Identification of Nuclear Import and Export Signals within Fli-1: Roles of the Nuclear Import Signals in Fli-1-Dependent Activation of Megakaryocyte-Specific Promoters

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The Ets factor Friend leukemia integration 1 (Fli-1) is an important regulator of megakaryocytic (Mk) differentiation. Here, we demonstrate two novel nuclear localization signals (NLSs) within Fli-1: one (NLS1) is located at the N terminus, and another (NLS2) is within the Ets domain. Nuclear accumulation of Fli-1 reflected the combined functional effects of the two discrete NLSs. Each NLS can independently direct nuclear transport of a carrier protein, with mutations within the NLSs affecting nuclear accumulation. NLS1 has a bipartite motif, whereas the NLS2 region contains a nonclassical NLS. Both NLSs bind importin alpha (IMP α) and IMP β , with NLS1 and NLS2 being predominantly recognized by IMP α and IMP β , respectively. Fli-1 also contains one nuclear export signal. Leptomycin B abolished its cytoplasmic accumulation, showing CRM1 dependency. We demonstrate that Ets domain binding to specific target DNA effectively blocks IMP binding, indicating that the targeted DNA binding plays a role in localizing Fli-1 to its destination and releasing IMPs for recycling back to the cytoplasm. Finally, by analyzing full-length Fli-1 carrying NLS1, NLS2, and combined NLS1-NLS2 mutations, we conclude that two functional NLSs exist in Fli-1 and that each NLS is sufficient to target Fli-1 to the nucleus for activation of Mk-specific genes.

Megakaryocyte (Mk) platelet progenitors are derived from hematopoietic stem cells by a complex differentiation process that is dependent on the interaction of hematopoietic progenitor cells, cytokines, and stromal elements (9, 40). Several transcription factors have been demonstrated to play essential roles in Mk differentiation and development. Among them is Friend leukemia integration 1 (Fli-1), a member of the Ets transcription factor family (2, 16). Fli-1 is a ubiquitously expressed transcription factor originally identified for its overexpression in erythroleukemia in mice infected with Friend's leukemia virus. Fli-1 and other Ets proteins share a conserved Ets domain that is responsible for its specific binding to a 10-bp-long DNA sequence centered over a GGA core (34, 39). Inactivation of the Fli-1 gene in mice is lethal at embryonic day 11.5, with death resulting from brain hemorrhage and endothelial cell dysfunction. Furthermore, Fli-1 knockout mice produce small, undifferentiated Mk progenitors with abnormal ultrastructural features, such as reduced α granules and disrupted demarcation membrane systems (16, 41). Levels of expression of Mk-specific genes (normally expressed late during differentiation), such as glycoprotein IX (GPIX) and glycoprotein Ib α (GPIb α), are also markedly reduced (16). Moreover, Fli-1^{-/-} embryonic stem cells are unable to produce Mk colonies or multilineage colonies in colony formation assays (25). A growing number of proteins have been shown to interact with Fli-1, modifying its functions. Previous work in our laboratory has revealed synergistic activation of Mk-specific gene expression by cooperative protein-protein interaction be-

tween Fli-1 and GATA-1 (11). In addition, Wang et al. demonstrated that the close association between Fli-1 and GATA-1 converts FOG-1 from a repressor of GATA-1 to an activator (45), and EKLF represses the Fli-1-dependent megakaryocytic GPIX gene transcriptional activity by its interaction with Fli-1 (42).

The nuclear transport of nuclear proteins involves cellular pathways of signal-mediated transport through nuclear pore complexes (NPC) embedded in the nuclear envelope, which are mediated by nucleocytoplasmic shuttling receptors of the karyopherin-importin (IMP) superfamily (12). Transport of proteins into and out of the nucleus relies on specific targeting signals. The IMPs interact with nuclear localization signals (NLSs) or nuclear export signals (NESs) on the cargo molecules and carry them through the NPC (5, 12, 33). In the first step of conventional nuclear protein import, the NLS-containing protein is recognized by the IMP α / β heterodimer through the NLS-binding IMP α subunit and targeted to the NPC by the IMP β subunit (1, 14). In the second step, the complex is translocated through the NPC by transient IMP β -mediated interactions with nucleoporins, the FG-repeat-containing (hydrophobic repeat) components of the NPC (4, 38). Finally, IMP α and the NLS-containing protein are released from IMP β into the nucleoplasm through binding of RanGTP to IMP β (13, 37). The IMPs are then recycled back to the cytoplasm for another round of transport. It should also be noted that while the IMP α / β 1 heterodimer is involved in the nuclear import of many well-known nuclear proteins, such as simian virus 40 large tumor antigen, most other pathways require IMP β 1 or an IMP β homologue, with no requirement for IMP α . The best-defined NLSs contain either a single stretch of basic amino acids or a bipartite sequence of basic amino acids (10). An increasing number of proteins have recently been described as

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having multiple NLSs. However, the importance of having multiple NLSs is unclear.

Gene transcription takes place in the nucleus; thus, Fli-1 must be imported into the nucleus from the cytoplasm. However, the regions within Fli-1 that serve as signals for its nuclear or cytoplasmic localization and the proteins that enable Fli-1 to become localized in the nucleus are unknown. In this report, we identify two novel nuclear import sequences within Fli-1, show that Fli-1 is recognized by both IMP α and IMP β by a series of Fli-1 NLS mutants, and analyze the role of NLSs in transcriptional regulation of the Mk-specific GPIX and GPIb α genes. Either NLS is sufficient to target a green fluorescent protein (GFP)–Fli-1 fusion protein to the nucleus. We have also identified one NES and have shown that it is CRM1 dependent. In this study, we also provide evidence that the binding of the specific DNA to the Ets domain dissociates the Ets domain from the IMPs, which are then free to return to the cytoplasm and to be used for the next round of nuclear import. Furthermore, the N-terminal NLS mutants do not abolish the activation of the Fli-1-inducible Mk-specific GPIX and GPIb α genes. Although the NLS2 mutation within the Ets domain dramatically reduced the activation of Fli-1-inducible Mk-specific genes, our data strongly suggested that the reduction of transcription was due to the mutation affecting the binding of Fli-1 to DNA. Collectively, our results indicate that either NLS is sufficient to target Fli-1 to its destination in the nucleus for activation of Mk-specific gene expression.

MATERIALS AND METHODS

Plasmid constructions. To generate GFP–Fli-1 fusion protein constructs, the full-length Fli-1 gene was linked downstream and upstream of the gene encoding GFP by a Gateway system using pcDNA-DEST53 and pcDNA-DEST47 vectors, respectively (Invitrogen, Carlsbad, Calif.). The Fli-1 fragments were subcloned upstream of GFP using pcDNA-DEST47. The pGex–Fli-1 (amino acids [aa] 269 to 373) construct and GPIX-567 and GPIb α -253 luciferase reporter constructs have been published previously (11). NLS1 fragment mutants, NLS2 fragment mutants, NLS1 mutants in full-length GFP–Fli-1, NLS2 mutants in full-length GFP–Fli-1, combined NLS1–NLS2 mutants in full-length GFP–Fli-1, and Ets domain NLS2 mutant GST fusion constructs were generated by site-directed mutagenesis (QuikChange kit; Stratagene, La Jolla, Calif.).

GST fusion proteins. IMP α (PTAC58) subunit, IMP β (PTAC97) subunit, and the Ets domain of Fli-1 (aa 369 to 371) and six Ets domain NLS2 mutants were expressed as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* strain BL21 (DE3) and purified as described previously (11, 18, 21, 22). GST-free IMP α and IMP β and the Ets domain were prepared by thrombin cleavage (18).

Cell culture. Rat hepatoma (HT) cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) as previously described (11, 18). Growth factor-dependent UT-7/TPO (purified recombinant human thrombopoietin) cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 1 ng of TPO/ml, as previously described (17). K562 cells were cultured in RPMI 1640 supplemented with 10% FBS.

Transient cell transfections. Lipofectamine–DNA complexes were formed and incubated with HT and HeLa cells according to the manufacturer's instructions (Invitrogen). The cells were allowed to express GFP–Fli-1 fusion proteins on coverslips and 48 h after transfection were fixed with 4% paraformaldehyde for examination with either a Leica or an Olympus confocal microscope. Leptomycin B (LMB) (Sigma, St. Louis, Mo.) was added to the transfected HT and HeLa cells at a concentration of 7.5 ng/ml, and the cells were cultured for 5 h.

Cell staining with propidium iodide. Following transfection, the DNAs of K562 and UT-7/TPO cells were stained red with propidium iodide (Sigma) as follows. The cells were washed with phosphate-buffered saline and resuspended in 2 ml of 0.5% Tween-20 (Bio-Rad, Hercules, Calif.) in RPMI and incubated in the dark on ice for 45 min. Following the incubation, 2 ml of 1% paraformaldehyde–0.5% Tween 20 in RPMI was added, and the cells were incubated in the dark on ice for 5 min. Subsequently, the cells were spun and resuspended in 0.5

ml of RPMI; 50 μ g of propidium iodide/ml was then added, and the cells were incubated in the dark overnight at 4°C. The following day, a cytospin was carried out and the cells were examined with an Olympus confocal microscope.

GST pull-down studies. ³⁵S-labeled Fli-1 fragments, NLS2 mutants, and NLS1 mutants were prepared by in vitro translation using the TNT T7 Coupled Reticulocyte Lysate System (Promega, San Luis Obispo, Calif.). GST interaction assays were performed as previously described (11). Briefly, 0.3 μ M GST and GST fusion proteins were incubated at room temperature for 90 min with 20 μ l of GST beads in a 100- μ l reaction volume of phosphate-buffered saline. For experiments (see Fig. 3B), 0.9 μ M RanGDP or RanGTP (Ran Q69L, a dominant GTP form of Ran) (Calbiochem, La Jolla, Calif.) was added to the above-mentioned reaction mixture. After incubation, the beads were washed with 500 μ l of GST pull-down buffer. Subsequently, 4 μ l of in vitro-translated product was incubated with the GST bead-bound protein in a 100- μ l reaction volume of GST pull-down buffer for 2 h at 4°C. The beads were then washed with GST pull-down buffer. The reaction mixture was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. After electrophoresis, the gel was dried and exposed to a phosphorimaging screen (Kodak, Rochester, N.Y.) overnight and developed on a Personal FX phosphorimager (Bio-Rad).

EMSAs. Electrophoretic mobility shift assay (EMSA) analysis was carried out as follows. The GPIX promoter DNA probes (GPIX Ets oligonucleotide, 5'-CAC TGG GGG GAT AAG CCA GGC TAT TTT CAT CAC TTC CTT CCG CCC G-3') and their complements were annealed and end labeled with polynucleotide kinase and [³²P]ATP, followed by purification on MicroSpin G-25 columns (Amersham Pharmacia, Little Chalfont, United Kingdom) according to the manufacturer's instructions. EMSA (see Fig. 6B) was performed with the probes and GST-free Ets domain in the presence of GST, GST-IMP α , or GST-IMP β . The Ets domain protein was preincubated for 20 min with 0.05, 0.5, or 5 μ g of GST, GST-IMP α , or GST-IMP β . Binding reactions with 0.2 fmol of [³²P]ATP-labeled GPIX promoter DNA probe were performed in a 20- μ l volume on ice for 30 min in 25 mM Tris-Cl (pH 5.7), 10% glycerol, 6 mM MgCl₂, 0.5 mM EDTA, 60 mM KCl, 0.5 mM dithiothreitol, and 200 μ g of bovine serum albumin/ml. EMSA (see Fig. 10A) was performed with the probes and Ets domain GST fusion protein or six Ets domain NLS2 mutants. After binding, the reaction mixture was subject to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 5% polyacrylamide gels in 0.5 $\times$ Tris-borate-EDTA at 4°C. The gels were dried and exposed to a Kodak phosphorimager screen overnight and developed on a Personal FX phosphorimager.

ELISA-based binding assay. An enzyme-linked immunosorbent assay (ELISA)-based binding assay (18, 19) was used to examine the affinity of binding of importin subunits (without GST moieties) to Ets domain protein in the presence or absence of 1 μ M GPIX promoter DNA probe or 1 μ M nonspecific DNA. Ninety-six-well microtiter plates were coated with the GST-free IMP α or GST-free IMP β and incubated with increasing concentrations of Ets domain, and bound Ets domain of Fli-1 was detected using a goat anti-Fli-1 primary antibody (BD Biosciences, San Jose, Calif.), an alkaline phosphatase-coupled rabbit anti-goat secondary antibody (Sigma), and the substrate *p*-nitrophenyl phosphate (Sigma). *A*₄₀₅ measurements were performed over 90 min using a plate reader (Molecular Devices), with values corrected by subtracting the absorbance both at 0 min and in wells incubated without Ets domain.

Luciferase reporter assays. Transient transfection of HT cells was carried out in six-well plates using Lipofectamine (Invitrogen) reagent according to the manufacturer's instructions. HT cells were transfected with 400 ng of the GPIX-567 and GPIb α -253 luciferase reporter plasmids and expression plasmids encoding Fli-1 or Fli-1 NLS mutants as indicated. The total amount of DNA in each transfection was kept constant at 1.2 μ g by adding the pcDNA-DEST47 backbone. Cells were harvested 48 h after transfection, and 10 μ l of lysate was assayed for luciferase activity using the Promega luciferase assay system kit. The results shown represent the average increase in firefly luciferase activity $\pm$ the standard error of the mean (SEM) from three experiments performed in triplicate.

Quantitation of subcellular distribution. As an initial approach to quantitation, slides were fixed 48 h after transfection, and 30 to 50 positive cells were scored as to whether nuclear fluorescence was greater than, equal to, or less than cytosolic fluorescence. Care was taken to avoid damaged, dead, or autofluorescent cells. The results from at least three independent transfections per construct were used for statistical analysis. As a second approach, 30 to 50 individual cells per construct were scored for cytosolic and nuclear fluorescence intensity using the Image J (National Institutes of Health) program. Cytoplasmic and nuclear values for each cell were summed to give total cellular fluorescence, and the percent fluorescence values for the nuclear compartment were calculated (15, 18, 29).

RESULTS

Fli-1 contains two nuclear import signals and one nuclear export signal. Although Fli-1 is well known as a nuclear transcription factor, the functions of its nuclear import and export signal motifs have not been investigated. Therefore, we first set out to map the regions of Fli-1 required for these processes. To visualize the cellular localization of Fli-1 by fluorescence microscopy, we linked the gene encoding GFP downstream and upstream of the full-length Fli-1 gene (Fig. 1A, a and b) and various N-terminal and C-terminal deletion fragments of Fli-1 (shown schematically in Fig. 1B). These GFP constructs were transfected into HT and HeLa cells, and the cellular localization of Fli-1, full length and fragments, was determined. The full-length Fli-1-GFP fusion proteins were translocated to the nucleus (Fig. 1A, B, and C). In addition, we found that two distinct regions of Fli-1 harbor an NLS, one within the N-terminal region from aa 62 to 126 (NLS1) (Fig. 1A and B, n) and an additional one within the Ets domain (NLS2) (Fig. 1A and B, s). The latter has been described for other Ets proteins (29, 44). Both NLSs can drive nuclear localization of the GFP fusion proteins effectively (Fig. 1A), suggesting that they are indeed functional NLSs. However, the NLS within the N-terminal domain appears to be more potent, since the NLS1 fragment leads to 86 to 89% nuclear localization in HT and HeLa cells, whereas the NLS2 fragment consisting of the Ets domain results in only 72 to 74% nuclear localization (Fig. 1A and C). Our results indicated that nuclear accumulation of full-length Fli-1 was very strong in both HT and HeLa cells (>94%) and was due to the combined functional effects of both NLS1 and NLS2 (Fig. 1A and C).

In addition, we also attempted to identify the region(s) within the Fli-1 protein capable of serving as an NES. The GFP-tagged Fli-1 fragment containing the residues from aa 127 to 276 (Fig. 1A and D, q) was excluded from the nucleus. It harbors the N-terminal activation domain of Fli-1. Elimination of the last 80 amino acids (Fig. 1A and D, fragment aa 127 to 196 [p]) leads to the GFP fusion protein being equally distributed throughout the nucleus and cytoplasm. These findings suggest that either the protein was not imported into the nucleus or it contains a functional NES. Further characterization revealed an increase in nuclear staining in HT and HeLa cells after treatment with the nuclear export inhibitor LMB, indicating that the protein was in fact imported into the nucleus but was subsequently exported. It further suggests that the nuclear export mediated by the NES is CRM1 dependent (Fig. 1D). However, the NES within the region aa 127 to 276 is not a strong signal in HT and HeLa cells. Although the GFP fusion protein containing this region showed cytoplasmic accumulation in most HT and HeLa cells, these cells still had some residual nuclear fluorescence (20%) (Fig. 1C).

Localization of Fli-1 in the megakaryocytic cell lines K562 and UT-7/TPO. All previous transient-transfection assays were carried out in the rat hepatoma cell line HT and the human epithelial cell line HeLa. However, since Fli-1 is involved in megakaryopoiesis, we examined the nuclear-cytoplasmic distribution of full-length Fli-1 and various Fli-1 fragment constructs in the megakaryocytic cells K562 and UT-7/TPO (Fig. 2A). The N-terminal fragment (aa 62 to 126) and the Fli-1 Ets domain fragment (aa 277 to 360) were chosen for transfection

because they both contain an NLS of Fli-1, and the fragments aa 127 to 196 and 1 to 75 were used as our negative controls because previous transfection results showed that these regions did not accumulate in the nucleus in HT and HeLa cells.

Figure 2B and C, the images of results obtained from K562 and UT-7/TPO cells showed that the full-length Fli-1 (a) and both the N-terminal fragment (aa 62 to 126) (b) and the Ets domain (c) accumulated in the nucleus, whereas the fragment aa 127 to 196 (d) and the fragment aa 1 to 75 (e) were located in both the nucleus and the cytoplasm (Fig. 2B and C), similar to the results observed in HT and HeLa cells (Fig. 1A). Thus, these data indicate that the two NLSs are also functional in K562 and UT-7/TPO cells.

Interaction of Fli-1 with IMP α and IMP β . Since two regions in Fli-1 containing nuclear import sequences have been identified, we investigated the nuclear import pathway used by these regions (Fig. 3A). In order to do this, we incubated GST-IMP α and GST-IMP β with in vitro-translated Fli-1 fragments in a pull-down assay to define nuclear transport receptors that bind to them. As shown in Fig. 3B, full-length Fli-1 (a) was able to bind to both GST-IMP α (lane 2) and GST-IMP β (lane 3). The NLS1 region (aa 62 to 126) (e), which was shown to contain an NLS from previous transfection results, bound more strongly to GST-IMP α , suggesting that its nuclear import may be via the IMP α / β -mediated pathway. The NLS2 Ets domain (aa 277 to 360) (g) was shown to bind predominantly to GST-IMP β (lane 3), implying that it may have a preference for the IMP β -mediated pathway. Conversely, the fragment aa 1 to 75, which does not contain an NLS (f), and the fragment aa 127 to 276 containing the NES (h) were unable to bind to either IMP α or IMP β . To demonstrate that importin-cargo interactions are the important determinants for nuclear import of Fli-1, additional experiments were carried out using RanGTP and RanGDP. As showed in Fig. 3C, RanGTP dissociated the binding of IMP β to full-length Fli-1 (a, lane 7), as well as the fragment containing NLS2 (g, lane 7); in contrast, addition of an equal amount of RanGDP did not affect the binding of IMP β to them (lane 6). As a negative control, addition of RanGTP or RanGDP to the IMP α lanes of both full-length Fli-1 (a, lanes 3 and 4) and the fragment containing NLS1 (e, lanes 3 and 4) had no effect, since IMP α does not bind to Ran. Taking these data together, it appears that Fli-1 is able to utilize both pathways, with NLS1 and NLS2 having a preference for IMP α / β - and IMP β -mediated pathways, respectively.

Mutational analyses of Fli-1 NLS1. As NLSs are typically made up of positively charged amino acids, to further map the two NLSs of Fli-1, the positively charged amino acids lysine and arginine within these two regions were mutated to the neutral amino acid alanine. The mutated fusion vectors were then transiently transfected into HT and HeLa cells, and the localization of the fusion proteins was examined (Fig. 4 and 5).

The mutants that disrupted nuclear accumulation of the NLS1-GFP fragment (aa 1 to 126) were characterized (Fig. 4B and C). Analysis of the point mutants K67A (c), R68A (d), K87A (f), and K90A (g) suggests that these residues play a critical role in nuclear accumulation of the NLS1 fragment. The mutants were clearly distinguishable from the wild-type (wt) NLS1-GFP fragment due to the balanced distribution of the mutated fusion proteins (their nuclear accumulation was

B

	Fli-1 fragments		Nuclear accumulation
	GFP		No
a	1 452		Yes
b	1 452		Yes
c	1 401		Yes
d	75 452		Yes
e	75 401		Yes
f	1 373		Yes
g	1 276		Yes
h	1 196		Yes
i	1 126		Yes
j	1 75		No
k	22 126		Yes
m	41 126		Yes
(NLS1) n	62 126		Yes
o	76 126		No
p	127 196		No
(NES) q	127 276		No
r	269 373		Yes
(NLS2) s	277 360		Yes

FIG. 1. Identification of two nuclear import signals and one nuclear export signal in the Mk-specific Fli-1 gene. (A) CLSM images of localization for full-length Fli-1 and Fli-1 fragments. Full-length Fli-1-GFP (a and b) and Fli-1-GFP deletion fragments (c to s) were transfected into HT and HeLa cells. The localization of GFP fusion proteins was determined 48 h after transfection. (B) Diagram of the NLS-NES mapping process. The N-terminal activation domain, the C-terminal activation domain, and the Ets domain (middle) are represented by black bars, and nuclear accumulation of each fragment is indicated. (C) CLSM images were analyzed to determine the percent nuclear accumulation of Fli-1 fragments (mean + SEM, for $n > 50$), with the arrows indicating percent nuclear accumulation of GFP alone. (D) CLSM images of localization of Fli-1 fragments (Fli-1-GFP, aa 127 to 196 [p], and Fli-1-GFP, aa 127 to 276 [q]) in the absence (–) or presence (+) of LMB (7.5 μ g/ml for 5 h).

~50%). In contrast, mutation of R77 (e), K109 (h), R122 (i), and R123 (j) had no effect on NLS1-GFP nuclear accumulation. In addition, mutation of R63 (b) had a mild effect (nuclear accumulation was ~68 and 71% in HT and HeLa, respectively). These results indicated that mutation of R63, K67, R68, K87, and K90 impaired the nuclear accumulation of the

NLS1 fragment and that the amino acids K67, R68, K87, and K90 are critical for the function of NLS1. Having established the roles of lysine and arginine in the nuclear accumulation of the NLS1-GFP fusion protein in HT and HeLa cells, we investigated whether the mutants would inhibit their ability to bind the IMPs using in vitro-translated

FIG. 2. NLSs of Fli-1 can target GFP-Fli-1 fusion protein into the nucleus of UT-7/TPO and K562 cells. (A) Schematic representations of Fli-1 fragments used in the Mk-specific cell lines. The full-length Fli-1-GFP (a), the NLS1-GFP fragment (b), the NLS2-GFP fragment (c), the Fli-1-GFP fragment aa 1 to 75 (d), and the Fli-1-GFP fragment aa 127 to 196 (e) were transfected into the above-mentioned cell lines. CLSM images of K562 and UT-7/TPO are shown in panels B and C, respectively. Lanes 1 show the GFP fusion proteins. Lanes 2 show propidium iodide staining of the nuclei. Lanes 3 show the merge of lanes 1 and 2. Lanes 4 show the transmission images.

GFP-NLS1 fragment fusion proteins in a GST pull-down assay (Fig. 4D). The binding of K67A, R68A, K87A, and K90A to IMP α (lane 2) and IMP β (lane 3) was negligible (c, d, f, and g). The binding of the IMPs to R63A (b) and the wt NLS1 region (a) was only slightly different, but the recognition of R77A (e), K109A (h), R122A (i), and R123A (j) by the IMP was similar to that of the wt GFP-NLS1 (a). These data suggest that the mutations (K67A, R68A, K87A, and K90A) strongly disrupted the IMP recognition of the NLS1 fragment. These results are consistent with the nuclear accumulation analysis findings described above (Fig. 4B and C).

The mutagenesis analysis clearly shows that NLS1 resembles a classical bipartite NLS motif, in that R63, K67, and R68 form the N-terminal arm while K87 and K90 constitute the C-terminal arm (Fig. 4A). The spacing between the two arms is significantly larger than that of a classical bipartite NLS (usually 10 to 12 amino acids). This is fully consistent with the strong physical interaction between IMP α and the NLS1 region (Fig. 4D). However, both arms of NLS1 are necessary for nuclear import; point mutation of K67 and R68 at the N-terminal arm and mutation of K87 and K90 at the C-terminal arm of NLS1 completely obliterate nuclear accumulation, as well as recognition of the IMPs.

FIG. 3. Direct interactions of Fli-1 and Fli-1 fragments with IMP α and IMP β . (A) Schematic representations of full-length Fli-1 and Fli-1 fragments used in GST pull-down assays. (B) GST pull-down results demonstrating the interaction of full-length Fli-1 and the Fli-1 fragments with IMP α or IMP β . Lane 1 contains 10% of the input in vitro-translated 35 S-labeled Fli-1-GFP protein. The other lanes show Fli-1-GFP protein retained by GST-IMP α , GST-IMP β , or GST protein. (C) GST pull-down results demonstrating the interaction of full-length Fli-1 (a), the NLS1 fragment (e), and the NLS2 fragment (g) with IMP α or IMP β in the presence or absence of Ran. Fli-1-GFP protein retained by GST-IMP α is shown in lane 2 (in the absence of Ran), lane 3 (in the presence of RanGDP), and lane 4 (in the presence of RanGTP). Fli-1-GFP protein retained by GST-IMP β is shown in lane 5 (in the absence of Ran), lane 6 (in the presence of RanGDP), and lane 7 (in the presence of RanGTP).

C

D

FIG. 4. Mutational analyses of NLS1 within the N-terminal domain. (A) Primary sequence of NLS1 region. The positively charged amino acids lysine and arginine are showed in boldface, with the boxes indicating the two arms of the bipartite NLS1. The positions of the lysine and arginine replaced by alanine in NLS1 mutants are indicated. (B) CLSM images of localization of the wt NLS1 (wt aa 1 to 126) (a) and its mutants R63A (b), K67A (c), R68A (d), R77A (e), K87A (f), K90A (g), K109A (h), R122A (i), and R123A (j) in HT and HeLa cells. (C) CLSM images were analyzed to determine the percent nuclear accumulation of the wt NLS1 and its mutants (mean + SEM, for $n > 30$). (D) GST pull-down was carried out as described in the legend to Fig. 3, with results demonstrating the interaction of the wt NLS1 and the mutants with IMP α or IMP β . Lane 1 contains 10% of the input in vitro-translated 35 S-labeled Fli-1-GFP protein. The other lanes show GFP fusion proteins retained by GST-IMP α , GST-IMP β , or GST protein.

FIG. 5. Mutational analyses of NLS2 within the Ets domain. (A) Primary sequence of Ets domain showing α -helix and β -sheet regions. The positively charged amino acids lysine and arginine are shown in boldface. The positions of the lysines and arginines replaced by alanines in the NLS2 mutants are indicated. (B) CLSM images of localization of the wt NLS2/Ets domain (wt aa 277 to 360) and its mutants in HT and HeLa cells. (C) CLSM images were analyzed to determine the percent nuclear accumulation of the wt NLS2/Ets domain and its mutants (mean + SEM, for $n > 30$). (D) GST pull-down results demonstrating the interaction of the wt NLS2/Ets domain and its mutants with IMP α or IMP β . Lane 1 contains 10% of the input in vitro-translated 35 S-labelled Fli-1-GFP protein. The other lanes show GFP fusion proteins retained by GST-IMP α , GST-IMP β , or GST.

FIG. 6. DNA target sites effectively compete with IMP α and IMP β for binding to the Ets domain of Fli-1. (A) GST-IMP α and GST-IMP β were incubated with in vitro-translated ^{35}S -labeled GFP-NLS2/Ets domain (aa 277 to 360) region (^{35}S Ets domain) in the presence (+) or absence (+ non) of the GPIX promoter DNA probe corresponding to the NLS2/Ets domain binding site (GPIXDNA) or to the nonspecific DNA (scrambled nucleotide sequence; nsDNA). (B) EMSA was performed with GPIXDNA and GST-free Ets domain protein in the presence or absence of GST-IMP α , GST-IMP β , or GST. The GST-free Ets domain was preincubated with 0.05, 0.5, and 5 mg of GST-IMP α , GST-IMP β , or GST for 30 min; ^{32}P -labeled GPIX promoter DNA probe (^{32}P GPIXDNA) was then added to the reaction mixtures. (C) ELISA was performed with GST-free Ets domain protein and GST-free IMP α or GST-free IMP β in the presence or absence of GPIXDNA or nsDNA. LB, low binding; OD, optical density.

TABLE 1. Binding parameters of Ets domain to IMP α and IMP β ^a

Ets $\pm$ DNA	K_d (nM)	
	IMP α	IMP β
Ets	124.1 $\pm$ 4.6 ^b (3)	25.0 $\pm$ 3.2 ^d (3)
Ets + GPIXDNA	LB ^c (3)	LB (3)
Ets + nsDNA	113.6 $\pm$ 13.0 ^b (2)	31.2 $\pm$ 7.2 ^d (2)

^a In the presence or absence of GPIX promoter DNA probe (GPIXDNA) or nonspecific DNA (nsDNA) as measured using an ELISA-based assay. The data represent the mean $\pm$ SEM (*n* in parentheses) for the apparent dissociation constant (K_d) determined as outlined in Materials and Methods.

^b Significant differences (Student's *t* test) (*P* < 0.05) were not observed between K_d s for IMP α in the absence or presence of nsDNA (*P* = 0.4255).

^c LB, low binding; K_d not able to be determined.

^d Significant differences were not observed between K_d s for IMP β in the absence or presence of nsDNA (*P* = 0.6265).

Mutational analyses of Fli-1 NLS2. Introduction of point mutations within the region of the DNA binding domain of Fli-1 produced proteins that either accumulated in the nucleus or were distributed throughout the nucleus and cytoplasm (Fig.

5A to C). For example, mutation of K325 (f), K334 (h), R337 (i), R340 (j), K350 (m), and R355 (o) inhibited accumulation in the nucleus, resulting in the NLS2-GFP fusion protein being equally distributed throughout the nucleus and cytoplasm (52 to 56% nuclear accumulation), whereas mutation of K310 (b), R319 (c), R324 (e), and K354 (n) had no inhibitory effect on NLS2-GFP nuclear accumulation. Mutation of R320 (d), K327 (g), and K345 (k) had only a slight inhibitory effect on GFP-NLS2 nuclear accumulation (65 to 67% nuclear accumulation), so that the majority of the proteins was located in the nucleus. However, the levels of nuclear accumulation were reduced compared to those of the wt NLS2-GFP. Thus, the data indicate that the positively charged amino acids R320, K327, and K345 play a modest role in NLS2 of Fli-1, whereas the amino acids K325, K334, R337, R340, K350, and R355 are critical for the function of NLS2.

As for NLS1 mutants, pull-down assays were also performed using in vitro-translated NLS2-GFP mutants (Fig. 5D). Compared to the wt NLS2 region (a), the mutants K310A (b),

FIG. 7. Mutational analyses of NLS1 within the full-length Fli-1 GFP fusion proteins. (A) CLSM images of localization of the wt full-length Fli-1 and Fli-1 NLS1 mutants. Wt Fli-1 (a), Fli-1-GFP K67A (b), Fli-1-GFP R68A (c), Fli-1-GFP K87A (d), and Fli-1-GFP K90A (e) were transfected into HT and HeLa cells. The localization of GFP fusion proteins was determined 48 h after transfection. (B) GST pull-down results demonstrating the interaction of wt full-length Fli-1 (a) and full-length NLS1 mutants with IMP α or IMP β . Lane 1 contains 10% of the input in vitro-translated ³⁵S-labeled Fli-1-GFP protein. The other lanes show GFP fusion proteins retained by GST-IMP α , GST-IMP β , or GST.

FIG. 8. Role of NLS1 within Fli-1 in activation of Fli-1-inducible GPIX promoter. The activation of the Mk-specific promoter GPIX by wt or mutant Fli-1 in HT cells is shown. HT cells were transfected with 400 ng of the GPIX-567 luciferase reporter plasmid alone (bars 1) or in combination with increasing amounts of Fli-1-GFP expression plasmid (bars 2 to 4; 200, 400, and 800 ng, respectively). Wt Fli-1 (A) or NLS1 mutants, namely, Fli-1-GFP K67A (B), Fli-1-GFP R68A (C), Fli-1-GFP K87A (D), and Fli-1-GFP K90A (E). Cells were harvested 48 h posttransfection in passive lysis buffer, and 10 μ l was used in the luciferase assay. Values are expressed as the mean increases plus standard deviations relative to a value of 1 for each reporter. The results shown are the means from three experiments performed in triplicate.

FIG. 9. Role of NLS1 within Fli-1 in activation of Fli-1 inducible GPIb α promoter. Luciferase reporter assays were carried out as described in the legend to Fig. 8. Activation of the megakaryocyte-specific promoter GPIb α by wt Fli-1 and its NLS1 mutants in HT cells was investigated. HT cells were transfected with 400 ng of the GPIb α -253 luciferase reporter plasmid alone (non) or in combination with 200 ng of Fli-1-GFP expression plasmid (wt Fli-1) or Fli-1-GFP expression plasmid carrying NLS1 mutants (Fli-1-GFP K67A, Fli-1-GFP R68A, Fli-1-GFP K87A, and Fli-1-GFP K90A).

R319A (c), R320A (d), R324A (e), K327A (g), K345A (k), and K354A (n) had little to no effect on the ability of GFP-NLS2 to bind GST-IMP α (lane 2) and GST-IMP β (lane 3). Whereas the NLS2-GFP mutants K325A (f), K334A (h), R337A (i), R340A (j), K350A (m), and R355A (o) were either weakly retained or unable to be retained at all by GST-IMP α (lane 2) or GST-IMP β (lane 3). These same point mutations abolished the nuclear accumulation of the NLS2 region in transient-transfection studies. This indicates that the nuclear accumulation of the Fli-1 Ets domain appears to be dependent on the interaction of these positively charged amino acids with the IMPs.

DNA target sites effectively compete with the IMPs for binding the Fli-1 Ets domain. The Ets domain of Fli-1 that is responsible for its specific DNA binding spans amino acids 277 to 360 (30). Our previous data (11), together with the findings of this study, suggest that this region of Fli-1 has a dual function in DNA binding and nuclear import. If the Ets domain recognizes both DNA and the IMPs, we reasoned that Fli-1 might not be able to accommodate occupancy by both DNA and the IMPs simultaneously. There is thus a possibility that the specific DNA bearing a Fli-1 domain binding site in the GPIX promoter (the GPIX promoter DNA probe) may compete with the IMPs for binding to the Ets domain. To investigate this, we evaluated the influence of the GPIX promoter DNA probe and the nonspecific DNA (oligonucleotide with a

scrambled sequence) on Ets domain binding to IMP α or IMP β using three different types of binding assays.

GST-IMP α and GST-IMP β were incubated with in vitro-translated radiolabeled GFP-NLS2/Ets domain (the fragment containing NLS2 and the Ets domain) in the presence or absence of the GPIX promoter DNA probe or with nonspecific DNA (Fig. 6A). The NLS2/Ets domain clearly interacted with the IMPs in the absence of the GPIX promoter DNA probe (lanes 2 and 5), but preincubation of NLS2-GFP with the GPIX promoter DNA probe effectively abolished its interaction with the IMPs (lanes 3 and 6). The nonspecific DNA with a scrambled nucleotide sequence did not impair the ability of the NLS2/Ets domain to bind to the IMPs (lanes 4 and 7). The results shown in Fig. 6A indicate that the IMPs cannot bind to the NLS2/Ets domain when it is bound to the GPIX promoter DNA probe. The finding suggests that the IMPs and the GPIX promoter DNA probe have overlapping recognition sites within the NLS2/Ets domain.

The strong interaction of the NLS2/Ets domain with the GPIX promoter DNA probe was confirmed by an EMSA (Fig. 6B). Recombinant NLS2/Ets domain protein was incubated with a radiolabeled GPIX promoter DNA probe, and the appearance of an NLS2/Ets domain-DNA complex was readily detected. To determine whether the IMPs could influence NLS2/Ets domain binding to the GPIX promoter DNA probe, an EMSA was repeated with incubation of recombinant NLS2/Ets domain protein and increasing amounts (0.05, 0.5, and 5 μ g) of recombinant GST, GST-IMP α , or GST-IMP β prior to addition of the GPIX promoter DNA probe. Prior binding of GST or GST-IMP α to the NLS2/Ets domain has little or no influence on the ability of the GPIX promoter DNA probe to associate with the Fli-1 Ets domain. Even a molar concentration of GST, GST-IMP α , or GST-IMP β $\sim$ 3 orders of magnitude greater than that of the specific DNA only slightly decreased but did not abolish DNA binding to the Ets domain (Fig. 6B). This result indicates that the DNA corresponding to the Ets target site in the GPIX promoter has a higher relative affinity for the NLS2/Ets domain than the IMPs.

To quantitatively compare the binding affinities of IMP α and IMP β to the NLS2/Ets domain and to characterize the role of the GPIX promoter DNA probe in the release of the IMPs from the NLS2/Ets domain, the binding affinities of IMP α and IMP β for the NLS2/Ets domain in the presence or absence of the GPIX promoter DNA probe or nonspecific DNA were examined using an ELISA-based assay. Although the NLS2/Ets domain recognized IMP α with only low binding affinity (Fig. 6C and Table 1) ($K_d > 100$ nM), it bound IMP β with much higher affinity, with a K_d of $\sim$ 25 nM (Fig. 6C and Table 1). Clearly, IMP β showed a higher binding affinity (>5 -fold) for the NLS2/Ets domain than IMP α . Binding of IMP α and IMP β to the NLS2/Ets domain was completely abolished (Fig. 6C and Table 1) ($K_d > 1,000$ nM) in the presence of the GPIX promoter DNA probe, indicating that the GPIX promoter DNA probe can strongly compete with the IMPs for binding to the NLS2/Ets domain and can induce the release of the IMPs from the NLS/Ets domain. The specificity of the effects was indicated by the fact that the affinity and the maximal binding for IMP α and IMP β were not affected by the presence of the nonspecific DNA.

FIG. 10. Mutational analyses of NLS2 within the full-length Fli-1-GFP fusion proteins. (A) EMSA was performed as described in the legend to Fig. 6B to show the interaction of GPIXDNA and Ets domain GST fusion protein or the Ets domain NLS2 mutants. Lane 1 shows the binding of the wt Ets domain to GPIXDNA. The other lanes show NLS2 mutants of the Ets domain interacting with GPIXDNA. (B) CLSM images of localization of the full-length wt Fli-1 and full-length Fli-1 NLS2 mutants. Wt Fli-1 (a), Fli-1-GFP K325A (b), Fli-1-GFP K334A (c), Fli-1-GFP R337A (d), Fli-1-GFP R340A (e), Fli-1-GFP K350A (f), and Fli-1-GFP R355A (g) were transfected into HT and HeLa cells. The localization of GFP fusion proteins was determined 48 h after transfection. (C) GST pull-down results demonstrating the interaction of full-length wt Fli-1 (a) and its full-length NLS2 mutants with IMPα or IMPβ. Lane 1 contains 10% of the input in vitro-translated ³⁵S-labeled Fli-1-GFP protein. The other lanes show GFP fusion proteins retained by GST-IMPα, GST-IMPβ, or GST. (D and E) A luciferase reporter assay was carried out as described in the legends to Fig. 8 and 9. Activation of the Mk-specific promoter GPIX (D) or GPIBα (E) by wt Fli-1 and its NLS2 mutants in HT cells was investigated.

FIG. 10—Continued.

The NLS1 mutation in full-length Fli-1 does not affect transcription regulation of Fli-1-inducible Mk-specific genes. To determine whether NLS1 of Fli-1 is needed for maximum activation of transcription of Mk-specific genes, the four critical positively charged amino acids (K67, R68, K87, and K90) of NLS1 were mutated to the neutral amino acid alanine in full-length Fli-1. The mutated NLS1 fusion proteins were still able to accumulate in the nuclei of HT and HeLa cells, probably as a result of the intact NLS2 sequence (Fig. 7A). As expected, IMP α recognition, but not IMP β recognition, by the full-length Fli-1 NLS1 mutants was strongly disrupted (Fig. 7B). The results are fully consistent with the pull-down assay data for the NLS1 fragment (Fig. 4D).

Transactivation assays were carried out to cotransfect the plasmids encoding Fli-1 cDNA (wt or mutant) and the GPIX or GPIb α luciferase reporter in HT cells. Consistent with our previous report (11), Fli-1 activated the reporter to a significant level (Fig. 8A, lanes 2 to 4). Replacement of the lysine with alanine on K67 of Fli-1 (Fli-1-GFP K67A) leads to a nonfunctional NLS1 but had no effect upon the ability of Fli-1 to activate the reporter (Fig. 8B). Similar results were observed for other NLS1 mutants, namely, Fli-1-GFP R68A (Fig. 8C), Fli-1-GFP K87A (Fig. 8D), and Fli-1-GFP K97A (Fig. 8E); thus, the ability of each Fli-1 mutant to activate the GPIX reporter is either not affected or in some instances is even slightly better than that of the wt Fli-1. Similar results were also obtained for another Mk-specific gene, the GPIb α gene, where mutation of the N-terminal NLS did not impair the ability of Fli-1 to activate the GPIb α gene promoter (Fig. 9).

The NLS2 mutation reduces Fli-1 Ets domain DNA binding activity and consequently down regulates the expressions of Fli-1-inducible Mk-specific genes. To assess the functional consequences of mutation of NLS2 on Ets domain DNA binding activity, we next compared the DNA binding capacities of wt and six critical positively charged amino acid mutants of the

Fli-1 Ets domain (K325A, K334A, R337A, R340A, K350A, and R355A) using EMSAs as shown in Fig. 10A. Binding of K325A, R337A, R340A, and K350A recombinant Ets mutant proteins to the GPIX promoter DNA probe was not detectable, indicating that the mutation abolished the Ets DNA binding activity. However, DNA binding activities of K334A and R355A were decreased by ~ 10 - and 4-fold, respectively. These results are consistent with the finding in Fig. 5 and confirm that NLS2 overlaps the Ets domain DNA target site.

We also made mutations to these six critical positively charged amino acids of NLS2 in full-length Fli-1. The mutated NLS2 fusion proteins were still accumulated in the nucleus of HT and HeLa, probably as a result of the intact NLS1 sequence (Fig. 10B), which was similar to that of NLS1 mutants in full-length Fli-1. Compared to the wt Fli-1 (Fig. 10C, a), the six mutants had little or no effect on the ability of Fli-1-GFP fusion protein to bind GST-IMP α (b to g, lane 2), whereas the mutants were either weakly retained or unable to be retained at all by GST-IMP β (b to g, lane 3). These same point mutations abolished the IMP β recognition of the NLS2 fragment, as shown in Fig. 5D. Interesting results were observed for NLS2 mutants in the luciferase reporter assays of Fli-1-inducible Mk-specific genes (Fig. 10D and E), namely, Fli-1-GFP K325A, Fli-1-GFP R334A, Fli-1-GFP R337A, Fli-1-GFP R340A, Fli-1-GFP R350A, and Fli-1-GFP R355A, in which the ability of each Fli-1 mutant to activate the GPIX reporter was reduced dramatically, with Fli-1-GFP K325A, Fli-1-GFP K334A, Fli-1-GFP R337A, and Fli-1-GFP K350A completely losing the ability to activate the GPIX promoter. Although Fli-1-GFP R334A and Fli-1-GFP R355A were slightly better than the other mutants, both the mutants' inducible luciferase activities were threefold lower than that of wt Fli-1. Similar results were also obtained for another Mk-specific gene, that for GPIb α , in which mutation of the NLS2 in the Ets domain impairs the ability of Fli-1 to activate the GPIb α promoter

(Fig. 10E). Thus, these mutants disrupted DNA binding of the NLS2/Ets domain and consequently failed to activate the Mk-specific genes effectively. Fli-1-GFP K334A and Fli-1-GFP R355A mutants still bound DNA (though less than wt Fli-1) and yet failed to accumulate in the nucleus (Fig. 5B, h and o), indicating that the nuclear retention of the Ets domain fragment is not due to DNA binding but to its functional NLS.

The combined mutations of NLS1 and NLS2 in full-length Fli-1 abolish its nuclear accumulation and expression of Fli-1-inducible Mk-specific genes. To determine whether other functional NLSs could exist in Fli-1, the four combined NLS1-NLS2 mutants were made in full-length Fli-1, namely, Fli-1-GFP K87A-R334A (Fig. 11A, b), Fli-1-GFP K87A-R337A (Fig. 11A, c), Fli-1-GFP K90A-R340A (Fig. 11A, d), and Fli-1-GFP K87A-R355A (Fig. 11A, e). These combined NLS1-NLS2 mutations are not capable of significant nuclear accumulation of the Fli-1-GFP fusion proteins (Fig. 11A). A pull-down assay was also carried out to determine the binding between IMPs and these combined NLS1 and NLS2 mutants. Our data clearly indicated that the combined mutants were not recognized by IMP α or IMP β . As noted, the full-length Fli-1 NLS1 mutants Fli-1-GFP K87A and Fli-1-GFP K90A showed strong Fli-1-inducible GPIX gene expression, whereas the full-length NLS2 mutants Fli-1-GFP R334A or Fli-1-GFP R355A each resulted in $\sim 30\%$ of wt Fli-1-inducible GPIX gene expression. The combined NLS1-NLS2 mutants Fli-1-GFP K87A-R334A and K87A-R355A showed further reduction, i.e., to absolutely no transactivation activity. However, with the other two full-length combined mutants, Fli-1-GFP K87A-R337A and K90A-R340A, it was not possible to show an additional reduction in the GPIX promoter transactivation activity, since the NLS2 mutants Fli-1-GFP R337A or Fli-1-GFP R340A already had no transactivation activity. Similar results were also obtained for the GPIB α promoter. Our mutagenesis analysis of full-length Fli-1 strongly suggests that there are only two functional NLSs.

DISCUSSION

In the present study, for the first time two NLSs and one NES within Fli-1 were identified using functional criteria, NLS1 (the N-terminal region aa 62 to 126), NLS2 (the Ets domain, aa 277 to 360), and NES (the region aa 127 to 276). Subsequent analysis showed that these sites were both sufficient and necessary for normal nuclear import or export. The work presented here indicates that NLS1 and NLS2 are functional in determining the subcellular localization of full-length Fli-1 or truncated Fli-1 proteins in HT and HeLa cells, as well as the megakaryocytic cell lines K562 and UT-7/TPO. Our results indicate that Ets domain binding to GPIX promoter DNA effectively releases the IMPs and that NLS2 within the Ets domain alone is sufficient to target Fli-1 into the nucleus for activation of the Mk-specific GPIX and GPIB α genes. Moreover, the combined NLS1 and NLS2 mutation in full-length Fli-1 abolished Fli-1-mediated nuclear accumulation, and consequently, these combined mutants failed to activate the Mk-specific genes.

Nuclear localization signals and nuclear export signal in Fli-1. In this study, we have provided evidence that NLS1 within the N-terminal region of Fli-1 is a bipartite NLS and

binds predominantly to IMP α . More specifically, our mutagenesis data for both the truncated protein (Fig. 4D) and the full-length Fli-1 (Fig. 7B) indicated that the positively charged amino acids K67 and R68 in the N-terminal arm, as well as K87 and K90 in the C-terminal arm, are absolutely critical for a functional NLS1. Although the two arms of NLS1 are distant in the primary amino acid sequence, they may be positioned close together in the three-dimensional structure, allowing them to fit into the two binding pockets on IMP α , as revealed in the crystal structure of IMP α (7, 27).

As Fli-1 belongs to a growing family of transcription factors that are highly homologous in their Ets domains, Fig. 12A illustrates the consensus amino acid sequence of NLS2 within the Ets domain and several other Ets family members (3, 8, 32, 35, 36, 46). An inspection of the sequence reveals that many, but not all, amino acids are conserved, indicating important functional roles for residues that are common to all family members. Furthermore, of the six amino acids that constitute NLS2 in the Fli-1 Ets domain, five (K325, K334, R337, R340, and K350) are common to all family members and R355 is common to most, and where it is not common, there is still a positively charged amino acid. Based on this observation, together with our results demonstrating critical roles for these positively charged amino acids in nuclear localization and IMP binding of Fli-1, we propose that the NLS2 in the Ets domain of Fli-1 could be a common NLS of all Ets family members. Our mutagenesis and ELISA data showed that NLS2 within the Ets domain region is a nonclassical type of NLS and is recognized predominantly by IMP β with a K_d of ~ 25 nM; the K_d values for the interaction between Fli-1 NLS2 and importin β are similar to those of other NLS motifs, such as human retinoblastoma protein, yeast transcription factor GAL4, and plant transcription factor maize opaque (18). This finding is confirmed by an additional RanGTP experiment, in which NLS2 is dissociated from IMP β by the addition of a dominant GTP form of Ran (RanQ69L). Our ELISA data indicated that IMP β bound to NLS2 with a K_d of ~ 25 nM. The role of the secondary structure of NLS2 in determining import capacity may help to explain the NLS2 mutagenesis results. The Ets domain-NLS2 region, as shown in Fig. 5A, forms three α -helices and a short four-stranded antiparallel β -sheet. An α -helix (aa 284 to 294) is located near the N-terminal region of the Ets domain, followed by two β -strands. Residues 316 to 325 form a second helix, followed by a loop leading to a third helix (aa 332 to 345). At the C-terminal region of the Ets domain, two more β -strands pair with the first two strands to form a four-stranded antiparallel β -sheet. However, K325 is located in the second helix; K334, R337, and R340 are located in the third helix; and K350 and R355 are located in the third β -sheet. Thus, it is unlikely that the mutation that specifically impaired nuclear import of the Ets domain changed its secondary structure. As shown in the three-dimensional structure modeling (Fig. 12B), these six amino acids are situated close together. Whether the structure of the Ets domain is critical for the functional roles of these amino acids remains to be determined. In summary, the data presented in this study have led to the identification of two NLSs within Fli-1. Furthermore, it appears that Fli-1 can utilize nuclear import pathways mediated by both IMP α/β and IMP β .

We showed that amino acids 127 to 276 contain a signal for

FIG. 11. Combined NLS1 and NLS2 mutational analyses within the full-length Fli-1 GFP fusion proteins. (A) CLSM images of localization of the full-length wt Fli-1 and its combined full-length NLS1 and NLS2 mutants. Wt Fli-1 (a), Fli-1-GFP K87A-K334A (b), Fli-1-GFP K87A-R337A (c), Fli-1-GFP K90A-R340A (d), and Fli-1-GFP K87A-R355A (e) were transfected into HT and HeLa cells. The localization of GFP fusion proteins was determined 48 h after transfection. (B) GST pull-down results demonstrating the interaction of wt Fli-1 (a) and the combined NLS1 and NLS2 mutants with IMP α or IMP β . Lane 1 contains 10% of the input in vitro-translated ³⁵S-labeled Fli-1-GFP protein. The other lanes show GFP fusion proteins retained by GST-IMP α , GST-IMP β , or GST. A luciferase reporter assay was carried out as described in the legends to Fig. 8 and 9. Activation of the megakaryocyte-specific promoter GPIX (C) or GPIb α (D) by wt Fli-1 and the combined NLS1 and NLS2 mutants in HT cells was investigated.

FIG. 12. Comparison of NLS2-containing segment in the Fli-1 Ets domain and homologous sequences of other Ets family members. (A) Positively charged amino acids required for NLS2 of Fli-1 are depicted as boldface characters, with the boxes indicating their conserved positions. The conserved lysine and arginine of other Ets family members, namely, PTF, SAP-1, Elk-1, GABP α , Ets-1, and NERF, in the same positions are also shown as boldface characters. (B) The Ets domain of Fli-1 comprises three α -helices and a short four-stranded antiparallel β -sheet. The critical amino acids of NLS2 are shown in yellow.

the nuclear export of Fli-1. Deletion of the sequences corresponding to amino acids 197 to 276 from the GFP fusion protein abolished its export, suggesting that the amino acids in this region are important in contributing to the activity of the NES. LMB treatment led to equal nuclear and cytoplasmic distributions of the GFP fusion protein containing this region, indicating that CRM1 is the export receptor for this NES. Fli-1 contains both NLS and NES, suggesting Fli-1 may be a nucleocytoplasmic shuttling protein. Each NLS or NES may be regulated independently of the other. Protein phosphorylation in the vicinity of NLSs or NESs has been repeatedly shown to play a role in regulating nuclear import and export (20, 23, 29). Although we have not yet characterized the regulatory pathways of Fli-1 nuclear transport, there is evidence that Fli-1 expression can be modulated by different signal pathways. However, the precise residues that are phosphorylated have not yet been identified. Several serines, threonines, and tyrosines are found in the regions containing these NLSs and NES. They are potential consensus phosphorylation sites. It is not known which, if any, of these phosphorylation events may regulate the function of the NLSs and NES within Fli-1.

The Ets domain of Fli-1 appears to have multiple functions:

serving as an NLS for nuclear import, interacting with other proteins (e.g., GATA-1 and EKLF), and binding DNA (11, 42). However, its nuclear import function is distinct from its DNA binding activity, as the R334A or R355A mutant, which still retains significant DNA binding capability (Fig. 10A), fails to show strong nuclear accumulation. The Ets domain may have additional functions. After Fli-1 has entered the nucleus as the Fli-1/IMP complex, the abilities of specific DNA sequences to compete effectively with the IMPs for binding to the Ets domain may play a role in appropriate targeting of Fli-1 to its destination in the nucleus and in releasing the IMPs from Fli-1. Following the IMPs' release from their Fli-1 cargos, they can be exported back to the cytoplasm and used for another round of nuclear import (12, 28). The position of the NLS within the DNA binding domain of Fli-1 suggests an elegant mechanism to regulate cellular localization of this activator of transcription.

Roles of the NLSs within Fli-1 in activation of the Mk-specific genes GPIX and GPIb α . As we found with Fli-1, an increasing number of proteins have been described as having multiple NLSs. These include ERF (29), 5-lipoxygenase (24), BRCA1 (6), Epstein-Barr virus DNase (31), and the herpes

simplex virus products ICP22 (43) and XPG nuclease (26). The importance of having multiple NLSs is unclear. In the case of Fli-1, each NLS is capable of mediating nuclear import of the full-length Fli-1-GFP fusion protein. Furthermore, the actions of multiple NLSs can be additive. This is evident from our quantitative confocal laser scanning microscopy (CLSM) analysis, in which NLS1 and NLS2 by themselves contribute ~74 and 86% nuclear accumulation of GFP-Fli-1, respectively. When they are combined, an additive effect is observed (95% nuclear accumulation). Full-length Fli-1 mutants defective in NLS1 are still able to accumulate in the nucleus and fully activate the Mk-specific GPIX and GPIIb α genes. Full-length Fli-1 mutants defective in NLS2 are also able to accumulate in the nucleus, but their abilities to activate the Mk-specific genes are greatly reduced. Our EMSA data showed that mutations to the six critical positively charged amino acids of NLS2 resulted in either reducing or completely disrupting DNA binding of the Ets domain. This result led us to suggest that the reduced or absent luciferase reporter activities of the Fli-1 NLS2 mutants are related to the reduced or markedly impaired DNA binding activities.

All four combined NLS1 and NLS2 mutants were distributed evenly between the nucleus and cytoplasm and lost the ability to activate the Mk-specific genes. We thus conclude that there are only two functional NLSs within Fli-1. Either NLS1 or NLS2 is sufficient to target full-length Fli-1 into the nucleus for activation of the Mk-specific genes. However, two NLSs in a protein such as Fli-1 could be designed for optimal activity. We speculate that two NLSs will ensure adequate nuclear import of Fli-1 if either of its NLSs is disrupted by gene mutation or masked by another protein. Indeed, the redundancy of having two NLSs in Fli-1 could be critically important when Fli-1 interacts with GATA-1 or EKLF, as we and others have shown that GATA-1 or EKLF interacts with Fli-1 via its Ets domain (11, 42). Transcription factors and nuclear proteins are differentially expressed in different cell types and at different stages of cell development, and they may bind Fli-1 at various sites. Thus, the NLS that is actually functional in a given cellular circumstance may depend on the site of Fli-1 that binds its interacting protein.

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