FGF-1 and FGF-2 Require the Cytosolic Chaperone Hsp90 for Translocation into the Cytosol and the Cell Nucleus*

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Similarly to many protein toxins, the growth factors fibroblast growth factor 1 (FGF-1) and FGF-2 translocate from endosomes into the cytosol. It was recently found that certain toxins are dependent on cytosolic Hsp90 for efficient translocation across the endosomal membrane. We therefore investigated the requirement for Hsp90 in FGF translocation. We found that low concentrations of the specific Hsp90 inhibitors, geldanamycin and radicicol, completely blocked the translocation of FGF-1 and FGF-2 to the cytosol and the nucleus. The drugs did not interfere with the initial binding of FGF-1 to the growth factor receptors at the cell-surface or with the subsequent internalization of the growth factors into endosomes. The activation of known signaling cascades downstream of the growth factor receptors was also not affected by the drugs. The data indicate that the drugs block translocation from endosomes to the cytosol implying that Hsp90 is required for translocation of FGF-1 and FGF-2 across the endosomal membrane.

FGF-1 and FGF-2 bind to and activate FGFRs on the surface of target cells. Several downstream signaling cascades such as the Ras/MAPK, phospholipase C-γ/PKC, and PI 3-kinase/Akt pathways are then initiated (1). FGF signaling is important in several cellular processes such as proliferation, angiogenesis, migration, survival, and differentiation (2, 3).

In addition, FGF-1 and FGF-2 have the peculiar ability to translocate through the endosomal membrane into the cytosol and then be transported into the nucleus (4). Several laboratories have presented data indicating that the nuclear targeting is involved in the proliferation of cells. Thus, elimination of the N-terminal nuclear localization signal of FGF-1 resulted in considerably reduced mitogenic activity even if binding and activation of the receptors was not much changed (5). When FGF-1 was introduced into the cytosol as a fusion protein with diphtheria toxin, it stimulated DNA synthesis in cells lacking FGFRs arguing for an intracellular role of the growth factor (6). Similarly, nuclear FGF-2 has been shown to activate rDNA transcription and to be associated with cell proliferation (7, 8). Nuclear FGF-2 has also been proposed to be involved in the survival of carcinoma cells important for lung metastasis (9).

We recently found that after endocytosis by the specific FGFRs, FGF-1 and -2 translocate from endosomes into the cytosol (10, 11). The positive inside membrane potential was found to be crucial for translocation across the endosomal membrane. Furthermore, we have shown that FGF-1 is then transported further from the cytosol to the nucleus by the concerted action of two nuclear localization signals (12). In the nucleus FGF-1 is phosphorylated by PKCδ and then rapidly exported to the cytosol by a leptomycin B-sensitive protein, probably exportin-1, and subsequently degraded (13). We have previously shown that the translocation of FGF-1 is prevented by PI 3-kinase inhibitors suggesting that it is regulated by signaling events (14).

Another class of proteins that are endocytosed and translocated from endosomes are certain protein toxins. They have targets in the cytosol and need to translocate an enzymatic moiety into the host cells. Several of them, e.g. anthrax and diphtheria toxin, utilize the low pH in endosomes to trigger translocation (15, 16). Interestingly, it was recently found that efficient translocation of diphtheria toxin catalytic domain requires the cytosolic chaperone Hsp90 (17). Hsp90 has also been shown to be essential for the translocation of Clostridium botulinum C2 toxin and iota-like toxins (18, 19).

In light of the recent findings that certain toxins depend on Hsp90 for efficient translocation from endosomes to cytosol, we decided to investigate whether transport of FGF-1 and FGF-2 is also dependent on this chaperone. We here demonstrate that Hsp90 inhibitors completely block the translocation of the growth factors from endosomes to the cytosol.

EXPERIMENTAL PROCEDURES

Materials, Media, and Buffers—[35S]Methionine (1000 Ci/mmol), [33P]phosphate (3000 Ci/mmol) were obtained from Amersham Biosciences. Geldanamycin was obtained from Calbiochem, and radicicol was from Sigma. Recombinant FGF-1 was prepared as described previously (20). The following buffers were used: HEPES medium, bicarbonate- and serum-free Eagle’s minimal essential medium buffered with HEPES to pH 7.4; dialysis buffer, 140 mM NaCl, 20 mM HEPES, and 2 mM CaCl2, adjusted to pH 7.0 with NaOH; lysis buffer, 0.1M NaCl, 20 mM Na2HPO4, 10 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide, pH 7.4; PBS, 140 mM NaCl and 10 mM Na2HPO4, pH 7.4.

Cell Cultures—NIH/3T3 and HUVE cells were propagated as earlier described (6). Cells were seeded into tissue culture plates the day preceding the experiments.

In Vitro Transcription and Translation—Plasmid DNA was linearized downstream of the encoding gene and transcribed with T3 RNA polymerase as described (21). The mRNA was precipitated with ethanol and dissolved in H2O containing 10 mM dithiothreitol and 0.1 unit/μL...
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RNAsin. The translation was performed for 1 h at 30 °C in micrococcal nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI). Radioactive proteins were prepared in lysates containing 1 μM [35S]methionine and a 25 μM concentration of the other 19 amino acids. Labeled methionine was replaced by 25 μM unlabeled methionine when nonradioactive proteins were synthesized. The amount of protein in the nonlabeled lysates was estimated as earlier described (22) by translating in parallel a small aliquot of the lysate in the presence of 5 μM [35S]methionine. The lysates were finally dialyzed against PBS to remove free [35S]methionine and reducing agents.

SDS-PAGE—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in 13.5% gels as described by Laemmli (23). After electrophoresis, the gel was fixed for 30 min in 25% methanol, 4% acetic acid and then incubated for 30 min in 1 M sodium salicylate, 2% glycerol, pH 5.8. Kodak XAR-5 film was exposed to the dried gel at ~80 °C.

Binding of 125I-Labeled FGF-1—Confluent NIH/3T3 cells growing on 12-well gelatinized microtiter plates were washed twice with ice-cold binding buffer (Dulbecco’s modified Eagle’s medium containing 50 mM HEPES, pH 7.4, 0.2% gelatin, and 10 units of heparin per ml). Cells were incubated with [125I]–FGF-1 for 2 h at 4 °C. Then they were washed twice with binding buffer, twice with PBS containing 10 units/ml heparin, once with 0.7 M NaCl, and once with HEPES medium. Cells were lysed in 0.5 M NaOH, and the solubilized radioactivity was measured using a γ-counter (1261 Multigamma, LKB Wallac). Recombinant FGF-1 was iodinated by the iodogen method (30). The specific activity of [125I]–aFGF was ~15,000 cpm/ng.

Measurement of Cytotoxicity—NIH/3T3 cells were incubated with increasing amounts of geldanamycin or radicicol for 6 h in HEPES medium without leucine. The cells were then transferred to the same medium containing 1 μCi/ml [3H]leucine and incubated for 20 min at 37 °C. The cells were then extracted with 5% trichloroacetic acid for 10 min, followed by a brief wash in 5% trichloroacetic acid, and subsequently dissolved in 0.1 M KOH. The cell-associated trichloroacetic acid-insoluble radioactivity was measured and reported as a percentage of radioactivity incorporated by cells untreated with the drugs.

Phosphorylation Assay in NIH/3T3 Cells—NIH/3T3 cells growing in 6-well tissue culture plates (2 × 104 cells per well) were incubated for 24 h in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 1% serum at 37 °C. The cells were then incubated with 5% trichloroacetic acid for 10 min, followed by a brief wash in 5% trichloroacetic acid, and subsequently dissolved in 0.1 M KOH. The cell-associated trichloroacetic acid-insoluble radioactivity was measured and reported as a percentage of radioactivity incorporated by cells untreated with the drugs.

Fractionation of NIH/3T3 Cells—To fractionate the cells, NIH/3T3 cells were incubated with radiolabeled proteins for 6 h at 37 °C and washed with high salt/low pH buffer (2 M NaCl in 20 mM sodium acetate, pH 4.0). The cells were then washed with PBS, and 20 μg/ml digitonin was added to permeabilize the cells. After 5-min incubation at 25 °C, the cells were kept on ice for additional 30 min to allow components of the cytosol to diffuse into the buffer. The medium was recovered and denoted the cytosolic fraction. The remainder of the cells was lysed with lysis buffer and scraped from the plastic to recover nuclei and centrifuged at 15,800 × g for 15 min. The supernatant was designated the membrane fraction. The pellet was washed three times in lysis buffer. After sonicating for 10 s on ice, the lystate was centrifuged for 5 min at 15,800 × g, and the supernatant was designated the nuclear fraction. The different fractions were adsorbed to heparin-Sepharose and subsequently analyzed by SDS-PAGE and fluorography.

RESULTS

Phosphorylation of FGF-1 Is Blocked by Hsp90 Inhibitors—When FGF-1 is added to NIH/3T3 cells, it is transported to the nucleus where it is phosphorylated by PKCδ (13). Phosphorylation of exogenous FGF-1 can therefore be used as an essay to monitor translocation of the growth factor to the nucleus of the cells.

We incubated serum-starved and [33P]-treated NIH/3T3 cells with recombinant FGF-1 for 6 h. The cells were then lysed and analyzed for phosphorylated growth factor. As seen in Fig. 1A, lane 2, a band corresponding to phosphorylated FGF-1 was observed. When the addition of FGF-1 was omitted there was no band observed (lane 1). We then added increasing concentrations of the Hsp90 inhibitor radicicol (24, 25). Already at low concentrations of radicicol (10 nM), the phosphorylated band disappeared completely, indicating that Hsp90 is required for one or more steps in the process leading to phosphorylation of the growth factor. Similar results were obtained with another Hsp90 inhibitor, geldanamycin (GA), which also completely blocked phosphorylation at low concentrations (Fig. 1B).

To test whether the drugs interfered with the phosphorylation process as such, we performed an in vitro phosphorylation assay. NIH/3T3 cells incubated with or without radicicol or geldanamycin and treated with 12-O-tetradecanoylphorbol-13-acetate to activate PKC were lysed and the active PKCδ was immunoprecipitated with an anti-PKCδ antibody. Recombinant FGF-1 was added to the immunopurified PKCδ in a kinase buffer containing [γ-33P]ATP either in the absence or presence of radicicol, geldanamycin, or other drugs as indicated. As can be seen in Fig. 1C, FGF-1 was phosphorylated by the activated and immunopurified PKCδ (lane 2). When an irrelevant antibody was used, anti-Sumo, there was no PKCδ purified and FGF-1 was not phosphorylated, as expected (lane 1). Addition of either radicicol or geldanamycin did not inhibit the in vitro phosphorylation of FGF-1.
FIGURE 1. Hsp90 inhibitors block phosphorylation of FGF-1. A, serum-starved NIH/3T3 cells labeled with 25 μCi/ml \([^{33}P]\) were incubated with increasing concentrations of radicicol (Rad) and 100 ng/ml FGF-1 in the presence of 20 units/ml heparin. In one case, the addition of FGF-1 was omitted (lane 1). After 6-h incubation, the cells were lysed in phosphate-free lysis buffer containing phosphatase and protease inhibitors. The lysate was treated with heparin-Sepharose for 2 h at 4 °C. The heparin-Sepharose with adsorbed material was then washed three times in PBS and incubated with 2 μg/ml TPCK-treated trypsin for 30 min at 25 °C to reduce the background of phosphorylated proteins. The pellet was then washed once with 0.5 M NaCl in PBS and once with PBS and subjected to SDS-PAGE and fluorography. B, serum-starved NIH/3T3 cells labeled with 25 μCi/ml \([^{33}P]\) were incubated with increasing concentrations of geldanamycin (GA) and 100 ng/ml FGF-1 in the presence of 20 units/ml heparin. After 6-h incubation, the cells were lysed and treated as described for A. C, serum-starved NIH/3T3 cells (10⁶ cells/sample) were treated with 20 nM TPA for 6 h in the absence or presence of 10 nM radicicol (Rad), 10 nM geldanamycin (GA), 5 μM rottlerin, or 1 μM Go6976 (Go6). 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-PKCδ antibody (goat IgG) (lanes 2–6). Then, the immunoprecipitated material was incubated in kinase buffer for 1 h at 37 °C with 1 μg of recombinant FGF-1 and 30 μCi of \([γ-^{32}P]ATP\) in the absence or presence of 10 nM radicicol (lane 3), 10 nM geldanamycin (lane 4), 5 μM rottlerin (lane 5), or 1 μM Go6976 (lane 6) and analyzed by SDS-PAGE. D, serum-starved NIH/3T3 growing in MatTek culture plates for live cell imaging were incubated with and without 100 nM geldanamycin or 100 nM radicicol for 6 h. Differential interference contrast images of the cells were obtained by scanning the living cells with a Zeiss LSM 510 confocal microscope. E, NIH/3T3 cells were incubated with increasing concentrations of geldanamycin or radicicol for 6 h. Then the ability of the cells to incorporate \([^{3}H]leucine\) was measured as described under “Experimental Procedures.” Symbols indicate the mean of three replicate samples, and error bars represent the S.D.
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FIGURE 2. The translocation of FGF-1 and FGF-2 to the cytosol is blocked by Hsp90 inhibitors. A, NIH/3T3 cells were treated with 20 μg/ml digitonin for 5 min at 25 °C to permeabilize them. The cells were kept for 30 min at 4 °C to allow cytosolic proteins to leak out into the medium. The material recovered in the medium was designated the cytosolic fraction (C). The remainder of the cells was lysed and fractionated into a membrane (M) and a nuclear fraction (N). Proteins from each fraction were analyzed by Western blotting with antibodies against Hsp90, lamin A, calreticulin, p44/42 MAPK and Rab5. B, serum-starved NIH/3T3 cells were incubated with [35S]methionine-labeled FGF-1 in the presence of 20 units/ml heparin. After 6-h incubation, the cells were fractionated as in A. FGF-1 was recovered by adsorption to heparin-Sepharose and analyzed by SDS-PAGE and fluorography. In some cases, 10 μM geldanamycin (GA), 10 μM radicicol, or the PI-3K inhibitor LY294002 (50 μM) was added during the incubation. C, serum-starved NIH/3T3 cells were incubated with [35S]methionine-labeled FGF-2 in the presence of 20 units/ml heparin and treated as in B. In one case, 10 μM geldanamycin (GA) was added. D, serum-starved NIH/3T3 cells were treated as in Fig. 1A with FGF-1 and heparin in the absence or presence of 10 μM geldanamycin or radicicol and 10 μM lactacystin.

Geldanamycin has been reported to be toxic to certain cells and we tested the toxicity of the drugs in NIH/3T3 cells. First, we checked the morphology of NIH/3T3 cells treated with the Hsp90 inhibitors. No obvious change in morphology of the cells was observed when they were tested the toxicity of the drugs in NIH/3T3 cells. First, we checked the morphology of NIH/3T3 cells treated with the Hsp90 inhibitors. No obvious change in morphology of the cells was observed when they were treated for 6 h with 100 nM of the inhibitors (Fig. 1D). We also tested whether the level of protein synthesis was changed in cells treated with the drugs. The cells were treated with increasing concentrations of the drugs for 6 h, and the ability of the cells to incorporate [3H]leucine into trichloroacetic acid precipitable material was used to estimate the rate of protein synthesis (Fig. 1E). Both compounds were toxic at 10 μM, but there was no detectable toxicity at lower concentrations. In our phosphorylation assay only a 10 nM concentration of the inhibitors was used, which is far below the concentration where toxicity was observed. We therefore conclude that the inhibition of phosphorylation of FGF-1 observed was not simply due to toxicity of the drugs.

Hsp90 Inhibitors Block Translocation of FGF-1 and FGF-2 to the Cytosol and the Nucleus—Since phosphorylation of FGF-1 takes place in the nucleus, we wanted to investigate more closely at which stage of the transport the inhibition occurred. We therefore chose to add radiolabeled FGF-1 to cells and fractionate them into membrane, cytosol, and nuclear fractions (20). For this purpose, we used digitonin to make holes in the plasma membrane to let cytosolic proteins leak out into the medium. The medium was then recovered and designated the cytosolic fraction. The remnants of the cells were lysed with 1% Triton X-100 and fractionated by centrifugation into a nucleus and a membrane fraction.

We verified first that the fractionation procedure resulted in separation of marker proteins from the different parts of the cell. The membrane markers calreticulin (endoplasmic reticulum) and Rab5 (endosomes) were found mainly, as expected, in the membrane fraction (Fig. 2A). The cytosolic proteins Hsp90 and MAPK were found both in the cytosolic and membrane fraction. That a part of the proteins was found in the membrane fraction was expected, since it is recommended to adjust the amount of digitonin used so that only 60–80% of the cytosolic components are released. At higher concentrations of digitonin internal membranes are affected as well. We measured the release of the cytosolic protein lactate dehydrogenase and adjusted the amount of digitonin to induce a release of 80% total lactate dehydrogenase. This means that the amount of proteins found in the cytosolic fraction is necessarily underestimated. In addition both Hsp90 and MAPK can associate with the cytoplasmic side of membranes and therefore be part of the membrane fraction. Importantly, the nuclear marker, lamin A, was found only in the nuclear fraction and none of the membrane and cytosolic markers were found in the nuclear fraction. In conclusion, the analysis of marker proteins shows that the fractionation procedure gives satisfactory separation of the three compartments of the cell.

The localization of radiolabeled FGF-1 in cells incubated with the exogenous growth factor was then studied using the same fractionation procedure. As seen in Fig. 2B, lane 1, after incubating for 6 h, FGF-1 was found in all three fractions (M, C, N). In the presence of geldanamycin (10 nM) or radicicol (10 nM) FGF-1 was only found in the membrane fraction (lanes 2 and 3). As a control we added LY294002, a PI 3-kinase inhibitor that blocks the translocation of FGF-1 to the cytosol and the nucleus (14). Also in this case (Fig. 2B, lane 4), FGF-1 was only found in the membrane fraction. We also tested the translocation of FGF-2 in the same kind of experiment. When added to serum-starved NIH/3T3 cells, FGF-2 was recovered from the membrane, cytosol, and nuclear fraction (Fig. 2C, lane 1). In contrast, in the presence of geldanamycin the growth factor was only found in the membrane fraction and was absent from the cytosol and nuclear fractions (lane 2).
Hsp90 has in some cases been found to stabilize proteins in the cytosol (26). Thus, when Hsp90 is inhibited by geldanamycin or radicicol, the client proteins of the chaperone have a reduced half-life. We therefore considered the possibility that the lack of FGF-1 in the cytosol and the nucleus could be due to degradation of the growth factor by proteasomes in the absence of functional Hsp90. We therefore tested whether phosphorylation of FGF-1 could be detected in the presence of lactacystin, a proteasome inhibitor. However, as can be seen in Fig. 2D, the addition of lactacystin together with geldanamycin or radicicol did not result in the appearance of phosphorylated growth factor. Reduced stability is therefore probably not the reason for the lack of the growth factor in the cytosol and in the nucleus.

Taken together, the data indicate that FGF-1 and FGF-2 are dependent on cytosolic Hsp90 to translocate to the cytosol and the nucleus.

**Binding and Internalization of FGF-1 Is Not Affected by the Drugs**

The drugs could also interfere with the binding of FGF-1 to the FGFRs on the cell-surface of NIH/3T3 cells or the subsequent endocytosis of the growth factor. To measure binding, we incubated NIH/3T3 cells with increasing amounts of $^{125}$I-FGF-1 in the presence of heparin. In some cases, we also added 100 nM geldanamycin or 100 nM radicicol to test whether the drugs interfered with the binding. As seen in Fig. 3A, there was no demonstrable effect of the drugs on the binding to the FGFRs. We also tested the binding in a competition assay by incubating NIH/3T3 cells with $^{125}$I-FGF-1 and increasing concentrations of unlabeled FGF-1 with or without geldanamycin or radicicol. Similar results were obtained in this experiment (Fig. 3B).

To analyze whether the endocytosis of FGF-1 was changed in the presence of the drugs, we labeled NIH/3T3 cells overexpressing FGFR-1 with the fluorophore Cy3 (27). FGF-1-Cy3 was added to NIH/3T3 cells transfected with a pcDNA3 vector encoding FGFR1 and incubated for 2 h at 37 °C. The live cells were then analyzed by confocal microscopy as described for Fig. 1B. The red channel image was superimposed onto a differential interference contrast image of the same cell. In some cases, 100 nM geldanamycin or 100 nM radicicol was added 30 min before the incubation with FGF-1-Cy3 and was present throughout the incubation period.
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IB: anti-phospho MAPK

IB: anti MAPK

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Akt

IB: anti-phospho Akt

IB: anti-Akt

FIGURE 4. FGF signaling in the presence of the inhibitors. A, serum-starved NIH/3T3 cells were preincubated in the absence (lanes 1, 2, 4, 6, and 8) or in the presence of a combination of 100 nM geldanamycin and 100 nM radicicol (lanes 3, 5, 7, and 9). The cells were left untreated (lane 1) or stimulated for 15 min (lanes 2 and 3 and 6 and 7) or 6 h (lanes 4 and 5 and 8 and 9) with 10 ng/ml FGF-1 or FGF-2 in the presence of 10 units/ml heparin. The cells were subsequently lysed and analyzed by Western blotting with anti-p44/42 MAP kinase antibodies (lower panel). The membrane was stripped and reprobed with anti-phosphorylated-p44/42 MAP kinase antibodies (upper panel). B, serum-starved NIH/3T3 cells were treated as described for A except that the membrane was probed with anti-phospho-Akt antibodies (upper panel), stripped, and reprobed with anti-Akt antibodies (lower panel).

absence or presence of 100 nM geldanamycin or radicicol for 2 h at 37°C. Intracellular red dots representing endosomes containing FGF-1 was then observed (Fig. 3C). We observed no difference in the endocytic uptake of FGF-1-Cy3 whether the inhibitors were added or not.

At the present time it is only possible to see uptake of FGF-1 in cells overexpressing the FGF receptors. Furthermore, the overexpression does not result in increased transport to the nucleus, and it is therefore not possible to convincingly detect FGF-1-Cy3 in the nucleus either.

It appears that the drugs do not affect binding and internalization of FGF-1, and the data point to the possibility that it is the translocation of the growth factors from endosomes to the cytosol that is affected.

Geldanamycin or Radicicol Does Not Affect FGF Signaling—Cell signaling could be implicated in the translocation of FGF-1 into the cells (14). We therefore investigated whether the Hsp90 inhibitors altered cell signaling from the FGF receptors. First, we checked whether the stimulation of the Ras/MAPK cascade, which is induced by the intrinsic tyrosine kinase of the activated FGF receptor, was affected. This was done by probing the phosphorylation status of MAPK using a phospho-MAPK-specific antibody. As seen in Fig. 4A, upper panel, a strong band corresponding to phosphorylated MAPK was observed after 15-min stimulation of NIH/3T3 cells by FGF-1 or -2. This activation of MAPK was similar whether 100 nM geldanamycin and radicicol were added or not. Also after 6-h continuous stimulation with FGF-1 or -2, MAPK was activated, and there was no difference in the absence and presence of the inhibitors. The lower panel is the same Western blot membrane probed with an antibody against total MAPK to demonstrate equal loading.

Next, we tested the activation of the PI 3-kinase cascade, which leads to phosphorylation of Akt. After 15-min stimulation of NIH/3T3 cells by FGF-1 or -2, phosphorylated Akt was observed (Fig. 4B, upper panel, lanes 2 and 6). The addition of 100 nM geldanamycin and radicicol did not affect the activation of Akt (lanes 3 and 7). In long term experiments, where the cells were stimulated for 6 h, Akt was still activated but to a lower extent than after 15 min (lanes 4 and 8). In this case, it also appeared to be somewhat less phosphorylated Akt when the drugs were present (lanes 5 and 9). However, the difference is small and only after a long incubation time (6 h). The results in the lower panel indicate that similar amounts of Akt were present in each case.

Altogether, the cell signaling events investigated are not much affected by the drugs. It therefore seems unlikely to be a disturbance in signaling that causes the complete block in translocation of FGF-1 and -2 into the cell when they are treated with Hsp90 inhibitors.

Time Dependence of Reactivation of Blocked Translocation by Monensin—We have earlier demonstrated that translocation of FGF-1 and FGF-2 to the cytosol is blocked by the inhibitors of vesicle-type proton pumps, bafilomycin A1 and concanamycin A (10, 11), probably because the electrical potential (positive inside) across the limiting membrane of intracellular vesicles is required for translocation to occur (Fig. 5A). However, even in the presence of bafilomycin A the translocation can be restored when the cells are treated with the ionophores monensin or nigericin that exchange monovalent cations across membranes in an electroneutral manner. This is probably due to restoration of the membrane potential by activation of the Na+/K+-ATPase present in the vesicular membrane. Thus, the additional treatment with the Na+/K+-ATPase inhibitor, ouabain, prevents translocation under these conditions.

Ten nM bafilomycin A1 completely blocked translocation assayed by phosphorylation of FGF-1 (Fig. 5B, lane 2). When monensin was present during the last hour of the experiment the translocation was restored (Fig. 5B, lane 3). Interestingly, it was sufficient to add monensin for the last 15 min to obtain efficient translocation (Fig. 5B, lane 5). Treatment with monensin for a short time period (15 min) was also sufficient to restore FGF-1 translocation, which had been blocked by concanamycin A, another inhibitor of the vesicle-type proton pump (Fig. 5C). The data indicate that during the block of translocation induced by bafilomycin A1 or concanamycin A, the growth factor accumulates in intracellular vesicles where it is unable to exit. Upon addition of monensin, the growth factor is rapidly translocated to the cytosol, presumably due to restoration of the membrane potential across the vesicular membrane.

We used this assay to test the time required for the Hsp90 inhibitors to block the membrane translocation step in the internalization pathway. The outline of the experiment is showed in Fig. 6A. Radiolabeled FGF-1 was added to cells in the absence (Fig. 6B, lane 1) or in the presence of bafilomycin (lanes 2–5) to inhibit translocation and allow accumulation of the growth factor in endosomes. Monensin was then added to relieve the block in translocation and, as expected, FGF-1 was now also found in the cytosolic and nuclear fractions (lane 3). When geldanamycin or radicicol was added as late as 30 min before monensin, the translocation into the cytosol and the nucleus was still completely blocked (lanes 4 and 5). As an internal control, there was no FGF-1 in the cytosolic or nuclear fraction when LY294002, a PI 3-kinase inhibitor, which blocks FGF-1 translocation (14), was added (lane 6). These
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results indicate that the Hsp90 inhibitors block the membrane translocation step as such and not earlier events.

**DISCUSSION**

Here we have provided evidence that Hsp90 is crucial for the translocation of FGF-1 and FGF-2 across cellular membranes. More specifically, it seems to be the membrane translocation from endosomes into the cytosol that requires Hsp90.

The effect of Hsp90 inhibitors on different steps in the uptake pathway taken by FGF-1 to reach the nucleus was studied. Neither the initial binding of the growth factors to the FGF receptors nor the endocytosis of FGF-1 was affected by the Hsp90 inhibitors. FGF-1 and FGF-2 seemed to be trapped in endosomes in the presence of the Hsp90 inhibitors. It therefore appears that it is the actual translocation across the endosomal membrane that is affected when Hsp90 is inhibited.

We have previously shown that FGF translocation is dependent on signaling by PI 3-kinase (14). Furthermore, Hsp90 is involved in the stability of Akt, which acts downstream of PI 3-kinase activation. The effect of Hsp90 inhibition on translocation could therefore be indirect by interfering with the signaling from the FGF receptors. However, we could not detect much change in signaling in cells treated with the inhibitors. Furthermore, when cells were treated with the inhibitors for a short time period (1 h), in the monensin-“pulse” experiments (Fig. 6), the translocation was still inhibited. We were treated with the inhibitors for a short time period (1 h), in the monensin-“pulse” experiments (Fig. 6), the translocation was still inhibited. We therefore think that the block of translocation by Hsp90 inhibitors is unlikely to be due to interference with signal transduction.

Geldanamycin and radicicol both inhibit the intrinsic ATPase activity of Hsp90 necessary for the proper function of the chaperone (26). Hsp90 binds generally to substrate proteins in the near native state and are not like many other chaperones thought as being involved in nascent protein folding. We have earlier provided evidence that FGF-1 does not unfold to a large extent during translocation, but it was not excluded that FGF-1 could assume a "molten globule" state that could more easily pass through the hydrophobic membrane bilayer (20). Thus, Hsp90 could potentially aid in the refolding to a native state after translocation.
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to the cytosol. However, since we could not detect even traces of FGF-1 in the cytosol when cells were treated with the Hsp90 inhibitors, it appears more likely that Hsp90 is required at a step in the translocation across the membrane before eventual refolding in the cytosol.

Hsp90 could be directly involved in the translocation of FGF across the membrane. In a generic membrane translocation system, the protein to be translocated is first targeted to the cis side of the membrane to a translocon (28). The polypeptide is then translocated across the membrane through a translocon. Often, chaperones are involved in the translocation process on the trans side of the membrane. For instance, the proper function of the ER translocon is dependent on the ER luminal chaperone Bip, which is involved in the movement of the polypeptide chain across the membrane (29). Chaperones also assist in the correct folding of the protein on the trans side of the membrane (28). In mitochondrial translocation, the chaperone mHsp70 plays a similar role, and in chloroplast translocation, the chaperones Hsp70-IAP and Hsp93 are involved (28). Also in FGF translocation, mechanistic energy for translocation could be provided by Hsp90. Maybe Hsp90 is directly involved in the pulling of FGF-1 across the membrane. However, we could not detect a direct binding of FGF-1 and -2 with Hsp90. Hsp90 is directly involved in the pulling of FGF-1 across the membrane. Maybe ones Hsp70-IAP and Hsp93 are involved (28). Also in FGF translocation, the chaperone involved in the movement of the polypeptide chain across the membrane is Bip in ER translocation, we should possibly not be able to see a stable interaction, since in this case, they only interact transiently. Possibly the FGFs only interact with Hsp90 transiently during translocation through the membrane.

Hsp90 protects several client proteins from degradation in the cytosol (28). We therefore tested whether degradation of FGF-1 after translocation was the reason for the disappearance of FGF-1 in the cytosol and nucleus when the cells were treated with geldanamycin and radicicol. However, in the presence of the proteasomal inhibitor lactacystin in addition to geldanamycin we could still not detect any FGF-1 in the cytosol or in the nucleus. This result is consistent with the hypothesis that it is the actual translocation across the membrane into the cytosol that is affected by the Hsp90 inhibitors. However, Hsp90 could potentially have an additional role in refolding of the growth factor and protection of FGF-1 from degradation after translocation.

It is striking that several toxins also depend on Hsp90 for efficient translocation into the cytosol. For both diphtheria toxin and C. botulinum C2 toxin Hsp90 seems to be crucial for the actual translocation step across the endosomal membrane (17, 18). Initial binding and endocytosis did not seem to be affected by the drugs. In the case of diphtheria toxin, Hsp90 was part of a bigger complex involving thioreductase and other chaperones that was proposed to act together in the translocation of the toxin (17). Perhaps, also in the case of FGF-1, a multiprotein complex may be involved in the membrane translocation.

It is interesting that certain toxins and the FGF growth factors seem to utilize a similar system involving Hsp90 for translocation across the endosomal membrane. The Hsp90-dependent translocation system is obviously not developed for the translocation of dangerous toxins. Possibly, the system was developed for growth factor translocation and exploited by toxins for efficient transport into the cell.

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FGF-1 and FGF-2 Require the Cytosolic Chaperone Hsp90 for Translocation into the Cytosol and the Cell Nucleus
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