Gut-enriched Krüppel-like Factor Represses Ornithine Decarboxylase Gene Expression and Functions as Checkpoint Regulator in Colonic Cancer Cells*

Zhi Y. Chen, Jue-Lon Shie, and Chi-Chuan Tseng‡
From the Section of Gastroenterology, Veterans Affairs Boston Healthcare System and Boston University School of Medicine, Boston, Massachusetts, 02118

Gut-enriched Krüppel-like factor (GKLF, KLF4) is an epithelial-specific transcription factor that expresses in the gastrointestinal tract and mediates growth arrest of colonic epithelium. The molecular mechanisms governing its growth inhibitory effect have not been fully elucidated. In the present study, we showed that induction of GKLF mRNA and protein expression by interferon-γ treatment was associated with reduction of ornithine decarboxylase (ODC) gene expression and enzyme activity in colon cancer HT-29 cells. Overexpression of GKLF in HT-29 cells significantly reduced ODC mRNA and protein levels as well as enzyme activity and resulted in growth arrest, indicating that ODC might be a downstream target of GKLF. This conclusion was further supported by data showing that GKLF mRNA and protein concentrations were the highest at the G1/S boundary of the cell cycle, where ODC mRNA and protein levels were the lowest and that overexpression of GKLF resulted in cell arrested at the G1 phase. Reporter gene transfection studies and electrophoretic mobility gel shift assays demonstrated that GKLF repressed ODC promoter activity and that these effects appeared to be mediated through interaction with a GC box in the proximal portion of the promoter. Transfection studies using reporter constructs and chromatin immunoprecipitation assays also demonstrated that GKLF inhibited transcription of the ODC gene by interfering with the binding of Sp1 to the ODC promoter. These results indicate that GKLF may function as a G1/S checkpoint regulator and exert its growth arrest effect through down-regulation of ODC gene expression. Furthermore, GKLF is a transcriptional repressor of the ODC gene, and these effects are mediated by interaction with the GC-rich region on the promoter.

Ornithine decarboxylase (ODC), a key regulatory enzyme of the biosynthesis of polyamines, is essential for cell proliferation and differentiation (1). The expression of ODC is highly regulated in cells and is responsive to a wide variety of growth-promoting stimuli (2, 3). Alternation in ODC gene expression resulting in polyamine accumulation has been demonstrated to associate with cell transformation and carcinogenesis (4). Furthermore, ODC has previously been shown to play a critical role in the progression of colon cancers. Luc and Baylin (5) examined polyps from familial adenomatous polyposis patients and demonstrated higher levels of ODC activity in dysplastic polyps than in nondysplastic ones. Porter et al. (6) also found levels of ODC activity in the carcinoma tissues 8-fold higher than in the adjacent normal colonic mucosa. In experimental animal models of colon carcinogenesis, both tumor-promoting agents and carcinogens induce ODC activity (7, 8). These data are consistent with the essential role of ODC in the tumorigenesis of the colon.

Gut-enriched krüppel-like factor (GKLF, KLF4) is a recently identified epithelial-specific transcription factor that expresses extensively in the gastrointestinal tract (9–12). Several in vivo and in vitro studies have shown that the expression of GKLF is associated with growth arrest, but the mechanisms in which GKLF functions as a negative regulator of cell growth have not been well defined. Recently, our laboratory has demonstrated that GKLF mRNA levels in human colon were significantly decreased in the precancerous polyps and cancerous tissues (12). These data indicated that down-regulation of GKLF expression might result in uncontrolled cell proliferation and tumor formation. In addition, our studies also demonstrated that GKLF inhibited cyclin D1 gene expression and resulted in a decrease in DNA synthesis in colon cancer cells (13). This study was undertaken to explore whether ornithine decarboxylase gene, another important regulator of cell growth, plays a role in GKLF-mediated functions. The molecular mechanisms of their interaction were also investigated.

MATERIALS AND METHODS

Cell Culture—The human colon carcinoma cell lines HT-29 and HCT116, as well as Chinese hamster ovary cell line were obtained from American Type Culture Collection (Manassas, VA). HT-29 and HCT116 cells were maintained in McCoy’s growth medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (Invitrogen) in an atmosphere of 95% air and 5% CO2 at 37 °C. Chinese hamster ovary cells were cultured in F-12K nutrient medium with 10% fetal bovine serum. The cells were subcultured at appropriate intervals to maintain a subconfluent density.

Plasmid Construction and Site-directed Mutagenesis—The sense human GKLF expression vector pcDNA3/GKLF (+292 to +1565) was constructed as previously described (12). An ODC promoter luciferase construct, pODC−1156/+13, containing 5′-flanking sequences from −1135 to +13 of the ODC gene, was kindly provided by Dr. Andrew P. Butler (Anderson Cancer Center, Science Park-Research Division, Smithville, TX). Various truncated promoter constructs including pODC−409/+13.Luc, pODC−179/+13.Luc, and pODC−90/+13.Luc were created by restriction endonuclease digestion or by PCR using appropriate primers. The ODC promoter mutants were

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‡ To whom correspondence should be addressed: Section of Gastroenterology, Veterans Affairs Boston Healthcare System and Boston University School of Medicine, Boston, Massachusetts, 02118. Tel.: 617-638-8330; Fax: 617-638-7785; E-mail: chichuan.tseng@bmc.org.

§ The abbreviations used are: ODC, ornithine decarboxylase; GKLF, gut-enriched Krüppel-like factor; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; IFN, interferon.

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generated by site-directed mutagenesis according to the manufacturer's protocol (Stratagene, La Jolla, CA). All of the constructs were confirmed by sequencing analysis and ligated to the pGL3-Luc plasmid containing a firefly luciferase reporter gene.

**Cell Transfection, Luciferase, and β-Galactosidase Assays**—All of the transfection experiments were performed using LipofectAMINE or LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. For transient transfection studies, the cells were transfected with 5 μg of the pcDNA3/GKLF or control vector pcDNA3 unless indicated otherwise. After 48 h, the cells were harvested for assay.

To create stable cell lines expressing GKLF or pcDNA3, HT-29 cells were transfected with pcDNA3/GKLF or pcDNA3 DNA and then grown in medium containing G418 (400 μg/ml). After 2–3 weeks, multiple neomycin-resistant colonies were isolated from each transfection and

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**Fig. 1.** Induction of GKLF expression by IFN-γ is associated with down-regulation of ODC in HT-29 cells. **A.** HT-29 cells were incubated with increasing concentrations of IFN-γ (0–200 units/ml) for 24 h. The levels of GKLF and ODC mRNA transcripts were examined by Northern blot analysis of 20 μg of total RNA with 32P-labeled GKLF or ODC probe. **B.** HT-29 cells were treated as described above and examined for GKLF and ODC protein expression by Western blot analysis. To normalize protein loading, the blot was stripped and rehybridized with 32P-labeled glyceraldehyde-3-phosphate (GAPDH) dehydrogenase probe. **C.** Cells were treated with increasing concentrations of IFN-γ (0–200 units/ml) for 24 h, and cell extracts were collected for ODC activity assay as described under "Materials and Methods." ODC activity was expressed as pmol of CO₂ released from [14C]ornithine/h/mg protein. The values were presented as the means ± S.E. of three separate experiments. *, p < 0.05 compared with untreated control.

**Fig. 2.** Overexpression of GKLF inhibits ODC activity and cell proliferation in HCT116 and HT-29 cells. **A.** Expression of GKLF protein in HCT116 and HT-29 cells transiently transfected with pcDNA3 or pcDNA3/GKLF. The protein was harvested at 48 h after transfection. **B.** ODC enzyme activity and total cell numbers were measured in HCT116 and HT-29 cells transfected with pcDNA3 (open bar) or GKLF (hatched bar). Approximately 1 × 10⁶ cells from each cell line were used for transfection, and the ODC enzyme activity and total cell numbers were measured 48 h later. The results represent the means ± S.E. of three separate experiments. *, p < 0.05 compared with pcDNA3-transfected cells.
were expanded into cell lines. Each cell line was examined for GKLF expression by Northern or Western blot analysis. A pcDNA3-expressed (pC-B-2) and a GKLF-expressed (pG-17) cell lines were selected and used for current studies.

To examine transcriptional regulation of ODC promoter by GKLF, the cells were transiently transfected with pCMV gal, ODC reporter plasmid in the presence of GKLF or control vector (pcDNA3). To determine luciferase activities, the transfected cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and then lysed in 200 μl of lysis buffer following the manufacturer’s instructions and as described previously (13). The luciferase activity was determined in triplicate and normalized to β-galactosidase activity to correct for transfection efficiency.

Electrophoretic Mobility Shift Assay—Nuclear extracts from pcDNA3 or pcDNA3/GKLF-transfected HCT116 cells were prepared as described previously (13). A double-stranded oligonucleotide probe corresponding to the ODC promoter −119 to −99 (5’-AGTCCCGGCCCTC-CCCCCG-3’) was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase. Assays were performed by incubating 5 μg of nuclear extracts in the binding buffer (Promega) containing 200,000 cpm of labeled probe for 20 min at room temperature. To confirm the specificity of DNA-protein binding, the nuclear extract was preincubated with excess unlabeled wild-type or mutated double-stranded oligonucleotides. For the supershift experiments, nuclear extracts were incubated with GKLF antiserum on ice for 30 min before adding to the binding reaction.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed according to the protocol from Dr. Farnham’s laboratory (14, 15). HCT116 cells (2 × 10⁷) were transfected with pcDNA3/GKLF or control pcDNA3 plasmid. Forty-eight hours later, the cells were cross-linked by the addition of formaldehyde directly into the medium to achieve a final concentration of 1% and incubated for 10 min at room temperature. Formaldehyde was then quenched with 0.125M glycine. The cells were washed and suspended in PIPES buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40), containing protease inhibitors. The cells were then pelleted and resuspended in nuclei lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitors. The lysates were then subjected to sonication to reduce DNA length to between 500 and 1,000 bp. The samples were then electrophoresed on 4% nondenaturing polyacrylamide gels with 0.5 TBE, and the gels were dried and exposed to x-ray films (Kodak X-AR).

FIG. 3. Effects of GKLF expression on ODC activity and cell growth in stably transfected HT-29 cells. A and B, representative Northern or Western blot autoradiograms used to measure GKLF, ODC, and glyceradehyde-3-phosphate dehydrogenase mRNA and GKLF, ODC, and β-tubulin protein levels in HT-29 cells stably expressed pcDNA3 (pC-B-2) or GKLF (pG-17). C, ODC enzyme activities in pG-17 (black bar) and pC-B-2 (hatched bar) cells were measured at day 4 after cells were plated. D, growth curve of pG-17 (closed square) and pC-B-2 (open circle) cells. The cell numbers were measured at different days as indicated. All of the measurements were made in triplicate. The results represent the means ± S.E. of three separate experiments. * p < 0.05 compared with control pC-B-2 cells.
heating at 65 °C. The samples were subjected to proteinase K treatment. DNA was recovered by phenol-chloroform extraction and ethanol precipitation and was used as a template for PCR using two primers (5'-GGCCACCGTGAGGGCACGGTTG-3' and 5'-CGGGCGCTACAG-GAGGGACTGACA-3'). These two primers were designed according to the sequences 5' and 3', respectively, to the putative Sp1-binding domain on the proximal portion of the ODC promoter. The DNA from "no antibody" fraction was designated as "total input," and 1× dilution buffer was used as a negative control (mock) for PCR. The PCR products were analyzed on a 1.0% agarose gel, visualized by ethidium bromide staining, and quantified by laser densitometry and integration of the images.

**ODC Activity Assay**—The cell extracts were prepared by washing cells with ice-cold PBS and then placed in ice-cold ODC reaction buffer (10 mM Tris, pH 7.4, 2.5 mM dithiothreitol, 0.3 mM pyridoxyl-5-phosphate, and 0.1 mM EDTA). The cells were scraped, collected, and homogenized, and the cell extracts were centrifuged at 12,000 × g for 20 min at 4 °C. Supernatant was collected and assayed for ODC activity as described (17). Briefly, the samples were aliquoted at 250 µl in triplicate, and the reactions were started by the addition of 0.1 µCi of 10 µCi [14C]L-ornithine to the cytosolic extracts. The tubes were capped with rubber stoppers fitted with metabolic wells containing 250 µl of [14C]CO2 trapping medium containing 10% serum. The cells were synchronized at the G1/S phase by treating G/S phase cells with 5 µg/ml aphidicolin (Sigma) for 24 h. To obtain cells synchronized at the S phase, the aphidicolin-treated cells were washed and maintained in the drug-free medium for 3 h. Mitotic cells were prepared by incubating aphidicolin-treated cells with 0.1 µg/ml nocodazole for 20 h. The cell cycle distribution of HT-29 cells was analyzed by using flow cytometry as described (18). Briefly, the cells were trypsinized, washed with PBS, and fixed in 70% ethanol. The fixed cells were washed with PBS, incubated with 1 µg/ml RNase A for 30 min at 37 °C, stained with propidium iodide (50 µg/ml), and analyzed on a Becton Dickinson fluorescence-activated cell sorter.

**Western Blot Analysis**—To obtain whole cell extracts, the cells were washed twice with ice-cold PBS, scraped and pelleted by centrifugation (200 × g). The cell pellets were then lysed in the standard RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. The protein concentrations were determined by Bio-Rad assay kits, and 80 µg of protein from each sample was separated on the 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Bio-Rad) at 100 V for 1.5 h at 4 °C. Monoclonal anti-ornithine decarboxylase (Sigma) and polyclonal anti-GKLF antibodies were used at 1:500 dilution (12). The protein levels were detected using horseradish peroxidase-conjugated secondary antibodies and ECL following the manufacturer's instructions (Amersham Biosciences).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated by the STAT-60™ method following the manufacturer's instructions (Leedo Medical Laboratories, Inc., Houston, TX). RNA samples (20 µg) were denatured, size-fractionated by electrophoresis on 1.2% agarose-formaldehyde gels, and transferred onto Zeta bind nylon membranes (CUNO, Inc., Meriden, CT). Hybridization was performed overnight at 42 °C using [α-32P]dCTP-labeled GKLF probe (Random primer labeling kit from Roche Molecular Biochemicals, Indianapolis, IN). The blots were washed with 2× SSPE, 0.1% SDS, followed by 0.1× SSPE (3.0 mM NaCl, 0.2 mM NaH2PO4, and 0.02 mM EDTA), 0.1% SDS. All blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech, Palo Alto, CA) to verify RNA loading.

**RESULTS**

**Induction of GKLF Expression by Interferon-γ Is Associated with Down-regulation of ODC**—As described above, endogenous ODC enzyme activity is enhanced during cell proliferation. We have previously shown that interferon-γ (IFN-γ) induced GKLF expression and resulted in growth inhibition of HT-29 cells (16). To examine whether GKLF-mediated growth inhibition is associated with a change of ODC gene expression, the effects of IFN-γ on the levels of ODC mRNA and protein expression as well as ODC activity were examined. As shown in
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Fig. 1. IFN-γ induced dose-dependent increases of GKLF mRNA and protein levels in HT-29 cells. Conversely, ODC mRNA and protein concentrations (Fig. 1, A and B) as well as ODC enzyme activities (Fig. 1C) were inhibited by IFN-γ in a dose-dependent manner. These results demonstrated a reciprocal effect of IFN-γ on GKLF and ODC gene expression and suggested a potential role of ODC in GKLF-mediated growth arrest.

Overexpression of GKLF in HT-29 Cells Reduces Levels of ODC mRNA, Protein, and Enzyme Activity and Results in Growth Arrest—To further delineate the interaction between GKLF and ODC, the effects of GKLF expression on ODC activity and cell growth were examined by transient transfection assay. HT-29 and HCT116 cells were transfected with either GKLF or control vector pcDNA3. After 48 h, the cells were harvested for the measurement of ODC activity and cell proliferation. As shown in Fig. 2A, cells transfected with GKLF expressed a prominent 60-kDa protein, corresponding to the expected molecular mass of GKLF, indicating that GKLF was overexpressed in these cells. Overexpression of GKLF significantly repressed ODC activity (Fig. 2B) and resulted in growth inhibition when compared with vector-transfected control cells (Fig. 2C). Similar results were observed in HT-29 and HCT116 cells, suggesting that the effects of GKLF on ODC activity and cell growth were not cell-specific. Interestingly, an additional 30-kDa protein was present in GKLF-transfected cells. These findings were also reported previously by another laboratory (10). The significance of this low molecular weight protein is currently unknown, and this may represent a nonspecific binding from the polyclonal GKLF antiserum used in our study or result from a rapid proteolysis of GKLF protein.

The effects of GKLF on ODC activity and cell growth were also examined in HT-29 cells stably expressed GKLF or control pcDNA3 DNA. As illustrated in Fig. 3 (A and B), the concentrations of GKLF mRNA and protein were significantly higher in GKLF-expressed (pg-17) than in control (pC-B-2) cells. Similar to those observed in transient transfection study, the levels of ODC mRNA (Fig. 3A), protein (Fig. 3B), and enzyme activities (Fig. 3C) were lower in GKLF-expressed than in pcDNA3-transfected cells. Moreover, pG-17 cells grew much more slowly than control pC-B-2 cells (Fig. 3D). These results were consistent with the growth inhibitory function of GKLF and suggested that these effects were likely mediated through down-regulation of ODC gene expression.

Expression of GKLF and ODC during Cell Cycle Progression in HT-29 Cells—Previous studies have shown that ODC played an important role during the G1/S transition of the cell cycle (19, 20). If ODC was a potential downstream target of GKLF, as suggested above, the expression of GKLF and ODC should closely relate to each other during cell cycle progression. To examine this hypothesis, HT-29 cells were synchronized to the different phases of cell cycle by serum starvation and cell cycle phase inhibitors, and GKLF and ODC mRNA and protein levels were measured. The distribution of HT-29 cells in cell cycle progression was confirmed by flow cytometric analysis (Fig. 4A). As shown in Fig. 4B, GKLF mRNA and protein levels were low in the exponentially growing cells, and the levels began to increase as cells entered the G1 phase and reached the maximum at the G1/S boundary. GKLF mRNA and protein concentrations were then rapidly decreased as cells entered the S and G2/M phases. In contrast, ODC mRNA and protein levels were high in the exponentially growing cells and in the S phase, and their concentrations began to decrease at the G1 and G1/S transition phases (Fig. 4B). The cyclin A mRNA concentration, as expected, reached the maximal level when cells entered the S phase.

Fig. 5. GKLF represses transcriptional activity of the ODC promoter. A, ODC luciferase construct pODC–1156/+13 (0.5 μg/well) and pcCMV-βgal (0.1 μg) were co-transfected with control pcDNA3 vector (open bar) or pcDNA3/GKLF (hatched bar) into HT-29, HCT116, and Chinese hamster ovary (CHO) cells. Luciferase and β-galactosidase activities were determined 48 h later. B, ODC luciferase construct pODC–1156/+13 (0.5 μg/well) was co-transfected with increased amounts of pcDNA3/GKLF, control pcDNA3 vector, or antisense GKLF (black bar) into HCT116 cells. Luciferase and β-galactosidase activities were determined 48 h later. The data are expressed as percentages of control (cells co-transfected with pcDNA3 only) and represent the mean ± S.E. of three separate experiments after correcting for differences in transfection efficiency by β-galactosidase activities. *, p < 0.01, compared with pcDNA3-transfected cells.

To further explore the potential role of GKLF in mediating cell cycle progression, the distribution of HT-29 cells was examined in HT-29 cells transfected with pcDNA3 or pcDNA3/GKLF. Overexpression of GKLF in HT-29 cells resulted in increases in cells arrested at the G1 phase from 31 to 57% (data not shown), suggesting that GKLF may function as a G1/S checkpoint regulator.

GKLF Represses Transcriptional Activity of ODC Promoter—To further investigate the molecular mechanisms of GKLF-regulated ODC gene expression, the effects of GKLF on ODC promoter activity were first examined in three different cell lines. As illustrated in Fig. 5A, co-transfection with GKLF significantly repressed ODC promoter activity in HT-29, Chinese hamster ovary, and HCT116 cells by 72, 78, and 63%,
respectively. In addition, GKLF dose-dependently inhibited ODC promoter activity, and antisense GKLF transfection resulted in an increase of ODC activity (Fig. 5B). These data indicate that GKLF functions as a transcriptional repressor of the ODC gene and that attenuation of GKLF suppression by antisense GKLF transfection results in an inappropriate activation of the ODC promoter.

To determine the region on ODC promoter responsible for transcriptional repressive effect of GKLF, a serial of truncated ODC promoter constructs were co-transfected with control pcDNA3 or pcDNA3/GKLF plasmid into HCT116 cells. As shown in Fig. 6A, transfection with GKLF resulted in an ~55% inhibition of pODC−1156/+13 promoter activity. Deletion of the ODC promoter sequence from −1156 to −179 did not significantly alter the repressive effect of GKLF, whereas the GKLF effect was completely abolished in pODC−90/+13 Luc construct. These data suggested that the GKLF binding domain on the ODC promoter was probably located at the region between −179 and −90.

The GC-rich Region in the Proximal Portion of ODC Promoter Is Essential for GKLF Binding—Previous reports have demonstrated a GC-rich region, located at −123 to −91 of the ODC promoter, consisting of a potential protein-binding site for at least three zinc finger transcription factors, including Sp1, WT1, and ZBP-89 (21, 22). To examine whether this GC-rich region also comprised a GKLF-binding domain, three mutated ODC constructs were created, and the sequences were shown on Fig. 6B. Mutation of the GC region between −114 and −110 (M1) or −104 and −100 (M3) has no or minimal effect on GKLF function, but the repressive effect of GKLF was completely abolished in ODC construct in which the GC region between −109 and −105 (M2) was mutated. These results indicate that the GC-rich area between −109 and −105 of the ODC promoter is required for GKLF function.

The GKLF-binding motif on the ODC promoter was further characterized by electrophoretic mobility shift assay. Electrophoretic mobility shift assay using a wild-type probe (nucleotides −116 to −99 of the ODC promoter) revealed the presence of four major DNA-protein binding complexes (designated as C1, C2, C3, and C4) in nuclear extracts from HCT116 cells (Fig. 7). Binding of all bands to this GC-rich region probe was reduced significantly upon competition with 25- or 50-fold molar excess of unlabeled probe (Fig. 7, lanes 4 and 5) but not with mutated M2 oligonucleotide (Fig. 7, lane 6), indicating a specific binding to the wild-type probe. To determine whether these complexes contained GKLF, supershift assays were performed using GKLF antibodies. As illustrated in Fig. 7, the C4 band was supershifted with GKLF antibodies (Fig. 7, lane 7), suggesting that the GC-rich region contained a functional binding element for GKLF. Interestingly, the intensity of the supershifted band appeared to be less than that of the C4 band. It is possible that GKLF antibody might interfere with or disrupt DNA-protein binding.

GKLF Represses Sp1-stimulated ODC Promoter Activity—
Previous studies on ODC promoter had shown that the GC-rich region also consisted of a Sp1-binding domain (21). To further characterize the function of GKLF on ODC gene, the effect of GKLF on Sp1-mediated ODC promoter activity was investigated. As illustrated in Fig. 8A, co-transfection with pCMV-Sp1 plasmid stimulated transcriptional activity of the ODC promoter in a dose-dependent manner, and these effects were attenuated by increased amount of GKLF DNA (Fig. 8B). These results indicated that GKLF might interact with the same or in the close proximity of the Sp1-binding element on the ODC promoter. The physical interaction between GKLF and Sp1 on the ODC promoter was further examined by a chromatin immunoprecipitation assay. Chromatin fragments from HCT116 cells transfected with pcDNA3 or pcDNA3/GKLF DNA were immunoprecipitated with or without monoclonal anti-Sp1 antibody. DNA from the immunoprecipitant was isolated and subjected to PCR analysis using primers specific to the putative Sp1-binding domain on the proximal portion of ODC promoter. As illustrated in Fig. 9A, an expected 212-bp DNA fragment was amplified in samples containing total input chromatin or Sp1 immunoprecipitant but not in the control sample (mock) containing dialysis buffer. Furthermore, the intensity of amplified DNA fragment is higher in pcDNA3- than in pcDNA3/GKLF-transfected cells (Fig. 9), suggesting that GKLF may compete with Sp1 for the same binding domain on the ODC promoter.

**DISCUSSION**

Colorectal cancer is a major cause of cancer deaths in the Western world. Although molecular analysis of colorectal tumours in the past few decades has resulted in remarkable progress in the identification of a number of genes that are mutated during colorectal carcinogenesis, the cellular and molecular events governing cell growth in the colon remain poorly understood. GKLF (KLF4) is a recently identified and developmentally regulated transcription factor (9). Several studies have shown that GKLF is a negative regulator of cell proliferation; however, the mechanisms of its action are still unclear. In a previous study, we have demonstrated that IFN-γ stimulated GKLF mRNA and protein levels in colonic cancer cells and that enhanced GKLF expression is associated with IFN-γ-promoted growth inhibition and apoptosis (14). In this study, we find that the induction of GKLF expression by IFN-γ is associated with down-regulation of ODC gene expression,
suggesting that ODC may function as a downstream target of GKLF that involves in growth inhibition of tumor cells.

Previous studies have shown that the expression of endogenous ODC was tightly coupled to mammalian cell proliferation (1, 2). Elevated cellular ODC expression and enzyme activity is essential for normal cellular DNA synthesis, and inhibition of ODC expression reduces cell proliferation (3, 4). Abnormal ODC expression may have detrimental effects on cell growth and result in malignant transformation and carcinogenesis. Therefore, to maintain normal growth and differentiation, cells must possess the ability to regulate ODC gene expression. The molecular mechanisms responsible for the regulation of the ODC gene are, however, not fully elucidated. In the current studies, overexpression of GKLF in colon cancer cells down-regulated ODC gene expression and its enzyme activity and resulted in growth inhibition. In addition, ODC promoter activity was significantly repressed by GkLF. These findings support our hypothesis that ODC is a downstream target of GkLF. Previously, our laboratory has shown that GkLF mRNA levels were significantly reduced in the colon polyps and cancer tissues (12). As stated above, many studies reported elevated ODC activities in the colon cancer tissues (5, 6). It is plausible that down-regulation of GkLF expression in the colon may induce ODC gene activity and result in cell hyperproliferation and, ultimately, colon cancer formation.

The eukaryotic cell cycle is a carefully regulated event, and the growth of mammalian cells is tightly controlled by checkpoints in the cell cycle. Two checkpoints, one at the G1/S transition and the other at the G2/M transition, have recently been described to control and ensure the order of events in the cell cycle and to integrate DNA repair with cell cycle progression. Several zinc finger-containing transcription factors are implicated in the regulation of cell cycle progression, and mutation of genes encoding components of cell cycle checkpoints has been shown to increase genetic instability and accelerate cellular evolution (20). Previous studies have demonstrated that upon stimulation of quiescent cultured cells by fresh medium, the levels of GkLF mRNA expression are decreased significantly. These data suggest that GkLF may exert a negative effect on cell cycle progression. In addition, ODC has also been shown to be an important marker for quiescent cells to progress through the G1 and into the S phase of the cell cycle (12). In this report, we have shown that the level of GkLF gene expression is the highest at the G1/S transition phase of the cell cycle. In contrast, ODC mRNA and protein levels were the lowest at the G1/S transient phase. Moreover, overexpression of GkLF in HCT-29 cells significantly reduced ODC mRNA and protein levels and resulted in an increase in cells arrested at the G1/S phase. Together, these data suggest that GkLF may function as a G1/S checkpoint regulator and that down-regulation of GkLF results in overexpression of ODC gene and allows cells to enter the proliferation phase of cell cycle. In our previous report we have shown that GkLF repressed cyclin D1 promoter activity and resulted in growth inhibition (13). It is possible that the effect of GkLF on ODC gene expression may result from alternation of cyclin D1 levels. However, our current report showing the interaction between GkLF and ODC promoter as well as the identification of a GkLF-binding element on ODC promoter suggests that the inhibitory property of GkLF on ODC is likely independent from the cyclin D1 gene.

The regulation of ODC gene expression may occur at different levels, including transcriptional and post-transcriptional mechanisms (21–24). It has been observed that hormones, growth factors, tumor promoters and several oncogenes, such as ras (25), fos (26), mos (27), and myc (28, 29), stimulated ODC activity in cells. Several DNA-binding domains, including sites for Sp1, CREB/ATF have recently been identified on the ODC promoter (22, 23); however, little is known about transcription factors that repress ODC promoter activity. Mosher et al. (30) and Li et al. (31) have demonstrated that Wilm’s tumor suppressor (WT1), a zinc finger transcription factor, repressed the transcriptional activity of ODC gene by interacting with multiple binding sites on the promoter. Li et al. (22) and Law et al. (23) showed that NF-ODC1 protein inhibited ODC promoter activity through a Sp1-independent mechanism, and ZBP-89, a DNA-binding protein, appeared to be a candidate protein responsible for NF-ODC1 binding. In this report, we showed that GkLF inhibited transcriptional activity of the ODC promoter, and these effects appeared to be mediated by interaction with the GC-rich region on the promoter. Although the putative GkLF-binding domain CCTCC on the ODC promoter is closely related to ZBP-89 binding element CCTCCCCC (21), the DNA-protein complexes formed by GkLF and ZBP-89 were quite distinct. Previously, Li et al. (22) showed that interaction between the GC-rich region and Jurket nuclear extracts resulted in four different DNA-protein complexes. They also reported that the C1 complex was primarily due to Sp1 binding and that the C3 complex was the result of NF-ODC1 binding (22). In our current study, four identical DNA-protein complexes were also observed when radiolabeled GC-rich oligonucleotide was incubated with nuclear extracts from HCT116 cells. Moreover, supershift study confirmed that the C4 DNA-protein complex might result from GkLF binding. Although our study did not specifically examine DNA-protein interaction among GkLF, Sp1, and ZBP-89, it was likely that the GkLF-binding domain overlapped or in the close proximity of Sp1- or ZBP-89-binding
region. These conclusions were further supported by our results showing that GKLF inhibited basal and Sp1-induced transcriptional activity of the ODC promoter and that GKLF co-transfection significantly reduced Sp1 binding on the ODC promoter by chromatin immunoprecipitation assay.

Although the precise physiological function of ZBP-89 is not clear, ZBP-89 has been shown to repressed epidermal growth factor-stimulated promoter activity of the gastrin gene in a GH4 pituitary cell line and may play a role in cell proliferation (32). As stated above, GKLF mediated growth arrest in colon cancer cells. Together, these data suggested that GKLF might interact with other transcription factors, such as ZBP-89 or Sp1, on the ODC promoter to regulate normal cell growth in the colon. These interactions warrant further investigation.

In summary, our data suggest that the growth inhibitory effect of GKLF result in part from down-regulation of ODC gene expression. GKLF functions as a G1/S checkpoint regulator, and this effect is likely mediated through ODC. Finally, GKLF inhibited the activation of the ODC promoter through interaction with the GC-rich region in the proximal portion of the promoter.

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