

## Role of the Nuclear Localization Sequence in Fibroblast Growth Factor-1-stimulated Mitogenic Pathways\*

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**Fibroblast growth factor-1 (FGF-1) is a potent mitogen for mesoderm- and neuroectoderm-derived cell types *in vitro*. However, a mutant FGF-1 with deletion in its nuclear localization sequence (NLS, residues 21–27) is not mitogenic *in vitro*. We demonstrated that synthetic peptides containing this NLS were able to stimulate DNA synthesis in a FGF receptor-independent manner after they were delivered into living NIH 3T3 cells by a cell-permeable peptide import technique. The stimulation of maximal DNA synthesis by these peptides required the presence of peptides during the entire G<sub>1</sub> phase of the cell cycle. The mitogenic effect was specific for the NLS of FGF-1 because a peptide with double point mutations at lysine residues was inactive in stimulating DNA synthesis. Our results suggest that the NLS plays an important role in the mitogenic pathway initiated by exogenous FGF-1 by its direct involvement in the nuclear transport and signaling of internalized FGF-1.**

Mitogenic signaling for many growth factors is triggered by their binding to the transmembrane receptor tyrosine kinases, for example, those for epidermal growth factor and platelet-derived growth factor. Upon ligand-receptor binding, these receptors are dimerized and autophosphorylated. The activated receptors further phosphorylate the receptor substrates to initiate intracellular kinase signaling cascades (1–3). It is evident, however, that there may be alternative signaling pathways for some growth factors involving their nuclear transport and signaling (for review see Ref. 4). In this context, subsequent to receptor-ligand internalization, growth factor ligands may translocate to the nucleus and directly function in mitogenic processes (5, 6). FGF-1<sup>1</sup> is one of two prototype members of the fibroblast growth factor family. It is a potent mitogen for many cell types and is involved in embryogenesis, angiogenesis, and neurite outgrowth (7, 8). The mechanism by which FGF-1 transmits mitogenic signals is still not entirely clear. It has been shown, however, that a mutant FGF-1 with deletion in its

nuclear localization sequence (NLS) Asn-Tyr-Lys-Lys-Pro-Lys-Leu (residues 21–27) failed to stimulate DNA synthesis and cell proliferation *in vitro* although it could still bind to the FGF receptor and induce intracellular receptor-mediated tyrosine phosphorylation and *c-fos* expression (5). The fact that FGF-1 (21–27) was able to direct  $\beta$ -galactosidase into the nucleus, as well as the evidence of nuclear localization of FGF-1 (9, 10), suggest that nuclear transport of FGF-1 following receptor-mediated internalization might be important for stimulating DNA synthesis by FGF-1 *in vitro*.

To examine directly the functional role of the NLS in FGF-1-stimulated mitogenesis, we have delivered the peptide encompassing this sequence into living cells by using our recently developed cell-permeable peptide import method (CPPI) (see Ref. 11). We demonstrated in this report that cell membrane-permeable peptides containing this NLS sequence can stimulate DNA synthesis in NIH 3T3 cells in a FGF receptor-independent manner. Our results together suggest the nuclear transport of FGF-1 plays an important role in the mitogenic pathway initiated by exogenous FGF-1. This may represent an important signaling mechanism for certain growth factors.

### MATERIALS AND METHODS

**Peptides and Antibodies**—Peptides listed in Fig. 1 were synthesized by a stepwise solid-phase peptide synthesis method and purified by C<sub>18</sub> reverse-phase high pressure liquid chromatography as described (11–13). The molecular weights of the purified peptides were verified by mass spectrometry analysis and were shown to agree with the calculated molecular masses. A polyclonal anti-SM peptide antibody raised against the SM peptide-keyhole limpet hemocyanin conjugate in rabbits (11) recognized in enzyme-linked immunosorbent assay not only SM peptide but also SA $\alpha$  peptide analogs.

**Indirect Immunofluorescence Assay**—Confluent NIH 3T3 cells grown on the chamber slides (Nunc) were treated with 0.5 ml of SA $\alpha$  peptide solution in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) under the conditions indicated in the figures. The intracellular peptide was detected by an indirect immunofluorescence assay using anti-SM peptide antibody and rhodamine-labeled anti-rabbit antibody as described (11). Coverslips with stained cells were mounted in Poly/Mount (Polyscience) and analyzed in an Olympus fluorescence microscope using a  $\times 100$  oil immersion lens.

**Mitogenic Assays**—Confluent 3T3 cells grown initially in DMEM containing 10% FBS were transferred to a low serum medium (DMEM containing 0.5% FBS) for 2 days. FGF-1 in the presence of heparin (5 units/ml) or the test peptides was added to a fresh low serum medium at the indicated concentrations. In thymidine incorporation assay, [<sup>3</sup>H]thymidine was added after 16 h, and 4 h later, the cells were washed with phosphate-buffered saline, treated with trichloroacetic acid, and solubilized with 0.15 M NaOH, and the radioactivity was determined in a liquid scintillation counter. In a DNA quantitative assay, after 20 h of incubation with peptide or FGF-1, cells were harvested, spun down, and washed with serum-free phosphate-buffered saline three times. The cells were fixed with methanol precooled to  $-20^{\circ}\text{C}$  for DNA analysis by the flow cytometric method.

**Translocation of <sup>125</sup>I-Labeled Peptides into NIH 3T3 Cells**—Both SA $\alpha$  and SA $\alpha$ M4 peptides were radiolabeled with <sup>125</sup>I by the IODO-GEN method (Pierce). The specific activities of both peptides were similar ( $1.44 \times 10^4$  and  $1.66 \times 10^4$  cpm/ng, respectively). The confluent NIH 3T3 monolayers ( $1.6 \times 10^6$  cells) on each dish were treated with 30 ng of <sup>125</sup>I-labeled peptide at 37  $^{\circ}\text{C}$  for 30 min. The cells were washed and lysed as described (11). The radioactivity in the cell lysate was counted in a Packard Auto-Gamma counter.

**Tyrosine Phosphorylation Studies**—To examine the tyrosine phosphorylation induced by FGF-1 and SA peptides, confluent 3T3 cells grown initially in DMEM containing 10% FBS were starved in DMEM containing 0.5% FBS for 2 days. FGF-1 in the presence of heparin (5 units/ml) or SA peptide was added to cells. After incubation for the

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<sup>1</sup> The abbreviations used are: FGF, fibroblast growth factor; NLS, nuclear localization sequence; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PLC, phospholipase C.



TABLE I

DNA synthesis stimulated by SA peptide as compared with FGF-1

Serum-starved NIH 3T3 cells ( $1.3 \times 10^6$  cells) were untreated (control) or treated with SA peptide or FGF-1 for 20 h, harvested, spun down, washed, and fixed for DNA analysis by the flow cytometric method. The data were the mean  $\pm$  S.E. of six experimental measurements and were analyzed for statistical significance by analysis of variance.

Stimuli	Diploid		G <sub>1</sub> /S ratio
	G <sub>1</sub> phase	S phase	
	%		
Control	86.8 $\pm$ 1.2	7.2 $\pm$ 0.7	12.1
SA (50 $\mu$ g/ml)	86.2 $\pm$ 3.1	6.7 $\pm$ 0.9	12.9
SA (100 $\mu$ g/ml)	76.5 $\pm$ 3.9	13.1 $\pm$ 0.5	5.8
	( <i>p</i> < 0.05)	( <i>p</i> < 0.05)	
FGF-1 (15 ng/ml)	60.2 $\pm$ 4.2	27.8 $\pm$ 2.3	2.2
	( <i>p</i> < 0.05)	( <i>p</i> < 0.05)	

trast to SA $\alpha$  peptide, both control peptides did not show any measurable functional activities when tested within comparable concentration ranges (Fig. 2B). These results suggest that neither the cell membrane-translocating sequence alone (SM peptide) nor the nuclear localization sequence alone (ANL peptide) was sufficient for stimulating DNA synthesis. Cell-permeable SA $\alpha$  peptide therefore was active because it carried the functional NLS of FGF-1 into cells. To clarify whether the functional activity of SA $\alpha$  peptide was contributed exclusively by the NLS of FGF-1, we also prepared and tested a cell-permeable analog of the SA $\alpha$  peptide (referred to as SA, see Fig. 1), which contained only the NLS of FGF-1 but not the carboxyl-terminal epitope tag. Fig. 2B indicates the functional importance of the NLS because the SA and SA $\alpha$  peptides were identical in their ability to stimulate thymidine incorporation. Both SA peptide analogs within the concentration range used were not cytotoxic as determined by staining with fluorescein diacetate/ethidium bromide (11, 14).

The mitogenic effect of the SA peptide was verified by flow cytometric analysis of the DNA distribution in each phase of the cell cycle in the SA peptide-treated 3T3 cells. As shown in Table I and Fig. 2C, the DNA content in the S-phase, which reflected the cell fractions in this phase, was significantly increased when the cells were treated for 20 h with the SA peptide at 100  $\mu$ g/ml, which coincided with the fully effective concentration in the thymidine incorporation assay (Fig. 2B). A similar, but stronger, stimulation was observed in the cells treated with FGF-1 containing the same NLS (Table I and Fig. 2C). These results support the important role of the NLS region of FGF-1 in inducing DNA synthesis.

**Functional Importance of Lysine Residues in the NLS of FGF-1**—The NLS of FGF-1 sequence contains 3 lysine residues (positions 22, 23, and 25 in SA $\alpha$  peptide in Fig. 1). To determine the functional importance of these basic residues, a series of cell-permeable peptides containing single or double point mutations of 3 lysine residues was prepared (Fig. 1) and examined in a thymidine incorporation assay. As shown in Fig. 3A, the peptides (SA $\alpha$ M1, SA $\alpha$ M2, and SA $\alpha$ M3) with a single point mutation in each of 3 lysine residues (Lys<sup>22</sup>  $\rightarrow$  Thr, Lys<sup>23</sup>  $\rightarrow$  Thr, and Lys<sup>25</sup>  $\rightarrow$  Thr, respectively) were still able to stimulate the [<sup>3</sup>H]thymidine incorporation in a manner similar to that of the SA $\alpha$  peptide, although the SA $\alpha$ M3 peptide was slightly less active. However, the peptide SA $\alpha$ M4 with double point mutations (Lys<sup>22</sup>  $\rightarrow$  Thr and Lys<sup>23</sup>  $\rightarrow$  Thr) exhibited impaired activity, suggesting that these basic residues are involved in the pathway leading to DNA synthesis. To exclude the possibility that the inactivity of the SA $\alpha$ M4 peptide was due to its lower cell membrane permeability, NIH 3T3 cells were incubated with either <sup>125</sup>I-SA $\alpha$  or <sup>125</sup>I-SA $\alpha$ M4, and the amount of cell-associated <sup>125</sup>I-labeled peptides was measured and compared.

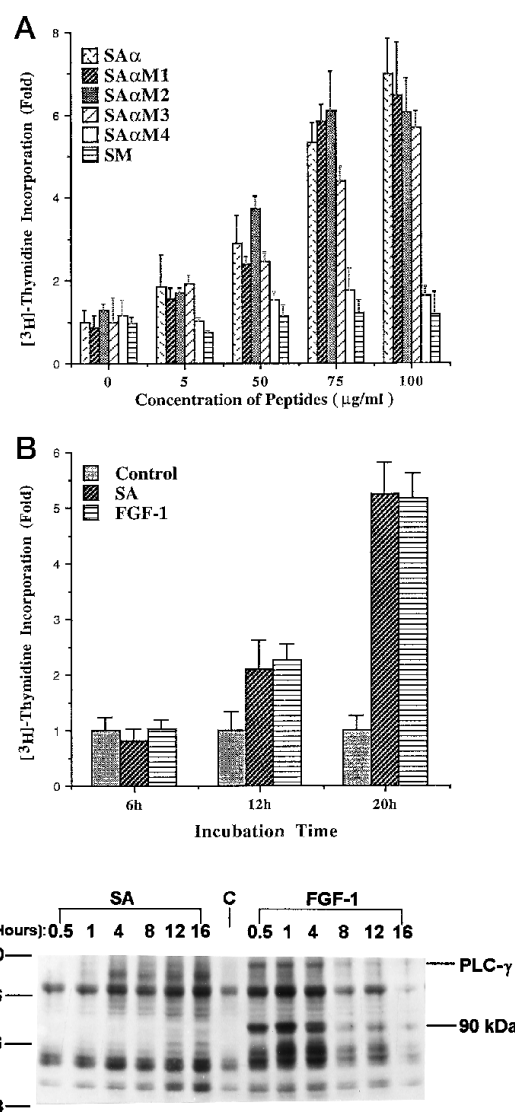


FIG. 3. A, the effect of NLS mutations on SA $\alpha$  peptide-stimulated [<sup>3</sup>H]thymidine incorporation by NIH 3T3 cells. Serum-starved NIH 3T3 cells were treated with various amounts of SA $\alpha$  or its mutant peptides. The rest of the procedure was the same as described in the legend to Fig. 2B. Bars represent the mean  $\pm$  S.D. of six samples and are calculated as multiplicity of counts in the tested sample over the control sample (untreated cells). The differences between SA $\alpha$  peptide and SA $\alpha$ M4 peptide and between SA $\alpha$  peptide and SA $\alpha$ M3 peptide at 75 and 100  $\mu$ g/ml were significant (*p* < 0.01 and 0.05, respectively) by analysis of variance. The experiment was repeated three times with similar results. B, long term peptide exposure required for the SA peptide-stimulated [<sup>3</sup>H]thymidine incorporation by NIH 3T3 cells. Serum-starved cells were untreated (control) or treated with SA peptide (100  $\mu$ g/ml) or FGF-1 (15 ng/ml)/heparin (5 units/ml) for the indicated time periods. The rest of the procedure was the same as described in the legend to Fig. 2B. Bars represent the mean  $\pm$  S.D. of triplicate samples and are calculated as multiplicity of counts in the tested sample over the control sample. The experiment was repeated three times with similar results. C, the induction of tyrosine phosphorylation by SA peptide or FGF-1 in NIH 3T3 cells. Serum-starved cells were untreated (lane C) or treated with SA peptide (100  $\mu$ g/ml) or FGF-1 (15 ng/ml) for the indicated time periods. The immunoblot of the equivalent amount of whole cell lysates was obtained with anti-phosphotyrosine antibody. The tyrosine-phosphorylated 90-kDa protein and 150-kDa PLC- $\gamma$  protein affirmed by anti-PLC $\gamma$  antibody were indicated.

No significant difference between <sup>125</sup>I-SA $\alpha$ - and <sup>125</sup>I-SA $\alpha$ M4-treated cells was observed in cell-associated radioactivity counts ( $18,120 \pm 1,933$  versus  $16,730 \pm 2,747$  cpm/ $1.6 \times 10^6$  cells, *p* > 0.05, *n* = 6, Student's *t* test). Therefore, our results from these assays indicate that the loss of mitogenic activity of

SA $\alpha$ M4 peptide is due to the lack of the two functional lysine residues in this FGF-1 sequence. Interestingly, subcellular fractionation of these cells showed that about 90% of the <sup>125</sup>I-labeled SA $\alpha$  peptide was associated with the nuclear fraction (data not shown). Therefore, lack of strong nuclear staining in the immunofluorescence assay (Fig. 2A) might have resulted from modification and/or association of the SA $\alpha$  peptide with specific nuclear constituents, which prohibited peptide recognition by the antibody.

**Stimulation of DNA Synthesis by the SA Peptide Required a Long Incubation Time**—We further examined whether the mitogenic stimulation by SA peptides was cell cycle-dependent. As shown in Fig. 3B, [<sup>3</sup>H]thymidine uptake by NIH 3T3 cells was not observed in the cells treated for 6 h (G<sub>1</sub> phase) with SA peptide at 100  $\mu$ g/ml. However, it became substantial after cells were treated for 20 h (S phase). A similar pattern of stimulation was seen in cells treated with the full-length FGF-1 *in vitro* (Fig. 3B). It has also been shown that stimulation of maximal DNA synthesis by FGF-1 in Balb/c 3T3 cells requires the presence of FGF-1 during the entire G<sub>1</sub> phase of the cell cycle (15).

**The Mitogenic Effect of SA Peptides Is FGF Receptor-independent**—It is known that exogenous FGF-1 binds to FGF receptors on NIH 3T3 cells and induces the tyrosine phosphorylation of a number of intracellular proteins (15–20). Among them, a 90-kDa protein and a 150-kDa phospholipase C- $\gamma$  (PLC- $\gamma$ ) are rapidly and strongly phosphorylated in FGF-1-stimulated NIH 3T3 cells and are often used as the indicators of FGF receptor activation (18–20). However, recent studies have suggested that phosphorylation of PLC- $\gamma$  and the resulting hydrolysis of phosphatidylinositol are not required for FGF-stimulated mitogenesis (21, 22). In Fig. 3C, we demonstrated that unlike FGF-1, SA peptide did not stimulate the early tyrosine phosphorylation of the 90-kDa protein and PLC- $\gamma$  in NIH 3T3 cells even at concentrations sufficient to induce DNA synthesis, suggesting that the mitogenic effect of SA peptides is FGF receptor-independent. Instead, SA peptide triggered the slight tyrosine phosphorylation of several unknown proteins at the late G<sub>1</sub> phase of the cell cycle (Fig. 3C). It is still not clear whether this low level phosphorylation is important for the SA peptide-induced activity.

#### DISCUSSION

In this report, we suggest a dissociation of FGF-1-stimulated mitogenesis from its receptor tyrosine kinase activation in NIH 3T3 cells. We have demonstrated that the peptide containing the NLS of FGF-1 can stimulate DNA synthesis after it is delivered into NIH 3T3 cells by using a cell-permeable peptide import method (11). Our finding is supported by a recent observation that a mutant FGF-1 with deletion in its NLS is not mitogenic *in vitro* (5). We thus propose that the NLS of FGF-1 may play two functional roles in exogenous FGF-1-stimulated mitogenesis. First, in mediating nuclear translocation of FGF-1, internalization of FGF-1 following its receptor binding may allow the association of the partitioned growth factor through the NLS with the intracellular machinery that facilitates nuclear transport of FGF-1. A number of cytosolic proteins have been known to mediate nuclear translocation of various NLS-containing proteins (for review see Ref. 23). This role of the NLS of FGF-1 may not be crucial (24) because internalized FGF-1 with a molecular size of 16.5 kDa should enter the cell nucleus by free diffusion. However, the NLS could be important if FGF-1 is transported to the nucleus in the form

of FGF-FGF receptor complex. As concerns the second role of the NLS of FGF-1, the functional ability of SA peptides in stimulating DNA synthesis suggests that the NLS is directly involved in the FGF-1-induced nuclear mitogenic signaling. Such signaling may be initiated by the binding of the positively charged NLS to specific molecules in the nucleus or on the nuclear membrane. The importance of the basic residues as demonstrated by our mutagenesis study is buttressed by our recent finding that cell-permeable peptides containing the NLS of nuclear factor  $\kappa$ B p50 protein or the NLS of v-Rel protein can also stimulate DNA synthesis in NIH 3T3 cells in a manner similar to SA peptides (data not shown). Because the basic cores of the two NLS sequences, KRQK (p50) and KRQR (v-Rel), are similar to that of the NLS of FGF-1, KKPK (Fig. 1), a 4-residue sequence motif, K-K(R)-X-K(R), may be functionally important. It is expected that this proposed sequence motif can be found in many intracellular NLS-containing proteins. However, it may become physiologically relevant only when a significant amount of molecules containing this motif is translocated into the nucleus, for example by receptor-mediated FGF-1 internalization or by cell-permeable peptides as shown in this study.

The mitogenic effect of SA peptides is not limited to NIH 3T3 cells. We found that SA peptides could also induce DNA synthesis in bovine hamster kidney-21 cells. However, the same peptides, unlike full-length FGF-1, were inactive in murine LE-II endothelial cells despite their good cell membrane permeability in this cell line (data not shown). These results thus suggest that different mechanisms may be involved in FGF-1-stimulated mitogenesis in various cell types.

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