Lung Krüppel-like Factor Contains an Autoinhibitory Domain That Regulates Its Transcriptional Activation by Binding WWP1, an E3 Ubiquitin Ligase*

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Lung Krüppel-like factor (LKLF/Krüppel-like factor 2), a member of the Krüppel-like factor family of transcription factors, is expressed predominantly in the lungs, with low levels of expression in other organs such as heart, spleen, skeletal muscle, and testis. LKLF is essential during pulmonary development and single-positive T-cell development and is indispensable during mouse embryogenesis. In this study, we performed a series of experiments to define the activation domain of LKLF as a means to further advance the understanding of the molecular mechanisms underlying transcriptional regulation by this transcription factor. Using deletion analysis, it is shown that LKLF contains a transcriptional activation domain as well as a strong autoinhibitory subdomain. The inhibitory subdomain is able to independently suppress transcriptional activation of other strong activators such as viral protein 16, VP16. This occurs either when the inhibitory domain is fused directly to VP16 or when the inhibitory domain is independently bound to DNA by Gal4 DNA-binding domain independent of the VP16 activator. Overexpression of the LKLF autoinhibitory domain alone potentiates transactivation by wild type LKLF, suggesting that the inhibitory domain binds a cofactor that prevents LKLF from transactivating. A yeast-two hybrid screen identified WWP1, an E3 ubiquitin ligase that binds specifically to the LKLF inhibitory domain but not to other transcription factors. In mammalian cells, WWP1 functions as a cofactor by binding LKLF and suppressing transactivation. These data demonstrate that LKLF contains multiple domains that either potentiate or inhibit the ability of this factor to function as an activator of transcription; moreover, regulation of LKLF transactivation is attenuated by an E3 ubiquitin ligase, WWP1.

Lung Krüppel-like factor (LKLF/KLF2)1 is a member of a multigene family of transcription factors called the KLF family. LKLF is expressed predominantly in fetal and adult lungs, with limited expression in other organs (1, 2). Analysis of chimeric mice derived from LKLF−/− embryonic stem cells demonstrated that LKLF is essential for late stages of normal lung development (3). In addition to pulmonary development, the programming of the quiescent state of single-positive (CD4+ or CD8+ ) T cells and the late-stage survival of these cells in the peripheral lymphoid organs and blood are also dependent on LKLF (4, 5). Early in T-cell development, single-positive thymocytes are produced and survive in the thymus without LKLF. However, the mature circulating cells undergo apoptosis in LKLF−/− mice, resulting in severely reduced numbers of peripheral T cells (4, 5).

Targeted disruption of LKLF through gene targeting in embryonic stem cells results in embryonic lethality (6, 7). LKLF homozygous null mice die in utero between 12.5 and 14.5 days of gestation due to severe hemorrhage. Defects in the blood vessel morphology, an abnormally thin tunica media, endothelial cell necrosis, and decreased deposition of the extracellular matrix surrounding the vessels collectively contribute to hemorrhage in LKLF−/− embryos (6, 7). Analogous to its role in other organs, LKLF does not appear to be an important regulator of the initiation or early stages of blood vessel morphogenesis; rather, it is active in the late stages of development including the cell-mediated assembly and stabilization of the blood vessel wall (6). Whereas the mechanism by which LKLF regulates blood vessel integrity is unknown, it has been suggested that LKLF may regulate a signaling pathway responsible for endothelial cell differentiation or survival required for the formation of the mature blood vessel wall (6).

LKLF was initially described as a lung-specific transcription factor (1). However, it is now clear that LKLF plays a pivotal role in blood vessel formation and T-cell activation in addition to pulmonary development. A unifying theme in these apparently diverse roles is that LKLF is essential for late stages of development but is not required for the initial steps. Despite the large amount of information about the biological role of LKLF, no specific target gene(s) or mechanisms of LKLF regulation have yet been identified. Determination of the molecular mechanisms that underlie LKLF function as a regulator of transcriptional pathways is paramount in providing additional insight into its roles in pulmonary function, maintenance of single-positive T cells, and embryogenesis.

One approach to deciphering the molecular mechanisms of transcriptional activation by this factor is to study its functional domains. Transcription factors are commonly composed of two distinct separable domains, an activation domain and a DNA-binding domain. Recent studies of two Krüppel-like factors, the erythroid (EKLF/KLF1) and gut-enriched (GKLF/EZF/KLF4) Krüppel-like factors, demonstrate that for transcriptional activation, only a small subdomain of the activation domain is required (8–10). This is in contrast to intestinal-enriched Krüppel-like
factor, which requires the entire activation domain (11). LKLF, EKLF, and GKLKF comprise a subfamily in the KLF family. All these transcription factors contain an inhibitory subdomain located adjacent to the zinc fingers (8–10). Deletion of the inhibitory subdomains in EKLF and GKLKF allows these factors to function as more potent activators of transcription. Despite the similarity in size, function, and physical location of these inhibitory subdomains, no obvious conservation in the amino acid sequence has been detected. Thus, activation domains are beginning to emerge as complex multifunctional domains with discrete subdomains that are likely involved in the regulation of transcription factors. By studying the biochemical attributes and configurations of the activation domains present in transcriptional activators we can better understand the mechanisms governing gene regulation and, ultimately, the role of tissue-specific transcriptional activators.

Our current studies demonstrate that LKLF contains a modular activation domain that can be separated into inhibitory and transactivating subdomains. The inhibitory domain of LKLF binds specifically to an E3 ubiquitin ligase that is able to attenuate transactivation by LKLF. The interactions between these two proteins likely represent one method by which this transcription factor is regulated.

MATERIALS AND METHODS

Plasmid Constructs—Full-length LKLF cDNA fragment was subcloned into expression vector pM (12) that contained the coding region of the GAL4 DNA-binding domain (DBD) to create a fusion protein between GAL4 DBD and LKLF. Six GAL4-LKLF mutants (GAL4-LKLF 1–267, GAL4-LKLF 1–257, GAL4-LKLF 1–141, GAL4-LKLF 1–110, GAL4-LKLF 1–88, and GAL4-LKLF 1–57) were then created by serial deletions utilizing the restriction enzyme site (BssHI, NotI, SacII, XbaIII, SacII, and BstXI, respectively) in the coding region of LKLF and a restriction site in the polylinker. After deletion of the 5′ end of LKLF cDNA, the staggered ends generated from the restriction digests were blunted with T4 polynucleotide kinase before ligation. Stop codons in the vector prevented any read through. Three additional LKLF internal deletion mutants (GAL4-LKLF Δ110–257, GAL4-LKLF Δ88–267, and GAL4-LKLF Δ57–215) were constructed in pGEM3 (Promega, Madison, WI) and then subcloned into pM vector. The internal deletion mutants were created by in vitro restriction endonuclease digestion of the subcloned constructs or PCR amplification of the zinc fingers, adding a silent mutation to create a unique restriction site. GAL4-LKLF Δ57–215 was created utilizing two internal NarI sites, and GAL4-LKLF Δ110–257 was created utilizing two XbaIII restriction sites. PCR was used to create GAL4-LKLF Δ88–267 by amplifying the zinc finger domain to introduce a second SacII restriction site by silent mutation at the 5′ end (5′-NNN-AAG-CTT-GGC-GGC-GCG-3′ and 5′-NNN-AAG-CTT-GCA-GTG-TGT-TTG-3′). Each of these constructs was created utilizing an interstitial activation domain that can be separated into inhibitory subdomains, no obvious conservation in the amino acid sequence has been detected. Thus, activation domains are beginning to emerge as complex multifunctional domains with discrete subdomains, no obvious conservation in the amino acid sequence has been detected. Thus, activation domains are beginning to emerge as complex multifunctional domains with discrete subdomains, no obvious conservation in the amino acid sequence has been detected. Thus, activation domains are beginning to emerge as complex multifunctional domains with discrete subdomains.
pAS-1-CYH2 vector from the pGAD10 vector. Isolated plasmids were transformed into HB101 E. coli and grown on M9 leucine-deficient selection medium with ampicillin. Isolated pGAD10 vector was then subjected to double-stranded nucleotide sequence analysis (Applied Biosystems). To eliminate the possibility that the interaction between WWP1 was fortuitous, an interaction between WWP1 and other transcription factors was examined. GAL4-WWP1 was co-transformed with pAS-1-CYH alone or with pAS-1-CYH containing cDNA for the inhibitory domain of EKLF, full-length EKLF, GATA 5, or GATA 6. In addition, WWP1 was also transformed by itself.

Immunoprecipitation of LKLF-HA and WWP1-FLAG—LKLF tagged with the HA epitope and WWP1 tagged with the FLAG epitope were transiently transfected into COS cells as described above. After co-transfection with WWP1 and LKLF, the cells were lysed with a hypotonic buffer containing Nonidet P-40. The cell lysates were split and incubated with either a polyclonal antibody against the HA antigen (Santa Cruz Biotechnology) or preimmune serum. The protein-antibody complex was then precipitated by the addition of protein G-Sepharose (Zymed Laboratories Inc.). After washing the absorbed beads in co-immunoprecipitation buffer, the precipitants were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore). Finally, Western blot analysis using a monoclonal antibody (M2) against the FLAG antigen (Sigma) fused to WWP1 was used to evaluate the association of the two proteins during the precipitation.

RESULTS
LKLF Contains Transcriptional Activation and Autoinhibitory Domains—Previous studies have indicated that some members of the Krüppel-like family of transcription factors consist of multiple domains that function in transcriptional activation, inhibition of activation, protein-protein interaction, DNA binding, and transcriptional repression (8–10, 15). To identify the functional domains of LKLF, a series of plasmids containing various portions of LKLF cDNA joined to the DBD of yeast transcription factor GAL4 were constructed (Fig. 1, A and D). Because GAL4 fusion proteins are of yeast origin, they have the advantage of little or no background interference in mammalian cells. In addition, GAL4 DBD directs proteins to the nucleus, alleviating the concern that deletion mutants might disrupt the natural nuclear localization signal of a protein. Both serial deletions and internal deletions were created and assayed for the ability to transactivate pG5-CAT, a reporter construct containing five GAL4-binding sites in front of the E1b minimal promoter (Fig. 1, A and D). Full-length LKLF (aa1–354) fused to the GAL4 DBD transactivated the reporter gene only modestly. However, because additional portions of the carboxyl terminus of LKLF were deleted, transcriptional activation increased. Removal of amino acids 111–354 resulted in a 25-fold increase in transcriptional activation (Fig. 1B). Further deletions of the activation domain resulted in a slight attenuation in transactivation. Equivalent results are observed when these experiments are conducted in a mouse lung adenoma cell line (LA4) expressing endogenous LKLF (data not shown). Western blot analysis of cell lysates was performed to demonstrate that the full-length protein and each mutant protein were made in the cell, ruling out the possibility that the inability of LKLF or LKLF deletion mutants to transactivate was the result of the protein not being synthesized. (Fig. 1C).

Amino acids 1–110 are able to function as a potent activator of transcription and transactivate significantly better than full-length LKLF, suggesting that an inhibitory domain is present...
outside this region of the protein. To determine whether the inhibitory domain is contained within the Cys₂-His₂ zinc fingers, internal deletion constructs were engineered that maintained this protein feature (Fig. 1D). The activity of the activating subdomain (aa 1–110) to enhance reporter gene expression is unaffected by the presence of the zinc fingers. This demonstrates that the zinc fingers do not contain the inhibitory subdomain (Fig. 1E) and further localizes this regulatory region to amino acids 111–267. A Western blot analysis once again demonstrated the presence of full-length LKLF and each mutant protein in the cells (Fig. 1F).

Whereas the natural DNA-binding domain of LKLF is located on the carboxyl terminus of the activation domain, the GAL4 DNA binding domain was attached to the amino-terminal portion. To rule out any positional effects from this configuration, additional LKLF deletion constructs were engineered to contain the GAL4 DBD on the carboxyl terminus of the activation domain. These deletion constructs were assayed for the ability to activate transcription from a reporter gene. In agreement with the above-mentioned results, removal of amino acids 111–267 increased transcriptional activation, although the total fold activation is slightly lower. Collectively, these data demonstrate that LKLF contains a potent transactivation domain in the first 110 amino acids. In addition, LKLF contains a region between amino acids 111–267 that is responsible for suppressing transactivation and represents a regulatory region in the protein.

**LKLF Inhibitory Domain Is Modular and Capable of Preventing Transcriptional Activation by VP16**—Deletion of amino acids 111–267 from LKLF allows this factor to function as a stronger activator of transcription, suggesting that this region functions as a regulator of transcriptional activation. To reinforce the deletion analysis and provide a positive assay for this function, we show that this LKLF inhibitory domain can also directly suppress other transactivators. LKLF inhibitory domain was fused to the heterologous protein Lex A-VP16 (Lex A-VP16-LKLF 111–267) and compared with unmodified Lex A-VP16 for the ability to transactivate a reporter gene. The LKLF inhibitory domain almost completely suppressed transactivation by VP16 (Fig. 2A). Western blot analysis confirmed that both Lex A-VP16 and Lex A-VP16-LKLF 111–267 proteins were being made in the cell (Fig. 2B). These results indicate that the LKLF inhibitory domain is not only capable of inhibiting transactivation by a strong transcriptional activator such...
as VP16 but is a modular protein region that can function independent of other regions of LKLF.

**LKLF Inhibitory Domain Is Independently Capable of Inhibiting Transactivation through Intermolecular Interactions—**The ability of the inhibitory domain to suppress transactivation may occur through an intramolecular or intermolecular mechanism. To examine the possibility that the LKLF inhibitory domain functions through an intermolecular mechanism, several chimeric proteins were generated that consist of the GAL4 DNA-binding domain fused to various regions of LKLF. The ability of these GAL4 fusion proteins to modulate transcriptional activation by the heterologous protein Lex A-VP16 when both factors are simultaneously bound to the promoter region was examined. In all, three GAL4-LKLF heterologous proteins were generated with either the inhibitory domain (GAL4-LKLF 111–267), the activation domain (GAL4-LKLF 1–110), or both domains (GAL4-LKLF 1–267) fused to the GAL4 DBD. Each of the three GAL4-LKLF chimeric proteins or the control GAL4 DBD alone was examined for the ability to modulate transactivation of Lex A-VP16. For these studies we utilized an artificial CAT reporter system in which five GAL4-binding sites are present adjacent to eight Lex A-binding sites (L8G5-CAT) (Fig. 3A) (13). In this system, Lex A-VP16 and a GAL4 DBD fusion protein are able to bind and occupy their respective response elements simultaneously. As expected, GAL4 DBD bound to the promoter in the absence of Lex A-VP16 but was unable to transactivate the CAT reporter gene, demonstrating that GAL4 DBD by itself does not have any transactivating capabilities (Fig. 3B). The addition of Lex A-VP16 even in the presence of GAL4 DBD resulted in strong transactivation (Fig. 3B). When GAL4 DBD was replaced with GAL4 DBD fused to LKLF inhibitory domain, transactivation by Lex A-VP16 was attenuated 6-fold (Fig. 3B). This demonstrates that the LKLF inhibitory domain is able to regulate transactivation by an intermolecular mechanism. Furthermore, the inhibition of the Lex A-VP16 transactivation by the inhibitory domains cannot be attributed to steric hindrance or to a physical block of transactivation. This is demonstrated with the analysis of Lex A-VP16 activation in the presence of GAL4 DBD fused to the LKLF activation domain (GAL4-LKLF 1–110). In this situation, transactivation was potentiated rather than suppressed (Fig. 3B). A similar result is observed when both domains are fused to GAL4 DBD, although, as one might expect, the potentiation was not as high as that of activator alone (Fig. 3B). Each of the GAL4 DBD fusion proteins and GAL4 DBD fused to VP16 was also tested for the ability to transactivate in the absence of Lex A-VP16. Essentially, this recapitulates the data from the earlier experiment but also demonstrates that the inhibitory domain had no transactivation potential associated with it (Fig. 3B). In addition, the LKLF activation domain and VP16 are able to transactivate the reporter gene with comparable efficiency (Fig. 3B).

In addition, the three GAL4-LKLF fusion constructs were tested for the ability to inhibit Lex A-VP16 when the GAL4 binding sites are absent from the promoter (L8-CAT) (Fig. 3C). In this situation, Lex A-VP16 was able to occupy the promoter; however, because the GAL4-binding sites are absent, the GAL4 DBD fusion protein was unable to bind the promoter. Neither GAL4 DBD alone nor any of the three GAL4 fusion proteins are able to modulate Lex A-VP16 when not bound to the promoter (Fig. 3D). This indicates that there is not a physical interaction that would allow the Lex A-VP16 protein to recruit the GAL4-LKLF fusion proteins to the promoter. Taken together, these data demonstrate that the LKLF inhibitory domain is able to suppress transactivation through an intermolecular mechanism.

**Amino Acids 111–267 Represent the Minimal Functional Inhibitory Domain—**Protein interactions often occur through small stretches of amino acids. For example, carboxyl-terminal-binding protein 2 binds to a Pro-Val-Asp-Leu-Thr motif in basic Krüppel-like factor (KLF3) (16). In an attempt to define the minimal functional region of the LKLF autoinhibitory domain, a series of amino- and carboxyl-terminal deletions of the autoinhibitory domain were fused to GAL4 DBD, producing chimeric proteins. Six unique chimeric proteins (LKLF 111–200, LKLF 111–150, LKLF 150–200, LKLF 150–267, LKLF 200–267, and LKLF 111–267 fused to the GAL4 DBD) were tested for the ability to repress Lex A-VP16 as described above using the L8G5-CAT reporter gene (Fig. 4A). The full-length inhibitory domain (aa 111–267) inhibited transactivation most efficiently (Fig. 4B). Deletions from either the carboxyl end or the amino end of the inhibitory domain still inhibit transactivation of Lex A-VP16, but these truncated regions did not attenuate transactivation as effectively as the full-length inhibitory domain (Fig. 4B). This suggests that both ends of the inhibitory domain are involved in the inhibition. Moreover, when a midregion of the inhibitory domain (aa 150–200) is fused to GAL4 DBD, no inhibition was observed. In addition, no inhibition was observed by any of the fusion proteins when the GAL4-binding sites are absent from the promoter (L8-CAT) (data not shown).

Therefore, amino acids 111–267 of LKLF represent the minimal region within the LKLF transactivation domain essential for its autoinhibition.

**Fig. 4.** LKLF inhibitory domain and serial deletion mutants of the inhibitory domain tethered to DNA simultaneously with Lex A-VP16. Lex A-VP16 fusion constructs and GAL4 with a LKLF inhibitory domain fusion construct or with serial deletions in the inhibitory domain were co-transfected with L8G5-CAT reporter construct into COS cells. A, a schematic of GAL4-LKLF inhibitory domain fusion constructs. B, normalized CAT activity of Lex A-VP16 and GAL4 DBD-LKLF inhibitory domain (aa 111–267) or GAL4 DBD-LKLF inhibitory domain with a deletion mutation using the L8G5-CAT reporter gene is represented in the bar graph (B; mean ± S.E.; n = 3 experiments).
Overexpression of the Inhibitory Region of LKLF Enhances Transcriptional Activation by LKLF—The ability of the LKLF inhibitory region to repress transcription by an intermolecular mechanism suggests an interaction with a co-repressor capable of suppressing transactivation. In such a case the inhibitory region may act as a docking site for a co-repressor. According to this hypothesis, one would predict that the co-repressor should be titrated away by overexpression of the inhibitory domain alone. As shown in Fig. 5, although LKLF can function as a weak activator of transcription, overexpression of GAL4-LKLF 111–269 increased the activation of the reporter gene in a dose-dependent manner (Fig. 5). These results are consistent with the LKLF inhibitory domain serving as an interactive site for a co-repressor protein that regulates the transactivation function of this transcription factor.

WWP1, an E3 Ubiquitin Ligase, Binds LKLF Inhibitory Domain—Our data suggest that a cofactor binds to LKLF to suppress its ability to transactivate. Therefore, to isolate such a putative cofactor, which might bind to the inhibitory domain of LKLF and regulate its ability to transactivate, a yeast two-hybrid screen was conducted. A rat lung cDNA library (CLONTECH) was used in conjunction with the LKLF inhibitory domain fused to GAL4-DBD during this screen.

The two clones that exhibited the strongest interaction both coded for the WW domains of an E3 ubiquitin ligase, WWP1 (17, 18). The interaction with this protein was considerably stronger than that of the other clones, as indicated by the enhanced growth in the presence of 3-amino 1,2,4-triazole (3-AT) when compared with the other clones. In addition to interacting with the LKLF inhibitory domain, WWP1 interacts with GAL4 DBD-LKLF (aa 1–354) but does not interact with five control baits, GATA 5, GATA 6, EKLF inhibitory domain (aa 195–354) (8), full-length EKLF, and GAL4 DBD (Fig. 6), demonstrating specificity between LKLF and WWP1. No interactions were observed between a second member of the Krüppel-like factor family, EKLF, or members of a second zinc finger family, the GATA family of transcription factors. To verify by an independent method that LKLF and WWP1 associate, co-immunoprecipitation of epitope-tagged proteins and Western analysis were conducted with mammalian cells. Equal amounts of LKLF tagged with the HA epitope (LKLF-HA) and FLAG-tagged WWP1 (WWP1-FLAG) were co-expressed in COS cells. As controls, we analyzed cells expressing LKLF-HA, WWP1-FLAG, or empty vector. LKLF was immunoprecipitated with anti-HA antibody from whole cell extracts or with preimmune serum as a negative control and examined by immunoblotting with anti-FLAG antibody to detect the presence of WWP1. Indeed, WWP1 associated with LKLF in mammalian cells, as demonstrated by co-immunoprecipitation (Fig. 6D). As expected, WWP1 was not precipitated when preimmune serum was substituted for anti-HA antibody or when LKLF was not co-expressed with WWP1 (Fig. 6E). The inhibitory domain of LKLF associates with the WW domains of WWP1 in yeast and mammalian cells.

WWP1 Suppresses LKLF-mediated Transactivation—To establish a functional consequence of the association between LKLF and WWP1, we examined the ability of LKLF to transactivate in the presence of WWP1 (Fig. 7). Interestingly, LKLF-mediated transactivation is suppressed in a dose-dependent manner by the presence of WWP1 (Fig. 7). It is unlikely that high concentrations of an E3 ligase are responsible for a general or nonspecific effect because E6AP, which is also an E3 ubiquitin ligase, does not significantly attenuate transactivation by LKLF. Not only does the inhibitory domain of LKLF engage in protein-protein interactions with WWP1, but these interactions also modulate the function of this protein.
**FIG. 7.** WWP1 attenuates transactivation by LKLF. COS cells were transfected with LKLF alone or with increasing amounts of WWP1 (0.25, 0.5, or 1.0 μg) or with increasing amounts of E6AP (0.25, 0.5, or 1.0 μg). The total amount of DNA in each transfection was kept constant with vector DNA. The percentage activation is represented in the bar graph. The percentage activation of LKLF was set at 100% (mean ± S.E.; n = 3 experiments).

**FIG. 8.** Comparison of the subdomains of LKLF, EKLF, and GKLF. The white box represents the transactivating subdomain, whereas the black box represents the inhibitory subdomain. Each transcription factor contains a putative nuclear localization signal (NLS) within the inhibitory domain. The Cys2-His2 zinc finger domain is represented by the shaded box.

**DISCUSSION**

The Krüppel-like family of transcription factors is comprised of a number of different members whose expression is restricted to specific tissues or cell types. Three members of this family, LKLF, GKLF, and EKLF, have been divided into a subfamily based on Jukes-Canton algorithm (19) and a conserved nuclear localization signal (20). It is now apparent that all three members of this subfamily also share a spatially conserved inhibitory domain located adjacent to the DNA-binding domain (Fig. 8) (8–10). When this region is deleted from any of the three transcription factors, it becomes a strong transcriptional activator. Although LKLF, EKLF, and GKLF contain inhibitory domains, such autoinhibitory domains are not present in all members of the Krüppel-like factor family. For example, the activation domain of intestinal-enriched Krüppel-like factor (KLF5) or of the ubiquitous Krüppel-like factor (UKLF/KLF7) possesses only an activator domain (11, 21). Thus, the inhibitory domains are limited to this subfamily, and deletion of this domain from these factors results in a similar increase in the transcriptional activation ability of each of these factors. Although the inhibitory domains among these three factors lack any homology, a common feature based on charge, hydrophobicity, or the proline-rich regions involved in protein-protein interactions could be sufficient for autoinhibition. This may explain why conservation in the amino acid sequence between LKLF, GKLF, and EKLF is not observed. However, the autoinhibitory domain in LKLF requires two regions for complete inhibition, suggesting that a more complex secondary structure is involved and raising the possibility of the existence of different interacting factors present in the tissue in which these individual members are expressed.

Whereas autoinhibitory domains are not commonly observed in transcription factors, there are several examples of transcription factors that contain such domains. One of the best-characterized examples is Ets-1, a member of the Ets family. Ets-1 contains an autoinhibitory domain that functions intramolecularly to repress DNA binding and thus prevents transactivation (22). Binding of core-binding factor alpha 2 to Ets-1 counteracts the autoinhibition by increasing the DNA binding affinity of Ets-1 (23). Similarly, activating transcription factor-2, a basic region leucine zipper transcription factor, also contains an autoinhibitory domain that has been proposed to regulate transactivation by masking a portion of the activation domain and thereby preventing transactivation (24). In contrast, the DNA binding affinity of EKLF is increased when its inhibitory domain is removed, suggesting that the inhibitory domain of EKLF acts through an intramolecular interaction (8). However, this type of intramolecular mechanism cannot account for the observation that the LKLF inhibitory domain is able to attenuate transactivation of VP16 when it is tethered to the DNA via the GAL4 DBD. This suggests that the inhibition must occur, at least in part, through an intermolecular mechanism and cannot be completely explained through intramolecular effects such as alterations in DNA binding affinity. However, an intermolecular mechanism involving protein-protein interaction does not totally preclude the involvement of intramolecular contributions. The LKLF inhibitory domain was able to suppress Lex A-VP16 more efficiently when fused directly to VP16 than when tethered to DNA, supporting the possibility of a dual mechanism. Alternatively, fusing the LKLF inhibitory domain directly to VP16 could be more efficient because it brings a putative inhibitory cofactor in closer proximity than when tethered to the DNA by GAL4 DBD.

One hypothesis that is directly supported by our data is that the LKLF autoinhibitory domain may directly interact with a corepressor that is involved in preventing transactivation by LKLF. The addition of increasing amounts of inhibitory domain fused to GAL4 DBD in cells expressing wild type LKLF allowed wild type LKLF to transactivate more efficiently. This suggests that the excess inhibitory domain can sequester a corepressor that is involved in the inhibitory process, supporting the idea that the inhibitory domain functions through intermolecular interactions through protein-protein interactions. In an effort to identify and characterize such a corepressor, a yeast two-hybrid screen was conducted using the LKLF inhibitory domain (aa 111–267). The 2 strongest of 15 clones code for the WW domains of an E3 ubiquitin ligase, WWP1 (17, 18). WWP1 contains four WW domains composed of 38–40-amino acid regions that are named after two highly conserved tryptophan residues characteristically spaced either 22 or 23 residues apart (25). These domains recognize and bind to target proteins that are rich in proline residues (18), a hallmark feature of the autoinhibitory domain of Krüppel-like factors. In addition, WWP1 contains a catalytic homology to E6 carboxyl terminus (HECT)
domain that catalyzes the transfer of ubiquitin moieties as a thiol intermediate from a conserved cysteine residue in this domain to a lysine residue in the target protein, resulting in their polyubiquitination, followed by degradation by the 26S proteasome complex (26). However, not all ubiquitinated proteins are destined for the proteasome degradation. In certain cases, ubiquitination also functions as a posttranslational modification that regulates the target protein (27, 28). WWP1 not only binds to LKLF in a yeast two-hybrid assay but also interacts physically in mammalian cells as demonstrated by coimmunoprecipitation of the two proteins and suppresses LKLF-mediated transactivation of a reporter gene.

It is well documented that ubiquitin E3 ligases function as transcriptional co-repressors with many transcription factors such as p53 (29), MATα2 (30), c-Myc (31), c-Jun (32), c-Fos (33, 34), estrogen receptor (35), progesterone receptor (36), signal transducers and activators of transcription 1 (37), nuclear factor κB (38, 39), and SMAD1 (40), and they modulate these factors through a variety of mechanisms. For example, c-Jun and c-Fos undergo rapid degradation mediated by ubiquitination of lysine-rich domains within the protein (32–34). Deletion of these regulatory domains in the oncoproteins, c-Jun and c-Fos, results in a more stable form of these oncoproteins. Conversely, the stability of MET4 is unaffected by ubiquitination; however, ubiquitinated MET4 fails to form functional transcription complexes, demonstrating that ubiquitination can repress function without degradation (41). Several lines of evidence suggest that MDM2 regulates p53 not only by ubiquitination but also by binding and masking the activation domain (42, 43). Progesterone receptor-mediated transactivation is potentiated in the presence of E6AP, an E3 ligase, demonstrating E3 ubiquitin ligase may function through multiple mechanisms in regulating gene expression by transcription factors (36). Regardless of whether proteins are targeted for degradation or not, ubiquitination plays a key role in cellular processes such as gene transcription.

Collectively, our data show that LKLF contains an inhibitory domain that functions by interacting with a repressive cofactor, WWP1. Removal of the inhibitory domain from LKLF results in a better transcriptional activator. Likewise, attachment of this domain to the strong viral activator VP16 suppresses VP16-mediated transactivation. Furthermore, overexpression of the inhibitory domain in conjunction with LKLF also potentiates LKLF-mediated transactivation. Each of these observations is consistent with the idea that the LKLF inhibitory domain functions at least in part by directly interacting with WWP1. Previously, other investigators have shown that LKLF degradation during T-cell development is preceded by an alteration in the electrophoretic mobility of the protein, suggesting that LKLF may undergo a protein modification such as ubiquitination that may target LKLF for degradation in T cells (4). In addition, we have established that both the amino- and carboxyl ends of the LKLF inhibitory domain are required for transcriptional repression. Both of these regions are rich in lysine residues, the target of ubiquitin ligase proteins. At this time, it is unclear whether these lysines are a target of ubiquitination or acetylation, but with the discovery that WWP1 binds LKLF, it is plausible that these sites are ubiquitinated. However, degradation alone cannot account for the ability of the inhibitory domain to attenuate VP16-mediated transactivation when the inhibitory domain is tethered to DNA near Lex A-VP16 but is not attached to Lex A-VP16. The inhibitory domain could have intrinsic inhibitory function in addition to providing a binding site for WWP1. Moreover, similar to p53 and MDM2, there may be multiple mechanisms by which WWP1 suppresses LKLF-mediated transactivation. The autoinhibitory domain represents a novel mechanism for regulating the function of this subfamily of transcription factors and may help understand the role of LKLF in the late stages of development.

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