Up-regulation of gut-enriched krüppel-like factor by interferon-γ in human colon carcinoma cells

Zhi Y. Chen, Jue-Lon Shie, Chi-Chuan Tseng*

Section of Gastroenterology, Boston VA Medical Center and Boston University School of Medicine, E.R.B.C. Room 513, 650 Albany Street, Boston, MA 02118, USA

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Abstract Interferon-γ (IFN-γ) induces growth arrest and apoptosis of tumor cells but the mechanisms for these functions are unknown. Recently, gut-enriched krüppel-like factor (GKLF) was found to possess similar biological properties. Treatment of HT-29 cells with IFN-γ inhibited cell proliferation and induced apoptosis, the effect was found to associate with GKLF expression. IFN-γ stimulates GKLF mRNA and protein levels in a dose- and time-dependent manner and this process is independent of p53, occurs rapidly and does not require de novo protein synthesis indicating that GKLF is an immediate-early IFN-γ-responsive gene. Moreover, overexpression of GKLF results in similar effect as IFN-γ suggesting that GKLF may function as a downstream target of IFN-γ. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Gut-enriched krüppel-like factor; Apoptosis; Growth arrest; HT-29 cell; p53

1. Introduction

Interferons (IFNs), including type I (IFN-α and -β) and type II (IFN-γ), are a family of cytokines with diverse biological functions, including antiviral, antiproliferative and immunomodulatory effects [1–6]. It has been postulated that IFNs exert their biological activities by inducing the expression of a number of effector genes (IFN-inducible genes or IFN-γ-response genes) on the target cell, and that these effects are modulated largely at the transcriptional level [7,8]. It has also been suggested that the intracellular signaling pathway triggered by the activation of IFNs receptors involved phosphorylation of Janus kinase (JAK), and signal transducer and activator of transcription (STAT) by tyrosine kinase [9,10].

Previous studies have demonstrated that IFN-γ inhibits cell growth and induces apoptosis in several tumor cells [11,12]. However, the mechanisms mediating its action are not fully understood. It has been reported that IFN-γ induces the expression of $p21^{WAF1/CIP1}$, a repressor of cyclin-dependent kinase, and inhibits cell growth by blocking the cell cycle progression [11]. In addition, Xu et al. showed that IFN-γ upregulates the expression of Fas and Fas ligand (FasL) on HT-29 cells and subsequently induces apoptosis of these cells [12]. They suggested that the growth inhibitory effect of IFN-γ is due to Fas- and FasL-mediated apoptosis. More recently, Chin et al. demonstrated that the activation of the STAT pathway is critical for IFN-γ-induced apoptosis [13]. Together, these data suggest that IFN-γ may induce growth arrest through multiple mediators.

Gut-enriched krüppel-like factor (GKLF) is a newly identified eukaryotic zinc finger transcription factor [14,15]. The amino acid sequence of the zinc finger portion of GKLF is closely related to other krüppel proteins, including the lung krüppel-like factor, and the erythroid krüppel-like factor, and the basic transcription element binding protein 2 [16,17]. The GKLF mRNA levels in NIH 3T3 fibroblasts were found to be increased during growth arrest by serum deprivation or contact inhibition [14]. Our laboratory has recently shown that GKLF expression was down-regulated in the dysplastic colonic epithelium suggesting that GKLF might be an important factor in controlling cell growth [18]. Furthermore, proinflammatory cytokines, particularly IFN-γ, have been shown to be up-regulated in patients with inflammatory bowel diseases in which active cellular turn-over occurs [19]. Whether GKLF is a potential downstream target of IFN-γ has not been investigated. In the present report, we demonstrate that GKLF mRNA expression in HT-29 cells was induced by IFN-γ, and that this induction is associated with IFN-γ-promoted growth inhibition and programmed cell death. Furthermore, up-regulation of GKLF expression by IFN-γ is time- and dose-dependent, and does not depend on p53 and de novo protein synthesis. This is the first report suggesting that GKLF is one of the target genes of IFN-γ.

2. Materials and methods

2.1. Cell culture and IFN-γ treatment

The human colon carcinoma cell line (HT-29) was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). Two additional human colon carcinoma cell lines, 40-16 (HCT116, p53+/+) and 379.2 (HCT116, p53−/−), were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University). These cells were cultured in modified McCoy's 5A medium containing 10% fetal bovine serum, penicillin and streptomycin, in an atmosphere of 95% air and 5% carbon dioxide at 37°C. Cells were subcultured at appropriate intervals to maintain subconfluent growth conditions. Human recombinant IFN-γ was purchased from Boehringer Mannheim (Indianapolis, IN), and used at a final concentration of 200 U/ml unless stated otherwise. IFN-γ treatments were performed by replacing the media with fresh complete medium with or without IFN and cells were harvested at appropriate time points for analysis. Human recombinant IFN-α and IFN-β were obtained from from Boehringer Mannheim (Indianapolis, IN).

2.2. Cell transfection

Transient transfection was performed by the lipofection method using the lipofectin reagent as recommended by the manufacturer (Life Technologies, Inc.). Transfections of 40-16 (HCT116, p53+/+) and 379.2 (HCT116, p53−/−) cells were accomplished using 2 μg/well pcDNA3 or pcDNA3-GKLF DNA in six-well plates. Two days fol-

*Corresponding author. Fax: (1)-617-638 7785.

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lowing transfection, cells were examined for specific protein expression by Western blot analysis.

2.3. Measurement of cell proliferation

Cell number was determined using a semi-automated tetrazolium-based colorimetric (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [20]. Briefly, HT-29 cells were seeded into a 24-well plate at a density of 5×10^4 cells/well and incubated overnight at 37°C. Cells were treated with IFN-γ for 24 h and at the end of treatment, 20 μl of MTT (2.5 mg/ml) was added to each well. Cells were incubated for an additional 4 h to allow the interaction between dye and mitochondrial dehydrogenase in the viable cells. After removal of the residual dye and medium, 500 μl of dimethylsulfoxide was added, and the absorbance at 540 nm was measured with a spectrophotometer.

2.4. Detection of DNA fragmentation

For analysis of DNA fragmentation, control and IFN-γ-treated cells were collected by centrifugation. Low molecular weight genomic DNA was extracted as described in [21]. Briefly, the washed cells were lysed in a solution of 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, and 0.5% Triton X-100. Samples were mixed and then centrifuged at $13\,000\times g$ for 10 min. The supernatant containing low molecular weight DNA was recovered and extracted twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v). DNA was precipitated in 0.3 M sodium acetate and two volumes of cold (−20°C) ethanol overnight. The pellets were resuspended in TE buffer containing 100 ng of DNase-free RNase and incubated for 30 min at room temperature before being subjected to electrophoresis in 2% agarose gels. DNA was visualized by ethidium bromide staining. Gels were photographed using a Polaroid camera with 667 film.

2.5. 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 in 1.2% agarose-formaldehyde gel, and transferred onto Zetabind nylon membranes (Cuno, Inc., Meriden, CT). Hybridization was performed at 42°C overnight using [α-^{32}P]dCTP-labeled GKLF probe (Random primer labeling kit from Boehringer Mannheim, Indianapolis, IN). Blots were washed with 2×SSPE/0.1% SDS, followed by 0.1×SSPE/0.1% SDS. All blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA probe (GAPDH; Clontech, Palo Alto, CA) to correct for RNA loading.

2.6. Western blot analysis

To obtain whole-cell extracts, cells were washed twice with ice-cold phosphate-buffered saline, scraped, and pelleted by centrifugation ($200\times g$). Cell pellets were then lysed in the standard RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. Protein concentrations were determined by Bio-Rad assays and 25–80 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. Polyclonal antibody to GKLF was used at 1:500 dilution, and the DO-1 p53 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:1000 dilution. Specific protein was detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) following the manufacturer's instruction (Amersham, Arlington Heights, IL).

2.7. Statistics

Results are expressed as mean ± S.E.M. Statistical analysis was performed using ANOVA and Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. IFN-γ-induced growth inhibition and programmed cell death are correlated with up-regulation of GKLF expression in HT-29 cells

The effect of IFN-γ on the growth of HT-29 cells was examined using the MTT assay. As shown in Fig. 1A, IFN-γ inhibited cell growth in a dose-dependent manner with significant inhibition observed at 20 and 200 U/ml. To examine whether the growth inhibitory effect of IFN-γ is associated with apoptosis of HT-29 cells, DNA fragmentation analysis was performed. As shown in Fig. 1B, at concentrations of 20 or 200 U/ml, IFN-γ was capable of inducing DNA fragmentation in HT-29 cells. In contrast, no fragmented DNA was observed in untreated control cells. These results are consistent with previous reports indicating that IFN-γ treatment induces growth arrest and apoptosis of HT-29 cells.

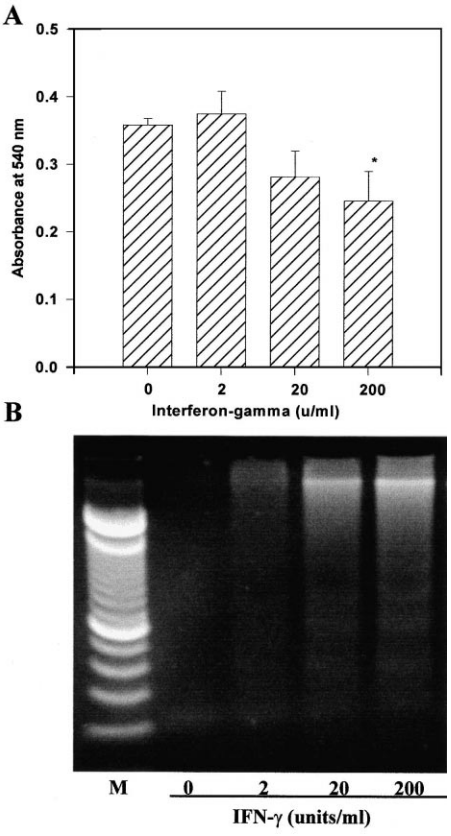

Fig. 1. IFN-γ induces growth inhibition and apoptosis of HT-29 cells in a dose-dependent manner. A: HT-29 cells were treated with different concentrations of IFN-γ for 24 h and cell growth was measured using the MTT assay as described in Section 2. Each data point represents the mean ± S.E.M. of three separate experiments and was expressed as optimal density (OD). $^*P<0.05$ vs. control (dose = 0). B: Low molecular weight genomic DNA extracted from IFN-γ-treated HT-29 cells was analyzed by agarose gel electrophoresis and DNA fragmentation was visualized by ethidium bromide staining.

To explore whether GKLF is involved in the IFN-γ-induced growth arrest and apoptosis, GKLF mRNA levels were examined in IFN-γ-treated or untreated HT-29 cells by Northern blot analysis. As shown in Fig. 2, GKLF mRNA levels were induced dose-dependently by IFN-γ treatment (Fig. 2A), and these increments were also time-dependent, with the most significant increase observed at 24 h after treatment (Fig. 2B). Moreover, the induction of GKLF mRNA expression was associated with increases in GKLF protein levels (Fig. 2C). These data suggest that GKLF is an IFN-γ-inducible gene, and may play a role in IFN-γ-promoted growth arrest and programmed cell death in HT-29 cells.

3.2. GKLF expression induced by IFN-γ is independent of p53

Although p53 protein has been shown to regulate the expression of several transcription factors, the p53 gene in HT-29 cells was known to be mutated. To determine whether the induction of GKLF expression by IFN-γ is mediated through p53, two human colonic adenocarcinoma cells, 40-16 (HCT116, p53+/+; possessing wild-type p53) and 379.2 (HCT116, p53−/−; a p53-deficient mutant), were used for the studies. As demonstrated in Fig. 3, IFN-γ increased GKLF mRNA levels in a dose-dependent manner in both 40-16 and 379.2 cells suggesting that IFN-γ-mediated GKLF expression is not dependent on p53 protein.

3.3. Specificity of IFN-γ-induced GKLF expression

Previous studies have shown that type I and type II IFNs induce the expression of several common genes, but the expression of certain genes can be selectively induced by either type I or II IFNs [22]. To examine the specificity of IFN-γ-induced GKLF mRNA expression, HT-29 cells were cultured in the presence of IFN-α or IFN-β. As shown in Fig. 4, at concentrations of 0–1000 U/ml, neither IFN-α (Fig. 4A) nor IFN-β (Fig. 4B) had a significant effect on GKLF mRNA levels in HT-29 cells, suggesting that IFN-γ-induced GKLF expression is type II IFN-specific. To further explore the potential contribution of other serum factors on IFN-γ-induced GKLF expression, HT-29 cells were cultured in the absence of

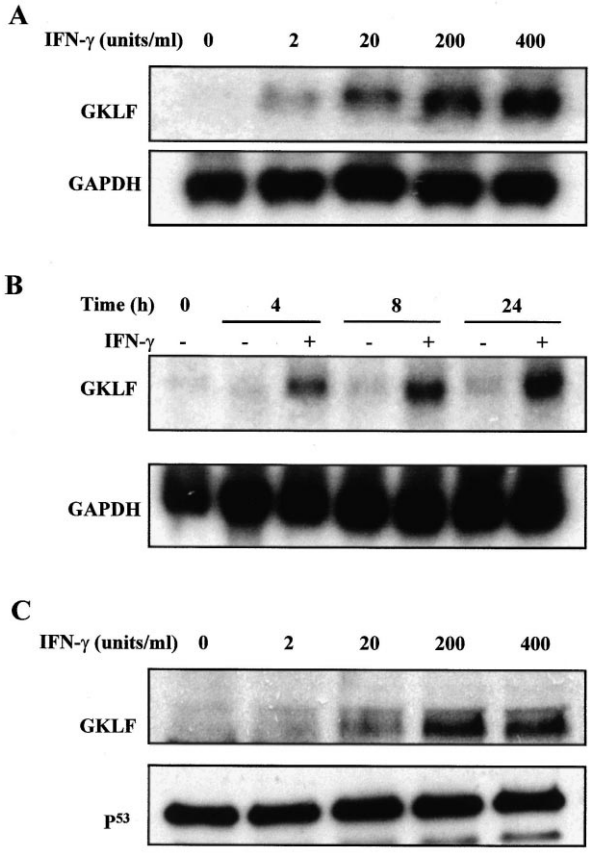

Fig. 2. IFN-γ induces up-regulation of GKLF mRNA and protein levels. A: HT-29 cells were incubated with increasing concentrations (0–400 U/ml) of IFN-γ for 24 h and total RNA was prepared and analyzed by Northern blot as described in Section 2. B: HT-29 cells were cultured with either medium alone (−) or with 200 U/ml IFN-γ (+) for 0–24 h. The expression of GKLF mRNA was examined by Northern blot analysis. To normalize the amount of loaded RNA, the blot was stripped and hybridized with a ^{32}P-labeled GAPDH cDNA. C: HT-29 cells were treated with various concentrations (0–400 U/ml) of IFN-γ for 24 h. The GKLF and p53 protein levels were examined by Western blot analysis as described in Section 2.

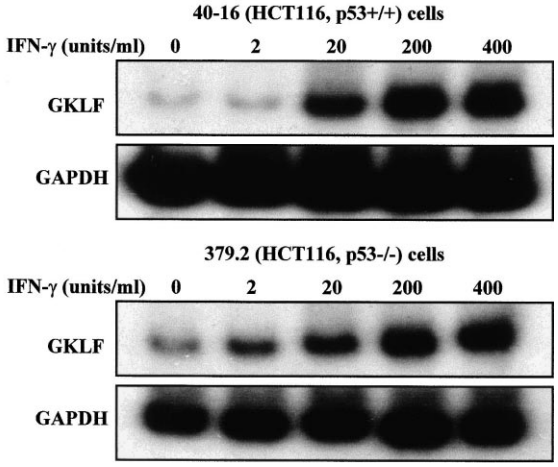

Fig. 3. GKLF mRNA expression induced by IFN-γ is independent of p53. The 40-16 (HCT116, p53+/+) and 379.2 (HCT116, p53−/−) cells were cultured in the presence of various concentrations of IFN-γ (0–400 U/ml) for 24 h. GKLF expression was determined by Northern blot analysis. To confirm the amount of RNA loaded, the same blot was hybridized with a GAPDH probe.

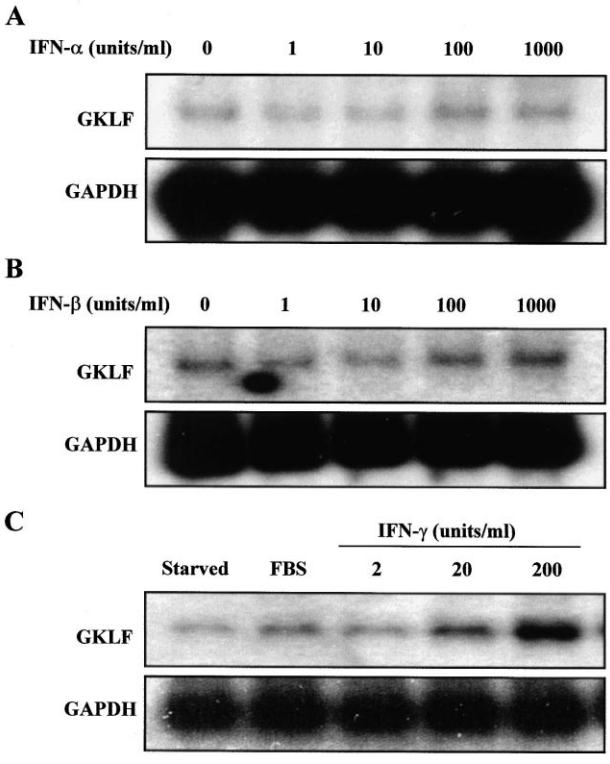

Fig. 4. Specificity of IFN-γ-induced GKLF expression in HT-29 cells. Cells were treated with different doses of IFN-α (A) or IFN-β (B) (0–1000 U/ml) for 24 h, and GKLF mRNA levels were determined by Northern blot analysis. C: HT-29 cells were starved for 12 h in the serum-free medium, and then treated with IFN-γ for an additional 24 h in the serum-free medium. Total RNA was extracted and analyzed for the presence of GKLF mRNA.

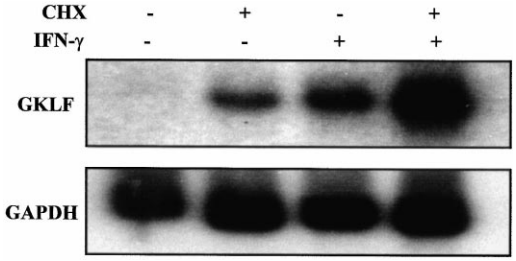

Fig. 5. GKLF mRNA induction by IFN-γ does not require de novo protein synthesis. HT-29 cells were preincubated with (+) or without (−) cycloheximide (CHX) for 2 h and then treated with (+) or without (−) IFN-γ for 24 h. GKLF mRNA levels in HT-29 cells were determined by Northern blot analysis. To confirm the amount of RNA loaded, the same blots were hybridized with a GAPDH probe.

serum for 12 h, and then treated with IFN-γ in the serum-free medium for an additional 24 h. As shown in Fig. 4C, GKLF mRNA levels were significantly increased in cells treated with 200 U/ml IFN-γ in the presence of serum-free medium, indicating that the effect of IFN-γ on GKLF expression was not mediated through other serum factors.

3.4. IFN-γ induces GKLF mRNA expression without new protein synthesis

To determine whether the induction of GKLF expression by IFN-γ is dependent on de novo protein synthesis, HT-29 cells were preincubated with cycloheximide (CHX), a protein synthesis inhibitor, and then treated with IFN-γ. As shown in Fig. 5, CHX did not inhibit the induction of GKLF by IFN-γ. In contrast, preincubation with CHX enhanced both basal and IFN-γ-stimulated GKLF mRNA levels in HT-29 cells. These results suggest that IFN-γ-induced GKLF expression is independent of new protein synthesis, and that GKLF may function as an immediate-early IFN-γ-responsive gene.

3.5. Kinetics of GKLF mRNA induction by IFN-γ

The kinetics of GKLF mRNA induction by IFN-γ was examined in HT-29 cells. In response to IFN-γ (200 U/ml), GKLF mRNA levels increased at 2 h, reached the maximal concentration at 24 h and then gradually decreased (Fig. 6A). The effect of IFN-γ treatment on GKLF mRNA stability was assessed by culturing HT-29 cells in the presence of IFN-γ for 24 h. Actinomycin D was then added to the medium to inhibit the synthesis of new mRNA and total GKLF mRNA levels were determined at different time points. As shown in Fig. 6B, the GKLF mRNA level decreased gradually during the next 24-h period and the estimated $t_{1/2}$ for GKLF mRNA turnover was approximately 6 h (Fig. 6C).

3.6. Overexpression of GKLF induces apoptosis in colon cancer cells

To determine whether GKLF overexpression affects the growth and apoptosis of colon cancer cells, 40-16 (HCT116, p53+/+), 379.2 (HCT116, p53−/−), and HT-29 cells were transiently transfected with pcDNA3/GKLF or pcDNA3 (control) for 48 h. Western blot analysis confirmed that cells transfected with pcDNA3/GKLF expressed a major (60 kDa) and a minor (30 kDa) protein (Fig. 7A), whereas very little native GKLF protein was identified in pcDNA3-transfected cells. Overexpression of GKLF significantly decreased cell proliferation in all three cell lines (Fig. 7C). Moreover, GKLF promoted apoptosis in both 40-16 and HT-29 cells but not in 379.2 cells (Fig. 7B). Although the mechanisms through which GKLF is involved in the induction of apoptosis in colon cancer cells are unknown, our results suggest that this process may be p53-independent.

4. Discussion

IFN-γ is a cytokine produced primarily by T lymphocytes and natural killer cells. In addition to its antiviral properties, IFN-γ has recently been shown to be an important regulator of cell growth, differentiation, and apoptosis [11–13]. However, the mechanism(s) mediating the IFN-γ effects has not been fully elucidated. GKLF is a newly identified eukaryotic

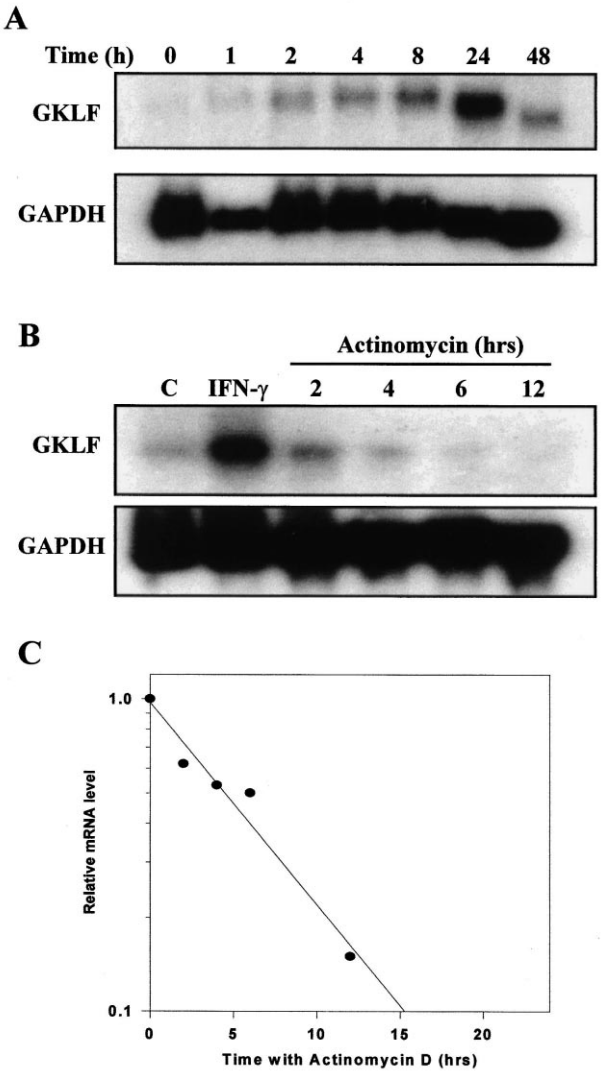

Fig. 6. Kinetics of GKLF mRNA induction by IFN-γ. (A). HT-29 cells were treated with IFN-γ (200 U/ml) and harvested sequentially for different durations. Total RNA was extracted and GKLF expression was analyzed by Northern blot. B: The stability of GKLF mRNA in IFN-γ-treated HT-29 cells was examined by treating cells with IFN-γ (200 U/ml) for 24 h before the addition of actinomycin D (10 μg/ml). Total RNA was harvested at the indicated times and examined for the expression of GKLF and GAPDH. C: The half-life ($t_{1/2}$) of GKLF mRNA turn-over was calculated from the above data.

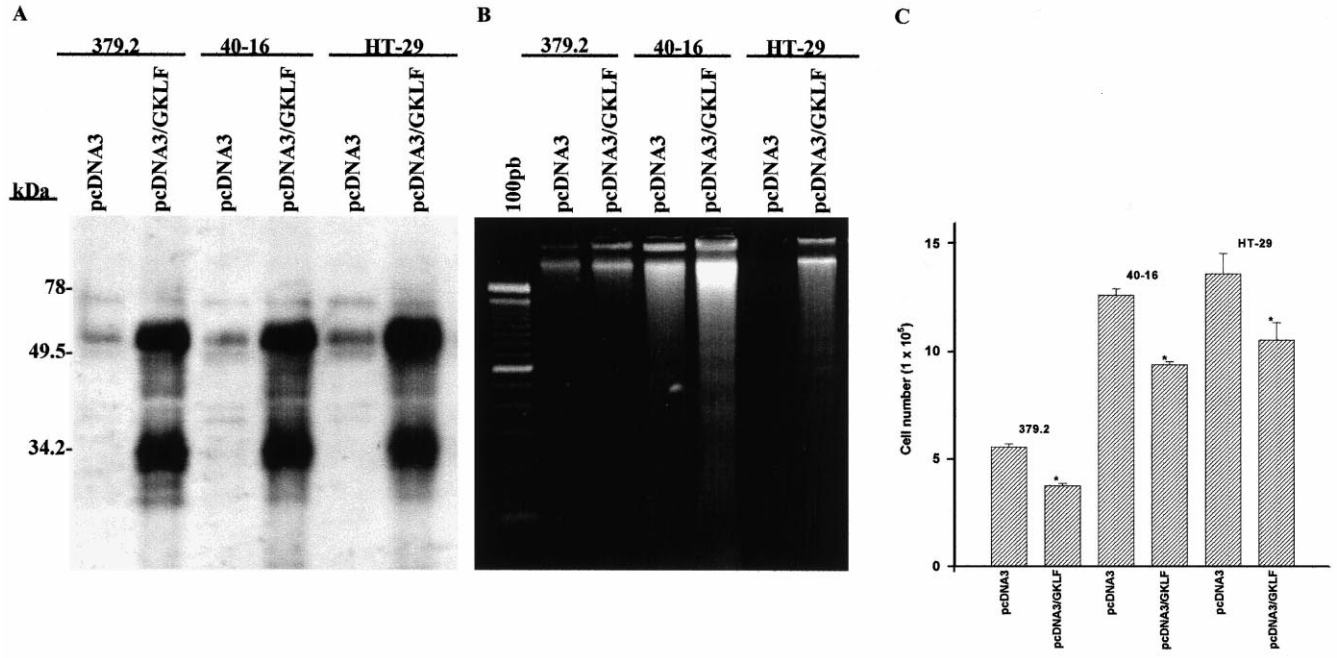

Fig. 7. Overexpression of GKLF induced growth arrest and promoted apoptosis. A: The 379.2, 40-16 and HT-29 cells were transiently transfected with pcDNA3-GKLF or pcDNA3 (control) cDNA. Total protein was harvested at 48 h and examined for the expression of GKLF. B: Transfected cells were harvested to examine for the presence of fragmented DNA as described in Section 2. C: Cells were transfected with GKLF or pcDNA3 (control) cDNA, and cell numbers were determined 48 h later. Each data point represents mean ± S.E.M. of three separate experiments. $*P < 0.05$ compared to pcDNA3-transfected cells.

transcription factor widely distributed in the gastrointestinal tract [14,15]. Previous studies have shown that the expression of GKLF is temporally associated with growth arrest of NIH 3T3 fibroblasts [14–17]. Recent studies from our laboratory, as presented above, have demonstrated that GKLF may play an important role in the regulation of cell growth in the gastrointestinal tract [18]. The mechanisms modulating GKLF expression have not been explored previously. In the present study, we show that GKLF mRNA and protein levels were low in the proliferating HT-29 cells and that IFN-γ treatment significantly enhanced GKLF expression at both the mRNA and protein level, suggesting that GKLF may be a downstream target gene of IFN-γ. Furthermore, IFN-γ treatment in HT-29 cells reduced cell number and promoted DNA fragmentation, and similar effects were observed in cells overexpressing GKLF DNA. These results suggest that up-regulation of GKLF expression may play an important role in IFN-γ-induced growth arrest and apoptosis of colon cancer cells.

In this report, we show that IFN-γ-induced up-regulation of GKLF expression occurs rapidly (2 h after treatment) and does not depend on de novo protein synthesis. Furthermore, co-incubation with cycloheximide fails to suppress GKLF induction and results in a further increase in GKLF mRNA levels, indicating that one or more factors which are sensitive to cycloheximide and are capable of affecting GKLF expression may already be present in HT-29 cells. This phenomenon is similar to what has been observed in other immediate-early response genes [23,24], and suggests that GKLF may function as an immediate-early IFN-γ-responsive gene. A series of immediate-early IFN-γ-responsive genes including *IRF-1*, *FcrR1*, *Ly6-A/E*, *GBP* and *ICSBP* have recently been identified [25–29]. These genes share some common features, i.e. their promoters contain a GAS sequence to which activated Stat 1α can bind; and the GAS sequence is essential for their transcriptional induction. Furthermore, the expression of immediate-early IFN-γ-response genes is mediated by tyrosine phosphorylation of the pre-existing Stat 1α, a mechanism accounting for their rapid induction. Whether these properties exist in the GKLF gene warrant further examination.

Mutations of the p53 gene are commonly found in various human cancers, including colon cancer. Loss of normal p53 activity leads to uncontrolled cell growth, suggesting that p53 is a tumor suppressor gene. Although the mechanism mediating the suppressive effect of p53 is not fully understood, p53 was found to bind to DNA in a sequence-specific manner and stimulated the transcription of genes downstream of the binding domains, such as $p21^{WAF1/CIP1}$ [30,31]. Recently, Yang et al. reported that wild-type p53 is necessary for the induction of GKLF by DNA damage induced by methyl methane sulfonate in mouse embryo fibroblasts [32]. In the present study, up-regulation of GKLF expression by IFN-γ was observed not only in HT-29 cells (p53 mutant) but also in 40-16 (HCT116, p53+/+) and 379.2 (HCT116, p53−/−) cells, suggesting that IFN-γ-induced GKLF expression is mediated through a p53-independent pathway. Using a transient transfection assay, we found that overexpression of GKLF reduces cell growth in all three cell lines. However, DNA fragmentation was only observed in 40-16 and HT-29 cells, but not in 379.2 cells. These results provide evidence that the growth arrest and apoptosis induced by GKLF in colon cancer cells may be mediated through a different pathway. Further investigation will be required to elucidate their mechanisms.

Previous studies have shown that type I and II IFNs induce the expression of several common genes, whereas the expression of certain genes is modulated differentially by the type I or II IFNs [22,25]. These effects may account for the similar

and different biological properties shared by two IFNs. In this report, we show that the induction of GKLF mRNA expression by IFN-γ is specific to type II interferon, as no significant change in GKLF mRNA level was observed in cells treated with IFN-α or -β. These data suggest that GKLF is a downstream target of IFN-γ that mediates its unique functions including growth arrest and apoptosis in the colon cancer cells.

In summary, the results of this study demonstrate that IFN-γ induced GKLF gene expression in HT-29 cells in a dose-dependent and time-dependent manners. This induction is rapid, p53-independent, and does not require de novo protein synthesis suggesting that GKLF is an immediate-early IFN-γ-responsive gene. Overexpression of GKLF in colon cancer cells induces similar effects as IFN-γ indicating that GKLF is the downstream target of IFN-γ. These data and our previous report [18] suggest that GKLF may play an important role in the physiological cell renewal by inducing cell proliferation, differentiation and cell death through apoptosis in the non-neoplastic colonic mucosa as well as in cancer cells.

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