



Published in final edited form as:

Int J Biochem Cell Biol. 2000 ; 32(11-12): 1103–1121.

The biology of the mammalian Krüppel-like family of transcription factors

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Abstract

Recent advances in molecular cloning have led to the identification of a large number of mammalian zinc finger-containing transcription factors that exhibit homology to the *Drosophila melanogaster* protein, Krüppel. Although the amino acid sequences in the zinc finger domains of these Krüppel-like factors (KLFs) are closely related to one another, the regions outside the zinc fingers of the proteins are usually unique. KLFs display seemingly different and broad biological properties with each functioning as an activator of transcription, a repressor or both. This review article provides a current phylogenetic classification of the identified KLFs to date. More importantly, the currently known biological activities of the KLFs in regulating transcription, cell proliferation, differentiation and development are summarized and compared. Further characterization of this interesting protein family should provide additional insights into their respective regulatory role in various important biological processes. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords

Activation; Gene expression; Krüppel-like factors; Repression; Transcription factors; Zinc fingers

1. Introduction

The regulation of tissue- and development-specific expression of eukaryotic genes is a fundamentally important process that represents the focus of research for numerous molecular biologists. A great deal of attention has been paid to the study of transcription factors, which directly participate in the regulation of gene transcription by interacting with *cis*-regulatory DNA elements in specific genes. Most transcription factors are classified based on the structural motifs that they use to bind to DNA (reviewed in Ref. [1]). The zinc finger motif is an important example of a DNA-binding motif. As the name implies, a zinc finger contains a single zinc atom, which serves as a critical structural component of the finger motif. The zinc atom is tetrahedrally coordinated by amino acids, such as cysteine and histidine. A frequently encountered zinc finger protein contains two cysteine and two histidine residues per finger and

is referred to as a C₂H₂ zinc finger. Each finger is a simple structure consisting of 25–30 amino acid residues that includes two β-pleated sheets in the amino terminal half and an α-helix in the carboxyl terminal half, held together at the base by the zinc atom. Many zinc finger transcription factors contain multiple fingers that are continuously aligned with one another, which fit in the major groove of DNA [1].

The C₂H₂ zinc finger motif has a remarkably conserved primary sequence. In the completely sequenced *Caenorhabditis elegans* genome, 138 proteins (0.7% of all proteins) contain the C₂H₂ motif [2,3]. In comparison, there are 352 (2.6%) C₂H₂ zinc finger proteins in the recently completed genome of *Drosophila melanogaster* [3,4]. It has been estimated that ≈1% of the human genome consists of genes encoding C₂H₂ type of zinc finger protein [5]. This would correspond to between 700 and 1000 genes encoding distinct zinc finger proteins [6]. It is therefore, not surprising that 434 matches of human zinc finger proteins were found in a recent search in the GenBank protein database for protein sequences related to the three C₂H₂ zinc fingers of the ubiquitous transcription factor, Sp1 [7].

A subset of C₂H₂ zinc finger proteins contains amino acid sequences that resemble those of the segmentation gene product of *D. melanogaster*, Krüppel [8]. In addition to the conserved amino acid sequence in the zinc finger, these proteins share a highly conserved seven-amino acid inter-finger spacer, TGEKP(Y/F)X, often referred to as a H/C link. Sp1, for example, exhibits homology to Krüppel. Many other transcription factors containing the Krüppel motif have been identified. Studies indicate that they play key roles in regulating a diverse range of biological processes, including cell growth, differentiation, embryogenesis and tumorigenesis. More recently, a family of Krüppel-like factors (KLFs) that are highly related to the Krüppel protein erythroid Krüppel-like factor (EKLF) [9] has been described [10,11]. This family is expanding rapidly such that since EKLF was first cloned in 1993, a total of 12 KLFs have been identified. The KLFs have been given numerical designations by the Human Gene Nomenclature Committee (HGNC) [12]. EKLF, for instance, is now KLF1. Despite the relatively short span of time since their discovery, this family of proteins has been shown to exhibit important tissue- or organ-specific regulatory functions. The present article will review the known biological activities of the KLFs identified thus far.

2. Phylogenetic classification of the human Krüppel-like factor family

Fig. 1 illustrates the phylogenetic relationship among the 12 human KLFs along with an additional six other Krüppel-like proteins that have not received a HGNC numerical designation. Table 1 provides information on the GenBank accession number, the Unigene number, the length of the polypeptide, and the chromosome localization, when known, for each of the 12 KLFs. All proteins in Fig. 1 contain three zinc fingers. With the exception of KLFs 9–11, the zinc fingers of the remaining nine KLFs are localized to the extreme carboxyl terminus and are followed by an additional one to three amino acid residues before the termination codon. It is also clear from the diagram that subfamilies, or clusters, of related proteins could be defined due to close homology. Thus, EKLF (KLF1), LKLF (KLF2), and GKLF (KLF4) are more closely related to one another than to other members of the Krüppel protein family. In fact, this close relationship has previously been documented based on a functional analysis of the motif required for nuclear localization of the different Krüppel proteins [13]. Another example is provided by the concurrent regulation of two closely related Krüppel-like factors, TIEG1 (KLF10) and TIEG2 (KLF11), by the transforming growth factor-β (TGF-β1) [14,15]. The latter example suggests that there are possibly functional, in addition to sequence, conservation within subfamilies of highly related Krüppel proteins.

3. The biology of mammalian Krüppel-like factors

3.1. Erythroid Krüppel-like factor (EKLF) (KLF1)

As the earliest identified KLF, EKLF represents a 'prototype' of mammalian Krüppel-like factors. Since its initial identification [9], a large body of literature has accumulated that describes the biochemical and physiological functions of EKLF (reviewed in Ref. [16]). EKLF was originally isolated by subtraction cloning of a cDNA library enriched for genes expressed in a mouse erythroleukemia cell line [9]. Expression of *EKLF* is predominantly restricted to cells of the erythroid lineage [9]. Moreover, EKLF binds to and activates a CACCC element in the promoter of the β -globin gene in humans and mice [9]. Because CACCC element is a functionally important and evolutionarily conserved *cis*-acting element of the β -globin gene as well as other erythroid cell-specific promoters and enhancers [17], EKLF is considered an important regulator of globin and other erythroid cell-specific gene expression. Indeed, EKLF has been shown to be critical for the cell-specific inducibility of the β -globin gene promoter and this activity is localized to the proline-rich transactivation domain of EKLF [18].

The biochemical mechanisms by which EKLF regulates transcription are well-established [16]. Human EKLF contains 362 amino acid (aa) residues [19], including three C_2H_2 zinc finger motifs near the very carboxyl (C) terminus. A short peptide sequence in the region immediately amino (N) terminal to the zinc fingers is very rich in basic aa residues. A similar basic region is found in the mouse GKLF, which was shown to be crucial for the nuclear localization of the protein [13]. The N-terminal half of EKLF, similar to GKLF and LKLF, is rich in proline (~ 15%), serine, threonine and acidic residues, all of which have been implicated in activation or repression of transcription [20]. EKLF binds strongly to the CACCC element in the β -globin promoter (5'-CCACACCCT) but to a much less extent to a similar element in the γ -globin promoter (5'-CTCCACCCA) [21]. One potential explanation for this difference is the presence of other neighboring *cis*-elements in the γ -, but not in the β -globin promoter that may prevent the recruitment of EKLF to the γ -globin promoter [22]. Added to the fact that *EKLF* is expressed at a higher level in adult erythroid tissue compared to the fetal tissue [21], it has been suggested that EKLF is involved in the human γ - to β -globin gene switch during development.

EKLF is an activator of transcription of the β -globin gene promoter [9]. The transactivation domain of EKLF has been localized to the N-terminal portion of the protein between aa residues 20 and 291 [18]. Further analyses of the mechanisms of transactivation by EKLF, however, indicate that they are far more complex than previously thought. Thus, the transactivation domain of EKLF contains distinct stimulatory and inhibitory subdomains [23]. The inhibitory subdomain resides between aa residues 196–291 and exerts its effect in *cis* by interfering with DNA binding [23]. In contrast, the stimulatory subdomain resides between aa residues 20–124, which coincides with an acidic aa-rich region of the protein that is also involved in interaction with other proteins [23]. Additional experiments suggest that either the conformation or phosphorylation status of this subdomain may be critical for such interactions [23]. Indeed, a casein kinase II (CKII) [24] site is present in the interaction domain of EKLF and can be phosphorylated by CKII [25]. Moreover, the ability of EKLF to transactivate gene expression has been shown to critically depend on the phosphorylation status of this CKII site [25].

An exciting recent advance in understanding EKLF's biochemical properties is in the area of chromatin remodeling. A clue to the overall contribution of chromatin structure to EKLF function came from the observation that the nucleosome structure in the mouse β -globin gene promoter is altered (remains in a closed configuration) in mice that are null for the EKLF allele when compared to their wild type littermates [26]. Using an in vitro chromatin assembly system, a protein complex named E-RC1 (for EKLF coactivator-remodeling complex 1) has been identified and shown to induce transcription of an assembled chromatin template in

collaboration with EKLK [27]. E-RC1 exhibits homology to the yeast SWI/SNF family of chromatin remodeling complexes, which are involved in the activation of transcription of a subset of genes [28]. The SWI/SNF complex functions in order to disrupt chromatin structure and facilitate binding of transcription factors to the remodeled chromatin [29,30]. Although the ultimate nature of E-RC1 remains to be defined, it is possible that EKLK's principal function as a transcriptional activator is to attract E-RC1 to the β -globin promoter.

Another important component of the EKLK transcription complex is a family of transcription co-activators that exhibits histone acetyltransferase (HAT) activity and modifies chromatin structures (reviewed in Ref. [31]). Thus, EKLK was shown to physically associate with HATs such as CBP, p300, and P/CAF [32]. Moreover, CBP and p300 acetylate at least two lysine residues in the inhibitory subdomain of EKLK immediately on the N-terminal side of the zinc fingers [23,32]. The interaction between EKLK and CBP/p300 leads to an enhancement of transcriptional activation of the β -globin promoter in erythroid cells [32]. Taken together, these observations indicate that EKLK is a tissue-specific transcription factor that undergoes post-translational modifications, including phosphorylation and acetylation and assists in the remodeling of chromatin structure by recruiting co-activator complexes to the β -globin gene cluster.

The *in vivo* function of EKLK can partially be elucidated by its pattern of expression during development [33]. The earliest detectable sign of *EKLK* expression in a developing mouse embryo is on embryonic day 7.5 (E7.5) in the primitive erythroid cells at the very beginning of blood island formation in the yolk sac. By E9, *EKLK* is expressed in the hepatic primordia and remains high in the developing liver, which becomes the sole source of EKLK mRNA in an E14.5 fetus. In the adult spleen, *EKLK* expression is strictly localized to the red pulp. These studies demonstrate that EKLK is a specific, early marker of erythroid differentiation and its expression in the adult is consistent with its requirement for later stage β -globin gene expression. More definitive proofs of the physiological functions of EKLK came from studies involving gene knockout. *EKLK*^{-/-} mice die in utero at E15 due to severe ineffective erythropoiesis [34,35]. The levels of β -globin mRNA in the fetal livers of *EKLK*^{-/-} mice are reduced 10-fold compared to heterozygous (*EKLK*^{+/-}) littermates, whereas the levels of α -globin mRNA are normal. In addition, the embryonic stage of hematopoiesis in the *EKLK*^{-/-} mice appears to be normal, including expression of the fetal and embryonic globin genes, ϵ and ξ [34,35]. It is not until hematopoiesis has been switched to the adult phenotype before the fatal anemia sets in. This stage-specific and β -globin-gene-specific requirement indicates that EKLK is necessary to facilitate the fetal-to-adult hemoglobin switch in mammals. The contribution of EKLK to red blood cell physiology is further documented by the inability of a human fetal A γ -globin transgene to rescue the abnormal red blood cells, therefore prenatal lethality, in *EKLK*^{-/-} embryos despite an apparent restoration of globin chain imbalance [36]. This last study suggests that other non-globin genes regulated by EKLK are essential for the normal progression of erythropoiesis.

3.2. Lung Krüppel-like factor (LKLK) (KLF2)

Using the zinc finger region of EKLK as a hybridization probe to screen a mouse genomic library, Lingrel et al. isolated a cDNA clone encoding a 354-aa polypeptide that they named LKLK [37]. Expression of the mouse LKLK gene is primarily found in the lung, heart and spleen [37]. The tissue distribution of the human LKLK transcript is similar to that of the mouse with the exception that the heart has a higher level of transcripts than the other organs [38]. Expression of *LKLK* is also developmentally regulated with expression first detected at E7 of the mouse embryo followed by a down-regulation at E11 and subsequent reactivation at E15 [37]. Similar to EKLK, the region of the protein outside its three zinc fingers has a high

percentage of proline residues. Lastly, LKLF is able to transactivate a human β -globin gene promoter containing a CACCC element [37].

To better understand the *in vivo* function of LKLF, gene knockout experiments have been performed. These studies yielded interesting and somewhat surprising results. LKLF-deficient ($-/-$) mice die *in utero* from severe intra-embryonic and intra-amniotic hemorrhaging between E12.5 and E14.5 of development [39,40]. This bleeding disorder is associated with specific defects in the morphology of blood vessels. Umbilical veins and arteries in the LKLF $^{-/-}$ embryos display an abnormally thin tunica media and aneurysmal dilatation before rupturing into the amniotic cavity. Similarly, vascular smooth muscle cells in the aorta from LKLF $^{-/-}$ animals fail to organize into a compact tunica media. These results indicate that LKLF is a necessary component in the assembly of the vascular tunica media and blood vessel stabilization during embryogenesis.

To circumvent the problem of embryonic lethality in LKLF-null mice, Lingrel et al. generated mouse aggregation chimeras by injecting LKLF $^{-/-}$ embryonic stem (ES) cells into blastocysts of wild type mice, and analyzed the contribution of LKLF-deficient cells to the formation of various internal organs [41]. In chimeric mice that survive after birth, LKLF $^{-/-}$ ES cells contribute significantly to all of the major organs except the lungs. Although some highly chimeric animals die at birth, histopathological examination of their lungs suggests an abnormality in lung development [41]. It is concluded that LKLF plays an important role in normal lung development.

Additional insights into the physiological functions of LKLF are provided by an independent study, also involving mouse aggregation chimeras. In addition to the aforementioned organs, *LKLF* expression is also found in lymphoid tissues [42]. Specifically, LKLF mRNA is present in both CD4 $^{+}$ and CD8 $^{+}$ single-positive (SP) quiescent T lymphocytes in the thymus and spleen, but absent in double-positive (DP) CD4 $^{+}$ CD8 $^{+}$ lymphocytes. Upon T cell receptor (TCR)-mediated activation of the quiescent splenic T cells, LKLF mRNA and protein are rapidly degraded, suggesting that LKLF play an important role in regulating the function of resting SP T cells *in vivo*. To address this issue and to circumvent the embryonic lethality in LKLF-null mice, Leiden et al. generated a model in which LKLF $^{-/-}$ ES cells are injected into recombina-activating gene 2-deficient (RAG2 $^{-/-}$) blastocysts to produce LKLF $^{-/-}$ RAG2 $^{-/-}$ chimeric mice [42]. Because mature B and T cells cannot develop in the absence of RAG2, all B and T cells in the chimeric mice are derived from the LKLF $^{-/-}$ ES cells. In control experiments, injection of LKLF $^{+/-}$ ES cells into RAG2 $^{-/-}$ blastocysts fully rescued the defects in B and T cell development in the RAG2 $^{-/-}$ animals. In contrast, chimeric mice produced by injection of RAG2 $^{-/-}$ blastocysts with LKLF $^{-/-}$ ES cells display profound peripheral T cell defects. Total splenic and lymph node T cell numbers are reduced by > 90%. In addition, the few LKLF $^{-/-}$ splenic and lymph node T cells display a constellation of cell surface markers that is characteristic of an activated phenotype, which leads to death from Fas ligand-induced apoptosis [42]. These results, therefore, indicate that LKLF is required to program the quiescent state of SP T lymphocytes and to maintain their viability in the peripheral lymph organs and blood. A more recent study showed that *LKLF* is reexpressed following culture of activated CD8 $^{+}$ T cells in certain cytokines (IL-2, IL-7) that are known to influence T cell development [43]. Evidence was presented that supports a role for the induction of *LKLF* reexpression in determining long-term T cell survival and development of memory T cells [43].

3.3. Basic Krüppel-like factor (BKLF) (KLF3)

In addition to EKLF, the CACCC element in the β -globin gene promoter interacts with a number of other proteins when incubated with nuclear extracts isolated from the murine erythroleukemia cell line, MEL [44]. One of these proteins is Sp1. To identify the remaining protein(s) that binds to CACCC, Crossley et al. screened a MEL cDNA library with a mixed

probe containing sequences in the zinc finger regions of EKLF and Sp1 [44]. Several of the identified positive clones encode a novel cDNA sequence, which is designated BKLF. The open reading frame of BKLF contains a protein of 344 aa that is subsequently shown to bind to the CACCC sequence in the β -globin promoter [44]. A recent report indicates that BKLF also binds to a CACCC element present in the promoter of the human C4 complement gene [45]. Similar to other KLFs, the region of BKLF outside the zinc fingers is rich in proline residues. However, a notable difference between BKLF and EKLF is the unusually high isoelectric point of 10.2 of BKLF, thus the name basic KLF, as compared to 7.0 for EKLF. The tissue distribution of BKLF in the adult mouse is also wider than that of EKLF and includes liver, lung, muscle and brain, in addition to hematopoietic tissues [44]. It is of interest to note that the level of BKLF is selectively reduced in fetal liver erythrocytes in mice lacking the EKLF gene due to knockout [44]. This suggests that BKLF may participate in the regulation of erythropoiesis in conjunction with EKLF. A preliminary report indicating that mice with BKLF knockout display abnormalities in hematopoiesis, albeit less severe than the EKLF^{-/-} mice, supports the notion that BKLF is involved in erythropoiesis [46].

BKLF can activate a reporter gene containing a CACCC element although the degree of activation is less than that caused by EKLF [44]. However, when the CACCC sequence is linked to a glucocorticoid response element (GRE), BKLF becomes a potent repressor of the reporter gene while EKLF remains a strong activator [47]. The repression domain is mapped to the N-terminal 74 aa of BKLF [47]. Using this region of BKLF as a bait in a yeast two-hybrid screen, Turner and Crossley isolated a cDNA clone encoding a co-repressor called C-terminal-binding protein 2 (CtBP2) [47]. A model of the mechanism of repression by BKLF and CtBP2 has recently been proposed [48]. Given that EKLF is highly expressed in late stages of erythroid development and that EKLF may be an activator of the BKLF gene, it is not surprising that BKLF is also highly expressed in similar stages. Relative to EKLF, BKLF binds to the CACCC element in the fetal γ -globin promoter with a much higher affinity [44]. It is, therefore, possible that one of the primary physiological functions of BKLF is the suppression of fetal and embryonic globin gene expression in adult tissues. This hypothesis is supported by the observation that there is an excess of fetal globin chains in the embryos of EKLF^{-/-} mice when the BKLF level is concomitantly decreased [34,35].

3.4. Gut-enriched Krüppel-like factor (GKLF) (KLF4)

In an effort to identify novel zinc finger-containing transcription factors with a role in regulating cell growth, Yang et al. screened a NIH 3T3 cDNA library at reduced stringency with the zinc finger portion of an immediate early transcription factor zif268 [49]. One of the positive clones contained a novel sequence, which was subsequently named gut-enriched Krüppel-like factor (GKLF) [50]. GKLF encodes a polypeptide of 483 aa and, similar to KLFs 1–3, contains three Krüppel-type zinc fingers in the very C-terminal end. The region immediately N terminal to the three zinc fingers is a 20-aa peptide containing a cluster of basic aa residues, which is essential for the nuclear localization of the protein [13]. Only two other KLFs, EKLF and LKLF, exhibit any degree of sequence homology to this basic region of GKLF, indicating that the three KLFs belong to a subfamily of closely related Krüppel proteins [13] (see Fig. 1).

The tissue distribution of GKLF is enriched for the gastrointestinal tract; hence the name gut-enriched KLF [50,51]. Expression of *GKLF* can also be found in a select number of other organs including the lung [50], testis [50], skin [52] and thymus [53], and in vascular endothelial cells [51]. In situ hybridization experiments indicate that expression of *GKLF* primarily occurs in the epithelial cells of the gut [50,52] and skin [52]. In these epithelial tissues, the GKLF transcript is primarily localized to the post-mitotic cells of the gut [50] and skin epithelium [52]. Similarly, *GKLF* expression has been correlated with maturation of thymus epithelial cells [53]. Collectively, these studies establish that expression of *GKLF* is primarily

associated with a terminally differentiated state of epithelial cells. This is supported by the observation that fetal *GKLF* expression is highest around E17 [52,53,55], a time during fetal development in which the gut, skin and thymus epithelial tissues undergo dramatic changes prior to maturation.

The biochemical properties of GKLF are well established. GKLF is able to interact with the CACCC element in the β -globin promoter [52,54]. Using an empirical approach, Shields and Yang identified a consensus DNA sequence to which GKLF binds [56]. This sequence is GC-rich and is similar but not identical to the binding site for Sp1. One of the naturally occurring *cis*-elements called the basic transcription element (BTE), found in the promoter and essential for the expression of a family of cytochrome P-450 drug-metabolizing genes [57], is highly related to the consensus binding sequence of GKLF and shown to interact with GKLF [58]. Indeed, GKLF can inhibit the promoter activity of one of the cytochrome P-450 genes, *CYP1A1*, in a BTE-dependent fashion [58]. This inhibition is due primarily to the ability of GKLF to physically compete with Sp1, a strong activator of the *CYP1A1* promoter [59], for binding to the BTE [58]. In contrast, other studies have shown that GKLF can act as an activator of transcription either by itself [51,56] or in conjunction with other Krüppel-like factors [60, 61]. GKLF is therefore a pleiotropic transcription factor with a context-dependent transcriptional activating or suppressing activity. A detailed characterization of the mechanism of transactivation by GKLF has recently been performed [62]. This study identified a number of acidic aa residues near the N-terminus that are crucial for the transactivating effect of GKLF [62]. In addition, GKLF physically interacts with the p300/CBP transcription co-activators through the same acidic residues and this interaction is necessary for the transactivating ability of GKLF [62]. In this regard, the mechanism of activation by GKLF is similar to EKLF [32].

The association of *GKLF* expression with a growth-arrested state *in vivo* has been demonstrated *in vitro* as well. Thus, in cultured NIH 3T3 cells, expression of *GKLF* is found in a growth-arrest state that is either induced by serum deprivation or contact inhibition [50]. In contrast, there is little or no *GKLF* expression in cells that are actively proliferating [50]. In cells rendered quiescent by serum deprivation then stimulated to reenter the cell cycle by serum addition, there is a temporal down-regulation of *GKLF* expression during a critical phase of DNA synthesis [50]. Conversely, forced expression of GKLF by transfection inhibits DNA synthesis [50]. These results suggest that GKLF is a potent negative regulator of cell growth. This conclusion is consistent with the finding that expression of *GKLF* is decreased in the intestine of a murine tumor model, the *Min* mouse, in which large numbers of intestinal adenomas form as a result of mutation of the tumor suppressor gene, *APC* [55]. More recently, it was shown that expression of *GKLF* is reduced in the intestinal adenomas of *Min* mice when compared to the surrounding normal mucosa, as well as in the colonic adenomas of patients with familial adenomatous polyposis, a hereditary colon cancer syndrome resulted from mutation in the *APC* gene [63]. Combined together, these observations strongly support a role of GKLF in regulating proliferation of the intestinal epithelial cells.

The involvement of GKLF in the control of the cell cycle is further supported by recent studies showing that *GKLF* is induced during cell cycle arrest due to DNA damage [64]. This induction is caused by the activation of the tumor suppressor, p53, and a consequence of this induction is the transcriptional activation of the gene encoding the cyclin-dependent kinase inhibitor, p21^{WAF/Cip1}, through a direct binding of GKLF to the p21^{WAF1/Cip1} promoter [64]. Overexpression of GKLF in the human colonic carcinoma cells, HT-29, also results in a reduction of cyclin D1 expression and that this reduction is due to a repressive effect of GKLF on the cyclin D1 promoter [65]. Moreover, treatment of HT-29 cells with interferon- γ inhibits proliferation and induces apoptosis, which is accompanied by a rise in the levels of GKLF mRNA [66]. It is of interest to note that the inductive effect of interferon- γ on *GKLF* expression, unlike that resulted from DNA damage, is independent of p53 [66]. Be that as it may, it is clear

that there appears to be two distinct classes of ‘target’ genes regulated by GKLf. One includes genes with functions primarily related to terminal differentiation such as CYP1A1 [58], keratin 4 [60] and keratin 19 [61]. The other group belongs to genes that are directly involved in the regulation of cell growth including p21^{WAF1/Cip1} [64], cyclin D1 [65], and even GKLf itself [67]. These studies suggest that GKLf may have a dually important function in regulating differentiation- and growth-specific gene expression in epithelial cells.

A hint of the physiological function of GKLf is provided by a recent study in knockout mice [68]. Mice null for the GKLf alleles are born normal but die within 24 h after birth due to a loss of skin barrier function [68]. This loss of barrier function occurs without significant morphological or biochemical alterations to the well-known structural features of epidermis that are essential for mechanical integrity. Instead, late stage differentiation structures are selectively perturbed, including the cornified envelope [68]. Although the guts of the GKLf^{-/-} mice are described to be normal, no histological or physiological information is provided to allow evaluation whether gastrointestinal function is perturbed due to the loss of GKLf.

3.5. Intestinal-enriched Krüppel-like factor (IKLF) (KLF5)

In an attempt to identify novel members of the Krüppel-like family of transcription factors, Lingrel et al. conducted a search of the expressed sequence tag database (dbEST) using the zinc finger region of LKLF [69]. Four overlapping cDNA clones were obtained that encode a 446-aa polypeptide, subsequently named IKLF [69]. Like *GKLf*, *IKLF* is primarily expressed in the gastrointestinal epithelium although its site of expression is toward the proliferating zone of crypt cells [69,70], as opposed to the more luminal expression of *GKLf* [50]. Thus, expression of *GKLf* and *IKLF* in the intestinal epithelium appears to be complementary, rather than redundant, to each other. The developmental expression of the two genes also seems to contrast each other. While expression of *GKLf* is higher in later stages of gestation (E16-E17), that of *IKLF* is higher in earlier stages (E7). Like GKLf, IKLF is able to bind to the CACCC element of the [β-globin promoter and activates a reporter gene linked to two copies of the CACCC sequence [69]. A human homologue of the mouse IKLF gene was recently identified based on its ability to bind to the epidermal growth factor response element (EGFRE) of the lactoferrin gene and found to be 93% identical [71]. Interestingly, this human IKLF represses expression of a reporter driven by the lactoferrin EGFRE [71].

It should be noted that IKLF is identical to a previously isolated protein called BTEB2 (for basic transcription element binding protein 2) with the exception that BTEB2 is only 219 aa in length [72]. It appears that the open reading frame of BTEB2 represents only a part of the IKLF as shown by Ohnishi *et al.* [70]. A cDNA clone encoding a rabbit homologue of BTEB2 has also been recently identified from a rabbit aorta smooth muscle cell line [73]. In that model, expression of the BTEB2 gene is highly induced in the neointimal smooth muscle cell of the rat aorta after balloon injury [73]. In cultured cells, the levels of BTEB2 mRNA are rapidly and persistently induced by treatment with phorbol ester and basic fibroblast growth factor [74]. This induction is mediated by the immediate early gene product Egr-1 through its binding to an Egr-1-binding site in the promoter of *BTEB2* [74]. It therefore appears that BTEB2 may be a mediator of the cellular responses to mitogenic stimulation.

3.6. Core promoter binding protein (CPBP)/GC-rich sites binding factor (GBF)/Zf9 (KLF 6)

Within the span of a year, three groups independently isolated cDNA clones encoding KLF6, which was given such different names as CPBP [75], GBF [76] and Zf9 [77]. Phylogenetically, KLF6 is most related to KLF7 [78] (Fig. 1). Human KLF6 contains 283 aa and is present in many tissues, including the placenta, heart, lung, liver and pancreas [75]. As an activator of transcription, KLF6 interacts with the core promoter element present in both TATA-less [75]

or TATA box-containing promoters [76]. The cellular 'target' genes of KLF6 identified to date include those encoding pregnancy-specific glycoprotein 5 (PSG5) [75], collagen $\alpha 1(I)$ [76], leukotriene C₄ synthase (LTC₄S) [79], urokinase plasminogen activator (uPA) [80], transforming growth factor (TGF)- $\beta 1$ [81], and types I and II TGF- β receptors [81]. In addition, KLF6 physically interacts with GSK3 β and the two coactivate the human keratin 4 promoter [60].

Although initially thought to be constitutively expressed in a diverse array of tissues, *KLF6* has recently been shown to be an inducible gene with characteristics resembling an immediate-early gene [80,82]. For example, the level of KLF6 protein increases significantly in liver stellate cells within 3 h of liver injury caused by CCl₄ [77]. Expression of *KLF6* is also transiently induced in 3T3-L1 preadipocytes stimulated to differentiate by various adipogenic hormones including serum, insulin, phorbol ester, and agents that elevate intracellular cyclic AMP levels [82]. Similarly, *KLF6* is induced by phorbol ester in bovine aortic endothelial cells [80]. These observations suggest that *KLF6* expression is regulated in a number of physiological processes and the induced production of KLF6 may mediate some of the subsequent physiological responses to these stimuli.

Studies by Friedman et al. have shed additional light on the function of KLF6. Activation of liver stellate cells is a physiological response to many forms of liver injury [83]. The activated stellate cells play a crucial role in the formation of extracellular matrix, which leads to subsequent liver fibrosis [83]. Induction of *KLF6* expression occurs rapidly during stellate cell activation and precedes induction of structural and cytokine genes, including collagens, platelet-derived growth factor (PDGF) receptor, and transforming growth factor- $\beta 1$ and its receptors [77]. Induction of *KLF6* occurs in distinct models of liver injury in vivo and in vitro, suggesting that the upregulation of *KLF6* is a general feature of stellate cell activation. The significance of this induction is further enhanced by the observation that KLF6 functions to activate the promoters of collagen $\alpha 1(I)$ [77], TGF- $\beta 1$ [81], and types I and II TGF- β receptors [81]. The combined expression of these down-stream genes may contribute to the process of hepatic fibrogenesis. Additional evidence supporting a role for KLF6 in mediating extracellular matrix homeostasis is provided by a recent study demonstrating the induction of *KLF6* in balloon-injured vascular endothelium [80]. A consequence of this induction is the KLF6-mediated transcriptional activation of the gene encoding urokinase plasminogen activator (uPA), which in turn causes an increased level of bioactive TGF- β via enhancement of proteolytic activation of latent TGF- β [80]. These studies suggest that KLF6 may have a wide range of physiological functions in mediating fibrogenic or fibrinolytic responses of various tissues to injury.

3.7. Ubiquitous Krüppel-like factor (UKLF) (KLF7)

Only one publication to date describes the identification and isolation of human ubiquitous Krüppel-like factor (UKLF) [78]. UKLF was originally identified during a search for novel Krüppel-like proteins expressed in endothelial cells using a PCR strategy designed to amplify EKLK-related sequences. After its cloning, it becomes clear that UKLF is present in almost all tissues surveyed, hence the name ubiquitous [78]. Like other KLFs, UKLF binds strongly to the CACCC element but weakly to the Sp1 element. In a manner similar to the mechanism of transactivation by GSK3 β [62], the activation domain of UKLF is localized to the 72 aa in the N-terminal portion of the protein in a region that is rich in acidic residues [78]. Among all the KLFs, UKLF is most related to KLF6 although the two genes are localized to different chromosomes [78].

3.8. Basic Krüppel-like factor 3 (BKLF3)/ZNF741 (KLF8)

Using the sequence for an expressed sequence tag, ZNF741, a cDNA clone named BKLF3 or KLF8 was isolated from the K562 cell line [84]. Like many KLFs, KLF8 is a CACCC-binding protein that exerts a repressive effect on a CACCC-dependent promoter [84]. At least part of this repressive effect can be attributed to the association of KLF8 with the co-repressor C-terminal binding protein (CtBP) [84]. It is interesting to note that all three closely related KLFs, KLF3, KLF8 and KLF12 (Fig. 1), contain a conserved motif that is used to contact CtBP [84]. This group of KLFs, therefore, is functionally conserved and behaves mostly as transcriptional repressors.

3.9. Basic transcription element-binding protein (BTEB) (KLF9)

Of the 12 KLFs, BTEB was the first identified [59]. Along with Sp1, BTEB was cloned based on its ability to bind to the basic transcription element (BTE), which is a single GC-box sequence in the promoter and necessary for the constitutive expression of the cytochrome P-450IA1 (CYP1A1) gene [85]. Human BTEB is 244 aa in length [86], the zinc fingers of which bind to BTE with an affinity equal to Sp1 [87]. In contrast to some of the other KLFs, such as EKLF and GKLF, the transcriptional activation domain of BTEB is localized to two regions that are rich in hydrophobic aa residues [88]. Interestingly, like GKLF, BTEB is a bifunctional transcription factor, capable of activating the expression of genes containing multiple GC box sequences in promoters such as the simian virus 40 early promoter, but repressing a BTE-containing promoter present in the CYP1A1 gene [59].

Although a BTEB-knockout model is not available, several recent studies have partially revealed its potential physiological functions. These studies suggest that BTEB has a regulatory role in diverse biological processes. For example, a recent study implicates BTEB in mediating collagen $\alpha 1(I)$ gene expression which is induced by acetaldehyde, the major active metabolite of alcohol, in rat liver stellate cells [89]. In this regard, BTEB is similar to Zf9, which also activates collagen $\alpha 1(I)$ gene expression in stellate cells during liver injury [77]. Since an excessive production of collagen matrix proteins is central to the pathogenesis of hepatic cirrhosis, which results from various forms of liver injury including those caused by alcohol, both BTEB and Zf9 appear to have fundamentally important roles in mediating the fibrotic responses in the liver. A different group provides evidence that BTEB may have a regulatory function in endometrial epithelial gene expression associated with pregnancy. First, BTEB is specifically localized to the porcine endometrial epithelium during different stages of pregnancy [90]. Second, in conjunction with progesterone receptor, BTEB is able to activate expression of the uteroferrin gene, which encodes a uterine endometrial secretory protein [91]. Although these studies are suggestive that BTEB may be involved in mediating hormone-regulated gene expression, they do not present direct evidence. In contrast, another recent study showed that *BTEB* expression is regulated by the thyroid hormone, T3, and that this regulation is specific to neuronal cells in the developing central nervous system [92]. Overexpression of *BTEB* in neuronal cells increases the number and length of neurites in a dose-dependent manner, suggesting that BTEB play a critical role in neural development.

3.10. Early growth response α (EGR α)/transforming growth factor- β -inducible early gene 1 (TIEG1) (KLF10) and transforming growth factor- β -inducible early gene 2 (TIEG2) (KLF11)

TIEG1/EGR α (KLF10) and TIEG2 (KLF11) are two highly related Krüppel-like factors (Fig. 1) (for a detailed recent review, see Ref. [93]). Human TIEG1 encodes a 480-aa ubiquitously expressed polypeptide. It was initially identified by differential display-polymerase chain reaction in a human osteoblastic cell line that had been treated with transforming growth factor- $\beta 1$ (TGF- $\beta 1$) [14]. In the same study, a select number of growth-stimulating factors other than TGF- $\beta 1$, including bone morphogenetic factor 2 (BMP2) and epidermal growth factor (EGF), have been shown to induce *TIEG1* gene expression [14]. A second group concomitantly

identified *TIEG1* as early growth response α ($EGR\alpha$) gene in several prostate cancer cell lines and showed that its expression is regulated by serum, phorbol ester, and EGF 8594. *TIEG1* and $EGR\alpha$ subsequently were confirmed to be encoded by the same gene [95]. In addition to being subjected to regulation by various growth factors, *TIEG1/EGR\alpha* expression is also influenced by various hormones. For example, the estrogen 17β -estradiol (E2) causes a rapid increase in expression of *TIEG1/EGR\alpha* in estrogen receptor (ER)-positive human fetal osteoblastic cells [96]. In contrast, androgens such as 5α -dihydroxytestosterone (DHT) inhibit expression of *TIEG1/EGR\alpha* in prostate cancer cell lines [94,97].

Consistent with its pattern of expression in response to growth factor and hormonal treatments, the presumed physiological function of *TIEG1/EGR\alpha* is its involvement in the regulation of cell growth. In synchronized prostate cells, for example, $EGR\alpha$ mRNA is highly expressed in the G1 phase of the cell cycle [94]. Similarly, the rapid induction in *TIEG1/EGR\alpha* expression in human fetal osteoblastic cells by estrogen is correlated with an estrogen-induced inhibition of DNA synthesis in the same cells [96]. A converse relationship between *TIEG1/EGR\alpha* expression and the state of cell growth is also illustrated by a decreased *TIEG1/EGR\alpha* mRNA level in breast cancer cells as compared to normal breast epithelium [98]. The expression of *TIEG1/EGR\alpha* has also been shown to be similarly decreased in prostate cancers compared to normal prostate [99]. Taken together, these studies are highly suggestive of an anti-proliferative nature of *TIEG1/EGR\alpha* activity.

TGF- β 1 is a potent inhibitor of cell growth in many different cell types, in part by causing apoptosis [100–102]. The fact that *TIEG1* expression is induced by TGF- β 1 suggests that *TIEG1* may be involved in the regulation of apoptosis. Indeed, overexpression of *TIEG1* in a TGF- β 1-sensitive pancreatic cell line, PANC1, led to the induction of apoptosis [103]. A similar apoptotic-promoting effect by *TIEG1* has been documented in another epithelial cell line of lung origin, Mv1Lu [104]. More recently, the mechanisms by which *TIEG1* causes apoptosis was defined in further detail in the liver cell line, Hep 3B, that has been rendered to undergo apoptosis by TGF- β 1 [105]. In these cells, the induction in *TIEG1* expression by TGF β 1 precedes that of any morphological features of apoptosis. The expression of genes encoding some of the classical apoptotic proteins, such as Bax and Bcl-X_L, does not change after treatment with TGF- β 1. Instead, TGF- β 1- and *TIEG1*-induced cell death are accompanied by an increase in the generation of reactive oxygen species and a loss of mitochondrial membrane potential preceding the morphological changes of apoptosis [105]. This mechanism by which *TIEG1* induces oxidative stress is reminiscent of the mechanism of action by p53 to generate reactive oxygen species. p53-Induced apoptosis is accompanied by the transcriptional induction of redox-related genes [106]. It is proposed that these gene products form reactive oxygen species, which lead to oxidative mitochondrial damage. Due to the similarities between *TIEG1*- and p53-dependent apoptosis, it is possible that *TIEG1* expression may also cause the transcriptional induction of redox-related genes.

A closely related cDNA to *TIEG1*, called *TIEG2* (KLF11), was recently identified based on the homology to *TIEG1* [15]. *TIEG2* shares 79% amino acid identity with *TIEG1* in the zinc finger region and 40% identity outside the zinc fingers. Similar to *TIEG1*, the tissue expression of *TIEG2* is ubiquitous but with an enrichment in pancreas and muscle [15]. Moreover, expression of *TIEG2* is similarly induced by TGF- β 1 and that its overexpression inhibits cell growth [15]. It is of interest to note that both *TIEG1* and *TIEG2* are repressors of transcription [15] and that their repressive activities are localized to three conserved repressor domains in the two proteins [107]. Combined together, these findings highly suggest that *TIEG1* and *TIEG2* are two closely related Krüppel-like proteins with similar physiological roles in mediating apoptosis caused by TGF- β 1.

3.11. AP-2rep (KLF12)

AP-2rep (KLF12) was first identified by its ability to bind to a crucial *cis*-element in the promoter of the gene encoding AP-2 α , an important mammalian transcription factor [108]. This element confers autoregulatory activity with a core consensus binding site for AP-2 and was found to interact with three different transcription factors, AP-2rep, BTEB (KLF9), and AP-2 [108]. Whereas BTEB and AP-2 are both activators of the AP-2 α promoter, AP-2rep is a potent repressor of the promoter activity [108]. There is also an excellent correlation between induction of AP-2rep mRNA expression and downregulation of AP-2 α mRNA during development of the kidney [108]. Moreover, AP-2rep is able to suppress the endogenous AP-2 α gene expression and is inversely negatively regulated by AP-2 α [109]. These findings therefore point to a role for AP-2rep as a transcriptional silencer and reveal reciprocal regulation of AP-2 α and AP-2rep.

4. Why so many related Krüppel-like factors?

It was merely 15 years ago when the zinc finger was first proposed to represent a stably folded structural domain that involves the binding of zinc to conserved cysteine and histidine residues in the repeated sequences of the *Xenopus* transcription factor TFIIIA [110,111]. At the time, Klug et al. noted that “it would not be surprising if the same 30 residue units were found to occur in varying numbers in other related gene control proteins” [110]. Indeed, the zinc finger motif proves to be one of the most prevalent structures in biology. As stated in Section 1, it has been estimated that human may have close to 1000 distinct C₂H₂ zinc finger protein-encoding genes, accounting for \approx 1% of the genome [6]. A significant portion of these proteins contains the Krüppel motif, making the 18 KLFs in Fig. 1 only a small fraction of all zinc finger proteins. An obvious question is why so many zinc finger proteins are in existence in the human genome. One possible answer may rest on the remarkably stable nature of the zinc finger structure, as demonstrated by numerous crystallographic studies (see for example Ref. [112]). The lack of redox activity of zinc and its characteristic binding kinetics make it an especially suitable metal ion to stabilize a peptide structure [113]. The tetrahedral coordination of zinc then facilitates the interactions between proteins and DNA. Moreover, since the zinc finger is modular in nature, the specificity of DNA sequence recognition can be modified by the specific residues in the finger used to contact DNA and by the number of repeats each protein may contain.

It is clear that the Krüppel-like factors described in this articles share a high degree of similarity in the DNA sequences to which they recognize. However, there appears to be two loosely divided subgroups of sequences, based on the DNA binding affinity for each KLF. The Sp family of proteins favor the classical GC-rich Sp1-binding box [114], whereas the remaining KLFs favor the CACCC-, or GT-, element. As both sequences are found in a relatively high frequency in the regulatory regions of various mammalian genes, it is not unreasonable to assume that the KLFs play an important role in the regulation of expression of myriad genes. The significance of these *cis*-sequences in modulating gene expression is underscored by their frequent presence in promoters containing CpG-islands that are subject to DNA methylation, an important mechanism for regulating cell growth and development [115]. The versatility of the DNA-protein interactions is further enhanced by the presence of certain *cis*-elements in the promoters of some genes that appear to have evolved to exhibit strong affinities for both subgroups of KLFs. For example, while GKLf binds strongly to a GC-rich, empirically determined consensus sequence, it binds much weaker to a classical GC-rich Sp1-binding sequence [56]. However, both GKLf and Sp1 bind with high affinity to the basic transcription element (BTE) [55], a single GC-box sequence present in the promoters of a family of cytochrome P450 genes, including *CYP1A1* [85]. It is of interest to note that the BTE resembles a composite sequence between the empirically determined GKLf-binding site and the classical

Sp1-binding site. Perhaps this is one mechanism by which regulatory sequences evolve in which to attract the participation of DNA-binding proteins with different sequence preferences.

Another reason for the existence of multiple KLFs with similar recognition sequences is the diverse biochemical mechanisms by which these proteins function. While some KLFs are potent activators of transcription, such as Sp1 [116], some are repressors of transcription, like KLF12 [108]. Some KLFs are bifunctional, acting as activators or repressors, depending on the circumstances, as exemplified by GKLF [51,58,62]. The mechanisms by which the KLFs modulate transcription are also diverse and may depend on the presence of intrinsic activation or repression domains, competition or cooperation with other KLFs, and interactions with co-activators or co-repressors. It is, therefore, not unreasonable to assume that the purpose of such a large repertoire has important biological implications, and may allow the fine-tuning of gene expression under various physiological conditions.

5. Conclusion

This article summarizes the biology of 12 mammalian Krüppel-like factors, the majority of which were discovered within the last 5 years. There is significant conservation in several aspects among the members of this family. For example, they share a high degree of sequence homology in the zinc finger regions. In addition, the zinc fingers of most of the KLFs are located to the extreme carboxyl terminal end of the proteins, which suggests that they may have originated from the same ancestral gene. All KLFs bind to a similar DNA sequence that has a CACCC homology or is rich in GC-content. As a result, there is a certain degree of overlap in the target genes that the KLFs regulate. In fact, it is not unusual for several KLFs to interact with the same *cis*-element in the same gene and perhaps with each other. Lastly, each of the KLFs appears to exert important regulatory functions on many biological processes, such as growth, development, differentiation and apoptosis. Despite these similarities, the majority of KLFs seem to have unique tissue-specific roles in an *in vivo* setting. Some of these roles have begun to be elucidated primarily by *in vivo* experiments involving gene knockout. It is anticipated that within the next few years, there will be further experimentation on these proteins that is likely to provide additional insights into the mechanisms of action of the KLFs and their physiological relevancy.

Acknowledgements

This work was in part supported by Grants DK10020, DK52230 and CA84197, from the National Institutes of Health, USA.

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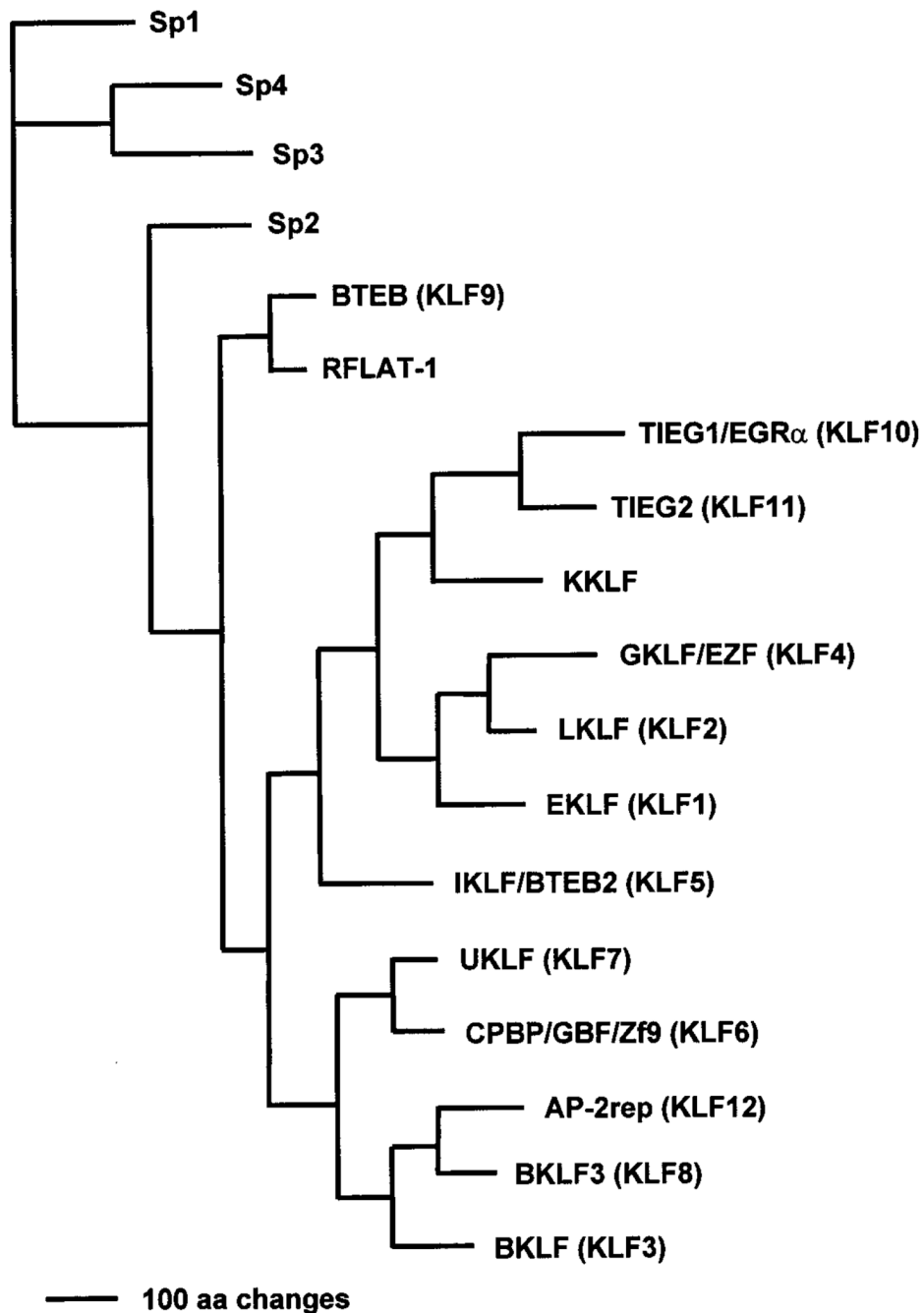


Fig. 1. Classification of related human Krüppel-like factors. Human KLF1 and related proteins were identified by BLAST searching, then multiple aligned using the PileUp program of GCG (Genetics Computer Group version 9.0, Madison, WI). The relationship of 18 Krüppel-like proteins was visualized as an unrooted phylogram using a heuristic search with Phylogeny Analysis Using Parsimony (PAUP version 4.0b3a; Sinauer Associates, Sunderland, MA). The horizontal bar in the left lower corner of the figure indicates 100 amino acid changes. Branch lengths are proportional to amino acid changes. For keys to abbreviations, see Table 1. RFLAT-1 and KKLf have not received a HNGC nomenclature. RFLAT-1 is RANTES (regulated upon activation, normal T cells expressed and secreted) factor of late activated T

lymphocytes-1 [117]. Its gene is expressed in T cells 3 days after activation and coincides with RANTES expression. KKLf represents kidney-enriched Krüppel-like factor whose identification was only recently described [118]. It functions to repress transcription of two kidney-specific chloride channel genes by blocking the activating effect of another transcription factor, MA2 [118].

Table 1

The 12 mammalian Krüppel-like factors^a

HGNC ^b name	Gene	# AA ^c	Accession No. (protein)	Unigene No.	Chromosome localization	References
KLF1	EKLF	362	Q13351	Hs.37860	19p13.12–p13.13	[9]
KLF2	LKLF	355	NP_006066	Hs.107740	19p13.11–p13.13	[37]
KLF3	BKLF	345	BAA92271	Hs.129026	ND ^d	[44]
KLF4	GKLF/EZF	470	NP_042226	Hs.182965	9q31	[50,52,54]
KLF5	BTEB2/IKLF	457	AAF18307	Hs.84728	ND	[69,71,72]
KLF6	CPBP/Zf9/GBF	283	AAC39929	Hs.4055	10p15	[75,76]
KLF7	UKLF	302	NP_003700	Hs.21599	2q32	[78]
KLF8	BKLF3	359	NP_009181	Hs.25442	Xp11.21	[84]
KLF9	BTEB	244	NP_001197	Hs.150557	ND	[59]
KLF10	TIEG1/EGR α TF	480	NP_005646	Hs.82173	8	[14,93]
KLF11	TIEG2	512	NP_003588	Hs.193776	ND	[15]
KLF12	AP-2rep	402	NP_009180	Hs.23510	13q22	[108]

^a EKLF, erythroid Krüppel-like factor; LKLF, lung Krüppel-like factor; BKLF, basic Krüppel-like factor; GKLF, gut-enriched Krüppel-like factor; EZF, epithelial zinc finger; BTEB2, basic transcription element binding protein 2; IKLF, intestinal-enriched Krüppel-like factor; CPBP, core promoter-binding protein; Zf9, Zinc finger 9; GBF, GC-rich sites binding factor; UKLF, ubiquitous Krüppel-like factor; BTEB, basic transcription element binding protein; TIEG1, TGF- β -inducible early gene 1; EGR α TF, early growth response α transcription factor; TIEG2, TGF- β -inducible early gene 2; AP-2rep, AP-2 repressor.

^b Human Gene Nomenclature Committee.

^c Amino acid.

^d Not determined.