Two Nuclear Localization Signals Required for Transport from the Cytosol to the Nucleus of Externally Added FGF-1 Translocated into Cells†

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ABSTRACT: Externally added FGF-1 is transported into the nucleus of cells. It was earlier shown that FGF-1 contains an N-terminal nuclear localization signal (NLS) implicated in the stimulation of DNA synthesis. We here provide evidence that FGF-1 contains a second putative NLS (NLS2), which is located near the C-terminus. It is a bipartite NLS consisting of two clusters of lysines separated by a spacer of 10 amino acids. A fusion protein of GFP and the bipartite NLS was more efficiently transported into the nucleus than GFP alone, indicating that it can act as an NLS in the living cell. FGF-1 mutated in the N-terminal NLS (NLS1) or in the first cluster of the bipartite NLS2 bound to heparin and FGF receptors and activated downstream signaling similarly to the wild-type growth factor. Mutations in the second cluster of NLS2 resulted in impaired interaction with heparin and reduced stability. When radiolabeled FGF-1 with mutated NLS1 or the first lysine cluster of NLS2 was added to NIH/3T3 cells, it was translocated into the cytosol, but not transported efficiently to the nucleus. Phosphorylation of FGF-1 occurs normally in the nucleus, and while wild-type FGF-1 was phosphorylated after addition to cells, the NLS mutants were not. It therefore appears that both NLS1 and NLS2 are important for efficient transport of FGF-1 to the nucleus. Stimulation of DNA synthesis by FGF-1 with mutations in both NLSs was reduced considerably indicating that efficient transport to the nucleus may be involved in the stimulation of DNA synthesis.

The family of fibroblast growth factors (FGF) comprises 22 members in humans (*1*). These signaling proteins are involved in several processes including cell proliferation, migration, angiogenesis, and development (*2*). The growth factors mediate their signal by binding to high-affinity FGF receptors (FGFR[1]) at the cell surface (*3*). Additional lower-affinity interaction with heparan sulfate proteoglycans (HSPG) modulates the interaction of the growth factors with the specific FGFRs (*4*, *5*). Binding of the fibroblast growth factors to the FGFRs causes dimerization of the receptors resulting in phosphorylation of the intracellular part of the receptor by its intrinsic kinase domain. This initiates further downstream signaling by mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI-3K), and phospholipase Cγ.

In addition to signaling through receptors from the cell surface, several members of the FGF family have been reported to have intracellular functions (*6*). When added externally to cells, FGF-1 was found to translocate into the nucleus where it apparently stimulated DNA synthesis (*7*, *8*). Several cytosolic and nuclear proteins that interact directly with FGF-1, e.g. CK2 (*9*), FIBP (*10*), mortalin (*11*), and p34 (*12*), have been identified. CK2 has been implicated in many processes including cell cycle progression, and FGF-1 may influence this signaling. FIBP, mortalin, and p34 may also be implicated in the signaling of FGF-1 inside the cell. However, the precise mechanism of how FGF-1 transduces the intracellular signal remains to be elucidated.

FGF-2 is also internalized and transported to the nucleus (*13*, *14*), and it has been found to interact with several intracellular proteins, e.g. CK2 (*15*), FIF (*16*), L6/TAXREB107 (*17*), and RPS19 (*18*). FIF is a nuclear putative antiapoptotic factor and may be implicated in FGF-2-induced protection of some cell types. L6/TAXREB107 and RPS19 are ribosomal proteins and suggest that FGF-2 may modulate ribosomal activity.

Finally, a third member of the FGF family, FGF-3, seems to play a role in the cell nucleus. When expressed, half of the FGF-3 pool is secreted and the other half is transported to the nucleus (*19*). The nuclear FGF-3 pool was proposed to inhibit DNA synthesis and proliferation, and it was also proposed that these effects were induced by binding to a nucleolar protein called NoBP (*20*).

It was noted early on that FGF-1 contains an N-terminal monopartite nuclear localization signal (NLS) (*7*). Deletion of this signal prevented transport of the growth factor to the nucleus assessed by cell fractionation. The induction of DNA

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[1] Abbreviations: FGF-1, fibroblast growth factor 1; FGFR, fibroblast growth factor receptor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NLS, nuclear localization sequence; HSPG, heparan-sulfate proteoglycan; MAPK, mitogen-activated protein kinase.

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synthesis was completely abolished when the NLS was deleted. However, later results showed that deletion of the N-terminal NLS sequence resulted in an unstable protein (*21*). When the lysines in the NLS were mutated rather than deleted, the stimulation of DNA synthesis was similar to wild-type FGF-1 (*22*). It therefore appeared that the N-terminal NLS was not crucial for the induction of DNA synthesis as earlier proposed and the concept of FGF-1 playing a role in the nucleus was questioned.

Later on additional evidence was presented demonstrating that FGF-1 is indeed transported into the nucleus of intact cells (*8*). In these experiments intracellular modifications of the growth factor were used to assess translocation of FGF-1 into the cytosol and the nucleus. In one approach a farnesylation signal, a CaaX-box, was fused to the C-terminus of FGF-1 (*23*). Since farnesyl transferase is localized to the cytosol and the nucleoplasm, farnesylation of exogenously added FGF-1-CaaX clearly indicates that FGF-1 can reach the interior of cells.

Furthermore, it was noticed that FGF-1 is phosphorylated by PKC when added to cells (*24*). Since PKC is only found in the cytosol or nucleus, this further underscores the translocation of FGF-1 into cells.

Using these methods to monitor translocation we have characterized in some detail the pathway utilized by FGF-1 to reach the nucleus. We have found that binding to and endocytosis by the specific FGFRs is necessary for translocation. Binding and endocytosis of FGF-1 in cells lacking FGFRs, but expressing HSPG, does not lead to translocation (*25*, *26*). Furthermore, FGF-1 is translocated across the endosomal membrane by the help of the vesicular membrane potential (*27*). In similarity to peroxisomal translocation, unfolding of the protein does not seem to be a prerequisite for translocation (*28*). It also seems that translocation of FGF-1 is a regulated process since inhibitors of PI-3 kinase block the translocation (*29*).

We here provide evidence that FGF-1 contains a second NLS, a bipartite one. We find that both NLSs are required for efficient transport of FGF-1 into the nucleus after its translocation to the cytosol. Furthermore, we find that mutations in either of the two NLSs have little influence of the stimulation of DNA synthesis, while mutating both NLSs reduces considerably the ability of FGF-1 to stimulate DNA synthesis.

EXPERIMENTAL PROCEDURES

Materials, Media, and Buffers. [^{35}S]Methionine (1000 Ci/mmol), [^{33}P]phosphate (3000 Ci/mmol), [γ^{33}P]ATP (2500 Ci/mmol), and [methyl-^{3}H]thymidine (25 Ci/mmol) were obtained from Amersham Biosciences. Other chemicals were from Sigma-Aldrich. The following primary antibodies were used for Western blotting: anti-Lamin A (133A2, Abcam), anti-HSP90 (BD Transduction Laboratories), anti-Calreticulin (SPA-600, Stressgen), anti-PKC δ (sc-937-G, Santa Cruz Biotechnology), anti Rab5A (sc-309, Santa Cruz Biotechnology), anti p44/42 MAP kinase (No. 9102, Cell Signaling Technology), and anti-Phospho-p44/42 MAPK (No. 9106, Cell Signaling Technology). 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 $CaCl_2$, adjusted to pH 7.0 with NaOH. Lysis buffer: 0.1 M NaCl, 20 mM NaH_2PO_4, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF and 1 mM NEM, pH 7.4. PBS: 140 mM NaCl and 10 mM NaH_2PO_4, pH 7.4. Restore Western blot stripping buffer was from Pierce.

Site-Directed Mutagenesis and Plasmid Construction. Plasmids coding for GFP fusion proteins were constructed by inserting a linker containing the sequences coding for the two NLS into the C-terminus of the EGFP coding frame in the vector pEGFP-C3 (Clontech).

Site-directed mutagenesis was performed using the Quickchange kit from Stratagene with pB-aFGF as a template to make pB-NLS1$^-$, pB-NLS2A$^-$, pB-NLS2B$^-$, pB-NLS1$^-$-NLS2A$^-$, and pB-NLS1$^-$NLS2B$^-$ and the appropriate oligonucleotides.

For the production of recombinant FGF-1 we used the plasmid pET-3C-FGF-1 (*7*). Site-directed mutageneses were performed using pET-3C-FGF-1 as a template to make the plasmid pNLS2A$^-$. pNLS1$^-$ was made by cloning the fragment from pB-NLS1$^-$ into pTrc99A. pNLS1$^-$NLS2A$^-$ was made by site-directed mutagenesis using pB-NLS1$^-$ as a template.

Cell Cultures. NIH/3T3 and HeLa cells were propagated as earlier described (*27*). Cells were seeded into tissue culture plates 2 days preceding the experiments.

FRAP Experiments. HeLa cells were seeded onto LabTek II (MatTek Corporation) tissue culture plates and transfected with pEGFP, pEGFP-NLS1, or pEGFP-NLS2 using the calcium phosphate method. The cells were incubated for 24 h to allow expression of the GFP proteins. The FRAP experiments were carried out at 25 °C using a Zeiss LSM510 META confocal microscope equipped with an argon laser and 63X Apochromat objective. The nucleus was selectively photobleached by choosing a region of interest (ROI) in the nucleus and scanning for ~30 cycles at maximum power. Since the diffusion of proteins is very high in the nucleus, the nucleus was homogeneously bleached. This was checked by three-dimensional reconstruction of photobleached cells showing that the whole nuclei had the same low intensity after bleaching.

After the photobleaching pulse, the recovery of fluorescence in the cells was recorded by time-lapse confocal microscopy. The fluorescence intensity inside the nucleus and in the cytosol was measured, averaged, and plotted as a function of time. The average fluorescence intensity was calculated using the Zeiss LSM510 software and represents the total fluorescence intensity measured in a given ROI, divided by the area of that ROI.

Photobleaching of fixed cells demonstrated that reversibility of the photobleached GFP was negligible (~5%). De novo synthesis of GFP did not influence the results, since the addition of cycloheximide did not change the recovery of fluorescence in the nucleus (data not shown).

In Vitro Transcription and Translation. Plasmid DNA was linearized downstream of the encoding gene and transcribed with T3 RNA polymerase as described (*30*). The mRNA was precipitated with ethanol and dissolved in H_2O containing 10 mM DTT and 0.1 unit/μL 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 [^{35}S]-methionine and 25 μM concentrations of the other 19 amino acids. Labeled methionine was replaced by 25 μM unlabeled

methionine when non-radioactive proteins were synthesized. The amount of protein in the nonlabeled lysates was estimated as earlier described (*31*) by translating in parallel a small aliquot of the lysate in the presence of 5 μM [^{35}S]-methionine. The lysates were finally dialyzed against PBS to remove free [^{35}S]methionine and reducing agents.

Expression and Purification of Recombinant Proteins. Expression of recombinant protein from the plasmids pET-3C-FGF1, pET-3C-NLS2A$^-$, pTrc-NLS1$^-$, and pTrc-NLS1$^-$-NLS2A$^-$ in *Escherichia coli* BL21DE was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The bacterial pellet was resuspended in 20 mM Na_2HPO_4, pH 7.5, 0.5 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, and sonicated. The supernatant was applied to a heparin-Sepharose column (Amersham), and the bound material was washed with 0.7 M NaCl and then eluted with 2 M NaCl in 20 mM Na_2HPO_4, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA.

SDS−PAGE. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in 13.5% gels as described by Laemmli (*32*). After electrophoresis, the gel was fixed for 30 min in 27% 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.

Cell Binding and Translocation Assay. The experiments were performed essentially as earlier described (*8*, *33*). To measure binding, dialyzed in vitro translated proteins were added to NIH/3T3 cells and incubated for 2 h at 4 °C. The cells were washed three times in HEPES medium and lysed in lysis buffer. Proteins were adsorbed to heparin-Sepharose and analyzed by SDS−PAGE and fluorography. 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. 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 an 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. The cells were scraped to recover nuclei and centrifuged at 15800*g* for 15 min. The supernatant was designated the membrane fraction. The pellet was washed three times in lysis buffer. After sonication for 10 s on ice, the lysate was centrifuged for 5 min at 15800*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. To compare the intensity of bands, the gels were exposed to phosphorimager screens, scanned with a Storm PhosphorImager, and analyzed by ImageQuant software (Amersham Biosciences, NJ). To correct for background, adjacent areas of the gels with the same size but containing no visible bands were scanned, and the values obtained were subtracted from those obtained with the bands of interest.

Measurement of DNA Synthesis. Cells growing in 24-well tissue culture plates (5×10^4 cells per well) were preincubated for 24 h in 0.5% NCS supplemented medium at 37 °C. Then the cells were treated with increasing concentrations of wild-type or mutant FGF-1 and the incubation was continued for 24 h at 37 °C. During the last 6 h the cells were incubated with 1 μCi/mL [methyl-^{3}H]thymidine as described (*7*), and the incorporated radioactivity was measured.

In Vitro Phosphorylation Assay. NIH/3T3 cells (5×10^5 cells/sample) treated for 1 h at 37 °C with 20 nM TPA were lysed in phosphate-free lysis buffer containing phosphatase- and protease-inhibitor cocktails. A polyclonal antibody against PKCδ was added (2 μg/sample) to each fraction and left for 2 h at 4 °C. The PKCδ immunopreciptates 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 $MgCl_2$, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1 mM ATP, and phosphatase- and protease-inhibitor cocktails). Then immunopreciptates were incubated in kinase buffer for 1 h at 37 °C with either FGF-1, NLS1$^-$, NLS2A$^-$, or FGF-1(K132E) in the presence of 30 μCi [γ-^{33}P]-ATP. The samples were centrifuged, and the 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 H_2O. The adsorbed material was subjected to SDS−PAGE and fluorography.

Phosphorylation Assay in NIH/3T3 Cells. In vivo phosphorylation was performed essentially as previously described (*28*). NIH/3T3 cells growing in 6-well tissue culture plates (2×10^5 cells per well) were starved for 24 h in DMEM medium containing 1% NCS at 37 °C. The starvation was continued for 12 h in phosphate-free DMEM containing 1% NCS and 25 μCi/mL [^{33}P]phosphate. The cells were then treated for 6 h with 100 ng/mL unlabeled FGF-1 or growth factor mutants and 10 units/mL heparin. Subsequently, the cells were washed in PBS and then lysed in P-lysis buffer (10 mM Tris, 50 mM NaCl, 5mM EDTA, 1% Triton X-100, and protease- and phosphatase-inhibitor cocktails, pH 7.4). The nuclei and membrane fractions were sonicated and incubated with heparin-Sepharose. To reduce the background of phosphorylated proteins, the heparin-Sepharose with adsorbed material was treated with 2 μg/mL trypsin for 15 min on ice. To terminate the digestion, the beads were washed twice with PBS containing PMSF. The adsorbed material was analyzed by SDS−PAGE and fluorography.

MAPK Activation Assay. Cells were serum starved for 24 h in DMEM + 0.5% NCS. Then the cells were treated with 10 units/mL heparin and 2−50 ng/mL FGF-1 for the indicated period of time, lysed in SDS sample buffer, and analyzed by SDS−PAGE and Western blotting using anti-p44/p42 MAP kinase antibodies. After stripping, the membrane was reprobed with antibodies against phospho-p44/p42 MAP kinase.

RESULTS

Identification of a Second Putative NLS in FGF-1. FGF-1 contains a monopartite NLS at its N-terminal end that is involved in the transport of the growth factor to the cell nucleus. By sequence analysis we identified a second putative NLS of the bipartite type (Figure 1A). The bipartite NLSs are characterized by two clusters of lysines separated by a spacer of 10 amino acids (*34*). Multiple alignment of the sequence of FGF-1 from different species showed that the lysines implicated in the two NLSs are conserved, indicating that they are important for the function of FGF-1 (Figure 1B). Importantly, the lysines involved in the putative bipartite NLS are exposed on the surface of FGF-1 (Figure 1C).

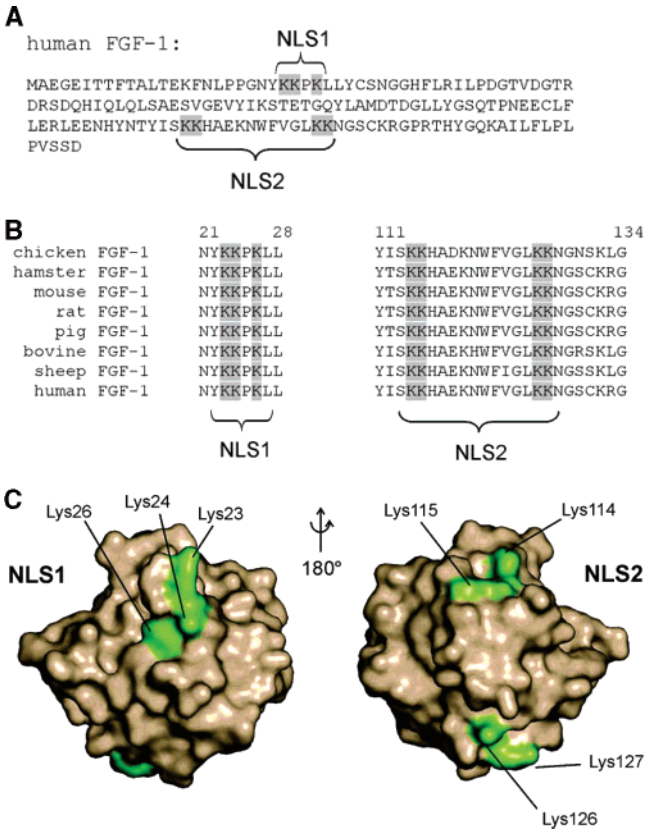

FIGURE 1: Sequence comparison of FGF-1 from different species. (A) The amino acid sequence of human FGF-1 is shown with the lysines implicated in the potential NLSs shaded in gray. Full-length FGF-1 is shown. However, we use the shorter form of FGF-1 (amino acids 21−154) that has all the characteristics of full-length FGF-1 (*7*). In addition, the amino terminal part is cleaved of during translocation resulting in only the shorter form inside the cells (*14*). (B) The sequence of different FGF-1 proteins was obtained from the Entrez search and retrieval system. A multiple alignment of the FGF-1 homologues was generated using the VectorNT software. Only the parts showing the putative NLSs are shown. The lysines implicated in the NLSs are shaded in gray. (C) The software PyMOL (*44*) was used to make a surface rendering of FGF-1 from the PDB file 1EVT (*42*). The lysines involved in the putative monopartite NLS (left) and bipartite NLS (right) were colored in green.

We fused the sequences coding for the two NLSs to GFP to test if they could act as NLSs experimentally (Figure 2). The plasmids coding for either GFP alone, GFP fused to the N-terminal NLS (GFP−NLS1), or the putative bipartite NLS fused to GFP (GFP−NLS2) were transiently transfected into HeLa cells. GFP and the two fusion proteins are small enough to diffuse freely through the nuclear pores, but by photobleaching the pool of GFP in the nucleus and following the recovery of fluorescence in the nucleus over time we were able to follow the import of GFP fusion proteins into the nucleus.

We noticed that the average intensity of the GFP fluorescence was higher in the nucleus than in the cytosol both for GFP−NLS1 and for GFP−NLS2, but the average intensity of GFP−NLS2 in the nucleus appeared to be less than for GFP−NLS1, indicating that the NLS2 signal may be weaker. With GFP alone the average intensity was the same in the cytosol and in the nucleus.

When the nucleus was photobleached and the recovery of fluorescence was recorded, we observed that an equal amount of fluorescence in the cytosol and the nucleus occurred after 120−140 s for both GFP−NLS1 and GFP−NLS2. For GFP alone, the signal reached equilibrium between the cytosol and nucleus only after ~400 s. Importantly, the reestablished levels of average fluorescence intensities in the nucleus were higher than in the cytosol in the case of GFP−NLS1 and GFP−NLS2 as they were before photobleaching. This argues against an accidental differential distribution of the GFP-fusion proteins at the moment the image was taken which could have occurred if only one picture was taken.

The results demonstrate that both NLSs are able to target GFP to the nucleus and that the two NLSs are functional when expressed in mammalian cells.

Characterization of the NLS Mutants. Even if NLS1 and NLS2 appear to act as NLSs when fused to GFP, it is essential to test if they can function in the context of the proper protein. Therefore, we made mutations in the two NLSs by changing lysines into alanines (Figure 3A). The constructs were expressed in a rabbit reticulocyte lysate to obtain radiolabeled growth factor mutants.

We first tested the binding of the mutated growth factor to heparin. Interaction of FGF-1 with cell-surface heparan sulfate proteoglycans (HSPG) or with the soluble equivalent heparin is involved in the binding to the high affinity FGF receptors. Heparin also stabilizes the growth factor, making it more resistant to degradation.

To test the interaction, we used Sepharose beads coated with heparin. Radiolabeled FGF-1 and FGF-1 mutants were added to heparin-Sepharose in the presence of increasing concentrations of NaCl. The mutants NLS1$^-$, NLS2A$^-$, and NLS1$^-$NLS2A$^-$ were bound to heparin-Sepharose equally well as wild-type FGF-1 (Figure 3B). On the other hand, the interaction of the mutants NLS2B$^-$ and NLS1$^-$NLS2B$^-$ with heparin-Sepharose was strongly reduced. Examination of the crystal structure of FGF-1 bound to heparin provides an explanation for these observations (*35*). The two lysines in NLS2B$^-$ are both involved in binding to heparin, while the other lysines mutated in NLS1$^-$ and NLS2A$^-$ do not seem to be involved. Thus, the experimental data fits with the structural data.

The introduction of mutations in a protein can change its conformation and consequently its susceptibility to proteases. We therefore tested the FGF-1 mutants for their resistance to degradation by trypsin. The mutants NLS1$^-$, NLS2A$^-$, and NLS1$^-$NLS2A$^-$ were similarly resistant as wild-type FGF-1, indicating that the introduced mutations did not influence their folding (Figure 3C). The mutants NLS2B$^-$ and NLS1$^-$NLS2B$^-$ were less resistant to trypsin and are probably not as stable as wild-type FGF-1.

Binding and Activation of FGFRs by the FGF-1 Mutants. We then tested the binding of the mutants to high affinity FGF receptors on NIH/3T3 cells. The experiments were done in the absence and presence of heparin. In the absence of heparin, FGF-1 binds to the HSPG as well as the FGFRs, while in the presence of heparin FGF-1 binds only to the FGFRs.

In the presence of heparin, all the mutants bound to the cells in similar amounts (Figure 4A). By adding an excess of unlabeled recombinant FGF-1 during the binding step, the radioactive band disappeared demonstrating that the binding could be competed out and indicating that the protein

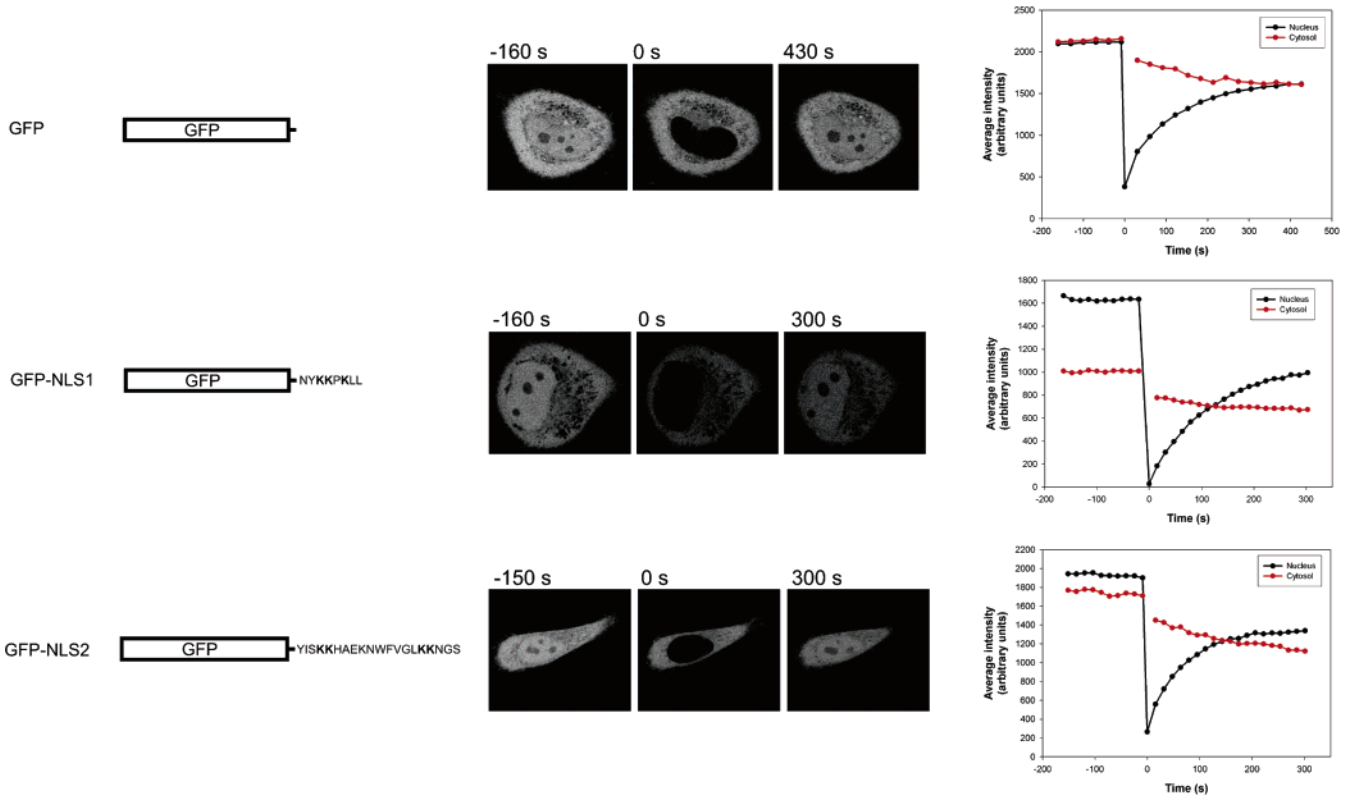

FIGURE 2: FRAP analysis of NLS1 and NLS2. HeLa cells transfected with the indicated plasmids coding for GFP or GFP−NLS fusion proteins were selectively photobleached in the nucleus, and the fluorescence recovery was measured by time-lapse confocal microscopy. The average fluorescence intensity in the nucleus and the cytosol was plotted as a function of time. The results shown are representative of 5 independent experiments.

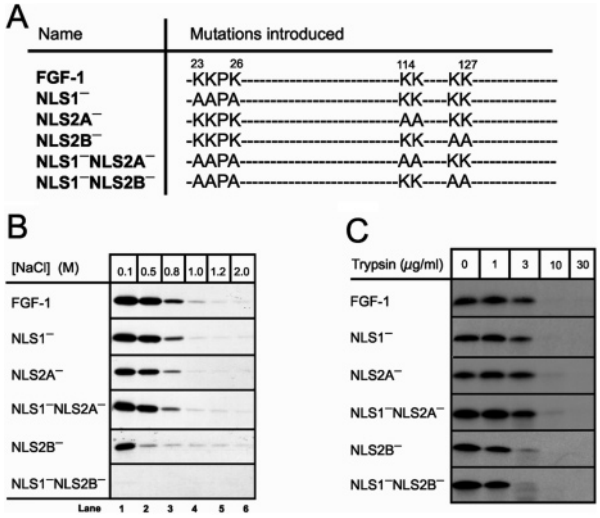

FIGURE 3: Characterization of the NLS mutants. (A) Schematic representation of the NLS mutants showing the location of the introduced mutations. (B) Heparin-Sepharose beads were incubated with radiolabeled growth factor mutants for 2 h at 4 °C. Increasing concentrations of NaCl were added to the samples. The heparin-Sepharose beads with adsorbed material were washed with PBS and analyzed by SDS−PAGE and fluorography. (C) The radiolabeled proteins were treated with increasing concentrations of trypsin for 30 min at 37 °C. The reaction was terminated by addition of 1 mM PMSF. The samples were then analyzed by SDS−PAGE and fluorography.

associated with the cells was indeed bound to specific FGFRs.

In the absence of heparin, higher amounts of the mutants NLS1⁻, NLS2A⁻, and NLS1⁻NLS2A⁻ were found associated with the cells, probably because in this case they bind to HSPG as well as to FGFRs. The mutants NLS2B⁻ and NLS1⁻NLS2B⁻ appear to bind only to the FGFRs because no more radiolabeled protein was bound in the absence of heparin. These results are in accordance with the finding that these mutants were impaired in their ability to bind heparin.

Since all the mutants bound to the receptor, we tested if they were able to activate the receptors and induce downstream signaling. We therefore chose to test if the MAPK cascade was initiated after serum-starved NIH/3T3 had been treated with FGF-1 or the FGF-1 mutants. After growth factor stimulation MAPK becomes phosphorylated and we used an antibody specific for the phosphorylated form of MAPK to probe for activation.

In Western blots using the phospho-MAPK-specific antibody there was no detectable band when the cells were serum-starved, indicating that MAPK was not phosphorylated (Figure 4B, lane 1). However when FGF-1 or FGF-1 mutants were added to cells for 10 min, a double band corresponding to phosphorylated MAPK was found in all cases (Figure 4B, lanes 2−7).

The Western blot was stripped and reprobed with an antibody against total MAPK to ensure that equal amounts of proteins had been loaded onto the gels (Figure 4B, lower panel). Thus, in short-term experiments, all the mutants stimulated the MAP kinase pathway at the same level as wild-type FGF-1.

Also in longer-term experiments (3 h), the growth factor mutants stimulated the phosphorylation of MAPK (Figure 4C). However, in this case, the mutant NLS1⁻NLS2B⁻ stimulated to a lesser degree than the others. This could be

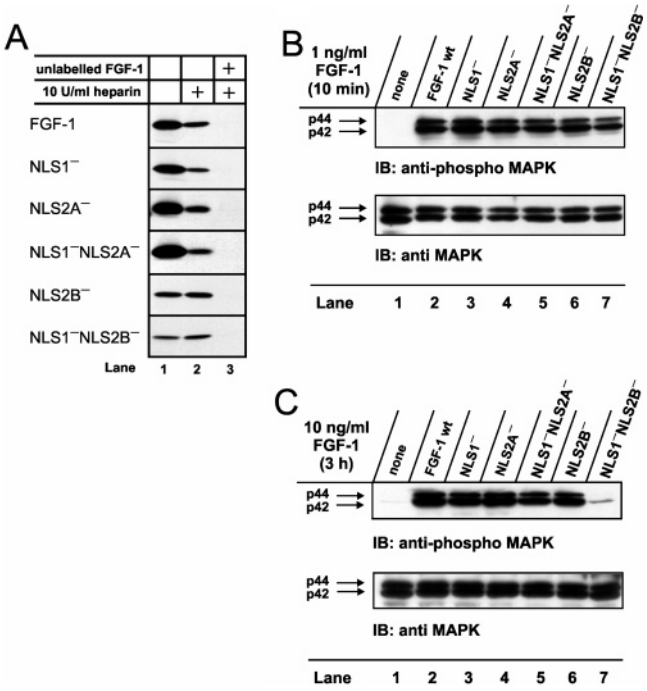

FIGURE 4: Binding of FGF-1 and NLS mutants to NIH/3T3 cells and stimulation of phosphorylation of MAP kinase. (A) NIH/3T3 cells were incubated with [^{35}S]methionine labeled wild-type FGF-1 and with mutants for 2 h at 4 °C in the absence or presence of 10 units/mL heparin. In some cases additional 5 μg/mL unlabeled FGF-1 was present. The cells were then washed in HEPES medium, lysed, and analyzed by SDS−PAGE and fluorography. (B) Serum-starved NIH/3T3 cells were treated with 1 ng/mL FGF-1 or NLS mutants in the presence of heparin and incubated for 10 min. 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). (C) Serum-starved NIH/3T3 cells were treated with 10 ng/mL FGF-1 or NLS mutants in the presence of heparin and incubated for 3 h. The cells were lysed and analyzed as in panel B.

due to the lack of binding to heparin or to the instability of this mutant.

In conclusion, the mutants NLS1$^-$, NLS2A$^-$, NLS2B$^-$, and NLS1$^-$NLS2A$^-$ seem to have the same activity as wild-type FGF-1, while NLS1$^-$NLS2B$^-$ seems to stimulate less in long-term experiments probably due to its poorer binding to heparin and its lower stability. FGF-1 with NLS2B mutations was not studied further as the impaired heparin binding was likely to influence its activity.

Translocation to the Cytosol and Further Transport to the Nucleus. We then wanted to test if the introduced mutations in the NLSs altered the transport of the growth factor mutants to the nucleus of living cells. For this purpose we used a previously described fractionation protocol (*28*). Radioactive FGF-1 and the mutant proteins were added to serum-starved NIH/3T3 cells and incubated for 6 h. The cells were then washed with low pH/high salt buffer to remove surface-bound radioactive proteins. The cells were treated with digitonin to permeabilize the cells and allow cytosolic proteins to leak out into the medium. In this way, we could detect translocation of the mutants to the cytosol. Then we lysed the remainder of the cells and fractionated the lysate into a nuclear and a membrane fraction.

First, we tested different concentrations of digitonin to find the optimal concentration to use in the permeabilization step.

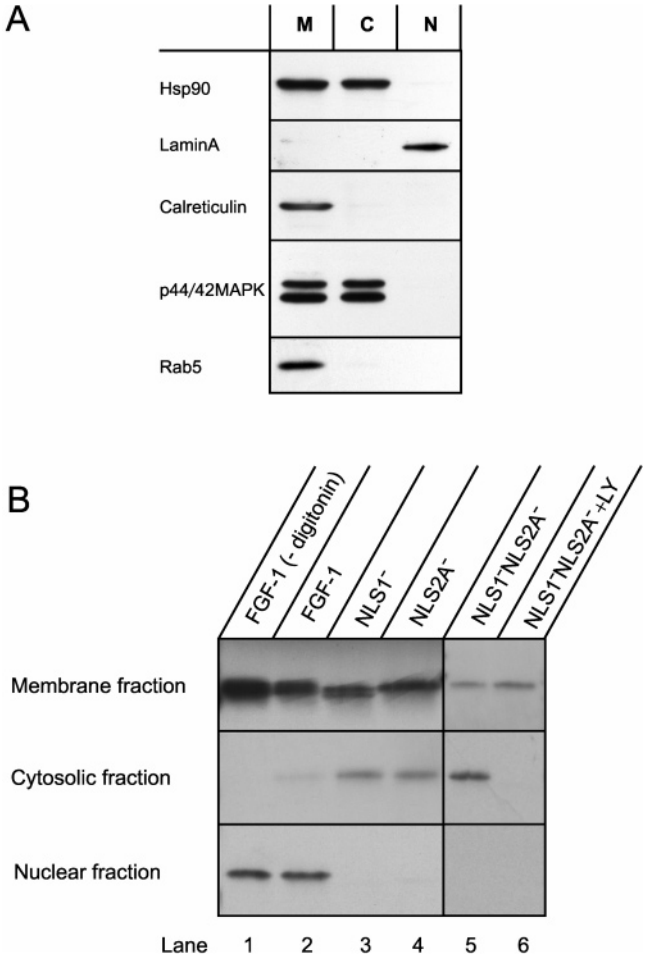

FIGURE 5: Translocation to the cytosol and transport to the nucleus of the NLS mutants. (A) NIH/3T3 cells were treated with 20 μg/mL digitonin for 5 min at 25 °C to permeabilize the cells. Then the cells were kept for 30 min at 4 °C to allow cytosolic proteins to leak out into the medium. The proteins recovered in the medium were designated the cytosolic fraction. The remainder of the cells was lysed and fractionated into a membrane and a nuclear fraction. Proteins from each fraction were analyzed by Western blotting with antibodies against HSP90, lamin A, calreticulin, p44/42 MAPK, and Rab5a. (B) Serum-starved NIH/3T3 cells were incubated with FGF-1 or growth factor mutants in the presence of 20 units/mL heparin. After 6 h incubation at 37 °C the cells were fractionated as in panel A. FGF-1 and the mutants were recovered by adsorption to heparin-Sepharose and analyzed by SDS−PAGE and fluorography. In one case, the PI-3K inhibitor LY294002 (50 μM) was added during the incubation.

An optimal concentration of digitonin releases 60−80% of cytosolic proteins. A higher concentration often affects internal membranes as well. We found that 20 μg/mL digitonin released ~80% of the cytosolic protein lactate dehydrogenase and decided to use this concentration (results not shown).

Second, we tested if the fractionation protocol resulted in separation of markers from different compartments. We fractionated the cells and analyzed marker proteins from each fraction by Western blotting (Figure 5A). The cytosolic proteins HSP90 and p44/42 MAPK were found in the cytosolic fraction and the membrane fraction as expected, since not all the cytosol is released from the cells. In addition HSP90 and p44/42 MAPK might interact with internal membranes and a fraction of the proteins will be in the membrane fraction. More importantly, the membrane mark-

ers, the ER protein calreticulin and the endosomal protein Rab5, were only found in the membrane fraction. The nuclear protein lamin A was only found in the nuclear fraction. In conclusion, it seems that the fractionation protocol results in good separation of proteins. The concentration of proteins in the cytosolic fraction will necessarily be underestimated since we used a concentration of digitonin that gave ~80% release. Furthermore, material from the nucleus could leak out into the membrane fraction during the fractionation procedure. It is therefore possible that the concentration of proteins found in the nucleus is underestimated.

Finally, we analyzed the translocation and transport of wild-type and mutant FGF-1. In all cases, radioactive growth factors were recovered from the membrane fraction. This includes material in endosomes that had not translocated to the cytosol or the nucleus (Figure 5B). Wild-type FGF-1 was in addition found in both the cytosolic and nuclear fraction (Figure 5B, lane 2). We quantified the bands in Figure 5B by phosphorimaging and ImageQuant software as described in Experimental Procedures. Approximately 20% of the total cell-associated FGF-1 was found in the nucleus, while only 4% was found in the cytosol.

When the treatment with digitonin was omitted, FGF-1 was not present in the cytosolic fraction as expected (lane 1). Interestingly, the mutants NLS1$^-$, NLS2A$^-$, and NLS1$^-$NLS2A$^-$ were found only in the cytosolic fraction (Figure 5B, lanes 3, 4, and 5), but not in the nuclear fraction. The amount in the cytosol represented approximately 10% of the total growth factor for NLS1$^-$ and NLS2A$^-$ mutants. It was somewhat higher for the double mutant. The amount of wild-type and mutant FGF-1 in the different fractions varies between experiments. Since the results for the double mutant were obtained on another day than the others, this may explain the difference in the relative amount in each fraction. However, all three NLS mutants were only found in the cytosolic fraction and never in the nucleus.

Addition of LY294002, an inhibitor of PI-3 kinase, prevented the appearance of the mutant growth factor NLS1$^-$NLS2A$^-$ in the cytosolic fraction. This is in accordance with earlier findings, which suggest that PI-3 kinase activity is necessary for FGF-1 translocation to the cytosol and the nucleus (*29*).

It appears that mutations in either of the two NLSs do not prevent translocation to the cytosol but reduce the transport of the growth factor to the nucleus. We conclude therefore that both NLSs are involved in the transport of the FGF-1 from the cytosol to the nucleus.

Phosphorylation in Vitro and in Vivo. To test further the ability of the mutant growth factors to translocate into the nucleus of cells, we took advantage of the fact that when FGF-1 is added externally to cells, it is phosphorylated by PKC after translocation into cells (*24*). Recent evidence indicates that the phosphorylation normally takes place only in the nucleus of the cells by PKCδ (*36*).

Even if the introduced mutations do not interfere directly with the PKC phosphorylation site, we wanted first to test if the mutants could be phosphorylated in vitro by PKCδ. We immunoprecipitated PKCδ from cells that had been activated by TPA, and then used wild-type and mutant FGF-1 as substrates for phosphorylation. The reaction was carried out in the presence of radioactive ATP to label the phosphorylated proteins.

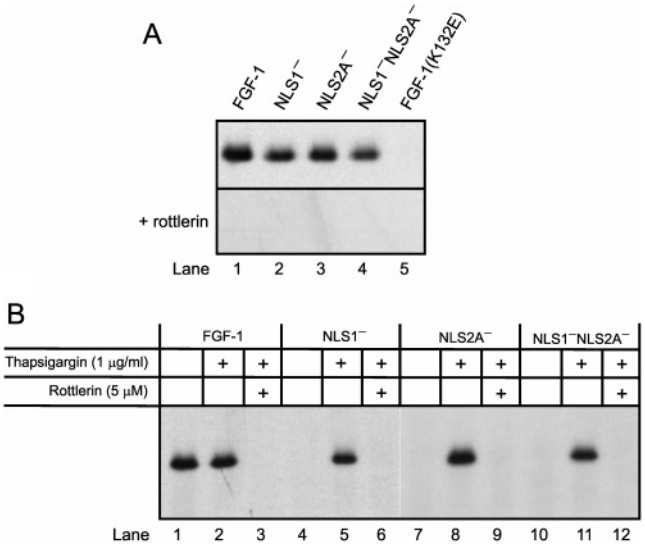

FIGURE 6: Phosphorylation of FGF-1 and NLS mutants. (A) PKCδ was immunoprecipitated from TPA activated NIH/3T3 cells and used in an in vitro assay to phosphorylate FGF-1 and the mutants. PKCδ was mixed with 1 μg of FGF-1, NLS1$^-$, NLS2A$^-$, or NLS1$^-$NLS2A$^-$ in kinase buffer and in the presence of [^{33}P]ATP. After 1 h incubation at 37 °C, sample buffer was added and the reaction mixture was analyzed by SDS−PAGE and fluorography. (B) Serum-starved NIH/3T3 cells were incubated with 100 ng/mL FGF-1, NLS1$^-$, or NLS2A$^-$ in the presence of 20 units/mL heparin and in the presence or absence of thapsigargin (1 μg/mL) or rottlerin (5 μM). After 6 h incubation at 37 °C, 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 3 times in PBS and incubated with 2 μg/mL 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.

As seen in Figure 6A, wild-type FGF-1, NLS1$^-$, NLS2A$^-$, and NLS1$^-$NLS2A$^-$ were phosphorylated by purified PKCδ, while the FGF-1(K132E) mutant that has an abrogated PKC phosphorylation site (*24*) was not. Furthermore, the PKCδ inhibitor, rottlerin, inhibited the phosphorylation (*36*). We therefore concluded that the phosphorylation sites of the mutants are functional in vitro, and we proceeded by looking at phosphorylation of the growth factor mutants in living cells.

Recombinant FGF-1 was added to serum-starved NIH/3T3 cells in the presence of radioactive phosphate and incubated for 6 h. The cells were then lysed and the growth factor was recovered by adsorption to heparin-Sepharose and then analyzed by SDS−PAGE and fluorography. In accordance with previous studies (*24*), a radiolabeled band was observed indicating that the growth factor had reached the nucleus and had been phosphorylated (Figure 6B, lane 1). We then tested if the mutants NLS1$^-$, NLS2A$^-$, or NLS1$^-$NLS2A$^-$ could also be phosphorylated after addition to NIH/3T3 cells. However, no phosphorylation of these mutants was observed (Figure 6B, lanes 4, 7, and 10), indicating that the NLS mutants were not efficiently transported to the nucleus.

It was recently found that, in NIH/3T3 cells treated with thapsigargin, activated PKCδ was not transported into the nucleus and this resulted in phosphorylation of FGF-1 in the cytosol (*36*). We therefore treated the cells with this drug to test if the mutants could be phosphorylated in the cytosol.

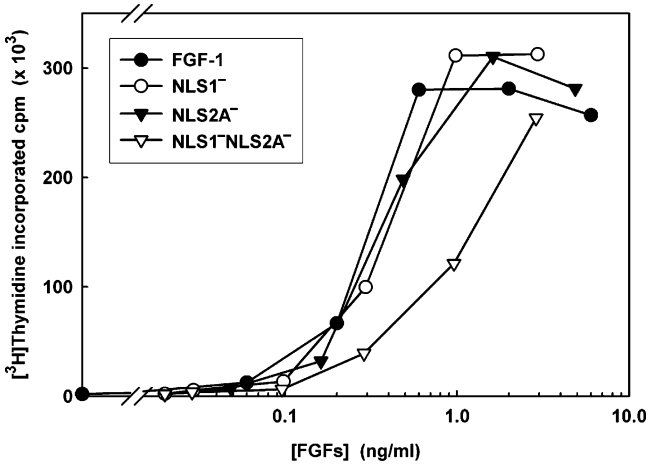

FIGURE 7: Ability of FGF-1 and the NLS mutants to stimulate DNA synthesis. NIH/3T3 cells growing in 24-well tissue culture plates were serum-starved for 24 h. The cells were treated with increasing amounts of FGF-1 or growth factor mutants and incubated for 24 h. During the last 6 h the cells were labeled with [^{3}H]thymidine and the incorporated radioactivity was measured. FGF-1, closed circles; NLS1$^-$, open circles; NLS2A$^-$, closed triangles; NLS1$^-$-NLS2A$^-$, open triangles.

Indeed, both wild-type and mutant FGF-1 were now phosphorylated in the cytosol (Figure 6B, lanes 2, 5, 8, and 11). Also in this case the phosphorylation could be inhibited by rottlerin (lanes 3, 6, 9, and 12).

Taken together, the data indicate that mutations in either of the two NLSs reduce the transport of the mutant growth factor to the nucleus without interfering with their ability to be translocated to the cytosol. This suggests that both NLSs are functional and involved in the efficient transport of the growth factor to the nucleus.

Stimulation of DNA Synthesis. It has been reported that transport of FGF-1 to the nucleus may be involved in the stimulation of DNA synthesis (*7*, *8*). We therefore tested the ability of the NLS mutants to stimulate DNA synthesis. Serum-starved NIH/3T3 cells were incubated with FGF-1 and the mutant proteins for 24 h at 37 °C. Radioactive thymidine was added during the last 6 h of the incubation period. The cell-incorporated radioactivity was counted and used as a measure of DNA synthesis. As seen in Figure 7, FGF-1 with mutations in either of the NLSs, NLS1$^-$ or NLS2A$^-$ stimulated the cells similarly to wild-type FGF-1. However, the dose−response curve for FGF-1 with mutations in both NLSs, NLS1$^-$NLS2A$^-$, was considerably right-shifted. This suggests that this mutant is less efficient in stimulating DNA synthesis than the wild-type FGF-1 and that higher concentrations of the mutant growth factor are needed to get full response.

DISCUSSION

We have here demonstrated that FGF-1 harbors two different NLSs that act together to bring externally added growth factor into the nucleus. The NLSs are not involved in the translocation to the cytosol, but are crucial for the transport from the cytosol to the nucleus. Mutations in either of the NLS had little influence on the ability of the growth factor to stimulate DNA synthesis, while mutations in both NLS decreased it considerably.

FGF-1 (16 kDa) is small enough to diffuse through the nuclear pores. However, mutations in the NLSs reduced considerably the rate of transport to the nucleus after 6 h incubation. They are therefore probably needed for the efficient transport into the nucleus and for accumulation of the growth factor in the nucleus. It has been reported that FGF-1 stimulates DNA synthesis in the nucleus (*7*, *8*). However, the stimulation of DNA synthesis was not much affected in mutants concerning either one of the NLSs, but there was a clear reduction of the amount of growth factor in the nucleus after 6 h incubation. Possibly, the 24 h incubation period used to measure stimulation of DNA synthesis allowed the mutant growth factors time to accumulate to a sufficiently high level in the nucleus. Also the double mutant stimulated DNA synthesis at higher concentrations, maybe because in this case free diffusion into the nucleus would provide enough growth factor in the nuclear compartment. However, other interpretations are possible. It can be speculated that nuclear translocation is not crucial for the stimulation of DNA synthesis on the basis of the results with the single NLS mutants. Maybe the activation of surface receptors and/or the presence of the growth factor in the cytosol are sufficient for the observed stimulation of DNA synthesis. At the present time it is not clear which role FGF-1 plays in the nucleus. The previously observed effect of nuclear FGF-1 on DNA synthesis and the effect of the double NLS mutant shown here may be indirect. Further work will be required to resolve this question.

We applied a previously used FRAP method to study the nuclear import of the NLS sequences fused to GFP (*37*, *38*). Since images from a laser scanning confocal microscope represent thin sections of similar thickness, the average intensity measured in a region of interest is directly proportional to the concentration of GFP−NLS fusion proteins. We believe therefore that the concentration of GFP−NLS fusion proteins was higher in the nucleus than in the cytosol because of the increased nuclear transport and not simply because the nucleus is just a thicker part of the cell.

Unfortunately, when FGF-1 and the mutants were fused to GFP and expressed in cells, the resulting fusion proteins were not imported into the nucleus, and we could therefore not apply the FRAP methodology to test the NLSs in the context of the whole protein (our unpublished data). Similar results were found by Zhan et al., who fused FGF-1 to β-galactosidase and studied the nucleocytoplasmic transport of the resulting fusion protein after expression in cells (*26*). The FGF-1 fusion protein was not efficiently transported to the nucleus, and it was proposed that FGF-1 contains a cytoplasmic retention signal. In agreement with what we have found in this study, they concluded that externally added FGF-1 is transported efficiently into the nucleus, while the intracellularly expressed growth factor is not. These results may reflect a mechanism provided by the cell to avoid nuclear accumulation of newly synthesized FGF-1 in the producing cell. The synthesized FGF-1 is kept in the cytosol and can then be secreted by the cell. In this way, intracrine stimulation in the FGF-1 producing cell is avoided and only receiving cells accumulate FGF-1 in the nucleus.

Even if the two NLSs are not sufficiently strong to import the whole pool of the GFP fusion proteins into the nucleus as shown in the FRAP experiments, they may act together to efficiently direct FGF-1 into the nucleus. This is supported by the results obtained with the mutated FGF-1 where

mutations in either of the NLSs reduced transport of the growth factor to the nucleus, indicating that both are required for efficient transport.

There are examples of other proteins with two NLSs, e.g. the glucocorticoid receptor (*39*) and the transcription factor NF-AT2 (*40*). In some cases, these NLSs seem to be differently regulated thereby modulating the nuclear transport of the protein. If this is the case with the two NLSs of FGF-1, is not clear at the present time, but phosphorylation of FGF-1 could potentially regulate the two NLSs differently and determine the strength of the import signal.

FGF3, a member of the FGF family, also contains two NLSs (*41*). FGF3 has in addition a signal for secretion, and it was proposed that two NLSs are necessary to compete with the opposing signal for secretion. A similar mechanism may also account for the two NLSs of FGF-1. There may be competing signals that direct FGF-1 to a different location (e.g. secretion signal or nuclear export signal). Turning on and off one or both of the NLSs by phosphorylation could then potentially change the distribution of FGF-1 inside the cell.

Examination of the crystal structure of FGF-1 in complex with heparin and the ectodomain of the FGFR-1 showed that none of the introduced mutations interfered with their binding to the receptor (*42*, *43*). When we tested the binding of the mutants to NIH/3T3 cells, which express FGFR-1 on the surface, there was no big difference in the binding consistent with the structural data. However, the two lysines mutated in NLS2B$^-$ (lys126 and lys127) are implicated in the binding to heparin (*35*). In accordance with this, these mutants eluted from heparin-Sepharose at lower NaCl concentrations than wild-type FGF-1 indicating lower affinity to heparin. Interestingly, the mutants with impaired heparin binding still bound to and activated the FGF receptors. Under our binding conditions, full affinity to heparin seems not to be required for binding to the FGF receptors.

In conclusion, FGF-1 contains two NLSs and seems to exploit the classic nuclear transport system for translocation into the nucleus. Thus, after translocation from endosomes into the cytosol, FGF-1 is probably picked up by importins that bring it into the nucleus where it may exert its effect. The nuclear transport induced by the two NLSs together is probably quite efficient since the major part of the translocated FGF-1 is found in the nucleus and only very little is found in the cytosol after 6 h incubation (Figure 5B).

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