The Kruppel-like KLF4 Transcription Factor, a Novel Regulator of Urokinase Receptor Expression, Drives Synthesis of This Binding Site in Colonic Crypt Luminal Surface Epithelial Cells*

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The urokinase-type plasminogen activator receptor (u-PAR) plays a central role in cell migration, growth, and invasion and is regulated, in part, transcriptionally. In mice, u-PAR expression is restricted to a few tissues, one of which is the colon. We therefore screened a colon expression library for regulators of u-PAR promoter activity and identified a zinc finger protein bearing consensus sequences to the Kruppel-like family of transcription factors and showing partial homology with one of the members, KLF4. Like u-PAR, KLF4 expression is predominant in the luminal surface epithelial cells of the colonic crypt, and we hypothesized that u-PAR synthesis in these cells is directed by this transcription factor. Colon cells from KLF4 null mice showed a dramatic reduction in u-PAR protein compared with wild-type mice. Conversely, KLF4 expression in HCT116 colon cancer cells increased the amount of u-PAR protein/mRNA. Transient transfection of KLF4 with a reporter driven by 5’-deleted u-PAR promoter fragments indicated the requirement of the proximal 200 base pairs for optimal expression. Mobility-shifting experiments demonstrated binding of KLF4 to multiple regions of the u-PAR promoter (−154/−128, −105/−71, and −51/−24), and chromatin immunoprecipitation assays confirmed the binding of KLF4 to the endogenous promoter. Deletion of the −144/−123 promoter region diminished but did not eliminate the ability of KLF4 to transactivate the u-PAR promoter, suggesting cooperativity of these binding sites with respect to activation of gene expression. In conclusion, we have identified KLF4 as a novel regulator of u-PAR expression that drives the synthesis of u-PAR in the luminal surface epithelial cells of the colon.

The urokinase-type plasminogen activator receptor (u-PAR),1 a 45–60-kDa glycosylated receptor linked to the cell surface via a glycolipid chain, is a multifunctional protein that plays a central role in proteolysis, cell migration, and growth control (1–4). The u-PAR contributes to these physiological functions via different mechanisms. First, the serine protease, urokinase, bound to this receptor activates plasminogen at a much faster rate than fluid-phase plasminogen activator, thereby augmenting extracellular matrix degradation. Second, the u-PAR interacts with the extracellular domain of integrins, thereby mediating cell adhesion and migration (1, 5, 6) as evident in re-epithelialization of skin wounds (7, 8). Third, the u-PAR stimulates cell growth via both epithelial growth factor receptor-dependent and independent signaling pathways (4, 9). Finally, it has been shown that the seven-transmembrane receptor, formyl peptide receptor-like receptor-1/lipoxin A4 receptor, a G protein-coupled receptor, directly interacts with a soluble cleaved form of u-PAR to induce chemotaxis (10).

In cancer, there is strong evidence implicating u-PAR expression in tumor growth and progression. Indeed, elevated u-PAR levels are evident in various malignancies (11–13) and correlate with growth (4) and tumor progression (12). Moreover, interfering with u-PAR expression or function retards growth and invasiveness of some cancers, including glioblastomas (14–16).

The amount of u-PAR protein is controlled mainly at the transcriptional level, although altered message stability (17, 18), translational efficiency (19), and receptor recycling (20) all contribute to the quantity of this gene product. Our laboratory and others have previously described several upstream transcriptional elements regulating u-PAR expression in tissue culture. Thus, Soravia et al. (21) were the first to report a GC-rich region (−99/−70) bound with Sp1, which was required for basal expression of the gene. We subsequently identified a novel NF-κB (−148/−124) bound with Sp1/Sp3 and an AP-2-related factor and was required for u-PAR expression in cultured colon cancer cells (22, 23). Additionally, a third region with a DNase I footprint spanned nucleotides −190/−171, containing a consensus AP-1 motif, and was necessary for the constitutively high or phorbol 12-myristate 13-acetate-inducible expression of u-PAR (22). In separate investigations, Hapke et al. (24) implicated a PEA3/Ets silencing motif located at −248, whereas Wang et al. (25) demonstrated a novel NF-κB element (located at −45) that was required for expression of this gene in cultured cells. In addition to these reports, our studies analyzing tissue-specific expression of u-PAR in transgenic mice indicated the requirement for a region residing between −1.5 and −8.5 kb for expression in the placenta (26).

One of the few tissues that constitutively express u-PAR in the healthy mouse is the colon where u-PAR protein/mRNA predominates in the upper region of the crypt (26). u-PAR localization to the surface epithelial cells may contribute to their detachment via proteolysis wherewithupon the epithelial cells undergo anoikis as a result of the absence of a basement membrane-generated survival signal (27); however, at the present time, the mechanism that drives expression of this gene in the colon is unknown. Consequently, we undertook expression

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‡The abbreviations used are: u-PAR, urokinase-type plasminogen activator receptor; EGFP, enhanced green fluorescence protein; FACS, fluorescence-activated cell sorting; EMSA, electrophoretic mobility shift assays; TGF-β, transforming growth factor-β; IP, immunoprecipitated.
KLF4, a Novel Regulator of Urokinase Receptor Expression

22675

cloning studies with a colon cDNA library to identify regulators of u-PAR expression in colon tissue.

EXPERIMENTAL PROCEDURES

Antibodies—A FLAG sequence flanked with HindIII, BamHI, XhoI, and NotI sites (AAG CTG ATG ATG TAC GAT TAC TAA AAA GAC GAT GAC GAC AAG GGA TCC CTC GAG TCT AGA GGG CCC (underlined nucleotides indicate restriction sites)) was synthesized and cloned between the HindIII and XhoI sites at the multiple cloning sites in the pcDNA3 vector generating the pcDNA/FLAG/BamHI vector. The KLF4 coding sequence was then isolated from pcDNA3.1-KLF4 and subcloned into the pcDNA/FLAG/BamHI vector. The KLF4-FLAG sequence was subsequently purified using HindIII and XhoI and cloned into the EcoRI/HindIII and XhoI-generated window of the pIRE2-EGFP (enhanced green fluorescence protein) vector (Clontech) to generate the KLF4-EGFP expression vector sequence. Sequencing was used to confirm the identity and orientation of this construct.

All u-PAR promoter fragments were cloned into the pGL3-Basic (Promega) reporter construct. Fragments −398/+52, −197/+52, and −8/+52 were recovered by restriction digestion from the corresponding chloramphenicol acetyltransferase constructs described previously (22). The −1469/+52 u-PAR promoter fragment was prepared by PCR from genomic DNA whereas fragments −154/+52, −105/+52, −88/+52, and −51/+52 were prepared by PCR using −398/+52 as a template. All fragments were blunt-ended and ligated into pGL3-Basic following Smal digestion and phosphorylation of the latter.

Antibodies to Sp1 (sc-59x), Sp3 (sc-64x), and GFKL (KLF4) (sc-20691x) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-FLAG antibody (F3165) was obtained from Sigma.

Expression Cloning—An arrayed human colon cDNA library (LCO-1001, Oigene Technologies, Rockville, MD) in which cDNAs were constructed in the pCMV6- XL4 expression vector was used. DH10B Escherichia coli bacteria were transformed with DNA from the library and screened on ampicillin LB agar plates to obtain −100 colonies per plate. Every plate was replicated using the replicating plating tool and velvetseed replicating of tissue with AbTech Products or by manual selection. The bacteria were transformed with DNA from the library and replica plated squares (Bel-Art Products) or by manual selection. The colonies were picked and grown into 96-well plates. After 24 h, cells were cotransfected (using LipofectAMINE 2000) with u-PAR promoter-driven luciferase reporter (0.2 μg) and an expression vector (pcDNA3.1) encoding KLF4. Where indicated, transfections included 0.03 μg of a β-actin-regulated Renilla luciferase reporter to normalize for varying transfection efficiencies. Cells were washed 24 h later, lysed, and assayed for luciferase activity. Statistical analyses (unpaired t test) were performed using the GraphPad Prism software (GraphPad Software, version 5).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts and EMISA were carried out as described by us elsewhere (23, 29). EMSA was performed using 10 μg of nuclear extract, 0.6 μg of poly(dI·dC), and 2 × 10^6 cpm of a [γ-32P]ATP labeled oligonucleotide.

Electron microscopy—Immunofluorescent immunoprecipitations—These assays were performed essentially as described previously (30) but with modifications. Cells were treated with 1% formaldehyde for 8 min to cross-link proteins to DNA. The cells were collected, washed twice in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and then washed sequentially for 10 min in ice-cold Solution 1 (0.2 M sucrose (v/v) Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 7.5) and thereafter in Solution 2 (0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 7.5). The pellet was resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxychlorohydrate, and protease inhibitors) and sonicated eight times in 15-s intervals.

Chromatin immunoprecipitations—These assays were performed essentially as described previously (26) but with the minor modification that blocking was accomplished with 5% normal horse serum and 1% normal goat serum and that 3,3′-diaminobenzidine tetrahydrochloride was used as a substrate for visualizing immunoreactive proteins. Counterstaining was achieved with hematoxilin.

Acian Blue Staining—Detection of acidic mucins was performed by staining with Alcian Blue as described elsewhere (26).

Western Blotting—Western blotting for u-PAR was performed as described previously (29). Briefly, cell extract (in a Triton X-100 buffer with protease inhibitors) was immunoprecipitated with a polyclonal anti-u-PAR antibody. The immunoprecipitated material was then subjected to Western blotting, and the blot was probed with 5 μg/ml anti-u-PAR monoclonal antibody (3931, American Diagnostica, Greenwich, CT) and a horseradish peroxidase-conjugated goat anti-mouse IgG. Bands were visualized by ECL.

For detection of KLF4, 80 μg of cellular protein was subjected to SDS-PAGE, and detection was achieved using an anti-FLAG M2 as primary antibody (Sigma) and an anti-mouse-horseradish peroxidase-conjugated secondary antibody (Promega). The KLF4 expression in colon tissue.

Northern Blotting—Total cellular RNA was isolated from 90% confluent cultures using TRIzol reagent (Invitrogen). The RNA was resolved in a 1% agarose-formaldehyde gel and transferred to a nylon membrane by capillary action using 20× SSC. The blot was probed at 65 °C with a randomly primed, radiolabeled 0.8-kb cDNA specific for human u-PAR mRNA. Stringency conditions were performed with 0.1× SSC, 0.1% SDS at 65 °C. Loading efficiencies were checked by reprobing the blot with a radiolabeled cDNA, which hybridizes with the glyceralde- hyde-3-phosphate dehydrogenase mRNA.

Transfections—Transfections were performed using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen). Briefly, 12 μg of DNA diluted into Opti-MEM I reduced serum medium (catalog no. 31985-070, Invitrogen) was mixed with LipofectAMINE 2000 (using Opti-MEM I reduced serum medium as diluent), and the mixture was added to 10⁶ cells. After 24 h, cells were either observed by fluorescence to determine transfection efficiency or harvested.

Fluorescence-activated Cell Sorting (FACS) Analysis—Cells were harvested with 3 mM EDTA, washed with phosphate-buffered saline, and stained with 0.1% normal horse serum albumin (Sigma) and 0.1% formaldehyde in phosphate-buffered saline. Subsequently, the cells were incubated for 30 min at room temperature with an anti-u-PAR monoclonal antibody (R4) or a control IgG1 antibody (5 μg/ml). After washing, all samples were incubated for 20 min at room temperature with a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (5 μg/ml). Samples were then fixed in 70% ethanol (4 °C) overnight, after which cell pellets were resuspended in 50 μg/ml propidium iodide and 20 μg/ml RNase. Cells were incubated at 37 °C for 20 min and then subjected to FACS analysis.

Reporter Assays—Cells (300,000) were subcultured into 24-well plates. After 24 h, cells were cotransfected (using LipofectAMINE 2000) with u-PAR promoter-driven luciferase reporter (0.2 μg) and an expression vector (pcDNA3.1) encoding KLF4. Where indicated, transfections included 0.03 μg of a β-actin-regulated Renilla luciferase reporter to normalize for varying transfection efficiencies. Cells were washed 24 h later, lysed, and assayed for luciferase activity. Statistical analyses (unpaired t test) were performed using the GraphPad Prism software (GraphPad Software, version 5).

Real Time (Quantitative) PCR—Real time PCR assays were performed essentially as described previously by this laboratory (31) but with the following modifications. The primers used in the reaction had a concentration of 100 nM. A threshold cycle (Ct) value was obtained from each amplification curve using the software provided by the manufacturer. A ΔCt value was calculated by subtracting the Ct value for the 2% input sample from the Ct value for the immunoprecipitated (IP) sample, i.e., ΔCt = Ct(NaCl) − Ct(IP). The percent amount for the IP sample was then calculated by raising 2 to the ΔCt power, i.e., the total percentage of IP sample = 2^ΔCt × 2 as described previously. The following primers, corresponding to the human u-PAR promoter sequence, were used for real time PCR: forward 5′-TTTA-
CAGCAGTGGAGAGCGATT-3' and reverse (5'-CCCTGACTCATGGAGTTGTGAT-3'). These primers correspond to sequences -286/-265 and -116/-134, respectively, of the u-PAR promoter. For conventional PCR, the identical primer pair was used with the following PCR cycle parameters: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, with a total of 35 cycles. To verify the amplicon size, PCR products were checked in a 10% polyacrylamide gel.

RESULTS

Expression Cloning of a Zinc Finger Protein That Regulates u-PAR Promoter Activity

To identify regulators of u-PAR expression in the colon, we screened an arrayed colon cDNA library representing ~500,000 clones. The library was subdivided such that primary screenings were undertaken with pools of 100 cDNAs. Accordingly, HCT116 colon cancer cells were cotransfected with cDNA pools and a luciferase reporter regulated by 1469 base pairs of u-PAR upstream sequence and were subsequently assayed for luciferase activity. We previously showed that this promoter sequence includes all the regulatory elements necessary for appropriate u-PAR expression in the colon (26). One of the primary screens (Fig. 1A) identified a pool (B4) stimulating u-PAR promoter activity. This pool was further subdivided and screened in two subsequent rounds, thereby identifying clone B4-7-5, which selectively increased u-PAR promoter activity (Fig. 1B). Sequencing of this clone followed by a NCBI BLAST search identified a cDNA encoding a 538-amino acid zinc finger protein (zinc finger protein 306, accession number BT007427.1). Interestingly, a conserved C_x3-C_x3-K_x3-F_x3-S_x3-L_x3-H_x3-H amino acid sequence in the predicted peptide identified zinc finger protein 306 as a mem-

Fig. 1. Expression cloning of a zinc finger protein that up-regulates u-PAR promoter activity. A, primary screen of cDNA pools. HCT116 were cotransfected with a u-PAR promoter-regulated luciferase reporter (50 ng), using cDNA pools (350 ng) and pRL-SV40 (1 ng) as internal controls. After 24 h, cells were lysed and analyzed for luciferase activity. All data are expressed relative to the internal control. B, tertiary screen of 10 individual clones derived from subpool B4-7 identified in a secondary screen. Transfections and analyses were carried out as described for A. C, amino acid homology between zinc finger (ZFP) 306 and KLF4. Bold text indicates the consensus sequence TEGKPYX characteristic of Kruppel-like transcription factors.
ber of the C2H2 subfamily of zinc finger proteins, and a TGEK-PYX sequence evident between zinc fingers further refined the cDNA within the Kruppel-like subfamily of the C2H2 group (32). Intriguingly, a homology search (www.ncbi.nlm.nih.gov/blast/bl2seq) combined with a literature review for colon-specific Kruppel-like transcription factors identified KLF4 (also known as GKLIP) as a 56-kDa Kruppel-like DNA-binding protein (33–35) showing partial homology with zinc finger protein 306 (Fig. 1C). Of particular interest was the observation that, like u-PAR (26), KLF4 expression is restricted to the luminal surface epithelial cells of the colon (33, 34). Consequently, we determined whether KLF4 directs u-PAR expression in the luminal surface epithelial cells of the colon.

**Fig. 2.** A, predominance of u-PAR immunoreactivity in the luminal surface epithelium of the colon. u-PAR immunoreactivity in transverse sections of the colon from adult wild-type mice was detected using 3,3′-diaminobenzidine tetrahydrochloride following incubation of the sections with or without 10 μg/ml antibody (Ab). Arrows indicate staining of the luminal surface colon epithelium. B, diminished u-PAR protein in the luminal surface epithelium of the colon cells from KLF4 null mice. Colon tissue from 1-day old postnatal wild-type (wt) or KLF4 null (−/−) mice was subjected to immunohistochemistry for u-PAR protein as described in A. Arrows point to pronounced epithelial immunoreactivity in cells at the mouth of the crypt. Magnifications are indicated. C, u-PAR protein localizes to the luminal surface epithelium whereas acidic mucins are basolateral. Sections of adult mouse colon from wild-type animals were either stained for u-PAR protein as described in A or for acidic mucins using Alcian Blue. Note the distinct locations of the u-PAR immunoreactivity (luminal surface, left panel, arrows) and the acidic mucins (basolateral surface, right panel, arrows).

**Diminished u-PAR Protein in the Colon from KLF4 null Mice**—The predominant expression of u-PAR mRNA in the luminal surface cells of the gastrointestinal tract has been previously reported (36), and as expected immunohistochemical staining for u-PAR protein in the adult mouse colon revealed intense staining in the upper region of the colonic crypt (Fig. 2A, left panel, arrows). Some staining was evident in the muscularis propria, but this was erratic and may represent u-PAR expression in smooth muscle as reported elsewhere (37, 38). If KLF4 regulates u-PAR expression in the epithelial cells at the luminal surface of the mouse colon, then the absence of u-PAR protein would be predicted for this tissue derived from KLF4 null mice. Mice lacking both alleles of the KLF4 gene died 1 day postnatally (28), and u-PAR protein expression was analyzed in sections of colon from these animals. Like the colon from the adult mouse, u-PAR immunoreactivity was detected in the cells located at the mouth of the colonic crypt (Fig. 2B, upper panels, arrows). u-PAR immunoreactivity was also evident in migrating immune cells (rectangles) (39, 40), the presence of these activated cells probably reflecting an inflammatory response to maternal feeding in the new-born mice. More importantly, u-PAR immunoreactivity was dramatically diminished in the luminal surface cells of the colon derived from the KLF4 null mice (Fig. 2B, lower panels). Thus, although the u-PAR immunoreactivity was consistently detected throughout the luminal surface epithelial cell layer of wild-type mice, few cells showed u-PAR positivity in the KLF4 null mice. Furthermore, long stretches of the surface epithelium were completely devoid of this protein in the KLF4 knock-out mice. These data would suggest that u-PAR expression in the normal colon surface epithelium is driven largely by the KLF4 transcription factor. However, there is a concern relating to the ability of KLF4 to promote goblet cell differentiation (28) in this tissue. Accordingly, it may be that the absence of u-PAR protein is secondary to the lack of maturation of these cells. To address this possibility, colon tissue from KLF4 wild-type mice was stained either for u-PAR protein or with Alcian Blue to indicate acidic mucins normally restricted to goblet cells (28). We found that u-PAR protein was predominantly in cells located at the mouth of the crypt (Fig. 2C, left panel, arrows), whereas mucin-positive cells were located at the base of the crypt (Fig. 2C, right panel, arrows). These separate cell locations would argue against the possibility that the lack of u-PAR protein in KLF4 null mice is the result of incomplete goblet cell differentiation.

**KLF4 Expression Increases u-PAR Expression in Cultured Colon Cancer Cells**—To corroborate the data generated with the KLF4 null mice, we determined whether expression of exogenous KLF4 up-regulated u-PAR expression. Toward this end, we first subcloned the FLAG-tagged KLF4 coding sequence into the bicistronic pIRES-EGFP expression construct upstream of an internal ribosome entry site and the EGFP coding sequence. Accordingly, expression of EGFP is indicative of transcription of the KLF4 coding sequence. HCT116 colon cancer cells, characterized by a low level of endogenous KLF4 (41), were transiently transfected with this KLF4-bearing ex-
pression vector, and the cell pool was subsequently analyzed. The successful expression was denoted by the presence of EGFP-fluorescing cells (Fig. 3A) and Western blot detection of a FLAG-tagged protein indistinguishable in size from the KLF4 protein (60 kDa) (41) in cell lysates (Fig. 3B). Furthermore, u-PAR protein levels were clearly elevated in the HCT116 cells pooled after transient transfection with KLF4 (Fig. 3C), and Northern blotting indicated that the increased u-PAR protein was the result of a larger amount of u-PAR mRNA (Fig. 3D). These data further support the contention that KLF4 regulates u-PAR expression.

KLF4 has previously been shown to block the progression of cells from G1 into S phase (42). If u-PAR expression is restricted to G1, this might account for the increased u-PAR protein in the KLF4-transfected cells. To address this possibility, after they were stained with an anti-u-PAR antibody, cycling HCT116 cells were subjected to FACS analysis, but 96% of the total cell population was positive for u-PAR immunoreactivity (Fig. 4B) indicating expression of u-PAR across all phases of the cell cycle. Additionally, the mean channel fluorescence (Fig. 4C), indicative of expression level, was lower in G1 (7.2) compared with S (8.9) and G2/M (12.6) phases. Thus, it is unlikely that elevated u-PAR expression evident in KLF4-transfected HCT116 cells reflects the accumulation of cells in G1.

Identification of the u-PAR Promoter Region Mediating the Inductive Effect of KLF4—To identify the region of the u-PAR promoter required for stimulation by KLF4, we first cotransfected the KLF4 expression construct with a luciferase reporter regulated by 1469 base pairs of 5′/H11032-3′-flanking sequence into RKO colon cancer cells previously characterized as deficient in this transcription factor (42). This regulatory sequence was used because our recent studies demonstrated efficacy in driving reporter expression in the luminal surface cells of the colon in transgenic mice (26). Increasing amounts of the KLF4 expression vector yielded a dose-dependent increase in u-PAR promoter activity; for example, an input of 30 ng produced more than a 15-fold elevation of reporter activity (Fig. 5A). Increasing the input DNA amount beyond 30 ng also yielded stimulated u-PAR promoter activity but to a lesser extent because of the stimulation of the reporter by the empty vector. Such a biphasic promoter response may reflect squelching as occurs with the AP-2α transcription factor (43).

Subsequently, RKO cells were cotransfected with the KLF4 expression construct and the luciferase reporter regulated by varying lengths of the u-PAR promoter (Fig. 5B). As expected, the reporter regulated by 1469 base pairs of upstream sequence was stimulated over 4-fold by KLF4 coexpression. Likewise, the reporter fused to 398 base pairs of upstream sequence was activated to a similar extent by KLF4 expression. Deletion of
200 base pairs, thus generating the −197 u-PAR-Luc construct, modestly affected promoter activity. However, further 5’ truncations of the u-PAR promoter reduced the stimulation by KLF4 such that the reporter regulated by 51 base pairs of flanking sequence was unresponsive to this transcription factor. The lack of stimulation of the −51 u-PAR promoter construct did not reflect the detection limits of the luciferase assay as seen by the 56,000–76,000 relative light units observed in the absence of the KLF4 expression construct. We conclude that multiple sequences residing between −197 and −51 me-
subjected to EMSA using an oligonucleotide corresponding to the major transcriptional start site) in this region. Accord-

ingly, to determine whether KLF4 binds to this region of the promoter were required for optimal stimulation, we

transfection studies revealed that the proximal 200 base pairs of the u-PAR promoter (Fig. 7). The oligonucleotides spanning −105/−76 and −51/−24 bound KLF4 efficiently (bracket) as evidenced by (a) the additional band (bracket) in nuclear extracts derived from cells transfected with a KLF4-encoding vector (lanes 8 and 16) but not the empty vector (pIRES2-EGFP) and (b) the ability of the anti-GKLF (KLF4) antibody to abolish (lanes 9 and 17) this DNA-protein complex. In contrast, the sequence spanning −88/−61 showed little affinity for this transcription factor. Nevertheless, these data would argue that KLF4 binds multiple sequences within the proximal 200 base pairs of the u-PAR promoter. Our observation of KLF4 binding to the −51/−24 region, probably to a consensus KLF4 recognition motif located at −45, was of interest because this sequence was unresponsive in reporter assays (see Fig. 5B). It may be that although KLF4 bound to this region is necessary for u-PAR activation, by itself, it is insufficient to stimulate this promoter.

Because the chromatin environment modulates transcription factor binding (44), an issue that cannot be addressed by EMSA, chromatin immunoprecipitation assays were undertaken to determine whether KLF4 was bound to the endogenous u-PAR promoter. HCT116 cells were transiently transfected with the KLF4-bearing plasmid, and transfection efficiency was determined to be −50%, based on EGFP expression (Fig. 8A). DNA-protein complexes in the KLF4-transfected cells were cross-linked in situ, and chromatin was isolated and sheared. Subsequently the chromatin was immunoprecipitated with the anti-KLF4 antibody, and the DNA was purified and subjected to real time PCR for quantification using u-PAR promoter-specific primers (Fig. 8B). Control experiments indicated that this primer set specifically amplified a fragment of the predicted size (Fig. 8B). Using quantitative PCR, we found that the anti-KLF4 antibody caused an enrichment of more than 4-fold of the u-PAR promoter (Fig. 8C) suggesting that this transcription factor is bound in vivo to the regulatory portion of the u-PAR gene. A single amplification product was evident in the melting curve (Fig. 8D) following the real time PCR, again confirming specificity of the assay for the u-PAR promoter. Taken together, these findings suggest that KLF4 regulates u-PAR expression by binding directly to multiple sites in the proximal 200 base pairs of the 5′ flanking region.

Deletion of the −148/−123 Region Reduces but Does Not Eliminate u-PAR Promoter Activation by KLF4—We previously showed that the −148/−123 promoter region was required for the constitutively elevated u-PAR levels in colon cancer cells and mediated the induction of u-PAR gene expres-

previously characterized two of the slower migrating bands (parenthesis and line) as Sp1 and Sp3, and incubation with respective antibodies (lanes 5 and 6) confirmed this earlier observation. More importantly, nuclear extract from the KLF4-transfected cells gave rise to an additional band (lane 7, bracket), and this DNA-protein complex was unaffected by the anti-Sp1 and -Sp3 antibodies (lanes 10 and 11, bracket). Conversely, inclusion of either an anti-GKLF (KLF4) antibody or an anti-FLAG antibody confirmed that this band was KLF4 because both antibodies abolished the DNA-protein complex (lanes 8 and 9, bracket) while having little effect on the binding of Sp1 or Sp3. Thus, these in vitro data would suggest that this GC-rich region of the u-PAR promoter binds KLF4 in addition to Sp1 and Sp3.

Although the EMSA studies indicated recognition of KLF4 by the u-PAR −148/−123 promoter region, nevertheless, because the shorter 5′-deleted fragments were still responsive to this transcription factor, we explored the possibility that KLF4 binds to other sites in the u-PAR promoter. To address this issue, we used EMSA to “walk” the u-PAR promoter sequence in the direction of the transcriptional start site (Fig. 7). The oligonucleotides spanning −105/−76 and −51/−24 bound KLF4 efficiently (bracket) as evidenced by (a) the additional band (bracket) in nuclear extracts derived from cells transfected with a KLF4-encoding vector (lanes 8 and 16) but not the empty vector (pIRES2-EGFP) and (b) the ability of the anti-GKLF (KLF4) antibody to abolish (lanes 9 and 17) this DNA-protein complex. In contrast, the sequence spanning −88/−61 showed little affinity for this transcription factor. Nevertheless, these data would argue that KLF4 binds multiple sequences within the proximal 200 base pairs of the u-PAR promoter. Our observation of KLF4 binding to the −51/−24 region, probably to a consensus KLF4 recognition motif located at −45, was of interest because this sequence was unresponsive in reporter assays (see Fig. 5B). It may be that although KLF4 bound to this region is necessary for u-PAR activation, by itself, it is insufficient to stimulate this promoter.

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Deletion of the −148/−123 Region Reduces but Does Not Eliminate u-PAR Promoter Activation by KLF4—We previously showed that the −148/−123 promoter region was required for the constitutively elevated u-PAR levels in colon cancer cells and mediated the induction of u-PAR gene expres-

![Picture](https://example.com/fig6.png)

**Fig. 6. Binding of KLF4 to the u-PAR promoter.** HCT116 cells were transiently transfected with the pIRES2-EGFP (pIRES2EGFP) vector or the vector encoding KLF4 as described in the legend to Fig. 3. After 24 h, nuclear extracts were generated, and 10 μg of protein was subjected to EMSA using an oligonucleotide corresponding to the −154/−128 u-PAR promoter sequence. Where indicated, 2 μg of the indicated antibodies was added to the nuclear extract. The data represent at least triplicate experiments.

KLF4 Is Bound to Multiple Regions within the Proximal 200 Base Pairs of the u-PAR Promoter—If KLF4 regulates u-PAR expression directly, then the binding of this transcription factor to the promoter would be predicted. Because the transient transfection studies revealed that the proximal 200 base pairs of the promoter were required for optimal stimulation, we focused on this region. Interestingly, we had previously identified a GC-rich sequence (−148/−123) in the u-PAR promoter that regulated expression in colon cancer cell lines (22), and computer analysis (www.cbil.upenn.edu/tess) indicated a consensus KLF4 binding site (RGGGGY, located at −138 relative to the major transcriptional start site) in this region. Accordingly, to determine whether KLF4 binds to this region of the u-PAR promoter, HCT116 cells were transiently transfected with the KLF4-bearing expression vector or the empty vector (pIRES2-EGFP), and nuclear extracts were generated and subjected to EMSA using a probe encompassing the consensus KLF4 binding site region mentioned above. Nuclear extract from the vector-transfected cells yielded three slowly migrating bands (Fig. 6 parenthesis, line, asterisk, respectively). We
sion by the pp60Vrc proto-oncogene (29). Furthermore, because this region contains a canonical KLF4 binding site, we determined the contribution of the −148/−123 region to u-PAR promoter responsiveness. Accordingly, HCT116 cells were co-transfected with the KLF4 expression construct and the luciferase reporter regulated either by 398 base pairs or this amount of upstream sequence harboring an internal deletion of nucleotides −144/−123. Interestingly, although this internal deletion impaired the activation of the u-PAR promoter, reporter activity was residually stimulated 2.5-fold (Fig. 9). These data would further argue that the multiple KLF4-binding sequences in the u-PAR promoter cooperate with respect to u-PAR promoter stimulation.

**DISCUSSION**

We report the identification of KLF4 as a novel regulator of u-PAR expression that drives expression of the urokinase binding site in the luminal surface colon epithelial cells of healthy mice. Several lines of evidence support this contention. First, both KLF4 and u-PAR are predominantly expressed in cells located at the mouth of the colonic crypt. Second, disruption of the KLF4 gene in knock-out mice greatly diminishes the amount of u-PAR protein in this tissue. Third, KLF4 overexpression induces u-PAR expression in cultured colon cancer cells. Fourth, KLF4 binds to multiple sites within the proximal 200 base pairs of the upstream u-PAR sequence both in vitro and in vivo.

Although our study was limited to the colon, our findings probably have broader significance. Indeed, it is more than likely that KLF4 also directs u-PAR expression in other barrier epithelium including the epithelial lining of the stomach (33, 36), based on colocalization of the respective mRNAs. Likewise, an identical regulatory mechanism may also be responsible for u-PAR expression in the remodeling epidermis where, again, transcripts for both u-PAR (45) and KLF4 (33) are detected in the suprabasal layers (7, 46). On the other hand, whereas u-PAR synthesis is evident in the spleen, KLF4 cannot account for its expression because this transcription factor is absent from this tissue (39).

Although KLF4 is clearly implicated in regulating u-PAR expression in the normal colon, it is unlikely that the elevated expression of u-PAR in colon tumors results from this transcription factor, for multiple reasons. First, the colon cancer cell lines we used (RKO and HCT116) express low levels of KLF4 but, nevertheless, constitutively express the u-PAR gene (47, 48). Second, in carcinogenesis models of the intestine (49), KLF4 expression is repressed (49) whereas conversely u-PAR expression is induced in both azoxymethane-induced colon cancer (50) and in resected colon cancer (51–53). Indeed, in colorectal malignancies, it is more likely that transcriptional activation of the u-PAR gene is caused by other DNA-binding proteins, including members of the AP-1, AP-2, and Sp1/Sp3 families as we and others have reported elsewhere (22, 54–56).

On the other hand, for other epithelial cancers, u-PAR expression may be the result of transcriptional activation by KLF4. In ovarian cancer, for example, KLF4 and u-PAR expression is coordinately increased in peritoneal effusions (57). Similarly, in oral squamous cell carcinoma, a malignancy also characterized by increased u-PAR (58, 59), KLF4 mRNA is consistently detected (60) at a higher level than in the adjacent normal mucosa.

Although our studies have clearly shown that u-PAR expression in the luminal surface epithelial cells of the colonic crypt reflects transactivation by KLF4, we do not know how this DNA-binding protein is increased in expression or activity. One possibility relates to the observation that TGF-β induces KLF4 synthesis (61). Because TGF-β is a well established regulator of epithelial cell differentiation in the colon and is predominantly localized to the luminal surface epithelial cells (62), it is tempting to speculate that this cytokine might be responsible for KLF4 expression and ultimately transcription of the u-PAR gene. Indeed, consistent with this notion is an earlier report (63) that TGF-β strongly up-regulates (15-fold) u-PAR expression.
Although KLF4 is bound by the proximal 200 base pairs and transactivates the u-PAR promoter via this region, it is also plausible that expression of the u-PAR gene in the normal mouse colon requires upstream regulatory sequences. Indeed, our recent transgenic studies demonstrated the additional requirement of a promoter region \((1.5/0.4\,\text{kb})\) for u-PAR expression in the surface colon epithelial cells (26). Interestingly, although this upstream region lacks both RRGGYGY and CACCC motifs, which bind KLF4, because KLF4 also recognizes Sp1 binding sites (33, 41), it is possible that multiple motifs \((637, 666, 698, 856, 1104, 1224,\) and \(1421\)) for this latter transcription factor contribute to the inductive effect of KLF4 on u-PAR expression in the colon. Additionally, binding of KLF4 to the \((105/76\,\text{u-PAR promoter region})\) might also be via an Sp1 recognition site at \(-98\) because this sequence lacks the RRGGYGY and CACCC motifs. One experimental observation, however, would argue against this supposition. Thus, the appearance of the KLF4 binding complex in EMSA experiments was not accompanied by a reduction in the Sp1-DNA complex as would be expected if two transcription factors were competing for a common binding site.

The requirement of the \(1.5/0.4\,\text{kb}\) regulatory region, as reported in our previous studies analyzing u-PAR promoter requirements in transgenic mice, would appear to diverge from the data generated in the current study in which sufficiency of the proximal 200 base pairs of u-PAR sequence for full KLF4

**FIG. 8.** *KLF4 binds to the endogenous u-PAR promoter.* HCT116 cells were transfected with the pIRES2-EGFP vector encoding the KLF4 coding sequence as described in the legend to Fig. 6. After 24 h, the transfection efficiency was determined visually by fluorescence from the EGFP (A). DNA proteins were cross-linked with formaldehyde and subjected to chromatin immunoprecipitation using an anti-KLF4 antibody and the indicated primers (B). Reaction products were resolved by electrophoresis (B) or quantified by real time PCR (C). After analysis, a melting curve was performed to confirm the amplification of a single product (D).

**FIG. 9.** Deletion of u-PAR promoter region \(-144/-123\) reduces KLF4-dependent activation of the u-PAR promoter. HCT116 cells were transiently cotransfected with the KLF4 expression construct (or empty vector) and a luciferase reporter driven by either 398 base pairs of a 5'-flanking sequence (wt) or the indicated internal deletion (del\(-144/-123\)) in context of this length of promoter. After 1 day, cells were harvested and analyzed for reporter activity. The data are expressed as -fold induction compared with the empty expression vector. The experiment was performed three times, and data are shown as mean values (±S.D.).

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The requirement of the \(-1.5/0.4\,\text{kb}\) regulatory region, as reported in our previous studies analyzing u-PAR promoter requirements in transgenic mice, would appear to diverge from the data generated in the current study in which sufficiency of the proximal 200 base pairs of u-PAR sequence for full KLF4
response was evident in transient transfections. One possibility is that the chromatin environment of the transgene, absent for the transiently transfected reporter constructs, accounts for the divergent regulated expression. Alternatively, it may be that whereas normal mouse colon and cultured colon cancer share common mechanisms for controlling u-PAR expression, regulatory mechanisms unique to non-malignant and malignant tissue are also operative.

In conclusion, we have identified KLF4 as a novel regulator of u-PAR expression and demonstrated that the predominant expression of this binding site in the luminal surface colonic epithelial cells is directed by this transcription factor. This regulatory mechanism may also drive u-PAR expression in other barrier epithelium including that of the stomach and the skin.

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REFERENCES

1. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., Doyle, J. M., Simon, D. I., Bodary, S. C., and Rosenberg, S. (2000) J. Biol. Chem. 275, 5823–5830.
2. Chen, X., Johna, D. E., Geiman, D. E., Marban, E., Dang, D. T., Hamlin, G., Son, R., and Yang, W. V. (2001) J. Biol. Chem. 276, 30423–30428.
3. Regan, P., Ebert, A., Chao, J. P., and Yim, S. O. (1998) Cancer Res. 58, 3521–3524.
4. Rabbani, S. A., and Gladu, J. (2002) Blood 100, 401–402.
5. Suzuki, S., Hayashi, Y., Wang, Y., Nakamura, T., Morita, Y., Kawasaki, K., Aoyama, N., Kim, S. R., Inoue, K., Kuroda, Y., and Doh, W. F. (1999) Onco. 43, 479–486.
6. Abe, J., Urami, T., Kanno, H., Erhan, Y., Tanaka, T., Nishino, N., Takada, A., and Nakamura, S. (1999) Cancer 86, 2605–2611.
7. Zannetti, A., Del Vecchio, S., Carraro, M. V., Fonti, R., Franco, R., Botti, G., D’Auito, G., Stopelli, M. P., and Valentino, S. (2000) Cancer Res. 60, 1546–1551.
8. Man, B., Gels, M., Wiedow, A., Hanski, M. L., Gratchev, A., Ilyas, M., Bodmer, W. F., Moyer, M. P., Rieckew, E. O., Buhri, H. J., and Hanski, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1603–1608.
9. Schewe, D., Leupoldt, J. H., Boyd, D. D., Lengel, E., Grootesm, N. K., Schild, B. W., Jann, K. W., and Allgayer, H. (2003) J. Biol. Chem. 278, 2267–2276.
10. Schauer, M. E., Ross, D. T., Carriao, G., Gorres, T., Tayanska, S., Diekmann, D., Mavit, M., Wagn, Y., Dura, G. E., Sikir, T. L., Caldeira, S., Skomedal, H., Tu, I.-P., Hernandez-Bousard, T., Johnson, S. W., D’Orey, D. W., Fero, M. J., and Matsuoka, G. (1999) J. Biol. Chem. 274, 18428–18445.
11. Schmitt, M., Gruenfelder, M., Muller, J., and Hoppe, F. (2000) Head Neck 22, 498–504.
12. Shiomi, H., Ercini, Y., Tani, K., Kodama, H., and Hatorti, T. (2000) Am. J. Pathol. 156, 567–575.
13. Foster, K. W., Ren, S., Luco, I. D., Bob-Luppen, S. M., McKee-Bell, P., Greely, H., Hayes, W. M., Ryder, L., and Ruppert, J. M. (1999) Cell Growth & Differ. 10, 423–434.
14. King, K. E., Iemere, V. P., Weissberg, P. L., and Shanahan, C. M. (2003) J. Biol. Chem. 278, 11559–11563.
15. Avery, A., Paraviks, C., Hall, P., Flanders, K. C., Sporn, M., and Moor, M. (1993) Br. J. Cancer 68, 137–139.
16. Lund, L. R., Romer, J., Ellis, V., Blasi, F., and Dano, K. (1999) EMBO J. 18, 3399–3407.
17. Rabbani, S. A., and Gladu, J. (2002) J. Biol. Chem. 277, 160, 5823–5830.