Synergistic activation of the rat laminin \(\gamma_1\) chain promoter by the gut-enriched Kruppel-like factor (GKLF/KLF4) and Sp1

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ABSTRACT

Laminin is a multifunctional heterotrimeric protein present in extracellular matrix where it regulates processes that compose tissue architecture including cell differentiation. Laminin \(\gamma_1\) is the most widely expressed laminin chain and its absence causes early lethality in mouse embryos. Laminin \(\gamma_1\) chain gene (LAMC1) promoter contains several GC/GT-rich motifs including the bcn-1 element. Using the bcn-1 element as a bait in the yeast one-hybrid screen, we cloned the gut-enriched Kruppel-like factor (GKLF or KLF4) from a rat mesangial cell library. We show that GKLF binds bcn-1, but this binding is not required for the GKLF-mediated activation of the LAMC1 promoter. The activity of GKLF is dependent on a synergism with another Kruppel-like factor, Sp1. The LAMC1 promoter appears to have multiple GKLF- and Sp1-responsive elements which may account for the synergistic activation. We provide evidence that the synergistic action of GKLF and Sp1 is dependent on the promoter context and the integrity of GKLF activation and DNA-binding domain. GKLF is thought to participate in the switch from cell proliferation to differentiation. Thus, the Sp1–GKLF synergistic activation of the LAMC1 promoter may be one of the avenues for expression of laminin \(\gamma_1\) chain when laminin is needed to regulate cell differentiation.

INTRODUCTION

Laminin is a high molecular weight multifunctional protein present in extracellular matrix. It consists of three polypeptide chains, \(\alpha\), \(\beta\) and \(\gamma\), held together by disulfide bonds forming a cruciform structure (1). There are five \(\alpha\) chains (\(\alpha_1–5\)), three \(\beta\) chains (\(\beta_1–3\)) and three \(\gamma\) chains (\(\gamma_1–3\)), which account for the 12 known laminin trimeric assemblies (Laminin 1–12). Laminins not only function as structural components, but also bind cell surface receptors such as integrins and \(\alpha\)-dystroglycan. Laminin-mediated interactions play a key role in forming cellular architecture through processes such as cell adhesion, spreading and migration (1–3). Laminins also regulate cell differentiation (2,4–7).

The \(\gamma_1\) chain, found in 10 out of the 12 known laminin isoforms, is the most widely expressed laminin chain (1). Laminin \(\gamma_1\) is required for basement membrane formation and its absence causes early lethality in mouse embryos (8,9). These observations suggest that the synthesis of \(\gamma_1\) chain is essential for the laminin heterotrimeric assembly. Because its role is so important, regulation of laminin \(\gamma_1\) chain (LAMC1) gene expression has generated considerable interest (7,10–14). LAMC1 gene expression is responsive to multiple extracellular signals, including glucose (14), IGF (14), IL-1\(\beta\), TGF-\(\beta\), phorbol esters (11) and retinoic acid (10). Considering the fact that laminin is a multifunctional protein, it is not surprising that LAMC1 gene expression is responsive to many signals.

Cloning of the 5′ region of the LAMC1 gene revealed that the human and rodent promoters of these genes do not have TATA or CAAT boxes, but contain several GC/GT motifs, an arrangement commonly seen in TATA-less promoters (13,15). The LAMC1 promoter contains binding sites for several transcription factors including NF-\(\kappa\)B (16,17), retinoic acid receptor (7,10), AP-2 (17), Sp1 (18) and TFE3 (19). This assortment of transcriptional elements renders the LAMC1 promoter responsive to multiple extracellular signals. The multiplicity of the transcriptional elements allows the LAMC1 gene to respond to various extracellular signals that call for laminin expression to fulfill one of its many specific cellular functions.

One of the highly conserved GC/GT-rich motifs within the LAMC1 promoter is the transcriptionally active bcn-1 element (11,12). We used the yeast one-hybrid screen (20) to identify transcription factors from mesangial cells that regulate activity of the bcn-1 element. The screen identified several cognate transcription factors including GKLF/KLF4. We demonstrate that GKLF regulates LAMC1 gene promoter activity in several cell lines. We provide evidence that the action of GKLF is exerted through its synergistic action with Sp1. This synergism depends on the promoter context, the integrity of the GKLF activation and DNA-binding domain.

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MATERIALS AND METHODS

Cell lines

Rat mesangial cell, SI-MC cells, were cultured and maintained as described previously (21). HeLa cells and *Drosophila melanogaster* Schneider SL2 cells were obtained from American Type Culture Collection. HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone). SL2 cells were maintained at 27°C in M3 medium (Sigma) supplemented with 10% FBS (Sigma) and antibiotics.

Construction of rat mesangial cell cDNA–activation domain (AD) fusion library for yeast one-hybrid screen

The rat mesangial cell line used for construction of the library was established from collagenase-treated glomeruli, and cells were characterized (16,22) and maintained as described previously (12). Total cellular RNA was prepared from phorbol 12-myristate 13-acetate-treated (1 h) rat mesangial cells as described previously (23). Poly(A)^+ RNA was reverse transcribed using an oligo(dT) primer with a XhoI site. The RNA strand of the mRNA-cDNA hybrid was replaced with the corresponding DNA strand by using *Escherichia coli* RNase H, *E.coli* DNA polymerase I and *E.coli* DNA ligase (23), and the cDNA was ligated to *Eco* RI linkers after both termini were blunt ended. After cleavage with *Eco*RI and *Xho*I, the cDNA was ligated to *Eco*RI linkers after both termini were blunt ended. After cleavage with *Eco*RI and *Xho*I, the cDNA was ligated to *Eco*RI/SalI-digested yeast expression plasmid pGAD424 (2 μg, Λeu 2) (Clontech). The ligation products were purified by ethanol precipitation, and electo-transformed into *E.coli* DH10B (Gibco) with *E.coli* Pulser (Bio-Rad) to generate the pGAD-MC cDNA library.

Yeast one-hybrid screen

Yeast one-hybrid screen was carried out according to the manufacturer’s protocol (MATCHMAKER One-Hybrid System; Clontech). Briefly, three tandem copies of the 17 bp bcn-1 motif (5′-ccgcggccgtcggcgcggag-3′) were inserted upstream of the HIS3 reporter gene by designing two antiparallel 3× bcn-1 oligonucleotides with *Eco*RI and *Xba*I or *Sal*I sites at the ends. Sense and antisense oligonucleotides were annealed and subcloned into the *Eco*RI/XbaI digested pPHIS1 reporter plasmids (Clontech). The ligation products were purified by ethanol precipitation, and electro-transformed into *E.coli* DH10B (Gibco) with *E.coli* Pulser (Bio-Rad) to generate the pGAD-MC cDNA library.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

EMSA was performed as described previously (11) with some modification. Briefly, oligonucleotides used for EMSAs were synthesized commercially (Gibco). EMSA probes were generated by end labeling 1 pmol double-stranded oligonucleotides with 10 μCi [γ-32P]ATP (3000 Ci/mmol) (DuPont NEN) using T4 polynucleotide kinase (Gibco). Labeled nucleotides were purified from unincorporated nucleotides using Micro Bio-spin p-30 Chromatography columns (Bio-Rad). Binding reactions were performed in 10 μl volumes containing 10 fmol (50 000–200 000 c.p.m.) of labeled probe, 2 μg of recombinant GKLF protein and 0.5 μg of poly(dI-dC) in binding buffer [10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT) and 4% glycerol]. Samples were incubated for 20 min at room temperature and then separated by electrophoresis on a 6% polyacrylamide gel [19:1 acrylamide:bis-acrylamide (Bio-Rad)] in 0.5× TBE (25 mM Tris, 24 mM Borate and 0.5 mM EDTA).

Plasmid constructs

The LAMC1 promoter fragment spanning the −1077 to −20 region of the gene (relative to the first codon), designated −1077/−20 LAMC1, was constructed by PCR amplification of the LAMC1 region −1104/+455 5′-flanking promoter region (13). The deletion constructs of LAMC1 promoter, −506/−20 LAMC1, −293/−20 LAMC1, −506/−197, −310/−197 LAMC1 and −293/−197 LAMC1, were generated by PCR using appropriate primers and the −1077/−20 LAMC1 promoter as template. The PCR products were ligated into pGEM-T Easy vector (Promega) and digested with *Sac*I and *Bgl*II. The *Sac*I–*Bgl*II fragments were ligated into pGL3 basic vector cut with *Sac*I–*Bgl*II using Rapid DNA Ligation Kit (Roche). The construction of −370/−20 LAMC1 and −370/−197 LAMC1 was carried out by digesting −506/−20 LAMC1 or −506/−197 LAMC1 with *Mlu*I and *Bgl*II, and the fragments were subcloned into *Mlu*I and *Bgl*II-digested pGL3 basic vector. The construction of −239/−20 LAMC1 was carried out by digesting with *Xho*I and *Bgl*II and the fragments were subcloned into *Xho*I and *Bgl*II-digested pGL3 basic vector.

The mammalian expression vectors containing full length mouse GKLF (pMT3-GKLF) (24), pMT3-GKLF(E93/95/96V) or pMT3-GKLF(D99/102/104V) (kindly provided by Dr Vincent Yang) were described previously (25). The pPac-0 and pPac-Sp1 were described previously (26,27). The GKLF constructs in pPac-GKLF, pPac-GKLF(1–401) and pPac-GKLF(350–483), pPac-GKLF(E93/95/96V) or pPac-GKLF(D99/102/104V), were generated by PCR using pMT3-GKLF, pMT3-GKLF(E93/95/96V) or pMT3-GKLF(D99/102/104V), as a template, respectively. PCR products were ligated to pGEM-T Easy vector and digested with *Bam*HI and *Xho*I. The fragments were ligated in *Bam*HI- and *Xho*I-digested pPac-0. To generate pGEX-GKLF, full length GKLF was amplified by PCR using pMT3-GKLF as template. The PCR products were subcloned into the pGEM-T Easy vector and digested with *Bam*HI and *Eco*RI. The digested fragment and *Bam*HI- and *Eco*RI-digested pGEX-KT (28) were ligated as above.

All constructs were confirmed by DNA sequencing.

Transient transfections and luciferase reporter gene assay

SI-MC cells and HeLa cells were plated at 4 × 10⁵ cells per well in 6-well plates and incubated in RPMI-1640 or DMEM, respectively, with FBS for 24 h. Cells were transiently transfected (in duplicates) with 1 μg of reporter plasmid, effector expression plasmid (indicated in each experiment) and 0.1 μg of pRL-null (Promega) using SuperFect transfection reagent (Qiagen). Cell extracts were prepared 48 h later in lysis buffer.

Production of recombinant proteins

GST–GKLF recombinant protein was produced in bacteria. A single colony of BL21(DE3) pLysS (Novagen) was transformed with pGEX-GKLF, and grown in 100 ml LB broth, containing ampicillin and chloramphenicol, to an optical density of 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (0.1 mM) was then added. The incubation was continued for an additional 3 h, at which time the bacterial cells were collected by centrifugation. The pellets were washed in 30 ml PBS, containing 1 mM EDTA and 150 mM NaCl, and incubated with 0.1 mg/ml lysozyme on ice for 30 min. After addition of 100 µl of 1 M DTT and 1.4 ml of 10% Sarkosyl, suspension was sonicated for 1 min. To bring the total volume to 20 ml, 4 ml 10% Triton X-100 was added. After removing debris by centrifugation, extracts were frozen and stored in aliquots at –70°C.

To purify GST fusion proteins, 1 ml of the bacterial lysate was added to a 300 µl bed of Glutathione Sepharose (Sigma) at 4°C for 1 h. The eluted solution was concentrated using Microcon YM-50 (Millipore) by centrifugation and then stored in aliquots at –70°C.

RESULTS

Cloning of the transcription factor GKLF, from a rat mesangial cell cDNA–AD fusion library, using the bcn-1 element as a bait in yeast one-hybrid screen

The rodent and human LAMC1 promoter contains a transcriptional element, bcn-1, that binds nuclear proteins from mesangial cells (11–13). We screened a cDNA fusion library, generated from rat mesangial cells, for a screen using the bcn-1 element as a bait in the yeast one-hybrid system. Two true positive cDNA clones represented the rat ortholog of the gut-enriched Kruppel-like factor (GKLF/KLF4) (24,30). Both of the clones, HL1012 and HL1022, were missing the poly(A) tail and the polyadenylation signal. As the rat GKLF cDNA had not been previously cloned, we used PCR to obtain the missing 3′ fragment of the cDNA. The final ORF potentially encodes a 482 amino acid protein with a mass of 52 kDa. The consensus sequence for polyadenylation, AATAAA (31), is found 16 bp upstream of the poly(A) tail. The deduced amino acid sequence of the rat and mouse GKLF is nearly identical (99%) and both share 89% similarity to the human sequence (Fig. 1). The deduced amino acid sequence predicts three tandem zinc finger motifs at the carboxyl end of the C2H2 type found in the Kruppel family of proteins (32) (bold) are identical in the three species.
domains of other transcription factors such as Egr-1 (33), BTEB2/IKLF (34), KKLF (35) and ZF9/CPBP (36).

Northern and western blot analyses showed that GKLF mRNA and protein are expressed in rat mesangial cells grown in culture (data not shown).

**Recombinant GKLF binds the bcn-1 motif in vitro**

To determine if GKLF directly binds to the bcn-1 motif, we synthesized GST–GKLF fusion protein in *E.coli* and tested its binding to 32P-labeled double-stranded bcn-1 oligonucleotide in EMSA. The gel shift assay revealed that GST–GKLF bound to 32P-labeled wild-type bcn-1 oligonucleotide (Fig. 2). To map the GKLF binding motif within the bcn-1 sequence, we tested a series of oligonucleotides in which two bases were mutated at a time. This analysis revealed that mutation of any one pair of bases in the middle of the CCCGCCCACCTCGCGC sequence abrogated the GST–GKLF binding (data not shown). To confirm the specificity of GKLF binding to the bcn-1 element we carried out competition experiments in which binding to the 32P-labeled wild-type bcn-1 element was carried out in either the absence or presence of synthetic oligonucleotide containing either wild-type (CCCCCCCCACCTGCCGC) or mutated (CCCCGCCgtCTCGCGC) bcn-1 element (Fig. 2). This experiment showed that an excess of oligonucleotide bearing the wild-type bcn-1, but not the mutated motif, abrogated the binding of GKLF to the 32P-labeled wild-type bcn-1. This confirms that GKLF binds in vitro to the bcn-1 element with a high degree of specificity. This result also suggests that the integrity of the CCCAC box, which is known to interact with Kruppel-like factors (37), is critical for the binding of bcn-1 to GKLF.

**Expression of GKLF activates LAMC1 promoter in rat mesangial and human HeLa cells**

GKLF contains both transcription activation and repression domains and is known to activate and repress gene expression (25,37,38). To test if GKLF activates or represses the LAMC1 promoter, rat mesangial cells were transiently transfected with firefly luciferase gene driven by the rat –1077/–20 LAMC1 promoter fragment with either pMT3 expression vector (−) GKLF or expression vector containing GKLF cDNA, pMT3-GKLF (+) GKLF. Renilla luciferase plasmid was used as a control for transfection efficiency. Forty-eight hours following transfection, cells were harvested and firefly and renilla luciferase activities were measured. Data are shown as mean ± SD of the ratios of firefly to renilla luciferase activities (n = 4).

**Figure 2.** Binding of recombinant GKLF to the bcn-1 motif in vitro. (A) The sequence of double-stranded synthetic oligonucleotide containing either wild-type or mutated bcn-1 element that was used as a competitor in the binding reaction. (B) Autoradiograph of the gel from EMSA. DNA-binding reaction was carried out in 10 µl binding buffer containing recombinant rat GST–GKLF and 32P-labeled double-stranded oligonucleotide containing wild-type bcn-1 element in the presence of either no competitor (none, lane 1), or 500-fold molar excess of double-stranded oligonucleotide containing either wild-type or mutated bcn-1 sequences as shown in (A). (C) Graph of the densitometric measurements of the shifted bands (B) expressed in digital light units (DLU).

**Figure 3.** GKLF-mediated activation of LAMC1 promoter in rat mesangial and human HeLa cells. Subconfluent rat mesangial and human HeLa cells were transiently transfected with firefly luciferase gene driven by the rat –1077/–20 LAMC1 promoter fragment with either pMT3 expression vector (−) GKLF or expression vector containing GKLF cDNA, pMT3-GKLF (+) GKLF. Renilla luciferase plasmid was used as a control for transfection efficiency. Forty-eight hours following transfection, cells were harvested and firefly and renilla luciferase activities were measured. Data are shown as mean ± SD of the ratios of firefly to renilla luciferase activities (n = 4).
within the LAMC1 promoter to test the role of bcn-1 in the GKLF-mediated activation of this promoter. As before, expression of GKLF activated the wild-type LAMC1 promoter in HeLa cells 6-fold. Mutation of the core bases within bcn-1 decreased the baseline activity of the LAMC1 promoter by 50%, but expression of GKLF still increased the activity of the mutated promoter 7-fold (Fig. 4). This suggests that bcn-1 is not required for the GKLF-mediated activation of the LAMC1 promoter in this cell line.

Synergistic activation of LAMC1 by GKLF and Sp1 in the Drosophila SL2 cells

Sp1 activates LAMC1 promoter in hepatocellular carcinomas (18). Expression of exogenous Sp1 increased the activity of LAMC1 promoter in HeLa cells by <2-fold (data not shown). The small effect in response to the exogenous expression of Sp1 may reflect either lack of responsiveness of the LAMC1 promoter to Sp1 in these cells or the fact that these cells already express high endogenous levels of Sp1. We used Drosophila SL2 cells (Fig. 5), which do not express Sp1 (26), to circumvent the potential interference by endogenous Sp1. Co-transfection of LAMC1-luciferase reporter construct into SL2 cells, with as little as 50 ng of the expression vector bearing Sp1 cDNA, increased the activity of the promoter 9-fold (Fig. 5A). With 1000 ng of transfected Sp1 DNA, the increase was >30-fold. These data show that the LAMC1 promoter is activated by Sp1. Unlike Sp1, expression of GKLF in SL2 cells had only a weak effect, where transfection of 1000 ng of expression plasmid bearing GKLF cDNA increased the LAMC1 promoter activity 2-fold (Fig. 5A).
The LAMC1 promoter is induced by and contains several putative GKLF- and Sp1-binding sites (TFSEARCH). Thus, we wondered whether these transcription factors cooperate in the regulation of the LAMC1 promoter. Sp1 expression plasmid (50 ng) increased LAMC1 promoter activity 10-fold in SL2 cells (Fig. 5A). The Sp1-mediated effect was potentiated by co-transfection of increasing amounts of expression plasmid bearing GKLF (Fig. 5B). For example, co-transfection of 50 ng Sp1 and 1000 ng GKLF DNA increased the LAMC1 promoter activity >100-fold. These results suggest that GKLF and Sp1 activate the LAMC1 promoter synergistically, meaning that the effect seen with GKLF and Sp1 co-transfection is greater than the sum of the effects seen when either GKLF or Sp1 is expressed alone [(GKLF&Sp1/GKLF+Sp1)>1] (Fig. 6). These results indicate that the GKLF-mediated activation of the LAMC1 promoter depends on the presence of Sp1.

**Functional mapping of LAMC1 promoter in SL2 cells**

To identify the regions required for the synergistic activation by Sp1 and GKLF, we tested the activity of a series of LAMC1 promoter deletion mutants in SL2 cells luciferase reporter assay without and with expression of Sp1 and/or GKLF. With 50 ng of expression plasmid, the –1077/–20 and –506/–20 fragments of the rat LAMC1 promoter were activated by Sp1 nearly 9-fold (Fig. 6). In contrast, expression of GKLF had only a marginal effect: 15–20% increase. When Sp1 and GKLF were expressed together, the activity of these fragments was increased 28–34-fold, a 3-fold synergism. The –370/–20 LAMC1 promoter fragment, which lacks the bcn-1 element, exhibited a similar level of Sp1–GKLF-mediated synergism (3.14-fold) as was seen with the –506/–20 LAMC1 fragment. This similarity indicates that the bcn-1 element might not be essential for the synergistic Sp1–GKLF activation of the LAMC1 promoter.

With further 5′ deletion, –293/–20 LAMC1 promoter fragment, there was a decrease in Sp1-mediated activation, but the degree of Sp1–GKLF-mediated synergism (3.02-fold) was maintained. This result suggests that the region between –370 and –294 might contain an Sp1-responsive element(s) that is not needed for synergism. With 5′ deletion of another 54 nt, the Sp1 responsiveness and Sp1–GKLF synergism was abrogated. This result suggests that the stretch between –293 and –239 contains an element(s) that is critical for Sp1 and the synergistic Sp1–GKLF activation of the LAMC1 promoter. Next we tested several fragments of the LAMC1 promoter lacking a portion of the 3′ end. The –370/–197 fragment exhibited diminished, but still potent, Sp1 and Sp1–GKLF responses compared with the –370/–20 fragment; Sp1-mediated increase was 5-fold compared with 7-fold, and Sp1–GKLF-mediated increase was 15-fold compared with 26-fold. These results suggest that the full Sp1–GKLF synergistic activation requires a core –293/–239 region in conjunction with either a 5′ or a 3′ adjacent domain, that is –370/–310 or –196/–20 fragments.

The deletion analysis (Fig. 6) suggests there is no single GKLF- and Sp1-responsive element within the LAMC1 promoter that can completely account for the cooperative activation of this promoter by these factors. Instead, there are likely multiple overlapping and non-overlapping GKLF- and Sp1-binding sites in the –370/–239 region that permit synergistic activation.

**The Sp1–GKLF synergistic activation depends on the promoter context**

Many gene promoters that are regulated by Sp1 and GKLF have multiple recognition sites for these factors, including the Keratin 19 (K19) promoter (39). We investigated the Sp1–GKLF synergism with other promoters that have multiple elements recognized by these transcription factors. Individual transfections of either 5 ng pPac-Sp1 or 50 ng pPac-GKLF had no effect, but when co-transfected the activity of the LAMC1 promoter increased 7-fold (Fig. 7A). Transfection of Sp1

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**Table 1**

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<th>Synergism</th>
<th>Sp1</th>
<th>GKLF</th>
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*Figure 6.* Functional mapping of the region responsible for the Sp1–GKLF-mediated synergistic activation of rat LAMC1 promoter in SL2 cells. The Drosophila SL2 cells were transiently co-transfected with a series of LAMC1 promoter deletion constructs in combination with 50 ng of either pPac-O (pPac), pPac-GKLF (GKLF), pPac-Sp1 (Sp1) or both expression (GKLF&Sp1) plasmids. Forty-eight hours following transfections, cells were harvested and both firefly and renilla luciferase activities in the cell lysates were measured. Activities of the promoter constructs were calculated as ratios of firefly to renilla luciferase activity (mean of n = 4). The numbers shown for each construct represent values that were normalized by dividing each firefly luciferase activity by the renilla luciferase activity (mean of n = 4).
DNA into SL2 cells activated the K19 promoter 5-fold (Fig. 7B). By itself GKLF (500 ng) had no effect, but when co-expressed with Sp1 the K19 promoter activity increased >8-fold. This indicates synergistic activation of the K19 promoter, but compared with the LAMC1 promoter the magnitude of the synergism was much lower (Fig. 7B). The SV40 promoter contains six GC-boxes that are responsive to Sp1. Exogenous Sp1 (14-fold) and, to a lesser extent, GKLF (6-fold) activated the synthetic promoter in SL2 cells, but when used together their effect was merely additive (20-fold induction) (Fig. 7B).

We wondered if Sp1–GKLF synergism could be observed if three tandem repeats of the bcn-1 sequence were inserted upstream of the SV40 promoter. We chose the SV40 promoter since it contains multiple Sp1-binding sites (40). The activity of the 3× bcn-1–SV40 synthetic promoter construct was similarly responsive to both GKLF (16-fold increase) and Sp1 (20-fold increase). When GKLF and Sp1 were co-expressed, the activity of the 3× bcn-1–SV40 synthetic promoter increased 37-fold, an additive effect rather than a synergistic response. Together these results suggest that the promoter context could be a key determinant of the Sp1–GKLF synergism of those target promoters that have multiple binding sites for these factors.

The GKLF activation domain and zinc fingers are required for the Sp1–GKLF synergistic induction of the LAMC1 promoter in SL2 cells

As is the case with most transcription factors, the GKLF structure is modular. GKLF contains two clusters of acidic
residues in the AD (25) and a region that harbors three zinc fingers (Fig. 8A). Several deletion and point mutants of GKLF were used in transfections of SL2 cells to define the domains that are required for the synergistic activation of LAMC1 promoter by GKLF and Sp1. As before, expression of the full-length GKLF (GKLF 1–483) potentiated the Sp1-induced activation of the LAMC1 promoter. In contrast, the GKLF deletion construct that lacked the C-terminus, GKLF 1–401, did not stimulate the Sp1-mediated activation of the LAMC1 promoter (Fig. 8B). These results indicate that the GKLF zinc fingers are required for the synergistic action of Sp1 and GKLF and that GKLF might need to bind to DNA for the synergism to be observed.

A lack of synergism was also observed with the deletion of the N-terminal part of GKLF (GKLF 350–483). This deletion of GKLF removed the transcriptional AD, a highly acidic region. Two clusters of acidic residues within the AD are required for GKLF transcriptional activity (25). To test if the AD of GKLF is required for the synergism, we used two GKLF constructs where the residues within either the glutamic or aspartic acid clusters within the AD were mutated to valines. Co-transfection of either of the two AD GKLF mutant constructs had no effect on the Sp1-mediated activation of LAMC1 promoter. These results suggest that the GKLF AD is also required for the synergistic activation.

**DISCUSSION**

We provide evidence that GKLF and Sp1 synergistically activate the LAMC1 promoter, a cooperative process not previously described between two members of the Sp/XKLF transcription factor family (Figs 5–8). Although the precise molecular mechanism of the Sp1–GKLF synergy in the activation of the LAMC1 promoter remains to be elucidated, we have identified several components that appear to be required. First, we show that the GKLF DNA-binding domain is required (Fig. 8). Since recruitment of factors to promoters is the key determinant of the rate of transcription (41), the requirement for DNA binding suggests that GKLF may aid in the recruitment of other factors to the LAMC1 promoter that are needed for the synergism. This may include co-activators such as CBP or even Sp1 itself, both known to physically interact with GKLF (25,42). Indeed, CBP appears to participate in the synergistic activation of LAMC1 promoter by GKLF and Sp1 (data not shown). Second, mutations within the GKLF AD abrogate the Sp1–GKLF synergism (Fig. 8). This result suggests that the AD interacts with another factor(s) that is necessary for the activation of the LAMC1 promoter. The logical candidate here is the co-activator CBP. Third, the LAMC1 promoter has several CACCC and CG-rich GKLF- and Sp1-putative binding sites (18), suggesting the LAMC1 promoter may simultaneously recruit GKLF and Sp1, and thus
be synergistically activated. However, as illustrated by the low level of cooperative Sp1–GKLF activation of the K19 promoter (Fig. 7), the mere simultaneous recruitment of GKLF and Sp1 to multiple sites may not be sufficient for effective synergistic activation. Thus, promoter context is also a key determinant. That means the arrangement of the GKLF-, Sp1- and other binding sites within the promoter is a key determinant that defines the overall topology of the promoter bound to cognate transcription factors and determines the strength of coupling to the general transcriptional apparatus. Since the C-terminal domain of Sp1 binds GKLF (42), a direct physical interaction between GKLF and Sp1 is also suggested for the synergistic activation. It is conceivable that there are several adjacent GKLF and Sp1 binding sites within the core domain responsible for the synergism that allow formation of Sp1–GKLF heterodimers resulting in a promoter topology conducive to cooperative activation.

GKLF belongs to the family of Sp/XKLF transcription factors that is composed of at least 16 different mammalian proteins (43). In addition to Sp1 (44) and GKLF, this class of factors also includes Sp2-4, EKLF, LKLF, BKLF, UKLF, BTEB and others. Although these factors are structurally similar and bind similar DNA sequences, their respective gene targets are diverse. This diversity can, in part, be explained by the ability of these proteins to interact with other factors of the same or different family of activators. For example, there are several reports describing the functional relationship between members of the Sp/XKLF family of factors, particularly those interactions involving Sp1. One of the better known examples is that of Sp1 and Sp3. In the regulation of several viral and cellular promoters, Sp3 represses Sp1 activity by competing for the same binding site (45). Similarly, GKLF has been shown to suppress the cytochrome P-450IA1 (CYP1A1) promoter in a manner that is both Sp1- and Sp3-dependent, an observation that, in part, could be explained by the physical interaction between these factors (42). The cooperative effects exhibited by GKLF and Sp1 (Figs 5–8), illustrate how synergism (determined by promoter context), recruitment of a co-activator(s), protein–DNA and protein–protein interaction, could explain specificity of gene induction and the diversity of physiological effects exhibited by closely related transcription factors such as members of the Sp1/XKLF family of proteins.

As illustrated in SL2 cells, Sp1 can activate the LAMC1 promoter, but its effectiveness is increased by GKLF (Figs 5 and 7). The same may be true for HeLa cells that express high levels of endogenous Sp1 but have little endogenous GKLF. As a result, exogenous GKLF is a potent activator of the LAMC1 promoter in these cells (Figs 3 and 4). Thus, GKLF may provide a specific avenue for potent activation of the LAMC1, but only in cells that also express Sp1. What could be the physiological settings for deployment of GKLF to activate the LAMC1 gene? It is thought that GKLF has a role in regulating genes whose expression is found in differentiated cells (37,46). The repertoire of genes activated in differentiating cells may include laminin, which is known to regulate cell differentiation (3,47). GKLF may mediate cell differentiation through the activation of the LAMC1 gene followed by the assembly of laminin. Thus, identification of GKLF as a regulator of LAMC1 gene expression may reflect a pathway for induction of γ1 chain when laminin is needed to regulate cell differentiation. Chang et al. (7) have demonstrated that there are enhancer elements within the first intron of the LAMC1 gene which greatly activate the LAMC1 promoter during retinoic acid induced differentiation of F9 cells. These observations suggest that the role of the LAMC1 gene in cell differentiation can be induced by several pathways.

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