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Opposing effects of Krüppel-like factor 4 (gut-enriched Krüppel-like factor) and Krüppel-like factor 5 (intestinal-enriched Krüppel-like factor) on the promoter of the *Krüppel-like factor 4* gene

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ABSTRACT

KLF4 (Krüppel-like factor 4 or gut-enriched Krüppel-like factor, GKLF) and KLF5 (Krüppel-like factor 5 or intestinal-enriched Krüppel-like factor, IKLF) are two closely related members of the zinc finger-containing Krüppel-like factor family of transcription factors. Although both genes are expressed in the intestinal epithelium, their distributions are different: *Klf4* is primarily expressed in the terminally differentiated villus cells while *Klf5* is primarily in the proliferating crypt cells. Previous studies show that Klf4 is a negative regulator of cell proliferation and Klf5 is a positive regulator of cell proliferation. In this study, we demonstrate that Klf5 binds to a number of *cis*-DNA elements that have previously been shown to bind to Klf4. However, while Klf4 activates the promoter of its own gene, Klf5 suppresses the *Klf4* promoter. Moreover, Klf5 abrogates the activating effect of Klf4 on the *Klf4* promoter and Klf4 abrogates the inhibitory effect of Klf5 on the same promoter. An explanation of this competing effect is due to physical competition of the two proteins for binding to cognate DNA sequence. The complementary tissue localization of expression of *Klf4* and *Klf5* and the opposing effect of the two Klfs on the *Klf4* promoter activity may provide a basis for the coordinated regulation of expression of the *Klf4* gene in the intestinal epithelium.

INTRODUCTION

Krüppel-like factors (KLFs) are zinc finger-containing transcription factors that exhibit homology to the *Drosophila melanogaster* segmentation gene product Krüppel (1). A subfamily of mammalian KLFs highly related to the erythroid Krüppel-like factor (EKLF/KLF1) has recently been described (2). This rapidly expanding subfamily currently has 12 members, which have been given numerical designations by the Human Gene Nomenclature Committee (3). KLFs can be transcriptional activators or repressors (4) and they bind to a similar DNA sequence that has either a CACCC homology or is rich in GC content (5). It is therefore not surprising that KLFs may interact with the same *cis*-element in the same gene. For example, both KLF12/AP2-rep and KLF9/BTEB1 bind to the CACCC element in the *AP2*α promoter (6). But, while KLF12 acts as a repressor, KLF9 acts as a strong activator of the *AP2*α promoter (6). In another example, both KLF6/Zf9 and KLF4/GKLF activate the human *keratin 4* promoter through the CACCC motif and the two factors physically interact (7). Finally, both KLF4/GKLF and KLF5/IKLF bind to the CACCC element in the α*-smooth muscle* and *SM22*α promoters (8). In this case, KLF4 represses while KLF5 activates the induction of these two promoters by TGFβ (8).

The reciprocal effect of KLF4 and KLF5 in regulating gene expression merits additional consideration. Aside from their opposing biochemical effects on several smooth muscle gene promoters as described above, the cellular distribution and regulatory functions of KLF4 and KLF5 also exhibit an opposite pattern. For example, while *Klf4* is mainly expressed in the post-mitotic differentiated villus epithelial cells of the intestinal tract (9), *Klf5* is found primarily in the proliferating crypt cells (10). The cellular distribution of the two genes in the epidermis of the skin also mirrors that in the intestinal epithelium (11–13). *In vitro*, expression of *Klf4* is associated with a process of growth arrest (9), while that of *Klf5* mainly accompanies cellular proliferation (14). Moreover, forced expression of *Klf4* leads to a G_1/S cell cycle arrest (15,16) but that of *Klf5* causes a transformed phenotype (14).

Because of the relatively restricted pattern of tissue expression of *Klf4*, we have been characterizing factors that regulate its expression. One such factor, Cdx2, previously shown to drive differentiation of intestinal epithelial cells (17), is shown to be a transactivator of the *Klf4* promoter (18,19). In addition, Klf4 is capable of transactivating the promoter of its own gene

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through three closely spaced GC-boxes within the promoter (18). The present study demonstrates that Klf5 binds to the same DNA *cis*-elements as Klf4, including those within the *Klf4* promoter. However, while Klf4 activates, Klf5 represses *Klf4* promoter activity. Furthermore, the two factors can abrogate each other's effect on the *Klf4* promoter. Lastly, the two factors compete with each other for binding to the same *cis*-element. The results of these studies suggest that Klf4 and Klf5 have opposing biochemical functions in regulating expression of the *Klf4* gene and may potentially be involved in a coordinated effort to orchestrate the proliferative and differentiated phenotype of the intestinal epithelium.

MATERIALS AND METHODS

Plasmid constructs

The eukaryotic expression construct containing full-length Klf4 (PMT3–Klf4) and the luciferase reporter construct containing 1.0 kb of the 5′ flanking promoter region of the *Klf4* gene (*Klf4*–pGL2–Luciferase) were previously described (18). The expression construct containing full-length Klf5, pBK CMV–Klf5, was generously provided by J. Lingrel (University of Cincinnati, College of Medicine) (10). An *Eco*RI–*Kpn*I fragment of the full-length *Klf5* cDNA from pBK CMV–Klf5 was subcloned into the PMT3 vector to create PMT3–Klf5. The two PMT3 constructs were digested with appropriate restriction endonucleases to release the zinc finger portions of Klf4 and Klf5, which were then subcloned back into the PMT3 vector to create PMT3–Klf4–ZF and PMT3–Klf5–ZF, respectively. The internal control for transfection, pRL–CMV, was purchased from Promega Corporation (Madison, WI).

The prokaryotic expression vector pET–16b containing the zinc finger portion of Klf4 (amino acids 350–483) has previously been described (20). The C-terminal portion of Klf5 between amino acids 242 and 446 was cloned into the prokaryotic expression vector pET101/D. Both recombinant proteins contained a 10-histidine tag at the N-terminus and were purified by nickel affinity chromatography as described before (20). The apparent molecular weight for the resultant recombinant protein was 18 and 26 kDa, respectively, for Klf4 and Klf5.

Preparation of nuclear extracts

Nuclear extracts containing full-length Klf4 or full-length Klf5 were prepared from COS-1 cells transfected with PMT3–Klf4 or PMT3–Klf5. Nuclear extracts from cells transfected with PMT3 alone were used as controls. Extracts from transfected cells were prepared as previously described (18). Briefly, transfected cells were rinsed with ice-cold phospate-buffered saline, scraped, harvested and pelleted. The pellets were washed with 4 pack cell volume (p.c.v.) of solution containing 10 mM Tris–HCl pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and a Complete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN). Following 10 min incubation on ice, the cells were lysed by 10 strokes of a Dounce homogenizer. Nuclei were collected by centrifugation (Sorvall Microspin) and resuspended in 2 p.c.v. of a lysis solution containing 420 mM KCl, 20 mM Tris–HCl pH 7.8, 1.5 mM $MgCl_2$, 0.5 mM DTT, 20% glycerol and a Complete Protease Inhibitor Tablet (Roche). After incubation for 1 h at 4°C with gentle agitation, the solution was centrifuged and the supernatant dialyzed twice against 500 ml of 20 mM Tris–HCl pH 7.8, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol and a Complete Protease Inhibitor Tablet (Roche). Extracts were divided into small aliquots and immediately cryopreserved.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed according to previously published protocols (9,20). The basic transcription element (BTE) probe contained a sequence in the promoter of the cytochrome *P4501A1* gene that had previously been shown to bind to Klf4 (21). The target detection assay (TDA) probe was a previously selected consensus-binding site for KLF4 (20) and closely resembled the three GC-boxes in the promoter of the *Klf4* gene (18). The GC-2 probe refers to the GC-box 2 sequence in the *Klf4* promoter between nucleotide –108 and –86 that has previously been shown to bind Klf4 (18). Probes were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. In each reaction, 0.5 pmol of labeled probe was used. Nuclear extracts from PMT3-, PMT3–Klf4- or PMT3–Klf5-transfected cells, or purified recombinant Klf4 or Klf5 were incubated in a volume of 30 µl containing 20 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 150 mM NaCl, 20 mM KCl, 5 µM $ZnCl_2$, 0.5 mM DTT, 5% glycerol and 2 µg poly(dI•dC) on ice for 30 min. The labeled probe was then added to the reaction and the incubation continued for another 15 min at room temperature. The DNA and DNA–protein complexes were resolved from one another by native 6.0% polyacrylamide gel electrophoresis and visualized by autoradiography.

Transfection and reporter assays

Transient transfection by lipofection of Chinese hamster ovary (CHO) cells with various DNA constructs (*Klf4*–pGL2–Luciferase, PMT3–Klf4, PMT3–Klf5, PMT3–Klf4–ZF and PMT3–Klf5–ZF) were performed as previously described (9,18,20). Transfections were performed in 6-well tissue culture dishes at 37°C for 16 h. All transfections included the internal standard pRL–CMV (Promega) to normalize for transfection efficiency. Luciferase and *Renilla* assays were performed using the Dual-Luciferase Assay (Promega).

RESULTS

Klf4 and Klf5 bind to similar *cis*-DNA elements

We first examined whether Klf4 and Klf5 bound to similar sequences by EMSAs using radiolabeled oligonucleotides containing either an established consensus sequence for Klf4 (TDA) (20) or the BTE (21). As shown in Figure 1A, two common DNA–protein complexes were formed when the TDA probe was incubated with nuclear extracts from PMT3-, PMT3–Klf4- and PMT3–Klf5-transfected cells. However, an additional complex was formed in the reaction that contained either PMT3–Klf4- or PMT3–Klf5-transfected cells. The Klf4–TDA complex is identified by * and the Klf5–TDA complex is identified by +. The specificities of all the DNA–protein complexes were demonstrated by their gradual disappearance upon addition of increasing amounts of unlabeled probe (Fig. 1A). A similar pattern was observed when BTE was used as a probe except that the intensities of the two common bands were stronger when compared with the TDA probe (Fig. 1B). The results suggest that Klf4 and Klf5 bind to

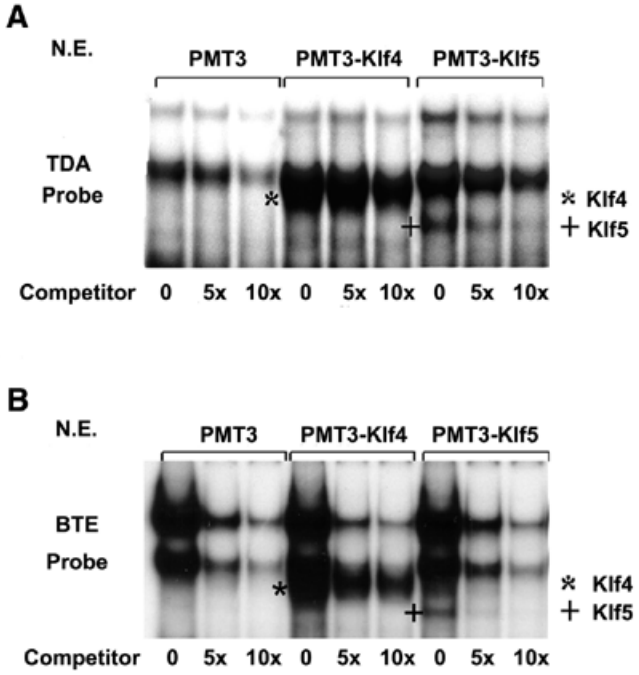

Figure 1. EMSAs of Klf4 and Klf5 on two *cis*-elements, TDA and BTE. EMSAs were performed using 10 µg nuclear extracts prepared from COS-1 cells transfected with an expression construct containing vector alone (PMT3), full-length Klf4 (PMT3–Klf4), or full-length Klf5 (PMT3–Klf5) and two radiolabeled oligonucleotide probes containing established *cis*-elements for Klf4: TDA **(A)** and BTE **(B)**. Where indicated, an unlabeled cognate oligonucleotide was used as a competitor at 5× and 10× molar excess of the labeled probe. * represents the shifted Klf4–probe complex and + represents the shifted Klf5–probe complex. N.E., nuclear extracts.

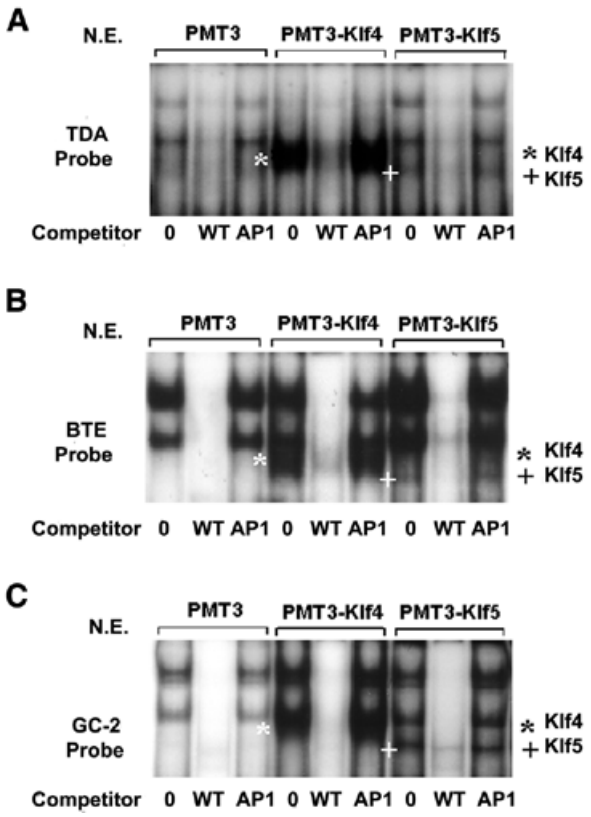

Figure 2. EMSAs of Klf4 and Klf5 on an additional *cis*-element, GC-2, that is present in the Klf4 promoter. EMSAs were performed with 10 µg nuclear extracts (N.E.) prepared from cells transfected with PMT3, PMT3–Klf4 or PMT3–Klf5 in the absence of any competitors (0), or in the presence of 25× molar excess of a cognate competitor (WT) or an unrelated competitor (AP1). Probes used include TDA **(A)**, BTE **(B)** and GC-2 **(C)**. * represents the shifted Klf4–probe complex and + represents the shifted Klf5–probe complex.

similar DNA elements. Furthermore, both Klf4 and Klf5 bound to the GC-2 probe (Fig. 2C) which represents one of three GC-box sequences in the *Klf4* promoter, all of which have been shown to bind Klf4 (18). The specificities of DNA–protein interaction with all three probes were further demonstrated by the failure of an unrelated AP1 probe to compete for the formation of the complexes (Fig. 2A–C).

Klf4 activates and Klf5 represses the *Klf4* promoter

Recent work from our laboratory showed that Klf4 activates the promoter of its own gene by interacting with the GC-boxes in the proximal promoter (18). To determine whether Klf5 also influences the promoter activity of the *Klf4* gene, we performed co-transfection studies using the *Klf4*–pGL2–Luciferase reporter and a eukaryotic expression construct containing either Klf4 or Klf5. As depicted in Figure 3, PMT3–Klf4 significantly activated the *Klf4* promoter when compared with PMT3 alone, confirming previous findings. In contrast, PMT3–Klf5 significantly suppressed *Klf4* promoter activity when compared with PMT3 alone. Thus, Klf4 and Klf5 have opposite effects on the activity of the *Klf4* promoter.

Klf4 and Klf5 compete to regulate the *Klf4* promoter

To further determine a possible interaction between Klf4 and Klf5 on the *Klf4* promoter, we performed co-transfection experiments using the *Klf4*–pGL2–Luciferase reporter, a constant amount of PMT3–Klf5 and increasing amounts of PMT3–Klf4 or PMT3–Klf4–ZF. As seen in Figure 4A, in the absence of any competing Klf4, Klf5 suppressed the reporter activity, as in Figure 3. The addition of Klf4 removed this inhibitory effect in a dose-dependent fashion (solid line). In contrast, a construct including only the zinc finger portion of Klf4 failed to reverse the inhibitory effect on the *Klf4* promoter (dashed line). Similarly, in a converse experiment with PMT3–Klf4 as the primary effector and Klf5 as the competing effector (Fig. 4B), Klf5 (solid line) but not Klf5–ZF (dashed line) abrogated the stimulatory effect of Klf4 on the *Klf4* promoter activity. These results indicate that Klf4 and Klf5 not only exhibit an opposite effect on the *Klf4* promoter, they actively compete with each other. Moreover, this competitive effect requires the full-length protein. The zinc fingers alone are not sufficient.

Klf4 and Klf5 compete with each other for DNA binding

One possibility that may explain the competing effects of Klf4 and Klf5 on the *Klf4* promoter is that the two proteins may compete with each other for binding to DNA. To address this possibility, we produced purified partial-length recombinant proteins that contained the zinc finger region of Klf4 and Klf5 (Fig. 5A). When incubated individually with a labeled GC-2 probe, each protein interacted avidly with the probe (Fig. 5B). We next performed EMSA of GC-2 using a constant amount of recombinant Klf4 and increasing amounts of Klf5. As seen,

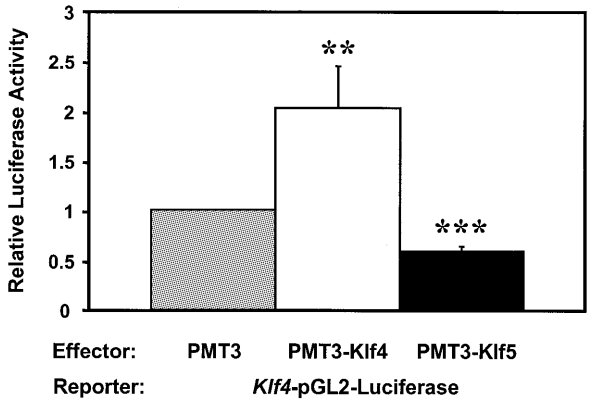

Figure 3. The opposite effects of Klf4 or Klf5 on the Klf4 promoter. Five micrograms each of the *Klf4*–pGL2–Luciferase reporter and PMT3, PMT3–Klf4 or PMT3–Klf5 and 2 μg pCMV–RL were co-transfected into CHO cells using Lipofectamine reagent (Promega). Firefly and *Renilla* luciferase assays were determined 24 h after transfection. Shown are the means of three independent experiments (n = 3) performed in triplicate. Relative luciferase activity was standardized with the internal control *Renilla* luciferase activity. Bars represent standard deviations. ** = $P < 0.01$, *** = $P < 0.001$ by Student's t-test compared with PMT3 vector control.

Klf5 reduced the binding of Klf4 to the probe in a dose-dependent fashion (Fig. 5C). Similarly, if an increasing amount of Klf4 was incubated with a constant amount of Klf5, the binding of Klf5 to the probe also gradually diminished (Fig. 5D). These results indicate that Klf4 and Klf5 compete with each other in binding to DNA.

DISCUSSION

The present study demonstrates how two closely related Klfs exert their effects on transcription of a target gene in an opposing manner. We show that Klf5 binds to several *cis*-elements previously established to be the binding sites for Klf4, including TDA, BTE and GC-2, the last a native *cis*-element in the *Klf4* promoter. However, while Klf4 activates the promoter activity of its own gene, Klf5 has exactly the opposite effect. Moreover, when present together, the two Klfs interfere with each other in modulating the *Klf4* promoter activity. We also show that a possible explanation for this interfering effect is due to competition of the two proteins for binding to DNA.

Opposing effects of other KLFs on the promoters of several other genes have previously been described. For example, Klf4 suppresses the activity of the cytochrome *p4501A1* (*CYP1A1*) promoter while Sp1 activates it in a BTE-dependent fashion (21). Also, similar to the present study, Klf4 abrogates the stimulatory effect of Sp1 on the *CYP1A1* gene in a dose-dependent manner. In this case, it was shown that the zinc fingers of Klf4 compete for binding to BTE with Sp1 (21). It is of interest to note that KLF5, also known as BTEB2 (10), is a strong activator of the *CYP1A1* promoter (22). The findings on the *CYP1A1* promoter would then be the first example in which KLF4 and KLF5 antagonize each other in regulating promoter activity. In another example, KLF4 and KLF5 bind to the same CACCC element present in the promoters of two smooth muscle genes, α-*smooth muscle actin* and *SM22*α (8). Here, KLF4 inhibits TGFβ-dependent stimulation of the *SM22*α promoter while KLF5 further activates TGFβ-dependent stimulation of the same promoter (8). Thus, evidence from literature to date indicates that KLF4 and KLF5 exert opposite effects on the promoters of the genes studied.

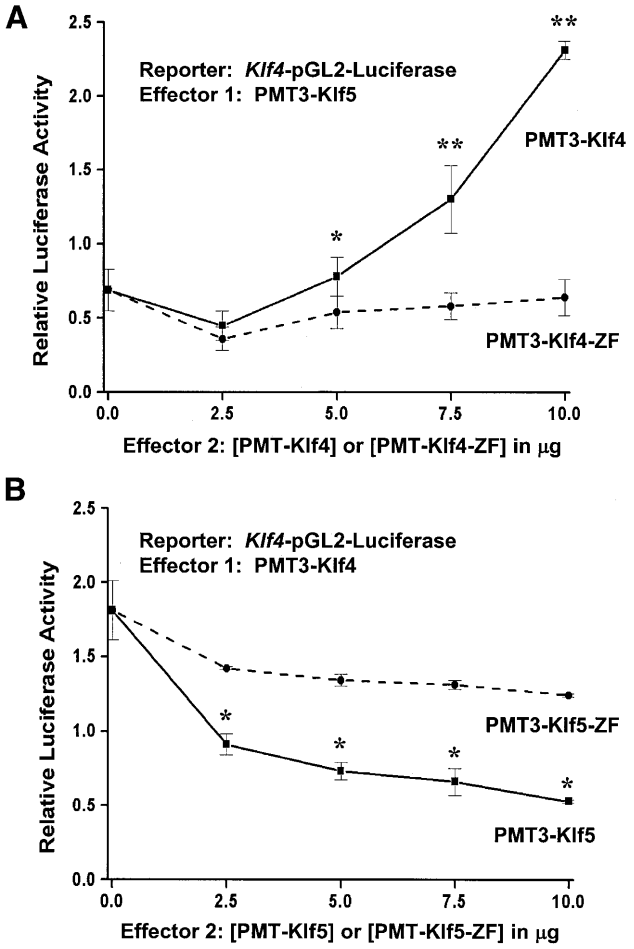

Figure 4. The competing effects of Klf4 and Klf5 on the Klf4 promoter. **(A)** Five micrograms each of *Klf4*–pGL2–Luciferase reporter and effector 1 (PMT3–Klf5) were co-transfected into CHO cells in presence of increasing amounts of effector 2: either PMT3–Klf4 (solid line) or PMT–Klf4–ZF (dashed line). **(B)** Five micrograms each of *Klf4*–pGL2–Luciferase reporter and effector 1 (PMT3–Klf4) were co-transfected into CHO cells with increasing amounts of effector 2: either PMT3–Klf5 (solid line) or PMT3–Klf5–ZF (dashed line). The total amount of DNA transfected was kept constant at 25 μg by the addition of PMT3 DNA. Firefly and *Renilla* luciferase assays were determined 24 h after transfection. Shown are the means of four independent experiments (n = 4) performed in triplicate. The relative activity was determined from dividing the reporter activity of effector 1-transfected cells by that of PMT3-transfected cells. Bars represent standard deviations. * = $P < 0.05$, ** = $P < 0.01$ by Student's t-test comparing full-length with zinc finger only-containing constructs.

It should be noted that the biochemical behavior of KLF4 and KLF5 described above is a reflection of their biological behavior. For example, the cellular distribution of the two Klfs in several epithelial tissues is complementary, rather than redundant, to each other. Thus, *Klf4* is mainly expressed in the post-mitotic villus epithelial cells of the intestine (9) and the suprabasal epidermal cells of the skin (11), while *Klf5* is expressed mostly in the proliferating cells of the crypt in the intestine (10) and the basal epidermal cells of the skin (13). Moreover, the pattern of expression of the two genes *in vitro*

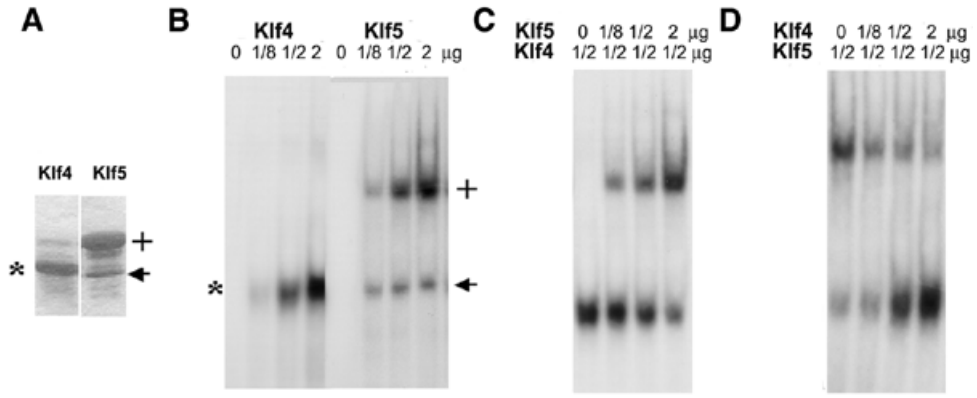

Figure 5. The competing effects of Klf4 and Klf5 on binding to DNA. **(A)** Purified recombinant proteins containing the zinc finger portion of Klf4 (*) and Klf5 (+) are shown in this Commassie blue-stained gel. The apparent molecular weight for Klf4 is 18 kDa and that for Klf5 is 26 kDa. The arrow points to a degradation product of Klf5, which apparently binds to DNA (B). **(B)** EMSAs of recombinant Klf4 and Klf5 on the GC-2 probes. Increasing amounts of purified recombinant Klf4 or Klf5 were used in the experiment. **(C)** EMSAs were performed using a constant amount of recombinant Klf4 and increasing amounts of Klf5. **(D)** EMSAs were performed with a constant amount of recombinant Klf5 and increasing amounts of Klf4.

contrasts each other. *Klf4* is mainly expressed in the growth-arrested cells due to serum deprivation (9), contact inhibition (9) or DNA damage (21). Expression of *Klf5*, on the contrary, is associated with a proliferative state as seen during serum or mitogenic stimulation (14). Based on the results of current and past studies, one may speculate that the lack of crypt expression of *Klf4* in the intestinal crypt epithelium may, in part, be due to the presence of Klf5 in this compartment.

A potential mechanism that may explain how Klf4 and Klf5 compete with each other to regulate the activity of the *Klf4* promoter appears to be exerted at the level of DNA–protein interaction. As demonstrated in Figure 5, Klf5 actively inhibits the binding of Klf4 to the GC-2 probe, and vice versa. However, the zinc finger portion of one Klf is not sufficient in reversing the effect of the other Klf on the activity of the *Klf4* promoter (Fig. 4). These results indicate that amino acid sequence outside the zinc fingers of both proteins is required for the full regulation of the *Klf4* promoter. Klf4 has previously been shown to be a pleiotropic transcription factor, with activating and repressing effects (23). Mutational studies have also located the activating and repressive domains to regions outside the zinc fingers in the Klf4 polypeptide (24). KLF5, although known to be a transcription activator in most studies (10,22), can act as a repressor (25). One might therefore expect that some of these domains involved in transcriptional regulation by the two proteins are responsible for the interplay noted in the current study.

The opposing effects of Klf4 and Klf5 are reminiscent of the opposing effects of Cdx1 and Cdx2 along the crypt–villus axis of the intestinal epithelium. CDX1 and CDX2 are homeodomain transcription factors with sequence homology to the *caudal* gene of *D.melanogaster*, which in mammals are specifically expressed in the epithelium of small intestine and colon (26–29). *Cdx1* is expressed in the proliferating crypt cells (27,30,31) while *Cdx2* is expressed predominantly in the differentiated villus cells (28,32). Several studies have shown that CDX1 regulates cell proliferation (33,34) and CDX2 regulates cell differentiation and growth arrest (17,35).

Of interest, we have recently reported that the intestinal gatekeeper adenomatous polyposis coli (APC) induces *Klf4* expression via Cdx2 (19). APC is a cytoplasmic protein that modulates the oncogenic Wnt signal transduction cascade by binding to β-catenin and promoting its phosphorylation by glycogen synthase kinase 3 β (GSK3β), for subsequent degradation (36). Our previous data, which showed that APC activation of *Klf4* via Cdx2 occurred through a β-catenin independent pathway, have led to our proposal that these three proteins form a part of an enterocyte-specific tumor suppressor pathway in the sequence of APC → CDX2 → KLF4 → growth arrest (19). This pathway may become active as epithelial cells migrate up the intestinal villus and undergo differentiation with the simultaneous loss of proliferative potential. Interestingly, Wnt, which is the product of a proto-oncogene with opposing effects to APC, can up-regulate *CDX1* but not *CDX2* expression (37). Moreover, Wnt has recently been reported to induce mouse *KLF5/BTEB2* through a β-catenin independent, protein kinase C dependent pathway (38). A search of the mouse *Klf5* promoter (DDBJ/EMBL/GenBank accession no. AF285184) reveals several potential CDX1 binding sites (39). We may postulate that *KLF5* is regulated by Wnt via CDX1 in the proliferating intestinal crypt cells while *KLF4* is regulated by APC via CDX2 in the differentiating intestinal villus cells. Crosstalk and negative regulation between these opposing pathways, at the level of KLF4 and KLF5 as shown in this paper, as well as possibly higher levels involving CDX1, CDX2, Wnt and APC, tightly coordinate the intestinal epithelial cells as they proliferate, migrate and differentiate. Current studies are underway to address these interactions.

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