Human EZF, a Krüppel-like Zinc Finger Protein, Is Expressed in Vascular Endothelial Cells and Contains Transcriptional Activation and Repression Domains*

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Members of the erythroid Krüppel-like factor (EKLF) multigene family contain three C-terminal zinc fingers, and they are typically expressed in a limited number of tissues. EKLF, the founding member, transactivates the β-globin gene as demonstrated by gene deletion experiments in mice. Using a DNA probe from the zinc finger region of EKLF, we cloned a cDNA encoding a member of this family from a human vascular endothelial cell cDNA library. Sequence analysis indicated that our clone, hEZF, is the human homologue of the recently reported mouse EZF and GKL. hEZF is a single-copy gene that maps to chromosome 9q31. By gel mobility shift analysis, purified recombinant hEZF protein bound specifically to a probe containing the CACCC core sequence. In co-transfection experiments, we found that sense but not antisense hEZF decreased the activity of a reporter plasmid containing the CACCC sequence upstream of the thymidine kinase promoter by 6-fold. In contrast, EKLF increased the activity of the reporter plasmid by 3-fold. By fusing hEZF to the DNA-binding domain of GAL4, we mapped a repression domain in hEZF to amino acids 181–388. We also found that amino acids 91–117 of hEZF confer an activation function on the GAL4 DNA-binding domain.

It has been estimated that 10% of the proteins within a cell are DNA-binding transcription factors that regulate important cellular processes such as cell lineage determination, growth and differentiation, and temporal or cell type-specific gene expression (1–3). After binding to cognate cis-acting elements, these transcription factors either activate or repress initiation of transcription (4, 5). Transcription factors are grouped into several classes, which include the helix-loop-helix, leucine zipper, homeodomain, and zinc finger protein families (2).

The zinc finger transcription factors can be classified further into subfamilies on the basis of the sequence and position of amino acid residues important for zinc binding (Cys2-His2, Cys4, or Cys3-His1), the spacing between the zinc-binding amino acids, and the transcription activation or repression domains (glutamine-rich, acidic, or proline-rich domains) (6–10). A new zinc finger subfamily was identified recently whose members are characterized by a highly conserved C-terminal region containing three Cys2-His2 zinc fingers and a proline rich N-terminal domain (8, 10–13). Members of this subfamily include the erythroid (EKLF), lung (LKF), and basic (BKL) Krüppel-like factors, and BTEB2 (or placental Krüppel-like factor). All four factors transactivate gene expression after binding to DNA.

The founder of this family, EKLF, was originally isolated as an erythroid cell-specific factor by subtractive cloning (8). It binds and transactivates via the CACCC site of the β-globin gene promoter (8, 9, 14). In vitro, EKLF plays an important role in human γ-globin to β-globin gene switching (11). This observation is consistent with data showing that disruption of the EKLF gene by homologous recombination in mice results in defective hematopoiesis in the fetal liver and lethal β-thalassemia (15, 16).

The other members of the EKLF family, LKLF, BKL, and BTEB2, were isolated by homology screening with the zinc finger regions of EKLF, Sp1, and BTEB (a GC box-binding zinc finger protein) (10, 12). LKLF is expressed highly in the lung and the spleen and transactivates the β-globin gene via the CACCC site (10). Although BKL is also expressed in hematopoietic precursor cells, its expression is less restricted than that of EKLF (13). Also, even though BTEB2 was isolated from a placental library with a BTEB probe, the BTEB2 zinc finger region is more homologous to the zinc finger region of EKLF than it is to that of BTEB or Sp1 (12).

To identify new members of the EKLF family that may be involved in the regulation of vascular endothelial cell function, we used the zinc finger region of EKLF to screen a human vascular endothelial cell cDNA library. We isolated a member of the EKLF family and found it to be the human homologue of mouse EZF and GKL (17, 18). Mouse EZF/GKL has been shown to be a nuclear protein. Its mRNA is expressed highly in...
quiescent fibroblasts. The growth-arresting nature of EZF/GKLF was demonstrated by its ability to inhibit DNA synthesis in cells that overexpress the gene (17). By in situ analysis, the mouse homologue was shown to be expressed at high levels in epithelial cells of the epidermis, tongue, palate, esophagus, stomach, and colon (18).

We show in this report that the human homologue (hEZF) is expressed in vascular endothelial cells of an endodermal origin, in contrast to the ectodermal origin of the mouse homologue in epithelial cells. We also demonstrate that purified, recombinant full-length hEZF protein binds specifically to a probe containing the CACCC core sequence in gel mobility shift assays. In contrast to other members of the family, which are transcriptional activators, hEZF functions as a transcriptional repressor, as demonstrated by its ability to repress reporter gene activity in transient transfection assays. By gene fusion experiments, we identified both the activation domain and the repression domain within hEZF.

EXPERIMENTAL PROCEDURES

Cloning of hEZF—A cDNA probe encoding the C-terminal zinc finger region of EKLF (bp 956–1146) was generated by reverse gene polymerase chain reaction (PCR) (19, 20). The forward primer (5'-GAACCTTGCCACTTAAAGGCAG-3') and reverse primer (5'-ACGCTCATGTGCAAGCTAAGTG-3') were designed according to the published sequence (8). The DNA fragment was labeled by random priming (Stratagene, La Jolla, CA) and used as a probe to screen a human umbilical vein endothelial cell cDNA library. Approximately 1.6 million phages were plated, transferred to nitrocellulose, and screened according to standard techniques with minor modification (20). The filters were washed initially with 0.5 × SSC (75 mM sodium chloride, 7.5 mM sodium citrate) and 0.1% SDS (sodium dodecyl sulfate) at 37 °C and then more stringently with 0.2 × SSC and 0.1% SDS at 65 °C. More than 40 clones were obtained that hybridized differentially. Six were isolated, three were sequenced, and one was characterized further. It included the entire coding region of hEZF. The cDNA was mapped by restriction digestion and sequenced from both orientations by the dyeoxy chain termination method with Sequenase version 2 (Amer sham, Arlington Heights, IL) or on an automated DNA Sequencer (Licor, Lincoln, NE) according to the manufacturer’s instructions. Sequence analysis was performed using the GCG software package (Genetics Computer Group, Madison, WI). Southern Blot Analysis and Chromosomal Localization of hEZF—High molecular weight genomic DNA was prepared from cultured human aortic endothelial cells (21). Genomic DNA (10 μg) was digested with several restriction enzymes, fractionated on 0.8% agarose, and screened according to standard techniques with minor modification (20). The ratio of CAT activity to β-galactosidase activity was assayed as described (22). The ratio of CAT activity to β-galactosidase activity in each sample served as a measure of normalized CAT activity.

RESULTS

Isolation and Characterization of the hEZF cDNA—To identify additional members of the EKLF family that may be involved in the regulation of vascular endothelial cell function, we screened a human umbilical vein endothelial cell cDNA library using a DNA probe containing the zinc finger region of EKLF under low-stringency conditions. One of the cDNAs isolated contained 1876 nucleotides and a deduced open reading frame coding for a 470-amino acid protein with an estimated pl of 9.2. Analysis of the amino acid sequence revealed three Cys 3-His 3- krüppel-type fingers at the C terminus, a proline- and serine-rich N terminus, and a potential nuclear localization signal at amino acids 371–377 (Fig. 1). A single transcript of 3.5 kilobases was detected by Northern blot analysis with the zinc finger region of EKLF under low-stringency conditions. One of the cDNAs isolated contained 1876 nucleotides and a deduced open reading frame coding for a 470-amino acid protein with an estimated pl of 9.2. Analysis of the amino acid sequence revealed three Cys 3-His 3- krüppel-type fingers at the C terminus, a proline- and serine-rich N terminus, and a potential nuclear localization signal at amino acids 371–377 (Fig. 1). A single transcript of 3.5 kilobases was detected by Northern blot analysis with the zinc finger region of EKLF under low-stringency conditions. One of the cDNAs isolated contained 1876 nucleotides and a deduced open reading frame coding for a 470-amino acid protein with an estimated pl of 9.2. Analysis of the amino acid sequence revealed three Cys 3-His 3- krüppel-type fingers at the C terminus, a proline- and serine-rich N terminus, and a potential nuclear localization signal at amino acids 371–377 (Fig. 1). A single transcript of 3.5 kilobases was detected by Northern blot analysis with the zinc finger region of EKLF under low-stringency conditions. One of the cDNAs isolated contained 1876 nucleotides and a deduced open reading frame coding for a 470-amino acid protein with an estimated pl of 9.2. Analysis of the amino acid sequence revealed three Cys 3-His 3- krüppel-type fingers at the C terminus, a proline- and serine-rich N terminus, and a potential nuclear localization signal at amino acids 371–377 (Fig. 1).
characterization of hEZF activation and repression domains

1. MAVDALLPSFTFAFAGPSKRTLQPGAKPRRWSRELKSHKMKLPFPVLP
   Mouse
   MAVDALLPSFTFAFAGPSKRTLQPGAKPRRWSRELKSHKMKLPFPVLP
   51
   Human
   A9PFL KỳAAATVQLEASAGACAGCGCTSMDFLPLVEPPEFLPOLLNMLDFTL
   Mouse
   M9PFL KYAAATVQLEASAGACAGCGCTSMDFLPLVEPPEFLPOLLNMLDFTL
   121
   Human
   SNSmH PRSVASXVSSsSASSSSSSGSAPASSCGTVYFAAGND
   Mouse
   SNSmH CRESVXVXSSSASSSSSSGSAPASSCGTVYFAAGND
   151
   Human
   HCVWFG3LYWLRASPPAPPFLDLIDVPSQGVAVLLRFLD
   Mouse
   HCVWFG3LYWLRASPPAPPFLDLIDVPSQGVAVLLRFLD
   201
   Human
   PTVY1FQQPCQDFQ033MLVFXKLSAPSGXVQSIYVSPVYSGFPGSN
   Mouse
   PTVY1FQQPCQDFQ033MLVFXKLSAPSGXVQSIYVSPVYSGFPGSN
   251
   Human
   PTVVAFVNGDFRKRCPKIEQAVSCTC....HLGACPLLENHRPAA
   Mouse
   PTVVAFVNGDFRKRCPKIEQAVSCTC....HLGACPLLENHRPAA
   301
   Human
   HOFGLQFQSPFRFPLGLVLVSELSRSCZFLPLPKFEPHGFQTSPFL
   Mouse
   HOFGLQFQSPFRFPLGLVLVSELSRSCZFLPLPKFEPHGFQTSPFL
   341
   Human
   HPQMFQCVPTPLQYEMPSGGCMLPPESRKGRSHRRKNTATHTAT
   Mouse
   HPQMFQCVPTPLQYEMPSGGCMLPPESRKGRSHRRKNTATHTAT
   391
   Human
   HTRFQPCQCPFCFARFrGSHLGCWWXVQVFRVFRFPL
   Mouse
   HTRFQPCQCPFCFARFrGSHLGCWWXVQVFRVFRFPL
   441

Fig. 1. Comparison of human and mouse EZF amino acids. hEZF amino acid numbers are indicated at the top of the sequence. The cysteine and histidine residues of the three zinc fingers (boxed) are highlighted in white type on a black ground.

Chromosomal Localization of the hEZF Gene—Hybridization of an hEZF cDNA probe with human genomic DNA that had been digested with BamHI, EcoRI, and PstI revealed a simple pattern of hybridization, indicating that hEZF is a single-copy gene in the human genome. To map the chromosomal location of hEZF, we carried out genomic PCR analysis against a GeneBridge 4 radiation hybrid panel with specific primers from the hEZF cDNA sequence. The results from the genomic PCR experiments were analyzed against a human genome data base of sequence-tagged sites at the Whitehead Institute/MIT Center for Genome Research worldwide web site. The human EZF gene mapped to chromosome 9q31. Thioredoxin and the disease locus TAL2 (T-cell acute lymphocytic leukemia-2) have been mapped to the same locus.

Binding of Recombinant hEZF to the CACCC Site of the β-Globin Gene—The high degree of sequence conservation among the zinc finger regions of EZF, EKLF, and LKLF suggests that hEZF may also bind to the CACCC sequence. Gel mobility shift analysis was performed with purified recombinant full-length hEZF protein and an oligonucleotide probe encoding a CACCC site derived from the β-globin gene (8). Incubation of hEZF with the probe resulted in a DNA-protein complex (Fig. 2). This complex was specific because it was competed away by an unlabeled identical probe but not by an unrelated probe. Mutation of the core CACCC sequence to CACCG has been shown to obliterate the binding and transactivation of EKLF (9). In our analysis (Fig. 2), an unlabeled probe with this single base mutation failed to compete for binding, indicating that hEZF binds specifically to the CACCC site.

hEZF Represses Transcription in Transient Transfection Experiments—All members of the EKLF family identified before hEZF function as transcriptional activators. In particular, EKLF, LKLF, and BKLF have been shown to transactivate reporter plasmids via the CACCC site (8–10, 13). Because hEZF bound to the CACCC site, we decided to determine the effect of hEZF on a CAT reporter plasmid (pCAC-tkCAT) that contains a single copy of the β-globin CACCC site upstream of the minimal thymidine kinase promoter (8). Cotransfection of pcDNA3-hEZF decreased the promoter activity of pCAC-tkCAT in a dose-dependent manner in BAEC (Fig. 3A). A 10 to 1 expression plasmid to reporter plasmid ratio resulted in a 6-fold repression. This repression was specific because cotransfection of the antisense plasmid pcDNA3-hEZF(AS) had no effect on activity. In contrast, cotransfection of pcDNA3-EKLF increased CAT activity by 3-fold in BAEC (Fig. 3B). These results demonstrate that hEZF functions as a transcriptional repressor in our transient transfection system.

hEZF Contains Transcriptional Activation and Repression Domains—To identify domains in hEZF that may mediate its transcriptional effect, we generated a series of plasmids containing various fragments of hEZF fused to the DNA-binding domain of the yeast transcription factor GAL4 (Fig. 4). The fusion plasmids were cotransfected with a reporter construct containing five GAL4-binding sites in front of the thymidine kinase minimal promoter (pGAL45tkCAT). The GAL4-hEZF plasmid containing hEZF amino acids 2–470 had little effect on reporter activity. In contrast, the plasmid coding for amino acids 2–388 (from which the three zinc fingers had been removed) increased transcription by 25-fold (Fig. 4). These data indicate the presence of a potential activation domain between amino acids 2 and 388 of hEZF (from which the three zinc fingers had been removed) increased transcription by 25-fold (Fig. 4B). These data indicate the presence of a potent activation domain between amino acids 2 and 388 of hEZF that is inhibited by the presence of the zinc finger domain. The ability to transactivate was retained when the N-terminal 90 amino acids were deleted (GAL4-hEZF (AS) had no effect on activity). In contrast, cotransfection of pcDNA3-EKLF increased CAT activity by 3-fold in BAEC (Fig. 3B). These results demonstrate that hEZF functions as a transcriptional repressor in our transient transfection system.

hEZF contains transcriptional activation and repression domains. The GAL4-hEZF constructs coding for hEZF amino acids 2–117 or amino acids N-terminal of the zinc fingers (GAL4(292–388)) or the zinc finger region alone (GAL4-hEZF(386–470)) did not affect CAT activity. These N-terminal deletion experiments mapping the hEZF activation domain to amino acids 91–114 (Fig. 4B) are supported by a series of C-terminal deletion experiments (Fig. 4B). GAL4-hEZF constructs coding for hEZF amino acids 2–117 or
2–180 were able to increase transcription by more than 40-fold. To determine whether the hEZF activation domain was modular, we made a fusion construct containing amino acids 91–117 of hEZF and the GAL4 DNA-binding domain. GAL4-hEZF(91–117) increased transcription by more than 30-fold (Fig. 4B), indicating that a potent modular activation domain is located between amino acids 91 and 117.

The diminished transcriptional activity of GAL4-hEZF(114–388) in comparison with that of GAL4(1–147) (Fig. 4B) suggested the presence of repression domains C-terminal of amino acid 114. Furthermore, the enhanced activation obtained with deletions GAL4-hEZF(2–180) and GAL4-hEZF(2–117) over that obtained with GAL4-hEZF(2–388) (Fig. 4B) is consistent with the loss of a domain important for repression. To identify the repression domain(s) in hEZF, we generated additional plasmids and assayed their effect on the pGAL45tkCAT reporter. Plasmids containing hEZF amino acids 140–388, 163–388, and 181–388 repressed transcription by 4–5-fold (Fig. 5). However, plasmids containing segments C-terminal of amino acid 240 showed no repression activity. The region containing the zinc fingers alone (GAL4-EZF(386–470)) or that containing the fingers in conjunction with the 47 amino acids N-terminal of them (GAL4-EZF(342–470)) did not repress transcription. Our data from the N-terminal deletion analysis suggest that hEZF amino acids 181–240 may contain the repression domain.

To further define the C-terminal border of this repression domain, we generated GAL4-fusion constructs containing hEZF amino acids 181–325 and 178–244. Neither construct repressed transcription (Fig. 5). Thus, the repression domain of hEZF appears to be contained within amino acids 181–388.

DISCUSSION

Using the zinc finger region from EKLF as a probe to screen a human endothelial cell cDNA library, we have isolated hEZF, a new member of the EKLF multigene family. hEZF maps to chromosome 9q31, close to the T-cell acute lymphocytic leukemia-2 disease locus. Further investigation will be required to determine whether hEZF is related to this disease. Although it has been shown that the zinc finger region of hEZF binds to DNA fragments containing the CACCC motif (18), our experiments are the first to show that the full-length hEZF protein binds to this sequence (Fig. 2). It has been shown that all
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previously known members of the EKLF family function as transcriptional activators: EKLF, LKLF, and BKLF activate transcription via the CACCC site of the β-globin promoter, and BTEB2 activates transcription via the promoter’s GC box (8, 10, 12, 13). We show here that in contrast to EKLF, hEZF represses transcription when transfected into vascular endothelial cells (Fig. 3A). The ability of hEZF to function as a transcriptional repressor is similar to that of several other Cys2-His2 zinc finger transcription factors, such as ZNF174 transcriptional repressor is similar to that of several other members of the Cys2-His2 zinc finger protein family (such as Egr-1 (27), WT-1 (36), Krüppel (37), and EKLF (35)). Like other members of the Cys2-His2 zinc finger protein family (such as Egr-1 (27), WT-1 (36), Krüppel (37), and EKLF (35)), hEZF contains activation as well as repression domains. The presence of activation and repression domains may allow Cys2-His2 zinc finger proteins to alter their function as the situation dictates (38, 39). A potential switch between a positive and negative transcriptional effect could depend on an interaction with other factors that may change the conformation of hEZF to expose either the activation or the repression domain (40–42). For example, the thyroid hormone receptor binds a corepressor to repress transcription in the absence of thyroid hormone. Hormone binding alters the receptor’s conformation and leads to the release of the bound corepressor and recruitment of a coactivator. Thus, the hormone-bound thyroid receptor acts as a transcriptional activator (41). Under conditions other than those examined here, hEZF may also act as an activator depending on its binding to other factors.

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