The Krüppel-like transcriptional factors Zf9 and GKLF coactivate the human keratin 4 promoter and physically interact

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Abstract Zf9/CPBP/KLF6 is a widely expressed member of the Krüppel-like family of transcriptional factors which regulates gene expression in hepatic stellate cells. Because of its ubiquitous expression including in the esophagus, we have explored its function in the esophageal squamous epithelium, a model system to study cellular proliferation and differentiation. Reverse transcription-PCR (RT-PCR) and Western blot analyses revealed that Zf9 was highly expressed in human esophageal squamous cancer cell lines. Additionally, Zf9 localizes to the esophageal squamous epithelium by immunohistochemistry. Using transient transfection, Zf9 transactivates the human keratin 4 (K4) promoter reporter gene construct in a subset of the esophageal cancer cell lines through indirect mechanisms. Co-transfection of Zf9 and GKLF/KLF4, which is also a member of the Krüppel-like factors and expressed in the esophageal squamous epithelium, leads to coactivation in an additive fashion. Furthermore, we demonstrate that there is a physical interaction between GKLF and Zf9, a novel finding for Krüppel-like family members.

Key words: Zf9/KLF6; Gut-enriched Krüppel-like factor (GKLF/KLF4); Keratin 4 promoter

1. Introduction

Transcriptional factors are grouped into several classes, which include the helix-loop-helix, leucine zipper, homeodomain, and zinc finger protein families. Some zinc finger transcriptional factors can be further subclassified based on their homology to the *Drosophila* Krüppel protein [1]. The Krüppel-like transcriptional factors so far cloned include erythroid Krüppel-like factor (EKLF/KLF1), lung Krüppel-like factor (LKLF/KLF2), basic Krüppel-like factor (BKLF/KLF3), gut-enriched Krüppel-like factor (GKLF/KLF4), basic transcription factor element binding protein 2 (BTEB2/KLF5), Zf9/CPBP/KLF6, ubiquitous Krüppel-like factor (UKLF/KLF7), fetal erythroid Krüppel-like factor (FKLF/KLF8), and basic transcription factor element binding protein (BTEB1/KLF9).

The Zf9 gene was cloned originally from cDNA libraries of activated rat stellate cells [2] and human placenta [3]. Zf9 contains three Cys2–His2 zinc fingers in its carboxyl-terminal domain and a unique amino-terminal domain that is rich in serine/proline clusters, which could be potential targets for post-translational modifications. Rat zf9 mRNA displays ubiquitous expression, with highest levels in lung and intestine. Zf9 protein expression is accelerated after CCl_4-induced acute liver injury in rats [2]. Since Zf9 expression is induced in activated stellate cells, it may be important in modulating immediate early response genes. To date, targets of Zf9 include the collagen α1 (I) promoter [2] and the promoters of the TGF-β types I and II receptors [4].

GKLF/KLF4 is also a member of the Krüppel-like transcriptional factors, originally cloned from an NIH3T3 library [5] and a primary mouse embryonic fibroblast library [6]. GKLF mRNA expression is relatively ubiquitous, however, there is correlation with expression in differentiating cells or differentiated compartments. For example, GKLF is enriched in epithelial cells located in the middle to upper crypt region of the colonic epithelium [5]. GKLF is expressed in the suprabasal compartment which is the early differentiated region of stratified squamous epithelium found in the skin, tongue and esophagus [6]. GKLF had higher expression in growth-arrested fibroblasts than in exponentially growing cells. Constitutive expression of GKLF in COS-1 cells resulted in the inhibition of DNA synthesis [5] and the GKLF mRNA level was significantly decreased in the small intestine of Min mice compared to wild-type mice [7], suggesting that GKLF may be a negative regulator of cell cycle progression.

The keratins are attractive models for investigating proliferation and differentiation in the stratified squamous epithelium [8]. While some keratins are ubiquitous, others are relatively tissue restricted. For example, keratin 4 (K4) is highly expressed in the esophageal and corneal squamous suprabasal cells and its expression is associated with the switch from proliferation to differentiation [9]. K4 homozygous null mice have a phenotype largely restricted to the esophagus and cornea where there is impairment of the normal differentiation program [10]. Thus, K4 appears to be important in maintaining the differentiation phenotype in a highly tissue-specific

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Abbreviations: GKLF, gut-enriched Krüppel-like factor; EKLF, erythroid Krüppel-like factor; LKLF, lung Krüppel-like factor; BTEB2, basic transcription factor element binding protein 2; CPBP, core promoter binding protein; K, keratin; bp, base pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; SCC, squamous cell carcinoma

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fashion. We have previously demonstrated that GKLF can transactivate the human K4 promoter through the binding to a CACCC motif [9]. Given the ubiquitous expression of Zf9 and the presence of multiple GC motifs in the human K4 promoter, we sought to determine the role of Zf9 in the esophageal squamous epithelium in regulating the early differentiation linked K4 gene transcription and in this context, identifying gene targets outside of stellate cells. We demonstrate herein that Zf9 transactivates the K4 promoter and there is an additive effect on K4 promoter activity by GKLF and Zf9. This may be mediated through physical interaction between Zf9 and GKLF.

2. Materials and methods

2.1. Tissue culture

TE-2, TE-4, TE-8, TE-9, TE-11, TE-12, T.T, and T.Tn are esophageal cancer cell lines derived from human esophageal squamous cell carcinomas and were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 100 mg/ml streptomycin (Sigma), 100 units/ml penicillin (Sigma), and L-glutamine (Life Technologies, Inc., Grand Island, NY, USA) at 37°C. HepG2 (liver cancer cell line), 3T3 (mouse fibroblasts), 293 (human embryonic kidney cells), CaCO2 (colon cancer cell lines), SaoS2 (osteosarcoma cell line), and SCC-13 (skin cancer cell line) (ATCC, Manassas, VA, USA) were maintained under similar conditions. OKF6 cells (primary human oral keratinocytes) were grown in keratinocyte serum-free medium supplemented with a specialized growth factor mixture (Life Technologies, Inc.). Primary human skin keratinocytes (KPC) and human esophageal keratinocytes (EPC) were grown as described previously [11]. Het1a cells (human esophageal cells immortalized with SV40 large T antigen) were maintained in BRFF-EPM2 medium (Biological Research Faculty and Facility, Inc., Ijamsville, MD, USA) as described previously [12].

2.2. DNA plasmids

The human K4 promoter reporter gene constructs (K4-1040, K4-940, K4-740, K4-540, K4-340, K4-200, and K4-140) have been described previously [13]. K4-105, K4-80, and K4-30 were generated using K4-1040 as a template for PCR with sense primers as follows: K4-105-S: 5′-GCG TCG ACT GTG CCA AGG CCA AGC CCA-3′; K4-80-S: 5′-GCG TCG ACT TTC AGA TTT TTC CCT TTA-3′; K4-30-S: 5′-GCG TCG ACA CAT ATA AGA GAC GAA GGC-3′.

The antisense primer sequence was as follows: 5′-GAA GAT CTG GCT GCA GAG AGC GAG CTG-3′.

The *Sal*I- and *Bgl*II-digested PCR products were ligated into the luciferase reporter gene promoterless vector, pXP2.

A histidine/Xpress-tagged GKLF expression vector, pcDNA3.1-HisB-GKLF, has been described previously [9]. A Zf9 expression vector cloned into pCIneo (Promega Corp., Madison, WI, USA), pCIneo-Zf9, was described previously [2]. All plasmids were purified by a modified alkaline lysis method (Qiagen Inc., Valencia, CA, USA).

2.3. Transfections

Transient transfection of all DNA constructs was performed by a liposome mediated method using 4 μl of lipofectamine and 6 μl of Plus reagent (Life Technologies, Inc.) containing 0.95 μg total DNA. Transfection efficiency was monitored by using 0.2 μg per plate of pCMV-βgal. Cells were harvested 48 h after transfection, washed twice with ice-cold phosphate-buffered saline (PBS), lysed in 200 μl of cell lysis reagent (Promega Corp.), and 100 μl of lysate was mixed with 100 μl of luciferase assay reagent (Promega Corp.). Each experiment was performed in triplicate and at least three sets of independent transfections were performed. Luciferase activity was normalized to β-galactosidase activity and the results were calculated as the mean ± S.E.M. values for luciferase activity.

2.4. Reverse transcription-PCR (RT-PCR)

Oligonucleotide primers were designed to amplify the amino-terminal region of the zf9 gene. The oligonucleotide primer sequences were as follows: Zf9-S (sense): 5′-ATG GAT GTG CTC CCA ATG TGT AGC ATC-3′ Zf9-AS (antisense): 5′-GTT AAA ATG GCA CCG ATG-3′. Total RNA was extracted as previously described [9] and then reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Perkin-Elmer, Norwalk, CT, USA) with random primers. Following a single cycle of reverse transcription at 42°C for 15 min, samples were subjected to 30 cycles of PCR (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) using the Zf9-specific primers and the PCR products were electrophoresed on a 1% agarose gel.

2.5. Immunoprecipitation and Western blotting

For detection of endogenously expressed Zf9 protein, lysates from the cell lines were prepared in a buffer (ELB) containing 50 mM HEPES (pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediaminetetraacetate (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and protease inhibitor tablets (Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA, USA). Total protein samples (10 μg/sample) were separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). The membrane was blocked in 5% milk (Bio-Rad Laboratories, Richmond, CA, USA) in 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20 (1×TBST) for 1 h at room temperature (RT). The membrane was incubated with primary polyclonal Zf9 antibody (R-173, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) (1:500 in 1×TBST) overnight at 4°C, followed by incubation with the anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA) (1:3000 in 1×TBST) for 1 h at RT. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus, Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA) and exposed to Kodak-X-Omat LS film (Eastman Kodak Special Products Division, Rochester, NY, USA).

For immunoprecipitation, TE-8 cells were harvested 48 h after cotransfection of 0.5 μg pCIneo-Zf9 and 0.5 μg pcDNA3.1-HisB-GKLF, washed three times with ice-cold PBS, scraped with a rubber policeman into 1 ml of ELB buffer, transferred to microtubes, and centrifuged at 14 000 rpm for 15 min at 4°C. After the supernatant was incubated with either 2 μg of anti-Zf9 antibody (R-173) or anti-Xpress antibody (Invitrogen, Carlsbad, CA, USA) for 1 h at 4°C, 20 μl of protein A/G Plus agarose beads (Santa Cruz Biotechnology, Inc.) were added to the lysates and incubated overnight at 4°C. The beads were subsequently washed three times with 500 μl ELB buffer, solubilized in SDS sample buffer (65 mM Tris, pH 6.8, 25% glycerol, 2% SDS, and 5% β-mercaptoethanol), boiled, and subjected to SDS–PAGE followed by Western blotting under the same conditions as described above. As a negative control, immunoprecipitation was conducted with control rabbit or mouse IgG (Sigma, St. Louis, MO, USA). For detection of GKLF protein, anti-rabbit GKLF serum (a gift from Dr. Vincent W. Yang) (1:2500 in 1×TBST) and anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech, Inc.) were used as the primary and secondary antibodies, respectively. For detection of Zf9 protein, anti-Zf9 antibody (R-173) (1:500 in 1×TBST) and anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech, Inc.) (1:3000 in 1×TBST) were used as the primary and secondary antibodies, respectively. To confirm the specificity of our immunoprecipitation and Western blot conditions, Western blot was performed with control rabbit or mouse IgG.

2.6. Immunohistochemistry

Normal mouse esophageal tissue was fixed in 4% paraformaldehyde and paraffin embedded. Paraffin sections were mounted on commercially provided charged slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA, USA) and dried at 60°C for 1 h. After routine dewaxing and rehydration, the sections were soaked in 10 mM citric acid (pH 8.0) and heated in a microwave oven for 14 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubating the sections with methanol containing 1.5% hydrogen peroxide for 15 min at RT. The sections were washed in PBS, treated with avidin and biotin blocking solutions (Vector Laboratories Inc., Burlingame, CA, USA), and incubated with protein blocking agent (Immunotech Inc., Westbrook, ME, USA). Subsequently, they were incubated with anti-Zf9 polyclonal antibody [2] (1:10 000 in PBS, 0.1% BSA, 0.2% Triton

X-100) or control serum (Vector Laboratories Inc.) at the same dilutions overnight at 4°C. After rinsing with PBS, the sections were incubated with biotinylated secondary antibody (1:200 in PBS, 0.1% BSA, 0.2% Triton X-100) for 45 min at 37°C and HRP-conjugated ABC solution (Vector Laboratories, Inc.) was applied for 40 min at RT. The peroxidase activity was developed with DAB solution (Vector Laboratories, Inc.). Counterstaining was done with hematoxylin.

3. Results

3.1. Zf9 expression in diverse cell types and normal esophagus

Since ubiquitous expression of rat zf9 mRNA in rat tissues has been previously reported [2,3], we determined expression of human zf9 mRNA in various cell lines by RT-PCR using zf9-specific primers. A 600 bp PCR product was detected in all cell lines examined, including normal and transformed esophageal cells (Fig. 1). Then we assayed Zf9 protein in cell lines, including of human esophageal squamous cancer origin, by Western blot analysis. We detected as expected a single 46 kDa band corresponding to Zf9 in all cell lines examined (Fig. 2), implying post-translational modification(s) of Zf9 such as glycosylation or phosphorylation in vivo, since in vitro translated Zf9 is approximately 32 kDa [2].

These data led us to determine the localization of Zf9 protein in normal murine esophageal tissue by an immunohistochemical approach with an antigen retrieval technique [14]. As shown in Fig. 3A, positive nuclear staining was predominantly in the epithelium, although no specific distribution was noted in this compartment. By contrast, esophageal tissue incubated with rabbit serum instead of anti-Zf9 antibody did not show any nuclear staining except weak cytoplasmic signals in the epithelium (Fig. 3B), suggesting cross-reactivity but confirming the specificity of the anti-Zf9 antibody. Collectively, the Zf9 expression data implied that Zf9 may have a physiological role in the esophagus, prompting us to explore its modulation of transcriptional regulatory mechanisms.

3.2. Zf9 transactivates the full-length K4 promoter in TE-8 and HepG2 cell lines

It has been shown previously that Zf9 can transactivate the collagen α1 (I) promoter with two GC-rich regions [2] and the promoters of the TGF-β types I and II receptors [4] in HepG2 and stellate cells. The K4 promoter is active in human esophageal cancer cell lines and contains some GC-rich elements [13]. We therefore explored Zf9's ability to influence K4 promoter activity in the TE series of esophageal cancer cell lines using the pCIneo-Zf9 expression vector, HepG2 cells served as a control for Zf9 transactivation of genes. Transient transfection of Zf9 showed significant transactivation of the full-length K4-1040 promoter luciferase construct compared with the empty expression vector (pCIneo) in TE-8 and HepG2 cell lines, but not in TE-2, TE-4, TE-9, or TE-12 cell lines (Fig. 4). This result indicates that Zf9 can transactivate the K4 promoter restricted to a limited number of esophageal cancer cell lines in spite of expression of endogenous Zf9, suggesting a requirement of cellular context for the activation of the K4 promoter by Zf9.

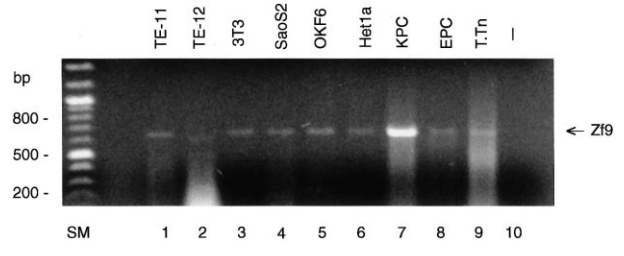

Fig. 1. Zf9 mRNA expression in various cell lines. cDNAs from TE-11, TE-12, NIH3T3, SaoS2, OKF6, Het1a, KPC, EPC, and T.Tn cells (lanes 1–9) were amplified by PCR using zf9-specific primers to generate 600 bp products. Lane 10 has no DNA template (negative control).

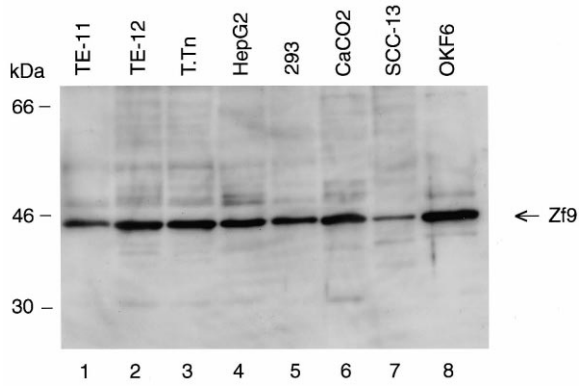

Fig. 2. Zf9 protein expression in various cell lines. Western blot of lysates from TE-11, TE-12, T.Tn, HepG2, 293, CaCO2, SCC-13, and OKF6 cells (lanes 1–8) was probed with anti-Zf9 antibody (1:100 dilution). Equal loading of protein was confirmed with Ponceau-S staining.

3.3. Zf9 mediated transactivation of the K4 promoter may be mediated through indirect effects

In order to determine the regions which are responsible for the transactivation of Zf9 in the K4 promoter, K4 promoter deletion constructs were co-transfected with pCIneo-Zf9 in TE-8 cells. Although the K4 promoter contains multiple potential Zf9 binding sequences flanking −815 and −803, −450 and −440, −278 and −243, and −229 and −220, Zf9 could consistently and surprisingly transactivate the K4−1040, −940, −740, −540, −340, −200, and −140 constructs about 4-fold compared with the pCIneo empty vector, indicating that these GC-rich elements may not be preferred binding sites for Zf9 (Fig. 5). In order to determine whether three GC-rich regions between positions −125 and −116, −102 and −88, and −60 and −40 could be pivotal elements for Zf9 binding, we synthesized K4−105, −80, and −30 constructs. The nearly 4-fold activation by Zf9 was attenuated gradually in parallel with a decrease of basal K4 promoter activity without an abrupt decrease in transactivation (Fig. 5). This result suggests that transactivation of the K4 promoter by Zf9 in part might originate from an indirect effect, possibly mediated by a co-factor(s).

3.4. Additive effect of Zf9 and GKLF on the K4 promoter

GKLF is a member of the Krüppel family of transcriptional factors and has been shown to be expressed in the suprabasal compartment of the esophageal squamous epithelium [6]. We have previously reported that GKLF can transactivate the K4 promoter in 293 cells through direct binding of a CACACCC motif between −340 and −140 nucleotides [9]. These facts prompted us to investigate the combinatorial effects of GKLF and Zf9 on the K4 promoter in TE-8 cells. As shown in Fig. 6, whereas Zf9 or GKLF expression vectors individually could transactivate the K4 promoter 4-fold, the combina-

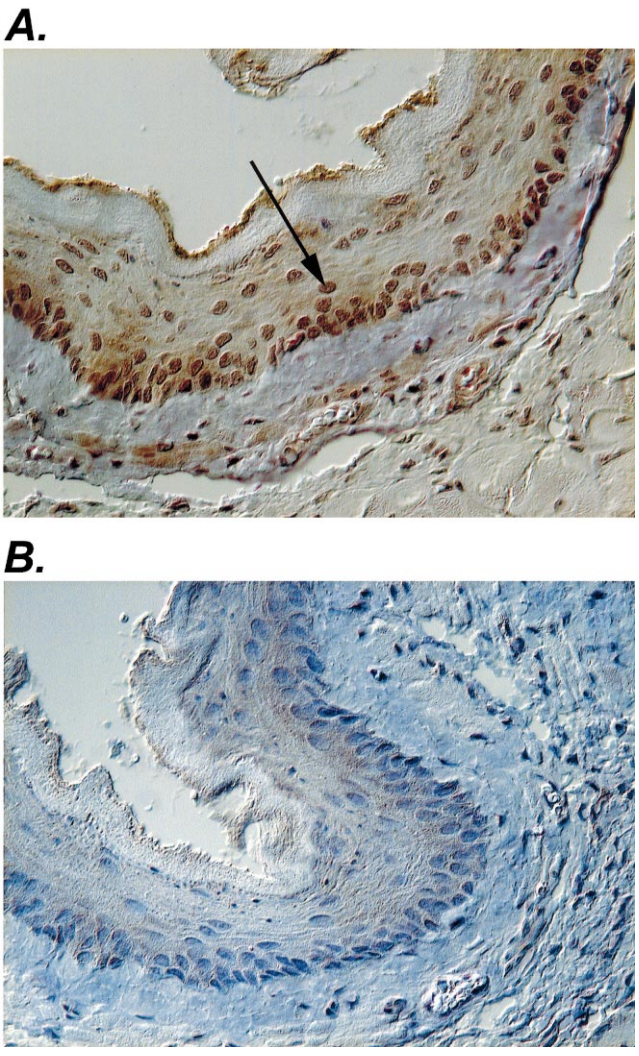

Fig. 3. Zf9 is expressed in the esophageal epithelium. Zf9 protein expression was examined in normal murine esophagus by immunohistochemistry using the anti-Zf9 antibody. Panel A is a representative section indicating Zf9 staining in nuclei (arrow) and also cytoplasm in the esophageal epithelium. Panel B shows a tissue section with rabbit serum and no nuclear staining. (Both figures at 400× magnification.)

tion of Zf9 and GKLF resulted in nearly 8-fold activation compared with the empty expression vectors. It should be noted that empty vectors were co-transfected with Zf9 and GKLF to adjust the total amount of transfected DNA and exclude the possibility of non-specific binding of Zf9 or GKLF to the CMV promoter. Overall, these results suggested that Zf9 and GKLF might directly or indirectly cooperate in transactivating the K4 promoter.

3.5. *Physical interaction of Zf9 and GKLF in esophageal squamous epithelial cells*

As a next step, we sought to explore the mechanisms underlying how Zf9 and GKLF might cooperate. One possibility was direct protein–protein interaction between Zf9 and GKLF. To that end, immunoprecipitation followed by Western blotting were performed after co-transfection of the Zf9 and histidine/Xpress-tagged GKLF expression vectors in TE-8 cells. Even though TE-8 cells expressed endogenous Zf9 (data not shown), we transfected both Zf9 and GKLF expression

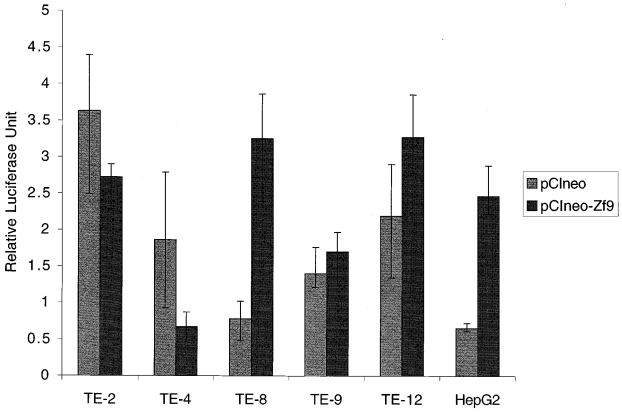

Fig. 4. Zf9 transactivates the K4 promoter in TE-8 and HepG2 cells. Transient co-transfection of the Zf9 expression vector (0.25 μg pCIneo-Zf9) and the full-length K4 promoter luciferase reporter construct (0.25 μg K4-1040) was carried out in the indicated cell lines and then assayed for luciferase activity. Experiments were performed in triplicate and repeated at least three times in independent experiments. Data represent means ± S.E.M.

vectors to reproduce the same conditions as in Fig. 6. When immunoprecipitation was conducted with anti-Xpress antibody and Western blotting was performed with anti-GKLF serum, we could detect an expected 65 kDa band for GKLF (58 kDa GKLF plus 7 kDa tag) and when anti-Zf9 antibody was used in a similar way, a 46 kDa band corresponding to Zf9 was detected, verifying both antibodies are appropriate for immunoprecipitation under the experimental conditions employed (Fig. 7 and data not shown). When cell lysates were immunoprecipitated with anti-Zf9 antibody and blotted with anti-GKLF serum, the specific band corresponding to GKLF (65 kDa) was appreciated (Fig. 7). When cell lysates were immunoprecipitated with anti-Xpress antibody and blotted with anti-Zf9 antibody, the specific band corresponding to Zf9 (46 kDa) was detected (data not shown). The specificity of this interaction was verified by the use of control anti-rabbit and mouse IgG as controls for immunoprecipitation and

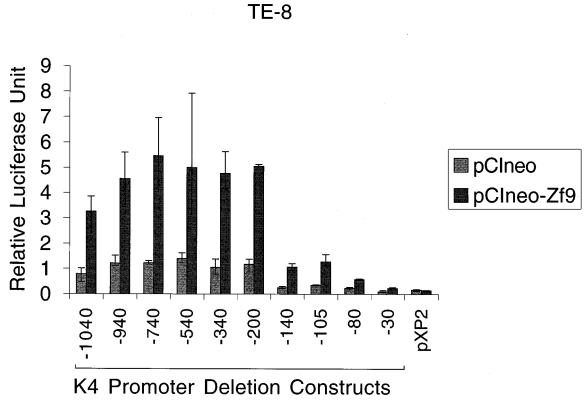

Fig. 5. Transient co-transfection of the Zf9 expression vector and the K4 promoter deletion constructs in TE-8 cells. The Zf9 expression vector (0.25 μg pCIneo-Zf9) was transiently co-transfected with 0.25 μg of the K4 promoter deletion constructs (K4-940, K4-740, K4-540, K4-340, K4-200, K4-140, K4-105, K4-80, and K4-30) in TE-8 cells. Experiments were performed in triplicate and repeated at least three times in independent experiments. Data represent means ± S.E.M.

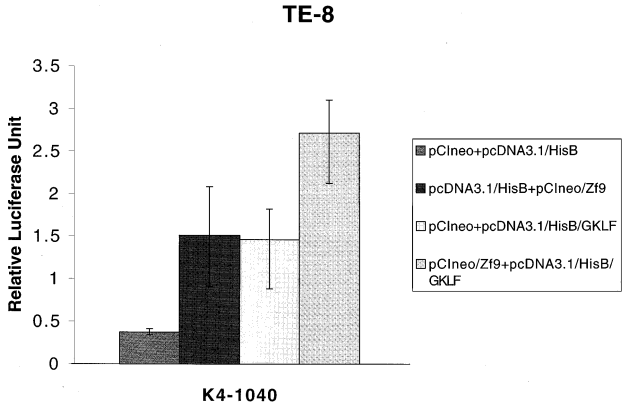

Fig. 6. Zf9 and GKLF have an additive effect on the K4 promoter. The Zf9 expression vector (0.25 μg pCIneo-Zf9) and histidine/Xpress-tagged GKLF expression vector (0.25 μg pcDNA3.1-HisB-GKLF) were transiently co-transfected with the full-length K4 promoter luciferase construct (0.25 μg K4-1040). The total amount of DNA was 0.5 μg by co-transfecting with the empty vector, pCIneo or pcDNA3.1-HisB. Experiments were performed in triplicate and repeated at least three times in independent experiments. Data represent means ± S.E.M.

Western blotting (Fig. 7 and data not shown). This result demonstrates that Zf9 and GKLF are able to interact.

4. Discussion

Zf9 is a member of the Krüppel-like transcriptional factors, originally cloned from a cDNA library of activated rat hepatic stellate cells. Its protein expression in stellate cells is induced after acute liver injury [2]. The same factor, alternatively named CPBP, was cloned from a cDNA library of human placenta [3]. zf9 mRNA expression is nearly ubiquitous, although cellular localization within tissue compartments has not been reported to date.

GKLF is another fairly ubiquitously expressed member of the Krüppel-like transcriptional factors. It has been shown that GKLF is expressed in esophageal suprabasal cells and transcriptionally activates the human K4 promoter which localizes to suprabasal cells as well and is important in the transition from proliferation to differentiation in this cell type [3,9,10]. The transcriptional regulation of the K4 promoter by GKLF is mediated through a CACC-like element. Since there are multiple GC-rich elements in close proximity of the GKLF binding site within the K4 promoter, we explored whether a GC-rich element binding transcription factor, such as Zf9, might further regulate the human K4 promoter. All these observations led us to postulate that Zf9 might be expressed in the esophagus, and might have a pivotal role in regulating a promoter which is involved in differentiation of esophageal basal cells.

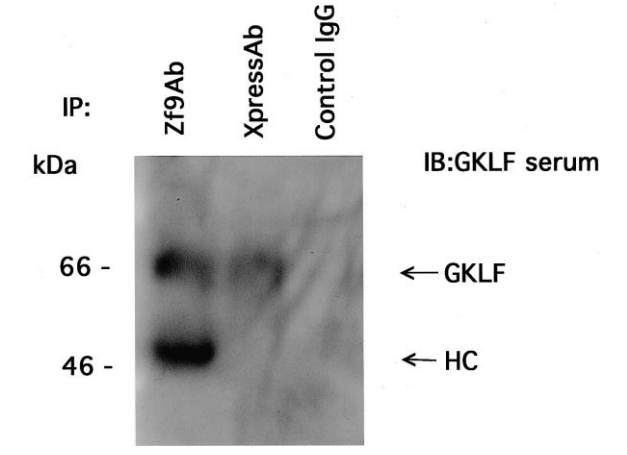

Fig. 7. Zf9 and GKLF proteins interact with each other. TE-8 cells were harvested 24 h after transient co-transfection of pCIneo-Zf9 (0.5 μg) and pcDNA3.1-HisB-GKLF (0.5 μg) and immunoprecipitated with either anti-Zf9 antibody or anti-Xpress antibody. The immunoprecipitated samples were subjected to SDS–PAGE followed by Western blotting with anti-rabbit GKLF serum. The upper band represent 65 kDa bands corresponding to histidine/Xpress-tag (7 kDa) and GKLF (58 kDa) and a lower band represents a product of immunoglobulin heavy chains (HC). Control IgG did immunoprecipitate either GKLF or Zf9.

RT-PCR using zf9-specific primers revealed that Zf9 mRNA was ubiquitously expressed in cell lines derived from human esophageal squamous carcinomas, immortalized human esophageal epithelial cells and primary human esophageal keratinocytes. Western blot analysis demonstrated robust Zf9 protein expression in these cell lines. Immunohistochemical analysis in normal murine esophagus revealed that Zf9 was predominantly expressed in the epithelium and in the nuclei of such cells, consistent with the notion that Zf9 is a transcriptional factor [2–4].

To investigate the biological role of Zf9, we then determined the effects of Zf9 upon the K4 promoter given the presence of GC regions and the role of the K4 protein in differentiation. To date, it has been shown that Zf9 can transactivate a reporter construct containing six GC-rich elements in *Drosophila* Schneider (S2) cells, the collagen α1 (I) promoter [2], and promoters of the TGF-β types I and II receptors [4]. Zf9 could transactivate the full-length K4 promoter luciferase construct in TE-8 and HepG2 cell lines, but not in TE-2, TE-4, TE-9, or TE-12 cell lines. This result suggests that the human K4 promoter can be transactivated by Zf9 in a limited number of esophageal squamous cancer cell lines, where an adequate cellular context might be required. Alternatively, exogenous Zf9 might not be able to overcome the effects of endogenous Zf9 which might be already abundant and saturates cognate DNA binding sites. Co-transfection of Zf9 with a series of K4 promoter deletion constructs failed to identify a region that might mediate Zf9 transactivation of the K4 promoter. This raised the possibility of indirect effects of Zf9 on the K4 promoter.

We investigated whether the K4 promoter activity could be affected by simultaneous transfection of other Krüppel-like factors, such as GKLF. Indeed, co-transfection of GKLF and Zf9 expression vectors in TE-8 cells had an additive effect on K4 promoter activity, raising the possibility that Zf9 and GKLF proteins might interact physically. We demonstrated that Zf9 and GKLF do interact based upon co-transfections followed by immunoprecipitation and Western blot analysis. Nevertheless, the manner by which the Zf9–GKLF interaction modulates K4 gene transcription is not clear. It is possible that GKLF recruits Zf9 and this heterodimeric complex binds DNA through GKLF. There might also be a more complicated interplay between Zf9 and GKLF with Sp1 in regulating gene transcription since both GKLF and Zf9 have been shown to bind Sp1 [4,15].

Overall, the K4 promoter is a newly identified gene target of Zf9 and underscores an important role for Zf9 in the squa-

mous epithelium as illustrated in the esophagus. Furthermore, Zf9 and GKLF interact physically and to our knowledge, this is the first evidence of protein–protein interaction between Krüppel-like transcription factors.

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