Enterocyte differentiation marker intestinal alkaline phosphatase is a target gene of the gut-enriched Krüppel-like factor

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Small intestinal epithelium is in a constant dynamic state of flux, replacing itself every 3–6 days (34). This continuous renewal of cells is necessary for the maintenance of normal gut structure and function, and it occurs through the highly coordinated and tightly regulated processes of proliferation, migration, differentiation, and apoptosis (17, 43). Although the exact mechanisms are poorly understood, the orderly progression from a rapidly dividing, pluripotent stem cell to a terminally differentiated, apoptotic enterocyte is thought to occur through the transcriptional regulation of a small subset of specific genes, which together comprise an overall differentiation program. Among these genes is the intestinal alkaline phosphatase gene (IAP). IAP is expressed by differentiated but not by undifferentiated enterocytes or by any other cell type. Thus IAP serves as an enterocyte differentiation marker and is a useful tool for the identification of factors that govern the overall enterocyte differentiation program.

Recently, a transcription factor enriched in gut epithelial tissues was identified and characterized on the basis of its zinc-finger homology to an immediate early transcription factor, zif268 (38). In fact, sequencing of this novel protein revealed zinc-finger domains that are closely related to a family of mammalian transcription factors that exhibit homology to the Drosophila melanogaster protein, Krüppel (38). This factor was found to be highly expressed in the gut, and it was designated gut-enriched Krüppel-like factor (GKLF). With the discovery of a large number of other KLFs, the Human Gene Nomenclature Committee has given them numerical designations; GKLF is now known as KLF4.

The Krüppel gene is responsible for segmentation in the developing fruit fly, and many of its mammalian counterparts are known to play roles in regulating cell proliferation, differentiation, and development (1). For instance, one of the first identified KLF, erythroid KLF (KLF1), has been shown to be a critical activator of the β-globin gene in erythroid cells and appears to be required for normal adult erythropoiesis (2, 30–33). Likewise, the early growth response-α/transforming growth factor-β inducible early gene 1 (KLF10) has been implicated in mediating cell cycle arrest and apoptosis (3, 15, 39, 41, 42). Additionally, the basic transcription element binding protein (KLF9) is thought to play a critical role in neural development (14).

KLF4 has been shown to be expressed in the small intestine and primarily in the differentiated gut epithelial cells (38). Its overexpression induces p21 expression and cell-cycle arrest, and its levels are downregulated in colonic neoplasms (7, 11). On the basis of these findings, it has been suggested that KLF4 likely plays a role in gut-epithelial differentiation. However, no specific target gene for KLF4 within the intestinal epithelium has been identified up to this point.

Previous work from our laboratory (29) described a critical DNA cis-element (IF-III) within the promoter region of the IAP gene. In the present study, we used IF-III in the yeast one-hybrid screen to identify KLF4 as a transcription factor that is able to bind the IAP promoter. We have explored the relationship of these two genes and report here that KLF4

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causes the transcriptional activation of the endogenous IAP gene, likely through the proximal promoter region containing the IF-III cis-element. These results strongly suggest that the gut differentiation marker gene IAP is a KLF4 target gene within the mammalian intestinal epithelium.

MATERIALS AND METHODS

Cell culture. HepG2, Cos-7, and RKO cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium ( Gibco, Grand Island, NY), ±10% fetal bovine serum (vol/vol), 2 mM L-glutamine, 1 × 10^{-5} U/l penicillin/streptomycin (GIBCO), and 5% CO_{2} at 37°C. Each experiment was performed with the cells at ~80% confluence. Cell culture media were replenished at the beginning of each experiment.

Establishment of stably transfected cell line RG24–2 (EcR-RKO/pAdLoxEGI-KLF4). A stable cell line containing an inducible KLF4 expression insert was established as previously described (7). Briefly, the pVgRXR plasmid was obtained from Invitrogen (Carlsbad, CA). This plasmid was generated to express a heterodimer of the DNA-binding domain of the D. melanogaster edysyne receptor (EcR) fused to a modified transactivation domain (TAD) of the herpes simplex virus VP16 protein and the retinoid X receptor (RXR) subunit. RXR is a natural partner of the D. melanogaster edysyne receptor, and binding of edysyne (or its analog ponasterone A) to the heterodimeric VgEcR and RXR results in a functional activator of the EcR element. Plasmid pAdLoxEGI-KLF4 was obtained by subcloning the full-length coding region of the KLF4 cDNA into pAdLoxEGI (18). This latter plasmid was constructed from the pAdlox plasmid by substituting the edysyne inducible promoter from pMD (Invitrogen) for the cytomegalovirus promoter and inserting an expression cassette containing the enhanced green fluorescence protein followed by an internal ribosome entry site (27, 45). The KLF4 cDNA was inserted between the Xhol and EcoRI sites in the multiple cloning sites after the internal ribosome entry site. The human colon cancer cell line RKO was then cotransfected with pVgRXR and pAdLoxEGI-KLF4 at a molar ratio of 1:20. Two days after transfection, 150 μg/ml Zeocin was added to the medium to select for resistant clones. Clones containing inducible KLF4 were identified by 24-h treatment with 5 μg ponasterone A followed by expansion of the green-fluorescent clones and sorting by Star Plus (Becton Dickinson). RG24–2 cells were maintained in Dulbecco’s modified Eagle’s medium (GIBCO), +10% fetal bovine serum (vol/vol), 1 × 10^{-5} U/l penicillin/streptomycin (GIBCO), 150 μg/ml Zeocin (Invitrogen), and 5% CO_{2} at 37°C.

Yeast one-hybrid screen. The yeast one-hybrid system was tested by using a positive control system from Clontech (Palo Alto, CA) as per the manufacturer’s protocol. Briefly, the p53HSI plasmid containing the p53 protein-binding site upstream of the HIS3 gene was integrated into a chromosome of the YM4271 yeast strain. The yeast was additionally transformed with the pAD53m plasmid that expresses the p53 protein hybridized to the yeast GAL4 protein. This hybrid protein is a strong transactivator for the p53HISi plasmid. Transformants were selected in the presence of 25 mM 3-amino triazole (3-AT, a competitive inhibitor of the yeast HIS3 protein) on histidine-free media so that only transformants expressing high levels of HIS3 would be selected. Once the efficacy of the system was verified, three direct repeats of the human IAP promoter sequence IF-III (see sequences below) were inserted upstream of the HIS3 gene and the resulting construct was transformed into YM4271 yeast. Integration was confirmed by growing yeast in complete media and checking for the maintenance of the HIS3 gene. The resulting strain was able to grow in the absence of histidine but not in the presence of 25 mM 3-AT. A plasmid cDNA library derived from 10.5-day-old mouse embryos was constructed as hybrids to the yeast GAL4 TAD and transformed into the IF-III-HIS3 yeast to produce 6.4 × 10^{4} independent transformants. Of these, 128 were capable of growth on histidine-free media in the presence of 3-AT (primary screen). An additional reporter construct containing three tandem repeats of the IF-III sequence upstream of the LacZ gene was then transformed into the 128 selected colonies and resulted in 12 transformants capable of producing a deep blue color in 30 min by β-galactosidase assay (secondary screen). cDNA in each of these transforming plasmids was sequenced and the results were analyzed by using the NIH BLAST program.

Production of recombinant protein. Bacterially expressed recombinant KLF4 protein (containing aa residues 350–483) was generated as described previously (26). Briefly, the prokaryotic expression plasmid pET-16b (Novagen, Madison, WI) containing KLF4 aa residues 350–483 was used to transform the Escherichia coli BL21(DE3) pLysS strain. Induction of recombinant protein production was accomplished by the addition of 1 mM isopropyl-β-D-thiogalactosidase to logarithmically growing cells for 4 h. After this, the bacteria were pelleted by brief centrifugation and placed in lysis buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 6 μM urea, 5 mM imidazole, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 20 μg/ml PMSF) on ice for 30 min. The lysate was then sonicated and purified by Ni^{2+}-NTA-agarose column (Qiagen, Valencia, CA) equilibrated with lysis buffer. After being washed extensively, bound protein was eluted with the same buffer containing instead 1 M imidazole. The eluted protein was serially dialyzed against a solution of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 μM ZnCl_{2}, and 10% glycerol and gradually decreasing concentrations of urea from 6 M to 0.

EMSA. Nuclear extract was obtained from the RG24–2 cells by the following protocol. Adherent cells were collected in 1 ml of PBS by scraping and centrifuged at 4°C at 3,000 rpm for 5 min. The supernatant was discarded, 0.4 ml of buffer (in mM: 10 HEPES, pH 7.9, 1.5 MgCl_{2}, 10 KCl, 0.1 EDTA, 0.1 EGTA, 1 DTT, 0.3 Na_{3}VO_{4}, 1 PMSF (all reagents from Sigma, St. Louis, MO), and 1 μl HALT protease inhibitor (Pierce, Milwaukee, WI)) was added to the pellet, and it was incubated on ice for 15 min. Fifty microliters of 10% Nonidet P-40 (Roche, Basel, Switzerland) were added and vortexed vigorously before centrifugation at 15,000 rpm for 30 s. The resulting supernatant was discarded, 50 μl of buffer (in mM: 20 HEPES, pH 7.9, 1.5 MgCl_{2}, 0.1 EDTA, 1 EGTA, 1 DTT, 0.3 Na_{3}VO_{4}, 1 PMSF, and 0.4 M NaCl, and 1 μl HALT protease inhibitor) were added to the pellet, and it was shaken vigorously for 15 min at 4°C before centrifugation at 4°C at 15,000 rpm for 5 min. Nuclear extract was obtained from both untreated RG24–2 cells and RG24–2 cells treated for 72 h with 5 μM of the edysyne analog ponasterone A (AG Scientific, San Diego, CA). Synthetic oligomers were obtained from Biosource International (Camarillo, CA), and the complimentary oligonucleotides were annealed and radiolabeled by using T4 polynucleotide kinase in the presence of [γ-32P]ATP or [γ-33P]ATP (New England Nuclear/Perkin-Elmer, Boston, MA) to a specific activity of ~1 × 10^{6} counts per μg of polyclonal anti-Sp1 (Upstate Biotech, Lake Placid, NY). An excess of unlabeled competitor oligomer (100 μM) was used in each binding experiment along with 8 μg of nuclear extract or 1–4 μg of purified KLF4 protein. Binding reactions were performed for 40 min at 4°C in gel shift binding buffer supplemented with 2 μg of poly(dl-dC) from Amersham Pharmacia Biotech (Little Chalfont, UK). An excess of unlabeled competitor oligomer (100 μM) was used to verify binding specificity and in some cases 1–5 μg of polyclonal anti-KLF4 or 1–2 μg of polyclonal anti-Sp1 (Upstate Biotech, Lake Placid, NY) was used in supershift analyses (38). The reaction mixtures were then electrophoresed at 4°C on 5% poly-
acrylamide gels in 0.5 \times \text{Tris-borate-EDTA} (Boston Bioproducts, Ashland, MA) after which the gels were dried and subjected to autoradiography.

**Northern blot analysis.** Total RNA was extracted from RG24–2 cells using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA was extracted from untreated cells and from cells treated for 24 h with ponasterone A at concentrations ranging from 1 to 25 \mu M. For Northern blot analyses, 15 \mu g of total RNA was combined with the appropriate amount of glyoxal loading dye (Ambion, Austin, TX), and the samples were electrophoresed through agarose gels prepared in Northern-Max Gly Gel-Prep/Running Buffer (Ambion). Equal loading was determined by examination of ethidium bromide-stained gels and by probing for the actin transcript. After electrophoresis, the RNA was transferred onto positively charged nylon membranes (Amersham Pharmacia Biotech) and cross-linked in a Stratalinker 1880-UV hybridization oven (Stratagene, La Jolla, CA) at the autocross-link setting according to the manufacturer’s protocol. Complementary DNA probes were \textsuperscript{32}P radiolabeled to a specific activity of \sim 5 \times 10^{4} \text{counts-min}^{-1} \text{\mu g}^{-1} \text{DNA}. The IAP probe is a 1.7-kb \text{PstI} fragment derived from the human IAP cDNA and was obtained from ATCC (20). The KLF4 probe is a 1.9-kb \text{EcoRI/XhoI} fragment derived from the mouse cDNA (16). The actin probe is a 1.0-kb \text{PstI} fragment derived from the mouse \beta-actin cDNA (10). Hybridizations were carried out by using the Rapid-hyb solution from Amersham Pharmacia Biotech at 65°C overnight. The membranes were then washed for 30 min twice at 65°C in a low stringency wash solution (2 \times \text{SSC}/0.1\% \text{SDS}) followed by a high stringency wash (0.1 \times \text{SSC}/0.1\% \text{SDS}) at 65°C for 30 min after which they were subjected to autoradiography.

**Transient transfection assay.** Cells were seeded at a density of \sim 5 \times 10^{5} \text{cells} per well in six-well cluster plates (Sigma). Transient transfections were accomplished by using the Superfect transfection kit (Qiagen) as per the manufacturer’s protocol. A 2.5-kb fragment of the human IAP 5’-flanking region along with various 5’- and internal deletions were placed in control of a firefly luciferase reporter gene and were transiently cotransfected with either wild-type or one of several mutated KLF4 expression vectors (13). These mutant KLF4 constructs were lacking the sequences required for the DNA binding domain (DBD), nuclear localization signals or TADs (16). The total amount of DNA was kept the same for each transfection by addition of nonspecific plasmid DNA. Each transfection sample also received 0.5 \mu g of a CMV Renilla luciferase plasmid (pRL-CMV; Promega, Madison, WI) to monitor transfection efficiency. The reported luciferase activities represent normalization with Renilla luciferase activities.

**Statistical analysis.** Statistical analyses were performed by using a standard one-way ANOVA with Dunnett’s posttest (InStat Software; GraphPad Software, San Diego, CA). \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**KLF4 binds to the IAP cis-element IF-III.** The yeast one-hybrid assay was used to screen a mouse embryonic cDNA library for proteins that bind to the IF-III element of the human IAP promoter. IF-III was chosen on the basis of previous work indicating a critical role for this DNA cis-element in IAP transactivation (29). The results of the yeast one-hybrid screen revealed five sequences showing near-perfect matches to previously reported sequences. Each identified gene was a member of either the Krüppel or nuclear receptor families of transcription factors. The genes were Sp3, KLF4, BKL, COUP-TF1, and TR4 (orphan nuclear receptor). Of the five, KLF4 was known to be expressed in gut epithelial cells and was therefore chosen for further study.

**Figure 1A** shows the results of EMSA using a synthesized double-strand oligonucleotide sequence corresponding to the IF-III sequence of the human IAP gene as well as the homologous sequence from the mouse IAP gene. Previous transfection studies had demonstrated that the mouse IF-III element was functionally equivalent to the human one (29). In both cases, the addition of purified KLF4 protein resulted in a shifted band that was competed away with excess unlabeled IF-III oligomer but not by excess of a same-sized unlabeled probe with an unrelated sequence (lanes labeled noncompetitor). This band was supershifted by the addition of anti-KLF4 antibody, confirming the nature of the shifted complex. These
EMSA experiments confirmed that the human and mouse IF-III IAP cis-elements do, indeed, contain a KLF4 binding site.

Proximal IAP promoter contains at least two KLF4 binding cis-elements. In previous work with DNase 1 footprinting, we (29) identified five binding regions for nuclear proteins, four of which contain a central GC-rich sequence. To clarify the presence and number of KLF4 response elements in the IAP promoter, we performed EMSA experiments by using synthesized double-strand oligonucleotides corresponding to the human IF-I, IF-II, IF-III, IF-IV, and IF-V sequences. Figure 1B demonstrates the affinity of the binding of KLF4 to each of these cis-elements. The binding is strongest with IF-II and IF-III. Using cold competitors, we have shown that the shifted complexes are similar in the cases of IF-II and IF-III. KLF4 appears to bind minimally to IF-I and IF-IV, although these are probably nonspecific interactions. No binding of KLF4 is seen in the case of IF-V. Interestingly, IF-V differs from the other elements in that it does not contain a central GC-rich sequence. These data indicate the presence of at least two KLF4 binding sites within the proximal IAP promoter.

IAP is upregulated by KLF4. We then sought to determine whether the endogenous IAP gene is truly a KLF4 target. To answer this question, an inducible KLF4 expression system was utilized. Northern blot analyses were performed on total RNA derived from RKO cells stably transfected with a plasmid engineered to overexpress KLF4 by stable transfection with a KLF4 expression vector (2). Neither KLF4 nor IAP was detected in the uninduced cells (Fig. 2, lane 0). Activation of the KLF4 insert by treatment with an ecdysone analog ponasterone A (Pon A) for 24 h resulted in KLF4 upregulation and detection of IAP. This effect is dose dependent as demonstrated by the increasing quantities of KLF4 and IAP detected as the concentration of ponasterone A is increased from 1 to 25 μM. Actin is included in Fig. 2, bottom, as a control for equal loading of the RNA. These results indicate that the endogenous IAP gene is a target of the KLF4 transcription factor.

KLF4 induction alters the protein binding with IF-III. We sought to ascertain the role of IF-III in IAP activation within the cell by using the system of inducible KLF4 expression and repeating EMSA experiments with nuclear proteins. Nuclear extracts derived both from uninduced and induced RG24–2 cells were used in the binding assays with IF-III and the results were compared. We noticed a significant difference in the binding pattern of nuclear proteins to the IF-III element with the appearance of a new band when using extract from ponasterone A-induced RG24–2 cells (Pon A +ve). This band was then supershifted by the addition of anti-KLF4 antibody, thus identifying the band as a KLF4-DNA complex (lane 11). Anti-KLF4 antibody had no effect on the binding with extract from uninduced cells (lane 6). Anti-Sp1 antibody was used to demonstrate the presence of Sp1 protein in both nuclear extracts (lanes 5 and 10). These experiments confirm that the KLF4 protein produced in the RKO cells is able to bind the IF-III element.

KLF4 transcriptionally activates IAP. A transient transfection system was then employed to further delineate the molecular mechanisms by which KLF4 activates the IAP gene. The results from transient cotransfections of a 2.5-kb IAP-luciferase reporter construct with increasing amounts of KLF4 expression vector are shown in Fig. 4. Baseline expression of the IAP construct was low in the HepG2 cells. KLF4 cotransfection caused transcriptional activation of the IAP reporter plasmid in a “dose-dependent” fashion as indicated by the increasing levels of reporter activity with increasing amounts of KLF4 vector transfected. At the highest amounts of KLF4, there was an ~5.5-fold increase in IAP-luciferase activity.

IAP promoter contains a KLF4 response element located between −224 and −114 bp upstream from translational start site. Figure 5 demonstrates the localization of the KLF4 response element within the human IAP promoter as determined by transient transfection of HepG2 cells by using various 5′ and internal deletions of the IAP-luciferase reporter construct. A significant degree of KLF4-induced IAP transactivation was observed until a segment of the IAP promoter sequence 5′ to −125 bp upstream from the translational start site was deleted. The shortest construct showing KLF4-induced IAP activation...
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Fig. 4. Transient cotransfections of HepG2 cells with a 2.5-kb IAP-luciferase reporter plasmid and increasing amounts of the KLF4 expression vector. Nonspecific plasmid DNA was used to maintain equal amounts of transfected DNA in all cases.

Included the 173-bp sequence 5′ from the start of translation, indicating that a KLF4 response element is located between −173 and −125 bp. Another construct that contains the 2.5-kb IAP promoter segment but with an internal deletion of the region between −224 and −114 bp demonstrated a minimal response to KLF4, confirming the importance of the proximal promoter region for the activation of IAP by KLF4. These transfection results indicate that KLF4 transactivates the IAP gene largely via one or more cis-elements located between −224 and −114 bp, a region that contains the KLF4-binding segments IF-II and IF-III (IF-II is located between −206 and −190 bp upstream from the translational start site and IF-III is located between −156 and −140 bp upstream from the translational start site). Results of transfections in Cos-7 cells were similar to those seen in HepG2 cells (data not shown).

Three KLF4 domains are required for full induction of IAP. Figure 6 shows the effects of mutant KLF4 proteins on the expression of the IAP gene. The 2.5-kb IAP-luciferase reporter construct was cotransfected with various mutant KLF4 plasmids producing truncated versions of the KLF4 protein. Basal expression of IAP was low and the full-length wild-type KLF4 caused the expected significant activation (~10-fold, P < 0.05). KLF4 constructs lacking the sequences for DBD or TAD did not cause significant IAP activation. Interestingly, constructs combining sequences for DBD with either nuclear localization sequences (NLS) or TAD sequences induced a level of IAP activity that was intermediate between the basal expression and transactivation observed with the full-length KLF4 construct. These results indicate that all three domains of the KLF4 protein (DBD, NLS, and TAD) are required for full induction of the IAP gene.

DISCUSSION

We have used the differentiation marker IAP as a tool to unravel the molecular mechanisms that govern the enterocyte differentiation program, and in the present work, have identified an important relationship between the recently described KLF4 and IAP. KLF4 is a member of the family of zinc-finger transcription factors that exhibit homology to the fruit fly Krüppel gene product (35). Krüppel is essential for segmentation in Drosophila and its mammalian counterparts, the KLFs, have been shown to play key roles in regulating the cell cycle, differentiation, and development of various cell lineages (1). KLF4 itself has been shown to cause a G1/S cell cycle arrest, likely through upregulation of p21 and downregulation of cyclin D1 (CD1) (7). KLF4 levels have also been shown to be decreased in human colon neoplasms (11). KLF4 null mice have been reported to die at birth, apparently because of a loss of skin barrier function (35). Interestingly, KLF4 null mice have been found to specifically lack goblet cells in the gut (28). Within the intact mammalian gut epithelium, KLF4 expression occurs in the differentiated cells as opposed to the undifferentiated cells (38). Based on all these features, it has been hypothesized that KLF4 may be a transcription factor involved in enterocyte differentiation. However, up to this point no gut-specific target gene for KLF4 had been identified. We were therefore intrigued when using a previously identified IAP cis-element in a yeast one-hybrid screen we identified KLF4 as a protein capable of binding this critical segment of the IAP 5′
regulatory region (29). The specificity of this binding interaction was independently confirmed by using EMSAs.

Presence of the KLF transcript in the mammalian small intestine has been demonstrated previously by Shields et al. (38) and was also identified by us (data not shown). With the use of an in vitro model system, butyrate-treated HT-29 cells, Shieh et al. (36) showed that KLF4 expression increases along with the expression of the differentiation marker, alkaline phosphatase. In the present work, we show that when RKO cells are engineered to express KLF4, there is a corresponding activation of the endogenous IAP gene and the degree of IAP activation mirrors the increasing levels of KLF4. This finding along with the previous data strongly suggests an actual physiological role for KLF4 regarding this important enterocyte differentiation marker.

Previously, we (29) identified five cis-elements (IF-I, II, III, IV, and V) in the proximal IAP promoter that bind nuclear proteins. Four of these elements contain relatively GC-rich sequences, which led us to speculate on the presence of multiple elements responsive to KLF4. EMSA performed with these sequences demonstrated that KLF4 did indeed bind with varying affinity to multiple cis-elements in the proximal IAP promoter and that the strongest binding was with IF-II and IF-III. The binding of nuclear extract to IF-I and IF-IV could not be efficiently competed away with cold competitors, indicating the probable nonspecific nature of these complexes.

Mechanisms of KLF4-mediated transcriptional regulation of the IAP gene were more precisely defined by using transient transfection studies. These experiments demonstrated that KLF4 is capable of causing the transcriptional activation of a reporter gene under the control of various segments of the human IAP promoter. Using a number of deletion constructs of the IAP 5’ regulatory region, we were able to localize the KLF4 response element to a segment of the IAP gene between −224 and −114 bp upstream from the translational start site. This segment contains the sequences for IF-II and IF-III thus demonstrating the functional relevance of these sequences to KLF4-mediated IAP activation. It is possible that other regions in the IAP 5’ flanking region may also contribute to the effects of KLF4 on IAP gene transcription. Taken together, these data indicate that KLF4 is an important transcriptional regulator of IAP and, furthermore, that KLF4 appears to function via a series of cis-elements that include the IF-III cis-element used in the yeast one-hybrid screen. Presence of multiple KLF4 binding sites regulating gene expression has been demonstrated by Higaki et al. (21) in the rat laminin-γ1 chain promoter. It is likely that in the case of the IAP promoter these multiple GC-rich cis-elements function in a coordinated fashion with KLF4, and perhaps with other factors, to achieve transcriptional regulation.

In this regard, we (29) have previously shown that other proteins, including Sp1 and Sp3 bind to the IF-III cis-element. In the present work, we have performed EMSAs by using nuclear extract derived from untreated and ponasterone A treated RG24–2 cells. Results of these experiments indicate a significant change from untreated to treated cells in the binding pattern of nuclear proteins with IF-III. This change can be attributed to binding of KLF4 protein to the IF-III sequence in cells expressing KLF4. As such, at least three proteins (KLF4, Sp1, and Sp3) bind to the IF-III element. Interestingly, interactions between KLF4 and Sp1 have been demonstrated in a number of promoters, and depending on the promoter, this interaction may take different forms. KLF4 and Sp1 work in synergy to activate the rat laminin-γ1 promoter (21). The K19 promoter contains an overlapping binding site for KLF4 and Sp1 through which they appear to exert a cooperative and additive stimulatory effect (5). In contrast, in the transcriptional regulation of the CD1 and the ornithine decarboxylase (ODC) promoters, KLF4 and Sp1 have opposing effects to each other and appear to compete for the same binding site (9, 37). Each of these genes is known to play a key role in either cellular proliferation (CD1 and ODC) or tissue architecture and cellular differentiation (laminin-γ1 and K19). In the case of IAP, a known marker of intestinal differentiation, the functional importance of these various transcription factors to the overall regulation of the gene will need further investigation.

In an effort to further elucidate the mechanism by which KLF4 activates IAP, multiple KLF4 expression constructs containing deletions of the sequences encoding its three major domains were used in transient transfections with the longest IAP reporter construct. These experiments demonstrated that to obtain full induction of IAP by KLF4, all three of the major domains of the KLF4 protein (DNA binding, nuclear localization, and transactivation) were required. Interestingly, two other constructs that combined the DBD with either the transactivation or nuclear localization domains resulted in an intermediate level of IAP activation. These results suggest that full activation by KLF4 requires the protein to bind to its DNA response element, cause transactivation through its TAD, and possibly interact with other proteins through its nuclear localization signal domain. KLF4 has been shown to affect transcription of other genes by working directly as a transcriptional activator, as well as through protein-protein interactions (6). Additional work will be necessary to determine the precise mechanisms by which KLF4 transcriptionally activates IAP.

Although IAP has been used extensively as a marker for enterocyte differentiation, little is known regarding its function. The IAP protein is a part of the surfactant-like particle found in the mammalian gut epithelia, and it has therefore been suggested that IAP may function in fat absorption (44). It is not known with certainty whether IAP null mice have not been reported in the literature. Regarding IAP regulation, our laboratory and others have documented the dramatic induction in IAP expression seen during postnatal development at the time of weaning (19, 22). In addition, we (22–24) found that IAP expression is generally repressed under atrophic conditions, e.g., with starvation and hypothyroidism. This repression of IAP expression has also been demonstrated to occur in vitro in models of serum starvation and postconfluent growth (25). Implications of this relationship between gut atrophy and IAP expression have not been elucidated, and it is unknown whether KLF4 plays any role in this aspect of IAP regulation. IAP is one of a family of alkaline phosphatase gene products, including liver/bone/kidney and placental forms. Interestingly, Chen et al. (8) have shown that both intestinal and placental alkaline phosphatase genes are induced in the RKO cells by KLF4. Furthermore, sequence alignment shows that the promoter regions of two genes display strong similarity. Whether the mechanism of KLF4-induced placental alkaline phosphatase gene activation is the same as we have shown for the intestinal form will require further study.
A variety of transcription factors appear to be involved in the activation of intestine-specific differentiation markers. Most notable are the caudal-related factors, Cdx1 and Cdx2, GATA factors, and HNF-1α, which appear to play critical roles in the expression of the brush-border enzymes sucrase and lactase (4, 40). Interestingly, no specific transcription factors have been previously identified in the context of IAP gene activation. As such, KLF4 is the first gut transcription factor found to regulate this important differentiation marker. Both KLF4 and IAP are expressed in enterocytes, and KLF4 directly regulates the expression of IAP in the RKO cell line. Hence it is very likely that KLF4 regulates endogenous IAP gene expression in enterocytes. It should be noted that Cdx2 might be an important activator of KLF4 (12). As such, it is possible that Cdx2 works directly on certain genes involved in the differentiation process (e.g., sucrase) while acting indirectly through KLF4 to activate other differentiation markers (e.g., IAP).

In summary, we have demonstrated that KLF4 is capable of specifically interacting with a critical regulatory region within the enterocyte differentiation marker gene IAP. This binding likely mediates the KLF4-induced activation of the endogenous IAP gene in an intestinal cell culture system. These results point to KLF4 as an important transcriptional regulator of the IAP gene. The recent work by Chen et al. (8) further demonstrates the dramatic changes in cell phenotype induced by KLF4. Further work should provide insights into the role that KLF4 may play in IAP gene activation in conjunction with other transcription factors, and perhaps more generally in the context of the overall enterocyte differentiation program.

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REFERENCES


