Members of high (22-, 22.5-, 24-, and 34-kDa) and low (18-kDa) molecular mass forms of fibroblast growth factor-2 (FGF-2) regulate cell proliferation, differentiation, and migration. FGF-2s have been previously shown to accumulate in the nucleus and nucleolus. Although high molecular weight forms of FGF-2 contain at least one nuclear localization signal (NLS) in their N-terminal extension, the 18-kDa FGF-2 does not contain a standard NLS. To determine signals controlling the nuclear and subnuclear localization of the 18-kDa FGF-2, its full-length cDNA was fused to that of green fluorescent protein (GFP). The fusion protein was primarily localized to the nucleus of COS-7 and HeLa cells and accumulated in the nucleolus. The subcellular distribution was confirmed using wild type FGF-2 and FGF-2 tagged with a FLAG epitope. A 17-amino acid sequence containing two groups of basic amino acid residues separated by eight amino acid residues directed GFP and a GFP dimer into the nucleus. We systematically mutated the basic amino acid residues in this nonclassical NLS and determined the effect on nuclear and nucleolar accumulation of 18-kDa FGF-2. Lys119 and Arg129 are the key amino acid residues in both nuclear and nucleolar localization, whereas Lys128 regulates only nucleolar localization of 18-kDa FGF-2. Together, these results demonstrate that the 18-kDa FGF-2 harbors a C-terminal nonclassical bipartite NLS, a portion of which also regulates its nuclear localization.

FGF-2 belongs to a large family of small polypeptides (17–34 kDa) that mediates cell proliferation, differentiation, and migration (reviewed in Ref. 1). A single FGF-2 mRNA encodes five protein isoforms. Differential initiation of translation from upstream CUG codons yields four high molecular mass isoforms of FGF-2 (22–34 kDa). The high molecular weight FGF-2s contain an N-terminal extension in which two nuclear localization signals are located (2, 3). The 18-kDa FGF-2 isoform is translated from the AUG codon and can be secreted through a pathway independent of the endoplasm reticulum and Golgi apparatus (reviewed in Ref. 4). The 18-kDa FGF-2 binds and activates cell surface FGF receptors. Activation of FGF receptors triggers downstream signal transduction and regulates gene expression (reviewed in Ref. 5). In addition to this signaling pathway, the presence of 18-kDa FGF-2, high molecular weight FGF-2s, and FGF-2 receptors in the nucleus suggests that FGF-2 participates in an intracrine signaling pathway (reviewed in Ref. 6).

Like other polypeptides, such as insulin and interleukin-1, 18-kDa FGF-2 can translocate into the nucleus after internalization (7). 18-kDa FGF-2, when added to adult bovine aortic endothelial cells, enters the nucleus and up-regulates the synthesis of ribosomal RNA (7, 8). Lysosomal inhibitors and microtubule-disrupting agents did not prevent accumulation of 18-kDa FGF-2 in the nucleus of human umbilical vein endothelial cells, indicating that nuclear translocation of 18-kDa FGF-2 is independent of lysosomes and microtubules (9). Recently, Hsia et al. (10) found that nuclear translocation of 18-kDa FGF-2 was dramatically inhibited by heparinase treatment or in heparin-deficient Chinese hamster ovary cells. These results suggest that heparan sulfate proteoglycans on the cell surface are involved in the nuclear translocation of exogenous 18-kDa FGF-2.

Endogenous 18-kDa FGF-2 also accumulates in the nucleus (11–17). In transfected baby hamster kidney (BHK-21) cells, 18-kDa FGF-2 concentrated in the nucleus, whereas in untransfected BHK-21 cells endogenous FGF-2 was detected in both cytoplasm and nucleus (11). Suramine, a compound that specifically inhibits the binding of FGF-2 to its receptors, blocked the endocytosis of FGF-2 in GM13732 bovine endothelial cells. However, the amount of 18-kDa FGF-2 in the nucleus of these cells did not change, suggesting that 18-kDa FGF-2 may translocate into the nucleus directly from the cytoplasm (12). Human and rat 18-kDa FGF-2 overexpressed in COS cells was present in both nuclei and cytoplasm (13, 14). 18-kDa FGF-2 tagged with fluorescence proteins (GFP or dsRed) localized in the nuclei of rabbit corneal endothelial cells (CEC) and rat Schwann cells (15, 16). Recently, Foletti et al. (17) reported that mouse and human 18-kDa FGF-2 accumulated in the nucleus when overexpressed in mouse fibroblast NIH 3T3 and human embryonic kidney 293T cells. However, other reports suggested that 18-kDa FGF-2 was predominantly cytoplasmic (18–20). Presta et al. (21) evaluated the nuclear localization capabilities of the sequence (27KDPRKR27) in the N terminus of 18-kDa FGF-2 based on its similarity to the NLS of FGF-1 (27YKKPR27) (22). However, altering basic amino acid residues to glutamine in the sequence did not prevent nuclear translocation of 18-kDa FGF-2 in COS-1 cells, suggesting that the NLS is located in another region (21). Claus et al. (16) found that mutating two arginine residues to glycine residues (R116G/R118G) in the C terminus of FGF-2 abolished nuclear
localization of both 18- and 23-kDa FGF-2 in rat Schwann cells. A serial deletion of the C terminus of mouse 18-kDa FGF-2 revealed that the nuclear localization of 18-kDa FGF-2 is regulated by C-terminal sequences, but the specific amino acid residues responsible for the localization were not identified (17). Those data suggest that the C terminus of FGF-2 contains an NLS.

The nucleus is the center of ribosomal biogenesis (reviewed in Ref. 23). Growth factors or growth-regulatory proteins such as FGF-1, FGF-2, FGF-3, angiogenin, and parathyroid hormone-related peptide have been detected in the nucleus (reviewed in Ref. 24). Exogenously added 18-kDa FGF-2 translocated into the nucleus and accumulated in the nucleus (7, 8). Similarly, endogenous 18-kDa FGF-2 overexpressed in the CEC and Schwann cells also localized to the nucleus (15, 16).

Studies of nuclear proteins revealed that signals are required for nuclear localization, but no consensus sequence has been determined (25–28). Several nuclear localization sequences, resembling NLSs (clusters of basic amino acid residues) have been identified in a nucleoacapsid protein of porcine reproductive and respiratory syndrome virus and ribosomal proteins S22, S25, and L22 (25–27). An interesting feature of these nuclear localization sequences is that they overlap with NLSs in these proteins (25–27). However, a nuclear localization sequence found in the N terminus of US11, a herpes simplex virus type 1 protein, lacks clusters of basic amino acid residues (28). Signals that are essential for nuclear localization of 18-kDa FGF-2 have not been reported.

Here we demonstrate that 18-kDa FGF-2 accumulates in the nucleus and nucleolus of COS-7 and HeLa cells. Examination of the sequence of FGF-2 revealed a nonclassical bipartite NLS in the 3′-end of the cDNA in pNLS-EGFP-N1. The resulting plasmid (pFGF-2-EGFP-N1) encodes 18-kDa FGF-2. To make mutants R116G/R118G, R116G, R116G/R118G, or R118G/K119G into the mutant K128G/R129G using the same primers for K128G/R129G, mutations were introduced into the forward and reverse primers for R118G to generate R116G/R118G, R116G/R118G/K119G, and R118G/K119G.

Site-directed Mutagenesis—To obtain a plasmid encoding wild type 18-kDa FGF-2, two stop codons were inserted by using HindIII and BsrGI to remove the cDNA of GFP K119G, and pN1-FGF-2-R116G/R118G/K119G/K128G/R129G) were untagged versions of FGF-2 (pN1-FGF-2) and FGF-2 mutants (pN1-FGF-2-R116G/K119G and pN1-FGF-2-R116G/R118G/R119G/R129G) were constructed by using HindIII and BsrGI to remove the cDNA of FGF-2 from the corresponding pEGFP-N1 plasmids described above and below (see “Site-directed Mutagenesis”). The overhangs were filled in with Klenow, and the resulting blunt ends were ligated.

A 17-amino acid sequence containing the NLS of the 18-kDa form of FGF-2 was fused to GFP by synthesizing two complementary DNA strands corresponding to the sequence 5′-TCCGAGGATCTGACTGCAG-3′ and 5′-GATCAGGATCTGACTGCAG-3′ containing a 5′-overhang of an XhoI site and a 3′-overhang of a BamHI site. After annealing, the double-stranded DNA was inserted into the corresponding sites at the 5′-end of GFP cDNA in plasmid pEGFP-N1. The two complementary DNA strands were 5′-TCCGAGGATCTGACTGCAG-3′ and 5′-GATCAGGATCTGACTGCAG-3′. The resulting amplified GFP cDNA was then digested with BamHI and inserted into the corresponding site at the 3′-end of GFP cDNA in pNLS-EGFP-N1. The plasmid pEGFP-N1 encoding a GFP dimer was constructed by first digesting pNLS-DEGF-P-N1 with XhoI and BamHI to remove the NLS, blunting the product with Klenow polymerase, and finally ligating the ends together.

**EXPERIMENTAL PROCEDURES**

Plasmid Construction.—To construct pEGFP-N1-FGF-2 encoding the fusion protein GFP-FGF-2, the full-length cDNA of human 18-kDa FGF-2 (gift of R. Florkiewicz) was amplified in a PCR using forward (5′-ATATGCTTTCCAGATCTCAGCGAGCATCACCC-3′) and reverse (5′-AGATGGCGGCGCTTAAATACGCTTTAGCAGA-3′) primers containing BamHI and NotI sites, respectively. The amplified DNA was digested with BamHI and NotI and then ligated to the corresponding sites at the 3′-end of GFP cDNA in pEGFP-N1 (Clontech). The full-length cDNA of FGF-2 was also placed at the 5′-end of GFP cDNA by first amplifying it using forward (5′-TTTATACTGCCCAGTTCCTCCCAGTGCCACATACCA-3′) and reverse (5′-TTTCTGAGCTTTAGTACCCAGAACATCG-3′) primers containing BamHI and NotI sites, respectively. The amplified DNA was digested with BamHI and NotI and then ligated to the corresponding sites at the 3′-end of GFP cDNA in pEGFP-N1 (Clontech). The two complementary DNA strands were 5′-TCCGAGGATCTGACTGCAG-3′ and 5′-GATCAGGATCTGACTGCAG-3′ containing a 5′-overhang of an XhoI site and a 3′-overhang of a BamHI site. After annealing, the double-stranded DNA was inserted into the corresponding sites at the 5′-end of GFP cDNA in plasmid pEGFP-N1.
For transient transfection, COS-7 or HeLa cells were trypanosized and counted. Cells (8 × 10^4) were plated onto a glass coverslip in a 24-well plate. After overnight incubation, 1 μg of plasmid DNA was transfected into cells using 1 μl of LipofectAMINE™ 2000 (Invitrogen) according to the manufacturer’s instructions.

**Immunoﬂuorescence Microscopy**—Immunoﬂuorescence microscopy was performed as described previously with minor modiﬁcations (29). In brief, transiently transfected cells on glass coverslips were ﬁxed in Buffer A (4% paraformaldehyde in full phosphate-buffered saline containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KHPO_4, 0.5 mM MgCl_2, and 1 mM CaCl_2) for 30 min at room temperature. Then cells were permeabilized in Buffer B (0.2% Triton X-100 in full phosphate-buffered saline) for 10 min at room temperature. Permeabilized cells were blocked in Buffer C (1% goat serum and 3% bovine serum albumin in full phosphate-buffered saline) for at least 1 h at room temperature. Mouse monoclonal FGF-2 antibody (1:20–100 diluted in Buffer C) (Oncogene) or mouse monoclonal nucleolin antibody (1:10 diluted in Buffer C) (gift from Dr. Piñol-Roma, New York) was incubated with cells for 16–24 h at 4 °C. Afterward, cells were incubated with Texas Red-conjugated goat anti-mouse IgG (1:100 diluted in Buffer C) (Jackson ImmunoResearch Laboratories) for 2 h at room temperature. For some experiments, FGF-2 and its fusion protein FGF-2-FLAG were visualized using ﬂuorescein isothiocyanate-conjugated goat anti-mouse IgG (1:50 diluted in Buffer C) (Jackson ImmunoResearch Laboratories), and the nuclei were stained with propidium iodide (4 μg/ml in Buffer C) (Sigma). Samples were mounted in SlowFade™ antifade solution (Molecular Probes, Inc., Eugene, OR) and observed using a Radiance 2000 confocal microscope (Bio-Rad). Confocal images (1024 × 768 pixels) were obtained using a 40× objective lens.

**Nuclear Localization Analysis**—NIH Image J version 1.30 software was used to quantify the nuclear localization of GFP fusion proteins. All confocal images were quantiﬁed using settings where the intensity of GFP fluorescence was linear and ranged from 10 to 160 pixel values (30). Usually, 8–10 sections encompassing an entire cell were taken at 2.0-μm intervals. To examine the nuclear localization of GFP and GFP fusion proteins, a square with an area of 225 pixels was used to measure the mean intensities of three different regions in the nucleus and the cytoplasm of a representative cell from each of three transfections. Thus, three representative cells yielded a total of nine areas from the nucleus and nine areas from the cytoplasm. Relative nuclear localiza- tion is reported as the ratio of the mean intensity of GFP fluorescence in the nucleus and cytoplasm (nucleocytoplasmic ratio).

**Protein Extraction and Western Blotting**—Transiently transfected cells were collected by scraping and then centrifuged for 5 min in a microcentrifuge. SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, 10% (v/v) glycerol) was added directly to the cell pellets. Cell lysates were clarified using sonication (10 s) and then centrifuged for 2 min in a microcentrifuge. Because heating converts pellets. Cell lysates were clarified using sonication (10 s) and then centrifuged for 2 min in a microcentrifuge. Because heating converts

**RESULTS**

18-kDa FGF-2 Accumulated in the Nucleus—Although the nuclear translocation of exogenous and endogenous 18-kDa FGF-2 has been reported (7–16), the targeting signals have not been deciphered completely. In order to investigate signals controlling the subcellular localization of 18-kDa FGF-2, we fused its cDNA to the 3‘-end of GFP cDNA. Plasmids encoding either GFP or FGF-GFP-2 were transfected into COS-7 cells, and expression of the proteins was veriﬁed using Western blotting. A 45-kDa protein was recognized by a GFP and an FGF-2 antibody, indicating that it was a fusion of GFP (27 kDa) and FGF-2 (18 kDa) (Fig. 1A, lanes 2 and 4). GFP, but not FGF-2, was detected in cells transfected with the control plasmid (Fig. 1A, lanes 1 and 3). Endogenous FGF-2 was not detected, because it is expressed at very low levels in COS-7 cells (34).

The subcellular localization of GFP and the FGF-2 fusion protein in COS-7 and HeLa cells was examined using ﬂuorescence microscopy. GFP was distributed throughout the cell (Fig. 1B, a), whereas GFP-FGF-2 was predominantly localized to the nucleus (Fig. 1B, d). The position of the nucleus was determined using propidium iodide (Fig. 1B, b and c). Merged images conﬁrmed that GFP-FGF-2 accumulated in the nucleus (Fig. 1B, e and f). Quantifying confocal images revealed that the nuclear/cytoplasmic ratio of GFP and GFP-FGF-2 in COS-7 cells was 2.4 and 10, respectively (Fig. 1C, gray bars). The same subcellular distribution of GFP and GFP-FGF-2 was observed in HeLa cells (Fig. 1C, white bars). We also placed FGF-2 cDNA at the 5‘-end of GFP cDNA to produce an FGF-2-GFP fusion protein (Supplemental Data, Fig. S4, lane 1). The fluorescence

**Nuclear Localization**

Although the nuclear translocation of exogenous and endogenous 18-kDa FGF-2 has been reported (7–16), the targeting signals have not been deciphered completely. In order to investigate signals controlling the subcellular localization of 18-kDa FGF-2, we fused its cDNA to the 3‘-end of GFP cDNA. Plasmids encoding either GFP or FGF-GFP-2 were transfected into COS-7 cells, and expression of the proteins was verified using Western blotting. A 45-kDa protein was recognized by a GFP and an FGF-2 antibody, indicating that it was a fusion of GFP (27 kDa) and FGF-2 (18 kDa) (Fig. 1A, lanes 2 and 4). GFP, but not FGF-2, was detected in cells transfected with the control plasmid (Fig. 1A, lanes 1 and 3). Endogenous FGF-2 was not detected, because it is expressed at very low levels in COS-7 cells (34).

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Nuclear and Nucleolar Localization of 18-kDa FGF-2

Identification of a Nuclear Localization Signal in 18-kDa FGF-2—The above results led us to hypothesize that 18-kDa FGF-2 contains an NLS. A putative NLS in the N terminus of 18-kDa FGF-2 had been proposed (22). However, mutations in this sequence did not prevent the nuclear accumulation of 18-kDa FGF-2 (22). To determine whether the N terminus (amino acids 1–120) of FGF-2 harbors a different putative NLS, we fused it to GFP. The resulting fusion protein did not accumulate in the nucleus, indicating that the N terminus lacks an NLS (Supplemental Data, Fig. S1, c–f).

The function of a putative NLS can be tested by fusing it to a cytoplasmic reporter protein or by mutating it. If the sequence is an NLS, it will drive the cytoplasmic reporter protein into the nucleus. In the latter case, a protein containing a defective NLS will fail to enter the nucleus. We synthesized a DNA fragment corresponding to the sequence (114TYRSRKYTSWYVALKR129) containing the putative NLS and then ligated it to the 5′ terminus of GFP cDNA, yielding NLS-GFP. This sequence directed GFP into the nucleus (Fig. 3A, compare a and c). The nuclear/cytoplasmic ratio of NLS-GFP in COS-7 and HeLa cells was about 4.4 and 3.4, respectively, whereas that of GFP was about 2.4 and 1.8, respectively (Fig. 3B). We also made a larger reporter protein, NLS-dGFP (60 kDa), a fusion protein containing the NLS fused to a GFP dimer. The 17-mer containing the NLS drove dGFP into the nucleus, whereas dGFP control distributed throughout the cell (compare Fig. 3A, compare a and d). Quantifying confocal images revealed that the nuclear/cytoplasmic ratio of NLS-dGFP in COS-7 and HeLa cells was about 2.8 and 3, respectively, whereas that of dGFP was about 1.4 (Fig. 3B). The expression of NLS-GFP, dGFP, and NLS-dGFP was confirmed by Western blotting (Supplemental Data, Fig. S4).

Together, these results indicate that the NLS is located in the C terminus of 18-kDa FGF-2 and that the NLS is a crucial determinant of nuclear localization.

Role of Basic Amino Acid Residues in Nuclear Localization of 18-kDa FGF-2—To determine the key elements of the NLS, we altered its basic amino acid residues using site-directed mutagenesis. Five basic amino acid residues form two clusters (Table I). We systematically altered each basic amino acid residue alone or in combination with others in the first cluster.
The NLS of FGF-2 drives GFP and GFP dimer into the nucleus. A, confocal images of COS-7 cells transfected with pEGFP-N1 (GFP; a), pEGFP-N1(dGFP; b), pNLS-EGFP-N1 (NLS-GFP; c), and pNLS-dEGFP-N1 (NLS-dGFP; d). B, the nuclear/cyttoplasmic (N/C) ratio was obtained from confocal images of GFP, NLS-GFP, dGFP, and NLS-dGFP expressed in COS-7 (gray bars) and HeLa (white bars) cells as described under "Experimental Procedures." The error bars represent S.D. of three determinations.

**Table I**

<table>
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<th>Wild type and mutant NLSs</th>
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<tr>
<td>NLS</td>
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(116RSRK118) and determined the effect on nuclear localization of 18-kDa FGF-2. The subcellular distribution of mutants R116G and R118G were similar to that of GFP-FGF-2 (compare Fig. 1B, d, and Fig. 4A, a and b). Quantifying confocal images revealed that the nuclear/cyttoplasmic ratio of R116G in COS-7 and HeLa cells was about 9 and 10, respectively, and that of R118G was about 13 in both COS-7 and HeLa cells (Fig. 5). In contrast, changing Lys119 into glycine moderately decreased nuclear localization of GFP-FGF-2 (compare Fig. 1B, d, and Fig. 4A, c). The nuclear/cyttoplasmic ratio of K119G in COS-7 and HeLa cells was about 6 and 5, respectively (Fig. 5). The expression of all mutants was confirmed by Western blotting (Supplemental Data, Fig. S5). Our results indicate that Lys119 plays a more important role than Arg116 and Arg118 in nuclear localization of 18-kDa FGF-2.

Changing both Arg116 and Arg118 into glycine residues moderately inhibited GFP-FGF-2 nuclear localization (Fig. 4A, d). The nuclear/cyttoplasmic ratio of R116G/R118G in COS-7 and HeLa cells was about 4 and 5, respectively (Fig. 5). Our results do not agree with those of Claus et al. (16), who reported that R116G and R118G mutations abolished nuclear localization of 18- and 23-kDa FGF-2s in Schwann cells. Changing both Arg116 and Lys119 into glycine (R116G/K119G) or mutating both Arg118 and Lys119 in this cluster (R118G/K119G) markedly reduced nuclear localization (Fig. 4A, e and f). The nuclear/cyttoplasmic ratio of R116G/K119G in COS-7 and HeLa cells was about 1.7 and 1.6, respectively, whereas that of R118G/K119G in HeLa and COS-7 cells was about 1.6 and 1.5, respectively (Fig. 5). Mutating all three basic amino acids in the first cluster (R116G/R118G/K119G) inhibited the nuclear accumulation of GFP-FGF-2 (Fig. 4A, g). Similarly, nuclear accumulation of untagged FGF-2-R116G/R118G/K119G was greatly reduced (Fig. 6, compare 6 and 7). The nuclear/cyttoplasmic ratio of this GFP-FGF-2 mutant in COS-7 and HeLa cells was about 1.4 and 1.3, respectively (Fig. 5), and that of the untagged FGF-2 mutant in COS-7 cells was 1.8 ± 0.6. Expression of this
and other untagged FGF-2 mutants was confirmed by Western blotting (Supplemental Data, Fig. S6B). These results confirmed that Lys^{129} is the most critical residue of the first cluster in the regulation of nuclear localization of 18-kDa FGF-2.

To understand the role of the second cluster in nuclear localization of 18-kDa FGF-2, we mutated each basic amino acid residue alone or together. The K128G mutant accumulated in the nucleus, whereas the nuclear localization of R129G and R116G/K128G mutants was moderately reduced (Fig. 4C). The nuclear/cytoplasmic ratio of K128G/R129G in COS-7 and HeLa cells was about 0.5 (Fig. 5). These results confirm that Lys^{129} does not play a major role in the nuclear localization of FGF-2. However, combining the R116G/R118G and R129G mutations dramatically reduced nuclear localization (Fig. 4C). The nuclear/cytoplasmic ratio in COS-7 and HeLa cells was about 1.2 (Fig. 5). These results confirm that Arg^{129} plays an important role in nuclear localization and indicate that both clusters work together in regulating nuclear localization of 18-kDa FGF-2. Compared with R116G, R118G, K119G, and K128G/R129G (Fig. 4, A–C) and B (C)), mutants R116G/K128G/R129G, R118G/K128G/R129G, and K119G/K128G/R129G remained in the cytoplasm (Fig. 5). Combining K128G/R129G mutations with any two basic amino acid residues in the first cluster (R116G/R118G/K128G/R129G, R116G/K119G/K128G/R129G, R118G/K128G/R129G, and K119G/K128G/R129G) blocked nuclear localization (data not shown). The nuclear/cytoplasmic ratio of these mutants in HeLa and COS-7 cells was less than or equal to 1 (Fig. 5). Changing all five basic amino acid residues into glycine residues resulted in the exclusion of the GFP-FGF-2 (Fig. 4C, c) or FGF-2 (Fig. 6f) mutants from the nucleus. The nuclear/cytoplasmic ratio of this FGF-2 mutant in HeLa and COS-7 cells was about 0.8 (Fig. 5), and that of the FGF-2 mutant in COS-7 cells was about 0.8 ± 0.1. These results confirm that both clusters are required for efficient nuclear localization of 18-kDa FGF-2. However, the first cluster is critical for nuclear localization. Our data also indicate that Lys^{119} and Arg^{129} are the key amino acid residues of the first and second clusters, respectively, in the nuclear localization of 18-kDa FGF-2.

**Nucleolar Localization of 18-kDa FGF-2**—Exogenously added 18-kDa FGF-2 has been shown to translocate into the nucleus and accumulate in the nucleolus (7, 8). The 18-kDa form of FGF-2 fused with fluorescent proteins also accumulated in the nucleolus of CEC and Schwann cells (16, 17). However, the motif that controls the nucleolar localization has not been determined. To visualize the nucleolus, we used an antibody that recognizes nucleolin (37, 38), one of the most abundant nucleolar proteins (S22, S25, and L22) overlap with their nuclear localization signals (25–27). Thus, we hypothesized that the sequence controlling the nucleolar localization of 18-kDa FGF-2 may also overlap with its NLS. To test this hypothesis, we examined the nucleolar localization of NLS-GFP and NLS-dGFP. Although NLS drove GFP into the nucleus, NLS-GFP and NLS-dGFP did not accumulate in the nucleolus (Fig. 3A, c and d). The position of the nucleolus was determined using a nucleolin antibody (data not shown).

The failure of the NLS to drive GFP into the nucleolus led us to hypothesize that the nucleolar targeting sequence may function only in the context of full-length FGF-2. A common feature...
of nuclear targeting sequences is the presence of basic amino acid residues (24–26). To investigate the role of basic amino acid residues of the NLS in nuclear localization, we reexamined our set of GFP-FGF-2 mutants. Changing Arg\textsuperscript{116} or Arg\textsuperscript{118} into glycine did not affect nuclear localization of FGF-2 (Fig. 7, compare A (d) and B (a and d)). The merged images confirmed their nuclear localization (Fig. 7B, c and f). However, changing both Arg\textsuperscript{116} and Arg\textsuperscript{118} into glycine residues essentially blocked their nuclear localization (Fig. 7B, p–r) but only moderately inhibited the nuclear localization of FGF-2 (Figs. 4A (d) and 5). These results indicate that Arg\textsuperscript{116} and Arg\textsuperscript{118} together, but neither alone, play an important role in nuclear localization of FGF-2. The K119G and R129G mutants did not localize to the nucleolus in COS-7 (Fig. 7B, g–i) and HeLa cells (data not shown), demonstrating that these amino acid residues are indispensable for nuclear localization. Furthermore, among the GFP-FGF-2 mutants studied (Table I), those containing K119G, R129G, or both mutations were blocked from the nucleolus (data not shown). The K128G mutant produced a unique pattern. This mutation did not change the nuclear localization of GFP-FGF-2 (Figs. 4B (a) and 5) but blocked nuclear targeting (Fig. 7B, m–o). Similarly, the untagged FGF-2-K128G mutant localized to the nucleus efficiently but did not accumulate in the nucleolus (Fig. 7C, q–i). Furthermore, all GFP-FGF-2 mutants containing a K128G alteration (Table I) failed to accumulate in the nucleolus (data not shown). These results indicate that Lys\textsuperscript{126} controls nuclear, but not nucleolar, localization of FGF-2.

**DISCUSSION**

We demonstrated above that the C terminus of 18-kDa FGF-2 harbors an unconventional bipartite NLS (116\textsuperscript{RSRRK-SWTVALKR}\textsuperscript{126}) that controls its nuclear and nucleolar localization. The NLS contains two small clusters of basic amino acid residues separated by a hydrophobic region containing eight amino acid residues. The first cluster consists of 116\textsuperscript{RSRK}, and the second cluster consists of 126\textsuperscript{KR}. Adding a 17-amino acid sequence containing this NLS to GFP drove the resulting fusion protein into the nucleus. Mutating basic amino acid residues in this sequence differentially modulated the nuclear localization of 18-kDa FGF-2. We demonstrated that basic amino acid residues Lys\textsuperscript{116} and Arg\textsuperscript{118} in this NLS are critical in regulating both nuclear and nucleolar localization of 18-kDa FGF-2, whereas Lys\textsuperscript{126} modulates only nuclear targeting. These findings reveal the key determinants of nuclear and nucleolar localization of 18-kDa FGF-2.

The subcellular distribution of FGF-2 has been controversial (7–20). Our results showing that 18-kDa FGF-2 is predominantly localized to the nucleus are consistent with most but not all (7–16) studies of subcellular distribution of 18-kDa FGF-2. Some reports show that 18-kDa FGF-2 is predominantly cytosolic (17–20). The reason for the discrepancy is not clear.

We showed above that NLS-directed fusion proteins into the nucleus but not as efficiently as full-length FGF-2. In the context of the full-length FGF-2, the NLS may adopt a conformation shown in a, d, g, j, m, and p. The position of the nucleoli (nucleolin) is shown by red fluorescence in b, e, h, k, n, and q. The extent of nuclear localization of GFP-FGF-2 mutants is indicated by yellow in the merged images c, f, i, o, and r. C, perturbation of nuclear localization of untagged FGF-2 and FGF-2-K128G. Confocal microscope images of COS-7 cells were taken 20 h after transfection with pEGFP-C1-nucleolin and either pN1 (a–c), pN1-FGF-2 (d–f), or pN1-FGF-2-K128G (g–i). FGF-2 and its mutant (red fluorescence) were detected using an FGF-2 antibody and a Texas Red-conjugated secondary antibody (b, e, and h). The nucleoli was located using autofluorescence of GFP-nucleolin (a, d, and g). The merged images (c, f, and i) reveal co-localization of GFP-nucleolin and FGF-2 but not FGF-2-K128G.
mation recognized by the nuclear translocation machinery. Most nuclear-cytoplasmic transport of proteins requires an NLS in the cargo and the nuclear transport receptors (karyoph-erins) that recognize NLSs (reviewed in Refs. 39 and 40). Recently, Fontes et al. (41) proposed a consensus sequence of a conventional bipartite NLS (KRX_{10–12}KRXX) for proteins tar-geted to the nucleus. Basic amino acid residues in this sequence are responsible for binding to the importin α receptor. Examination of the three-dimensional model of wild type FGF-2 (Protein Data Bank code 1BLA) (42) indicates that the two basic clusters of NLS may not be as accessible or in the same con-formation as they are in the full-length FGF-2 fused to GFP (GFP-FGF-2), thereby resulting in less efficient targeting.

The motif harboring the NLS of 18-kDa FGF-2 is multifunc-tional. It overlaps with binding sites for its receptors and for casein kinase II (43–45). A model for the nuclear activity of FGF-2 has been proposed (44–47). Binding of FGF-2 to casein kinase II triggers nuclear translocation of casein kinase II and casein kinase II-dependent phosphorylation of nucleolin. Nucleolin, but not phosphorylated nucleolin, inhibits rDNA transcription. However, our findings raise the possibility that the NLS reported here affects rDNA transcription by regulating the interaction between FGF-2 and its nuclear and nucleo-lar targets. The role of these residues in nuclear and nucleolar activities of 18-kDa FGF-2 is under investigation.

Claus et al. (16) reported that mutating two arginine resid-ues to glycine residues in the C terminus of FGF-2 (Arg^{148} and Arg^{151}) in 23-kDa FGF-2, Arg^{116} and Arg^{118} in 18-kDa FGF-2) abolished nuclear localization of both 18- and 23-kDa FGF-2s in rat Schwann cells. However, our results demonstrate that the NLS extends from Arg^{116} to Arg^{129} and is bipartite. It is possible that the mechanism of nuclear translocation of 18-kDa FGF-2 in various cell types is different.

Several growth factors have been detected in the nucleolus, but the functional relevance of this translocation remains unclear (28). Exogenously added FGF-2 was found to accumulate in the nucleolus and stimulate the synthesis of ribosomal RNA (7, 8). We also found that FGF-GFP-2 and FGF-2 expressed in COS-7 and HeLa cells accumulated in the nucleolus. This finding is consistent with that of Claus et al. (16) using rat Schwann cells. Nuclear and nucleolar localization of FGF-3, another FGF family member with less than 50% homology in amino acid sequence to FGF-2, is governed by different motifs within the protein (48). However, we found that the accumulation of 18-kDa FGF-2 in the nucleus and nucleolus is con-trolled by one motif. Furthermore, our analysis of basic amino acid residues in the NLS revealed that Lys^{119} and Arg^{129} are key elements for nuclear and nucleolar localization, whereas Lys^{128} modulates only nucleolar localization.

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