Destabilization of Krüppel-Like Factor 4 Protein in Response to Serum Stimulation Involves the Ubiquitin-Proteasome Pathway

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Abstract

Although the zinc finger transcription factor Krüppel-like factor 4 (KLF4) has been shown to be a negative regulator of cell proliferation, the mechanisms underlying the posttranslational modification of KLF4, especially at the level of protein degradation, are poorly understood. Here, we show that KLF4 protein levels in quiescent cells were high, but decreased rapidly as cells entered the proliferating stage following serum stimulation. This decrease was partially reversed by pretreatment with MG132, a proteasome inhibitor. Moreover, KLF4 was an unstable protein that underwent rapid turnover, and exhibited a relatively short half-life ($t_{1/2} \sim 120$ minutes). To investigate the involvement of the ubiquitin-proteasome pathway in the regulation of the stability of KLF4, HCT116 cells were treated with proteasome inhibitors. Our results showed that, following lactacystin treatment, levels of endogenous KLF4 increased in a time- and dose-dependent manner. Using a cell-free system, in vitro–translated $^{35}$S-labeled KLF4 protein was degraded by protein extracts prepared from exponentially growing HCT116 cells in the presence of ATP. These effects were prevented by pretreatment with MG132 or replacement of ATP with ATP-$\gamma$-S, a nonhydrolyzable analogue of ATP, suggesting that ATP is required for KLF4 degradation by the 26S proteasome. In addition, KLF4 was subject to ubiquitination when cells were treated with the proteasome inhibitor or transfected with exogenous ubiquitin. Collectively, these results indicate that destabilization of KLF4 following serum stimulation is mediated, at least in part, through a ubiquitin-proteasome pathway. (Cancer Res 2005; 65(22): 10394–100)

Introduction

The ubiquitin-proteasome pathway is responsible for the degradation of many short-lived regulatory proteins in vivo (1–3). Targeting of cellular proteins for proteasomal proteolysis is a highly complex and tightly regulated process that is comprised of two distinct steps: the covalent attachment of multiple ubiquitin molecules to the protein substrate, and the degradation of the polyubiquitinated protein by the 26S proteasome complex (4–6). The formation of ubiquitin-protein conjugates involves the sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2 or Ubc), and ubiquitin-protein ligases (E3). Generally, eukaryotic cells contain a single type of E1, multiple E2s, and a large number of E3s (7). The first enzyme, E1, activates ubiquitin in an ATP-dependent reaction by forming a thio ester bond with the COOH-terminal glycine of ubiquitin. The activated ubiquitin is then transferred to an active site cysteine of an E2. For the most part, the final step requires an E3 that recognizes E2 and facilitates the transfer of the activated ubiquitin from E2 to a lysine residue of a bound substrate, forming an isopeptide bond, or, in some cases, forming a thio ester with ubiquitin such as the HECT (homologous to E6-AP COOH terminus) family E3s (5, 6, 8). E3s play important roles in catalyzing the formation of chains of ubiquitin molecules on substrates. Once the polyubiquitin chain is assembled on a protein substrate by the cooperation of E1, E2, and E3 enzymes, the target protein is recognized and degraded by the 26S proteasome complex (9, 10).

Gut-enriched Krüppel-like factor, also known as Krüppel-like factor 4 (KLF4) or epithelial zinc finger, is a member of the Krüppel-like transcription factor family (11–14). The expression of KLF4 is primarily localized to epithelial cells of the gastrointestinal tract and several other organs such as the lung and skin (14–16). It has been shown that overexpression of KLF4 is associated with growth arrest (17). In response to antiproliferative stimuli or serum starvation, KLF4 expression is up-regulated in cultured fibroblasts as well as in colon cancer cells (14, 18). Conversely, stimulation of quiescent cells with serum leads to down-regulation of KLF4 expression (14). Moreover, forced expression of KLF4 by transfection inhibits DNA synthesis and blocks cell cycle progression during the G1 phase (19). These effects seem to be mediated through activation of the p21$^{WAF1/Cip1}$ and/or suppression of the cyclin D1 promoter (20, 21).

More recently, results from clinical and experimental studies have indicated that KLF4 is a tumor suppressor for both gastric and colorectal cancers. Loss of KLF4 in mice alters the proliferation and differentiation processes of gastric epithelia and results in the formation of precancerous lesions (22). Wei et al. (23) also found that KLF4 plays an important role in human gastric cancer development and progression. Loss of KLF4 expression in the primary tumors is significantly associated with poor survival, whereas restoration of KLF4 expression inhibits cell growth and tumor formation. Previous studies from our laboratory also showed that KLF4 levels are significantly decreased in adenomatous polyps and colon cancer tissue (24). Thus, these data strongly suggest that decreased KLF4 expression may contribute to tumorigenesis in the gastrointestinal tract. However, the mechanisms for regulation of KLF4 expression, especially at the postranslational level, remain poorly understood.

In this report, we show for the first time that down-regulation of KLF4 protein levels in response to serum stimulation is mediated, at least in part, by the ubiquitin-proteasome degradation system.

Materials and Methods

Reagents. The proteasome inhibitors lactacystin, MG132 (N-carboxyoxylt-leuciny1-t-leucinyl-t-norleucinyl, LLI), and MG115 (N-carboxyoxylt-leuciny1-t-leucyl-t-norvalinyl, LLnV) were purchased from Calbiochem (San Diego, CA). t-$^{[35]}$S)Methionine (>1,000 Ci/mmol; ~50 mCi/mL) for in vitro translation was obtained from Amersham Biosciences (Piscataway, NJ). Monoclonal antibodies recognizing Flag, HA, or His epitopes and anti-ubiquitin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies recognizing KLF4 were from Abcam (Cambridge, MA) and Zymed (South San Francisco, CA). The monoclonal antibodies recognizing the COOH-terminal glycine of ubiquitin were from Santa Cruz Biotechnology.
Cruz, CA). Cycloheximide, ubiquitin, phosphocreatine, creatine phosphokinase, ATP, ATPγS, and other reagents were purchased from Sigma (St. Louis, MO).

**Cell culture and synchronization of cells.** The human colon cancer cell lines, HCT116 and HT-29, were obtained from the American Type Culture Collection (Rockville, MD). Two additional human colon carcinoma cell lines, 40 to 16 (HCT116, p53+/+), and 37/2.9 (HCT116, p53−/−), were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University). These cells were maintained in McCoy’s 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/L streptomycin, and 100,000 units/L penicillin in an atmosphere of 95% air and 5% CO2 at 37°C. The human embryonic kidney cell line 293T (from American Type Culture Collection) was cultured in DMEM supplemented with 10% FBS. To synchronize cells, proliferating HCT116 cells were rendered quiescent by maintenance in McCoy’s 5A medium containing 0.5% FBS for 5 days, and then stimulated with fresh medium containing 20% FBS for 0 and 24 hours to allow cells to reenter the cell cycle. The cells were harvested at each time point and used for FACS (fluorescence-activated cell sorter) and Western blot analysis.

**Plasmids and transfection.** The Flag-tagged KLF4 construct was prepared by subcloning human KLF4 into the pFLAG-CMV-2 vector. The mammalian expression plasmids pTMT107 (His6-tagged ubiquitin) and pMTT23 (HA-tagged ubiquitin) were kindly provided by Dr. Dick Bohmann (University of Rochester Medical Center, Rochester, NY; ref. 25). All transfection experiments were done using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Western blot analysis and immunoprecipitation.** To obtain whole-cell extracts, cells were washed twice with ice-cold PBS, scraped, and pelleted by centrifugation (200 × g). Pellet were then lysed in the standard radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1.0% NP40, 0.5% sodium deoxycholate, and 0.1% SDS] containing protease inhibitors. Cell lysates were resolved by SDS-PAGE and following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Protein bands were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence following the manufacturer’s instructions (Amersham, Arlington Heights, IL). For immunoprecipitation, cells were lysed in Triton X-100 buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, and 1% Triton X-100] in the presence of protease inhibitors on ice. Cell debris was cleared by centrifugation at 14,000 × g for 15 minutes. The supernatants were incubated with the primary capturing antibody at 4°C for 2 hours prior to the addition of protein A/G beads (Santa Cruz Biotechnology) and incubation was continued at 4°C overnight. The washed immunoprecipitate was then electrophoresed, blotted, and coimmunoprecipitating proteins detected on Western blots. For the ubiquitin binding assay, His-tagged ubiquitin and KLF4 complexes were purified from 293T cells using nickel nitrilotriacetic acid agarose (Qiagen, Valencia, CA) and KLF4 was detected by Western blot analysis as described above.

**RNA isolation and Northern blot analysis.** Total RNA was isolated by the STAT-60 method following the manufacturer’s instructions (Leedo Medical Laboratories, Houston, TX). RNA samples (20 μg) were denatured and size-fractionated by electrophoresis on 1.1% agarose formaldehyde gels, and transferred onto Hybond-N nylon membranes (Amersham Biosciences). Hybridization was then done overnight at 42°C using a 450 bp Apa-I/Pst1 fragment of the human KLF4 DNA that was radio labeled with [32P]-dCTP (random primer labeling kit from Boehringer Mannheim, Indianapolis, IN). Blots were washed with 2 × saline-sodium phosphate-EDTA and 0.1% SDS, followed by 0.1 × saline-sodium phosphate-EDTA and 0.1% SDS. The blots were also stained with ethidium bromide and photographed to verify RNA loading.

**In vitro KLF4 degradation assay.** [35S]Methionine-labeled human KLF4 protein was synthesized using an in vitro transcription/translation kit according to the manufacturer’s protocol (Promega, Madison, WI). Protein extracts were prepared from exponentially growing HCT116 cells in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl 1% Triton X-100, 0.1% SDS, and 1 mmol/L EDTA], and used for KLF4 degradation assays done as described previously (26). Briefly, 1 μL of [35S]-labeled KLF4 protein was incubated at 37°C for 4 hours with 25 to 100 μg protein extract in reaction buffer [50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L MgCl2, 3 mmol/L DTT, 10 mmol/L ATP, 10 mmol/L phosphocreatine, 10 μg/mL creatine phosphokinase, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10% glycerol, and 2 μg/mL ubiquitin]. After incubation, the samples were subjected to gel electrophoresis and autoradiography.

**In vitro ubiquitination assay.** GST-KLF4 was purified using standard affinity purification strategies. Cell extracts from the serum-starved and proliferating HCT116 cells were prepared as previously described (27, 28). The resulting lysate was subjected to centrifugation at 100,000 × g for 4 hours at 4°C, and the supernatant was used for in vitro ubiquitination assays. Briefly, GST-KLF4 was incubated with the serum-starved or proliferating HCT116 cell extracts in the presence of an ATP-regenerating system [50 mmol/L Tris-HCl (pH 8.3), 5 mmol/L MgCl2, 5 mmol/L ATP, 10 mmol/L creatine phosphate, 0.2 units/mL creatine kinase] together with 1 μg/mL ubiquitin, 100 μg/mL ubiquitin aldehyde, 2 mmol/L DTT, and a protease inhibitor mixture (10 μg/mL each pepstatin, leupeptin, and phenylmethylsulfonyl fluoride). The reactions were carried out at 37°C for 1 hour and terminated by adding SDS sample buffer. Each reaction mixture was subjected to SDS-PAGE on a 7.5% gel, followed by Western blot analysis with anti-KLF4 antibody as described above.

**Statistical methods.** Results are expressed as means ± SE, and a two-way ANOVA with Dunnett’s post-test (Instat software, GraphPad software) was done. Differences between group means are analyzed by Student’s t test and are considered significant at P < 0.05.

**Results**

**Down-regulation of KLF4 protein levels by serum stimulation in HCT116 cells involves proteasomal function.** The levels of KLF4 protein in response to serum stimulation were examined in HCT116 cells. Proliferating HCT116 cells were synchronized to a quiescent state by serum starvation (0.5% FBS) for 5 days, and then allowed to reenter the proliferating stage by the addition of fresh medium containing 20% FBS. At the indicated time points (from 0 to 24 hours), cells were harvested and subjected to FACS or Western blot analysis. As illustrated in Fig. 1A, 90% of cells were arrested at the G1 phase during serum starvation, and after serum stimulation, cells progressed to the proliferating stage. Western blot analysis revealed that the amount of KLF4 protein was the highest in serum-starved HCT116 cells (time 0), but decreased rapidly as cells reentered the proliferating stage (Fig. 1B). Interestingly, the pattern of decrease in KLF4 protein levels resembled that of the cyclin-dependent kinase inhibitor p27Kip1, but was opposite to that of either Skp2 or cyclin A (Fig. 1B), indicating a reciprocal effect of serum stimulation on KLF4 and Skp2 expressions in these cells.

Earlier studies have reported that expression of KLF4 mRNA is low in proliferating NIH3T3 cells, but increases markedly after serum starvation. To determine whether accumulation of endogenous KLF4 protein in serum-deprived HCT116 cells resulted from transcriptional or translational control could not be completely ruled out. Proteasome-mediated degradation has been shown to be an important mechanism to control the turnover of many proteins. To examine whether down-regulation of KLF4 protein levels during serum stimulation is associated with proteasomal function,
serum-starved HCT116 cells were pretreated with MG132, a proteasome inhibitor, and then stimulated with serum. As shown in Fig. 1D, serum stimulation–induced decreases in KLF4 protein levels were attenuated by pretreatment with MG132, suggesting that the proteasomal pathway might be involved in the regulation of KLF4 protein turnover.

KLF4 exhibits a relatively short half-life in HCT116 cells. To assess the stability of endogenous KLF4, we examined the rate of KLF4 turnover in HCT116 cells by inhibiting new protein synthesis with cycloheximide. HCT116 cells were treated with 100 μg/mL cycloheximide for 0, 30, 60, 120, and 240 minutes, and the amount of KLF4 protein was assessed by Western blot analysis. In the presence of cycloheximide, KLF4 was rapidly degraded and exhibited a relatively short half-life ($t_{1/2}$ ~ 120 minutes; Fig. 2A and B). When HCT116 cells were pretreated with the proteasome inhibitor MG132, degradation of KLF4 was inhibited (Fig. 2C). These results suggest that KLF4 is an unstable protein, and that it is degraded through the proteasomal pathway.

Degradation of KLF4 is mediated by an ATP-dependent proteasomal pathway. To confirm that degradation of KLF4 might result from proteasome-dependent proteolysis, the effects of other proteasome inhibitors, including lactacystin, MG132, and MG115 on KLF4 levels were examined in HCT116 cells. Cells were incubated with these inhibitors or control vehicle for 16 hours and were then harvested for measurement of KLF4 protein levels. When cells were treated with all proteasome inhibitors, endogenous KLF4 protein levels were significantly increased.

**Figure 1.** Down-regulation of KLF4 protein levels in response to serum stimulation involves proteasomal function. A, proliferating HCT116 cells (P) were synchronized to a quiescent state (at time 0) by serum starvation (0.5% FBS) for 5 days and then stimulated with serum (20% FBS) to induce cell cycle progression. Cells were harvested at the indicated time points and subjected to FACS. The FACS profile at each time point is shown and the percentage of cells at each phase of the cell cycle is listed below the profiles. B, levels of KLF4, p27Kip1, Skp2, and cyclin A proteins at each time point were evaluated by Western blot analysis using the indicated antibodies. C, the effect of serum starvation on KLF4 mRNA expression. Proliferating HCT116 cells were cultured in medium containing 0.5% FBS for 1, 3, and 5 days, total RNA was extracted and levels of KLF4 mRNA were measured by Northern blot analysis. Blots were also stained with ethidium bromide to show expression levels of 28S RNA and to verify RNA loading. D, proliferating HCT116 cells (Pro-) were rendered quiescent by maintenance in medium containing 0.5% FBS for 5 days. The serum-starved cells were pretreated with the proteasome inhibitor MG132 (30 μmol/L) for 2 hours and then stimulated with serum (20% FBS). After 24 hours, cells were harvested and subjected to Western blot analysis to detect KLF4 protein levels. Cells treated with MG132 alone are shown as a positive control. The expression of β-tubulin was used as a loading control. Three separate experiments were done with similar results.

**Figure 2.** Half-life of endogenous KLF4 in HCT116 cells. A, HCT116 cells were incubated with 100 μg/mL cycloheximide for the indicated times, after which cell lysates were subjected to Western blot analysis for detection of KLF4. B, the immunoblots in (A) were subjected to densitometric measurement, and the amount of protein is expressed as a percentage of that at 0 minute, which was set at 100%. The graph was plotted as log% protein remaining versus time (minutes). C, HCT116 cells were treated with 30 μmol/L MG132 (+) or solvent DMSO (−) for 2 hours, and the protein synthesis inhibitor cycloheximide was then added. Cells were harvested at the indicated time points after the addition of cycloheximide and examined for the expression of KLF4 or β-tubulin by Western blot analysis. Three separate experiments were done.
compared with controls (Fig. 3A, top). To verify the action of the proteasome inhibitors, the same blots were stripped and reprobed with anti-ubiquitin antibodies. As expected, the high–molecular mass polyubiquitinated protein complex was also markedly increased in cells treated with these inhibitors (Fig. 3A, middle). Furthermore, treatment of HCT116 cells with lactacystin resulted in the accumulation of KLF4 in a time-dependent manner (Fig. 3B). A significant increase in KLF4 protein levels was first detected 8 hours after the addition of lactacystin, with the maximal increase observed at 16 hours and a decline thereafter (Fig. 3B). In addition, incubation of HCT116 cells with increasing concentrations of lactacystin (0.2-20 μmol/L) for 16 hours also caused a dose-dependent accumulation of KLF4 (Fig. 3C). These results strongly suggest that the proteasome-dependent degradation pathway is involved in the regulation of endogenous KLF4 protein levels in HCT116 colon cancer cells.

To further investigate the mechanisms involved in KLF4 degradation, a cell-free KLF4 degradation assay was developed using in vitro–translated, 35S-labeled KLF4 protein as substrate. In this assay, 1 μL of 35S-labeled KLF4 protein was incubated with protein extracts prepared from exponentially growing HCT116 cells. As illustrated in Fig. 3D, KLF4 protein was degraded by treatment with cell lysate in a dose-dependent manner (Fig. 3D). This effect was abolished by pretreatment with increasing concentrations of MG132 (Fig. 3E). Moreover, this process also required ATP, as replacement of ATP with ATP-γ-S, a nonhydrolyzable analogue of ATP, prevented 35S-labeled KLF4 from being degraded (Fig. 3F).

Accumulation of endogenous KLF4 induced by proteasome inhibitors is independent of p53. Previous studies have shown that KLF4 expression upon DNA damage is activated in a p53-dependent fashion (17). Because inhibition of proteasome activity also results in the accumulation of p53 protein in cells (29), we examined whether accumulation of KLF4 in HCT116 cells following treatment with proteasome inhibitors is associated with an increase in p53. KLF4 and p53 protein levels were examined in p53 wild-type (HCT116 p53+/+), p53 null (HCT116 p53−/−), or p53 mutated (HT-29) cells after treatment with or without lactacystin. Treatment with this inhibitor induced significant p53 accumulation in HCT116 p53+/+ cells (Fig. 4A), but not in HT-29 cells (Fig. 4C). By contrast, significant increases in KLF4 protein levels were observed all these cell lines (Fig. 4A, B, and C), indicating that the accumulation of endogenous KLF4 induced by proteasome inhibitors is independent of p53.

KLF4 is subject to ubiquitination. If KLF4 is degraded through a ubiquitin-mediated pathway, inhibition of proteasomal activity could result in the accumulation of ubiquitinated KLF4. To test this possibility, protein extracts of HCT116 cells treated with lactacystin or vehicle alone were immunoprecipitated with anti-KLF4 antibodies, followed by Western blot analysis using an anti-ubiquitin antibody. As shown in Fig. 5A, increased levels of a high molecular weight smear, characteristic of ubiquitinated proteins (UbKLF4), were observed in HCT116 cells treated with lactacystin. To confirm the interaction between ubiquitin and KLF4 in HCT116 cells, immunoprecipitation of ubiquitin–protein conjugates was done on lysates from cells treated with or without MG132. Western blot
analysis revealed that KLF4 was present in immunoprecipitated proteins in MG132-treated cells, but not in untreated control cells (Fig. 5B). Next, we examined whether KLF4 could be ubiquitinated by introducing exogenous ubiquitin using 293T cells transiently transfected with expression vector containing Flag-tagged KLF4 alone, or together with a HA-tagged ubiquitin plasmid. After 24 hours, cells were treated with or without MG132. Flag-tagged proteins were immunoprecipitated with anti-Flag antibodies, followed by Western blot analysis using anti-ubiquitin antibodies and a high molecular weight smear was detected in cells cotransfected with Flag-KLF4 and HA-ubiquitin, but not in cells transfected with Flag-KLF4 alone (Fig. 5C). Moreover, this effect was enhanced after treatment with MG132 (Fig. 5C). To further determine whether the high molecular weight smear contained ubiquitin, a specific assay for the detection of ubiquitinated proteins was employed (30). After affinity purification of His-tagged ubiquitin-KLF4 complexes on nickel nitritetriacetic acid agarose beads, KLF4 was present in the pull-down samples in cells transfected with both KLF4 and ubiquitin (Fig. 5D, top) and the amount of ubiquitin-KLF4 conjugates in cell lysates increased after MG132 treatment (Fig. 5D, middle). These results provide direct evidence that KLF4 is subject to ubiquitination.

To further determine the role of ubiquitination in serum-mediated KLF4 degradation, an in vitro ubiquitination assay was done in serum-starved and proliferating cells (Fig. 5E). In this study, proteasomes were removed from cell extracts by untracentrifugation at 100,000 × g for 4 hours. The proteasome-depleted supernatant (S100 pr-) from serum-starved (S), or proliferating (P) cells was incubated with GST-KLF4, in the presence of an ATP-regenerating system together with ubiquitin and ubiquitin aldehyde. When the reaction mixture was subjected to SDS-PAGE, followed by Western blot analysis using anti-KLF4 antibodies, ubiquitinated KLF4 complexes were more prominent in the proliferating than serum-starved cells, indicating an essential role for ubiquitination in serum-mediated KLF4 degradation (Fig. 5E).

Discussion

Although we and others have shown that levels of KLF4 protein are important in regulating cell growth (14, 15, 19, 23), little is known about the posttranslational modification of KLF4, especially at the level of protein degradation. In the present report, we show that the cellular abundance of KLF4 changed during the transition from quiescence to the proliferative state. In quiescent HCT116 cells, KLF4 protein levels were high, but decreased markedly after cells were induced to proliferate by serum stimulation. This decrease was partially reversed by pretreatment of HCT116 cells with proteasome inhibitors, suggesting the involvement of proteasomal activity in this process. Noticeably, down-regulation of KLF4 protein levels in serum-stimulated cells closely resembled that of p27kip1, but was contrary to the up-regulation seen with Skp2 and cyclin A. Skp2, an F-box protein in the SCFkip1 ubiquitin ligase complex, has recently been shown to specifically recognize p27kip1, and target it for ubiquitin-mediated degradation (31–33). Our results suggest that Skp2 may be involved in the degradation of KLF4 and further investigation of this association is warranted.

Cellular proteins differ widely in their stability. Our results show that KLF4 is an unstable protein in HCT116 colon cancer cells. When new protein synthesis was blocked, KLF4 underwent rapid turnover and exhibited a relatively short half-life, whereas pretreatment of HCT116 cells with MG132 led to a dramatic stabilization of KLF4, suggesting that the proteasome-dependent degradation machinery is involved in the turnover of KLF4. Recently, it has been reported that rapid turnover of proteins is often caused by signals that induce protein degradation. In some cases, the signal is part of the primary sequence of the protein itself. Using the PESTfind program (http://www.at.embnet.org/embnet/tools/bio/PESTfind/), we have recently determined that human KLF4 is a PEST-containing protein (with PEST score of 9.01). Although PEST sequences have been found in many short-lived regulatory proteins and serve as a signal targeting protein for ubiquitin-dependent degradation (34–36), a number of other ubiquitin-independent proteolytic mechanisms have also been described. For example, the IV.Box-PEST sequence has been shown to act as a modular domain to promote the physical interaction with and subsequent degradation by μ-calpain, a calcium-dependent cytosolic protease responsible for most nonlysosomal-targeted proteolysis (37). Whether this specific sequence in KLF4 protein affects its stability in colon cancer cells will be the subject of future studies.

In the present work, we also show that KLF4 is a target for the ubiquitin-proteasome degradation pathway. Treatment of HCT116 cells with specific proteasome inhibitors led to a significant increase in endogenous KLF4 protein. In an in vitro study, ATP-dependent cell-free KLF4 degradation was observed after incubating [35S]labeled KLF4 with HCT116 cell protein extracts, a process that could be blocked by the addition of a proteasome inhibitor. Moreover, inhibition of the proteasome activity and cotransfection with exogenous ubiquitin promoted the formation of ubiquitinated KLF4 in HCT116 cells. These observations were further confirmed by affinity purification of His-tagged ubiquitin-KLF4 conjugates on nickel agarose beads. Taken together, these results support the
hypothesis that KLF4 is degraded in a manner that is dependent on the ubiquitin-proteasome system. Furthermore, ubiquitinated KLF4 complexes were observed at higher levels in proliferating than in serum-starved cells, indicating an essential role of ubiquitination in serum-mediated KLF4 degradation.

Recent studies have shown that KLF4 exhibited both growth-inhibitory and stimulatory properties. Our laboratory has previously shown that KLF4 protein levels were down-regulated in colon cancer tissue, and that forced expression of KLF4 in colon cancer cells resulted in growth inhibition (18, 19, 24). As stated above, Wei et al. (23) and Kate et al. (22) have recently shown an important role of KLF4 in the growth and differentiation of gastric mucosa and have shown that down-regulation of KLF4 resulted in the promotion of tumor growth in the stomach. These data indicate that KLF4 may function as a tumor suppressor in the gastrointestinal tract. However, in primary rat kidney cells, KLF4 has been shown to have transforming activity (38) and its expression is increased in primary breast ductal carcinoma and oral squamous carcinoma (39, 40).

These results suggest that KLF4 exhibits dual functions and that the level of KLF4 protein plays a critical role in regulating cell growth.

Although other factors may also contribute to the down-regulation of KLF4 in the gastrointestinal tract, our work shows a potential role for posttranslational modification in controlling KLF4 expression levels. In view of the diverse growth-promoting properties of KLF4 in different tumors, it is intriguing to postulate that alteration of the KLF4 degradation process in different cells may affect its function.

In summary, although the precise role of KLF4 in controlling cell growth is still poorly understood, our work has indicated that destabilization of KLF4 protein in response to serum stimulation is mediated, at least in part, by a ubiquitin-proteasome degradation system. Further investigation will be required to unravel the potential contribution of KLF4 stability to abnormal cell growth and carcinogenesis.

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