Biochemical and Biophysical Research Communications **283,** 205–208 (2001)
doi:10.1006/bbrc.2001.4762, available online at http://www.idealibrary.com on IDEAL®

# A Nucleus-Localization-Deficient Mutant Serves as a Dominant-Negative Inhibitor of Gut-Enriched Krüppel-like Factor Function

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Received March 26, 2001

**Cancer cells differ from normal cells in many aspects, including loss of differentiation and uninhibited cell proliferation. Recent studies have suggested that gut-enriched Krüppel-like factor (GKLF) played an important role in the regulation of cell growth in the colon. Studies from this laboratory have shown that GKLF protein predominantly expressed in the cytoplasm but not the nucleus of colon cancer cells, suggesting that impaired nuclear translocation of GKLF might contribute to cancer formation. In this report, a region containing putative nuclear localization signal (NLS) of GKLF (PKRGRR; amino acids 385–390) was investigated. Mutation of KR to WT had no effect on the inhibitory properties of GKLF on cyclin D1 promoter activity and [$^3$H]thymidine uptake in HT-29 cells, whereas mutation of RR to GL abolished GKLF function completely. Additional mutation analyses demonstrated that Arg$^{390}$ is the most critical moiety within this region that mediated GKLF function and its nucleus localization. Cotransfection of Arg$^{390}$ mutant (RR/RS) completely inhibited wild-type GKLF function, and GFP-RR/RS GKLF fusion proteins failed to translocate to the nucleus. The results from this study demonstrate that Arg$^{390}$ confers the NLS of GKLF and that the nucleus-localization-deficient mutant serves as dominant-negative inhibitor of GKLF function.** © 2001 Academic Press

***Key Words:*** **NLS; GKLF; KLF4; cyclin D1 promoter.**

Gut-enriched Krüppel-like factor (GKLF/KLF4) is an eukaryotic zinc finger protein that expresses extensively in the gastrointestinal tract (1). Recent study has suggested that GKLF may play an important role in the regulation of cell growth and differentiation of colonic epithelium (1). Although GKLF protein is expressed in the cytoplasm and the nucleus of the normal colonic epithelial cells, its distribution in the cancerous cells appears to be different (1). The mechanism and the significance of this disparity are currently unknown.

The majority of eukaryotic transcription factors possess nuclear localization signal (NLS) which directs transcription factors to the nuclear pore complex and subsequently translocate across the nuclear membrane (2, 3). Two types of nuclear localization signals have been described in the literature, one consists of four or more arginine or lysine residues within a hexapeptide; and the other contains two clusters of basic amino acids separated by a nonbasic peptide (4). Examining GKLF cDNA sequence, we have identified a cluster of basic amino acids, PKRGRR (amino acids 385–390), which may confer its nuclear localization signal. In this report, the structure-function relationships of GKLF with regard to its nuclear localization signal were examined by mutation analyses and immunolocalization studies.

## MATERIALS AND METHODS

*Cell culture and transfection.* The human embryonic kidney (L-293) cells and human colonic adenocarcinoma (HT-29) cells (obtained from ATCC, Rockville, MD) were cultured in the minimal essential and the McCoy's 5A media (Sigma, St. Louis, MO), respectively at 37°C in a 95% air + 5% $CO_2$ atmosphere. Media were supplemented with 10% fetal bovine serum, 100 $\mu$g/ml streptomycin, and 100 units/ml penicillin. Cells were transfected with DNA using the Lipofectamine method according to the manufacturer's protocol (GIBCO, Gaithersburg, MD) and as described previously (1). After transfection, cells were incubated for an additional 48 h before analysis.

*Site-directed mutagenesis.* Mutation analysis of the putative GKLF nuclear localization signal was accomplished by using *Quik*Change Site-directed mutagenesis kit from Stratagene (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, two synthetic oligonucleotide primers encoding 14 complimentary base pairs on either side of a single or multiple bases mutation were annealed to the wild-type DNA and the mutated DNA

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was synthesized using DNA polymerase. The product was subjected to *Dpn*I digestion to remove wild-type DNA template and the synthesized DNA containing mutation was then transformed into the competent cells. The base pair changes were analyzed and confirmed by DNA sequencing. Inserts containing the mutations were subcloned into pCDNA-3 or pEGFP-N1 (Clontech Laboratories, Inc.) to generate green fluorescence protein-GKLF fusion proteins.

*Luciferase and β-galactosidase measurements.* To examine transcriptional regulation of the cyclin D1 (CD1) promoters by GKLF, HT-29 cells were transiently transfected with pCMV gal, pCD1-1745 Luc (containing 1745 bp upstream of the transcription initiation site of the CD1 promoter) DNAs in the presence of wild-type and/or mutant GKLF or control plasmid (pCDNA-3). For luciferase assay, transfected cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and then lysed in 500 μl of lysis buffer following the manufacturer's instructions (Analytical Luminescence, San Diego, CA). β-galactosidase activity in 40 μl of the cell lysate was determined after a 5–30 min incubation at 37°C with 2 mM chlorophenol red β-galactopyranoside (Boehringer Mannheim) in 2 nM $MgCl_2$, 0.1 mM $MnCl_2$, 45 mM 2-mercaptoethanol, and 100 mM $NaHPO_4$, pH 8.0. The reactions were terminated by adding 500 μl of 0.5 M EDTA, pH 8.0, and the absorbance at 570 nm was measured using a spectrophotometer.

*[$^3$H]thymidine incorporation.* For [$^3$H]thymidine incorporation experiment, HT-29 cells were transfected with wild-type or/and mutant GKLF cDNAs for 48 h. After culturing in the presence of serum-free medium overnight, cells were incubated with 1 μCi/ml [$^3$H]thymidine (20 Ci/mmol) at 37°C for 4 h before harvesting. After washing twice with cold PBS, cells were fixed with 10% trichloroacetic acid (TCA) at 4°C for 30 min, rinsed with 10% TCA, solubilized with 1 N NaOH, and neutralized with HCl. Aliquots equal to 0.1 volume of the solubilized material were counted in triplicate by liquid scintillation. Dishes that contained no cells were labeled and counted to provide background counts.

*Immunolocalization of GKLF.* The wild-type and mutant GKLF cDNAs were fused to the carboxyl terminus of the expression vector, pEGFP-N1, to generate green fluorescence protein (GFP)-GKLF fusion proteins. All constructs were sequenced to confirm the correctness of the reading frames. To examine the expression of GFP-GKLF proteins, L-293 cells were transfected with the empty vector or GFP fusion constructs using the Lipofectamine method as described above. After transfection, cells were incubated for an additional 48 h, fixed in 4% paraformaldehyde, and visualized with a Nikon Optiphot microscope equipped with epifluorescence.

*Statistics.* Results were expressed as mean ± SE. Statistical analysis was performed using ANOVA and Student's *t* test. *P* value less than 0.05 was considered to be statistically significant.

**TABLE 1**

Amino Acid Sequence (385–390) of Wild-Type and Mutants GKLF and Their Effects on Cyclin D1 (CD1) Promoter Activity and [$^3$H]Thymidine Incorporation in HT-29 Cells

| Constructs | Inhibition on CD1 promoter activity (%) | Decrease on [$^3$H]-thymidine incorporation (% of control) |
|---|---|---|
| PKRGRR (wild-type) | 67 ± 15 | 35 ± 12 |
| P**WT**GRR | 70 ± 10 | 32 ± 8 |
| PKRG**GL** | −10 ± 25** | −15 ± 12* |
| PKRG**S**R | 50 ± 22 | 40 ± 16 |
| PKRGR**S** | −5 ± 15** | −12 ± 20* |
| PKRGR**P** | −13 ± 10** | −5 ± 16* |

*Note.* The sequence of the different mutants is presented below the wild-type sequence (the bold type and underline indicate the mutated residues). Data are expressed as the mean ± SE of at least three separate experiments. *$P < 0.05$, **$P < 0.01$, compared to the wild-type.

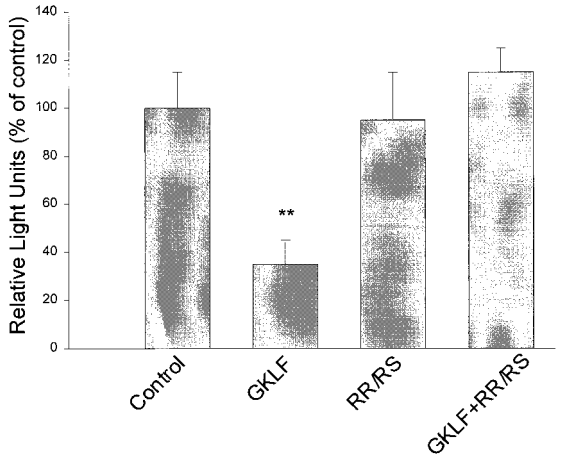


**FIG. 1.** Effect of wild-type and mutant GKLF on transcriptional activity of CD1 promoter in HT-29 cells. HT-29 cells were transfected with CD1 promoter (1 μg) and pCMV galactosidase (0.5 μg) DNAs in the presence of pCDNA-3 (control), wild-type or/and RR/RS mutant GKLF (0.1 μg) plasmids. After incubation for 48 h, cells were harvested for analyses of luciferase and β-galactosidase activities. Data represent means ± SE of 3 separate experiments after correcting for differences in transfection efficiencies by β-galactosidase activities. **$P < 0.01$, compared to control.

## RESULTS

*Attenuation of the inhibitory effect of GKLF on CD1 promoter by RR/RS mutation.* Previous studies from this laboratory have demonstrated that GKLF repressed CD1 promoter activity (5). To examine whether the NLS of GKLF contributed to this function, several mutations of the putative NLS, (PKRGRR; amino acids 385–390) were performed (see Table 1). As shown in Fig. 1, wild-type GKLF significantly reduced CD1 promoter activity to 33 ± 15% of that of control; whereas RR/RS mutant had no effect on the CD1 promoter activity. Cotransfection of RR/RS mutant with wild-type GKLF DNAs completely abolished the inhibitory effect of GKLF indicating that RR/RS mutant functioned as a dominant-negative inhibitor of GKLF function.

*Effect of RR/RS mutation on DNA synthesis.* Studies from this and other laboratories have suggested that GKLF mediated growth arrest (1, 6). To assess the involvement of NLS on GKLF-mediated cell growth, HT-29 cells were transfected with GKLF, and/or RR/RS mutant plasmids and DNA synthesis was determined by examining [$^3$H]thymidine incorporation in these cells. As demonstrated in Fig. 2, overexpressed GKLF in HT-29 cells resulted in a 35 ± 12% decreased

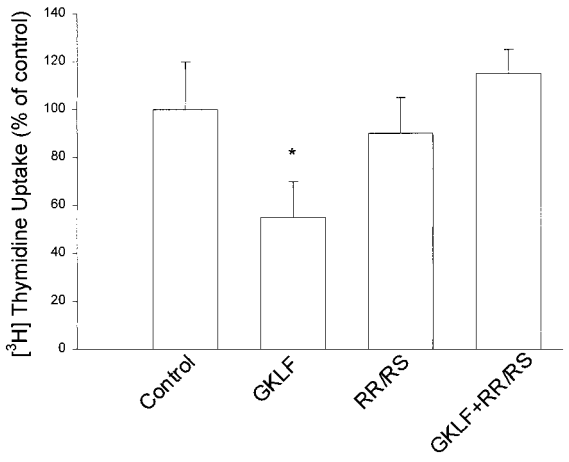


**FIG. 2.** Percent change of [$^3$H]thymidine incorporation in HT-29 cells transfected with pCDNA-3 (control), wild-type or/and mutant RR/RS GKLF (1 $\mu$g) DNAs. Data were expressed as % change over control and represented means ± SE of 5 separate experiments with each value determined in triplicate. *$P < 0.05$, compared to control.

in [$^3$H]thymidine incorporation. These effects were completely abolished by cotransfection with the RR/RS mutant consisting with the dominant-negative inhibitory function of this construct.

*$Arg^{390}$ is essential for GKLF function.* To explore the contribution of other basic amino acids within the PKRGRR (amino acids 385–390) region to GKLF function, additional mutations were performed as shown in Table 1. The effect of these mutants on CD1 promoter activity and DNA synthesis were examined as described above. As illustrated in Table 1, mutation of $Lys^{386}$-$Arg^{387}$ to Try-Thr (KR → WT) has no effect on the inhibitory function of GKLF on CD1 promoter activity and [$^3$H]thymidine incorporation, whereas mutation of $Arg^{389}$-$Arg^{390}$ to Gly-Leu (RR→ GL) completely abolished GKLF activities. When $Arg^{389}$ was mutated to Ser (R→ S), GKLF function was not affected, and substitution of $Arg^{390}$ with Ser or Pro (P) significantly diminished GKLF activities. These data suggest that $Arg^{390}$ is the most important amino acid within this region that mediates the growth arrest effect of GKLF.

*Localization of GKLF protein.* To examine intracellular distribution of GKLF protein, the wild-type and mutant GKLF cDNAs were fused to the carboxyl terminus of the expression vector, pEGFP-N1, to generate green fluorescence protein (GFP)-GKLF fusion proteins. In cells transfected with empty pEGFP-N1 vector, GFP was detected both in the nucleus and the cytoplasm (Fig. 3A). In contrast, the GFP-wild-type GKLF fusion proteins were localized predominantly to the nucleus of the L-293 cells (Fig. 3B), whereas the GFP-RR/RS GKLF mutant proteins were weakly visualized in the nucleus (Fig. 3C, arrow). In the majority of cells, no GFP-RR/RS GKLF fusion proteins were detected in the nucleus (Fig. 3C, inset).

## DISCUSSION

Several mechanisms that are responsible for nuclear translocation of eukaryotic transcription factors have been proposed. It is postulated that transcription factors are delivered to the nucleus by a selective transport mechanisms rather than by simply diffusion. Peptides composed of karyophilic clusters of arginines and lysines that signal transcription factors to specialized transporter molecules in the pore complex of the cytoplasm are named nuclear localization signals (NLS). Two types of NLSs have been described in the literature, one contains four arginines and lysines within a hexapeptide and the other comprise two clusters of basic amino acids separated by a nonbasic peptide (7, 8). Boulikas proposed that NLS served two functions: (1) to bind to transporter cytoplasmic or pore proteins and be transported to the nucleoplasm; and (2) to interact with regulatory genes in the nucleus through protein-protein interaction or by binding to a specific sequence on the DNA (8).

In this report, we examined a region containing a cluster of basic amino acids (PKRGRR; amino acid 385-390) that is located immediately upstream to the first zinc finger protein of GKLF. This area has previously been suggested to be the NLS of GKLF (9). When $Arg^{390}$ was mutated to either serine or proline, GKLF protein was weakly translocated to the nucleus. These

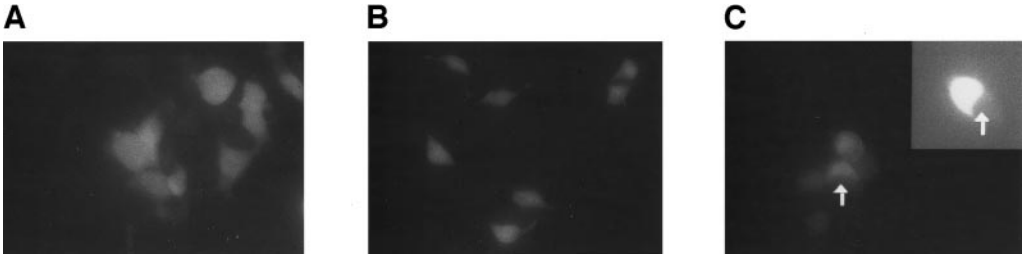


**FIG. 3.** Localization of green fluorescence protein (GFP)-GKLF fusion proteins in L-293 cells. L-293 cells were transfected with (A) empty GFP vector, (B) GFP-GKLF, or (C) GFP-RR/RS GKLF mutant plasmid (1 $\mu$g/$10^5$ cells) and were examined under fluorescence microscopy. (→, nucleus)

data suggested that $Arg^{390}$ was the most critical moiety within this region that mediates nuclear translocation of GKLF. Furthermore, co-transfection of this nucleus-localization-deficient mutant DNA with wild-type GKLF completely abolished the effects of GKLF on CD1 promoter activity and on DNA synthesis suggesting that the mutant served as a dominant-negative inhibitor of GKLF. The mechanisms of these dominant-negative effects are currently unknown. It is possible that the mutant protein may interfere with the interaction between the wild-type GKLF protein and its transporter protein or by inhibiting their transfer to the nucleoplasm. This is the subject of our current investigation.

Previous studies from this laboratory have shown that GKLF expression in the colon is important for the regulation of cell growth and differentiation of the colonic epithelium (1). Overexpression of GKLF in HT-29 cells resulted in cell arrested at G1 phase and down regulation of GKLF expression led to increase in DNA synthesis (1). When HT-29 cells achieved more differentiated phenotype after short-chain fatty acid stimulation, GKLF mRNA levels increased (1). Moreover, in an *in-vitro* transfection system, GKLF inhibited transcriptional activity of the cyclin D1 gene (5). These data suggest that upregulation of GKLF is essential for colonic cells to become differentiated and that down-regulation of GKLF may result in uncontrolled cell growth, probably through enhanced CD1 promoter activity. Using immunohistochemical staining, GKLF protein was found to localize in the cytoplasm and the nucleus of normal colonic epithelium, but was located predominantly in the cytoplasm of the cancerous cells. The significance of this differential expression of GKLF protein in the normal and cancerous cells is not clear. In the current report, we have shown that the nucleus-translocation-deficient GKLF mutant failed to exhibit the inhibitory effects on CD1 promoter. These results suggests that impaired translocation of GKLF protein to the nucleus, as seen in the cancerous cells, may result in uninhibited CD1 activity and ultimately hyperproliferation of the colonic epithelium. These hypotheses warrant further exploration.

In summary, our results indicate that $Arg^{390}$ plays an important role in mediating nucleus localization of GKLF protein and that the nucleus-localization-deficient mutant functions as a dominant-negative inhibitor of GKLF. We hypothesize that failure to translocate GKLF protein to the nucleus may result in uninhibited CD1 activity and enhanced DNA synthesis.

## ACKNOWLEDGMENT

This work was supported by United States Public Health Service Grant DK-52186 to C-C. Tseng.

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