Phosphorylation-regulated Nucleocytoplasmic Trafficking of Internalized Fibroblast Growth Factor-1

Antoni Więdołcha,* Trine Nilsen, Jørgen Wesche, Vigdis Sørensen, Jędrzej Małecki, Ewa Marcinkowska, and Sjur Olsnes

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

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Fibroblast growth factor-1 (FGF-1), which stimulates cell growth, differentiation, and migration, is capable of crossing cellular membranes to reach the cytosol and the nucleus in cells containing specific FGF receptors. The cell entry process can be monitored by phosphorylation of the translocated FGF-1. We present evidence that phosphorylation of FGF-1 occurs in the nucleus by protein kinase C (PKC) δ . The phosphorylated FGF-1 is subsequently exported to the cytosol. A mutant growth factor where serine at the phosphorylation site is exchanged with glutamic acid, to mimic phosphorylated FGF-1, is constitutively transported to the cytosol, whereas a mutant containing alanine at this site remains in the nucleus. The export can be blocked by leptomycin B, indicating active and receptor-mediated nuclear export of FGF-1. Thapsigargin, but not leptomycin B, prevents the appearance of active PKC δ in the nucleus, and FGF-1 is in this case phosphorylated in the cytosol. Leptomycin B increases the amount of phosphorylated FGF-1 in the cells by preventing dephosphorylation of the growth factor, which seems to occur more rapidly in the cytoplasm than in the nucleus. The nucleocytoplasmic trafficking of the phosphorylated growth factor is likely to play a role in the activity of internalized FGF-1.

INTRODUCTION

Fibroblast growth factor-1 (FGF-1 or acidic fibroblast growth factor) belongs to a large family of polypeptide growth factors that exhibit a wide spectrum of biological activities (Mason, 1994; Powers *et al.*, 2000). Among the 22 identified members of the family, FGF-1 and FGF-2 (basic FGF) are distinguished by the lack of a recognizable amino-terminal signal sequence (Mason, 1994; Olsnes *et al.*, 2003). After synthesis, the growth factors reside in the cytosol and nucleus of the producing cells (Olsnes *et al.*, 2003; Więdołcha and Sørensen, 2004), and they are released from the cells by a mechanism bypassing the classical endoplasmic reticulum (ER)-Golgi secretory pathway (Jackson *et al.*, 1992; Prudovsky *et al.*, 2003).

The biological action of the growth factors is exerted through binding to and activation of high-affinity cell surface receptors (FGFRs) that have intrinsic tyrosine kinase activity (Klint and Claesson-Welsh, 1999). FGF-1 contains a heparin sulfate-binding domain. Binding of the growth factor to heparin sulfates protects it against proteases and regulates binding to and activation of FGFRs (Ornitz and Itoh, 2001). There are four known FGF tyrosine kinase receptors and a number of splicing variants for each of them that could, at least partly, account for the different responses induced by the ligand (Powers *et al.*, 2000). In addition, FGF-1 binds to cysteine-rich transmembrane receptors without tyrosine kinase activity and with unknown function (Zhou *et al.*, 1997).

Accumulating evidence indicates that FGF-1 acts on cells by a dual mechanism. The growth factor activates the cell surface receptors inducing activation of intracellular second messengers (Powers *et al.*, 2000). In addition, the receptor-bound growth factor is endocytosed and translocated across the vesicular membrane to reach the cytosol and the nucleus (Olsnes *et al.*, 2003; Więdołcha and Sørensen, 2004). Localization to the nucleus seems to depend on the presence of a nuclear localization sequence in FGF-1 (Imamura *et al.*, 1990). Moreover, it has been shown that FGF-1 interacts specifically with intracellular proteins such as FIBP, p34, CK2, and mortalin (Kolpakova *et al.*, 1998; Skjerpen *et al.*, 2002a; Skjerpen *et al.*, 2002b; Mizukoshi *et al.*, 1999). These proteins may be involved in the intracellular action of the growth factor.

Translocation of exogenous FGF-1 to the cytosol and nucleus requires binding to FGFRs. Although the growth factor binds abundantly to surface heparan sulfate proteoglycans even in cells lacking FGFRs, it is not translocated to the cytosol and nucleus in these cells (Olsnes *et al.*, 2003). It also was demonstrated that endocytosed complexes of FGF-1 and FGFR4 (Citores *et al.*, 1999) or FGFR1 (Prudovsky *et al.*, 1996) are not rapidly degraded in lysosomes, but accumulate in juxtanuclear organelles.

Evidence for membrane translocation of exogenous FGF-1 was obtained by treatment of FGFR-positive cells with a growth factor mutant containing a C-terminal farnesylation signal, a CAAX-box (Więdołcha *et al.*, 1995). Because the farnesyl transferase is located exclusively in the cytosol and nucleus, the appearance of farnesylated FGF-1-CAAX indicates that the growth factor has crossed cellular membranes.

Another method used to assess membrane translocation takes advantage of the fact that FGF-1 has only one functional phosphorylation site (Ser 130) and that it is phosphorylated by protein kinase C (PKC), an enzyme that is present

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* Corresponding author. E-mail address: antoni.wiedołcha@labmed.uio.no.

in the cytosol and nucleus but not at the cell surface or inside vesicular compartments (Klingenberg *et al.*, 1998). Phosphorylation at this site indicates that FGF-1 must have been translocated to the cytosol and/or nucleus.

Further evidence that this is the case was obtained in experiments where the plasma membrane was permeabilized with streptolysin O under conditions where membranes of intracellular organelles remained intact. Under these conditions, the phosphorylated FGF-1 leaked into the medium together with soluble cytosolic proteins (Wesche *et al.*, 2000).

Using these methods, we could demonstrate that phosphatidylinositol (PI)3-kinase activity is required for translocation of externally added FGF-1 to the cytosol and nucleus. When the kinase was inhibited, FGF-1-CAAX was not farnesylated and FGF-1 was not phosphorylated (Klingenberg *et al.*, 2000). Recently, we have obtained evidence that translocation of FGF-1 to the cytosol occurs from the lumen of intracellular vesicles possessing vacuolar proton pumps and that an electrical potential across the vesicular membrane is required for translocation (Matecki *et al.*, 2002, 2004).

Although farnesylated CAAX-tagged FGF-1 was found to a large extent in the nucleus, phosphorylated growth factor was mainly present in the cytosol (Klingenberg *et al.*, 2000). This suggested that phosphorylation could be involved in the action of the internalized growth factor.

Nucleocytoplasmic transport of proteins is crucial for communication between the cytoplasm and the nucleus. It occurs by active or passive mechanisms, depending on the requirements for energy, size of the molecule, and the presence of a targeting signal recognizable by soluble receptors specific for nuclear import or export (Conti and Izaurralde, 2001). Nuclear pore complexes (NPCs) are the only known gates for transport across the nuclear envelope (Vasu and Forbes, 2001), but the mechanism of translocation through NPCs is still not completely understood.

Ca²⁺ depletion of the ER and of the nuclear envelope by calcium ionophores or by the calcium ATPase inhibitor thapsigargin has been found to block passive diffusion as well as active signal-mediated transport into the nucleus (Greber and Gerace, 1995; Greber *et al.*, 1997). It was suggested that the inhibition is due to a steric block of the central and peripheral NPC transport channels (Greber and Gerace, 1995; Perez-Terzic *et al.*, 1999).

In attempts to elucidate where in the cell internalized FGF-1 is phosphorylated, we studied phosphorylation in the absence and presence of thapsigargin, which was found not to block translocation of externally added FGF-1 into the cytosol. We here report that exogenous FGF-1 added to cells is phosphorylated by PKC δ in the nucleus, followed by rapid export to the cytosol. Nonphosphorylated FGF-1 remains in the nucleus. Treatment of the cells with thapsigargin or leptomycin B blocks the export of the phosphorylated FGF-1 from the nucleus to the cytosol, indicating that the intracellular localization of internalized FGF-1 is regulated by phosphorylation. The observed effect of leptomycin B indicates that exportin-1 is involved in the rapid export of the phosphorylated growth factor from the nucleus.

MATERIALS AND METHODS

Materials

[³⁵S]methionine (1000 Ci/mmol) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, United Kingdom); [³²P]phosphoric acid (~200 mCi/mmol) and [³³P]phosphoric acid (~3000 Ci/mmol), protein A-Sepharose and heparin-Sepharose were from Pharmacia AB (Uppsala, Sweden); and protease inhibitor cocktail Complete was from Roche Diagnostics

(Mannheim, Germany), phosphatase inhibitor cocktail, LY294002, rottlerin, and Gö 6976 were from Calbiochem, La Jolla. Anti-Rab5A (rabbit), anti-FGF-1 (goat), anti-Sumo-1 N-19 (goat), anti-PKC α (rabbit), anti-protein kinase C ζ (rabbit), and anti-PKC δ (goat and rabbit) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKC δ (mouse) and anti-Hsp-90 (mouse) were from BD Transduction Laboratories (Lexington, KY). Anti-phospho-extracellular enzyme-regulated kinase (ERK) 1/2 (mouse), anti-ERK 1/2 (rabbit), and anti-phospho-Thr505-PKC δ (rabbit) were from Cell Signaling Technology (Beverly, MA). Anti-lamin A (mouse) was from Abcam (Cambridge, United Kingdom). Anti-calreticulin (rabbit) was from Stressgen Biotechnologies (Victoria, BC, Canada). Anti-PKC δ antibody blocking peptide (sc 937) was from Santa Cruz Biotechnology. The Western blot stripping buffer was from Pierce Technology (Iselin, NJ). Other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell Cultures

NIH/3T3 cells were grown in Quantum 333 medium containing 2% calf serum. CPAE and HeLa cells were grown in DMEM containing 10% newborn calf serum. Mammary carcinoma cells MDMA-MB-453 were grown in L-15 (Leibovitz) medium supplemented with 10% fetal calf serum (FCS). Cells were seeded into Costar (Cambridge, MA) tissue culture plates the day preceding the experiment.

In Vitro Transcription and Translation

The plasmids encoding FGF-1, FGF-1(S130A), and FGF-1(S130E) and the preparation of the corresponding proteins from bacteria were described earlier (Klingenberg *et al.*, 1999). For in vitro transcription and translation, plasmid DNA was linearized downstream of the encoding gene and transcribed with T3 RNA polymerase as described previously (McGill *et al.*, 1989). The mRNA was precipitated with ethanol and dissolved in H₂O containing 10 mM dithiothreitol (DTT) and 0.1 U/ μ l RNasin. The translation was performed for 1 h at 30°C in micrococcal nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI). Radioactive FGF-1 was prepared in lysates containing 1 mM [³⁵S]methionine and 25 μ M of each of the other amino acids. The lysate was finally dialyzed against phosphate-buffered saline (PBS) to remove free [³⁵S]methionine and reducing agents.

Cell Fractionation

After lysis in lysis buffer (50 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and protease and phosphatase inhibitor cocktails), cells were centrifuged at 720 $\times$ g for 15 min at 4°C. The supernatant was centrifuged again for 5 min at 15,800 $\times$ g and designated the cytosol/membrane fraction. The pellet was washed twice by resuspension in lysis buffer containing 0.3 M sucrose that was layered on lysis buffer containing 0.8 M sucrose and centrifuged at 720 $\times$ g for 15 min at 4°C. The samples were then sonicated in lysis buffer containing 0.5 M NaCl and centrifuged for 5 min at 15,800 $\times$ g. The supernatant after the last centrifugation was designated the nuclear fraction.

Cells were incubated for 6 h with [³⁵S]methionine labeled FGF-1 and then washed twice with 1 M NaCl in 20 mM sodium acetate, pH 4.0, and twice with HEPES medium. Treatment with 20 μ g/ml digitonin for 5 min at room temperature and then for 30 min on ice was performed to allow leakage of cytosolic proteins into the medium (cytosolic fraction). The cellular pellet was lysed in lysis buffer. The nuclei were sedimented, and the supernatant was designated the membrane fraction. The nuclei were then sonicated and insoluble debris was removed by centrifugation. Subsequently, all fractions were incubated for 2 h at 4°C with heparin-Sepharose and then washed with 0.7 M NaCl. Finally, the adsorbed material was subjected to SDS-PAGE.

In Vivo Phosphorylation and Digitonin Assay

In vivo phosphorylation was performed essentially as described previously (Wesche *et al.*, 2000), with some modifications. Cells were starved for 24–48 h in DMEM medium containing 1% FCS (fetal bovine serum [FBS]). Starvation was continued in phosphate-free DMEM containing 1% FBS and 50 μ Ci/ml [³²P]phosphate or 25 μ Ci/ml [³³P]phosphate for 8–12 h. The cells were then treated with 10 U/ml heparin and 100 ng/ml FGF-1 for 6 h and washed twice with HEPES-containing medium before lysis in phosphate-free lysis buffer (25 mM Tris, pH 7.5, 20 mM NaCl, 2 mM DTT, 1 mM EDTA, 1% Triton X-100, and protease and phosphatase inhibitor cocktails). The lysates were fractionated into nuclear and cytosolic fractions by centrifugation and the nuclear fraction was sonicated in phosphate-free lysis buffer containing 0.5 M NaCl. All fractions were then incubated for 2 h at 4°C with heparin-Sepharose to adsorb the growth factor and protect it from being degraded by trypsin. The growth factor adsorbed onto the heparin-Sepharose was then subjected to trypsin digestion (2 μ g/ml) for 30 min at room temperature to degrade contaminating labeled proteins. Finally, the heparin-Sepharose aliquots were washed twice with lysis buffer containing 0.7 M NaCl and 1 mM phenylmethylsulfonyl fluoride, and once with H₂O. The samples were analyzed by SDS-PAGE and fluorography.

To analyze the in vivo localization of exogenously added FGF-1 that had been phosphorylated in the cells, the cells were subjected to digitonin treatment. The plasma membrane was permeabilized with 20 μ g/ml digitonin in the presence of 5 U/ml heparin for 5 min at room temperature. Then, the cells

were briefly washed to remove unbound digitonin. Subsequently, the cells were kept on ice for an additional 30 min, to allow the cytosol to leak into the medium, and then centrifuged. The supernatant was designated the cytosol fraction. The cellular pellet was lysed with 1% Triton X-100, and the nuclei were sedimented. The supernatant from this second centrifugation was taken as the membrane fraction. The nuclear pellet was sonicated in lysis buffer containing 0.5 M NaCl, and centrifuged as described above. The final supernatant was designated the nuclear fraction. All fractions were incubated with heparin-Sepharose, washed with 0.7 M NaCl in PBS containing 1% Triton X-100, and the adsorbed material was subjected to SDS-PAGE.

In some cases, the cells were treated directly with the buffer containing Triton X-100. In this case, the soluble fraction was termed the cytoplasmic fraction because it contains both cytosol and solubilized membranes.

In Vitro Phosphorylation

Cells (5×10^5 cells/sample) either untreated or treated for 6 h at 37°C with 1 μ g/ml thapsigargin or 100 ng/ml FGF-1, or both, or with 100 ng/ml FGF-1 and 5 μ M rottlerin, or 20 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were lysed in phosphate-free lysis buffer containing phosphatase and protease inhibitor cocktails and then fractionated into cytoplasmic and nuclear fractions. Then, a polyclonal antibody against PKC δ was added (2 μ g/sample) to each fraction and incubated for 2 h at 4°C. The PKC δ immunoprecipitates were collected using protein A-Sepharose and washed twice with lysis buffer and once with kinase buffer (50 mM MOPS, pH 7.0, 10 mM MgCl₂, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1 mM ATP, and phosphatase and protease inhibitor cocktails). Then, immunoprecipitates were incubated in kinase buffer for 1 h at 37°C with either 1 μ g of pure recombinant FGF-1 or

with the FGF-1(K132E) mutant in the presence of 30 μ Ci [γ -³³P]ATP. The samples were centrifuged, and supernatants were collected and submitted to adsorption to heparin-Sepharose for 2 h at 4°C. The heparin-Sepharose with the adsorbed material was washed once with 0.7 M NaCl in lysis buffer and once with H₂O. The adsorbed material was subjected to SDS-PAGE. In case of anti-protein kinase C α immunoprecipitates, 5 μ g of myelin basic protein per sample was used as kinase substrate instead of FGF-1. The reaction was stopped by adding 2 $\times$ SDS sample buffer and heating to 100°C for 5 min.

Detection of Phosphorylated-PKC δ

Cells were serum starved for 24 h and for additional 2 h in fresh medium (+ 0.5% serum). Then, cells were treated with 20 U/ml heparin and 100 ng/ml FGF-1 for the indicated period. Subsequently, the cells were scraped from the plastic, harvested by centrifugation and washed in PBS. The cells were lysed in lysis buffer containing protease and phosphatase inhibitors cocktail. The soluble material was separated from Triton X-100-insoluble material by centrifugation, and designated the cytoplasmic fraction. The remaining pellet was washed with lysis buffer, sonicated, centrifuged, and designated the nuclear fraction. Fractions were analyzed by SDS-PAGE and Western blotting by using anti-PKC δ antibodies (mouse). The membrane was stripped and reprobed with other antibodies in the following order: anti-phospho-Thr505-PKC δ , anti-lamin A, anti-ERK 1/2, and anti-phospho-ERK 1/2.

PKC δ Immunostaining

Serum-starved NIH/3T3 cells were either untreated or treated with 10 U/ml heparin and 100 ng/ml FGF-1 for 15, 90, and 240 min. After stimulation, the cells

Figure 1. (A) Effect of thapsigargin on intracellular localization of FGF-1. Serum-starved NIH/3T3 cells were incubated for 6 h with 10 U/ml heparin and 5 ng/ml [³⁵S]methionine-labeled FGF-1 in the absence (lane 1) or presence of 50 μ M LY294002 (lane 2), 10 nM bafilomycin A1 (lane 3), 10 nM bafilomycin A1, and 1 μ M monensin (lane 4), or increasing concentrations of thapsigargin (lanes 5–7). After the incubation, cells were permeabilized with digitonin and fractionated into membrane (M), cytosolic (C), and nuclear (N) fractions as described in *Materials and Methods*. Fractions were then adsorbed onto heparin-Sepharose and analyzed by SDS-PAGE and fluorography. (B) Controls for absence of cross-contamination during the cell fractionation procedure. The cells were fractionated as in A into membrane (M), cytosolic (C), and nuclear (N) fractions. The fractions were analyzed by Western blotting, by using anti-calreticulin, anti-ERK 1/2, and anti-lamin A antibodies.

Figure 2. Effect of thapsigargin on nucleocytoplasmic distribution of phosphorylated FGF-1. (A) Serum-starved NIH/3T3 cells were preincubated with [32 P]phosphate and treated for 6 h with 10 U/ml heparin and 100 ng/ml FGF-1. Increasing concentrations of thapsigargin were added 30 min before growth factor treatment. After incubation, cells were lysed, fractionated into cytoplasmic and nuclear fractions, and FGF-1 was adsorbed onto heparin-Sepharose and analyzed by SDS-PAGE and autoradiography. (B) Serum-starved cells were treated as in A, except that thapsigargin was present only during the last 2 h of the incubation. (C) Serum-starved NIH/3T3 cells were treated as in A with (lane 1) or without (lane 2) 10 U/ml heparin and 100 ng/ml FGF-1 or as in B in the presence of thapsigargin (lanes 3 and 4).

were washed twice in PBS and fixed in 3% paraformaldehyde for 50 min at 4°C. The reaction was quenched by treatment with 50 mM NH_4Cl in PBS for 15 min in room temperature followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. Background staining was reduced by treatment with 5% FCS for 20 min. Phosphorylated PKC δ and total PKC δ were detected by incubating the samples with rabbit anti-phospho-Thr505-PKC δ (Thr505) and mouse anti-total-PKC δ primary antibodies, respectively, for 20 min. After three washes in PBS the samples were incubated with appropriate secondary antibodies conjugated to a Cy2 fluorophore for 20 min and washed again. Coverslips were finally mounted onto glass slides using Mowiol and the samples were analyzed with a Leica confocal microscope (Leica, Wezlar, Germany).

REV-Green Fluorescent Protein (GFP) System and In Vivo Nuclear Export Assay

The plasmids pRev(1.4)-GFP and pRev(NES)-GFP have been described previously (Henderson and Eleftheriou, 2000) and were kind gifts from Dr. Beric Henderson (University of Sydney, Sydney, Australia). HeLa cells were seeded onto sterile glass coverslips and when the confluence had reached ~60%, they were transfected with 1 μg of DNA of either pRev(1.4)-GFP or pRev(NES)-GFP by using the FuGENE 6 reagent as described by the manufacturer (Roche Diagnostics). After expression of the GFP fusion proteins for 48 h, transfectants were either treated with leptomycin B (2 ng/ml) for 3 h or left untreated. Cycloheximide (15 μg/ml) was added to all samples 30 min before additional treatments to ensure that cytoplasmic GFP was a result of nuclear export and not of newly translated proteins. The coverslips were washed once in PBS and fixed for 30 min in 3% paraformaldehyde on ice. After two additional washes with PBS the coverslips were mounted onto glass slides by using Mowiol, and the samples were analyzed with a Leica confocal microscope.

Measurements of DNA Synthesis

NIH/3T3 cells were grown on 24-well microtiter plates (5×10^4 cells/well), serum starved for 24 h, and then treated with 10 U/ml heparin and the indicated amount of FGF-1 and the growth factor mutants for 24 h. During the last 6 h of incubation, 1 μCi/ml of [^3H]thymidine was present in the medium. Next, the cells were extracted with 5% trichloroacetic acid (TCA), and the radioactivity incorporated onto TCA-insoluble material was measured, as described previously (Imamura *et al.*, 1990).

Uptake and Intracellular Transport of FGF-1

NIH/3T3 cells growing on six-well plates (2.5×10^5 cells/well) were serum starved for 24 h and treated for 6 h with 10 U/ml heparin and ~10 ng/ml [^{35}S]methionine-labeled FGF-1, FGF-1(S130E) or FGF-1(S130A) mutants. After incubation, the cells were washed once with 2 M NaCl in 20 mM sodium acetate, pH 4.0, and once in HEPES medium. Then the cells were permeabilized with 20 μg/ml digitonin for 5 min at room temperature and then kept for 30 min on ice in the presence of 5 U/ml heparin. Next, the cells were lysed and subjected to fractionation procedure. In some cases after the washing, cells were incubated for an additional period before the digitonin treatment. Finally, the material in the membrane, cytosolic, and nuclear fractions was adsorbed onto heparin-Sepharose and analyzed by SDS-PAGE and fluorography.

RESULTS

Phosphorylation of FGF-1 Occurs Preferentially in the Nucleus

Intracellular Localization of Externally Added FGF-1. To study the intracellular distribution of internalized FGF-1,

Figure 3. (A) Ability of digitonin to release phosphorylated FGF-1 from cells. Serum-starved NIH/3T3 cells were preincubated with [33 P]phosphate and then incubated for 6 h at 37°C in the presence of 10 U/ml heparin and 100 ng/ml unlabeled FGF-1 (lanes 1–12). Thapsigargin (1 μ g/ml) was either absent (lanes 1–3) or present during the last 2 h (lanes 4–6) or during the entire incubation (lanes 7–9). The cells were subsequently incubated with 20 μ g/ml digitonin for 5 min at room temperature, washed in PBS, and then kept at 0°C for 30 min to allow the cytosolic material to leak out into the medium (C). The remaining cell components were lysed and fractionated into a Triton X-100-soluble (M) and a nuclear (N) fraction. All fractions were then incubated with heparin-Sepharose and analyzed by SDS-PAGE and autoradiography as described in *Materials and Methods*. (B) Serum-starved NIH/3T3 cells were incubated for 6 h at 37°C with 10 U/ml heparin and ~15 ng/ml [35 S]methionine-labeled FGF-1 and 10 nM bafilomycin A1. Then the cells were treated with 20 μ g/ml digitonin and processed further as in A. (C) Controls for cross-contamination during the cell fractionation procedure. The cells were fractionated as in A into membrane (M), cytosolic (C), and nuclear (N) fractions. The fractions were analyzed by Western blotting, by using anti-Hsp-90, anti-calreticulin, anti-Rab 5, and anti-lamin A antibodies.

cells were incubated with [35 S]methionine-labeled FGF-1 for 6 h and then treated with a low concentration of digitonin to selectively permeabilize the plasma membrane (Adam *et al.*, 1990). The cells were subsequently fractionated into three fractions: 1) a cytosolic fraction, which leaks into the medium upon treatment of cells with digitonin; 2) a membrane fraction obtained by further treatment with Triton X-100; and 3) a nuclear fraction obtained by centrifugation. In the absence of inhibitors, the labeled growth factor was found in all three fractions, with the major part located in the membrane fraction (Figure 1, lane 1). This fraction includes growth factor attached to the cell surface and FGF-1 present in endosomes. A considerable part was present in the nuclear fraction and somewhat less was found in the cytosolic fraction.

The PI3-kinase inhibitor LY294002, which inhibits translocation of the growth factor into cells (Klingenberg *et al.*, 2000), prevented the appearance of the growth factor in the cytosolic and nuclear fractions, whereas having little effect on the amount of growth factor in the membrane fraction (Figure 1, lane 2).

Translocation of FGF-1 also is blocked in the presence of a low concentration of bafilomycin A1, an inhibitor of the vesicular proton pump (Małeck *et al.*, 2002). As shown in lane 3, the growth factor was also in this case absent from the cytosolic and nuclear fractions.

The inhibition of translocation induced by bafilomycin A1 is due to a dissipation of the vesicular membrane potential (Małeck *et al.*, 2002), which is normally generated by the vesicular type proton pump present in the endosome membrane. Treatment with monensin, an ionophore specific for monovalent cations, is able to restore the membrane potential by providing luminal K^+ and thereby activating the latent Na^+K^+ -ATPase present in the vesicular membrane (Małeck *et al.*, 2002, 2004). When both bafilomycin A1 and monensin were present, the growth factor was in both the cytosolic and nuclear fractions (Figure 1, lane 4). The amount of growth factor in the cytosol and nucleus was, in this case, increased (compare with lane 1), possibly due to reduced proteolytic activity. Bafilomycin A1 and monensin increase the luminal pH in acidic vesicles and thereby inhibit vesicular proteases.

Thapsigargin has been reported to be an unspecific inhibitor of nucleocytoplasmic transport for certain karyophilic proteins (Greber and Gerace, 1995; Love *et al.*, 1998). We found that thapsigargin present throughout the incubation with the growth factor had no apparent effect on the transport of the total [35 S]methionine labeled FGF-1 to the cytosol and nuclear fractions (Figure 1, lanes 5–7).

No cross-contamination was found in the different cell fractions when analyzed for the presence of marker proteins (Figure 1B). Additionally, we observed that the nuclear fraction was not contaminated by either of the cytoplasmic proteins, Hsp 90 and Rab 5 (also see Figure 3C).

Figure 4. Ability of rottlerin to inhibit phosphorylation of FGF-1 in cytosol and nucleus. (A) Serum-starved NIH/3T3 (top) or MDA-MB-453 cells (bottom) were preincubated with [33 P]phosphate and then either treated with carrier (dimethyl sulfoxide) alone (lane 1) or treated for 6 h with 100 ng/ml FGF-1 (lane 2), 100 ng/ml FGF-1, and 5 μ M rottlerin (lane 3), 100 ng/ml FGF-1 and 1 μ M Gö 6976 (lane 4), 100 ng/ml FGF-1, 1 μ M Gö 6976 and 10 nM bafilomycin A1 (lane 5), or 100 ng/ml FGF-1 and 1 μ M Gö 6976 and 10 nM bafilomycin A1 and 1 μ M monensin (lane 6). Heparin (10 U/ml) was present in each case. After the incubation, the cells were lysed, sonicated, treated with heparin-Sepharose, and the adsorbed material was analyzed by SDS-PAGE and fluorography. (B) PKC α in vitro phosphorylation assay. Serum-starved NIH/3T3 cells were treated with 20 nM TPA for 30 min. Next, the cells were lysed and sonicated and subjected to immunoprecipitation with anti-PKC α antibody. Then, the immunoprecipitated material was incubated in kinase buffer for 1 h at 37°C with 5 μ g of myelin basic protein and 30 μ Ci of γ -[33 P]ATP in the absence (lane 1) or presence 1 nM (lane 2) and 10 nM (lane 3) Gö 6976, and 5 μ M rottlerin (lane 4). (C) Serum-starved NIH/3T3 cells were preincubated with [33 P]phosphate and treated for 6 h with 10 U/ml heparin and 100 ng/ml FGF-1 and increasing concentrations of the PKC δ -inhibitor rottlerin (lanes 3–5 and 7–9). Thapsigargin (1 μ g/ml) was either present during the entire incubation (lanes 2–5) or only during the last 2 h (lanes 6–9). After incubation, cells were lysed, fractionated into cytoplasmic and nuclear fractions, adsorbed onto heparin-Sepharose, and analyzed by SDS-PAGE and fluorography (top) or Western blot (bottom) by using anti-FGF-1 antibodies.

Effect of Thapsigargin on the Intracellular Localization of Phosphorylated FGF-1

In experiments where translocation of FGF-1 into cells was monitored by phosphorylation of the growth factor, a completely different picture emerged. The phosphorylated FGF-1 was mainly found in the cytoplasmic fraction (Figure 2A, lanes 1 and 5). When thapsigargin was present throughout the incubation with FGF-1, for a total of 6 h, labeling was strongly increased (Figure 2A, lanes 2–4). In this case, there was no detectable phosphorylated growth factor in the nuclear fractions (Figure 2A, lanes 6–8).

In the next set of experiments, the phosphorylation was allowed to proceed for some time before thapsigargin was added. Figure 2B demonstrates that when 1 $\mu\text{g}/\text{ml}$ thapsigargin was added 2 h before the end of the incubation period, phosphorylated growth factor was found mainly in the nuclear fraction (lanes 4 and 8). In contrast, when cells were incubated with the growth factor in the absence of thapsigargin, the majority of the phosphorylated FGF-1 was recovered in the cytoplasmic fraction (Figure 2B, lanes 1 and 5). At intermediary concentrations of thapsigargin, the ^{32}P -labeled protein was observed in both fractions (Figure 2B, lanes 2 and 3 and 6 and 7). Possibly, growth factor that had entered the nucleus and become phosphorylated was trapped in the nucleus by the thapsigargin treatment during the final 2 h of incubation.

To ensure that the bands observed represent phosphorylated FGF-1, we performed a control experiment as shown in Figure 2C. No phosphorylated proteins migrating ~ 16 kDa (lanes 1 and 2) were detected in untreated ^{32}P -labeled cells (lane 2) or when cells were treated with thapsigargin only (lanes 3 and 4). The higher molecular weight bands visible in the cytosolic fraction are unrelated to the growth factor or thapsigargin treatment.

To test further the effect of thapsigargin on the nucleocytoplasmic transport of the phosphorylated growth factor, we carried out experiments with digitonin. The plasma membrane is selectively permeabilized under these conditions, leading to leakage of soluble cytosolic proteins into the medium.

In the absence of thapsigargin, the phosphorylated growth factor was found in the cytosolic fraction (Figure 3A, lanes 1–3). When the cells were treated with thapsigargin for the final 2 h of incubation, phosphorylated growth factor was detected only in the nuclear fraction (lane 6). On the other hand, when thapsigargin was added before FGF-1 and remained present throughout the incubation period (6 h), the phosphorylated FGF-1 was released into the medium by the digitonin treatment (lane 7), indicating that it was in the cytosol. Similar results were obtained when streptolysin O was used instead of digitonin for selective permeabilization of the plasma membrane (our unpublished data). Similarly to the digitonin procedure used in Figure 1, SLO treatment can be used to distinguish between proteins free in the cytosol and proteins present in vesicular compartments.

In the presence of bafilomycin A1, which blocks the translocation of the growth factor from endosomes to cytosol, we were unable to detect phosphorylated FGF-1 in any fraction (Figure 3A, lanes 10–12). Figure 3B shows that in the presence of bafilomycin A1 [^{35}S]methionine-labeled FGF-1 was detectable only in the membrane fraction and neither the cytosolic nor the nuclear fraction was contaminated with the endocytosed radiolabeled growth factor. Also, in these experiments we did not detect cross-contamination in the different cell fractions when analyzed for the presence of marker proteins (Figure 3C).

The data indicate that when thapsigargin is added to FGF-1-treated cells during the final part of the incubation period, the export of phosphorylated FGF-1 from the nucleus to the cytosol is blocked.

Evidence That FGF-1 Is Phosphorylated by PKC δ

Effect of Rottlerin. Previously, we have provided evidence that after translocation into cells, FGF-1 is phosphorylated by PKC (Klingenberg *et al.*, 1998). Because the above-mentioned experiments suggested that the growth factor is preferentially phosphorylated in the nucleus, we focused on PKC isoforms containing a nuclear localization sequence, viz., α , δ , and ζ (DeVries *et al.*, 2002). For this reason, we tested the effect of rottlerin, which at low concentration (3–10 μM) is a selective inhibitor of PKC δ (Gschwendt *et al.*, 1994; Blass *et al.*, 2002; Deb *et al.*, 2003; Clark *et al.*, 2003; Kajimoto *et al.*, 2004), and Gö 6976, which selectively inhibits PKC α and β 1 isozymes but does not inhibit PKC δ , ϵ , and ζ (Martiny-Baron *et al.*, 1993). The data in Figure 4A demonstrate that FGF-1 externally added to NIH/3T3 cells (top) is phosphorylated in the absence (lane 2) but not in the presence of 5 μM rottlerin (lane 3). Gö 6976 (1 μM) did not inhibit the phosphorylation (lane 4). The presence of both 1 μM Gö 6976 and 10 nM bafilomycin A1 prevented the phosphorylation of FGF-1 as expected, because translocation of the growth factor was blocked due to inhibition of the vesicular membrane proton pumps by bafilomycin A1 (lane 5). When 1 μM monensin was present as well, the vesicular membrane electric potential was regenerated and FGF-1 was translocated into the cells and was phosphorylated, even in the presence of Gö 6976 (lane 6). Similar results were obtained when MDA-MB-453 cells were used instead of NIH/3T3 (Figure 4A, bottom).

To confirm the *in vivo*-observed effect of the PKC inhibitors used, we also carried out an *in vitro* phosphorylation experiment. NIH/3T3 cells were treated with 20 nM TPA for 30 min before lysis, and the ability of immunoprecipitated PKC α to phosphorylate myelin basic protein (MBP) was checked. Figure 4B shows that PKC α immunoprecipitated from TPA-treated cells phosphorylates MBP in the *in vitro* phosphorylation assay both in the absence (lane 1) or presence (lane 4) of 5 μM rottlerin, but not in the presence of 10 nM Gö 6976 (lanes 2 and 3).

In accordance with the data mentioned above, we found that when thapsigargin was present throughout the incubation with FGF-1, labeled growth factor was present exclusively in the cytoplasmic fraction (Figure 4C, top, lane 2). When thapsigargin was present only during the last 2 h of incubation, phosphorylated growth factor was detected solely in the nuclear fraction (lane 6). In both cases, rottlerin prevented phosphorylation of the growth factor (lanes 3–5 and 7–9). Also at a concentration of 5 μM , rottlerin inhibited strongly the phosphorylation (our unpublished data). Similar inhibitory effect on phosphorylation of intracellular FGF-1 also was observed when FGF-1-treated cells were exposed to 10 nM BIM, a general PKC inhibitor (our unpublished data).

Western blot with anti-FGF-1, which detects total FGF-1 (phosphorylated and unphosphorylated), demonstrated that in both cases FGF-1 was present in the nuclear fraction even in the presence of rottlerin (Figure 4C, bottom, lanes 7–9). The data suggest that PKC δ can phosphorylate FGF-1 in the cytosol as well as in the nucleus.

Figure 5. Ability of PKC δ to phosphorylate FGF-1. (A) Serum-starved NIH/3T3 cells (10^6 cells/sample) were treated with 10 U/ml heparin and 100 ng/ml FGF-1 for 6 h. After extensive washing, the cells were lysed and sonicated. Next, the lysates were subjected to immunoprecipitation with anti-sumo-1 antibody (goat IgG) (lane 1) or anti-PKC δ antibody (goat IgG), in the presence (lane 3) or absence (lanes 2, 4, 5, and 6) of 3 μ g of anti-PKC δ antibody blocking peptide. Then, the immunoprecipitated material was incubated in kinase buffer for 1 h at 37°C with 1 μ g of recombinant FGF-1 and 30 μ Ci of γ -[32 P]ATP in the presence of 1 μ M Gö 6976 (lane 4), 5 μ M rottlerin (lane 5), or 10 nM bafilomycin A1 (lane 6) and analyzed by SDS-PAGE. (B) Top, serum-starved CPAE cells (5×10^5 cells/sample) were left untreated (lane 1) or treated for 6 h with 1 μ g/ml thapsigargin (lane 2), 100 ng/ml FGF-1 (lane 3), 100 ng/ml FGF-1 and 1 μ M Gö 6976 (lane 4), 100 ng/ml FGF-1 and 1 μ g/ml thapsigargin (lane 5), 100 ng/ml FGF-1 and 5 μ M rottlerin (lane 6), or 20 nM TPA (lane 7). Heparin (10 U/ml) was always used together with FGF-1 treatment. Next, cells were lysed, fractionated into cytoplasmic

Pretreatment of Cells with FGF-1 Induces PKC δ Activity in Nuclei

To test further the possibility that PKC δ phosphorylates the growth factor, we applied an *in vitro* phosphorylation assay where pure, recombinant growth factor was used as a substrate for PKC δ that had been immunoprecipitated from lysed cells or from the nuclear and cytoplasmic fractions of cells treated in various ways. Figure 5 A shows that PKC δ activity is present only in anti-PKC δ immunoprecipitates (lanes 1 and 2) and that the kinase cannot be precipitated in the presence of peptide specifically blocking anti-PKC δ antibody (lane 3). Moreover the phosphorylation activity was not inhibited by 1 μ M Gö 6976 (lane 4), but it was prevented in the presence of 5 μ M rottlerin (lane 5). Also, the *in vitro* phosphorylation of FGF-1 was not affected when 10 nM bafilomycin A1 was present in the reaction mixture (lane 6).

It should be noticed that the inhibitors in this experiment were added directly to the kinase buffer after immunoprecipitation. Inhibitors were therefore present only during the phosphorylation of FGF-1 *in vitro* and absent during the treatment of the cells with the growth factor, thus excluding interference with the kinase-activating process.

We also carried out experiments where the different compounds were added to living cells. After incubation for 6 h, the cells were fractionated into a cytoplasmic and a nuclear fraction. We here found that both unstimulated (serum-starved) and unstimulated, thapsigargin-treated CPAE cells contained detectable PKC δ activity only in the cytoplasmic fraction (Figure 5B, top, lanes 1 and 2). In contrast, when the cells had been treated with FGF-1 for 6 h, kinase activity was detected in the nuclear fraction as well (lane 3). The nuclear and cytoplasmic activity of PKC was not inhibited in the presence of Gö 6976 (lane 4). When cells were treated both with FGF-1 and thapsigargin, PKC δ activity was no longer detectable in the nuclear fraction (lane 5), suggesting that thapsigargin blocked the entry of active PKC δ into the nucleus.

Treatment of the cells with FGF-1 and 5 μ M rottlerin inhibited the PKC activity in both fractions (lane 6). On the

other hand, stimulation of the cells with 20 nM TPA led to an increase of the *in vitro* phosphorylation of FGF-1 in the anti-PKC δ immunoprecipitates from sonicated nuclei (lane 7). The activity induced by TPA also was blocked by 5 μ M rottlerin (our unpublished data). In cells stimulated with 10% FCS, we could not detect PKC δ activity exceeding that in the untreated control (our unpublished data). Very similar data were obtained, when NIH/3T3 cells were applied to this kind of experiment (our unpublished data).

The bottom of the figure shows that the nuclear fraction was not contaminated by either of the cytoplasmic proteins ERK 1/2 or calnexin.

When antibodies against the PKC α and ζ were used instead of anti-PKC δ for immunoprecipitation preceding the *in vitro* phosphorylation assay, we were unable to detect FGF-1 phosphorylation with the nuclear and cytosolic extract of cells stimulated with FGF-1. When the K132E mutant of the growth factor, which lacks the PKC phosphorylation site was used as a substrate, no labeling was obtained, even with the anti-PKC δ immunoprecipitate (not demonstrated).

Activation of PKC δ is associated with phosphorylation of the enzyme (Kronfeld *et al.*, 2000; Kikkawa *et al.*, 2002; Kajimoto *et al.*, 2004), and it is not always accompanied by apoptosis (Ohmori *et al.*, 1998; Kronfeld *et al.*, 2000). As demonstrated in Figure 5C, a strong band of phosphorylated PKC δ was found in the nuclear fraction from cells that had been treated with FGF-1 for 15 min or longer (lanes 2–4, bottom) but not from untreated cells (lane 1). This FGF-1-inducible effect on PKC δ was not observed in the cytosolic fraction (lanes 1–4, top), although phosphorylation of ERK 1/2 as a response of the cells to FGF-1 treatment was easily detected. Staining with anti-lamin A antibody (nuclear fraction) or anti-PKC δ and anti-ERK1/2 antibodies (cytosolic fraction) demonstrates equal loading.

After increasing periods of FGF-1 stimulation of NIH/3T3 cells, the immunostaining pattern of fixed cells obtained with anti-pT505-PKC δ antibody differed from that of untreated cells. Significant increase of labeling was observed in the nucleoplasm and the nuclear envelope (Figure 5D, top). On the other hand, the pattern of immunostaining obtained with anti-total-PKC δ antibody did not change with time of FGF-1 treatment (Figure 5D, bottom). Together, it seems that nuclear PKC δ activity is a specific response to treatment of cells with FGF-1. Together with the results in Figure 4, the data indicate that upon FGF-1 stimulation, active PKC δ translocates from the cytosol to the nucleus and that thapsigargin inhibits this process.

Phosphorylated FGF-1 Is Exported from the Nucleus and Dephosphorylated in the Cytosol

Export of FGF-1 That Is Phosphorylated in the Nucleus. When cells are incubated with FGF-1 in the presence of bafilomycin A1 to prevent translocation of FGF-1, the growth factor accumulates in intracellular vesicular compartments. From here, it can be induced to rapidly translocate to the cytosol by treatment of the cells with monensin, which restores the electrical potential across the vesicle membrane that is required for translocation (Małecki *et al.*, 2002, 2004). After accumulation of FGF-1 in endosomes for 6 h in the presence of bafilomycin A1 (or concanamycin A; our unpublished data), addition of monensin for the final 30 min (Figure 6A) allowed vesicular FGF-1 to become phosphorylated and located to the cytosol (Figure 6B, lane 3). We used this approach to study the localization of phosphorylated growth factor shortly after translocation was induced.

Figure 5 (cont.) (C) and nuclear (N) fractions and subjected to immunoprecipitation with anti-PKC δ . The immunoprecipitated material was incubated in kinase buffer for 1 h at 37°C with 1 μ g of recombinant FGF-1 wt or FGF-1 (K132E) (our unpublished data) in the presence of 30 μ Ci of γ -[³²P]ATP. Next, the growth factor was adsorbed onto heparin-Sepharose and analyzed by SDS-PAGE and fluorography. Bottom, controls for cross-contaminating during cell fractionation procedure. The cells were fractionated into cytoplasmic (C) and nuclear (N) fractions and the fractions were analyzed by Western blotting, by using anti-calnexin, anti-ERK 1/2 and anti-lamin A antibodies. (C) Serum-starved NIH/3T3 cells were treated with 10 U/ml heparin and 100 ng/ml FGF-1 for the indicated periods of time, harvested, and fractionated as described in *Materials and Methods*. The fractions were analyzed by SDS-PAGE and Western blotting by using monoclonal mouse antibody against PKC δ . The membrane was stripped and reprobed sequentially with anti-phospho-Thr505-PKC δ , anti-lamin A, anti-ERK1/2, and anti-phospho-ERK1/2. (D) Serum-starved NIH/3T3 cells were either left untreated (0 min) or treated with 10 U/ml heparin and 100 ng/ml FGF-1 for 15, 90, and 240 min. Phosphorylated PKC δ and total PKC δ were detected by incubating the samples with rabbit anti-phospho-Thr505-PKC δ and mouse anti-total-protein kinase C δ primary antibodies, respectively, for 20 min. After three washes in PBS, the samples were incubated with appropriate secondary antibodies conjugated to Cy2 fluorophore for 20 min and washed again. Coverslips were finally mounted onto glass slides using Mowiol, and the samples were analyzed with a Leica confocal microscope.

Figure 6. Intracellular localization of FGF-1 phosphorylation. (A) Schematic diagram representing the time course of the experiment. (B) Serum-starved NIH/3T3 cells were preincubated with [32 P]phosphate and treated for 6 h with 10 U/ml heparin and 100 ng/ml FGF-1 in the absence (lanes 1 and 6) or presence of 10 nM bafilomycin A1 (all other lanes). Where indicated, 1 μ M monensin was added for the last 30 min of incubation (lanes 3–5 and 8–10). In some cases, 1 μ g/ml thapsigargin was added for the final 1 h (lanes 4 and 9) or was present during the entire incubation (lanes 5 and 10). After incubation, cells were lysed and fractionated into cytoplasmic and nuclear fractions, adsorbed onto heparin-Sepharose, and analyzed by SDS-PAGE and fluorography. (C) Control of the nuclear export inhibiting activity of thapsigargin. A HIV-Rev mutant deficient in nuclear export, Rev(1.4), was used to generate the construct pRev(1.4)-GFP (Henderson and Eleftheriou, 2000). Export activity was restored to the level observed for the wild-type when the original Rev NES was inserted between the Rev(1.4) mutant and the GFP encoding regions, producing pRev-NES-GFP (Henderson and Eleftheriou, 2000). HeLa cells were transiently transfected with pRev-NES-GFP and protein expression was allowed to proceed for 48 h. After fixation in 3% paraformaldehyde and mounting the coverslips on glass slides the samples were analyzed for GFP localization by confocal microscopy. The right panel represents a sample treated with 1 μ g/ml thapsigargin for 3 h. All samples were treated with 15 μ g/ml cycloheximide to avoid increased cytosolic GFP staining due to novel protein synthesis.

When thapsigargin was added 30 min before monensin, i.e., present for the last hour of the incubation (Figure 6A), the phosphorylated growth factor was found in the nucleus

(Figure 6B, lane 9), whereas when monensin was added in the absence of thapsigargin, the 32 P-labeled FGF-1 was found in the cytosol (lanes 3). Also, when thapsigargin was

Figure 7. Ability of leptomycin B to inhibit nuclear export of phosphorylated FGF-1. (A) Schematic diagram representing the time course of the experiment. (B) Serum-starved NIH/3T3 cells were preincubated with [33 P]phosphate and treated for 6 h with 10 U/ml heparin and 100 ng/ml FGF-1 in the absence (lanes 1, 6, 7, and 12) or presence of 5 ng/ml leptomycin B (all other lanes). Where indicated, leptomycin B was present during the entire incubation either alone (lanes 2 and 8) or in combination with 1 μ g/ml thapsigargin (lanes 4 and 10) or was added only for the last 2 h of incubation (lanes 3 and 9). In some cases, cells were incubated with 10 nM baflomycin A1 and then for the last 2 h leptomycin B was added (lanes 5 and 11) or not (lanes 6 and 12), and finally 1 μ M monensin was added for the last 30 min of incubation. After incubation cells were lysed and cellular material was treated as in Figure 6B. (C) HeLa cells were transiently transfected with pRev-NES-GFP (left and middle) and pRev(1.4)-GFP (without NES) (right). The sample of Rev-NES-GFP-expressing cells represented in the middle panel was treated with 2 ng/ml leptomycin B for 3 h to inhibit exportin-1-dependent nuclear export. All samples were treated with 15 μ g/ml cycloheximide to avoid increased cytosolic GFP staining due to novel protein synthesis. After fixation in 3% paraformaldehyde and mounting the coverslips on glass slides, all samples were analyzed for GFP localization by using a confocal microscope.

added before FGF-1 and was present during the whole experiment, the phosphorylated FGF-1 was in the cytosol fraction (lane 5). The data indicate that translocated exogenous FGF-1 is transported into the nucleus where a subset of the molecules is phosphorylated and then rapidly exported to the cytosol. The nonphosphorylated FGF-1 either remains in the nucleus or its kinetics of nucleocytoplasmic transport is very different from that of the phosphorylated form.

Because thapsigargin can block nuclear export of HIV Rev protein (Love *et al.*, 1998), we decided to check as a control its activity in a Rev-NES-GFP-expressing system (Henderson and Eleftheriou, 2000). Figure 6C demonstrates an inhibitory activity of thapsigargin on nuclear export in HeLa cells expressing the Rev-GFP fusion protein. In untreated cells, Rev-NES-GFP was localized mainly in the cytoplasm of transfected cells (left). However, in the presence of 1 μ g/ml thapsigargin the fusion protein was present exclusively in the nuclei (right).

Nuclear Export of Phosphorylated FGF-1 Is Blocked by Leptomycin B

Because the phosphorylated FGF-1 seemed to be rapidly exported from the nucleus to the cytosol, we tested whether the export is sensitive to leptomycin B, a specific inhibitor of exportin-1-dependent nuclear export (Kudo *et al.*, 1999). In the absence of the inhibitor, the majority of the phosphorylated FGF-1 was recovered in the cytoplasmic fraction (Figure 7, A and B, lane 1). However, when leptomycin B was added, the phosphorylated growth factor was detected only in the nuclear fraction (lane 8).

Similarly to the thapsigargin experiments, addition of leptomycin B for the last 2 h resulted in accumulation of phosphorylated FGF-1 in the nuclear fraction (lanes 3 and 9). However, when thapsigargin and leptomycin B were present throughout the incubation period, the 33 P-labeled FGF-1 was found in the cytosolic fraction (lane 4). This supports the view that when thapsigargin is present from the beginning of the incubation period, the translocated growth factor is phosphorylated in the cytosol and is then unable to enter the nucleus.

In experiments where addition of monensin was used to trigger translocation of FGF-1 that had accumulated in vesicles of bafilomycin A1-treated cells, addition of leptomycin B 90 min before monensin resulted in nuclear localization of the labeled FGF-1 (lane 11). As anticipated, omission of leptomycin B resulted in cytoplasmic localization of the phosphorylated growth factor (lanes 6), which is most likely due to rapid export from the nucleus. Treatment of the cells with leptomycin B also led to enhanced 33 P-labeling of the growth factor (compare lanes 1 and 8).

To test as a control the activity of leptomycin B, we again used the Rev-NES-GFP system. As demonstrated in Figure 7C, in untreated HeLa cells expressing the construct, Rev-NES-GFP was localized in cytoplasm (left), whereas in leptomycin B-treated cells it was present exclusively in the nuclei (middle) similarly to Rev1.4-GFP lacking the NES (right). Together, the data indicate that exportin-1 is the carrier involved in the exclusion of phosphorylated FGF-1 from the nucleus.

Inhibition of Nuclear Export of Phosphorylated FGF-1 by Leptomycin B Stabilizes the Labeled FGF-1

Because leptomycin B blocked nuclear export of 33 P-labeled FGF-1, we decided to study whether the intracellular localization of the phosphorylated growth factor alters the stability of the protein. To test this, cells were incubated with FGF-1 for 6 h at 37°C without or with leptomycin B. The cells

Figure 8. Effect of leptomycin B and okadaic acid on the stability of phosphorylated FGF-1. (A) Serum-starved NIH/3T3 cells were preincubated with [33 P]phosphate and treated for 6 h with 10 U/ml heparin and 100 ng/ml FGF-1 in the absence (lanes 1–3) or presence of 5 ng/ml leptomycin B (lanes 4–6). After incubation, cells were washed and lysed (lanes 1, 4) or were washed and incubated further for 2 h (lanes 2 and 5) or 4 h (lanes 3 and 6) either in the absence (lanes 2 and 3) or presence of 5 ng/ml leptomycin B (lanes 5 and 6), and then lysed. All lysates were treated as in Figure 6B. (B) Serum-starved NIH/3T3 cells were preincubated with [33 P]phosphate and treated for 6 h with 10 U/ml heparin and 100 ng/ml FGF-1 in the absence (lane 1) or presence of 0.5 μ M okadaic acid (lanes 2 and 3), 1.0 μ M okadaic acid (lanes 4 and 5), or 10 nM bafilomycin A1. As indicated, okadaic acid was added for the last 2 h (lanes 2 and 4) or the last 4 h of incubation (lanes 3 and 5). After incubation, cells were lysed, and cellular material was treated as in Figure 6B.

were then washed to remove unbound growth factor, and the incubation was subsequently continued for either 2 or 4 h in the absence and presence of leptomycin B. As shown in Figure 8A, lanes 4–6, leptomycin B clearly increased the stability of the phosphorylated FGF-1 trapped in the nucleus. In the absence of leptomycin B, the 33 P-labeled growth factor was undetectable already after 2 h of additional incubation (lanes 1–3).

The presence of lactacystin did not change the stability of 33 P-labeled FGF-1 in the cytoplasmic fraction (our unpublished data). This indicates that the disappearance of the 33 P-labeled growth factor exported to the cytosol is not due to degradation by the proteasomes. We therefore tested the effect of the phosphatase inhibitor, okadaic acid, on the

stability of the labeled FGF-1. As shown in Figure 8B, 0.5 μ M okadaic acid clearly increased the intensity of the bands representing 33 P-labeled FGF-1 in the cytosol (lanes 2 and 3), and the intensity was increased even more at 1 μ M (lanes 4 and 5). Because in the presence of 10 nM bafilomycin A1 there was no detectable 33 P-labeled FGF-1 (lane 6), the observed effect of okadaic acid concerns only translocated growth factor. The data indicate that FGF-1 phosphorylated in the nucleus is actively exported to the cytosol where it is dephosphorylated.

Experiments with FGF-1 Mutated in the Phosphorylation Site

We have earlier described mutants of FGF-1 that cannot be phosphorylated (Klingenberg *et al.*, 1999). In FGF-1(S130A), the serine residue that in wild-type FGF-1 is phosphorylated by PKC is mutated to alanine, whereas in FGF-1(S130E) it is changed to glutamic acid that could mimic the phosphorylated serine residue.

FGF-1(S130A) was found to be less able to stimulate DNA synthesis in starved NIH/3T3 cells than the wild-type FGF-1 (Skjerpen *et al.* 2002a; Figure 9A), whereas FGF-1(S130E) was equally effective (Figure 9A) or even somewhat more effective (Skjerpen *et al.* 2002a) than wild-type FGF-1.

Wild-type FGF-1 and the two mutants were labeled with [35 S]methionine and added to serum-starved cells. After 6 h incubation at 37°C, the cells were washed with 2 M NaCl in 20 mM sodium acetate, pH 4.0, to remove unbound and cell surface-bound labeled ligand and then they were washed with HEPES medium. Subsequently, the cells were permeabilized with digitonin and fractionated into a cytosolic fraction, a membrane fraction and a nuclear fraction, which were each analyzed for the presence of labeled growth factor.

The data in Figure 9B demonstrate that wild-type FGF-1 was present in all fractions (lane 1), although somewhat degraded in the case of the membrane fraction. FGF-1(S130A) was found in the nuclear fraction (lane 3), whereas FGF-1(S130E) was mainly found in the cytosolic fraction (lane 2).

In bafilomycin A1-treated cells, the 35 S-labeled proteins were found only in the membrane fraction in its undegraded form (Figure 9B, lanes 4–6). The radiolabeled material in the membrane fraction represents mainly 35 S-labeled FGF-1 that is endocytosed but not translocated into cells. Prevention of acidification of the vesicles by bafilomycin A1 apparently inhibits its degradation.

In the next experiments, we expanded the incubation period by 4 h after the acid/high salt wash after 6 h. After this prolonged incubation, the wild-type FGF-1 was not detectable in the nuclei and only a very weak band was still present in the cytosolic fraction (Figure 9C, lane 1). On the other hand, a strong band of FGF-1(S130E) was found in the cytosolic fraction (lane 2). FGF-1(S130A) was present only in the nuclear fraction (lane 3). When the experiment was carried out in the presence of leptomycin B, all growth factors were found in the nucleus (Figure 9C, lanes 4–6).

The data indicate that FGF-1(S130E) is largely exported from the nucleus by a mechanism that can be blocked by leptomycin B, whereas the mutant FGF-1(S130A) is not exported from the nucleus. It seems that the signal necessary for export from the nucleus to the cytosol is a negatively charged residue at position 130, brought about either by phosphorylation of the serine residue in the wild-type growth factor or by changing this residue to glutamic acid. The finding that the mutant S130A is less active than wild-type FGF-1 in stimulating DNA synthesis in serum-starved cells indicates that a negative charge in position 130 is of

functional importance. It should be noticed that the mutants of FGF-1 bind to FGF receptor and activate ERK 1/2 kinases as efficiently as the wild-type growth factor (Skjerpen *et al.*, 2002a).

To further explore our finding that PKC δ phosphorylates nuclear FGF-1 and that this process induces the active, leptomycin B-sensitive nuclear export of the growth factor to the cytosol, additional experiments with the wild-type and the S130E mutant of FGF-1 were carried out.

In the experiment described in Figure 9D, the distribution of 35 S-labeled FGF-1 and FGF-1(S130E) between the cytosolic and the nuclear fraction was assessed after 6 h incubation with the growth factors as well as after an additional 4 h incubation period after removal of external growth factors by a high salt/low pH wash. The data in Figure 9D (6 h panel) demonstrate that after 6 h of incubation, FGF-1 was present in the nuclear fraction in the absence (lane 1) or presence (lane 3) of 5 μ M rottlerin, whereas FGF-1(S130E) was detectable only in the cytosolic fraction (lanes 2), even in the presence of the inhibitor (lane 4). In the case of treatment with rottlerin and leptomycin B, both FGF-1 and FGF-1(S130E) were present only in the nuclear fraction (lanes 5 and 6).

When the assessment was carried out after additional 4 h of incubation after removal of the growth factor from the medium and the cell surface (Figure 9D, 10 h panel), the wild-type growth factor was detectable in the nuclear fraction only in the presence of rottlerin (lane 3) but not when the inhibitor was absent (lane 1). On the other hand, the FGF-1(S130E) was always present in the cytosolic fraction in the absence (lane 2) and presence (lane 4) of rottlerin. This mutant was only in the nuclear fraction under leptomycin B treatment (lane 6). It also should be noticed that after this period of incubation, the wild-type growth factor was poorly detectable in the cytosolic fraction and undetectable in the nuclear fraction (lane 1).

These data indicate that after export from nucleus to the cytosol the growth factor is dephosphorylated and then degraded. FGF-1(S130E) does not disappear from the cytosolic fraction, because it has a stable negative charge and cannot be "dephosphorylated." The strong effect of rottlerin on the stability and intracellular localization of wild-type FGF-1 indicates that the growth factor can be exported from nucleus only when it is phosphorylated and that its degradation in the nucleus is slow. On the other hand, dephosphorylation of FGF-1 in the cytosol induces degradation.

DISCUSSION

The main findings here presented are that FGF-1 translocated into cells from the exterior is to a large extent found in the nucleus where fraction of it is phosphorylated, apparently by PKC δ and that the phosphorylated growth factor is rapidly exported to the cytosol by a mechanism sensitive to leptomycin B and then dephosphorylated and probably degraded (for schematic illustration, see Figure 10).

Entry of FGF-1 into the nucleus does not require phosphorylation. Thus, the FGF-1(K132E) mutant, which is not phosphorylated (Klingenberg *et al.*, 1998), and FGF-1(S130A) (this article) are transported into the nucleus as efficiently as the wild-type FGF-1. This, and the observed effect of thapsigargin on the intracellular location of the phosphorylated growth factor, indicates that FGF-1 translocated into the cytosol is transported to the nucleus where it is phosphorylated and then exported back into the cytosol. Thapsigargin had no demonstrable effect on the intracellular localization of bulk [35 S]methionine-labeled FGF-1, indicating that the

Figure 9. Stimulation of DNA synthesis and nucleocytoplasmic trafficking of mutant growth factors. (A) Serum-starved NIH/3T3 cells were treated with heparin and increasing amounts of FGF-1 or FGF-1(S130E), or FGF-1(S130A), or FGF-1(K132E) for 24 h. During the last 6 h of incubation, 1 μ Ci/ml [³H]thymidine was present in the medium. The cells were extracted with 5% TCA and the radioactivity incorporated into TCA-insoluble material was measured. (B) Serum-starved NIH/3T3 cells were treated with heparin and 10 ng/ml [³⁵S]methionine-labeled FGF-1 or FGF-1(S130E), or FGF-1(S130A) for 6 h at 37°C in the presence (lanes 1–3) or absence (lanes 4–6) of 10 nM bafilomycin A1. After incubation, the cells were washed with 2 M NaCl in 20 mM sodium acetate, pH 4.0, and in HEPES medium and then permeabilized with digitonin in the presence of 5 U/ml heparin and fractionated into membrane (M), cytosolic (C), and nuclear (N) fractions as described in *Materials and Methods*. Fractions were adsorbed onto heparin-Sepharose and analyzed by SDS-PAGE and fluorography. (C) Serum-starved NIH/3T3 cells were treated with heparin and 10 ng/ml [³⁵S]FGF-1 or its mutants for 6 h in the presence (lanes 1–3) or absence (lanes 4–6) of 5 ng/ml leptomycin B. Then, the cells were washed with 2 M NaCl in 20 mM sodium acetate, pH 4.0, and HEPES medium, and the incubation was continued for 2 h at 37°C in the presence or absence of leptomycin B. Finally, the cells were treated with digitonin in the presence of 5 U/ml heparin. Then, the cells were fractionated and processed as in Figure 6B. (D) Serum-starved NIH/3T3 cells were treated with 10 U/ml heparin and 10 ng/ml [³⁵S]methionine-labeled FGF-1 or FGF-1(S130E) for 6 h at 37°C in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 5 μ M rottlerin, or in the presence of rottlerin and leptomycin B (lanes 5 and 6). Then, the cells were washed with 2 M NaCl in 20 mM sodium acetate, pH 4.0, and HEPES medium, and the incubation was continued for 4 h at 37°C. Next, the cells were treated with digitonin in the presence of 5 U/ml heparin and then fractionated and processed further as in Figure 6B.

majority of the translocated growth factor is not in the phosphorylated form.

The function of the phosphorylated growth factor in the nucleus is not clear. Translocation of FGF-1 to the nucleus is associated with stimulation of DNA synthesis (Imamura *et al.*, 1990; Wiedlocha *et al.*, 1994). The mutant (S130A) was less active in stimulating DNA synthesis than wild-type growth factor, whereas S130E was at least as active as the

wild-type in this respect. Possibly, this is due to the negatively charged residue in the latter mutant, which could mimic phosphorylation. The position of the negative charge seems to be critical. Thus, we have earlier demonstrated that the mutation K132E did not interfere with the accumulation of the growth factor in the nucleus (Klingenberg *et al.*, 2000).

Eukaryotic cells control many processes by regulating the movement of proteins into and out of the nucleus. Phos-

Figure 10. Schematic presentation of intracellular transport of exogenous FGF-1. After binding to and activation of specific tyrosine kinase receptors (1), FGF-1 is internalized by receptor-mediated endocytosis (2), reaching an endosomal compartment with a vesicular membrane potential generated by the vesicular type of proton pumps (3). The endocytosed growth factor is then translocated to the cytosol (4). In the next step, FGF-1 can reach the nucleus (5). A part of the externally added nuclear FGF-1 is phosphorylated by nucleus-located PKC δ (6). The kinase is activated (1a) and translocated (1b) to the nucleus after FGF receptor activation. The nuclear translocation of PKC δ can be blocked by thapsigargin. FGF-1 phosphorylated in the nucleus is subsequently relocated to the cytosol by an active nuclear export mechanism sensitive to leptomycin B treatment (7). The nuclear export of the phosphorylated FGF-1 also is blocked by thapsigargin. In the cytosol, the modified growth factor is dephosphorylated (8) and then degraded (9).

phorylation of karyophilic proteins is a way of regulating the transport. A number of transcription factors and kinases can be imported into the nucleus only in a phosphorylated form (Kaffman and O'Shea, 1999).

Phosphorylation is thought to enhance nuclear exit of proteins as well. An example is the transcription factor NF-AT2 that is exported from the nucleus as a phosphorylated protein, because only in this conformational state is

its nuclear export signal (NES) accessible for binding to exportin-1 (Beals *et al.*, 1997). In yeast, the nuclear import/export carrier Kap142 can export only phosphorylated cargo (Macara, 2001, and references therein). It also was shown that MAPKAP kinase 2 is translocated from the nucleus to the cytosol under stress-induced phosphorylation of the kinase in a leptomycin B-sensitive manner (Engel *et al.*, 1998).

Our data indicate that only the transport of phosphorylated FGF-1 is blocked under thapsigargin treatment, whereas transport of the nonphosphorylated growth factor is not. This indicates distinct mechanisms for crossing of NPC by phosphorylated and nonphosphorylated FGF-1. The data suggest that phosphorylation regulates the intracellular localization of the growth factor and possibly the life span of the protein.

Further evidence supporting this is provided by experiments with leptomycin B. The finding that leptomycin B is able to specifically inhibit the nuclear export of the modified FGF-1 suggests that the phosphorylated growth factor is exported from the nucleus by exportin-1 (Crm1), a karyopherin-mediated nuclear export protein sensitive to leptomycin B. Exportin-1 binds directly to cargo containing a leucine-rich NES. Because FGF-1 does not have an obvious NES, it is possible that it binds indirectly to exportin-1 via an NES-containing interaction partner.

The primary effect of thapsigargin is to inhibit the ER-associated Ca^{2+} -ATPase and thereby release Ca^{2+} from intracellular stores, mainly from the ER and the nuclear envelope. It has been suggested that depletion of the nuclear envelope of calcium blocks passive diffusion and signal-mediated transport through NPCs (Greber and Gerace, 1995). Although there is evidence for direct regulation of the NPC by stored Ca^{2+} (Greber and Gerace, 1995; Greber *et al.*, 1997; Perez-Terzic *et al.*, 1999; Love *et al.*, 1998), there are also reports demonstrating lack of a selective effect on the transport of nuclear proteins and ions of Ca^{2+} depletion caused by thapsigargin or calcium ionophores (Strubing and Clapham, 1999; Shanin *et al.*, 2001). A selective thapsigargin-induced inhibitory effect was observed in the case of the HIV Rev protein where the drug blocked only the export of Rev from the nucleus to the cytoplasm but not nuclear import (Love *et al.*, 1998). Therefore, it is not clear whether reduced luminal Ca^{2+} in the nuclear envelope blocks or only modifies the function of NPC. It is possible that thapsigargin treatment renders NPCs much less permeable for some proteins than for others.

It also was reported that depletion of the nuclear envelope of Ca^{2+} can induce distinct conformational changes in the NPC in different cell types, indicating structural differences in NPCs (Perez-Terzic *et al.*, 1999; Shanin *et al.*, 2001). Thapsigargin inhibited nuclear import of activated MAPK kinase in HL-60 cells (Marcinkowska *et al.*, 1997).

The finding that rottlerin, but not Gö 6976, inhibits *in vivo* phosphorylation of FGF-1 in three different cell lines, suggests that PKC δ phosphorylates the growth factor. The finding that FGF-1 can be phosphorylated *in vitro* by immunopurified PKC δ obtained from the nucleus of FGF-1-treated cells, but not from starved (Figure 5B) or epidermal growth factor-treated cells (our unpublished data) indicates that FGF-1 treatment induces transport of active PKC δ into the nucleus.

When thapsigargin is added to the cells before FGF-1, it is likely that FGF-1 is phosphorylated by PKC δ in the cytosol. This would imply that thapsigargin blocks FGF-1-induced translocation of active PKC δ into the nucleus. When thapsigargin was present only during the 2 h of treatment with FGF-1, the phosphorylation took place in the nucleus, suggesting that the activated PKC δ was transported into the nucleus during the first 4 h of treatment with the growth factor.

No clear rules have been established for subcellular distribution of PKC isoforms (Asaoka *et al.*, 1992). They can be differentially localized in different cell types and be present in the nucleus of resting cells. For this reason, the possibility

still exists that FGF-1 does not induce nuclear translocation of active PKC δ but only activates a nuclear pool of the enzyme and thapsigargin might block entry into the nucleus of the activating signal, e.g., diacylglycerol (Asano *et al.*, 1994).

The regulatory role of FGF-1 phosphorylation in nuclear export of the protein was supported by experiments using [^{35}S]methionine-labeled growth factors. The FGF-1(S130E) mutant, which mimics phosphorylated growth factor was constitutively transported to the cytosol, whereas the S130A mutant, which cannot be phosphorylated by PKC δ , remained in the nucleus. Moreover, the S130E mutant was exported from the nucleus even in the presence of rottlerin, whereas the export of the wild-type growth factor was inhibited. Additionally, leptomycin B blocked export of both FGF-1 and FGF-1(S130E). Because the stability of the S130E mutant as such, and the wild-type cytosolic FGF-1 in the presence of rottlerin, was much increased compared with the wild-type growth factor in the absence of the inhibitor, these experiments also indicate that dephosphorylation of FGF-1 in the cytosol induces degradation of the growth factor. Finally, it seemed that in the presence of okadaic acid the life span of the phosphorylated FGF-1 in the cytosol was increased, indicating phosphatases as crucial regulators of FGF-1 degradation in the cytosol.

The nuclear export of FGF-1 seems to be a tightly regulated process because controlled at least by two steps: 1) phosphorylation of FGF-1 and then 2) an export mechanism sensitive to leptomycin B.

Together with previous findings (Olsnes *et al.*, 2003; Więdołcha and Sørensen, 2004), these results contribute to the emerging picture that FGF-1 has important and regulated intracellular function.

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