of the FGF family (bFGF, FGF3) have been found in the nucleus of cells producing the growth factors (Kiefer *et al*, 1994; Bonnet *et al*., 1996; Stachowiak *et al*., 1996a; Pintucci *et al*., 1996; Riese *et al*., 1995; Cao *et al*., 1993; Imamura *et al*., 1992). Evidence is now accumulating that also externally added aFGF, after binding to its surface receptors, enters the cell and apparently the nucleus (Imamura *et al*., 1990; Zhan *et al*., 1992, 1993; Imamura *et al*., 1994; Więdłocha *et al*., 1994).

The receptor-bound growth factor is taken up by endocytosis and appears to be transported along with the receptor to a perinuclear compartment (Prudovsky *et al*., 1994). It is not clear where translocation across the membrane takes place. Also, it is not known if the growth factor is first translocated to the cytosol and then to the nucleus, or if it is translocated directly into the nucleus from a perinuclear compartment. There is also some evidence that aFGF as well as bFGF are localized in the nucleus together with their receptor (Johnston *et al*., 1995; Maher, 1996; Stachowiak *et al*., 1996a).

Direct evidence for translocation of the growth factor into cells was obtained in experiments where aFGF was engineered to contain a C-terminal farnesylation signal, a CAAX-motif (Więdłocha *et al*., 1995). Since the farnesyl transferase is located in the cytosol, and possibly in the nucleus (Lutz *et al*., 1992; Sinensky *et al*., 1994), the fact that externally added growth factor was farnesylated by the cells indicates that exogenous aFGF is translocated across cellular membranes to a compartment where farnesylation takes place. Further experiments are required to elucidate if the translocation occurs at the level of the plasma membrane, or from intracellular vesicles and cisternal compartments, such as endosomes, the Golgi apparatus or the endoplasmic reticulum.

Recently we reported that once inside the cells the growth factor is able to stimulate DNA-synthesis in tumor cells without detectable levels of FGF receptors. Thus, when aFGF was fused to diphtheria toxin and translocated along with the toxin to the cytosol of cells lacking FGF receptors and resistant to the intracellular action of diphtheria toxin, it stimulated cellular DNA synthesis in monkey kidney (Vero) cells and in a human osteosarcoma (U2OSDr1) cell line (Więdłocha *et al*., 1994). The stimulating effect depended upon the nuclear localization sequence present in the N-terminal part of the growth factor (Imamura *et al*., 1990). When this sequence was removed, the fusion protein did not migrate to the nucleus and there was no stimulation of DNA synthesis (Więdłocha *et al*., 1994).

In attempts to elucidate mechanisms involved in the transport of aFGF to the nucleus, we have in the

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present work studied the role of the cytoplasmic tail and of the tyrosine kinase of FGF receptor 4 in internalization of the growth factor and in its transport to the nuclear fraction.

Genistein is an inhibitor of tyrosine kinases (Akiyama *et al*., 1987) and it has been described to inhibit receptor-mediated endocytosis and mitogenic response to growth factors, although the concentrations of the drug required to inhibit mitogenesis are commonly found to be lower than those required to inhibit tyrosine phosphorylation in the cells (Koroma and de Juan, Jr, 1994; Linassier *et al*., 1990). We have here tested the effect of genistein on endocytic uptake and on transport of the growth factor to the nuclear fraction.

Results

Binding of aFGF to untransfected and transfected U20SDr1 cells

To test the role of the FGF receptor on transport of aFGF to the nucleus, U2OSDr1 human osteosarcoma cells, which do not express measurable amounts of endogenous FGF receptors (Więdłocha *et al*., 1996), were stably transfected with FGF receptor 4 (FGFR4), with a kinase-negative mutant receptor 4 (FGFR4-K503R) or with a deletion mutant (FGFΔR4) where most of the cytoplasmic part had been removed (Figure 1a). The transfection was verified by reverse transcription of cellular mRNA followed by PCR as described by Brogi *et al*. (1993) (data not shown) as well as by covalent cross-linking of ^{125}I-aFGF to the receptors (Figure 1b). In untransfected cells (U2OSDr1) no crosslinked material was observed (lanes 1–3) whereas in cells transfected with wild-type FGFR4 (U2OSDr1R4) a labelled band migrating corresponding to ~130 kD (lane 6) was observed. This appears to represent ^{125}I-aFGF (molecular mass 16 kD) crosslinked to FGFR4 (molecular mass ~110 kD). While heparin did not prevent the crosslinking (lane 4), excess unlabelled aFGF in the presence of heparin completely prevented crosslinking of ^{125}I-aFGF to the receptor (lane 5).

In cells transfected with the deletion mutant (U2OSDr1ΔR4), the crosslinked material migrated corresponding to ~95 kD (lanes 7, 8), as expected from the predicted MW of aFGF crosslinked to the deleted receptor. Also in this case unlabelled aFGF prevented crosslinking of ^{125}I-aFGF to the receptor (lane 9).

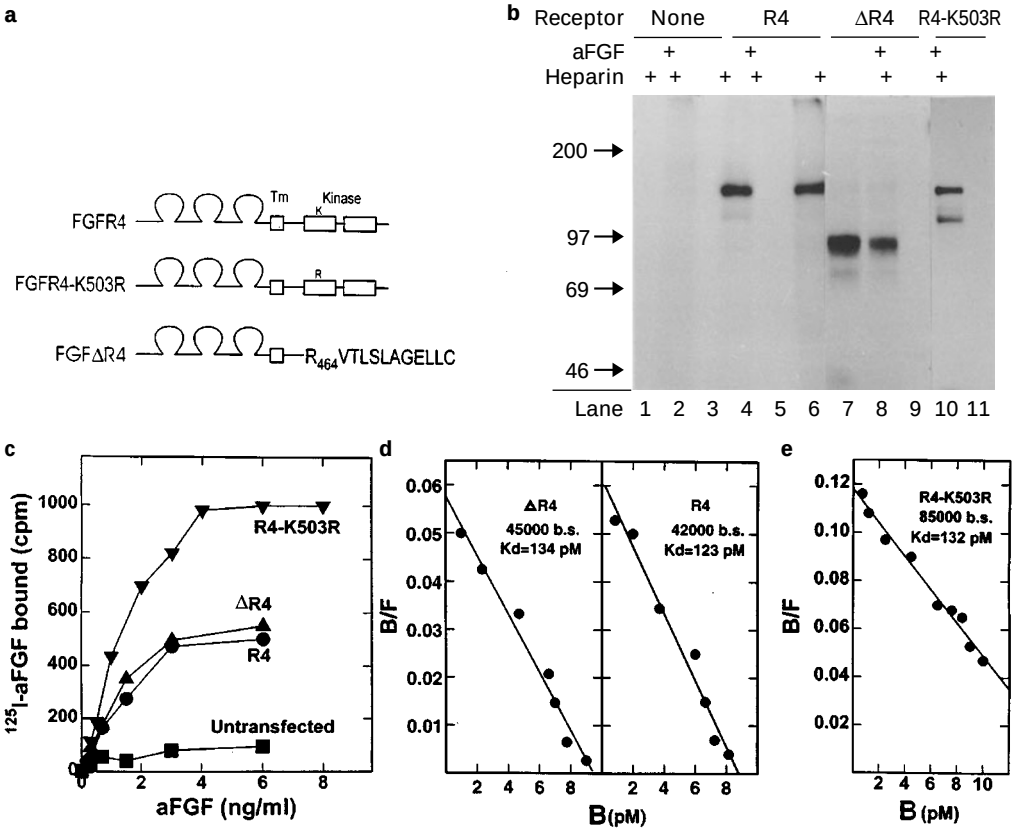

Figure 1 Ability of aFGF to bind to U2OSDr1, U2OSDr1R4 and U2OSDr1ΔR4 cells. (**a**) Schematic representation of wild-type (FGFR4), kinase-negative (FGFR4-K503R) and deleted (FGFΔR4) receptor. Tm, transmembrane domain; kinase, split tyrosine kinase domain; (**b**) U2OSDr1 cells were transfected with wild-type (FGFR4), with the deletion mutant (FGFΔR4) and with the kinase-negative (FGFR4-K503R) receptor. ^{125}I-aFGF was added to the cells for 4 h at 4°C. In some cases 10 U/ml of heparin and a 100-fold excess of unlabelled aFGF was present. Then the cells were treated for 20 min at 4°C with 0.3 mM disuccinimidyl suberate to induce crosslinking, and analysed by SDS–PAGE and autoradiography. (**c**) Binding of ^{125}I-aFGF to untransfected and transfected cells was measured by adding increasing concentrations of the labelled growth factor to cells at 4°C in the presence of 10 U/ml of heparin. After 4 h, unbound growth factor was removed by washing and the amount of radioactivity associated with the cells was measured (Więdłocha *et al*., 1996). (**d**, **e**) The binding data in **c** were analysed according to Scatchard (1949)

In cells transfected with the kinase-negative mutant (U20SDr1R4-K503R) crosslinking gave a band of ~130 kD in the absence, but not in the presence of excess unlabelled growth factor (lanes 10, 11).

U2OSDr1R4 and U2OSDr1ΔR4 cells bound approximately the same amount of ^{125}I-aFGF, whereas U20SDr1R4-VK503R bound approximately twice as much (Figure 1c). Scatchard analysis of the data indicated that the binding affinity was about the same in all cases (Figure 1d and e).

Effect of genistein on synthesis of protein and DNA

In experiments to be described below, we tested the effect of genistein, an inhibitor of tyrosine kinases, on the transport of aFGF into cells. For this purpose, we first tested to what extent the inhibitor was toxic to the U2OSDr1 cells. At low concentrations genistein did not substantially inhibit protein synthesis in serum-starved, aFGF-stimulated cells, and at 50 μM it reduced [^{3}H]leucine incorporation only to about the half (Figure 2a). On the other hand, [^{3}H]thymidine incorporation in response to aFGF in serum-starved cells was inhibited at much lower concentrations of the drug. In unstarved cells 1.5–2 times higher genistein concentrations were required for inhibition of [^{3}H]thymidine incorporation (not demonstrated). Genistein (74 μM) did not interfere with the binding of aFGF to its receptor (Figure 2b).

Effect of receptor mutation and of genistein on translocation of the growth factor into cells

Cytoplasmic proteins carrying a C-terminal CAAX motif (C = Cys, A = an aliphatic amino acid, X = any amino acid) are isoprenylated (farnesylated or geranylgeranylated (Casey and Seabra, 1996)). The sequence CVIM signals farnesylation. Since the farnesyl transferases are located in the cytosol and possibly in the nucleus (Chang *et al*., 1993; Lutz *et al*., 1992), farnesylation of an extracellular protein carrying a CAAX sequence can be taken as evidence for translocation of the protein into the cytosol or the nucleus. We have earlier demonstrated that upon incubation with cells containing the appropriate receptors, both diphtheria toxin and aFGF carrying a C-terminal CAAX-tag become farnesylated, indicating their translocation to the cytosol (Więdłocha *et al*., 1995; Falnes *et al*., 1995).

When aFGF modified to contain the C-terminal CAAX-motif, CVIM, was incubated with U2OSDr1R4 cells or U20SDr1R4-K503R cells, the growth factor was farnesylated (Figure 3a, lane 2). Growth factor lacking the CAAX tag was not farnesylated (lane 1). We here found that the farnesylation of the growth factor was inhibited with increasing concentrations of genistein (lanes 3–7). In the presence of 19 μM genistein it was virtually abolished. Genistein did not inhibit prenylation of a ~20 kD protein, probably a

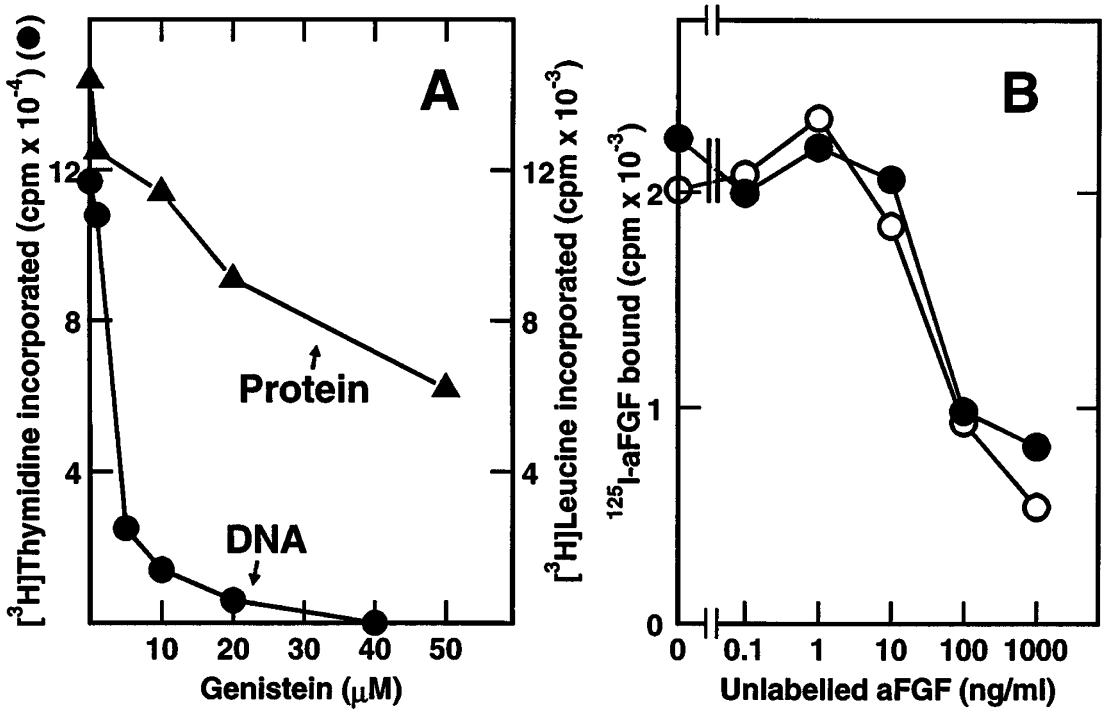

Figure 2 Effect of genistein on incorporation of [^{3}H]thymidine and [^{3}H]leucine in U2OSDr1R4 cells, and on binding of ^{125}I-aFGF to the receptor. (**a**) Cells were preincubated for 72 h in serum-free medium at 37°C, then increasing amounts of genistein, 10 ng/ml of aFGF and 10 U/ml heparin was added and the incubation was continued for 24 h more. During the last 6 h, the cells were incubated with 1 μCi/ml [^{3}H]thymidine, and the incorporated, TCA-precipitable radioactivity was measured. To measure [^{3}H]leucine incorporation, cells were serum-starved and incubated with aFGF and genistein as above. After 24 h the cells were washed three times with leucine-free medium, 1 μCi/ml [^{3}H]leucine was added and the incubation was continued for 30 min. Finally, the amount of TCA-precipitable radioactivity associated with the cells was measured. (**b**) Binding of ^{125}I-aFGF was measured by adding to cells 10 ng/ml of ^{125}I-aFGF and increasing concentrations of unlabelled aFGF in medium containing 10 U/ml heparin and in the absence (○) and presence (●) of 74 μM genistein. After slight agitation for 4 h at 4°C, the cells were washed and the amount of radioactivity associated with the cells was measured

small G-protein, that adhered to the immunoprecipitates. Furthermore, control experiments where CAAX-tagged diphtheria toxin (Falnes *et al*., 1995) was incubated with the cells in the presence of 74 μM genistein resulted in farnesylation of the toxin upon translocation to the cytosol (data not shown). These findings exclude the possibility that genistein inhibits the farnesylation reaction as such. In U2OSDr1ΔR4 cells there was no detectable farnesylation of the CAAX-tagged growth factor (data not shown).

When cells containing FGF-receptors are incubated with labelled aFGF, part of the growth factor is recovered from the nuclear fraction (Więdłocha *et al*., 1994). The data in Figure 3b show that this was also the case with U2OSDr1R4 cells. The presence of 19 μM genistein blocked transport to the nuclear fraction (lane 6). No labelled aFGF was recovered from the nuclear fraction of U2OSDr1ΔR4 cells (lane 14). In all cases labelled material was found in the cytoplasmic fraction, which contains material from intracellular vesicular organelles in addition to material in the cytosol.

Also in U20SDr1R4-K503R cells a considerable part of the cell-associated growth factor was found associated with the nucleus in the absence, but not in the presence of genistein (Figure 3c). In this case the growth factor in the cytosolic fraction was partially degraded. Degradation of aFGF in the cytosolic fraction was observed in many experiments also with the wild-type receptor and can therefore not be attributed to the mutation. The growth factor in the nuclear fraction was never visibly degraded. In the presented experiments the incubation with the labelled growth factor was for 10 h, but also after incubation for only 2–4 h growth factor was detectable in the

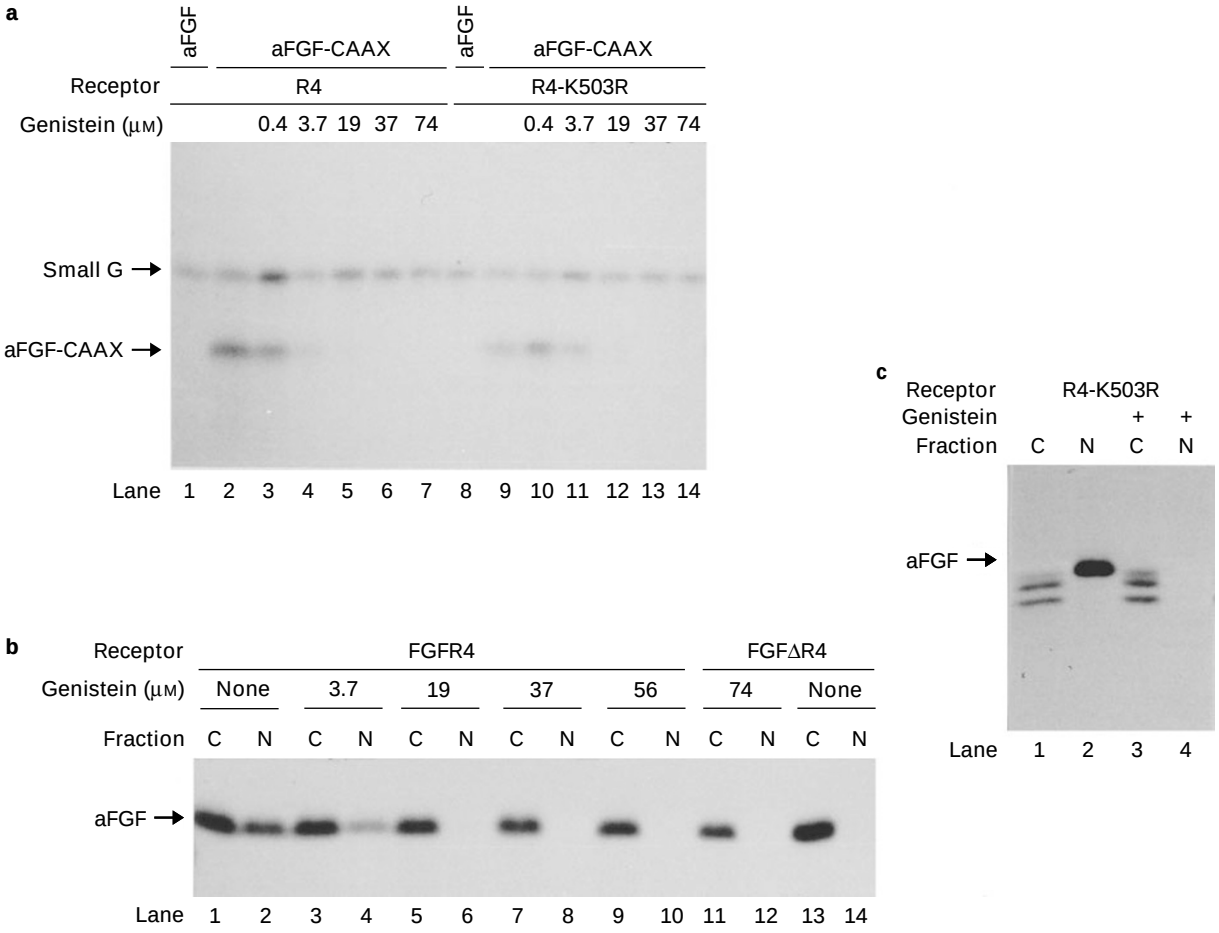

Figure 3 Effect of genistein and of receptor mutation on farnesylation of aFGF-CAAX by transfected cells (**a**), and on transport to the nuclear fraction of labelled aFGF (**b**, **c**). (**a**) Cells growing in 25 cm^2 flasks were serum-starved for 48 h and then preincubated over night in serum-free medium containing 5 μCi/ml [^{3}H]mevalonic acid, 1 μCi/ml [^{14}C]mevalonic acid and 10 μg/ml lovastatin. aFGF or aFGF-CAAX (100 ng/ml) was added and the cells were incubated for 10 h. The cells were then washed twice with HEPES medium containing 5 U/ml heparin, and three times with HEPES medium without heparin, then lysed and centrifuged for 5 min at 12 000 *g* at 4°C. The supernatant was taken as the cytoplasmic fraction. The nuclear pellets were sonicated and extracted with 0.7 M NaCl. After clarification by centrifugation for 5 min at 12 000 *g* at 4°C, the extract was diluted in PBS containing 0.1% Triton X-100, mixed with the cytosolic fraction and treated with anti-aFGF immobilized on Protein A-Sepharose-4B. The bound material was analysed by SDS–PAGE and fluorography. (**b**, **c**), Subconfluent U2OSDr1R4 and U2OSDr1ΔR4 cells (**b**) or U2OSDr1R4-K503R cells (**c**) were starved for 48 h in DMEM without serum. The cells were incubated for 10 h in the presence of 10 U/ml of heparin and 10 ng/ml [^{35}S]methionine-labelled aFGF. Then the cells were washed twice with incubation medium containing 5 U/ml heparin and three times with medium without heparin, and subsequently lysed in lysis buffer containing 1 mM PMSF and 1 μg/ml of aprotinin. The lysate was fractionated by centrifugation into a cytosolic (C) (supernatant) and a nuclear (N) fraction (pellet). The nuclear fraction was washed twice by resuspension in lysis buffer containing 0.3 M sucrose and 1 mM $MgCl_2$, layered over 0.8 ml of 0.7 M sucrose, and centrifuged for 15 min at 3000 r.p.m. at 4°C. Then the nuclei were sonicated and extracted by treatment with 0.7 M NaCl in PBS. The cytosolic fraction and the nuclear extract were subjected separately to Heparin-Sepharose adsorbtion and analysed by SDS–PAGE and fluorography

nuclear fraction in the absence, but not in the presence of genistein (data not shown).

The data indicate that the cytoplasmic tail of FGFR4, but not the active tyrosine kinase is required for translocation of the growth factor across cellular membranes, and that genistein inhibits the transport.

Endocytosis and degradation of aFGF in transfected cells

The possibility exists that translocation of aFGF across cellular membranes is initiated by endocytic uptake of the growth factor. The tyrosine kinase of the receptor has been implicated in endocytosis and receptor down-regulation (Ullrich and Schlessinger, 1990). The lack of farnesylation of CAAX-tagged aFGF and transport to the nuclear fraction could therefore be due to inhibition of endocytic uptake of aFGF in U2OSDr1ΔR4 cells, and in genistein-treated U2OSDr1R4 cells. To test this, we measured endocytic uptake of aFGF and its degradation by the cells under the different conditions.

When ^{125}I-aFGF was bound to transfected cells and the cells, after washing, were incubated at 37°C, endocytic uptake of the surface-bound ^{125}I-aFGF, measured as radioactive material that could not be removed with 1 M NaCl, pH 4.0, occurred at a lower rate in U2OSDr1ΔR4 cells than in U2OSDr1R4 cells (Figure 4a and b). In both cases 74 μM genistein inhibited the endocytosis only moderately.

In experiments where the cells were incubated with ^{125}I-aFGF at 37°C for longer periods of time, the uptake in U2OSDr1ΔR4 cells was also somewhat lower than in U2OSDr1R4 cells. Also here 74 μM genistein had little effect (Figure 4c and d).

Degradation of aFGF, measured as TCA-soluble radioactivity in the medium, occurred both in U2OSDr1R4 and in U2OSDr1ΔR4 cells (Figure 5a and b). When cell-associated trichloroacetic acid precipitable material was analysed by SDS–PAGE, degradation products were visible after 2 h (Figure 5c). When the incubation was at 24°C, the degradation was somewhat reduced, but clearly visible (Figure 5d). At 16°C there was, however, no evidence for degradation of the endocytosed ^{125}I-labelled growth factor. This is in accordance with the observation that transport of endocytosed material to lysosomes is blocked at 16°C (Dunn *et al*., 1980). Also the presence of monensin or NH_4Cl inhibited the degradation (data not shown). Degradation occurred to approximately the same

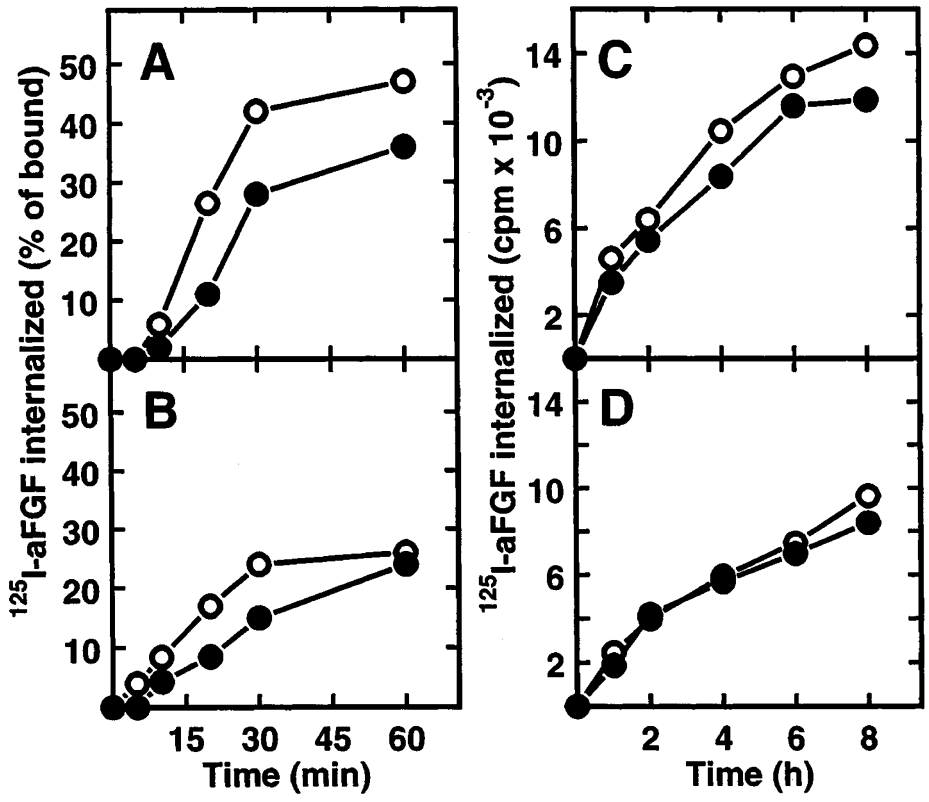

Figure 4 Endocytic uptake of ^{125}I-aFGF in U2OSDr1R4 (upper panels) and U2OSDr1ΔR4 cells (lower panels). (**a**, **b**) Cells in HEPES medium containing 0.2% gelatin, 10 U/ml heparin and 10 ng/ml ^{125}I-aFGF were kept at 4°C for 4 h. Then the cells were washed twice with ice-cold binding medium in the presence or absence of 74 μM of genistein for 10 min and subsequently incubated for increasing periods of time at 37°C in the absence (○) and presence (●) of genistein. At the indicated times the cells were washed once with PBS, twice with 25 mM HEPES, pH 7.5, containing 1 M NaCl, and twice with sodium acetate, pH 4.0, in 1 M NaCl. The cells were lysed in 0.2 M KOH and the radioactivity associated with the cells was measured. (**c**, **d**) Cells were incubated in DMEM containing 0.2% gelatin, 10 U/ml heparin and 10 ng/ml ^{125}I-aFGF at 37°C for increasing periods of time in the absence (○) and presence (●) of 74 μM genistein. When genistein was present, a 30 min preincubation with 74 μM genistein was included before the addition of ^{125}I-aFGF. After the indicated periods of time, the incubation medium was removed, and the cells were washed twice with PBS and twice with 25 mM HEPES, pH 7.5, containing 1 M NaCl. Ligand bound to FGF receptors was subsequently removed with 1 M NaCl in 20 mM sodium acetate, pH 4.0. The radioactivity remaining with the cells represents the amount of internalized ligand

extent in U2OSDr1R4 cells and U2OSDr1ΔR4 cells, and in neither case did genistein inhibit the degradation substantially.

It may be concluded that endocytosis and degradation occurs not only in cells transfected with wild-type receptor, but also in cells transfected with the deletion mutant, although at a lower rate. Furthermore, genistein does not interfere much with these processes. The data suggest that the absence of transport of aFGF to the cytosol and to the nuclear fraction in cells transfected with the deleted receptor and in U2OSDr1R4 cells treated with genistein is due to interference with a step occurring after or independent of endocytic uptake of the growth factor.

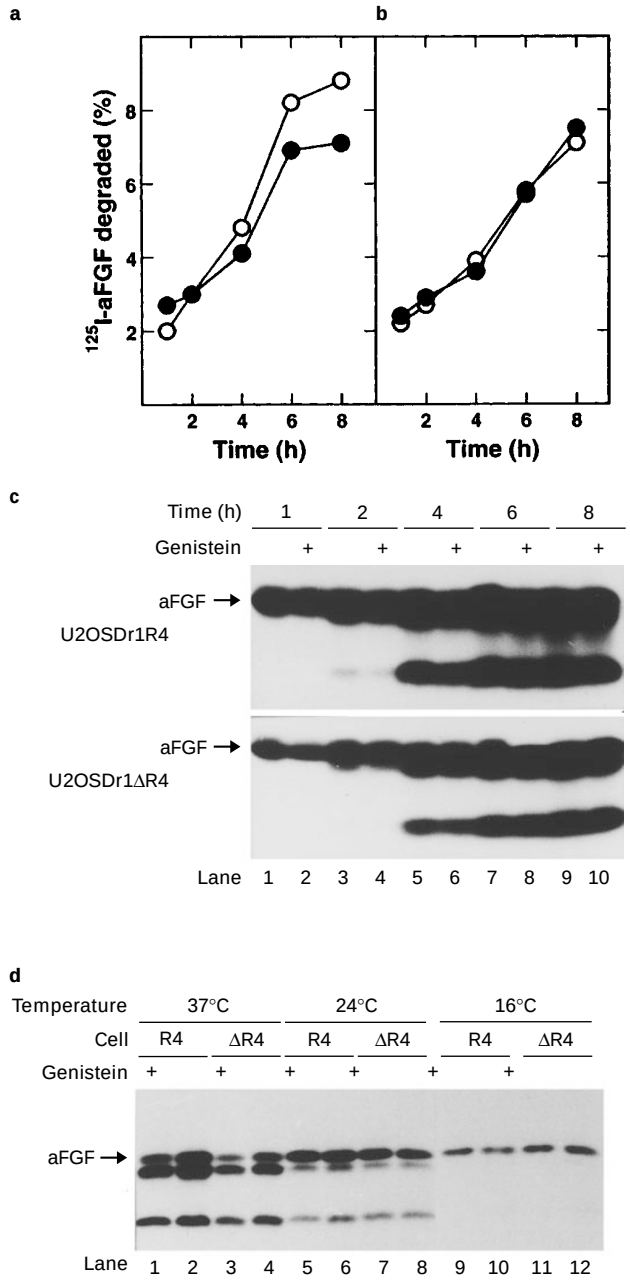

Figure 5 Degradation of ^{125}I-aFGF by U20SDr1R4 and U20SDr1ΔR4 cells. (**a**, **b**) U20SDr1R4 (**a**) and U20SDr1ΔR4 (**b**) cells were incubated with ^{125}I-aFGF as in Figure 4c,d in the absence and presence of genistein. After increasing periods of time the incubation medium was assayed for degraded ^{125}I-aFGF as TCA soluble radioactive material and expressed as per cent of the total radioactivity in the culture. (○), no genistein; (●), 74 μM genistein. (**c**) The cells, after an acid/salt wash, were lysed and the TCA-precipitable material in the cell lysate was analysed by SDS–PAGE and autoradiography. (**d**) Cells were incubated with ^{125}I-aFGF as above in the absence and presence of 74 μM genistein at 37°C, 24°C and 16°C for 24 h. Then the cells were lysed and the TCA-precipitable material was analysed by SDS–PAGE

Effect of genistein on FGF-receptor phosphorylation

Genistein has been shown to inhibit tyrosine kinases (Akiyama *et al*., 1987). To test if it inhibits aFGF-induced autophosphorylation of FGFR1, we incubated serum-starved NIH3T3 cells with different concentrations of genistein and stimulated the cells with aFGF. Then the cells were lysed, and subjected to immunoprecipitation with anti-phosphotyrosine. The immunoprecipitated material was analysed by SDS–PAGE and Western-blotting with a rabbit antibody against the C-terminus of FGFR1. The data in Figure 6a show that aFGF induced strong labelling of the receptor and that this process was not inhibited by genistein up to 74 μM. The two bands observed may represent different glycosylation states of the receptor or expression of receptors with both 2- and 3-immunoglobuline-like domains in NIH3T3 cells (Stachowiak *et al*., 1996b; Landgren *et al*., 1995).

Similar experiments were carried out with U2OSDr1R4 cells. In this case the receptor was precipitated from the cell lysate with anti-FGFR4 and the Western blot was probed with anti-phosphotyrosine. As shown in Figure 6b the labelling of the receptor was not inhibited by genistein.

In another approach we took advantage of the observation that when aFGF-treated cells expressing FGFR4 are lysed and immunoprecipitated with an antibody against the C-terminus of the receptor, an 85 kD serine kinase is associated with the immunoprecipitate due to its interaction with the activated and autophosphorylated FGFR4 (Vainikka *et al*., 1996). Upon subsequent incubation of the immunoprecipitate with [γ-^{32}P]ATP, the receptor is specifically labelled at serine residues. Phosphorylation of the receptor *in vitro* therefore indicates that the receptor had become tyrosine phosphorylated *in vivo* (Vainikka *et al*., 1996).

The data in Figure 6c show that in U2OSDr1 cells lacking receptors and in U2OSDr1ΔR4, no labelling was obtained corresponding to the migration rate of the receptor, whether the cells were treated with growth factor or not (lanes 1–4). Also cells transfected with wild-type receptor (U2OSDr1R4) showed no labelling at this position in the absence of aFGF (lanes 5, 6). On the other hand, when these cells were treated with aFGF, a strong band was observed migrating corresponding to ~110 kD, as expected for FGFR4 (lane 7). Two non-identified bands migrating corresponding to 43 and 46 kD were also observed. With cells transfected with the kinase negative mutant (U20SDr1R4-K503R) there was no evidence for phosphorylation of the receptor (Figure 6d).

The presence of genistein reduced the aFGF-induced labelling of the FGF-receptor somewhat, particularly at the higher concentrations (Figure 6c, lanes 8–10). At 19 μM genistein, which completely blocked farnesylation of aFGF-CAAX (Figure 3a) and transport of

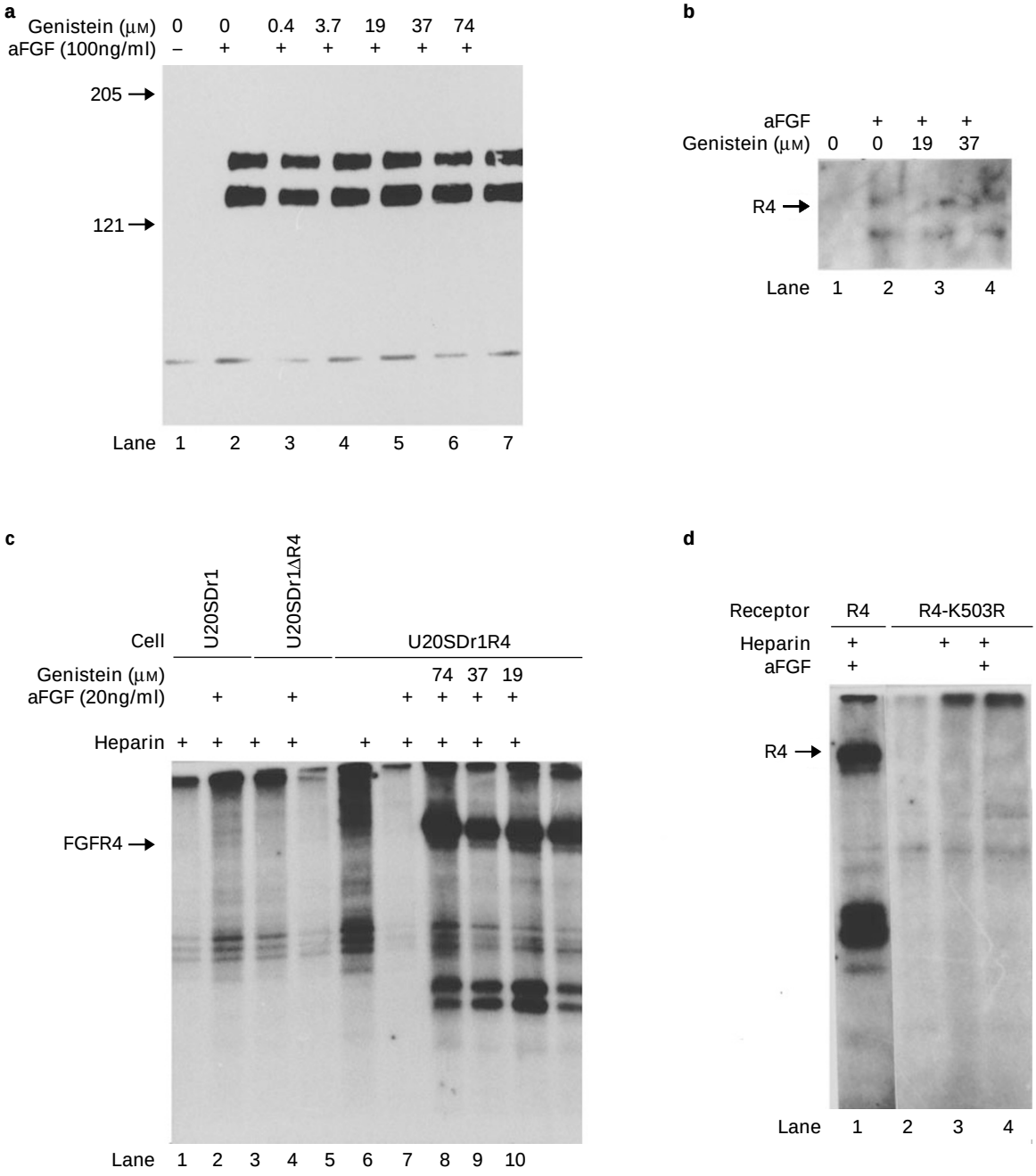

Figure 6 Effect of genistein on receptor tyrosine kinase activity in NIH3T3 (**a**) and U20SDr1R4 (**b**–**d**) cells. (**a**) Serum-starved NIH3T3 cells (expressing endogenous (FGFR1) were preincubated for 30 min at 37°C in the presence of heparin and different concentrations of genistein. The cells were then treated for 10 min with aFGF or left untreated in the same medium. Subsequently, the cells were washed, lysed and centrifuged for 5 min at 15 600 *g* at 4°C. The supernatant was submitted to immunoprecipitation with anti-phosphotyrosine-agarose and analysed by SDS–PAGE and Western-blotting with a rabbit antibody against FGFR1. (**b**) U20SDr1R4 cells were incubated and lysed as in **A**, but anti-FGFR4 was used for immunoprecipitation and anti-phosphotyrosine was used to probe the blots. (**c**, **d**). Untransfected and transfected U20SDr1 cells as indicated, were treated for 10 min with 20 ng/ml aFGF and 10 U/ml heparin or with 10 U/ml heparin alone. Where indicated, the cells were preincubated for 1 h at 37°C with increasing concentrations of genistein which was also present during the 10 min aFGF treatment. The cells were lysed and the nuclei were removed by centrifugation. The FGFR4 and associated proteins were immunoprecipitated from the post-nuclear supernatant with antibodies against the C-terminal end of the receptor. The immunoprecipitate was incubated with [γ^{32}P]ATP for 10 min at 30°C and finally analysed by SDS–PAGE and autoradiography

the growth factor to the nuclear fraction (Figure 3b), there was not much reduction of receptor phosphorylation (Figure 6b and c). The data are consistent with the possibility that inhibition of receptor phosphorylation is not the reason for the inhibitory effect of low concentrations of genistein on translocation of aFGF to the cytosol and to the nucleus.

Effect of genistein on farnesylation and transport of aFGF to the nuclear fraction in NIH3T3 cells

We also tested the effect of genistein on farnesylation of CAAX-tagged aFGF and transport of the growth factor to the nuclear fraction in NIH3T3 cells which express endogenous FGFR1. In these

cells the aFGF-stimulated DNA synthesis was reduced to less than 20% of the initial value at 19 μM genistein, whereas higher concentrations of genistein were required to inhibit serum-induced DNA synthesis. Even higher concentrations were required to inhibit protein synthesis (Figure 7a). Genistein did not inhibit binding of aFGF to NIH3T3 cells (Figure 7b).

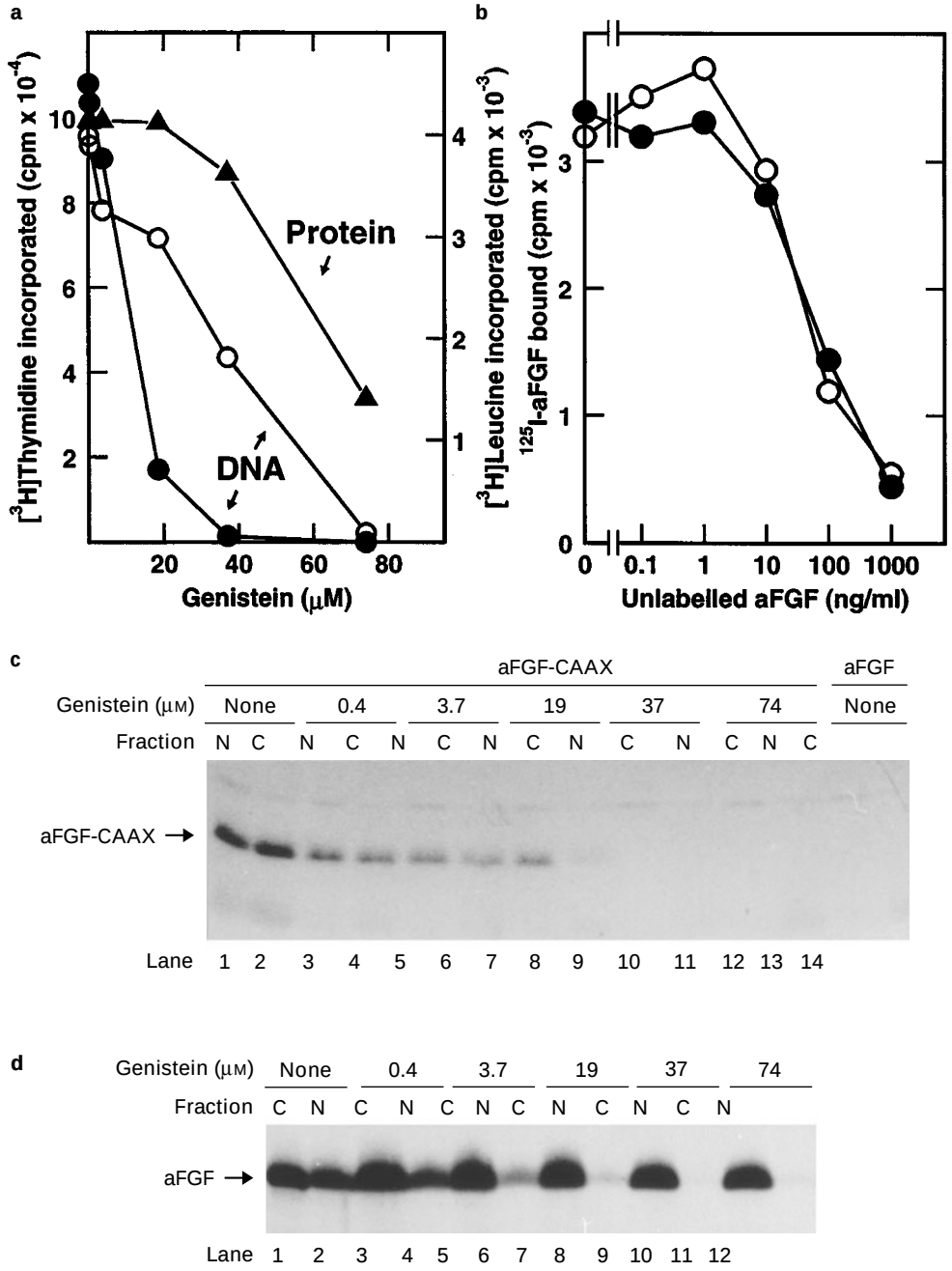

Figure 7 Effect of genistein on [^{3}H]leucine incorporation and on the ability of aFGF to stimulate DNA-synthesis and to become farnesylated and transported to the nucleus in NIH3T3 cells. (**a**) To measure [^{3}H]thymidine incorporation, cells were preincubated for 48 h at 37°C in serum-free medium containing insulin and transferrin, then increasing amounts of genistein and 5 ng/ml of aFGF (●) or 10% FCS (○) was added in the presence of 10 U/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 [^{3}H]thymidine, and the incorporated radioactivity was measured. To measure [^{3}H]leucine incorporation, cells were serum-starved and incubated with aFGF and genistein as above. After 24 h the cells were washed with leucine-free medium and incubated for 30 min in leucine-free medium containing 1 μCi/ml [^{3}H]leucine. The amount of TCA-precipitable radioactivity associated with the cells was measured. (**b**) Binding of ^{125}I-aFGF to cells was measured by adding 10 ng/ml of ^{125}I-aFGF and increasing concentrations of unlabelled aFGF in medium containing 10 U/ml heparin in the absence (○) and presence (●) of 74 μM genistein and kept at 4°C for 4 h with gentle shaking. After washing, the cell-associated radioactivity was measured. (**c**) Serum-starved cells were incubated with 10 U/ml heparin and 100 ng/ml aFGF or aFGF-CAAX overnight in the presence of lovastatin and labelled mevalonic acid. The cells were lysed and the post-nuclear supernatant was treated with Heparin-Sepharose which was subsequently washed with 0.7 M NaCl. The absorbed material was analysed by SDS–PAGE and fluorography. The nuclear pellet was treated as in Figure 3b. (**d**) *In vitro*-translated, [^{35}S]methionine-labelled aFGF (5 ng/ml) was added to cells in 6-well microtiter plates and incubated for 10 h at 37°C. The cells were then washed, lysed, fractionated into a cytosolic and a nuclear fraction as in Figure 3b and adsorbed to Heparin-Sepharose as described in Materials and methods. The adsorbed proteins from the cytoplasmic fraction (C) and from the sonicated and extracted nuclear fraction (N) were analysed by reducing SDS–PAGE and fluorography

When the cells were incubated with aFGF-CAAX, the growth factor was farnesylated (Figure 7c) and genistein at 19 μM strongly reduced the modification. Transport of the radiolabelled growth factor to the nuclear fraction was inhibited at 3.7–19 μM of the drug (Figure 7d).

Altogether, the data demonstrate that both in NIH3T3 cells and U2OSDr1R4 cells farnesylation of CAAX-tagged aFGF and transport of aFGF to the nuclear fraction are inhibited by genistein.

Discussion

We and others have earlier provided evidence that aFGF added to the cell culture medium is able to translocate into cells (Imamura *et al*., 1990, 1994; Zhan *et al*., 1992, 1994; Więdłocha *et al*., 1994). Translocation to the nuclear fraction appears to be associated with mitogenic activity. We have here studied the requirements for such transport. Our main finding is that transport of aFGF to the cytosol and to the nuclear fraction can be prevented by removing the major part of the cytoplasmic tail of FGFR4, but not by inactivating the kinase of the receptor by mutation. The transport was also inhibited when the cells were treated with genistein at doses that did not interfere much with the kinase activity of the receptor. The effect of genistein on aFGF transport to the nuclear location is probably not due to inhibition of the tyrosine kinase of the receptor, but could possibly be due to inhibition of some other kinase required for aFGF translocation.

Endocytosis and intracellular degradation of aFGF was not strongly inhibited either by genistein or by removal of the cytoplasmic part of the receptor, and certainly not sufficiently to account for the total inhibition of transport of the labelled growth factor to the nuclear fraction and the lack of farnesylation of the CAAX-tagged aFGF. It therefore appears that the inhibition of translocation by genistein and by deletion of the cytoplasmic receptor tail either occurs at a step after the endocytic uptake, or that the translocation of aFGF takes place at the level of the plasma membrane.

If endocytic uptake is a prerequisite for translocation into cells, aFGF may be transported to a late endocytic compartment where translocation can take place. The cytoplasmic tail of the receptor may contain a signal directing the receptor-bound aFGF to the appropriate location. Furthermore, genistein could interfere with this transport or, alternatively, it could inhibit the translocation process as such. In fact, genistein was recently shown to inhibit uptake of glucose and dehydroascorbic acid by the glucose transporter GLUT1 (Vera *et al*., 1996).

Genistein has been shown to be a competitive inhibitor of ATP binding to the catalytic domain of tyrosine kinases and to inhibit the tyrosine kinase activity of several growth factor receptors (Akiyama *et al*., 1987; Fallon *et al*., 1994). In the present work we show that genistein had little inhibitory effect on FGF receptor autophosphorylation even at comparatively high concentrations, whereas it inhibited aFGF-induced DNA synthesis at low concentrations. This is in agreement with observations made by other authors in different systems (Linassier *et al*., 1990; Lamaze *et al*., 1993).

Down-regulation of the PDGF receptor was found to be inhibited by genistein (Hill *et al*., 1990). We here found that comparatively high concentrations of genistein did not strongly inhibit the endocytosis of aFGF. Receptors that undergo efficient internalization, such as the transferrin receptor and the low density lipoprotein receptor, contain specific regions in the cytoplasmic domain that function as internalization signals (Trowbridge, 1991) interacting with protein components of coated pits, the adaptins (Robinson, 1992). Activated epidermal growth factor receptor also interacts with adaptins (Sorkin and Carpenter, 1993; Sorkin *et al*., 1991) and the receptor tyrosine kinase activity may be required for the induced uptake (Ullrich and Schlessinger, 1990). It is not clear whether the activated tyrosine kinase phosphorylates a specific substrate that mediates receptor internalization, or if autophosphorylation of the receptor molecule itself constitutes the signal for endocytosis. It is, however, clear that receptor autophosphorylation and sequences located in the carboxyl terminus of the receptor play a role in the internalization of EGF receptors (Sorkin *et al*., 1993; Chang *et al*., 1993). Mutation of an autophosphorylation site (Tyr-766) of FGFR-1 resulted in decrease in FGF receptor internalization, as well as in reduction in both ligand-induced FGF receptor down-regulation and degradation (Sorokin *et al*., 1994). Also deletion of the C-terminal 58 amino acids comprizing Tyr-766 resulted in strongly reduced endocytosis. In the present work we find that deletion of the major part of the cytoplasmic domain of FGFR4 reduced only moderately the rate of endocytosis. The reason for the different behaviour of FGFR1 and FGFR4 in this respect is not known.

Altogether, the data indicate that both the cytoplasmic tail of the receptor and a genistein-sensitive process different from the receptor kinase are required for translocation of aFGF into cells.

Materials and methods

Cells and materials

The human osteosarcoma cell line U2OSDr1 and the transfected derivatives U2OSDr1R4 (transfected with FGFR4) and U2OSDr1ΔR4 (transfected with FGFΔR4) have been described earlier (Więdłocha *et al*., 1995, 1996). FGFΔR4 encodes a FGFR4 molecule where most of the cytoplasmic domain, including the whole kinase domain has been deleted. It ends with amino acid Arg_{464} followed by the sequence Val-Thr-Leu-Ser-Leu-Ala-Gly-Glu-Leu-Leu-Cys obtained due to reading into the untranslated 3′ end until the first in-frame stop codon. The kinase-negative receptor FGFR4-K503R was formed by PCR using as forward primer 5′-GACCAAGCCAGCACTGTGGCCG-TCCGGATGCTCAAAGACAACGCC-3′ and as reverse primer 5′-GGTGAAGATCTCCCATAGCAG-3′. The forward primer contained a restriction site for *Bsp*EI in order to distinguish the mutated receptor from the wild type. The amplified material was cut partially with *Sma*I and to completion with *Eco*RI and cloned into pFGFR4Δsma (Więdłocha *et al*., 1996) which had been cut with the same enzymes. The plasmid was transfected into U2OSDr1 cells to yield U2OSDr1R4-K503R. These cells and NIH3T3 cells were propagated as earlier described (Więdłocha *et al*.,

1994). The cells were seeded into microtiter plates the day before the experiment. Unlabelled aFGF was produced in bacteria (Więdłocha *et al*., 1996) and labeled chemically with ^{125}I (Fraker and Speck Jr, 1978) and purified on a heparin-Sepharose column as described (Więdłocha *et al*., 1996). aFGF metabolically labelled with [^{35}S]methionine was synthesized in a cell-free system as earlier described (Więdłocha *et al*., 1994).

Binding of ^{125}I-labelled aFGF to cells

Confluent cells growing on 12 well gelatinized plates were washed twice with ice-cold binding buffer (DMEM containing 50 mM HEPES, pH 7.4, 0.2% gelatin, and 10 U/ml heparin). Cells were incubated with increasing concentrations of ^{125}I-aFGF for 4 h at 4°C. Then they were washed twice with ice-cold binding buffer, twice with phosphate-buffered saline (PBS) and once with 1 M NaCl in PBS. Cells were lysed in 0.5 M NaOH, and the solubilized radioactivity was measured. Nonspecific binding was estimated by the incubation of cells in the presence of a 100-fold molar excess of unlabelled pure recombinant aFGF. Receptor dissociation constants were estimated by the method of Scatchard (Scatchard, 1949).

Cross-linking of ^{125}I-aFGF to receptors

Cells (2×10^6) grown in gelatinized 60 mm plates were washed twice with ice-cold binding buffer and incubated for 3.5 h at 4°C with binding buffer containing 25 ng/ml of ^{125}I-aFGF in the presence or absence of a 100-fold molar excess of unlabelled pure recombinant aFGF. After washing once with cold binding buffer and once with PBS, the cells were treated for 20 min at 4°C with 0.3 mM disuccinimidyl suberate (Pierce, diluted from a stock solution of 30 mM dissolved in dimethyl sulfoxide) in PBS. After cross-linking, the cells were scraped from the wells, centrifuged for 30 s at 15 600 *g* in an Eppendorf centrifuge and washed with cold 25 mM Tris buffer, pH 7.4. They were then lysed by vortexing in 30 μl of buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μg/ml PMSF and kept on ice for 10 min. The lysates were centrifuged at 15 600 *g* for 10 min, and 10 μl of Laemmli sample buffer was added to the supernatant. The lysates were subjected to SDS–PAGE on 7.5% gels, and the ligand-receptor complexes were detected by autoradiography.

Internalization of ^{125}I-aFGF

Confluent cultures of U2OSDr1R4 and U2OSDr1ΔR4 cells grown in 35 mm gelatinized plates were incubated with DMEM containing 0.2% gelatin, 10 U/ml of heparin, 10 ng/ml ^{125}I-aFGF, and with or without 74 μM genistein at 37°C. The genistein treated cells had also been preincubated for 30 min with 74 μM genistein. At the indicated time points the medium was removed and assayed for degraded ^{125}I-aFGF as trichloroacetic acid (TCA)-soluble radioactive material. The monolayers were washed once with cold phosphate-buffered saline (PBS), twice with 1 M NaCl in 25 mM HEPES, pH 7.5, and finally twice with 1 M NaCl in 20 mM sodium acetate, pH 4.0, to remove cell surface-bound ^{125}I-aFGF. After the final acid/salt wash the cells were removed from the plate by treatment with 0.1 mg/ml pronase, washed once in HEPES-medium (DMEM where the bicarbonate was replaced with 20 mM HEPES, adjusted to pH 7,5) with 1 mM PMSF and 1 μg/ml aprotinin and resuspended in lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin). The amount of ^{125}I-aFGF present in the lysate, i.e. the amount of internalized ligand, was determined. TCA-precipitable material in the cell lysate was also analysed by SDS–PAGE and autoradiography.

To measure rapid internalization of ^{125}I-aFGF, cells were washed twice with ice-cold binding buffer and incubated for 4 h at 4°C in the same buffer containing 10 ng/ml ^{125}I-aFGF. The cells were washed twice with ice-cold binding buffer for 10 min in the absence or presence of 74 μM genistein. The cells were then incubated at 37°C in binding buffer with or without genistein. At the times indicated the buffer was removed, the surface-bound ^{125}I-aFGF was assayed as above and the cells were lysed in 0.2 M KOH. The solubilized radioactivity was measured.

Analysis of in vivo *farnesylation*

Cells growing in 25 cm^2 flasks were serum-starved for 36 h and then preincubated overnight in serum-free medium containing 3–6 μCi/ml [^{3}H]mevalonic acid (Du Pont) and 1 μCi/ml [^{14}C]mevalonic acid and 10 μg/ml lovastatin. aFGF or aFGF-CAAX (100 ng/ml) was added and the cells were incubated for 10 h. The cells were then washed twice with HEPES medium containing 5 U/ml heparin and three times with HEPES medium without heparin, then lysed and centrifuged for 5 min at 15 600 *g* The supernatant was centrifuged once more, and the second supernatant (the cytosolic fraction) was rotated for 2 h at 4°C with 30 μl of Heparin-Sepharose. The nuclear pellets were sonicated and extracted with 0.7 M NaCl. After clarification by centrifugation for 5 min at 15 600 *g* at 4°C, the extract was diluted five times in PBS containing 0.1% Triton X-100 and subjected to Heparin-Sepharose adsorbtion either alone or together with the cytosolic fraction, as indicated. The adsorbed material was analysed by SDS–PAGE and fluorography (Więdłocha *et al*., 1995).

Alternatively, the nuclear extract and the clarified cytosolic fraction were rotated for 2 h at 4°C with 30 μl of protein A-Sepahrose-CB that had previously been treated with 2 μl of rabbit anti-aFGF (Sigma).

Tyrosine phosphorylation of FGF-receptor

NIH3T3 cells were serum-starved for 24 h in the presence of 2.5 μg/ml insulin and 2.5 μg/ml transferrin. Cells were washed in HEPES medium with 10 U/ml heparin and preincubated for 30 min at 37°C in the presence of different concentrations of genistein or carrier (0.2% DMSO) in the same medium. Where indicated, aFGF (100 ng/ml) was added for 10 min. Cells were washed and lysed in the presence of phosphatase and protease inhibitors (Więdłocha *et al*., 1994) and centrifuged for 10 min at 15 600 *g* at 4°C. Tyrosine phosphorylated proteins in the supernatant were collected with mouse anti-phophotyrosine immobilised on agarose (Sigma), subjected to SDS–PAGE and transferred to a PVDF membrane. The membrane was probed with a rabbit anti-FGFR1 antibody (Santa Cruz) and visualised with a HRP-conjugated second antibody and enhanced chemiluminesence.

U2OSDr1 cells transfected with FGFR4 were serum-starved for 4 h, preincubated with different concentrations of genistein at 37°C for 30 min and then treated for 10 min with or without 50 ng/ml of aFGF in the presence of 10 U/ml heparin. Cells were lysed as above and subjected to immunoprecipitation with anti-FGFR4. Western blots were probed with anti-phosphotyrosine (Santa Cruz). To check that similar amounts or receptor had been immunoprecipitated in each case, the membrane was subsequently stripped and probed with anti-FGFR4 (not demonstrated).

In vitro *kinase assay*

Cells were starved for 3 h in serum-free medium and were, when indicated, stimulated for 10 min at 37°C with 20 ng/ml aFGF in presence of 10 U/ml heparin. After stimulation, the cells were lysed on ice for 15 min in Tris-lysis buffer (25 mM Tris, pH 7.5, 20 mM NaCl, 2 mM DTT, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.5% Triton X-100, 1 μg/ml antipain, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptine, 1 μg/ml pepstatin). The lysates were centrifuged, and the supernatants were incubated for 2 h at 4°C with anti-FGFR4 antibody (Santa Cruz Biotech). The immunocomplexes were then adsorbed to Protein A-Sepharose CL-4B (Pharmacia Biotech) and were washed three times in the same buffer and once in kinase buffer (20 mM Tris, pH 7.6, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 mM EGTA, 0.1 mM Na_3VO_4, 1 μg/ml aprotinin). For the kinase reaction, immunoprecipitates were incubated at 30°C for 10 min in 50 μl kinase buffer containing 1 μCi of [γ-^{32}P]ATP (Vainikka *et al*., 1996). The reactions were stopped by boiling in sample buffer and the samples were then analysed by SDS–PAGE and autoradiography.

Measurement of DNA-and protein synthesis

NIH3T3 and human osteosarcoma (U2OSDr1) cells growing in 24-well microtiter plates (5×10^4 cells/well) were preincubated for 48 h in serum-free medium with and without 5 μg/ml insulin and 5 μg/ml transferrin, respectively. The cells were then treated with 5–10 ng/ml of aFGF and 10 U/ml heparin in presence of increasing concentrations of genistein for 24 h at 37°C. To measure DNA synthesis, 1 μCi/ml [^{3}H]thymidine was present the last 6 h (Więdłocha *et al*., 1996). To measure protein synthesis, after the 24 h incubation the cells were washed three times with leucine-free medium and incubated with 1 μCi/ml of [^{3}H]leucine in leucine-free medium for 30 min. Finally, the amount of TCA-precipitable radioactivity associated with the cells was measured (Więdłocha *et al*., 1992).

Cell fractionation and analysis of material in the nuclear fraction

Near confluent cells growing in gelatinized 6 wells plates or 25 cm^2 flasks were serum-starved for 24–48 h. Then [^{35}S]methionine-labelled aFGF was added in the presence of 10 U/ml heparin. After 10 h incubation at 37°C, the cells were washed twice with HEPES medium containing 5 U/ml heparin and three times with HEPES medium without heparin. Then the cells were lysed in lysis buffer and centrifuged twice for 5 min at 15 600 *g* at 4°C. The supernatant (cytoplasmic fraction) was rotated for 2 h at 4°C with 30 μl of Heparin-Sepharose. The nuclear pellets were washed once in the same buffer and twice in lysis buffer containing 1 mM EGTA (instead of EDTA) 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.3 M sucrose and 1 mM $MgCl_2$, layered over 0.8 ml of 0.7 M sucrose and centrifuged for 15 min at 720 *g* at 4°C. The nuclei were then sonicated and extracted with 0.7 M NaCl. After clarification by centrifugation for 5 min at 15 600 *g* at 4°C, the extract was diluted in PBS containing 0.5% Triton X-100 and subjected to Heparin-Sepharose adsoption or immunoprecipitation using anti-aFGF antibody bound to protein A-Sepharose-4B. Finally, the Heparin-Sepharose was washed with 0.7 M NaCl to remove less strongly bound material and the remaining material was subjected to SDS–PAGE and fluorography.

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