Basic Residues within the Kruppel Zinc Finger DNA Binding Domains Are the Critical Nuclear Localization Determinants of EKLF/KLF-1∗

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EKLF/KLF-1 is an erythroid-restricted transcription factor essential for expression of the adult β-globin gene. EKLF/KLF-1 is a 358-amino acid nuclear protein with an amino-terminal proline-rich domain and a carboxy-terminal DNA binding domain. The nuclear localization signal (NLS) of EKLF/KLF-1 has not been empirically determined. We generated a series of epitope-tagged deletion and point mutants and assessed their subcellular localization. Our results delimit the NLS to the 83-amino acid (amino acids 276–358) DNA binding domain that consists of three Kruppel zinc fingers. All three zinc fingers are necessary for efficient nuclear localization; deletion of any individual finger results in cytoplasmic accumulation. Fusion of the three zinc fingers to green fluorescent protein (GFP) targeted GFP to the nucleus, demonstrating that the zinc finger domain is sufficient for nuclear localization. EKLF/KLF-1 containing histidine to alanine mutations that disrupt the structure of all three fingers retains appropriate nuclear localization, indicating that neither the tertiary structure of the zinc fingers nor specific DNA binding are necessary for nuclear localization. We demonstrate that basic residues within the fingers are the critical determinants for nuclear localization; mutations of these basic residues to alanine resulted in cytoplasmic mislocalization. The basic residues of all mammalian Kruppel zinc fingers are highly conserved; therefore we propose that these basic residues are a common NLS shared by all Kruppel family members.

The Sp/KLF family of transcription factors is an important family of proteins that plays critical roles in diverse aspects of mammalian development (1–3). Individual members function as transcriptional activators, repressors, or both and exert a wide range of molecular effects in cellular proliferation, differentiation, and malignancy. Gene knockout studies of several members have defined crucial roles in embryonic (Sp1, KLF1, KLF2) and post-natal (Sp4, KLF4) development (reviewed in Refs. 1 and 3). The family is defined by a highly homologous DNA binding domain (DBD) situated at or very close to the carboxyl terminus. The DBD consists of three C2H2-type zinc fingers that are similar to the Drosophila Kruppel protein. A second similarity shared by the family members is a characteristic amino acid (aa) sequence designated the Kruppel-link that connects individual zinc fingers (4). The members display no other significant homology in any other part of the protein. EKLF/KLF1 was one of the founding members of this family (reviewed in Ref. 5). This erythroid-specific transcription factor binds the β-globin promoter and activates high level expression. The β-globin promoter is a 358-aa protein consisting of an amino-terminal proline-rich transactivation domain (aa 1–275) and a carboxy-terminal DBD (aa 276–358). The DBD, consisting of three C2H2 Kruppel-like zinc fingers, binds specifically to the CCACACCT motif at −90 of the β-globin promoter. Gene ablation studies have demonstrated a critical role for EKLF/KLF1 in consolidating the switch between the fetal γ- to the adult β-globin gene expression; EKLF/KLF1−/− embryos display drastically reduced β-globin gene expression and die at E15.5 due to severe lethal anemia (6, 7). EKLF/KLF1 is also thought to exert important functions at the β-globin locus control region and other as yet undetermined erythroid target genes whose expression is necessary for survival (8, 9).

Although a large amount of information suggests a crucial role for EKLF/KLF1 in globin gene expression and adult hematopoiesis, the molecular mechanisms underlying its functions are not clearly understood. Several studies focus on a structure-function analysis of EKLF/KLF1 to yield insights into its functions (10–12). These studies identify discrete domains involved in transactivation (aa 1–104 and 140–225), chromatin remodeling (aa 225–358), and inhibition of DNA binding (aa 176–271). However, despite all this information, the sequences necessary for its nuclear localization are unknown. Studies conducted on a closely related family member GKLK/KLF4 have led to the proposal that the nuclear localization signal (NLS) of EKLF/KLF1 localizes to a 12-aa basic stretch at position 260 (13); however, this has not been experimentally confirmed. In this report we have identified and characterized the NLS of EKLF. The NLS localizes to the zinc finger DBD and is both necessary and sufficient for nuclear localization. We also demonstrate that the basic residues within this region are the critical determinants for nuclear localization. Comparison of the zinc finger sequences among the Kruppel family members indicate that the basic residues are almost perfectly conserved among them, suggesting that these residues could be part of an NLS common to all Kruppel members.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The plasmids HA-EKLF, HA-Δ44–254, HA-Δ255–358, and HA-Δ139–225 have been described previously (12). Unless noted otherwise, the plasmid HA-EKLF was used as the parental plasmid for the generation of all mutants. Restriction enzymes SmaI, MsII, BsaI, BspMI, and PflM1 cut at positions corresponding to codons

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† The abbreviations used are: DBD, DNA binding domain; aa, amino acids; NLS, nuclear localization signal; HA, hemagglutinin; kb, kilobase; bp, base pair; NES, nuclear export signal; PIPES, 1,4-piperazinediethanesulfonic acid; LMB, leptomycin B; GFP, green fluorescent protein.
Fig. 1. EKLF/KLF1 DNA binding domain is necessary for nuclear localization. A, schematic of HA-EKLF deletion constructs. The DNA binding domain is shown as three striped boxes at the carboxyl terminus, with each box depicting a Kruppel-like zinc finger. The heavy striped box at the amino terminus shows the HA epitope. The solid box represents a basic sequence at position aa280 previously proposed to be the NLS for EKLF. The subcellular localization of each mutant is summarized on the right. \( N \) represents completely nuclear localization (see C, panels D–F, for representative staining). \( C=N \) represents diffuse straining throughout the cell (see C, panels J–L, for representative staining). B, protein expression levels of the deletion mutants. COS cells were cotransfected with the indicated plasmid HA-\( \Delta256–276 \) and 256, 276, 277, 298, and 333, respectively. Plasmid HA-\( \Delta276–358 \) was generated by ligating two fragments, a 0.8-kb EcoRI-MsI I fragment and a 4.2-kb EcoRI-BamHI fragment (the BamHI end was blunted with Klenow). The plasmid HA-\( \Delta256–276 \) was generated in two steps. First, an intermediate plasmid was generated by ligating two fragments, a HA-\( \Delta130–250 \) fragment and a 170-bp BsoI-PfI fragment. A 330-bp MslI-BamHI fragment from this plasmid was then ligated with a 5.1-kb MslI-BamHI fragment from HA-EKLF.

HA-\( \Delta256–276 \) was generated by ligating two fragments, a 0.9-kb EcoRI-MsI I fragment and a 4.2-kb EcoRI-BspMI fragment (the BspMI end was blunted with mung bean nuclease). HA-\( \Delta276–358 \) was generated by digesting the HA-EKLF plasmid with BspMI and PfI. The BspMI end was blunted with Klenow, and the PfI end was blunted with mung bean nuclease. The 5.1-kb BspMI(blunt)-PfI(blunt) fragment was gel-purified and self-ligated. Plasmid HA-\( \Delta276–358 \) was generated by ligating a 0.9-kb EcoRI-MsI I fragment with a 4.2-kb EcoRI-PfI fragment (the PfI end was blunted with mung bean nuclease). HA-\( \Delta276–358 \) was generated by digesting the HA-EKLF plasmid with BspMI and BamHI. The BspMI and BamHI ends were blunted with Klenow, and the 5.1-kb BspMI(blunt)-BamHI(blunt) fragment was gel-purified and self-ligated. Plasmid HA-\( \Delta276–358 \) was generated by ligating two fragments, a 0.9-kb EcoRI-MsI I fragment and a 4.2-kb EcoRI-PfI fragment (the PfI end was blunted with mung bean nuclease). HA-\( \Delta276–358 \) was generated by digesting the HA-EKLF plasmid with BspMI and BamHI. The BspMI and BamHI ends were blunted with Klenow, and the 5.1-kb BspMI(blunt)-BamHI(blunt) fragment was gel-purified and self-ligated. Plasmid HA-\( \Delta276–358 \) was generated by ligating two fragments, a 0.9-kb EcoRI-MsI I fragment and a 4.2-kb EcoRI-PfI fragment (the PfI end was blunted with mung bean nuclease).

The HA-H295A, H325A, H353A plasmid was generated in several steps. First, plasmid HA-H295A was generated using PCR-based mutagenesis utilizing the following primers: upstream, 5'-GGTGCGCGCCAGGCTGCGTGCTGACT-3'; downstream, 5'-CTGACACTTAGCTCTGGCCATGCGTGTGCGTGCGCAGGGCGCGCTTGAG-3'. The mutant codon changing histidine to alanine is underlined. The PCR product was digested with SacI and BamHI, and self-ligated. Plasmid HA-H325A was generated by ligating a double stranded oligomer with SacI ends (top, 5'-GGACCTGCGCTGCTGCAATGAGAGGACAGACAGGAGTTG-GAGCAG-3') with a 5.9-kb PfI fragment (from HA-EKLF). The mutant codon changing histidine to alanine is underlined. The HA-H325A plasmid was generated by ligating a double-stranded oligomer with BamHI and PfI ends (top, 5'-GGACCTGCGCTGCTGCAATGAGAGGACAGACAGGAGTTG-GAGCAG-3'; downstream, 5'-AGTCCAGTGGGCTGAGAGGACAGACAGGAGTTG-GAGCAG-3') with a 5.9-kb PfI fragment and a 4.9-kb MslI-BamHI fragment. The mutant codon changing histidine to alanine is underlined. Plasmid HA-H325A was generated by ligating three fragments, a 0.9-kb EcoRI-FspI fragment (from HA-H295A plasmid), a 0.3-kb FspI-XbaI fragment (from HA-H325A plasmid), and a 4.2-kb EcoRI-XbaI fragment. Finally, a 1.0-kb EcoRI-PfI fragment (from HA-H295A, H325A plasmid) was ligated with a 4.2-kb EcoRI-PfI fragment (from HA-H325A) to generate HA-H295A, H325A, H353A. Plasmid HA-H295A, H325A, H353A was generated by ligating two fragments, a 0.3-kb SapI-BamHI fragment (from HA-H295A, H325A, H353A) and a 4.9-kb SapI-BamHI fragment from HA-\( \Delta256–276 \).

Oligomer-based mutagenesis was used to generate HA-EKLF mutant constructs together with CMV-Lac-Z and protein extracts (~10 \( \mu \)g) were analyzed by immunoblotting with anti-HA antibody. The protein extracts were normalized by \( \beta \)-galactosidase activity to control for transfection efficiency. Protein markers are depicted on the left. C, subcellular localization of wild type and mutant HA-EKLFs. COS cells were grown on coverslips and transfected with the indicated HA-EKLF mutant construct. After 48 h, indirect immunofluorescence was performed using 12CA5 anti-HA primary antibody and fluorescein isothiocyanate (FITC) anti-mouse secondary antibody. Mounting media contained propidium iodide, and cells were viewed through a fluorescence microscope using different color filters. The fluorescein isothiocyanate filter depicts the cells expressing HA-tagged protein in green (panels A, D, G, J, M, and P); the Texas red filter depicts propidium iodide stained nuclei in red (panels B, E, H, K, N, and Q); and two-color merge demonstrates co-localization (panels C, F, I, L, O, and R). A representative cell for each construct is shown, and the subcellular localization for each is indicated on the right. One hundred cells/translation were analyzed in three independent experiments, and the predominant localization category is indicated for each mutant. Table 1 lists a detailed analysis of the scoring for the subcellular localization of each construct.
Nuclear Localization Sequence of EKLF/KLF1

TABLE I
Subcellular distribution of mutant EKLFs

| Construct | % N | % N>C | % C=N |
|-----------|-----|-------|-------|
| HA-EKLF | 99 ± 1 | 1 ± 1 | 0 |
| HA-Δ4-254 | 96 ± 2.6 | 3.6 ± 2.5 | 0.3 ± 0.5 |
| HA-Δ355-358 | 0 | 1 ± 1 | 99 ± 1 |
| HA-Δ256-276 | 97 ± 2 | 2.6 ± 2.6 | 0.3 ± 0.5 |
| HA-Δ276-358 | 0 | 0.33 ± 0.5 | 99 ± 0.5 |
| HA-ZF1 | 14.2 ± 7 | 85.7 ± 0.7 | 0.3 ± 0.5 |
| HA-ZF2 | 6.3 ± 5.5 | 92.5 ± 5.5 | 0 |
| HA-ZF3 | 4 ± 3 | 96 ± 3 | 0 |
| HA-ZF1,2 | 1 ± 1 | 17.6 ± 8.6 | 81.3 ± 9.3 |
| HA-ZF2,3 | 1 ± 1 | 8.6 ± 2.5 | 90 ± 4 |
| HA-ZF1 | 17.6 ± 4.6 | 2.6 ± 4.6 | 92.3 ± 5 |
| HA-EKLF(mZF1) | 96.6 ± 2.5 | 3.3 ± 2.5 | 0 |
| HA-EKLF(mZF2) | 23.6 ± 5.5 | 75.6 ± 6.1 | 0.6 ± 1.1 |
| HA-EKLF(mZF3) | 20.6 ± 6.5 | 79 ± 6.5 | 0.3 ± 0.5 |
| HA-EKLF(mZF1,2) | 3 ± 2 | 68 ± 10.1 | 29 ± 1 |
| HA-EKLF(mZF2,3) | 5.6 ± 3.5 | 92.6 ± 4.5 | 1.3 ± 1.5 |
| HA-EKLF(mZF2,3) | 96.6 ± 1.5 | 2.35 ± 1.5 | 0 |
| HA-EKLF(mZF1,3) | 99 ± 1 | 0.3 ± 0.5 | 0.6 ± 0.5 |
| HA-ZF1,2,3 | 95.3 ± 4 | 4.3 ± 3.5 | 0.3 ± 0.5 |

GCGTCTTTTTTGGCGGCGTCCGCCTCGAATGGCAGATCGCCTCAGTTGGCGG-3' into plasmid pEGP-C2 digested with EcoRI and BamHI. The sequence encoding the human immunodeficiency virus Rev NES is shown in bold and represents the peptide LPLLELTL, whereas the sequence encoding the NLS is designated in italics and represents the NLS peptide PKKTRVK from SV40 large T antigen protein. All plasmids were verified by sequencing.

**Nuclear Fractionation**

Nuclear fractionation was performed as described in Greenwood and Johnson (14). Briefly, 48 h after transfection, COS cells were washed and harvested in phosphate-buffered saline. The cell pellet was lysed two times in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.05% Nonidet P-40, 1 mM EDTA). The nuclei were then spin-dissolved by isotropic centrifugal force for 5 min and washed twice with wash buffer (300 mM sucrose, 10 mM PIPES, pH 6.8, 3 mM MgCl₂, 1 mM EDTA, 25 mM NaCl). The nuclei were then purified over a sucrose cushion (1 ml sucrose in wash buffer) and resuspended in Buffer B (wash buffer with 0.5% Triton X-100). The nuclei were then incubated briefly on ice and spun at 1.2 relative centrifugal force for 5 min. The supernatant yielded the nucleosol fraction. The cell pellet was lysed two times in radioimmune precipitation buffer. Anti-tubulin antibodies (catalog #MAB052), and GFP plasmid (pEGFP-C2) was purchased from CLONTECH.

**RESULTS**

We used our previously established assay to define the sequences necessary for nuclear localization of EKLF/KLF1 (12). In this assay, HA epitope-tagged EKLF/KLF1 (wild type or mutant) constructs were transfected into COS cells, and HA-EKLF/KLF1 proteins were detected by indirect immunofluorescence with a fluorescein isothiocyanate-labeled anti-HA antibody (Fig. 1C, shown in green). Propidium iodide staining (red) was utilized to define the nucleus, and a two-color merge was used to assess subcellular localization. As shown in Fig. 1C, the wild type EKLF/KLF1 protein (HA-EKLF) localized exclusively to the nucleus (panels D, F) in more than 99% of transfected cells (Table I). Cells transfected with vector alone show very little background (Fig. 1C, panels A-C).

We had previously demonstrated that all signals necessary for nuclear localization are contained within a carboxyl-terminal region (aa 255–358) (12). A mutant (HA-Δ255–358, Fig. 1A) with this region deleted is located in the cytoplasm (Fig. 1C, panels J–L), whereas a mutant (HA-Δ4–255, Fig. 1A) containing only this sequence is localized to the nucleus (Fig. 1C, panels I–K).
panels G–I). This region consists of the DBD (aa 275–358) and a putative basic NLS-like sequence PKRSRRTLAPKR at position aa 260. This basic sequence has been proposed to be the NLS for EKLF/KLF1 because it is highly homologous to the NLS of the closely related family member GKL/KLF4 (13). To test this hypothesis, we deleted a 20-aa region encompassing NLS of the closely related family member GKLF/KLF4 (13). To find exclusively in the nucleus (Fig. 1C, panels M–O), indicating that this sequence is dispensable for nuclear localization. However, a mutant with the DBD deleted (HA-Δ276–358, Fig. 1A) was found to accumulate in the cytoplasm (Fig. 1C, panels P–R). These results indicate that the DBD (aa 275–358) is necessary for nuclear localization.

We next carried out experiments to further delimit the NLS within the DBD. This domain consists of three Kruppel zinc finger motifs that are highly homologous to each other. We evaluated the contribution of each zinc finger motif to nuclear localization. A series of mutants were generated that deleted any one or any two zinc fingers. Fig. 2A illustrates a schematic of these deletions. Western blot analysis conducted on cell extracts transfected with these mutants indicated that the mutants were expressed at wild type levels (Fig. 2B). Results from indirect immunofluorescence show that deletion of any one zinc finger leads to partial mislocalization of the protein (Table I and Fig. 2C, panels D–F, for representative staining). Deletion of any two zinc fingers results in diffuse staining throughout the cell (Table I and Fig. 2C, panels G–I, for representative staining). These experiments indicate that all three zinc fingers are necessary for efficient nuclear localization of EKLF/KLF1. We next determined if the three zinc fingers were sufficient for nuclear localization. The three zinc fingers were fused to GFP (GFP/ZF1,2,3, see Fig. 6A) and examined for subcellular distribution. GFP alone is found in the cytoplasm and gives rise to diffuse staining throughout the cell (Fig. 6B, panel A). As shown in Fig. 6B (panel B) the three zinc fingers targeted GFP predominantly to the nucleus. Taken together, these results demonstrate that the three zinc fingers of EKLF/KLF1 encode an NLS that is both necessary and sufficient for nuclear localization.

The NLS of several zinc finger-containing proteins are localized to their zinc fingers (15–20), and the tertiary zinc finger structure is crucial for nuclear targeting activity for some of them (16, 18). To determine whether zinc finger structure is essential for EKLF nuclear localization, we generated histidine to alanine point mutations in the first zinc-chelating histidine of all three zinc fingers (HA-H295A, H325A, H353A, Fig. 3A). These mutations have been shown previously to destabilize zinc finger tertiary structure (18, 21). The mutant protein was expressed at wild type level (Fig. 3B). When tested for subcellular distribution, the results demonstrate that the mutant protein was localized exclusively to the nucleus (Fig. 3C, panels D–F). To verify that the mutations did destabilize the zinc finger structure, we tested the transactivation capacity of this mutant in a transient transactivation assay. The histidine to alanine mutations should inhibit the formation of a stable zinc finger structure and, thus, prevent the protein from binding and activating the β-globin promoter. Although wild type EKLF/KLF1 activated the β-promoter 500-fold relative to controls, the mutant failed to transactivate the β-globin promoter (Fig. 3D). This result confirms that the histidine mutations destabilized zinc finger structure and suggests that the NLS is independent of zinc finger tertiary structure and sequence-specific DNA binding. Alternatively, the nuclear accumulation of this mutant could result from a redundant nuclear-targeting activity encoded by the NLS-like sequence at position aa 260.
Fig. 3. Tertiary zinc finger structure is not necessary for nuclear localization. A, schematic of different HA-EKLF mutant constructs. The first zinc-chelating histidines of all three fingers are mutated to alanine and are denoted by an asterisk. The shaded boxes represent the same features described in Fig. 1. The subcellular localization of each mutant is summarized on the right. Table I lists a detailed analysis of the scoring for subcellular localization of each construct. B, protein expression levels of the mutants. The indicated constructs were transfected into COS cells, and Western blot analysis was conducted as described in Fig. 1B. C, subcellular localization of wild type and mutant HA-EKLFs. Indirect immunofluorescence was conducted on the indicated constructs and analyzed as described in Fig. 1C. FITC, fluorescein isothiocyanate. D, histogram of transactivation analysis. The schematic of the reporter HS2γ-Luc-β/CAT is depicted at the top. This construct has been described previously and contains a human 1.5-kb KpnI-BglII HS2 fragment linked to a human γ-globin gene promoter (−299 to +37) driving the firefly luciferase (Luc) gene and a human β-globin promoter (−265 to +48) driving the chloramphenicol acetyltransferase (CAT) gene. This reporter was cotransfected with the indicated constructs together with CMV-Lac-Z into K562 cells. Chloramphenicol acetyltransferase activity was normalized to the β-galactosidase levels. The level for vector alone was set at 1×, and the activities of the wild type and mutant constructs are expressed relative to this value. E, biochemical fractionation of the nuclei. COS cells were transfected with the indicated constructs for 48 h. The nuclei were isolated and fractionated into chromatin (lanes 1, 3, and 5) and nucleosol (lanes 2, 4, and 6) fractions. The top panel shows an SDS/PAGE conducted on the different fractions (protein equivalent from 50,000 cells) and stained with Coomassie to show distinct protein species present in each fraction. The second panel depicts a Western blot analysis performed on the fractions using an anti-histone antibody. The third panel shows a Western blot analysis using anti-tubulin antibody. The bottom panel depicts a Western blot using anti-HA antibody as described in Fig. 1. The same blot was stripped and re-probed with the different antibodies. Whole cell extracts (WCE) from untransfected cells were run in lane 7 as a control. The protein markers are illustrated on the left of each panel.
To test this possibility, we constructed a mutant that deleted the putative NLS-like sequence (aa 256–276), whereas retaining the three histidine point mutations (HA-Δ256–276, H295A, H325A, H353A, Fig. 3A). When tested for subcellular distribution, our results demonstrate that this mutant protein is localized exclusively to the nucleus (Fig. 3C, panels G–I). These data exclude a role for this sequence (aa 256–276) in nuclear localization. All of these data indicate that the NLS

Fig. 4. Role of basic residues of the zinc finger region in nuclear localization. A, zinc finger amino acid sequences of different mutant constructs. The amino acid sequence of wild type zinc fingers is shown at the top (HA-EKLF). The basic residues are highlighted in bold. The amino acid sequences of different mutants are depicted below the wild type. Basic residues within indicated zinc fingers were mutated to alanine and are shown in bold. The dashes represent amino acids that were not changed and, thus, represent the wild type amino acid at that position. The subcellular localization of each mutant is summarized on the right. A representative staining of each category is shown in C. Table I lists a detailed analysis of the scoring for subcellular localization of each construct. B, protein expression levels of the mutants. The indicated constructs were transfected into COS cells, and Western blot analysis was conducted as described in Fig. 1 B, C, subcellular localization of representative categories. Indirect immunofluorescence was conducted on the indicated constructs and analyzed as described in Fig. 1 C. Panels A–C illustrate a representative example for nuclear staining (N; HA-EKLF), panels D–F illustrate a representative example of predominantly nuclear staining (N>C; HA-EKLF(mZF2)), and panels G–I illustrate a representative example of diffuse staining throughout the cell (C>N; HA-EKLF(mZF1,2,3)). FITC, fluorescein isothiocyanate.
encoded by the three zinc fingers is independent of its tertiary structure and sequence-specific DNA binding.

The nuclear localization signals of many transcription factors are localized to the DBD, and LaCasse and Lefebvre (22) propose that nuclear localization is a result of nonspecific binding to DNA (for review, see Ref. 22). These investigators suggested that sequence-independent protein-DNA associations result in selective retention and accumulation of transcription factors in the nucleus. If this hypothesis is true for EKLF/KLF1, one would predict that both wild type and zinc finger mutant (HA-H295A,H325A,H353A) proteins, which are observed in the nucleus, would be associated with chromatin. To test this prediction, we performed a biochemical fractionation assay to assess the sub-nuclear localization of these proteins. Cells were transfected with constructs encoding wild type or mutant EKLF; nuclei were then purified and fractionated into nucleosomal and chromatin fractions. Fig. 3E illustrates these fractions after electrophoresis on a SDS-PAGE gel and staining with Coomassie Blue. The purity of different fractions was confirmed by conducting Western blot analysis using chromatin (histone) and cytosolic (tubulin) markers. As shown in the Fig. 3E, histone proteins were present only in the chromatin fractions, and tubulin was absent from both chromatin and nucleosolic fractions. When the chromatin and nucleosolic fractions were tested for EKLF/KLF1 proteins, the results demonstrated that both wild type and mutant proteins were associated exclusively with the chromatin fraction (Fig. 3E, bottom panel). These results suggest that protein-DNA associations are important for nuclear accumulation. Biophysical studies of zinc finger proteins have demonstrated that nonspecific protein-DNA interactions are retained in the absence of tertiary zinc finger structures (23). Our observations that tertiary zinc finger structures are dispensable for EKLF/KLF1 nuclear localization are consistent with these studies. All of these data suggest that the zinc finger region may function as an NLS due to nonspecific associations with DNA.

Structural and biophysical studies of DNA-protein interactions show that nonspecific interactions with DNA are predominantly mediated through basic residues, which interact with the negatively charged phosphodiester backbone of DNA (24–27). The primary amino acid sequence of EKLF/KLF1 zinc fingers contains a total of 15 basic residues; four, six, and five basic residues are present in the first, second, and third zinc fingers, respectively (Fig. 4A). We directly assessed the roles of these basic residues for nuclear localization. All basic residues in any one, any two, or all three zinc fingers were mutated to alanine. Interestingly, mutations of basic residues in zinc finger 2 or zinc finger 3 resulted in a partial mislocalization of the protein to the cytoplasm (HA-EKLF(mZF2), HA-EKLF(mZF3), Fig. 4C, panels D–F, Table I). Importantly, the mutation of all 15 basic residues contained within the three zinc fingers resulted in a protein that is predominantly localized to the cytoplasm (HA-

Fig. 5. Cytoplasmic mislocalization of the mutant HA-EKLF(mZF1,2,3) does not result from exposure of a cryptic NES. COS cells were transfected with either HA-EKLF(mZF1,2,3) (panels A and B) or GFP/NES/NLS (panels C and D) for 48 h, and recombinant proteins were visualized by immunofluorescence microscopy. Cells were either untreated (panels A and C) or treated with 10 ng/ml LMB for 4 h (panels B and D). GFP/NES/NLS consists of the human immunodeficiency virus-Rev NES (LPPLERLTL) and SV-40 NLS (PKKKRKV) fused to GFP. This protein dynamically shuttles into and out of nucleus. Treatment of cells with the nuclear export inhibitor LMB inhibits nuclear export, leading to accumulation of the protein within the nucleus. This construct serves as a positive control for the effects of LMB. The subcellular localization of each panel is indicated on the top right of the panel. One hundred cells/transfection were analyzed in three experiments, and more than 95% of transfected cells showed the representative staining.

Fig. 6. The three zinc fingers are sufficient for nuclear localization. A, schematic of different constructs. The GFP/ZF1,2,3 construct is a fusion of the three zinc fingers to GFP. GFP/mZF1,2,3 is the same as GFP/ZF1,2,3 except that all basic residues within the zinc fingers are mutated to alanine (each change is denoted by an asterisk). The subcellular localization category of each mutant is summarized on the right. B, subcellular localization of the different constructs. COS cells were transfected with the indicated constructs, and direct immunofluorescence was conducted as described in Fig. 5. Representative cells from each construct are shown. The subcellular localization of each mutant is summarized on the right. One hundred cells/transfection were analyzed in three experiments, and more than 80% of them showed the representative staining.

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Fig. 7. Nuclear Localization Sequence of EKLF/KLF1

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FIG. 7. Basic residues of the zinc fingers are highly conserved among the Kruppel family members. The primary amino acid sequence of the EKLF/KLF1 zinc fingers are depicted at the top (EKLF ZF), and the consensus amino acid sequence of the 17 Kruppel members (adapted from Turner and Crossley (4)) are shown at the bottom. Basic residues are depicted in bold, and a box indicates their conserved positions.

EKLF(mZF1,2,3), Fig. 4C, panels G–I). These results indicate that the basic residues within the DBD play an important role in nuclear localization.

We next tested whether cytoplasmic accumulation of HA-EKLF(mZF1,2,3) results from a cryptic NES that is exposed as a consequence of the mutations. Cells transfected with the mutant were treated with the nuclear export pathway inhibitor leptomycin B (LMB). This compound binds and inhibits the function of Crm1 protein, a critical mediator of nuclear export pathways (28, 29). Interestingly, LMB treatment had no effect on the cytoplasmic accumulation of this mutant protein (Fig. 5, panels A and B). This result suggests that cytoplasmic accumulation of the mutant protein results from NES disruption and not from exposure of a cryptic NES. As a positive control for LMB activity, we generated a construct GFP/NES/NLS that fuses GFP with a NES (LPPPLERLTL) from human immunodeficiency virus Rev protein and a NLS (PKKKRKV) from SV40 large T antigen. GFP/NES/NLS shuttles continuously into and out of the nucleus, resulting in diffuse staining throughout the cell (Fig. 5, panel C). As expected, LMB treatment of these cells resulted in the nuclear accumulation of GFP/NLS/NES (Fig. 5, panel D).

Finally, all 15 basic residues within the three zinc fingers of the nuclear protein GFP/ZF1,2,3 were mutated to alanine (Fig. 6A). When a construct encoding this mutant protein was transfected into cells, GFP/mZF1,2,3 distributed diffusely throughout the cell (Fig. 6B, panel C). This result further demonstrates that these basic residues are directly involved in nuclear localization of EKLF/KLF1.

**DISCUSSION**

This report demonstrates that the NLS of EKLF/KLF1 is contained specifically within the three Kruppel zinc fingers. Deletion of the three zinc fingers results in a protein that is localized to the cytoplasm (HA-ΔZF1–276, panels A–C). All three zinc fingers are necessary for efficient nuclear localization; deletion of any one zinc finger results in partial loss of nuclear targeting (Fig. 2A, HA-ΔZF1, HA-ΔZF2, HA-ΔZF3), whereas deletion of any two zinc fingers resulted in predominant cytoplasmic accumulation (Fig. 2A, HA-ΔZF1,2, HA-ΔZF2,3, HA-ΔZF1,3). The three zinc fingers were sufficient to direct a heterologous protein to the nucleus (Fig. 6B, compare GFP/ΔZF1,2,3 with GFP). The ability of the zinc fingers to target EKLF/KLF1 to the nucleus is independent of finger tertiary structure and sequence-specific DNA binding; histidine to alanine mutations in the zinc fingers resulted in appropriate nuclear localization (Fig. 3C, HA-ΔH295A, H295A, H353A). Our results also demonstrate that the putative NLS-like sequence at position aa 260 does not play a critical role in nuclear targeting; EKLF/KLF1 proteins lacking this sequence are faithfully localized to the nucleus (Fig. 1C, HA-Δ256–276, panels M–O; Fig. 3C, panels G–I).

The NLS of several zinc finger proteins (Zif268, neuron restrictive silencer factor (NRSF), Wilms’ tumor 1 (Wt1), JAZ, mouse orphan receptor (TR2), tristetraprolin (TTP), and EGFR response factor 1 (CMG1)) are localized to their zinc finger regions (15–20). However, these proteins appear to have different sequence requirements for nuclear localization. For example, the NLS of Wt1 was delimited to the first (of four) zinc fingers, whereas the second (of two) zinc fingers of TR2 was sufficient for nuclear localization (15, 19). By contrast, all three zinc fingers are necessary for efficient nuclear localization of EKLF/KLF1.

Results obtained for Zif268 and JAZ demonstrated that mutations which disrupt the tertiary structure of zinc fingers also abrogated nuclear localization (16, 18); disruption of any one of the three zinc fingers of Zif268 resulted in cytoplasmic mislocalization. In contrast, the tertiary structures of all three zinc fingers in EKLF/KLF1 were dispensable for nuclear localization. In this respect, EKLF/KLF1 is similar to NRSF, TTP, and CMG1; the NLS of these proteins was also independent of zinc finger tertiary structure (17, 20).

Our results also demonstrate that the basic residues within the zinc fingers are a critical determinant for nuclear localization. Mutations of these residues in the three fingers resulted in predominant accumulation in the cytoplasm. This localization was not due to exposure of a cryptic NES, because treatment with the nuclear export inhibitor LMB had no effect on the cytoplasmic accumulation of this mutant. Our results also demonstrate that the basic residues are directly involved in nuclear localization; mutations in basic residues of GFP/ZF1,2,3 resulted in cytoplasmic accumulation.

Basic residues within the zinc fingers could function in several ways to affect nuclear localization. DNA and RNA binding domains are thought to function as nuclear retention signals based on their ability to bind DNA (22). This is consistent with a survey showing that the NLS of ~70% of nucleic acid-binding proteins are coincident with the nucleic-acid binding domain (22). Biophysical studies demonstrate that electrostatic interactions between positively charged basic residues and the negatively charged phosphoate DNA backbone are the predominant mediators of nucleic acid binding (24–27). Therefore, the basic residues of EKLF/KLF1 could mediate nonspecific interactions with DNA, leading to nuclear accumulation. Binding studies demonstrated that the nonspecific associations with DNA are independent of tertiary structure, at least for zinc finger domains (23). This is also consistent with our results demonstrating that the tertiary zinc finger structure is dispensable for nuclear localization and chromatin co-localization of EKLF/KLF1. Alternatively, the basic residues within the zinc fingers might be necessary for interacting with other chromatin-associated proteins and thus co-localize to chromatin. Indeed, the zinc fingers of EKLF/KLF1 can associate with components of SWI/SNF chromatin-remodeling complex in vitro (30). However, this possibility is not likely because protein-protein interactions mediated by zinc fingers require tertiary zinc finger structure, and our results demonstrate that finger tertiary structure is dispensable for nuclear localization. There is a formal possibility that the zinc finger basic residues constitute an unusually long basic-type NLS. However, most basic-type NLSs
constitute a short stretch (4–6 aa) of basic residues (classical) or two smaller clusters (~4 aa) separated by 6–10 residues (bipartite) (31). The 15 basic residues of EKLF/KLF1 are dispersed over a relatively large region (83 amino acids). Therefore, the likelihood that this sequence constitutes an atypically long basic-type NLS is remote, although not impossible.

EKLF/KLF1 belongs to a growing family of transcription factors that are highly homologous in their zinc finger region. Fig. 7 illustrates the consensus amino acid sequence of the zinc finger region of 17 SP/KLF family members (adapted from Turner and Crossley (4)). 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. Interestingly, a comparison between the consensus and EKLF/KLF1 zinc finger sequences demonstrates that 14 of the 15 basic residues are perfectly conserved in all 17 members. Based on this observation together with our results demonstrating a critical role for the basic residues in nuclear localization, we propose that the basic residues of Kruppel zinc fingers are a common NLS shared by all Kruppel family members. Consistent with this proposal, the zinc fingers of GKLF/KLF4 encode a potent NLS (13), and the NLS for Sp1 has been delimited to the zinc finger region (32). Our data suggest that mutation of basic amino acids in the GKLF/KLF4 and Sp1 zinc fingers will inhibit the NLS activity of these domains.

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Critical Nuclear Localization Determinants of EKLF/KLF-1

Basic Residues within the Kruppel Zinc Finger DNA Binding Domains Are the Critical Nuclear Localization Determinants of EKLF/KLF-1

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