Dean's Lecture

Probing the Onset and Regulation of Erythroid Cell-Specific Gene Expression

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Abstract

Cell-restricted transcriptional modulators play a crucial role in the process of selective gene regulation during hematopoiesis. Erythroid Krüppel-like factor (EKLF; KLF1) is a transcription factor, originally identified in this laboratory, which plays a crucial role as a transcriptional activator at the adult ß-globin locus. This review will describe a subset of experiments and approaches related to deciphering its mechanism of action and the means by which it itself is induced.
Key Words: Transcription, EKLF, erythroid cell, gene regulation.

Introduction

UNRAVELING THE MOLECULAR EVENTS that confer the ability to express lineage-specific genes upon an initially uncommitted, pluripotent hematopoietic stem cell remains a major unresolved issue in cell differentiation. This article will describe some of the experiments performed at my lab at the Mount Sinai School of Medicine that address this issue and will show how characterization of a cell-specific transcription factor has opened a window on the processes that lead to erythroid differentiation.

Erythroid Krüppel-like factor (EKLF) was originally isolated by a differential screening approach whereby mRNA that was specifically expressed in erythroid cells was identified after subtraction with common messages expressed in a myeloid cell line (1). Specifically, murine erythroleukemia (MEL) cells provided the erythroid mRNA sample, and the J774 monocyte-macrophage cell line provided the subtracting mRNA sample. This removed 99% of MEL mRNA from consideration. The remaining material was directly cloned. Most of these clones were related to Friend virus genes, metabolic clones (e.g., carbonic anhydrase), and transcription factors (e.g., c-myb and GATA1), but some clones were novel at the time. A Northern blot analysis of adult murine tissues directed the focus to one particular clone uniquely expressed in bone marrow and spleen. Further analysis of this clone (EKLF) indicated that it was expressed only in erythroid cell lines, but not in any lymphoid or myeloid lines, with the exception of a small amount of expression in mast cell lines.

A significant clue as to the function of this clone resulted from the completion of its primary sequence, which indicated that it contained three C2H2-type zinc fingers at the extreme carboxyl end, raising the possibility that it was a DNA-binding protein (1). More precisely, it was most closely homologous to the Krüppel family of transcription factors, which are named after the segmentation gene involved in *Drosophila* body patterning (2). This was fortuitous, as a variety of finger-swapping experiments had been performed with other members of this family (e.g., Zif 268, Sp1, Krox 20) with the aim of predicting its target DNA-binding site. The molecular analysis culminated in the crystallographic structure of Zif 268 bound to its target site (3). Of critical importance for predictive purposes were the interactions of basic residues, at specific amino acid locations, of Zif 268 with guanines within the DNA-binding sequence (4). In addition, each finger interacted with a triplet of nucleotides. This information enabled us to predict that EKLF would interact with 3'GGNGNGGGN5'.

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Glossary

BAF = BRG associated factors
BMP = bone morphogenetic protein
BMPR = BMP receptor
CBF = copper-binding protein
CFU-E = colony-forming unit-erythroid
DN = dominant negative
EB = embryoid bodies
E-RCI = EKLF remodeling complex-1
ES = embryonic stem
EKLF = Erythroid Krüppel-like factor
HS = hypersensitive site
LCR = locus control region
MEL = murine erythroleukemia
P/CAF = p300/CBP associated factor
SUMO = Small Ubiquitin-like Modifier

A subset of this sequence, known as the 5'CACCC3' element, was already recognized as one of a trio of elements that are repeated within erythroid enhancers and promoters. In particular, the CAC site within the ß-globin gene (5'CCA-CACCCT3') precisely matched the predicted EKLF consensus binding sequence. An oligonucleotide containing this sequence was directly tested and found to bind EKLF *in vitro* with high affinity. In addition, transfection studies demonstrated that EKLF functioned as a transcriptional activator (not repressor) when bound to this site. These studies provided the initial isolation and characterization of this transcription factor (1).

Functional Role of EKLF in Erythroid Expression

The ß-globin gene CACCC element is the site of thalassemia-causing point mutations in humans (5). Given this fact, individual examples of these elements were directly tested for their ability to interact with EKLF (6). The results were dramatic: Not only was EKLF unable to transactivate through these mutated sites, but also the binding affinity was decreased 40- to 100-fold. Molecular modeling of these interactions indicated that both specific and non-specific hydrogen bond interactions were adversely affected by what appeared to be minor changes in the sequence of the CACCC element. Methylation interference assays further demonstrated that each of the guanines on the G-rich strand in the nine-base-pair ß-globin CACCC element (3'**GG**T**G**T**GGG**A5') were critical for optimal binding by EKLF.

To understand the potential importance of these properties, a brief summary of ß-globin regulation is needed. Expression of the ß-like globin genes is regulated in a specific temporal sequence during mammalian development (5, 7, 8). In humans, the first blood cells formed within the yolk sac express the embryonic (epsilon) globin variant. After the site of hematopoiesis switches to the fetal liver, the fetal (gamma) globin genes are expressed. This is followed by yet another switch in expression to the adult (ß) globin gene after the bone marrow becomes the major site of hematopoiesis. In mice, there is a single switch in molecular expression from embryonic (y, bh0, bh1) globin genes in the yolk sac to the adult (ß) globin variant in the fetal liver and the adult bone marrow and spleen. Regulation of this large ß-like globin cluster is thought to occur by competition of each globin promoter member (embryonic, fetal, and adult) for interaction with the locus control region (LCR), thus enabling high-level activity of only one member at its appropriate time during development. In reality, this control is more complex, as an endogenous silencing mechanism also plays a vital role in this process (7 – 10).

Hints that EKLF's role in ß-globin regulation is complex followed from the point mutation analysis described above. Specifically, the murine embryonic (and human fetal) CACCC elements differ in one of the important guanines within the interaction sequence, 3'G**A**GGTGGGT5' (11). Given the deleterious effects of the thalassemia-causing point mutations, it was felt that this change would also adversely affect binding. This hypothesis was directly tested, and competition assays demonstrated that the embryonic CACCC element had an 8-fold lower affinity for EKLF compared to the adult CACCC element (12). The effects of such a mutation were tested *in vivo* by using K562 cells, a human erythroleukemic cell line that expresses gamma, but not ß, globin. In this line, EKLF had minimal effects on the activity of an intact gamma globin promoter, but could specifically activate the ß-globin promoter more than 1000-fold when it was linked in cis to the gamma promoter. These molecular data raised the possibility that EKLF may be important for the switch from fetal to adult ß-like globin expression and led to the prediction that an EKLF-null mutation would lead to deficiency in adult, but not in embryonic or fetal, globin expression, a conjecture that was borne out by its genetic knock-out in mice (13, 14). This led to an embryonic-lethal phenotype by day 14, due to lack of onset of ß-globin transcription, resulting in

extreme anemia and prenatal death, precisely at the time of the switch; embryonic globins were expressed at normal levels. These effects were unusually specific, since alpha-globins and other red-cell-specific genes were unaffected by the lack of EKLF expression. Additional studies that utilized EKLF-null embryonic stem cells to generate chimeric adult mice indicated that these cells did not contribute to the mature erythrocyte population, and that they were defective by virtue of a globin chain imbalance, leading to Heinz body formation, a shortened lifespan, and apparent clearance from the bloodstream (15).

These data revealed that EKLF is critical for the onset of adult ß-globin expression. To address the effects on the other linked globin-like genes in the cluster, EKLF-null mice were crossed with a murine line that carries the complete human ß-like globin cluster (16, 17). As seen with the murine locus, adult ß-globin expression was completely dependent on EKLF. However, gamma-globin levels were 5-fold higher, and although they were eventually silenced (as the mice still died from lack of adult ß-globin expression), this shut-off occurred significantly later than that observed in the presence of EKLF. In addition, most EKLF-null, colony forming unit-erythroid (CFU-E) colonies were reprogrammed to high gamma-globin expression. The mode of EKLF action thus appears to involve consolidating the switch from fetal to adult globin, possibly by enabling the ß-globin promoter to form a structure that is able to compete for interaction with the LCR to a greater extent than the embryonic- or fetal-globin promoters.

Mechanism of EKLF Transcriptional Activation

Two aspects of EKLF function were studied. One was the possible role that post-translational modification plays in EKLF activity, since the minimal activation and inhibitory domains each contain conserved sites for serine/threonine phosphorylation (18). In fact, one of the double-amino acid mutations (at aa 43–44) that disrupted the *in vivo* competitive ability of EKLF overlapped with a casein kinase II site (threonine at aa 41). As expected, immunoprecipitation of EKLF from erythroid cells revealed that EKLF is a phosphoprotein *in vivo*, exclusively at serine and threonine (no evidence was seen for modification of tyrosine). In addition, EKLF is a suitable substrate for casein kinase II *in vitro*. Further, the double mutant, or a direct mutant of thr41, was not able to transactivate a reporter *in vivo*. This loss was not observed when the charge at aa 41 was retained (i.e., after conversion to aspartic acid). The location of this important phosphorylation site within the interaction domain of the minimal activation region raises the possibility that EKLF interactions and activity may be modulated by phosphorylation.

The second aspect of EKLF function that was considered in the deletional studies was the importance of protein-protein interactions. The possibility that EKLF may also be involved in chromatin structure at the ß-globin locus led to directly testing whether it interacted with the class of molecules known as histone acetyltransferases (19). These are known to modify histones via acetylation of the amino terminal tails, leading to a more open chromatin structure at that region, thus enabling the transcription machinery to be fully recruited and active (20, 21). The finding that some transcriptional co-activators (such as CBP, p300, and P/CAF) harbor intrinsic histone acetyltransferase activity suggests a mechanism to directly link transcriptional activation and alteration of chromatin structure (22). In the present case (19), EKLF was found to associate with p300, CREB-binding protein (CBP), and p300/CBP associated factor (P/CAF) *in vivo*. Interestingly, EKLF is itself acetylated by p300 and CBP *in vitro*, but not by P/CAF. These sites have been mapped to two lysines (288 and 302). Mutation of lys-288 (but not lys-302) decreases EKLF transactivation of the ß-globin promoter and disables its ability to be superactivated by p300 and CBP (23). EKLF interactions with proteins, rather than with DNA, are affected by its acetylation. In addition to these post-translational modifications (Fig. 1), EKLF is also SUMOylated (SUMO: Small Ubiquitin-like Modifier) (Siatecka and Bieker, unpublished) and ubiquitinated (Quadrini and Bieker, unpublished).

An additional area in which EKLF-protein interactions may be playing an important role is in chromatin remodeling (24, 25). Using an *in vitro* chromatin reconstitution/transcription system, EKLF and a MEL-derived cell extract (EKLF remodeling complex-1; E-RC1) were both

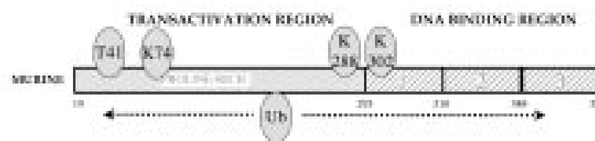

Fig 1. Post-translational modification of EKLF protein.
A schematic diagram of EKLF shows its transactivation (amino terminal) and DNA binding (carboxyl terminal) regions. The DNA binding region contains three C2H2 zinc fingers. Also indicated are the locations of important phosphorylation (T41), sumoylation (K74), acetylation (K288 and K302), and ubiquitination (throughout) sites discussed in the text.

required to provide an open chromatin structure and transcription at the ß-globin promoter (26). In the absence of either component, or by using a mutated EKLF binding site, the chromatin structure remained closed and refractory to transcription. Use of an EKLF deletion that had removed its amino terminal interaction domain still bound DNA and yielded an open chromatin structure, but no transcription was observed, thus uncoupling the two events. Intriguingly, purification of E-RC1 helped identify mammalian components of the SWI-SNF chromatin remodeling proteins, including BRG1, BAF (BRG1 associated factor) 170, BAF155, BAF47, and BAF57. Immunoprecipitation or immunoneutralization of this complex from the MEL fraction again led to an inability to activate the promoter. Interaction of EKLF with BRG1 appears critical for interaction with SWI-SNF (27).

These studies, taken together, implicate EKLF as a central player in the integration of a variety of signals that lead to protein modification, protein-protein interactions, opening of chromatin, and activation of transcription. There is ample precedent for each of the steps in this pathway from other systems, and a testable challenge for future studies will be to determine just how EKLF functions. The biological relevance of these studies has followed from studies that crossed the EKLF-null mice with mice that are transgenic for the complete human ß-like globin locus (17). These studies demonstrated that the hypersensitive site (HS) at the ß-globin promoter is absent when EKLF is not present. In addition, HS3 within the LCR was adversely affected. Thus, not only is EKLF required for transcription of ß-globin *in vivo*, but it also plays a role in forming the correct higher-order structure at the globin locus (28). EKLF interactions with histone acetyltransferases and with chromatin remodeling proteins provide a mechanism that partially accounts for how this may occur.

Regulation of EKLF Expression

Analysis of EKLF biological expression has also provided critical information about its role (29). EKLF is not expressed in embryonic stem (ES) cells, but becomes apparent during embryoid body differentiation in culture. Analysis of developing murine embryos indicated that EKLF is first expressed at the neural plate stage (i.e., day 7.5) within the extraembryonic mesoderm of the visceral yolk sac, the earliest morphologically identifiable time of blood island formation, after which its level continues to expand. It is then expressed at day 9.0 within the hepatic primordia, and by day 14.5 the fetal liver is the only site of EKLF expression. In the adult mouse spleen, it is expressed solely within the red pulp.

Because of these erythroid cell-specific properties, EKLF's own regulation has been investigated (30 – 32). Both the murine and human genomic clones have been isolated, sequenced, and mapped (33, 34). A surprisingly short region of the promoter is sufficient to reconstitute erythroid specificity to an adjacent reporter (30). Although there is no TATA sequence, the proximal promoter region contains GATA and CCAAT sites. The importance of these sites was verified by transfection assays in erythroid cells (MEL and 32DEpo1 lines). More distal elements have been implicated by identifying erythroid-specific DNAse hypersensitive sites at the EKLF genomic locus (31). Two sites within 1000 bp 5' of the transcription start site were identified by this approach. The sequence surrounding the more distal hypersensitive site not only is highly conserved with the human EKLF promoter (Lohmann and Bieker, unpublished), but also behaves as a strong enhancer element, even when placed adjacent to a non-erythroid promoter. However, erythroid specificity can be reestablished when this element is placed adjacent to the EKLF promoter, with the proximal GATA site proving critical for this enhancement (31). Experiments with transgenic mice that contain a ß-galactosidase reporter whose expression is directed by EKLF promoter elements reveal that this short piece is sufficient to generate expression in the blood islands as well as in the fetal liver (35). In addition, both hypersensitive sites, along with the proximal promoter, are all required for this effect. Experiments with 32DEpo1 cells have directed attention to a 49 bp sequence within the most conserved, distal hypersensitive site, which contains the core sequence responsible for enhancement and contains three protein-binding sites *in vitro* (31).

Conditions to differentiate embryonic stem cells to embryoid bodies (EBs) in the absence of serum were established in order to identify cytokines responsible for induction of EKLF expression during early development (36). These studies, in conjunction with expression of dominant negative proteins, enabled us to conclude that the BMP (bone morphogenetic protein) 4/BMP receptor/Smad pathway plays a critical role in EKLF induction (Fig. 2).

Additional Unresolved Issues and Future Directions

A paradox follows from the observation that EKLF message is expressed in both primitive (yolk sac) and definitive (mouse fetal liver) erythroid cells, yet is functionally only required for definitive erythropoiesis. Since EKLF protein is present in both populations, it has been proposed

Fig. 2. Models of EKLF induction by BMP4.
Our induction (BMP (bone morphogenetic protein) 4/BMPR (BMP Receptor)/Smad1/4 (dimer of Smad1 and Smad4 proteins)) and stable inhibitor (BMPR/1B-DN (dominant negative BMP Receptor 1B) and iSmad6 (inhibitory Smad6)) studies (36) implicate the BMP pathway as indicated. The delayed nature of this effect leads to postulating a required novel protein (circle) that is synthesized independently (a) or dependent (b) on the Smad complex. At the same time, the Smad1/4 complex itself may still play a role in EKLF induction in conjunction with its own target novel protein (not shown).

that post-translational modification or changes in protein-protein interactions might yield functionally altered EKLF states at different stages in development. This conundrum extends even deeper, as EKLF message is detected quite early in hematopoietic differentiation, since it is already transcribed both in multipotential cell lines (37, 38) and in enriched primary progenitors (early differentiating CD34+LIN- cells [39]). Again, the reason why EKLF should be expressed so precociously when its requirement is considerably later in differentiation, and whether this is functionally important, remains to be resolved.

Another issue of intense interest is whether there are any other downstream targets of EKLF besides ß-globin. This issue arises primarily because a large number of erythroid gene promoters contain CACCC elements that theoretically should interact with EKLF.

The importance of both the DNA binding and transactivation modules of EKLF in regulating ß-globin transcription and chromatin structure has inspired approaches to take advantage of these structures, to alter globin regulation. For example, modifying the EKLF fingers to bind the defective CAC site, thus providing a therapeutic agent that increases levels of delta-globin expression in ß-thalassemic or sickle cell patients. Alternatively, changing the EKLF activation module to a transrepressor could alter the structure of the locus enough to silence ß- yet reactivate the gamma-globin gene, thus achieving the same end (Manwani and Bieker, unpublished).

Summary

EKLF clearly plays a crucial role in the controlled events that lead to developmental regulation of the ß-like globin cluster (Fig. 3). As a result, structural and functional analysis of EKLF has enabled us to open a molecular window on the process of ß-globin gene regulation. Each EKLF module plays an important part in its functional specificity. The zinc fingers discriminate among very similar CACCC elements, and the transactivation domain plays a critical role in transcriptional activation and in opening the surrounding chromatin. Protein-protein interactions are pivotal for this process; in this context, it will be critical to determine whether post-translational modification (phosphorylation, acetylation, sumoylation, and ubiquitination) of EKLF is regulated during hematopoiesis and/or during development, and how this affects the efficiency of these interactions, and thus of the onset of ß-globin expression. Any tie-in of these changes to the action of extracellular effectors will be critical for understanding the complete transduction cascade involved in the onset of ß-like globin transcription during development.

Fig. 3. Summation of EKLF structure/function.
Diagram of EKLF, demarcation of its zinc fingers and their relation to its predicted binding site, and location of its actual ß-globin CACCC element binding site. The numbers below the protein scheme denote amino acids, while those above the ß-globin gene denote the base position upstream to the transcriptional start. A recapitulation of EKLF's role in erythroid gene control as discussed in the text is outlined.

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