Different abilities of the four FGFRs to mediate FGF-1 translocation are linked to differences in the receptor C-terminal tail

Vigdis Sørensen*, Antoni Wiedlocha, Ellen Margrethe Haugsten, Denis Khnykin, Jørgen Wesche and Sjur Olsnes

The Department of Biochemistry, Institute for Cancer Research, The University of Oslo, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

*Author for correspondence (e-mail: vigdis.sorensen@labmed.uio.no)

Accepted 19 July 2006
Journal of Cell Science 119, 4332-4341 Published by The Company of Biologists 2006
doi:10.1242/jcs.03209

Summary

Members of the fibroblast growth factor family bind to one or more of the four closely related membrane-spanning FGF receptors. In addition to signaling through the receptors, exogenous FGF-1 and FGF-2 are endocytosed and translocated to the cytosol and nucleus where they stimulate RNA and DNA synthesis. Here we have studied the ability of the four FGF receptors to facilitate translocation of exogenous FGF-1 to the cytosol and nucleus. FGFR1 and FGFR4 were able to mediate translocation, whereas FGFR2 and FGFR3 completely lacked this ability. By analyzing mutant FGFRs we found that the tyrosine kinase domain could be deleted from FGFR1 without abolishing translocation, whereas the C-terminal tail of the FGFRs, constituted by approximately 50 amino acids downstream of the kinase domain, plays a crucial role in FGF-1 translocation. Three amino acids residues within the C-terminal tail were found to be of particular importance for translocation. For FGFR2, the two amino acid substitutions Q774M and P800H were sufficient to enable the receptor to support FGF-1 translocation. The results demonstrate a striking diversity in function of the four FGFRs determined by their C-terminal domain.

Key words: FGF, FGFR, Nucleus, Translocation

Introduction

The FGF family comprises 22 members in humans and mediates a variety of biological responses including cell growth and proliferation, migration and differentiation. Each FGF binds to one or more of the four high-affinity transmembrane tyrosine kinase receptors designated FGFR1, FGFR2, FGFR3 and FGFR4. The receptors share between 55% and 72% homology at the protein level (Johnson and Williams, 1993) and they can be expressed as several different isoforms generated by alternative splicing. Each splice variant of FGFR is activated by a unique subset of FGFs (Ornitz et al., 1996; Powers et al., 2000). The prototype FGF, FGF-1, binds with similar affinity to all splice variants of FGFRs.

The large number of FGF and FGFR variants and their widespread expression provides the FGF/FGFR signaling system with functional diversity and reflects their many important roles during development as well as in the adult organism. FGFs stimulate growth of many cell types, e.g. fibroblasts, endothelial cells and chondrocytes. In the adult organism they function as homeostatic factors and are involved in wound healing, tissue repair and angiogenesis. FGFs and FGFRs are also involved in the development of several pathological conditions including cancers (Ornitz and Marie, 2002; Grose and Dickson, 2005).

The FGFs also bind avidly to cell surface heparan sulphate proteoglycans (HSPG) and to heparin. The HSPGs, or heparin, are directly involved in the binding of FGF to FGFR by stabilizing the receptor-ligand complex (Mohammadi et al., 2005). Binding of ligand to FGFR induces receptor dimerization and activation of the receptor tyrosine kinase domain by autophosphorylation, followed by activation of downstream effectors. FGF is known to activate the Ras-MAPK, the PLCγ-PKC, the PI3K-Akt and the p38 MAPK pathways (Boilly et al., 2000).

FGFR1 possesses seven tyrosine residues, which can be phosphorylated, in its intracellular region. The tyrosine residues that appear to be the most important for signaling (Tyr653, Tyr654, Tyr766) are conserved between FGFR1 through FGFR4 (Powers et al., 2000). The different FGFRs seem to mediate activation of the same targets and differ only in the strength of their tyrosine kinase activity (Raffioni et al., 1999). It has therefore not been clear whether the different forms of FGFRs contribute significantly to diversity in the FGF/FGFR signaling system other than in providing differential extracellular binding sites for the various FGFs.

Exogenous FGF-1 and FGF-2 that bind cell surface receptors can also be translocated across cellular membranes to reach the cytosol and nucleus (Olsnes et al., 2003; Wiedlocha and Sorensen, 2004). This is not unique for FGF as several growth factors and cytokines have been reported to internalize and subsequently translocate to the cell nucleus (Jans and Hassan, 1998; Olsnes et al., 2003). Internalization and nuclear localization seems to be an additional mechanism by which certain growth factors mediate signaling.

Journal of Cell Science

It has been shown that FGF-1 interacts specifically with intracellular proteins, such as FIBP (Kolpakova et al., 1998), p34 (Skjerpen et al., 2002b), CK2 (Bonnet et al., 1996; Skjerpen et al., 2002a) and mortalin (Mizukoshi et al., 1999) and translocated FGF has been found to stimulate RNA and DNA synthesis (Bouche et al., 1987; Wiedlocha et al., 1994; Wiedlocha et al., 1996). Intracellular FGF-2 has been shown to interact directly with upstream binding factor (UBF) and stimulate rRNA transcription (Sheng et al., 2005). Nuclear import is directed by two nuclear localization sequences in FGF-1 (Imamura et al., 1990; Wiedlocha et al., 1994; Imamura et al., 1994; Friedman et al., 1994; Wesche et al., 2005). The translocated FGF-1 is phosphorylated at a single site, at S130, by PKCδ. This phosphorylation appears to occur mainly in the nucleus and regulates the nucleocytoplasmic trafficking of FGF-1 (Wiedlocha et al., 2005).

Translocation of exogenous FGF-1 or FGF-2 into cytosol and nucleus is a regulated process, which has been found to occur in the G1 phase of the cell cycle (Bouche et al., 1987; Baldin et al., 1990; Zhan et al., 1993; Imamura et al., 1994; Malecki et al., 2004) and it requires PI3K activity (Klingenberg et al., 2000a; Malecki et al., 2004). It has been found that translocation of both FGF-1 and FGF-2 occurs from the lumen of intracellular vesicles possessing vacuolar proton pumps, most likely early endosomes and that it depends on the electrical potential across the vesicular membrane (Malecki et al., 2002; Malecki et al., 2004). Although FGF binds abundantly also to cell surface HSPG, FGF is not translocated to the cytosol in cells lacking FGFR (Wiedlocha et al., 1995; Klingenberg et al., 1998) indicating a crucial role for FGFRs in this process. Previously, we found that removal of the 11 most C-terminal amino acids of FGFR4, but not removal of the kinase domain, prevented translocation of FGF-1 in FGFR4-expressing COS-1 cells (Klingenberg et al., 2000b).

In the work we present in this paper, we have compared the four types of FGFRs with respect to their ability to facilitate translocation of exogenous FGF-1 to cytosol and/or nucleus. We find that FGFR1 and FGFR4 are both able to mediate translocation of FGF-1, while FGFR2 and FGFR3 completely lack this ability. Mutational analysis demonstrates the importance of the C-terminal tail of the receptors in FGF-1 translocation.

Results

FGFR expression in COS-1 cells and uptake of FGF-1

To compare the function of the different FGFRs under similar conditions, we used a common expression system. cDNA encoding IIIc splice variants of full-length human FGFR1, FGFR2, FGFR3 and FGFR4, as well as the shorter splice variants FGFR1β and FGFR2β (lacking Ig-like domain I) were cloned in the pcDNA3 expression vector and expressed by transient transfection in COS-1 cells. We have previously shown that exogenous FGF-1 can be translocated to the cytosol in COS-1 cells transfected with FGFR4 (Klingenberg et al., 2000a). To maintain the cells in a state that allows a reproducible response to FGF stimulation, they were propagated in low serum conditions (defined medium for fibroblasts supplemented with 2% serum) before the experiment.

The FGFRs were expressed in the COS-1 cells as proteins with molecular mass of ⩾100 kDa while no FGFR could be detected in untransfected cells (Fig. 1A). Incubation of the cells with [^{35}S]methionine-labeled FGF-1 (^{35}S-FGF-1) showed similar abilities of the various FGFRs to bind and endocytose FGF-1 (Fig. 1B). Heparin is a co-ligand for strong and stable binding of FGF-1 to FGFRs and was therefore always added together with FGF-1. FGF-1 can also bind to cell surface HSPG (low affinity-receptors) and be endocytosed after such

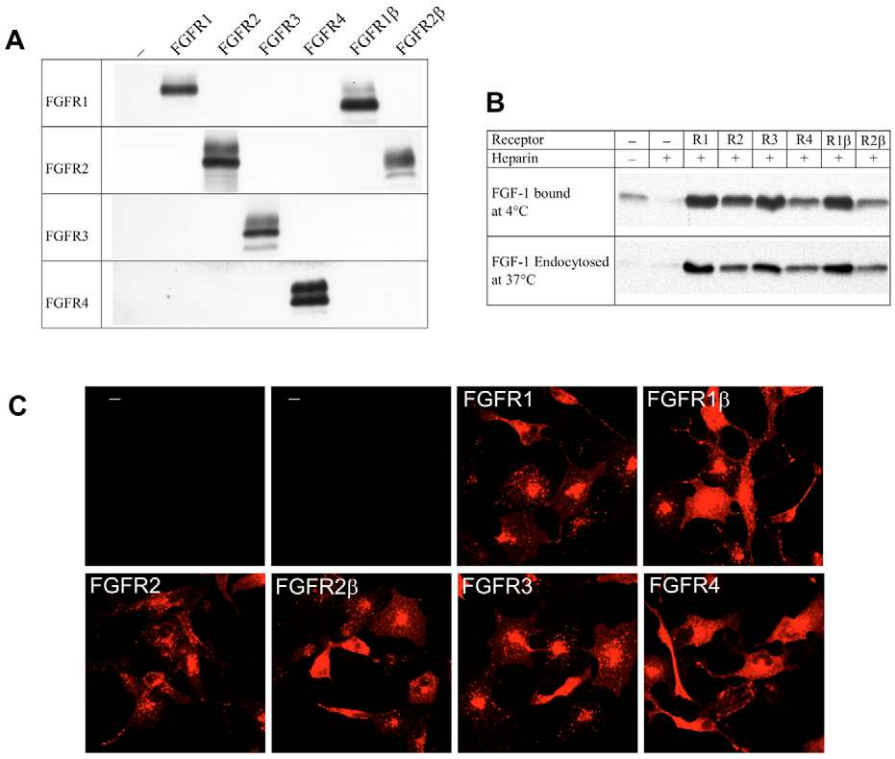

Fig. 1. Expression of FGFRs and uptake of FGF-1 in COS-1 cells. (A) Untransfected COS-1 cells and COS-1 cells transfected with FGFR1, FGFR2, FGFR3, FGFR4, FGFR1β, or FGFR2β, respectively, were lysed and analyzed by SDS-PAGE and immunoblotting. The membrane was probed sequentially with antibodies specific for FGFR1, FGFR2, FGFR3 and FGFR4. (B) To cells transfected with FGFR as indicated, ^{35}S-FGF-1 was allowed to bind at 4°C for 30 minutes and then the cells were washed and lysed immediately (upper panel) or further incubated at 37°C for 30 minutes to allow endocytosis of ^{35}S-FGF-1 to take place and then washed and lysed (lower panel). ^{35}S-FGF-1 was extracted from the lysates by binding to heparin-Sepharose and analyzed by SDS-PAGE and autoradiography. In the names of the receptors, FGFR is abbreviated to R. (C) COS-1 cells, untransfected or transfected with FGFR1, FGFR2, FGFR3, FGFR4, FGFR1β or FGFR2β as indicated, were incubated with Cy3-FGF-1 and heparin (except for the first image, which is without heparin) for 2 hours, fixed and analyzed by confocal fluorescence microscopy.

binding. However, 30 U ml^{-1} heparin, as we have used in these experiments, largely abolished binding to HSPG (Fig. 1B). Incubation of cells with Cy3-labeled FGF-1 (Cy3-FGF-1) for 2 hours and examination by confocal microscopy showed that the growth factor had accumulated in intracellular vesicles in cells transfected with FGFR but not in untransfected cells (Fig. 1C). The transfection frequency for the different receptors was similar and the intracellular structures containing Cy3-FGF-1 corresponds to endosomes, lysosomes and the juxtanuclear recycling endosomal compartment as previously described (Haugsten et al., 2005). We were unable to detect Cy3-FGF-1 in the nucleus neither after 2 hours of incubation as in Fig. 1C, nor after longer incubation times (not shown).

Only FGFR1 and FGFR4 support translocation of FGF-1

Translocation of externally added FGF-1 to the cytosol and nucleus can be monitored by phosphorylation of FGF-1 (Klingenberg et al., 1998). FGF-1 contains only one functional phosphorylation site, a PKC site at Ser130. PKC is only found in the cytosol and nucleus and not in endosomes (Mellor and Parker, 1998). Furthermore, endogenous FGF-1 is not made in measurable amounts in COS-1 cells. Phosphorylation of exogenously added FGF-1 can therefore be taken as evidence that the growth factor has reached the cytosol or the nucleus. Earlier, we have found that externally added FGF-1 is phosphorylated by PKCδ in the nucleus and subsequently transported to the cytosol (Wiedlocha et al., 2005).

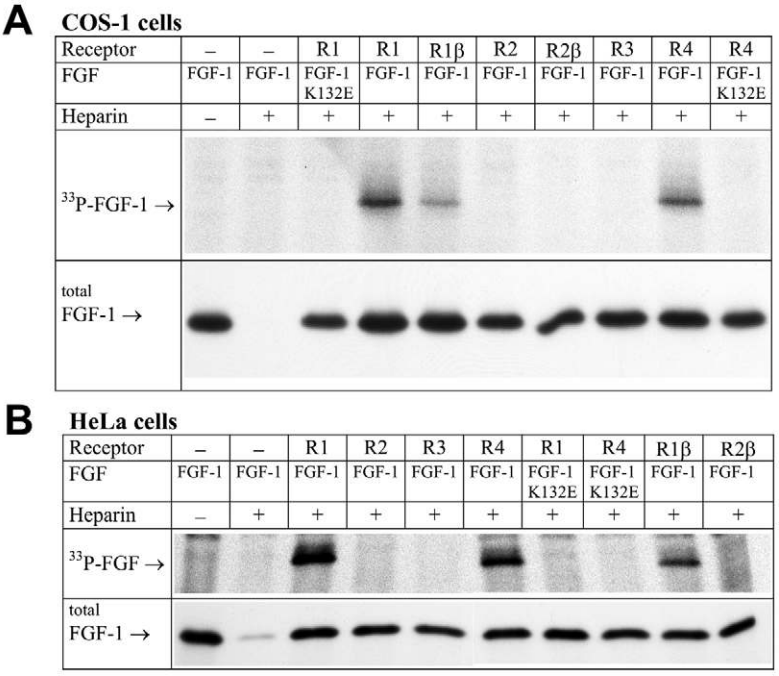

Fig. 2. Phosphorylation of exogenous FGF-1 in FGFR-transfected cells. (A) COS-1 cells and (B) HeLa cells were transfected with FGFRs as indicated, pretreated with [^{33}P]phosphate and incubated for 6 hours with FGF-1 or FGF-1 K132E and with heparin where indicated. FGF-1 was extracted from cell lysates as described in Materials and Methods and then separated by SDS-PAGE and blotted onto Immobilon-P membrane. ^{33}P-phosphorylated FGF-1 was detected by autoradiography (upper panel) and the total cell-associated FGF-1 was detected by anti-FGF-1 antibodies (lower panel).

To test the ability of the different FGFR variants to translocate FGF-1 to the cytosol and nucleus, transfected COS-1 cells were pre-incubated with [^{33}P]phosphate and then treated with FGF-1 and heparin for 6 hours. FGF-1 was extracted from cell lysates with heparin-Sepharose, fractionated by SDS-PAGE and transferred to an Immobilon-P membrane. First, ^{33}P-phosphorylated proteins on the membrane were visualized by autoradiography (Fig. 2A, upper panel) and then the membrane was exposed to immunodetection with anti-FGF-1 to detect the total amount of FGF-1 extracted from the cells (Fig. 2A, lower panel). In cells transfected with FGFR1, FGFR1β and FGFR4, a band strongly labeled with [^{33}P]phosphate and migrating as FGF-1 was found. This band did not appear in FGFR1- or FGFR4-transfected cells incubated with the non-phosphorylatable, yet receptor-binding mutant, FGF1 K132E (Klingenberg et al., 1998), arguing against the possibility that the band represents an endogenous protein that is phosphorylated in cells stimulated by FGF-1.

Immunodetection with anti-FGF-1 showed that the total amount of FGF-1 accumulated in the cells, which includes non-phosphorylated FGF-1 from endosomes, was similar in all transfected cells. This indicates that only FGFR1, FGFR1β and FGFR4 are capable of facilitating translocation of exogenous growth factor across cellular membranes to reach the cytosol and nucleus.

We also tested the translocation capability of the four human receptors in HeLa cells with similar results (Fig. 2B). Furthermore, full-length mouse versions of FGFR2 and FGFR3 were also found unable to facilitate translocation of FGF-1 (results not shown).

Translocation of FGF-1 across endosomal membrane and transport to the nucleus

The electrical membrane potential across vesicular membranes was previously found to be necessary for translocation of FGF-1 and FGF-2 in NIH3T3 cells (Malecki et al., 2002; Malecki et al., 2004). This membrane potential can be dissipated by treating the cells with bafilomycin A1, which inhibits the vesicular proton pumps, while the membrane potential can be restored and allow translocation of FGF to occur if, in addition, the cells are treated with the ionophore monensin. As demonstrated in Fig. 3A, bafilomycin A1 blocked translocation of FGF-1 in COS-1 cells transfected with either FGFR1 or FGFR4 and monensin allowed translocation to occur even in the presence of bafilomycin A1. This indicates that both FGFR1 and FGFR4 facilitate translocation of FGF-1 from endosomes.

Previously it was shown that in the presence of the nuclear export inhibitor leptomycin B (LMB), FGF-1 that is phosphorylated in the nucleus is prevented from being transported to the cytosol (Wiedlocha et al., 2005). Fig. 3B shows an experiment where the cells were treated with or without LMB and the harvested cells were fractionated into a cytoplasmic fraction (that contains both cytosol and lysed endosomes) and a nuclear fraction. Assessment of the purity

of the fractions is shown in Fig. 3C. The translocated and phosphorylated FGF-1 was found mainly in the cytoplasmic fraction in the absence of LMB (Fig. 3B, upper panel), while in cells transfected either with FGFR1 or with FGFR4 translocated FGF-1 was found mainly in the nucleus in the presence of LMB (Fig. 3B, second panel). These results (Fig. 3A,B) show that FGFR1 and FGFR4 function similarly in the translocation of FGF-1 to cytosol and/or nucleus, implying engagement of the same mechanisms. The results also indicate that the FGF-1 translocation mechanism and nuclear transport operating in the COS-1 cell used here, which are transiently transfected to express high levels of FGFR, is similar to that previously observed for NIH3T3 cells, which express a much lower level of endogenous FGFR1.

The data in Fig. 3B also demonstrate that the ^{33}P-labeled band is usually stronger in the presence than in the absence of LMB, indicating that phosphorylated FGF-1 is more stable in the nucleus than in the cytosol. In spite of this increase in sensitivity of the detection system, we could not detect phosphorylated FGF-1 in cells transfected with FGFR2 or FGFR3, strengthening the finding that these two receptors are devoid of translocating capability. Immunodetection with anti-FGF-1 showed that the total uptake of FGF-1 in the cells (including endocytosis) was similar for the different receptors (Fig. 3B, third panel).

FGF-1 translocated by FGFR1 and FGFR4 and trapped in the nuclear fraction by LMB could also be detected by anti-FGF-1 (Fig. 3B, bottom panel), as could the FGF-1 K132E mutant, which was translocated and localized to the nuclear fraction in the presence of LMB, although it cannot be phosphorylated. Since the nuclear fraction in the type of experiment shown in Fig. 3B is not contaminated by FGF-1 derived from endosomes and since the FGF-1 can be detected with anti-FGF-1, this method provided an opportunity to quantify the amount of FGF-1 that was translocated to the nucleus in the presence of LMB. The experiment was repeated for FGFR1 (Fig. 3D) and FGFR4 (not shown) and the amount of FGF-1 in the nuclear fraction was quantified by comparison to known amounts of FGF-1 run in the same gel. The results indicated that approximately 1-2 ng of FGF-1 had been translocated and could be recovered from the nuclear fraction in each experiment. Since 1.5 ng corresponds to 5.6×10^{10} molecules of FGF-1 and the number of FGFR1-expressing cells in each experiment was approximately 2×10^{5} cells, it follows that each of these cells had translocated approximately 280,000 molecules of FGF-1 into the nucleus. If this were distributed in the whole cell volume (~3 picolitres) the concentration would be 0.16 μM, which is close to the FGF-1 uptake previously estimated for NIH3T3 cells by a different approach (Malecki et al., 2002).

Translocation kinetics

It was previously found that translocation of FGF-1 into cytosol and nucleus in serum starved NIH3T3 cells occurs in

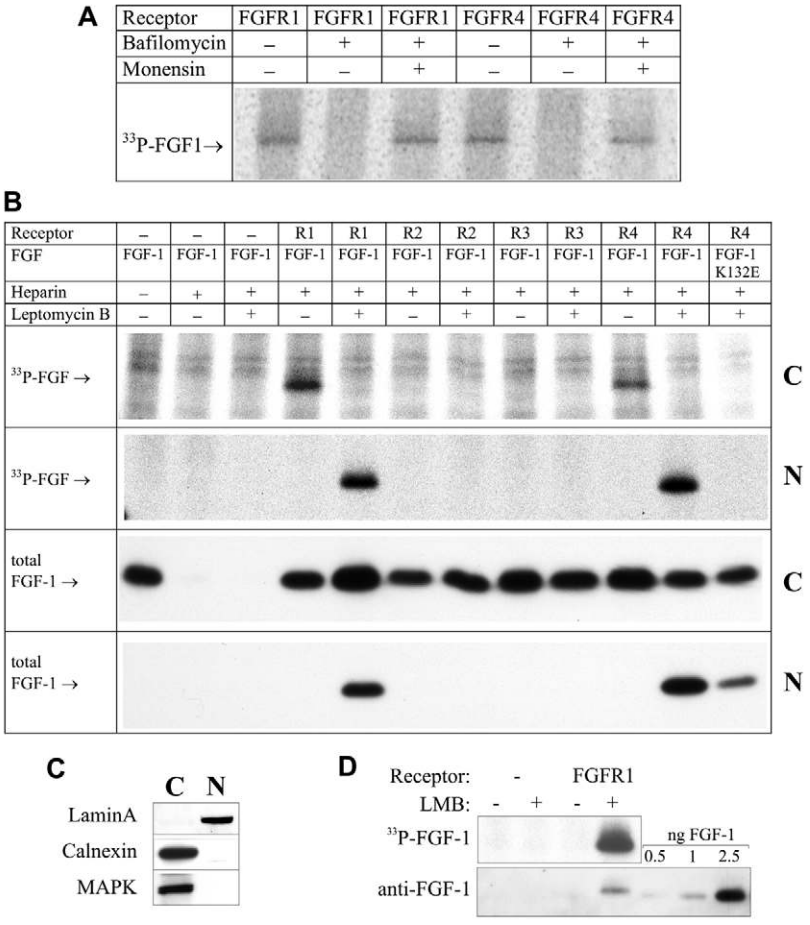

Fig. 3. Inhibition of FGF-1 translocation by bafilomycin A1 and FGF-1 accumulation in the nucleus by LMB treatment. (A) COS-1 cells were transfected with FGFR1 or FGFR4, treated with [^{33}P]phosphate and incubated for 6 hours with FGF-1 and heparin in the presence or absence of bafilomycin A1 and monensin, as indicated. FGF-1 was extracted from the cell lysates and assessed for phosphorylation by SDS-PAGE and autoradiography. (B) COS-1 cells were transfected with FGFR as indicated, pretreated with [^{33}P]phosphate and incubated for 6 hours with FGF-1 or FGF-1 K132E and with LMB where indicated. The cells were fractionated into cytoplasmic (C) and nuclear (N) fractions and FGF-1 was extracted as described in Materials and Methods and then separated by SDS-PAGE and blotted onto Immobilon-P membrane. ^{33}P-phosphorylated FGF-1 in the cytoplasmic (upper panel) and nuclear (second panel) fractions was detected by autoradiography and the total cell associated FGF-1 in the cytoplasmic (third panel) and nuclear (bottom panel) fractions was detected by anti-FGF-1 antibodies. (C) Cytoplasmic and nuclear fractions of lysed COS-1 cells were analyzed for their purity by western blotting and antibodies against the cytosolic protein MAPK, the ER resident protein Calnexin and the nuclear protein LaminA. (D) The experiment was performed as described in (B) but only nuclear fractions were analyzed. In addition, known amounts of FGF-1 was loaded on additional lanes of the gel to estimate the amount of FGF-1 that had been extracted from the nuclear fraction. Upper panel, autoradiography. Lower panel, anti FGF-1 immunodetection.

the late G1-phase of the cell cycle and peaks 6 hours after the start of FGF-1 stimulation (Malecki et al., 2004). COS-1 cells do not enter complete growth arrest upon serum starvation and we therefore studied the kinetics of translocation of FGF-1, monitored by phosphorylation, in FGFR1- and FGFR4-transfected COS-1 cells and compared it with the kinetics of the total uptake (mainly endocytosis) of FGF-1. The results demonstrate that phosphorylated FGF-1 could be detected after 4 hours of incubation with FGF-1, it reached a maximum at approximately 6 hours and declined after 8 hours (Fig. 4A). The kinetics of phosphorylation of FGF-1 was very similar for FGFR1 and FGFR4, but considerably delayed (by ~3 hours) when compared with the total uptake of FGF-1, which reached a half maximum level already during the first hour of incubation (Fig. 4B). The kinetics of phosphorylation are here considered to reflect closely the kinetics of the translocation process as such, since previous studies have shown that FGF-1 is phosphorylated rapidly (within 5-15 minutes) after its translocation (Wesche et al., 2006). Also, the kinetics of nuclear entry of FGF-1 in LMB treated cells were found to be similar whether measured by FGF-1 phosphorylation or by anti-FGF-1 antibodies (data not shown). The delay in translocation of FGF-1 compared with its uptake by endocytosis could be due to a requirement for activation or assembly of components assisting in the translocation process.

To investigate whether the different translocation abilities of FGFR-1 through -4 are related to differences in the activation of downstream signaling molecules by the four receptor tyrosine kinases, we analyzed the activated state of p44/42 MAPK, p38 MAPK, Akt, PLCγ1 and Stat1 (data not shown) in total cell lysates of COS-1 cells expressing either of the four FGFRs. We observed a similar activation of all of the tested signaling molecules, even in the absence of FGF-1 stimulation, probably due to the high expression level in the COS-1 cells that leads to auto-activation of the receptor tyrosine kinase. We were therefore unable to link the lack of FGF-1 translocation abilities of FGFR2 and FGFR3 to any deficit in activation of signaling molecules.

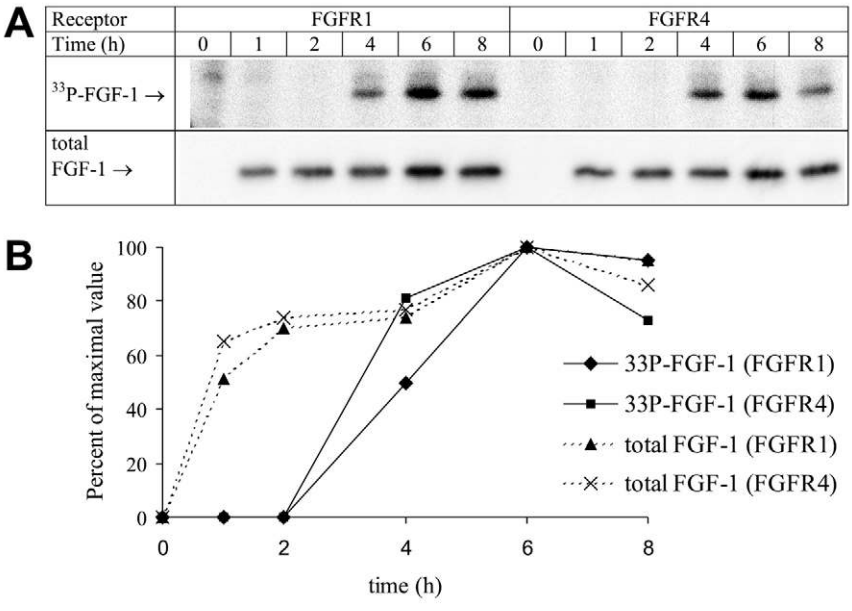

Fig. 4. Kinetics of uptake and phosphorylation of FGF-1. (A) COS-1 cells transfected with FGFR1 or FGFR4 were labeled with [^{33}P]phosphate and incubated with FGF-1 and heparin for the time indicated. FGF-1 was then extracted from the cell lysates and analyzed as in Fig. 2. The amount of ^{33}P-phosphorylated FGF-1 (upper panel) and total intracellular FGF-1 (detected by anti-FGF-1, lower panel) was quantified and plotted against time as percent of the maximal values (B).

Facilitation of translocation is associated with the C-terminal tail of the receptor

To determine which part of the receptor is important for FGF-1 translocation, we first investigated whether the ability to facilitate translocation is determined by structural features of the extracellular or intracellular regions. Two FGFR1-FGFR2 chimeric receptors were constructed that consist of the extracellular region of FGFR1 and the intracellular region of FGFR2, with the breakpoint between the FGFR1 and FGFR2 sequences being in a conserved region either immediately upstream or immediately downstream of the transmembrane region [FGFR1/FGFR2(375) and FGFR1/FGFR2(398), respectively]. Two other chimeric receptors were also constructed consisting of the extracellular region of FGFR2 and the intracellular region of FGFR1 with the breakpoint between the FGFR2 and FGFR1 sequences either immediately upstream or immediately downstream of the transmembrane region [FGFR2/FGFR1(376) and FGFR2/FGFR1(399), respectively]. Fig. 5A shows an overview of the chimeric and deleted receptor constructs we studied. Chimeric receptors were given names that include a number in parenthesis that refers to the number of the amino acid at which the switch between the two receptor types was made (see Materials and Methods for details).

In Fig. 5B-E the upper panels show the FGF-1 translocation, indicated by the appearance of ^{33}P-phosphorylated FGF-1, while the lower panels show that the various receptors were expressed to a similar level, indicated by a similar ability to endocytose FGF-1 (total FGF-1). As shown in Fig. 5B, when the intracellular region in FGFR2 was replaced by the intracellular region of FGFR1, the receptor gained the ability to facilitate the translocation of FGF-1, while FGFR1 lost its ability to translocate FGF-1 when it contained the intracellular part of FGFR2. This indicates that the translocation of FGF-1 depends on features of the intracellular part of the FGFRs.

Previously, it was found that the 11 most C-terminal amino acids, but not the kinase domain of FGFR4, were necessary for the FGF-translocating abilities of this receptor (Klingenberg et al., 2000b). We tested if this would also be the case for FGFR1. Indeed, deletion of amino acids 421-753, a region that encompasses the kinase domain and also disrupts the FRS2 binding site at amino acids 419-426 upstream of the kinase domain, did not abolish the ability of FGFR1 to mediate FGF-1 translocation (mutant FGFR1Δ421-753, Fig. 5C). But, when the deletion in FGFR1 was extended to include a further 27

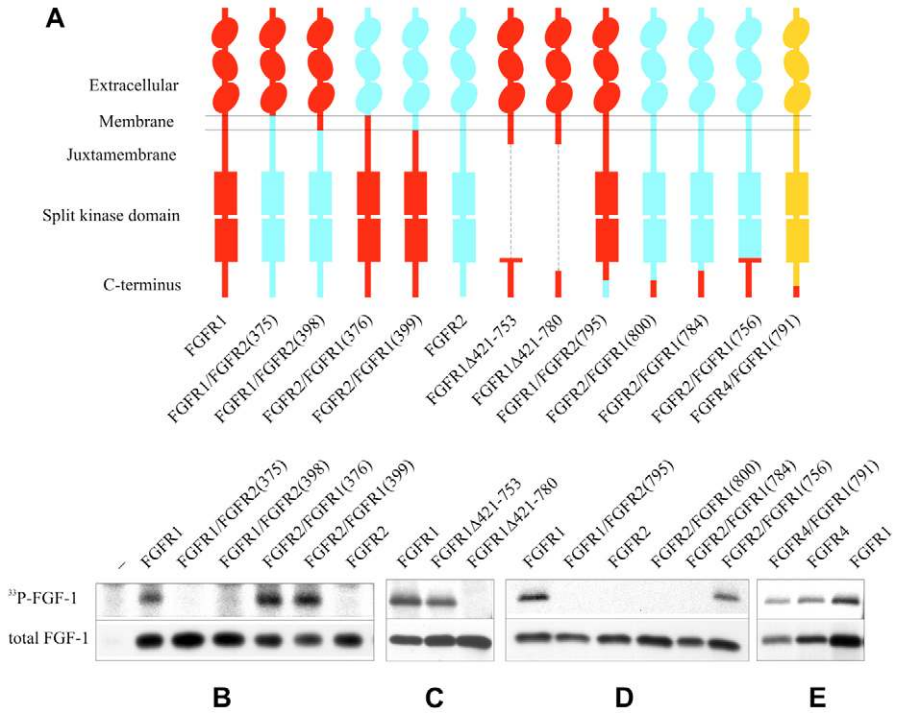

Fig. 5. Ability of chimeric and deleted receptors to translocate FGF-1. (A) Schematic overview of the FGFR mutants. Red indicates FGFR1-derived sequences, blue indicates FGFR2 and yellow indicates FGFR4. Deletions are indicated by a broken line. (B-E) COS-1 cells were transfected with FGFR-constructs as indicated. The cells were labeled with [^{33}P]phosphate and incubated with FGF-1 and heparin for 6 hours. FGF-1 was then extracted from the cell lysates and analyzed as in Fig. 2. Upper panels show phosphorylated FGF-1 detected by autoradiography and the lower panels show the total of endocytosed FGF-1, detected by anti-FGF-1.

downstream amino acids, translocation was completely abolished (mutant FGFR1Δ421-780, Fig. 5C).

We then investigated whether the C-terminal tail region downstream of the kinase domain could determine the translocation ability. An FGFR1 mutant where the 24 most C-terminal amino acids were replaced by the corresponding 21 C-terminal amino acids from FGFR2 [mutant FGFR1/FGFR2(795)] was unable to translocate FGF-1 (Fig. 5D), indicating that this region contains amino acid residues crucial for translocation. Introducing the 24 most C-terminal amino acids from FGFR1 into FGFR2 [mutant FGFR2/FGFR1(800)] was however, not sufficient to make FGFR2 translocation competent. We also introduced the last 41 and 69 amino acids from FGFR1 into FGFR2 [mutants FGFR2/FGFR1(784) and FGFR2/FGFR1(756), respectively]. The FGFR2/FGFR1(756) mutant, but not FGFR2/FGFR1(784), was able to translocate FGF-1 similarly to FGFR1 (Fig. 5D) suggesting that amino acid residues from the entire region downstream of the kinase domain could be important for translocation.

We also tested whether the C-terminal amino acids of FGFR1 could mediate translocation when transplanted into FGFR4. Previously it was shown that the 11 most C-terminal amino acids of FGFR4 are crucial for translocation of FGFR-1. When the 11 C-terminal amino acids in FGFR4 were replaced by the corresponding C-terminal amino acids from FGFR1 [mutant FGFR4/FGFR1(791)], this mutant translocated FGF-1 similarly to FGFR4 (Fig. 5E). Thus, despite their sequence dissimilarities, the C-terminal regions of FGFR1 and FGFR4 can mediate the same function.

Identification of single amino acid residues in the C-terminal tail of the FGFRs crucial for FGF-1 translocation

To investigate further which amino acids in the C-terminal tail determine the translocation ability of the FGFRs we performed more detailed analyses by mutating single amino acid residues. First, we used the non-translocating construct FGFR2/FGFR1(784) and changed selected amino acids in the first half of the C-terminal tail. Considering the aligned sequences of the FGFRs in several vertebrate species (Fig. 6A), we made the FGFR2 construct more FGFR1-like by introducing the single amino acid alterations E767Q or Q774M (marked by an asterisk in Fig. 6A; position 774 in FGFR2 corresponds to position 771 in FGFR1). As in previous experiments, in Fig. 6B-D the ability of the receptors to mediate translocation of FGF-1 is indicated by ^{33}P-FGF-1 (upper panels) and the expression level of the receptors is indicated by the total uptake of FGF-1 (lower panels). While E767Q had no effect, Q774M conferred FGF-1 translocation ability to the mutant receptor [FGFR2/FGFR1(784) E767QM and FGFR2/FGFR1(784) Q774M, respectively, Fig. 6B]. When introduced into the wild-type FGFR2, however, the Q774M mutation was not sufficient to confer translocation ability to the receptor (FGFR2 Q774M).

Another striking difference in the FGFR1 and FGFR2 sequences is the position Pro800 in FGFR2, which is histidine in FGFR1 and FGFR4 (marked by two asterisks in Fig. 6A). When Q774M was combined with the mutation P800H, the FGFR2 mutant (FGFR2 Q774M, P800H) efficiently translocated the growth factor (Fig. 6B). Thus, only two amino acid residues are responsible for the difference in the ability of FGFR1 and FGFR2 to mediate FGF-1 translocation. To test to what extent these two amino acids were important for translocation, we introduced into FGFR1 the opposite mutations, i.e. M771Q and H798P. The mutation M771Q strongly reduced the translocating ability of FGFR1, although not completely, as some translocated FGF-1 could be observed in some experiments (not shown). The mutation H798P completely blocked FGF-1 translocation by FGFR1 (Fig. 6C).

Despite the importance of the two amino acids Met771 and His798 in FGFR1, there might also be other amino acids that

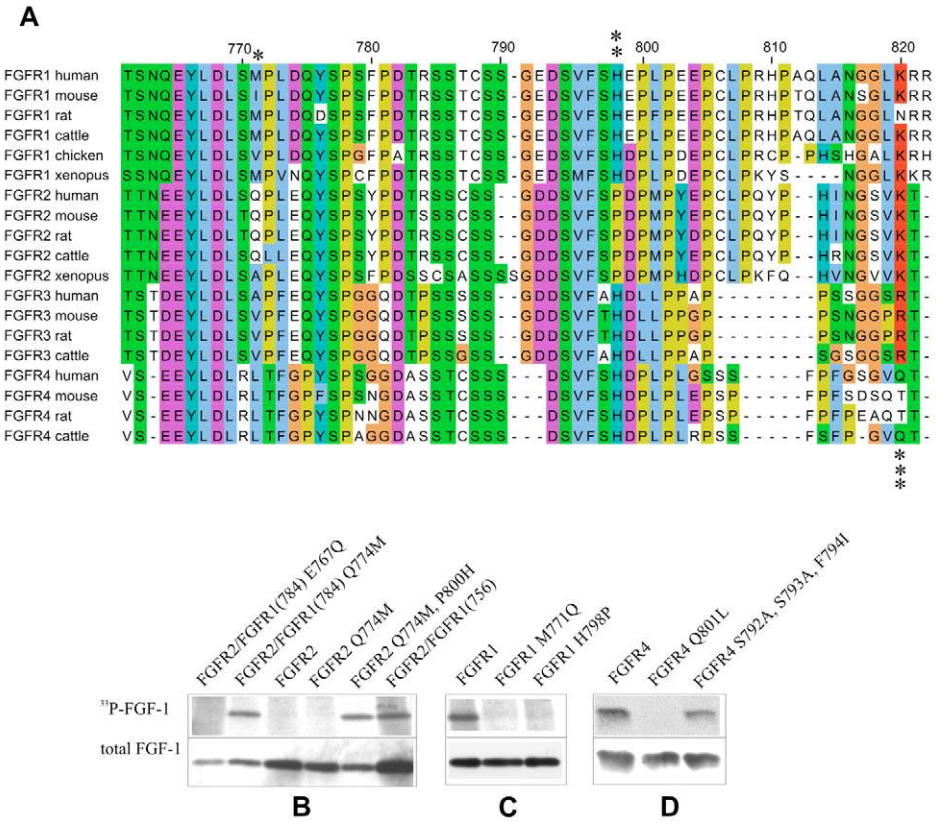

Fig. 6. Analyses of the C-terminal tails of the FGFRs. (A) ClustalW alignments of the C-terminal regions of FGFRs from various species as indicated. Numbering on top refers to the amino acids numbering here used for human FGFR1. Amino acid positions of special importance for FGF-1 translocation are indicated by *, ** and ***. (B-D) Ability of wild type and mutated receptors to translocate FGF-1. COS-1 cells were transfected with FGFR-constructs as indicated. The cells were labeled with [^{33}P]phosphate and incubated with FGF-1 and heparin for 6 hours. FGF-1 was then extracted from the cell lysates and analyzed as in Fig. 2. Upper panels show phosphorylated FGF-1 detected by autoradiography and the lower panels show the total of endocytosed FGF-1, detected by anti-FGF-1.

are necessary for translocation of FGF-1 (already present in FGFR2), as suggested by the importance of the 11 most C-terminal amino acids in FGFR4 (Klingenberg et al., 2000b). By further mutational analysis of the FGFR4 C-terminus we found a single amino acid residue crucial for FGF-1 translocation. As shown in Fig. 6D, when the penultimate Gln in FGFR4 was mutated to Leu (FGFR4 Q801L), translocation was completely blocked. Conversely, three point mutations in positions 792-794 of FGFR4 (mutant FGFR4 S792A, S793A, F794I) did not affect translocation. Altogether the data demonstrate functional diversity of the four FGFRs due to small differences in the C-terminal tail of the receptors.

Discussion

The main finding reported here is that FGFR1 and FGFR4 are able to facilitate translocation of exogenous FGF-1 to the cytosol and nucleus while FGFR2 and FGFR3 are unable to do so. This demonstrates a previously unrecognized diversity in the functions of the four FGFRs.

Our results also demonstrate a crucial role of the intracellular, C-terminal tail of the FGFR in the FGF-1 translocation process. The kinase domain of FGFR1 could be deleted without abolishing translocation. Conversely, the C-terminal tail of FGFR1, comprising approximately 50 amino acids downstream of the kinase domain, was necessary for translocation and when it was introduced into FGFR2 it enabled the FGFR2-FGFR1 chimeric receptor to translocate FGF-1. By mutating selected single amino acid residues, the difference between the translocating FGFR1 and the non-translocating FGFR2 was demonstrated to reside in only two amino acid residues within the C-terminal tail. The mutations Q774M and P800H converted FGFR2 into a fully translocating receptor. Either of the opposite mutations in FGFR1, M771Q or H798P, drastically reduced the translocation of FGF-1. Mutational analysis of the C-terminal region of FGFR4 furthermore revealed a crucial role of the penultimate amino acid Gln801.

The mutational analyses described here identify three amino acid residues of crucial importance for the ability of the FGFR to mediate FGF-1 translocation and also indicate how FGFR2 may have diverged from FGFR1 during evolution to become unable to mediate FGF-1 translocation. By considering the multiple sequence alignment of the FGFRs from several species (Fig. 6A), the important amino acids are in positions corresponding to Met771, His798 and Lys820 in human FGFR1. The first position is occupied by methionine, isoleucine, valine, or leucine, over a range of FGFR1 or FGFR4 molecules from different species suggesting that for FGF-1 translocation a hydrophobic amino acid is preferred in this position. The second position is occupied by histidine in FGFR1, FGFR4 and also FGFR3, from different species, but is replaced by proline in the FGFR2 molecules. This could indicate that the histidine in this position is a key residue in mediating FGF-1 translocation. The third position is lysine, aspartic acid, glutamine, or threonine in FGFR1 and FGFR4 molecules and lysine or arginine in FGFR2 and FGFR3 suggesting that a polar or positively charged amino acid in this position is required for translocation of FGF-1. Apparently, the inability of FGFR3 to mediate FGF-1 translocation is not governed by unfavorable amino acids in the above-mentioned positions, but is possibly determined by other features of the C-terminal tail.

Not many functions have been ascribed to the C-terminal tail of the FGFRs. FGF stimulation is known to induce autophosphorylation of Tyr766 within this C-terminal region and this recruits PLCγ (Mohammadi et al., 1991). However, since the kinase domain of FGFR1 (this study) and FGFR4 (Klingenberg et al., 2000b), can be deleted without abolishing FGF-1 translocation, it is unlikely that Tyr766/PLCγ plays any role for the FGF-1 translocation.

The crystal structure of the kinase domain of FGFR1, comprising amino acids 456-765, has been solved (Mohammadi et al., 1996), while the three-dimensional structure of the downstream region is not known. The ability of the C-terminal tail to mediate FGF-1 translocation even when the kinase domain is deleted, suggests that it constitutes a stable functional entity or domain.

The C-terminal domain, particularly the last ~20 amino acids, is the region in the different types of FGFRs that displays the most sequence divergency. Despite their sequence dissimilarities, the C-terminal domain of FGFR1 and FGFR4 apparently mediate a similar function, possibly because they adopt similar three-dimentional structures. This is supported by our finding that the 11 C-terminal amino acids of FGFR4 can be replaced by the corresponding C-terminal amino acids of FGFR1 without loosing FGF-1 translocation ability.

That exogenous FGF-1 and FGF-2 can translocate to the cell cytosol and nucleus has been recognized for several years (Olsnes et al., 2003), although the mechanism by which translocation occurs and the intracellular action of the growth factors have been more elusive. However, during recent years several intracellular proteins have been found to interact with FGF (Kolpakova et al., 1998; Mizukoshi et al., 1999; Skjerpen et al., 2002a; Skjerpen et al., 2002b; Sheng et al., 2005) and it is also becoming clear that both the translocation to cytosol and the nucleocytoplasmic trafficking of the growth factors are tightly regulated processes (Malecki et al., 2002; Malecki et al., 2004; Wiedlocha et al., 2005). Several observations suggest that the translocated FGF has mitogenic activity (Imamura et al., 1990; Wiedlocha et al., 1994; Wiedlocha et al., 1996; Klingenberg et al., 1998; Bossard et al., 2003).

Binding of FGF to FGFR induces receptor signaling and also receptor mediated endocytosis and intracellular trafficking of receptor and ligand. Translocation of FGF-1 and FGF-2 to cytosol has been found to occur from endosomes and depends on the membrane potential across the vesicle membrane (Malecki et al., 2002; Malecki et al., 2004). However, endocytosis as such is clearly not sufficient for translocation since FGF-1 endocytosed by cell surface HSPGs or by the high affinity FGFR2 or FGFR3 is not translocated.

Previous studies demonstrated that FGF-1 endocytosed by FGFR1 is for a large part sorted to lysosomes whereas FGFR4 shows a high degree of recycling through the recycling endosomal compartment. FGFR2 and FGFR3 are sorted similar to FGFR1, but with slightly higher extent of recycling (Haugsten et al., 2005). From this there appears to be no link between the described FGFR sorting pathways and the facilitation of FGF-1 translocation. This can be taken as an indication that the translocation occurs from early endosomes.

Expression of either FGFR type in the COS-1 cells used in this study leads to activation of p42/44 MAPK, p38 MAPK, Akt, PLCγ and Stat1. Thus, translocation of FGF-1 could not be correlated with activation of specific downstream effectors. In accordance with this, we find that deletion of the kinase domain as well as the FRS2 binding site in FGFR1 did not abolish FGF-translocation indicating that COS-1 cells do not depend on receptor signaling to facilitate FGF-1 translocation. COS-1 cells might be unusual in this sense since they have a high basal activity of signaling molecules, such as MAPK. Whether receptor signaling plays a role for FGF-1 translocation in more physiologically relevant types of cells needs further elucidation.

We show here that translocation of FGF-1 facilitated by FGFR1 or FGFR4 is similarly sensitive to inhibitors that manipulate the membrane potential of vesicles and has a similar time course. This suggests a common mechanism of translocation for the two types of receptor. These characteristics of the translocation are also common for the translocation of FGF-1 into other cell types expressing endogenous FGFRs (Malecki et al., 2002; Malecki et al., 2004). Compared with the rate of endocytosis, translocation of FGF-1 occurred after a delay of 2-3 hours. Possibly, this is the time required to assemble a translocon for FGF-1. A translocon would have to be built from pre-fabricated components, as translocation is not inhibited by the presence of cycloheximide during the incubation with FGF (Malecki et al., 2004). Possibly, the difference between translocating and non-translocating receptors is due to different abilities to recruit necessary translocon components by adsorption to their C-terminal tail.

Although the different types of FGFRs activate common downstream signaling pathways, they do differ in the strength of the receptor tyrosine kinase activities (Raffioni et al., 1999), in their intracellular sorting after endocytosis (Haugsten et al., 2005) and, as shown here, in their ability to facilitate translocation of FGF-1 to cytosol and/or nucleus. The functional differences between the different types of FGFRs may thus, account for different cellular responses to FGF stimulation.

Materials and Methods

Plasmids

pcDNA3 plasmids encoding the full length human FGFR4 (IIIc) and the mutant FGFR4 K503R have been described earlier (Munoz et al., 1997; Klingenberg et al., 2000b). cDNA encoding full-length human FGFR1 (IIIc) was cut out from pSV7d-FGFR1 (Wennstrom et al., 1991) with *Eco*RI and *Xba*I and ligated into pcDNA3. cDNA encoding the FGFR1β variant was obtained by deletion of the Ala31-Ser119 fragment from the full-length FGFR1 construct. cDNA encoding full-length human FGFR2 (IIIc) was cut out from the pCMV6-XL4 cDNA clone (Origene Technologies) as an *Eco*RI-*Xba*I fragment and ligated into pcDNA3. cDNA encoding human FGFR2β (IIIc) was cut out from pBluescript (RZPD, Clone ID: IMAGp998N0911701Q3) with *Not*I and *Spe*I and ligated into pcDNA3. The pcDNA3-hFGFR3 (IIIc) construct was a generous gift from Dr Avner Yayon, ProChon Biotech, Israel (Adar et al., 2002). Hybrid and deleted mutants of the FGFRs as described below, were made using combinations of standard techniques such as PCR, a Quick change site-directed mutagenesis kit (Stratagene) and subclonings. All constructs were confirmed by DNA sequencing. The hybrid receptor FGFR1/FGFR2(375) consists of amino acids Met1-Leu375 from FGFR1 and Glu377-stop from FGFR2. FGFR2/FGFR1(376) consists of amino acids Met1-Leu376 from FGFR2 and Glu376-stop from FGFR1. FGFR1/FGFR2(398) consists of amino acids Met1-Lys398 from FGFR1 and Met400-stop from FGFR2. FGFR2/FGFR1(399) consists of amino acids Met1-Arg399 from FGFR2 and Met399-stop from FGFR1. The construct FGFR1Δ421-753 is FGFR1 with a deletion of amino acids Ile421-Asp753. FGFR1Δ421-780 is FGFR1 with a deletion of amino acids Ile421-Phe780. The hybrid receptor FGFR1/FGFR2(795) consists of amino acids Met1-Val795 from FGFR1 and Phe798-stop from FGFR2. FGFR2/FGFR1(800) consists of amino acids Met1-Pro800 from FGFR2 and Glu799-stop from FGFR1. FGFR2/FGFR1(784) consists of amino acids Met1-

Pro784 from FGFR2 and Asp782-stop from FGFR1. FGFR2/FGFR1(756) consists of amino acids Met1-Asp756 from FGFR2 and Leu754-stop from FGFR1. FGFR4/FGFR1(791) consists of amino acids Met1-Ser791 from FGFR4 and Pro808-stop from FGFR1.

Cell culture and transfection

COS-1 cells and HeLa cells were propagated in Quantum 333 Complete medium for Fibroblasts (PAA Laboratories) supplemented with 2% (v/v) bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a 5% CO_2 atmosphere at 37°C. The cells were transfected with pcDNA3 plasmids with appropriate inserts using the Fugene-6 transfection reagent (Boehringer Mannheim) according to the procedure given by the company. Six hours after transfection the cells were transferred to a serum-free medium for overnight starvation before further experiments were performed.

FGF-1 preparation

Recombinant FGF-1 and FGF-1 K132E, [^{35}S]methionine labeled FGF-1 (^{35}S-FGF-1) and Cy3-labeled FGF-1 (Cy3-FGF-1) were produced as previously described (Wiedlocha et al., 1994; Wiedlocha et al., 1996; Klingenberg et al., 1998; Citores et al., 1999).

Binding and endocytosis of FGF-1

Cells were incubated with [^{35}S]-FGF-1 and 30 U ml^{-1} heparin in HEPES medium for 1 hour on ice. To analyze the cell-surface bound FGF-1 the cells were then washed with PBS and lysed in lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1 mM EDTA). To analyze endocytosis, the cells were transferred to 37°C and further incubated for 30 minutes, then washed with a high salt, low pH buffer (2 M NaCl, 20 mM Na-acetate, pH 4.0) to remove surface bound FGF-1 and lysed in lysis buffer. FGF-1 was extracted from the cell lysates by binding to heparin-Sepharose beads (Amersham Biotech) and then analyzed by SDS-PAGE. For microscopy, cells were grown on coverslips and incubated with Cy3-FGF-1 and 30 U ml^{-1} heparin for 2 hours, then washed with PBS and fixed with 3% paraformaldehyde in PBS for 15 minutes at room temperature. The coverslips were mounted with mowiol and examined with a Zeiss LSM510 META confocal microscope. The same settings of laser intensity and detector gain were used for all captured images.

FGF-1 phosphorylation assay

After transfections, cells were incubated for 16 hours in serum and phosphate free medium supplemented with [^{33}P]phosphate (Amersham Pharmacia Biotech). The cells were then treated with 100 ng ml^{-1} recombinant FGF-1 or FGF-1 K132E and 30 U ml^{-1} heparin (unless otherwise stated) for 6 hours, or as long as indicated. Where indicated, 10 nM bafilomycin A1, 1 μM monensin, or 5 ng ml^{-1} LMB was also present during this incubation. At the end of the incubation period the cells were washed with high salt, low pH buffer to remove surface bound FGF-1, once with Hepes medium and then lysed and scraped off the plate in lysis buffer supplemented with phosphatase inhibitors (Complete, EDTA-free Protease Inhibitor Cocktail Tablets from Roche Applied Science). The lysate was centrifuged and the supernatant was designated the cytoplasmic fraction of the cells. Sometimes the insoluble pellets, which contained the cell nuclei were further processed, i.e. washed once with lysis buffer, then sonicated to dissolve the nuclei and centrifuged to remove undissolved debris. FGF-1 was extracted from cytoplasmic and nuclear fractions by binding to heparin-Sepharose. To remove unspecific phosphoproteins that absorb to heparin-Sepharose, the beads were treated with 2 μg ml^{-1} TPCK-treated trypsin for 30 minutes at room temperature and washed three times with lysis buffer. Then FGF-1, which is highly resistant to trypsin treatment when bound to heparin, was eluted in SDS-PAGE sample buffer containing PMSF.

SDS-PAGE and western blotting

Samples were fractionated by standard SDS-PAGE and electroblotted onto Immobilon-P membrane (Millipore). For detection of radioactively labeled bands, the membrane was exposed to autoradiography film or scanned by a phosphor-imager. Immunodetection of bands on the membrane was performed using appropriate primary antibodies, HRP-labeled secondary antibodies and Super signal substrate solutions (Pierce). The chemiluminescent signal was detected on film or scanned using the Chemigenious Bio Imaging system (Syngene). Quantification of signals obtained by phosphor-imager or Chemigenius-scanning was done using the ImageQuant 5.0 software.

Antibodies

Antibodies against FGFR1, FGFR2, FGFR3, FGFR4, FGF-1 and phospho-PLCγ1 (Tyr783) were from Santa Cruz Biotechnology. Phospho-p38 MAPK (pT180/pY182) antibody was from BD transduction Laboratories. Phospho-FGFR (Y653/654), Phospho-p44/42 MAPK (T202/Y204), Phospho-Akt (S473), Phospho-Stat1 (Y701), Calnexin and MAPK antibodies were from Cell Signaling Technology. Lamin A antibody was from Abcam. Secondary antibodies were anti-mouse, -rabbit or -goat–HRP from Jackson Immuno-Research Laboratories.

Sequence analysis

To align the amino acid sequences of FGFRs from various species we used the JalView multiple alignment editor (Clamp et al., 2004) and Clustal W multiple sequence alignment (Thompson et al., 1994).

We thank Ken Rosendal for help with amino acid sequence analyses. V.S. and J.W. are postdoctoral fellows and E.M.H. is a predoctoral fellow of the Norwegian Cancer Society. This work was supported by the Research Council of Norway, by Novo Nordisk Foundation, Blix Fund, Rachel and Otto Kr. Bruuns Fund, Torsteds Fund and by the Jahre Foundation.

References

Adar, R., Monsonego-Ornan, E., David, P. and Yayon, A. (2002). Differential activation of cysteine-substitution mutants of fibroblast growth factor receptor 3 is determined by cysteine localization. *J. Bone Miner. Res.* **17**, 860-868.

Baldin, V., Roman, A. M., Bosc-Bierne, I., Amalric, F. and Bouche, G. (1990). Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. *EMBO J.* **9**, 1511-1517.

Boilly, B., Vercoutter-Edouart, A. S., Hondermarck, H., Nurcombe, V. and Le Bourhis, X. (2000). FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev.* **11**, 295-302.

Bonnet, H., Filhol, O., Truchet, I., Brethenou, P., Cochet, C., Amalric, F. and Bouche, G. (1996). Fibroblast growth factor-2 binds to the regulatory beta subunit of CK2 and directly stimulates CK2 activity toward nucleolin. *J. Biol. Chem.* **271**, 24781-24787.

Bossard, C., Laurell, H., Van den Berghe, L., Meunier, S., Zanibellato, C. and Prats, H. (2003). Translokin is an intracellular mediator of FGF-2 trafficking. *Nat. Cell Biol.* **5**, 433-439.

Bouche, G., Gas, N., Prats, H., Baldin, V., Tauber, J. P., Teissie, J. and Amalric, F. (1987). Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0–G1 transition. *Proc. Natl. Acad. Sci. USA* **84**, 6770-6774.

Citores, L., Wesche, J., Kolpakova, E. and Olsnes, S. (1999). Uptake and intracellular transport of acidic fibroblast growth factor: evidence for free and cytoskeleton-anchored fibroblast growth factor receptors. *Mol. Biol. Cell* **10**, 3835-3848.

Clamp, M., Cuff, J., Searle, S. M. and Barton, G. J. (2004). The Jalview Java alignment editor. *Bioinformatics* **20**, 426-427.

Friedman, S., Zhan, X. and Maciag, T. (1994). Mutagenesis of the nuclear localization sequence in EGF-1 alters protein stability but not mitogenic activity. *Biochem. Biophys. Res. Commun.* **198**, 1203-1208.

Grose, R. and Dickson, C. (2005). Fibroblast growth factor signaling in tumorigenesis. *Cytokine Growth Factor Rev.* **16**, 179-186.

Haugsten, E. M., Sorensen, V., Brech, A., Olsnes, S. and Wesche, J. (2005). Different intracellular trafficking of FGF1 endocytosed by the four homologous FGF receptors. *J. Cell Sci.* **118**, 3869-3881.

Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J. A., Hla, T. and Maciag, T. (1990). Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. *Science* **249**, 1567-1570.

Imamura, T., Oka, S., Tanahashi, T. and Okita, Y. (1994). Cell cycle-dependent nuclear localization of exogenously added fibroblast growth factor-1 in BALB/c 3T3 and human vascular endothelial cells. *Exp. Cell Res.* **215**, 363-372.

Jans, D. A. and Hassan, G. (1998). Nuclear targeting by growth factors, cytokines and their receptors: a role in signaling? *BioEssays* **20**, 400-411.

Johnson, D. E. and Williams, L. T. (1993). Structural and functional diversity in the FGF receptor multigene family. *Adv. Cancer Res.* **60**, 1-41.

Klingenberg, O., Wiedlocha, A., Rapak, A., Munoz, R., Falnes, P. and Olsnes, S. (1998). Inability of the acidic fibroblast growth factor mutant K132E to stimulate DNA synthesis after translocation into cells. *J. Biol. Chem.* **273**, 11164-11172.

Klingenberg, O., Wiedlocha, A., Citores, L. and Olsnes, S. (2000a). Requirement of phosphatidylinositol 3-kinase activity for translocation of exogenous aFGF to the cytosol and nucleus. *J. Biol. Chem.* **275**, 11972-11980.

Klingenberg, O., Wiedlocha, A., Rapak, A., Khnykin, D., Citores, L. and Olsnes, S. (2000b). Requirement for C-terminal end of fibroblast growth factor receptor 4 in translocation of acidic fibroblast growth factor to cytosol and nucleus. *J. Cell Sci.* **113**, 1827-1838.

Kolpakova, E., Wiedlocha, A., Stenmark, H., Klingenberg, O., Falnes, P. O. and Olsnes, S. (1998). Cloning of an intracellular protein that binds selectively to mitogenic acidic fibroblast growth factor. *Biochem. J.* **336**, 213-222.

Malecki, J., Wiedlocha, A., Wesche, J. and Olsnes, S. (2002). Vesicle transmembrane potential is required for translocation to the cytosol of externally added FGF-1. *EMBO J.* **21**, 4480-4490.

Malecki, J., Wesche, J., Skjerpen, C. S., Wiedlocha, A. and Olsnes, S. (2004). Translocation of FGF-1 and FGF-2 across vesicular membranes occurs during G1-phase by a common mechanism. *Mol. Biol. Cell* **15**, 801-814.

Mellor, H. and Parker, P. J. (1998). The extended protein kinase C superfamily. *Biochem. J.* **332**, 281-292.

Mizukoshi, E., Suzuki, M., Loupatov, A., Uruno, T., Hayashi, H., Misono, T., Kaul, S. C., Wadhwa, R. and Imamura, T. (1999). Fibroblast growth factor-1 interacts with the glucose-regulated protein GRP75/mortalin. *Biochem. J.* **343**, 461-466.

Mohammadi, M., Honegger, A. M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C. A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991). A tyrosine-

phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol. Cell Biol.* **11**, 5068-5078.

Mohammadi, M., Schlessinger, J. and Hubbard, S. R. (1996). Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. *Cell* **86**, 577-587.

Mohammadi, M., Olsen, S. K. and Ibrahimi, O. A. (2005). Structural basis for fibroblast growth factor receptor activation. *Cytokine Growth Factor Rev.* **16**, 107-137.

Munoz, R., Klingenberg, O., Wiedlocha, A., Rapak, A., Falnes, P. O. and Olsnes, S. (1997). Effect of mutation of cytoplasmic receptor domain and of genistein on transport of acidic fibroblast growth factor into cells. *Oncogene* **15**, 525-536.

Olsnes, S., Klingenberg, O. and Wiedlocha, A. (2003). Transport of exogenous growth factors and cytokines to the cytosol and to the nucleus. *Physiol. Rev.* **83**, 163-182.

Ornitz, D. M. and Marie, P. J. (2002). FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.* **16**, 1446-1465.

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G. and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15292-15297.

Powers, C. J., McLeskey, S. W. and Wellstein, A. (2000). Fibroblast growth factors, their receptors and signaling. *Endocr. Relat. Cancer* **7**, 165-197.

Raffioni, S., Thomas, D., Foehr, E. D., Thompson, L. M. and Bradshaw, R. A. (1999). Comparison of the intracellular signaling responses by three chimeric fibroblast growth factor receptors in PC12 cells. *Proc. Natl. Acad. Sci. USA* **96**, 7178-7183.

Sheng, Z., Liang, Y., Lin, C. Y., Comai, L. and Chirico, W. J. (2005). Direct regulation of rRNA transcription by fibroblast growth factor 2. *Mol. Cell Biol.* **25**, 9419-9426.

Skjerpen, C. S., Nilsen, T., Wesche, J. and Olsnes, S. (2002a). Binding of FGF-1 variants to protein kinase CK2 correlates with mitogenicity. *EMBO J.* **21**, 4058-4069.

Skjerpen, C. S., Wesche, J. and Olsnes, S. (2002b). Identification of ribosome-binding protein p34 as an intracellular protein that binds acidic fibroblast growth factor. *J. Biol. Chem.* **277**, 23864-23871.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Wennstrom, S., Sandstrom, C. and Claesson-Welsh, L. (1991). cDNA cloning and expression of a human FGF receptor which binds acidic and basic FGF. *Growth Factors* **4**, 197-208.

Wesche, J., Malecki, J., Wiedlocha, A., Ehsani, M., Marcinkowska, E., Nilsen, T. and Olsnes, S. (2005). Two nuclear localization signals required for transport from the cytosol to the nucleus of externally added FGF-1 translocated into cells. *Biochemistry* **44**, 6071-6080.

Wesche, J., Malecki, J., Wiedlocha, A., Skjerpen, C. S., Claus, P. and Olsnes, S. (2006). FGF-1 and FGF-2 require the cytosolic chaperone Hsp90 for translocation into the cytosol and the cell nucleus. *J. Biol. Chem.* **281**, 11405-11412.

Wiedlocha, A. and Sorensen, V. (2004). Signaling, internalization and intracellular activity of fibroblast growth factor. *Curr. Top. Microbiol. Immunol.* **286**, 45-79.

Wiedlocha, A., Falnes, P. O., Madshus, I. H., Sandvig, K. and Olsnes, S. (1994). Dual mode of signal transduction by externally added acidic fibroblast growth factor. *Cell* **76**, 1039-1051.

Wiedlocha, A., Falnes, P. O., Rapak, A., Klingenberg, O., Munoz, R. and Olsnes, S. (1995). Translocation of cytosol of exogenous, CAAX-tagged acidic fibroblast growth factor. *J. Biol. Chem.* **270**, 30680-30685.

Wiedlocha, A., Falnes, P. O., Rapak, A., Munoz, R., Klingenberg, O. and Olsnes, S. (1996). Stimulation of proliferation of a human osteosarcoma cell line by exogenous acidic fibroblast growth factor requires both activation of receptor tyrosine kinase and growth factor internalization. *Mol. Cell Biol.* **16**, 270-280.

Wiedlocha, A., Nilsen, T., Wesche, J., Sorensen, V., Malecki, J., Marcinkowska, E. and Olsnes, S. (2005). Phosphorylation-regulated nucleocytoplasmic trafficking of internalized fibroblast growth factor-1. *Mol. Biol. Cell* **16**, 794-810.

Zhan, X., Hu, X., Friesel, R. and Maciag, T. (1993). Long term growth factor exposure and differential tyrosine phosphorylation are required for DNA synthesis in BALB/c 3T3 cells. *J. Biol. Chem.* **268**, 9611-9620.