A Nuclear Export Sequence Located on a β-Strand in Fibroblast Growth Factor-1*

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Receptor-bound and endocytosed fibroblast growth factor-1 (FGF-1) is able to cross the vesicle membrane and translocate to cytosol and nucleus. This suggests an intracellular role of FGF-1, which also signals by activating transmembrane FGF receptors. Phosphorylation of internalized FGF-1 by nuclear protein kinase Cδ induces rapid export from the nuclei by a leptomycin B-sensitive pathway. In the present work, we have searched for and identified a Leu-rich nuclear export sequence (NES) at the C terminus of FGF-1 required for its nuclear export and able to confer nuclear export activity to a reporter protein in an in vivo system. Mutants where hydrophobic amino acids within the NES were exchanged for alanine exhibited reduced or abolished nuclear export. As demonstrated in co-immunoprecipitation experiments, a complex containing FGF-1, exportin-1, and its co-factor Ran-GTP, was formed in vitro. Formation of this complex in vivo was demonstrated by a peroxisomal targeting assay. Formation of the FGF-1-exportin-1-Ran-GTP complex in vitro as well as nuclear export of FGF-1 in vivo was dependent on phosphorylation of FGF-1, and it was abolished by leptomycin B. The FGF-1 NES was found to be situated along a β-strand, which has not been reported before, since NESs usually are α-helical.

Fibroblast growth factor-1 (FGF-1) is a member of a superfamily of growth factors involved in numerous cellular processes, such as differentiation, proliferation, and migration. The activation of high affinity cell surface receptors and the subsequent downstream effects are currently the best understood aspects of FGF-1 signaling (2). However, in addition to its external action, exogenous FGF-1 can be translocated into the cytosol and nucleus of cells expressing appropriate FGF receptors (3, 4).

Phosphatidyl 3-kinase activity and a transmembrane electrical potential across the limiting membrane of endosomes have been found to be required for FGF-1 translocation (5, 6), whereas the tyrosine kinase of the FGF receptor and extensive unfolding of the growth factor are not (3, 7–9). In addition to this, the identification of several intracellular interacting partners of FGF-1, such as mortalin, FIBP, CK2, and p34 (10–13), suggests an intracellular role for the growth factor.

The translocated growth factor can be found in the nucleus, presumably after arriving first in the cytosol. Imamura et al. (14, 15) obtained evidence for the presence of exogenously added FGF-1 in the nucleus of BALB/c 3T3 and HUVE cells, and they identified an N-terminal nuclear localization sequence required for the appearance of the growth factor in the nucleus. Recently, it was shown that FGF-1, similarly to FGF-2 (16), contains an additional C-terminal nuclear localization sequence (17).

All nucleocytoplasmic traffic occurs through the nuclear pore complexes. Molecules smaller than ~40 kDa may diffuse passively through the nuclear pore complexes, whereas transport of larger structures are mediated by nuclear transport receptors of the importin-β and -α families (18, 19). These receptors shuttle between the nucleus and cytosol and transfer multiple cargo molecules in a signal-mediated and energy-dependent way.

Several nuclear export receptors have been characterized, which mediate export of different types of RNA as well as protein cargo (20). Exportin-1/CRM (chromosomal region maintenance)-1 exports cargo containing a Leu-rich nuclear export sequence (NES) initially identified in the HIV-1 Rev protein and in protein kinase A inhibitor (21, 22).

So far, more than 75 experimentally validated Leu-rich NESs have been identified (NESbase version 1.0 (available on the World Wide Web) (23). Exportin-1-dependent transport is blocked by leptomycin B (LMB), a Streptomyces metabolite that covalently attaches to a cysteine residue adjacent to the NES recognition motif in the central region of exportin-1 (24).

The accessibility of the NES to the nuclear export machinery can be regulated by conformational shifts in the protein structure or by masking the NES by overlapping binding of additional factors (25, 26). Post-translational modifications, such as phosphorylation and acetylation, have been found to influence the accessibility of nuclear trafficking signals (27, 28).

We recently observed rapid nuclear export of the phosphorylated form of FGF-1 (29). This export was blocked by LMB,
suggesting that phosphorylated FGF-1 is actively exported from the nucleus by exportin-1. However, no NES responsible for the export has been identified within the FGF-1 molecule.

We here demonstrate a functional and transferable FGF-1 NES, which contains hydrophobic amino acids of both crucial and moderate importance to nuclear export. Additionally, we provide evidence that FGF-1 is able to form a complex with exportin-1 and RanGTP both in vitro in co-immunoprecipitation experiments and in vivo by employing a peroxisomal targeting assay (30). The complex formation is phosphorylation-dependent, and this modification, which is known to occur in the nucleus, may be a signal for regulated FGF-1 export from the nuclei.

**EXPERIMENTAL PROCEDURES**

**Materials**—FuGENE 6 transfection reagent was purchased from Roche Applied Science. Dulbecco’s modified Eagle’s medium (DMEM), streptomycin, penicillin, and the plasmid pcDNA3 were obtained from Invitrogen. Restriction enzymes were from New England Biolabs. Rabbit reticulocyte lysate and RNasin were obtained from Promega. [3H]Thymidine, [35S]methionine, [33P]phosphate, protein A-Sepharose, heparin-Sepharose, and ECL Western blot developing reagents were purchased from Amersham Biosciences AB. Protease inhibitor mixture, phosphatase inhibitor mixture, cycloheximide, heparin, actinomycin D, digitonin, Mowiol, Triton X-100, diethylthreitol, and LMB were purchased from Sigma. Alkaline phosphatase was obtained from Roche Applied Science. Rottlerin was purchased from Sigma. LMB was obtained from Molekulare Biologie Heidelberg, Germany. pTriEx2-Ran-Q69L was provided by Dr. Gorlich (Zentrum fur Molekulare Biologie Heidelberg, Germany). pTriEx2-Ran-Q69L-His was constructed from pQE32-Ran-Q69L by subcloning. pYFP-CRM-1 was a kind gift from Dr. Rodriguez (Department of Medical Oncology, Vrije Universiteit University Medical Center, Amsterdam, The Netherlands). The QuikChange site-directed mutagenesis kit (Stratagene) was used to construct the FGF-1 mutants L149A/V151A, F146A, and I144A/L145A/F146A. pcDNA3- myc-FGF-1-S130D-pts was constructed by inserting the cDNA for FGF-1 into the multiple cloning site of the pcDNA3 vector, where the Myc tag was added to the C terminus of FGF-1 by PCR. The peroxisomal targeting signal (pts), consisting of the three amino acids SKL, was then added to the C terminus by PCR. Finally, the point mutation S130D was added by using the QuikChange site-directed mutagenesis kit. pcDNA3- myc-FGF-1-S130D-pts was constructed by substituting Ile144, Leu145, and Phe146 to alanine using the QuikChange site-directed mutagenesis kit with pcDNA3- myc-FGF-1-S130D-pts as a template. Similarly, pcDNA3- myc-FGF-1-S130A-pts was constructed by substituting Ser130 to alanine.

**Cell Cultures**—NIH/3T3 cells were propagated in Quantum 333 medium containing 1% calf serum. COS and HeLa cells were grown in DMEM containing 10% fetal calf serum. Penicillin (100 units/ml) and streptomycin (10 μg/ml) were added, and cells were incubated in a 5% CO2 atmosphere at 37 °C.

**Protein Expression and Purification**—Recombinant FGF-1 was expressed from pET21d in the E. coli strain Rosetta 2 (DE3) PlysS and purified using a heparin column, HiTrap Heparin HP (GE Health care). An E. coli strain TG1 containing pQE60-CRM-1 and an E. coli strain M15 [pREP4] containing pQE32-Ran-Q69L were used to express exportin-1/CRM-1 and Ran-Q69L, respectively. The proteins were subsequently purified on a Ni2+-chelating column (GE Healthcare).

**In Vitro Transcription and Translation**—[35S]Methionine-labeled proteins were produced in a rabbit reticulocyte lysate system as described previously (29).

**In Vivo FGF-1 Phosphorylation**—When the NIH/3T3 cell monolayer was ~60% confluent, the cells were starved for 24 h in DMEM containing 1% fetal calf serum. Starvation was continued in phosphate-free DMEM containing 1% fetal calf serum for 6 h. Then 25 μCi/ml [33P]phosphate was added, and the cells were incubated for another 18 h. Appropriate reagents, recombinant FGF-1 (100 ng/ml), and heparin (10 units/ml) were then added, and the cells were incubated for 6 h to allow FGF-1 to translocate into the cytosol and nucleus. The cells were washed twice in HEPES buffer and lysed in lysis buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4) containing protease and phosphatase inhibitor mixtures. After scraping the cells off the plastic and a 5-min centrifugation at 15,800 × g, the supernatant was collected and concentrated by the cytoplasmic fraction. The pellet was sonicated in lysis buffer and centrifuged at 15,800 × g for 15 min. The supernatant was designated the...
nuclear fraction. Heparin-Sepharose was then added, and the samples were rotated for 2 h at 4 °C in order to adsorb the growth factor. After washing twice in PBS with 0.1% Triton X-100, the samples were treated with trypsin (2 μg/ml) for 30 min at room temperature in order to remove contaminating factors. After washing with PBS containing 0.1% Triton X-100 and protease inhibitors, the samples were subjected to SDS-PAGE and fluorography.

Cell Fractionation—NIH/3T3 cells were incubated in serum-free DMEM for 24 h prior to the addition of [35S]methionine-labeled FGF-1. The incubation was continued for 6 h, and then growth factor remaining at the cell surface was removed with 1 M NaCl in 20 mM sodium acetate (pH 4.0) for 10 min on ice. The cells were rinsed in HEPES buffer (pH 7.4) and then further incubated for 4 h at 37 °C in DMEM. The cytosolic fraction was purified by permeabilizing the plasma membrane by digitonin (20 μg/ml) for 5 min at room temperature and then for 30 min on ice. The medium containing the cytosol was collected and centrifuged at 15,800 × g for 5 min to remove cell debris. The cell remnants were then treated with 600 μl of lysis buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4) containing protease and phosphatase inhibitor mixtures for 15 min on ice, scraped off the plastic, and centrifuged for 5 min at 15,800 × g. The supernatant, containing the membrane fraction, was removed, and the nuclear pellet was sonicated in 600 μl of lysis buffer containing protease and phosphatase inhibitors. After centrifugation for 15 min at 15,800 × g, the supernatant was collected and designated the nuclear fraction. All fractions were finally treated with heparin-Sepharose for 2 h at 4 °C, and the absorbed material was subjected to SDS-PAGE and fluorography.

Activation of Signaling Pathways—NIH/3T3 cells were starved in DMEM containing 0.5% fetal calf serum for 24 h and then treated with growth factor (5 ng/ml) and heparin (10 units/ml) for either 15 min or 6 h. The cells were lysed in SDS-sample buffer and analyzed by SDS-PAGE and Western blot using phospho-specific antibodies or anti-Erk1/2 for loading control. Between each detection step, the membranes were stripped in stripping buffer.

Measurement of DNA Synthesis and Cell Proliferation—The measurement of [3H]thymidine incorporation in response to stimulation with growth factors was performed as described by Klingenberg et al. (35). For cell proliferation studies, NIH/3T3 cells were seeded at 2 × 104 cells/well, serum-starved for 24 h at 37 °C, and then treated with 10 units/ml heparin and wild type or mutant FGF-1 for up to 72 h. 50 ng/ml of FGF-1 was added at the time points 0, 24, 36, 48, and 60 h. The cells were counted using a Coulter counter (Beckman Z1) at the time points 0, 24, 36, 48, and 70 h.

Co-immunoprecipitation—[35S]Methionine-labeled wild type or mutant FGF-1 (1.0 μg), Ran-Q69L-His (0.75 μg), and exportin-1 (0.75 μg) were mixed in 400 μl of PBS containing 5 μl of lysis buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4) containing protease and phosphatase inhibitor mixtures and incubated for 2 h at 4 °C. An anti-His antibody (800 ng/sample) was attached to protein A-Sepharose and added to the samples followed by 4 h of incubation at 4 °C. After sedimentation, supernatants were collected, and the beads were then washed three times with PBS containing 0.1% Triton X-100. The samples were analyzed by SDS-PAGE and fluorography. The supernatants were incubated with heparin-Sepharose to adsorb unbound [35S]methionine-labeled FGF-1 and analyzed as above to ensure that sufficient labeled growth factor had been added to each sample.

In Vivo Export Assay—HeLa or COS cells were seeded onto sterile glass coverslips and, when the cell monolayer was ~50% confluent, transfected with the plasmids pRev-1.4-GFP, pRev-NES-GFP, or pRev-FGF-NES-GFP using the FuGENE 6 reagent, as described by the manufacturer. After expression of the GFP fusion proteins for 48 h, the cells were incubated for 3 h with either actinomycin D (5 μg/ml) or LMB (3 ng/ml) or left untreated. To ensure that cytoplasmic GFP was a result from nuclear export and not newly translated proteins, cycloheximide (15 μg/ml) was added to all wells 30 min prior to additional treatments. The coverslips were then washed once in PBS and fixed for 30 min in 3% paraformaldehyde on ice. After two additional washes with PBS, the coverslips were mounted in Mowiol and analyzed using a Zeiss LSM 510 META confocal microscope. The images were prepared by Adobe Photoshop 7.0. For cell fractionation, transfected HeLa cells were lysed in lysis buffer, and the soluble fraction was designated the cytoplasmic fraction. The insoluble fraction was washed in lysis buffer and sonicated, and the soluble fraction was designated the nuclear fraction.

In Vivo Protein-Protein Interaction Assay by Peroxisomal Targeting—HeLa cells were seeded on sterile coverslips and transfected with the appropriate constructs when ~50% confluent. Following protein expression for 24 h at 37 °C, the plasma membrane was permeabilized for 10 min with a 40 μg/ml concentration of the cholesterol-specific detergent digitonin. The peroxisomal membranes do not contain cholesterol and will remain intact upon this treatment. Cytosolic material is washed out in PBS for 3 × 5 min, the cells were fixed in 3% paraformaldehyde for 50 min at 4 °C, and the reaction was quenched by incubation for 10 min in 50 mM NH4Cl. The cholesterol-deficient peroxisomal membranes are then permeabilized with 0.1% Triton X-100 for 5 min and blocked for 20 min with 5% fetal calf serum to avoid subsequent nonspecific antibody binding. Finally, the cells were incubated with primary and secondary antibodies, and the coverslips were mounted in Mowiol. Samples were analyzed using a Zeiss LSM 510 META confocal microscope (Thornwood, NY), the images were prepared by Adobe Photoshop 7.0 (San Jose, CA).

RESULTS

Characterization of the Putative Nuclear Export Sequence by Amino Acid Substitutions—Upon visual examination of the amino acid sequence of FGF-1, we noticed a cluster of hydrophobic amino acids at the C terminus (Ile144, Leu145, and Phe146) for alanine. We also constructed the single mutant

Cell Fractionation—NIH/3T3 cells were incubated in serum-free DMEM for 24 h prior to the addition of [35S]methionine-labeled FGF-1. The incubation was continued for 6 h, and then growth factor remaining at the cell surface was removed with 1 M NaCl in 20 mM sodium acetate (pH 4.0) for 10 min on ice. The cells were rinsed in HEPES buffer (pH 7.4) and then further incubated for 4 h at 37 °C in DMEM. The cytosolic fraction was purified by permeabilizing the plasma membrane by digitonin (20 μg/ml) for 5 min at room temperature and then for 30 min on ice. The medium containing the cytosol was collected and centrifuged at 15,800 × g for 5 min to remove cell debris. The cell remnants were then treated with 600 μl of lysis buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4) containing protease and phosphatase inhibitor mixtures for 15 min on ice, scraped off the plastic, and centrifuged for 5 min at 15,800 × g. The supernatant, containing the membrane fraction, was removed, and the nuclear pellet was sonicated in 600 μl of lysis buffer containing protease and phosphatase inhibitors. After centrifugation for 15 min at 15,800 × g, the supernatant was collected and designated the nuclear fraction. All fractions were finally treated with heparin-Sepharose for 2 h at 4 °C, and the absorbed material was subjected to SDS-PAGE and fluorography.

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FGF-1-F146A. To study the transport (translocation) of exogenous FGF-1 to the cytosol and nucleus of cells, we have established a method that relies on the phosphorylation of FGF-1. FGF-1 possesses a single phosphorylation site, Ser[^1](30), which can be phosphorylated by PKC[^2](29, 33). Since PKC is only found in the cytosol and nucleus of cells and not in endosomes, phosphorylation of exogenously added FGF-1 indicates that it was translocated across cellular membranes to reach the cytosol/nucleus. Wild type and mutated growth factors were added to serum-starved and [^3]P-preloaded NIH/3T3 cells, and the growth factor was allowed to translocate into the cytosol/nucleus for 6 h. The cells were then fractionated into cytoplasmic and nuclear fractions, and the growth factor was adsorbed to heparin-Sepharose, as described under "Experimental Procedures." Finally, the samples were analyzed by SDS-PAGE and fluorography. All phosphorylated wild type FGF-1 was detected in the cytoplasmic fraction (Fig. 1A, lane 1). On the other hand, the phosphorylated mutants were only found in the nuclear fraction (Fig. 1A, lanes 2 and 3). Phosphorylated wild type FGF-1 was detected in the nucleus when the nuclear export was inhibited by LMB (Fig. 1A, lane 4). To further analyze the hydrophobic cluster of amino acids, we exchanged leucine at position 147 for alanine. Also, here all phosphorylated FGF-1 L147A was trapped in the nucleus (Fig. 1B, lane 4).

Finally, we studied the nuclear export of phosphorylated FGF-1 when the two most C-terminal hydrophobic amino acids were mutated. Modifying similar hydrophobic amino acids within an NES have efficiently abolished nuclear export of other proteins (22, 23). We constructed FGF-1-L149A/V151A and analyzed the subcellular localization after internalization and phosphorylation. In contrast to the mutants described above, phosphorylated FGF-1-L149A/V151A was detected in both the cytoplasmic and nuclear fractions (Fig. 1C, lane 7).

Bafilomycin A1 (Baf A1) inhibits the translocation of exogenous FGF-1 to cytosol/nucleus (6). To ensure that the phosphorylation of FGF-1 occurred in cytosol/nucleus and therefore reflects proper translocation, Baf A1 was used as a negative control for all mutants. No phosphorylated FGF-1 was detected in any of the subcellular fractions when Baf A1 was present, here demonstrated in Fig. 1B, lane 1.

The data above demonstrate that the active nuclear export was abolished when the most N-terminal hydrophobic residues (Ile[^1](144), Leu[^1](145), Phe[^1](146), and Leu[^1](147)) were mutated. Substituting similar hydrophobic amino acids in the NES have efficiently abolished nuclear export of other proteins (22, 23). We constructed FGF-1-L149A/V151A and the mutants F146A and I[^1](144)/L[^1](145)/F[^1](146) with and without LMB. Wild type FGF-1 and the mutant L147A. A negative control using the inhibitor of FGF-1 translocation, Baf A1. C, wild type FGF-1 and the mutants F146A and L149A/V151A with and without LMB. Wild type and mutants were also analyzed after a 4- or 8-h chase. D, 10 µl of both cytoplasmic (Cyt) and nuclear (Nuc) fractions were analyzed by SDS-PAGE and Western blotting using anti-Erk1/2 or anti-lamin-A antibodies to control for pure samples.
ated wild type FGF-1 was not detectable after the chase periods (Fig. 1C, lanes 2 and 3). On the other hand, phosphorylated FGF-1-Leu149/Val151 was still present in the cytoplasmic sample after a 4-h chase period (Fig. 1C, lane 8). The mutants where Phe146 had been substituted for alanine were still detectable in the nuclear fraction even after an 8-h chase period (Fig. 1C, lanes 5 and 6). In the presence of LMB, both wild type and mutant growth factors were retained in the nuclear fraction after an 8-h chase (Fig. 1C, lanes 10–12).

Controls for pure fractions were performed by analyzing 10 μl of cytoplasmic and nuclear samples by SDS-PAGE and Western blotting using anti-Erk1/2 (cytosolic) and anti-lamin A (nuclear) antibodies. No cross-contamination was observed (Fig. 1D).

The data indicate that the hydrophobic residues within the presumed FGF-1 NES are crucial for active nuclear export of phosphorylated FGF-1. Mutating these amino acids to alanine protected against nuclear export and cytosolic dephosphorylation and thereby increased the detection period of cytosolic/nuclear, phosphorylated growth factor. The fact that LMB inhibits nuclear export of wild type FGF-1 and protects against the dephosphorylation in the cytosol, supports the hypothesis that exportin-1 is involved.

The experiments above have the limitation that we can only follow the growth factor as long as it is phosphorylated, which occurs at the single site Ser130 (35). NIH/3T3 cells were therefore incubated with [35S]methionine-labeled growth factors for 6 h, and then the surface-bound material was removed by a high salt/lowl pH wash. The internalization process was allowed to continue for 4 h more, and then the cells were fractionated into cytosolic and nuclear fractions as described under “Experimental Procedures.” Wild type FGF-1 and a S130E mutant mimicking phosphorylated FGF-1 were only detectable in the cytosolic fraction (Fig. 2, lanes 1 and 10). The majority of FGF-1-L149A/V151A could also be detected in the cytosolic fraction, but a significant portion was also present in the nuclear fraction (Fig. 2, lane 4). As expected, FGF-1-F146A was only observed in the nuclear fraction (Fig. 2, lane 7). All FGF-1 variants remained in the nucleus in the presence of LMB (Fig. 2, lanes 3, 6, 9, and 12).

Rottlerin at a concentration of 5 μM inhibits selectively the δ isoform of PKC (36), which appears to be the isoform that phosphorylates internalized FGF-1 in the nucleus (29). Upon rottlerin treatment, wild type and FGF-1 with the substitutions F146A and FGF-1-L149A/V151A could only be found in the nuclear fraction (Fig. 2, lanes 2, 5, and 8), supporting the contention that nuclear export is phosphorylation-dependent. On the other hand, the FGF-1-S130E mutant was exported in the presence of the PKCδ inhibitor, presumably due to the negative charge mimicking phosphorylation (Fig. 2, lane 11).

**Functional Characterization of the NES Mutants**—Substituting amino acids introduce the risk of altering the protein structure and thereby the function of the protein. We tested the FGF-1 mutants in regard to their function as extracellular ligands and ability to activate the high affinity cell surface receptors. By Western blot, we tested the induction of phosphorylation of the downstream signaling proteins FRS2, Erk1/2, Akt, and phospholipase Cγ using phospho-specific antibodies. NIH/3T3 cells were serum-starved to make the Ras/mitogen-activated protein kinase protein kinase cascade quiescent and then treated with 5 ng of wild type or mutant FGF-1 for a period of 15 min or 6 h as indicated in Fig. 3A. Very little signaling activity was observed in untreated cells, whereas wild type and mutant FGF-1 induced similar levels of phosphorylated FRS2, Erk1/2, Akt, and phospholipase Cγ both after 15 min and after 6 h. Based on repeated experiments, we were not able to detect any differences in activation of these signaling molecules by wild type and mutant FGF-1. These results indicate that binding to and activation of high affinity FGF receptors at the cell surface are not affected by the modifications introduced by mutation.

The FGF-1 NES mutants were then tested for the ability to stimulate DNA synthesis. NIH/3T3 cells were treated with increasing concentrations of mutant and wild type FGF-1 for 24 h, and the incorporation of [3H]thymidine was measured. All mutants were able to induce DNA synthesis to similar levels as the wild type (Fig. 3B).

To further investigate whether the mutations had an effect on the biological activity of FGF-1, we studied the proliferation of NIH/3T3 cells stimulated with FGF-1 for up to 72 h. First, the cells were stimulated with 200 ng/ml wild type or mutant FGF-1 and then incubated for 72 h. We then found that the NES mutants had a reduced ability to stimulate proliferation compared with FGF-1 wild type (results not shown). To exclude the possibility that these results were affected by a reduced activity/stability of the FGF-1 mutants that had gone unnoticed in our previous experiments, we repeated the experiment but this time added 50 ng/ml fresh FGF-1 every 12 h for a total of 72 h. Also, in this case, the NES mutants FGF-1-L147A and FGF-1-I144A/F145A/F146A, as well as the phosphorylation-deficient FGF1-S130A mutant, gave a reduced proliferation compared with FGF-1 wild type (Fig. 3C). A possible explanation for these observations is that although the wild type and the NES mutants of FGF-1 stimulate DNA synthesis through activation of FGF receptors equally well, the nuclear accumulation of FGF-1 has an inhibitory effect on proliferation.

**Test of the Putative Export Sequence in an in Vivo Nuclear Export Assay**—Although the hydrophobic residues found to influence the nuclear export of phosphorylated FGF-1 are situated within an NES-like region, this does not provide decisive evidence that FGF-1 contains a functional NES. To test if the putative FGF-1 NES has a transferable and functional export activity, we employed a transfection-based in vivo assay (31).
wild type or mutant FGF-1 was added every 12 h, and the cells were incubated. Cidine incorporated by the cells during the last 6 h of a 24-h stimulation period.

pho-Akt, and anti-phospholipase C using anti-phospho-FRS2, anti-phospho-Erk1/2, anti-total Erk1/2, anti-phospho-PLCγ, and anti-total PLCγ antibodies. Western blotting using an anti-GFP antibody (Fig. 4B) was determined using fixed cells. To exclude the possibility that the fixation procedure could influence the results, we also performed the experiment in live cells. The GFP localization pattern obtained was in this case similar to that reported in Fig. 4, B and C. Nuclear export of Rev-NES-GFP was clearly inhibited by LMB treatment. Cells overexpressing Rev-FGF-1-GFP had increased cytoplasmic GFP staining as compared with the negative control. This tendency was further enhanced in the presence of actinomycin D, demonstrating that the putative FGF-1 NES is able to confer export activity. When treated with LMB, the nuclear GFP staining in cells overexpressing Rev-FGF-1-GFP was increased. This supports the view that exportin-1 is the nuclear export receptor for FGF-1.

The localization of the GFP fusion proteins described above was determined using fixed cells. To exclude the possibility that the fixation procedure could influence the results, we also performed the experiment in live cells. The GFP localization pattern obtained was in this case similar to that reported in Fig. 4, B and C (supplemental materials).

As a control, fractionation of HeLa cells expressing Rev-1.4-GFP, Rev-NES-GFP, or Rev-FGF-1-GFP into cytoplasmic and nuclear samples was performed after appropriate treatments with cycloheximide, LMB, and actinomycin D, as indicated. The samples were analyzed by SDS-PAGE and Western blotting using an anti-GFP antibody (Fig. 4D). The variations in the amount of GFP observed between the different samples were in accordance with the results obtained from the screen using confocal microscopy (Fig. 4, B and C). Cytoplasmic samples were not cross-contaminated with nuclear proteins or vice versa, since Erk1/2 was only detected in the cytoplasmic fraction, and lamin A was only detected in the nuclear fraction (Fig. 4E).

Test of the Export Signal in Vitro—We performed co-immunoprecipitation experiments to test if FGF-1 is able to interact with the export complex consisting of exportin-1 and Ran in vitro and if the FGF-1 mutations impairing the export activity have any effect on the formation of such a complex. Exportin-1

FIGURE 3. Functional characterization of the NES FGF-1 mutants. A, FGF receptor activation. Serum-starved NIH/3T3 cells were left untreated or treated with 5 ng of wild type (wt) or mutant FGF-1 for 15 min or 6 h. Cells were then lysed in SDS sample buffer and analyzed by SDS-PAGE and Western blot using anti-phospho-FRS2, anti-phospho-Erk1/2, anti-total Erk1/2, anti-phospho-Akt, and anti-phospholipase Cγ antibodies. B, the FGF-1 mutants were assayed for the ability to stimulate DNA synthesis in NIH/3T3 cells. Growth factor concentrations (ng/ml) are plotted against the amount of [3H]thymidine incorporated by the cells during the last 3 h of a 24-h stimulation period. C, stimulation of cell proliferation. To serum-starved NIH/3T3 cells, 50 ng of wild type (wt) or mutant FGF-1 was added every 12 h, and the cells were incubated for up to 72 h. At the indicated time points, the number of cells was counted. Values are averages from four samples, and the error bars represent S.D.
and cargo interaction occurs cooperatively with Ran-GTP (20). We expressed and purified a well described Ran mutant, Ran-Q69L, which is unable to hydrolyze GTP (41). Growth factors were [35S]methionine-labeled using the rabbit reticulocyte lysate system and added to the co-immunoprecipitation reactions together with recombinant exportin-1 and Ran-Q69L-His, as described under “Experimental Procedures.”

Using an anti-His antibody, both the wild type FGF-1 and the S130E mutant mimicking the phosphorylated form of FGF-1 were able to co-immunoprecipitate in a complex with exportin-1 and Ran-Q69L-His, as described under “Experimental Procedures.”

Using an anti-His antibody, both the wild type FGF-1 and the S130E mutant mimicking the phosphorylated form of FGF-1 were able to co-immunoprecipitate in a complex with exportin-1 and Ran-Q69L-His (Fig. 5A, lanes 2 and 4). The increased band intensity observed for FGF-1-S130E may reflect the stable negative residue mimicking phosphorylation as compared with wild type FGF-1, where only partial phosphorylation is expected to occur in the rabbit reticulocyte lysate. The FGF-1-S130A mutant was unable to form a complex (Fig. 5A, lane 3), indicating that under normal conditions, phosphorylation of Ser130 is necessary for the interaction.

Supernatants were collected after the first centrifugation following complex formation and incubated with heparin-Sepharose to collect unbound [35S]methionine-labeled growth factor. The resulting bands in the lower panels show that excess FGF-1 was present in each sample (Fig. 5, A and B).

If the interaction between wild type FGF-1 and exportin-1-Ran-Q69L is caused by phosphorylation of the growth factor in the rabbit reticulocyte lysate, phosphatase treatment should reduce or abolish this binding. [35S]Methionine-labeled wild type FGF-1 (10 μl) and the S130E mutant (10 μl) were pre-

**FIGURE 4.** In vivo nuclear export activity of the FGF-1 NES. A, putative NESs are inserted between the coding regions of an export-deficient mutant of the HIV-Rev protein and GFP. B, the Rev-1.4-GFP, Rev-NES-GFP, and Rev-FGF-1-GFP fusion proteins were transiently expressed in either HeLa or COS-1 cells. All samples were treated with cycloheximide (15 μg/ml) and with either actinomycin D (5 μg/ml) or LMB (3 ng/ml), as indicated. A minimum of 200 cells were screened for each sample and scored for nuclear only (Nuc), nuclear and cytoplasmic (Nuc & Cyt), or cytoplasmic only (Cyt) GFP localization. The experiment was repeated three times. C, representative images for the treatments described in B. The cell nucleus was visualized by staining with Draq5 (Alexis Biochemicals). D, the Rev-1.4-GFP (lane 1), Rev-NES-GFP (lanes 2 and 3), and Rev-FGF-1-GFP (lanes 4 – 6) fusion proteins were transiently expressed in HeLa cells. All samples were treated with cycloheximide (15 μg/ml) and with either actinomycin D (5 μg/ml) or LMB (3 ng/ml), as indicated. The cells were fractionated into cytoplasmic and nuclear samples and analyzed by SDS-PAGE followed by Western blotting using an anti-GFP antibody. The blots were stripped and reprobed with antibodies against Erk1/2 or lamin A to ensure equal loading. E, 10 μl of both cytoplasmic and nuclear fractions were analyzed by SDS-PAGE and Western blotting using anti-Erk1/2 or anti-lamin A antibodies to control for pure samples.
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**FIGURE 5. Interaction of phosphorylated FGF-1 with the exportin-1-Ran-Q69L complex in vitro.** A, [35S]methionine-labeled FGF-1 wild type (WT) and mutants were incubated with exportin-1 and Ran-Q69L-His and pulled down using protein A-Sepharose prebound to an anti-His antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). During washing, all supernatants were collected after the first sedimentation to adsorb unbound growth factors to heparin-Sepharose (HS). All samples were analyzed by SDS-PAGE and fluorography. Lane 1 shows 0.5 μl of [35S]methionine-labeled FGF-1. B, as described in A, but where indicated, incubations were in the presence of LMB, or samples were pretreated with alkaline phosphatase (AP). Treated with alkaline phosphatase (1 unit) for 1 h at 37 °C. The alkaline phosphatase treatment completely abolished the interaction between wild type growth factor and exportin-1-Ran-Q69L-His (Fig. 5B, lane 2) but had no effect on FGF-1-S130E binding (Fig. 5B, lane 6). Attempts at complex formation in the presence of LMB failed (Fig. 5B, lanes 3 and 7). Mutants with impaired export activity (L149A/V151A and F146A) were not able to form a complex with exportin-1-Ran-Q69L-His (Fig. 5B, lanes 8 and 9).

In summary, these co-precipitation experiments support the view that FGF-1 interacts with exportin-1 and Ran-GTP in a phosphorylation-dependent manner. Importantly, complex formation failed when export-impaired FGF-1 mutants were used, indicating a direct interaction between FGF-1 and exportin-1.

**Assay for Complex Formation of FGF-1, Exportin-1, and RanGTP in Vivo**—We employed a newly developed system for assaying protein-protein interactions in living mammalian cells to test for in vivo binding between phosphorylated FGF-1, exportin-1, and Ran-GTP (12, 30, 42). This method exploits the remarkable import capacity of peroxisomes, where large protein oligomers are able to traverse the peroxisomal membrane in a fully folded state (43). Such protein import is directed by a pts consisting of three C-terminal amino acids, Ser-Lys-Leu (43). This transferable signal is able to redirect soluble intracellular proteins into the peroxisomes and, importantly, also their interacting partners lacking a pts in a “piggy-back” fashion. Hence, by adding a pts tag to the protein of interest, its interacting partners can be co-transported into the organelle. Co-localization in these distinct compartments is confirmed by confocal microscopy and provides evidence for protein-protein interaction in the cytosol of living cells before the complex is translocated into the peroxisomal lumen.

To assay for in vivo interaction with exportin-1 and Ran-Q69L, we added a C-terminal pts tag to FGF-1-S130D, which mimics phosphorylation with a negative charge at residue 130 to yield FGF-1-S130D-pts (Fig. 6A). To ensure that FGF-1-S130D-pts was properly targeted to the peroxisomes, we co-stained cells transfected with pcDNA3-myc-FGF-1-S130D-pts for the peroxisomal marker protein catalase, as described under “Experimental Procedures.” When merging the images representing FGF-1-S130D-pts (red) and catalase (green), we could clearly see co-localization represented as a yellow punctate staining pattern (Fig. 6B). A merging technique has been implemented to test the specificity of co-localization in samples with some nonspecific background staining (42). By merging the images askew with approximately one peroxisomal diameter, the staining pattern indicating co-localization reverts to its original colors, here red and green (Fig. 6B). It has earlier been demonstrated that FGF-1 lacking a pts tag is not able to enter the peroxisomes (12).

Cells co-expressing FGF-1-S130D-pts, Ran-Q69L-His, and YFP-CRM-1 by triple transfection were stained for FGF-1-S130D-pts (red) and Ran-Q69L-His (blue), as described under “Experimental Procedures,” whereas the YFP-CRM-1 autofluorescence is observed in the green channel. Upon merging the images representing FGF-1-S130D-pts and YFP-CRM-1, yellow peroxisomes were clearly observed, indicating co-localization (Fig. 6C, i). When the images representing Ran-Q69L-His and FGF-1-S130D-pts from the same cell were merged, Ran-Q69L-His could also be observed in the peroxisomes together with FGF-1-S130D-pts (Fig. 6C, ii). When the technique of merging the images askew was implemented, both images showing co-localization reverted to their original colors, ensuring specificity (Fig. 6C, i and ii). Peroxisomal co-localization in triple-transfected cells after the addition of a pts tag to FGF-1-S130D provides evidence for in vivo binding between FGF-1-S130D, exportin-1, and Ran-Q69L-His.

Two negative controls were implemented to test if the co-localization observed between FGF-1-S130D-pts and exportin-1-Ran-Q69L-His in the peroxisomes is specific (supplemental materials). First, we substituted the three most N-terminal amino acids in the putative FGF-1 NES of FGF-1 S130D pts for alanine, yielding FGF-1-S130D/I144A/L145A/F146A-pts. Second, a phosphorylation-deficient mutant where serine 130 was exchanged for alanine, yielding FGF-1-S130D/I144A/L145A/F146A-pts. This indicates a requirement for an intact NES in FGF-1 for the in vivo interaction with exportin-1-Ran-Q69L-His and that the interaction is dependent on phosphorylation of Ser130.

**Conserved Structural Topology for Leu-rich Export Sequences**—It has been predicted that Leu-rich NESs display an α-helical secondary structure, where the first three hydrophobic amino acids residues reside on one side of an amphipathic α-helix while the fourth is placed on the opposite side. The classical consensus sequence for Leu-rich NESs (LX2–3LX2–3LX) fulfills the requirements for this hypothesis.

For six proteins in the NESBase (available on the World Wide Web), the three-dimensional structure is known, either for the protein itself or for a homologous protein. The molecular graphics and modeling program PyMol (DeLano Scientific LLC) was used to analyze and compare the NESs of these pro-
teins and of FGF-1 (Fig. 7). All six NESs have an overall α-helical structure in contrast to the NES of FGF-1, which almost exclusively spans a β-strand (Fig. 7B). In all NESs analyzed, the three initial hydrophobic residues in the NES are skewed on one face of a surface. This indicates that these residues are the most important factors for a functional NES. The fourth hydrophobic residue that is diagonally placed on the opposite side of the surface is absent in the case of eIF4a and Smad1, suggesting that other nonhydrophobic amino acids can compensate for the function in this position. Alternatively, this residue is not strictly important for NES function. Despite the difference in secondary structure between FGF-1 NES and these proteins, the spatial arrangement of the hydrophobic residues important for NES function is strikingly similar. Not only is the overall arrangement similar, but also the spacing between the residues, which is in the range of about 6–7 Å measured from α-carbon to α-carbon. Taking the rotational flexibility of the amino acids into account, the actual difference in structural topology between the α-helical and β-strand residing NESs is rather small. This similarity in structural topology among α-helical NESs has been pointed out earlier (44), and we now expand this hypothesis to also include NESs that are located on β-strands.

The nuclear export of FGF-1 is induced by PKCδ-dependent phosphorylation of Ser130 (29). This amino acid residue is placed about 20 Å away from the NES and hidden from the NES by two loops (Fig. 7A). In the primary structure, Ser130 and the NES are only separated by a short flexible loop. The flexibility of this loop is reflected in the relatively high B values in the x-ray structures of free FGF-1. It is therefore likely that phosphorylation at Ser130 can induce conformational changes that favors binding to exportin-1. This has recently been described for Nrf2, where its nuclear export is regulated by phosphorylation of a tyrosine residue located near the NES (45).

DISCUSSION

We here report that FGF-1 contains a functional Leu-rich type NES at the C terminus that enables exportin-1 interaction when FGF-1 is phosphorylated in the nucleus. In contrast to other NESs with a characterized secondary structure, FGF-1 NES is located almost entirely along a β-sheet.

Visual inspection of the amino acid sequence of FGF-1 drew our attention to a hydrophobic cluster (144ILFLPLPV151) located near the C terminus of FGF-1. This cluster resembles a Leu-rich NES but does not have an exact match with the original consensus sequence proposed for Leu-rich NESs (L_X2–3L_X2–3L_XL), where X represents any amino acid, and Leu can be substituted for other large hydrophobic amino acids (46). However, numerous experimentally validated NESs deviating from the consensus have been reported during the past decade. In fact, La Cour et al. (47) found that the majority of the Leu-rich type NESs that they analyzed deviated from this consensus.

When analyzing the FGF-1 amino acid sequence with the NES predictor, NetNES (47), FGF-1 Leu149 received a score for being a hydrophobic residue at the anchor position (most
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FIGURE 7. The secondary structure of nuclear export sequences. Proteins found in the data base for nuclear export signals, NESbase (available on the World Wide Web) or proteins homologous to a NESbase protein with known molecular structure were analyzed using the molecular graphics and modeling program PyMol (DeLano Scientific LLC). A, a ribbon diagram of FGF-1 (Protein Data Bank code 1JQZ). Hydrophobic amino acid residues involved in nuclear export are shown in blue. The FGF-1 residue phosphorylated by PKCβ, Ser130, is shown in green. B, ribbon diagrams showing the structural topology of FGF-1 NES and other characterized NES structures. (Protein Data Bank codes as follows: p53, 1AIE; eIF4a (homologous to ATP-dependent RNA helicase p54 in NESbase), 1QDE; Yap1p, 1SSE; IkBα, 1NFI; Smad1, 1KHU; Rad24, 109E.)

C-terminal amino acid within the NES). The score was relatively low (0.367), but residue Leu839 in BRCA1, which has been shown to be crucial for the nuclear export activity of BRCA1, received a similar score (0.354) (48). Since the NetNES prediction tool is still based on a very limited amount of data, it is here used as a supplement to experimental analysis. Molecular flexibility and local unfolding are thought to have a major impact on NES functionality. It is therefore difficult to predict nuclear export signals entirely based on amino acid sequence.

Post-translational modification has been reported to be able to regulate NES accessibility. Upon MAPKAP kinase 2 phosphorylation, the protein structure is believed to be altered so that the NES is made available for the export machinery (27). Also, phosphorylation of the nuclear factor Nrf2 at Tyr668 was recently found to be required for exportin-1 interaction and is thought to induce structural changes that expose the NES for exportin-1 interaction (45). Since PKCβ phosphorylation of FGF-1 induces rapid nuclear export, whereas nonphosphorylated growth factor remains in the nucleus (29), it is likely that phosphorylation is the regulating mechanism for active nuclear export of FGF-1 as well.

Nuclear export signals show a considerable variation in activity, where strength and accessibility of the sequence are thought to be determining factors (31). The putative FGF-1 NES provided export activity when tested in the pRev-GFP in vivo assay system. Here the accessibility of the NES peptide is optimized, which has the advantage of bypassing requirements such as local protein unfolding to expose the NES. Additionally, the Rev-GFP assay allows work in highly defined conditions due to (i) the controlled shuttling mechanisms of the HIV-1 Rev protein, (ii) the actinomycin D treatment that inhibits nuclear reimport of Rev-GFP, and (iii) the cycloheximide treatment controlling background cytoplasmic GFP staining. These are clear advantages as compared with conventional localization studies, where test peptides are merely attached to a reporter molecule like GFP (27, 49).

By employing the pRev-GFP assay, we could estimate the strength of the FGF-1 NES as compared with other NES sequences previously tested in the same system. Nuclear export sequences with very low activity, as identified in Hdm2 and p53, increased the cytoplasmic GFP staining only in the presence of actinomycin D (31), whereas the FGF-1 NES gave an increase in the absence of the drug. This indicates that the FGF-1 NES is more efficient in nuclear export than those present in Hdm2 and p53. On the other hand, the signals found in PKI and HIV-1 Rev are able to completely shift the GFP staining to the cytoplasm without actinomycin D treatment. Such a dramatic GFP relocation was not observed for the FGF-1 NES peptide. Altogether, the NES present in FGF-1 seems to possess an export activity of intermediary strength.

Exportin-1 binds cargo with relatively low affinity compared with other export receptors (50). This seems to be a necessity for efficient dissociation of the cargo from the export complex and release from the nuclear pore complex (51). Also, exportin-1 has a certain affinity for hydrophobic peptides in vitro, which must be taken into consideration when identifying leucine-type NESs. In our study, we have used several complementary in vitro and in vivo methods to show that FGF-1 does indeed contain an exportin-1-interacting NES at the C terminus. The requirement for phosphorylation observed both in vitro and in vivo, supports the contention of a regulated and specific interaction between exportin-1 and FGF-1.

The secondary structure of the FGF-1 NES differs from previous reports. However, when comparing the spatial arrangement of the amino acids with other NESs where the structure is known, a striking similarity becomes apparent (Fig. 7B). Since the presentation of the NES is generally thought to be the determining factor, the hydrophobic sequence found in FGF-1 might well serve as a nuclear export signal. The FGF-1 NES described herein is to our knowledge the first NES consisting entirely of a leucine-type NESs. In our study, we have used several complementary in vitro and in vivo methods to show that FGF-1 does indeed contain an exportin-1-interacting NES at the C terminus. The requirement for phosphorylation observed both in vitro and in vivo, supports the contention of a regulated and specific interaction between exportin-1 and FGF-1.

The secondary structure of the FGF-1 NES differs from previous reports. However, when comparing the spatial arrangement of the amino acids with other NESs where the structure is known, a striking similarity becomes apparent (Fig. 7B). Since the presentation of the NES is generally thought to be the determining factor, the hydrophobic sequence found in FGF-1 might well serve as a nuclear export signal. The FGF-1 NES described herein is to our knowledge the first NES consisting entirely of a β-sheet, and since structural information about the proteins containing an experimentally validated NES is still very limited, similar reports of NES secondary structure should be anticipated.

To our knowledge, the FGF-1 NES is also the first functional nuclear export sequence reported within the FGF family. Several other family members, such as FGF-2, FGF-3, and the fibroblast growth factor homologous factors (or FHFs, also denoted FGF-11 to -14), have been found to localize to the
nucleus and possess classical nuclear localization signals (52). The possibility is therefore not excluded that these growth factors possess an NES. In fact, a sequence similar to the FGF-1 NES is also present at the FGF-2 C terminus (ILFLPM).

Although the exact biological role of nuclear FGF-1 remains elusive, several intracellular proteins have been identified as binding partners for FGF-1 (53). FGF-2, which is produced as several different isoforms, is recognized as a nuclear factor binding machinery, we were able to provide evidence for in vivo binding between the mutant FGF-1-D130D, which mimics phosphorylated FGF-1, exportin-1, and Ran-GTP. An advantage of the peroxisomal targeting assay is the ability to detect interactions dependent on post-translational modifications, such as phosphorylation. Another phosphorylation-dependent interaction previously detected by this method is the interaction between cytokine-independent survival kinase and 3-phosphoinositide-dependent protein kinase 1 (42). Binding regulated by such post-translational modifications can be difficult to detect in vitro or in bacterial systems.

So far, the peroxisomal targeting assay has only been used to detect protein complexes consisting of two components (12, 42). We here show that the method is also able to detect a protein complex consisting of at least three components.

In conclusion, exportin-1 can be added to the list of intracellular binding partners of FGF-1. This interaction is part of a regulatory mechanism for nuclear exclusion of phosphorylated FGF-1. PKC8 phosphorylates FGF-1 in the nucleus, and we hypothesize that this modification results in a conformational shift that exposes the NES for binding to exportin-1.

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