Role of gut-enriched Krüppel-like factor in colonic cell growth and differentiation

JUE-LON SHIE,1 ZHI Y. CHEN,1 MICHAEL J. O’BRIEN,2 RICHARD G. PESTELL,3 MU-EN LEE,4 AND CHI-CHUAN TSENG1

1Section of Gastroenterology, 2Department of Anatomic Pathology, Boston Veterans Affairs Medical Center and Boston University School of Medicine, Boston 02118; 4Cardiovascular Biology Laboratory, School of Public Health, Harvard University, Boston, Massachusetts 02115; and 3Department of Development and Molecular Biology and Medicine, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461

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Shie, Jue-Lon, Zhi Y. Chen, Michael J. O’Brien, Richard G. Pestell, Mu-En Lee, and Chi-Chuan Tseng. Role of gut-enriched Krüppel-like factor in colonic cell growth and differentiation. Am J Physiol Gastrointest Liver Physiol 279: G806–G814, 2000.—Cancer cells differ from normal cells in many aspects, including hyperproliferation and loss of differentiation. Recent research has focused on the role of transcription factors in regulating abnormal cell growth. Gut-enriched Krüppel-like factor (GKLF) is a newly identified eukaryotic zinc finger protein expressed extensively in the gastrointestinal tract. In the current study, we demonstrated that GKLF mRNA levels were significantly decreased in the dysplastic epithelium of the colon, including adenomatous polyp and cancer. GKLF immunostains in the normal colon were higher at the surface epithelium and gradually decreased toward the crypt, but this gradient was not present in the adenomatous and cancerous mucosa. Constitutive overexpression of GKLF DNA in a human colonic adenocarcinoma cell line (HT-29) decreased [3H]thymidine incorporation, whereas suppression of GKLF gene increased DNA synthesis, indicating that downregulation of the GKLF gene might contribute to cellular hyperproliferation. Cyclin D1 (CD1) protein level and CD1-associated kinase activity were decreased in HT-29 cell overexpressed GKLF cDNA, and CD1 promoter activity was profoundly suppressed by GKLF. When HT-29 cells were cultured in the presence of sodium butyrate, GKLF mRNA levels increased as cells acquired more differentiated phenotypes. These results suggest that GKLF plays an important role in regulating cell growth and differentiation in the colonic epithelium and that downregulation of GKLF expression may cause colonic cells to become hyperproliferative. Furthermore, GKLF appears to be a transcriptional repressor of the CD1 gene.

colon cancer; adenoma; proliferation; transcription factor

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MATERIALS AND METHODS

Subject population. The expression of GKLF in normal colon, colonic polyps, or tumors was examined in this study. Study participants were drawn from patients who underwent colonoscopy examination at the Boston Veterans Affairs Medical Center (BVAMC). Participants entered the study if they agreed to have biopsy specimens taken from their large bowel during the course of a clinically indicated colonoscopy. Two biopsy specimens were taken from either polyps or tumors and adjacent normal-appearing colonic mucosa and were used for RNA extraction. The study was approved by the BVAMC Research and Development Committee. The tissue specimen from each polyp or tumor was reviewed by a pathologist and classified as malignant, adenomatous, or nondysplastic based on standard criteria.

GKLF antibodies. A peptide was synthesized based on the human GKLF sequence (amino acids 30–47) and was used to raise antiserum (27). This peptide displayed no sequence homology to other known proteins on computer-based analysis. The peptide was conjugated to KLH and injected into New Zealand White rabbits subcutaneously (Covance Research Product, Denver, PA). To characterize GKLF antiserum, GKLF and EKLF proteins were prepared from in vitro transcription and translation of GKLF and EKLF cDNAs (EKLFCDNAs) kindly provided by Dr. Bieker, Mount Sinai School of Medicine) in the presence of [35S]methionine (0.2 mCi/ml; Amersham) using a rabbit reticulocyte lysate system (TNT7T7 quick coupled transcription/translation system, Promega).

Immunoprecipitation of GKLF antiserum and [35S]methionine-GKLF or EKLF protein was performed in PBS (pH 7.4) containing 0.1% Triton X-100. This was prepared by incubating 5 μl (300,000 dpm) of [35S]methionine protein in the PBS/0.1% Triton X-100 buffer with a 1:500 dilution of GKLF antiserum. The mixture was incubated at room temperature for 30 min with continuous mixing and then added to 50 μl of a 50% slurry of protein A-Sepharose 6 MB beads (Pharmacia) for 30 min with continuous mixing and then added to 50 μl of a 50% slurry of protein A-Sepharose 6 MB beads (Pharmacia) and incubated further for 30 min. The beads were washed twice in PBS/0.1% Triton X-100 before the addition of 50 μl of sample buffer (2% SDS containing 10% β-mercaptoethanol) in preparation for SDS-10% PAGE. The gels were dried, and the location of [35S]methionine protein was determined after exposure to X-ray film for 24 h.

To further characterize GKLF antiserum and to exclude potential cross-reactivity between GKLF antiserum and other tissue proteins, the stomach, small intestine, and colon tissues were obtained from Sprague-Dawley rats and subjected to Northern and Western blot analyses (as described below) for the presence of GKLF mRNA and protein in those tissues.

Immunohistochemistry. Tissue sections from normal colon, adenomatous poly, and colon cancer were deparaffinized, rehydrated, and immersed in 3% hydrogen peroxide-methanol solution for 10 min at room temperature to inhibit endogenous peroxidase activity. The anti-GKLF antibody was diluted in 0.2% crystalline grade BSA in PBS, and slides were incubated in a humidified chamber at 4°C for 16–18 h. The sections were sequentially incubated with biotinylated goat anti-rabbit IgG, streptavidin-horseradish peroxidase (Pharminen, San Diego, CA), the chromogenic substrate diaminobenzidine (DAB), and hematoxylin counterstaining with intervening acetate buffer washes. Control slides were prepared in an identical manner, except that the tissue sections were incubated with GKLF antiserum and either 10^-4 M GKLF or alkaline phosphatase (Sigma Chemical) peptide.
Dickinson, San Jose, CA), using the appropriate dichroic mirror and emission filter. The data were analyzed using Acycyte software (Phoenix Flow System, San Diego, CA). The Multicycle program (Phoenix) was used for analysis of cell cycle distribution.

Luciferase and β-galactosidase measurements. To examine transcriptional regulation of cyclin D1 (CD1) and cyclin A (CA) promoters by GKLF, the CD1 promoter (1745 bp upstream from the transcription initiation site) and the CA promoter (3200 bp upstream from the transcription initiation site) were ligated to the pA3-Luc plasmid containing a firefly luciferase reporter gene (26). HT-29 cells were transiently transfected with pCMV galactosidase and CD1-Luc, CA-Luc, or pA3-Luc DNAs in the presence of GKLF or control pCDNA3 plasmid. For luciferase assays, cells were washed twice with PBS and then lysed in 500 μl of lysis buffer following the manufacturer's instructions (Analytical Luminescence, San Diego, CA). To assay luciferase activity, we mixed 100 μl of the cell lysate with 100 μl of luciferase substrate solution A (Analytical Luminescence). Using a luminescence meter with automatic injection, we then added 100 μl of substrate solution B (Analytical Luminescence) and measured luciferase activity as the light emission over a 30-s period.

β-Galactosidase activity in 40 μl of the cell lysate was determined after a 5- to 30-min incubation at 37°C with 2 mM chlorophenol red β-galactopyranoside (Boehringer Mannheim) in 2 mM MgCl₂, 0.1 mM MnCl₂, 45 mM 2-mercaptoethanol, and 100 mM NaHPO₄, pH 8.0. The reactions were stopped by adding 500 μl of 0.5 M EDTA, pH 8.0, and the absorbance at 570 nm was measured using a spectrophotometer. With each experiment, luciferase activity was determined in duplicate and normalized to β-galactosidase activity for each dish.

Western blot analysis. SDS-PAGE was performed according to the method of Laemmli (14). Protein samples were dissolved in loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, and 0.01% bromphenol blue), heated to 100°C for 3 min, and loaded onto the gel in electrophoresis buffer containing 25 mM Tris-HCl, pH 8.3, 250 mM glycine, and 0.1% SDS. At the completion of electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond enhanced chemiluminescence, Amersham Life Science). The membrane was incubated overnight in the blocking buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% nonfat powdered milk. The membrane was immunoblotted with CD1 or PCNA antiserum (Santa Cruz Biotech, Santa Cruz, CA). After incubation with the secondary antibody, the membrane was visualized with enhanced chemiluminescence (Amersham).

CD1-associated kinase activity. CD1-associated kinase activities were examined in wild-type and stable HT-29 cells. Cells were lysed in ice-cold modified radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.0, 100 μM sodium vanadate, 50 mM sodium fluoride, 50 μM leupeptin, 1% aprotinin, 2 mM EDTA, and 1 mM dithiothreitol). The samples were then sonicated and centrifuged in the cold room for 10 min, and the supernatants were collected and stored at -80°C. Total protein (100–500 μg) was subjected to immunoprecipitation with the CD1 antibodies. The immunocomplexes were collected with protein A-agarose and washed four times with RIPA buffer and three times with kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, and 1 mM dithiothreitol). Twenty-five microliters of kinase buffer with purified glutathione-S-transferase-retinoblastoma (GST-Rb) protein, 10 mM ATP, and 10 μCi [γ-32P]ATP (3,000 Ci/mmol, Amersham Pharmacia Biotech) were added, and the reactions were incubated at room temperature for 30 min. The reaction was stopped by addition of 20 μl of 5× Laemmlli buffer, boiled for 10 min, and separated on a 12.5% acrylamide gel. The amount of radioactive incorporation was analyzed by autoradiography and densitometry.

Alkaline phosphatase assay. After culture, cells were lysed with 0.25% sodium deoxycholate and total protein was determined (3, 12). Standard alkaline phosphatase assay (ALP) solutions ranging from 0 to 70 U/ml were prepared (Sigma enzyme control). One-hundred-microgram aliquots of each sample and standards were measured by adding 7 mM p-nitrophenyl phosphate, 0.1 mM NaHCO₃, and 5 mM MgCl₂ and then incubated at 37°C for 25 min with protection from light. Reactions were terminated by addition of 1 ml of 0.1 N NaOH to each tube. Enzyme concentrations were determined by colorimetric assay measuring the absorbance at 410 nm and calculated from the standard curve. Each sample was assayed in duplicate.

Statistics. Results were expressed as means ± SD. Statistical analysis was performed using ANOVA and Student's t-test. P < 0.05 was considered to be statistically significant.

RESULTS

Expression of GKLF transcript in normal colon, adenomatous polyps, and cancer. Northern blot analysis was performed on total RNA isolated from colonic tissue using a 32P-labeled probe prepared from human GKLF or cyclophilin cDNA. A representative Northern blot autoradiogram is shown in Fig. 1, where a major 3.5-kb band was noted for GKLF. This band is consistent with the predicted size published previously (7, 21, 27). The levels of GKLF mRNA, expressed as the ratio of GKLF mRNA to cyclophilin mRNA, were significantly lower in cancer and adenomatous polyps.
Characterization of GKLF antiserum. To examine the specificity of GKLF antiserum, GKLF and EKLF proteins prepared from in vitro transcription and translation were immunoprecipitated with anti-GKLF antibodies. As demonstrated in Fig. 3, a major 60-kDa band was visualized in GKLF but not in EKLF cDNA transcribed protein, indicating the lack of cross-reactivity between GKLF antiserum and EKLF protein. In rat tissues, GKLF mRNA message was detected in the gastric fundus, duodenum, jejunum, ileum, and colon (Fig. 4A). These findings were confirmed by the presence of GKLF protein in Western blot analysis (Fig. 4B). Together, these data support the specificity of GKLF antiserum.

Immunolocalization of GKLF in colon. Histology sections from normal colon, adenomatous polyp, and colon cancer were examined for the expression of GKLF using antiserum characterized above. In normal colon (Fig. 5, A, C, and G), GKLF protein was expressed primarily in the epithelium, and its level was higher at the surface epithelium and gradually decreased toward the crypt. Few GKLF-stained cells were scattered in the lamina propria. GKLF immunostains were predominantly cytoplasmic at the normal surface epithelium (Fig. 5, A and C) but were present in the cytoplasm and the nucleus of crypt epithelium (Fig. 5, A, C, and G). Moreover, GKLF immunostains in the crypt epithelium appear to be heterogeneous with some cells staining negative for GKLF. In adenomatous polyp, the location of GKLF immunostain was mainly nucleic and no surface-to-crypt gradient was observed (Fig. 5H). GKLF immunostains in colon cancer were diffusely distributed in the majority of cancer cells (Fig. 5, D and F). GKLF immunostains in normal and cancerous tissues were abolished by blocking sections with GKLF (Fig. 5, B and E) but not with the nonspecific peptide alkaline phosphatase (Fig. 5, C and F), demonstrating the specificity of the GKLF immunostains. Similar

![Fig. 2. Steady-state levels of GKLF mRNA in normal colon, carcinoma, and adenomatous polyps. GKLF mRNA concentrations in the polyps or tumors were expressed as % of normal control tissue and calculated as means ± SD of the ratio of GKLF mRNA to cyclophilin mRNA to correct for gel loading; n = 6–8 for each group. *P < 0.05 compared with normal colon.](http://ajpgi.physiology.org/)

![Fig. 3. SDS-PAGE analysis of GKLF (lane A) and erythroid Krüppel-like factor (EKLF) (lane B) proteins immunoprecipitated with anti-GKLF antiserum. GKLF and EKLF proteins were synthesized from in vitro transcription and translation of each individual cDNA as described in MATERIALS AND METHODS. Size standards are indicated at left.](http://ajpgi.physiology.org/)

![Fig. 4. Expression of GKLF in the rat gastrointestinal tract. Twenty micrograms of total RNA and 40 μg of protein from fundus (lane 1), duodenum (lane 2), jejunum (lane 3), ileum (lane 4), and colon (lane 5) were subjected to either Northern (A) or Western (B) blot analysis using 32P-labeled GKLF cDNA or GKLF antiserum as probes. Size standards for RNA and protein are indicated at left.](http://ajpgi.physiology.org/)
results were observed in the adenomatous mucosa (data not shown).

Effect of GKLF expression on DNA synthesis. The results of the above studies suggest that downregulation of GKLF in the colonic epithelium may result in uninhibited cell growth and malignant transformation. To assess the effect of GKLF on cell growth, wild-type HT-29 cells and HT-29 cells stably expressing sense or antisense GKLF cDNA were cultured in the absence of serum for 24 h. Cells were then exposed to media containing 10% fetal bovine serum for 4 h, at which time DNA synthesis was determined by [3H]thymidine incorporation. Figure 6 shows the results of five independent experiments. Overexpression of sense GKLF in HT-29 cells resulted in a 66 ± 12% decrease in the [3H]thymidine uptake, whereas downregulation of GKLF expression in antisense-transfected cells led to a 25 ± 11% increase in [3H]thymidine incorporation compared with wild-type cells.

Effect of GKLF on cyclin expression. The data from preceding studies suggest that the expression of GKLF is temporally associated with cell growth. To examine whether these growth effects were associated with changes in cyclin expression, the levels of CD1, cyclin E, and cyclin A mRNA were examined in wild-type and sense- or antisense-transfected HT-29 cells. As shown in Fig. 7, CD1 mRNA levels were significantly decreased in sense GKLF-transfected cells, but increased in antisense GKLF-transfected cells, with levels of 48 ± 25% and 135 ± 17%, respectively of the wild-type control. No significant change in the expression of cyclin A, cyclin E, or cyclophilin transcript was observed in those cells (Fig. 7).

CD1 protein and CD1-associated kinase activity. To examine the molecular mechanisms responsible for GKLF-mediated growth arrest, CD1 protein and CD1-associated kinase activity were measured in wild-type and GKLF-transfected HT-29 cells. As shown by Western blot analysis, CD1 protein levels were significantly decreased in sense GKLF-expressed cells but increased in antisense GKLF-transfected cells (Fig. 8). Similar changes in CD1-associated kinase activity, as determined by GST-Rb phosphorylation, were observed in those cells. These results indicate that the growth arrest effect of GKLF is associated with the decrease in CD1 protein level and in CD1-associated kinase activity.

Effect of GKLF on cell cycle progression. In mammalian cells, CD1 is normally expressed during the G1 interval and is believed to be an important factor in driving cells through a G1 restriction and entering S phase. To explore the effect of GKLF on cell-cycle evolution, wild-type and sense GKLF-transfected
HT-29 cells were subjected to cell-cycle analysis. As shown in Fig. 9, compared with wild-type cells, overexpression of GKLF resulted in an increase in cells arrested at G1 phase, suggesting a potential role for GKLF in modulating cell cycle progression.

Inhibition of CD1 promoter activity by GKLF. To determine the mechanism(s) by which GKLF inhibited CD1 mRNA expression, the effect of GKLF on CD1 promoter activity was further assessed. HT-29 cells were transiently transfected with CD1-Luc or CA-Luc (2 μg/well) reporter plasmid and pCMV β-galactosidase DNA (0.1 μg/well; control for transfection efficiency) in the presence or absence of GKLF DNA (0.05 μg/well). As shown in Fig. 10, transfection of HT-29 cells with CD1-Luc DNA increased luciferase activity to ~100-fold over the promoterless control (pA3-Luc), and this activity was significantly repressed by GKLF (90%). In contrast, GKLF had no effect on either the CA promoter or the basal reporter activity, indicating the specificity of its inhibitory effect on the CD1 promoter.

Increased GKLF mRNA levels in differentiated HT-29 cells. To explore the potential role of GKLF in modulating differentiation of the colonic mucosa, the expression of GKLF mRNA message was examined in HT-29 cells during the short-chain fatty acid-promoted differentiation process (18). In the presence of sodium butyrate, HT-29 cells exhibited more differentiated phenotypes as indicated by the increase in alkaline phosphatase activities and the decrease in PCNA levels from day 3 to day 7 (Fig. 11). GKLF mRNA levels were gradually increased as cells became more differentiated (Fig. 11). Moreover, the basal alkaline phosphatase levels in HT-29 cell overexpressing sense GKLF appeared to be higher than those in wild-type HT-29 cells (28 ± 6 and 12 ± 5 U/100 μg protein, respectively). These data indicate that GKLF may also play an essential role in regulating differentiation of the colonic epithelium.

DISCUSSION

Although many genetic alterations have been attributed to the development of colon cancer, the early events leading to colonic carcinogenesis are not clear. Moreover, hyperproliferation of the colonic mucosa has been observed to precede the formation of colonic polyps or tumors, but the mechanisms mediating cellular hyperproliferation have not been fully elucidated. In this report, we have demonstrated that the expression
of GKLF mRNA is significantly decreased in neoplastic colonic tissues including adenoma and carcinoma. These results are consistent with findings from Ton-That et al. (23), who demonstrated that the level of GKLF transcript is decreased in the intestine of multiple intestinal neoplasia mice during a period of tumor formation. Together, these data suggest that down-regulation of GKLF expression may contribute to malignant transformation of the colon.

Previous studies (4, 5) using in situ hybridization have shown that GKLF is expressed extensively in the gastrointestinal tract. By Northern blot analysis, we also found that GKLF mRNA was present in the rat stomach, small intestine, and colon. Despite these findings, whether GKLF exhibits any significant role in modulating cell growth in the gastrointestinal tract has not been reported. In current study, constitutive overexpression of GKLF in human adenocarcinoma cells resulted in a decrease in $[^{3}H]$thymidine uptake, whereas inhibition of GKLF expression led to an increase in DNA synthesis, suggesting that GKLF may play an essential role in controlling growth arrest in the colon. Furthermore, these results also imply that downregulation of GKLF may result in uninhibited cell growth and are consistent with findings from Shields et al. (21), who showed that GKLF mRNA levels in NIH 3T3 cells were significantly decreased when cells were rendered to proliferate (21).

The mechanism of GKLF-mediated growth arrest in the colonic epithelium is currently unknown. The eukaryotic cell cycle, consisting of four major phases (G1, S, G2, and M), is a series of carefully regulated events (10). When quiescent cells are stimulated, a cascade of cellular events takes place, resulting in DNA synthesis and subsequent cell division (10, 15, 16, 22). In addition, eukaryotic cells also possess proteins, such as tumor suppressor p53, which exhibit negative control on cell growth (9). Recently, another group of genes (p21, p27, p16, and p15) was found to inhibit the activities of the cyclin and cyclin-dependent kinases, both of which are essential for the progression of the cell cycle (20). In this study, we have shown that the expression of GKLF is closely related to that of CD1 and CD1-associated kinase activity. Moreover, GKLF suppresses the CD1 promoter activity. Although the causative role of CD1 in the growth inhibitory effect of GKLF has not yet been established, our results suggest that the function of GKLF may be mediated, at least in part, through the repression of the CD1 gene. This notion is further supported by the identification of multiple potential GKLF binding domain, CACCC motif, on the CD1 promoter (26). Whether GKLF regulates the expression of other cyclins or cyclin-dependent kinases warrants further investigation. Moreover, in contrast to our findings, Jenkins et al. (13) recently reported that GKLF increased transcriptional activity of the human keratin 4 and Epstein-Barr virus ED-L2 promoters and suggested that GKLF might function as a transcriptional activator in the esophageal squamous epithelium to regulate cell differentiation. Yet et al. (27) recently provided evidence showing that GKLF possessed both transcriptional activation and repression domains. It is feasible that GKLF may function as either transcriptional activator or repressor and that this property is promoter or cell specific.

Cancer cells differ from normal cells in many characteristics, including loss of differentiation, which arises from uninhibited cell growth and uncontrolled cellular evolution (9). In the dysplastic colonic epithelium, GKLF mRNA levels were significantly decreased. In contrast, GKLF mRNA levels increase as HT-29 cells gain more differentiated phenotype in response to short-chain fatty acid stimulation. These findings suggest that GKLF may also play a role in controlling the switch from proliferation to differentiation in the colonic epithelium. It is possible that upregulation of GKLF is essential for colonic cells to become differentiated and that downregulation of GKLF allows cells to enter the cell cycle and result in uncontrolled cell growth. These hypotheses warrant further exploration.

In the normal colonic mucosa, GKLF immunostain was found to locate primarily in the mature surface epithelium, and its level of expression gradually decreased toward the crypt cells. When colonic epithelia become dysplastic, as seen in the adenomatous or carcinous tissues, the surface-to-crypt gradient of GKLF immunostain disappears. These findings are consistent with a recent report from Foster et al. (6), who demonstrated that, in oral squamous mucosa, the expression
of GKLFT was predominantly located in the differentiating epithelia and that GKLFT immunostains were diffusely distributed when cells became dysplastic. Foster et al. (6) suggested that misexpressed GKLFT in the basal compartment of the squamous epithelium may result in tumor genesis. This conclusion is similar to our findings showing that GKLFT immunostains appear to localize in the proliferating epithelium of colonic adenomatous polyps and cancer. Whether aberrant expression of GKLFT results in hyperproliferation of colonic epithelium requires further investigation. Moreover, the immunostain of GKLFT in the crypt appears to be heterogenous. Whether these cells stained positive for GKLFT represent active proliferating cells also require further study.

In summary, the results of this study suggest that GKLFT may play an important role in modulating the switch of proliferation and differentiation in the colonic epithelia and that its level of expression is reduced in the adenomatous polyps and cancers. Furthermore, downregulation of GKLFT results in uninhibited cellular growth that may contribute to malignant transformation of the colonic mucosa. Finally, GKLFT appears to be a transcriptional repressor of the CD1 gene.

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