Regulation of Erythroid Krüppel-like Factor (EKLF) Transcriptional Activity by Phosphorylation of a Protein Kinase Casein Kinase II Site within Its Interaction Domain*

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Erythroid Krüppel-like factor (EKLF) is a red cell-specific activator whose presence is crucial for establishing high levels of adult β-globin expression in definitive cells during erythroid ontogeny. However, its simple presence within the erythroid lineage is not sufficient to activate the β-globin promoter. One explanation that may account for this is that post-translational modification of EKLF differs within erythroid cell populations and regulates its activity. We have therefore addressed whether phosphorylation plays a role in modulating EKLF action. First, in vivo analyses implicate serine/threonine kinases as important players in the terminal differentiation of MEL cells, and demonstrate that EKLF is phosphorylated at serine and threonine residues within its transactivation region. Second, directed disruption of a protein kinase casein kinase (CK) II site, located within the EKLF interaction domain, abolishes EKLF transactivation and in vitro competition activity. Third, in vitro assays demonstrate that CKIIα interacts with EKLF, and that the EKLF interaction domain is phosphorylated by CKII only at Thr-41; however, the CKII-site mutant is not phosphorylated. Finally, the transactivation capability of EKLF is augmented by cotransfection of CKIIα. We conclude that EKLF is a phosphoprotein whose ability to transcriptionally activate an adjacent promoter is critically dependent on the phosphorylation status of a specific site located within the EKLF interaction domain, and that serine/threonine kinases play an important role in this process.

Molecular models that describe the regulation of the β-like globin cluster require that developmental controls, in addition to those that impart erythroid cell specificity, be accommodated. This is because the β-like globin cluster combines tissue specificity with developmentally regulated switching in expression of its members (1). At a primary level, the identification of tissue-specific DNA-binding proteins that direct globin transcription have begun to illuminate the molecular mechanisms that establish and maintain this process (2). Although a number of these factors have been isolated and help to explain the tissue specificity of globin expression, the details of how and when they interact to establish the correct temporal expression of individual members remain elusive (3). In addition, these events occur within the context of chromatin, where structural constraints, and their attendant modulators, add yet another layer of potential cellular control points for regulation (4–6).

Current models of transcriptional regulation invoke protein-protein interactions as being of primary importance for establishing stereospecific arrays both at the promoter (7) and within more complex, long-range structures, such as that thought to form at the β-like globin locus (8–13). This is an open, erythroid cell-specific chromatin domain that encompasses the distal locus control region (LCR) and the set of β-like globin genes. Correct developmental regulation of each gene in the cluster is thought to occur by a dynamic competition of individual globin promoters for interaction with the LCR. As a result, these models not only postulate interactions between erythroid-enriched transcription factors and coactivators, but also with the surrounding histones and other chromatin-associated proteins. Post-transcriptional alterations that modulate the formation and stability of these complexes (e.g. phosphorylation (14)) can therefore provide another powerful layer of control upon the activity of tissue-specific regulators at specific times in development, leading to changes in transcript levels of selected globin target genes.

One of these tissue-specific regulators is erythroid Krüppel-like factor (EKLF). EKLF is a zinc finger DNA-binding protein that interacts with the CACCC element in the adult β-globin promoter and, through its proline-rich transactivation domain, directs high-level expression of β-globin expression (15, 16). Its interaction with the adult β-globin CACCC element is quite specific (17), as EKLF does not interact well with the closely related bh1/γ CACCC element and is thereby implicated as a potential globin switching factor (18). Consistent with this idea, genetic ablation of EKLF leads to profound β-thalassemia and embryonic lethality yet leaves expression of yolk sac β-like globins unaffected (19–21). In addition, EKLF expression is critical for consolidating the switch from fetal to adult globin and for generating an open chromatin structure around the adult β-globin transcription unit (22, 23).

However, a paradox that has emerged from these studies is that EKLF is expressed within erythropotential tissues (24) and cell lines (25–27) that do not produce adult β-globin. For example, murine EKLF is first expressed during development within the blood islands of the day 7.5 embryo, which produce primitive erythroid cells that only express the embryonic glo-

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The abbreviations used are: LCR, locus control region; EKLF, erythroid Krüppel-like factor; CAT, chloramphenicol acetyltransferase; HMBA, hexamethylene bisacetamide; GST, glutathione S-transferase.
EKLF Phosphorylation by Casein Kinase II

bina (24). EKLF is then expressed later in the fetal liver, where it helps generate optimal adult globin expression within the definitive erythroid cell compartment. Translational regulation cannot account for this difference in effect, as EKLF protein is present in both primitive and definitive erythroid populations (24).

Relevant to these observations is the fact that the EKLF transactivation region contains subdomains that are important for both inter- and intra-molecular interactions (16, 28). We have postulated that phosphorylation might play a role in either/botth of these processes (28), as there are a number of consensus phosphorylation sites in both the minimal activation and the inhibitory subdomains (29). To address whether post-translational modification plays a role in differential EKLF activity, we investigated whether changes in EKLF phosphorylation alter its transactivation properties in vivo. Our studies point to the status of a protein kinase CKII site within the interaction domain as being particularly important for EKLF transcriptional activity. As CKII is known to modulate a number of transcription factors (30, 31), we more directly examined its role in EKLF function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—pSG5/EKLF, pGAL/EKLF (which contains the EKLF transactivation domain (amino acids 20–291) fused to GAL–1–147), and pGAL/EKLF/24–124 have been described (15, 16, 28). pGAL/EKLF/mut A23024 and pGAL/EKLF/mut A43G44 were generated by site-directed mutagenesis of pGAL/EKLF as described previously (28). pGAL/EKLF/20–124/mut T41A and pGAL/EKLF/20–124/mut T41D were generated with the Quick-Change mutagenesis kit as per the manufacturer (Stratagene). pCKIIa has been described (32). All fragments were routinely purified (GeneClean) by agarose gel electrophoresis prior to ligation. pHis-EKLF was constructed by polymerase chain reaction cloning of an EKLF fragment that contained amino acids 20–376 into the NdeI/BamHI site of pET-15B (Novagen). pGST-EKLF/20–60 and pGST/EKLF/20–60/A43G44 were constructed by cloning of the 120-base pair BamHI/blunted ApaI (T4 DNA polymerase) fragment isolated from pGAL–147/EKLF/20–124 and pSGS/EKLF/360–195/M4/E43D4/A43G44, respectively, into the BamHI/Smal sites of pGEX5X (Pharmacia). pFLAG/EKLF/Zn was constructed in two steps, first by insertion of the BamHI/blunted ApaI fragment into pGAL/EKLF (which contains the transactivation region) in-frame into the Smal site of a FLAG sequence-containing vector (pBFT4 from J. Licht). This places the FLAG sequence at the amino terminus of the EKLF transactivation region. The XbaI/HindII (filled-in) fragment containing the fusion protein was then cloned into the BamHI/blunted site of pGStacSMH-T11A (33).

**Cell Manipulation**—Transient transfections of 32DEpo1 cells and CAT assays were as described (28). Results are the average of multiple experiments after normalization of CAT activity to growth hormone receptor activity. We investigated whether changes in EKLF phospho-

**In Vivo Labeling of MEL Cells, Immunoprecipitation, and Analysis—**

**In vivo** labeling of MEL cells. Immunoprecipitation, and analysis—actively growing MEL cells (cell line M4D3) that had been stably transfected with the expression vector for the FLAG-EKLF fusion protein under the control of metallothionein promoter (pFL/EKLFEZn) were resuspended at 6 × 10⁶ cells/ml in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum that contained 160 μM ZnCl₂. After 16 h, the cells were washed twice with phosphate-free Dulbecco’s modified Eagle’s medium, incubated for 2 h in phosphate-free Dulbecco’s modified Eagle’s medium containing 10% phosphate-free fetal calf serum (dialyzed against 20 mM HEPES, pH 7.4), and then incubated for 4 h with [32P]orthophosphate (0.5 μCi/ml). The cells then were washed with phosphate-buffered saline and resuspended in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KC1, 1.5 mM MgCl₂, 3 μM β-mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After incubation on ice for 15 min, 1/5 volume of ice-cold 2.5 mM NaCl, 50% glycerol was added, mixed, and further incubated on ice for 30 min. The lysate was centrifuged at 4°C for 15 min at 75,000 rpm in a TL-100 microcentrifuge and the supernatant was incubated with anti-FLAG monomlonal antibody M5 (Eastman Kodak) for 4 h followed by incubation with protein G-Sepharose (Pharmacia) for another hour. The beads were washed 3 times with lysis buffer containing 0.5 M NaCl and once with lysis buffer. Proteins on the beads were resolved by SDS-polyacrylamide gel electrophoresis and electrotransfer to Immobilon-P by established procedures (36). Phosphoamino acid analysis of the labeled band was as described (37).

**Fusion Protein Purification—**

The His-tagged EKLF expression vector (pHIS/EKLF) were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Bacterial cell pellets were collected and lysed in buffer A (6 mM guanidine-HCl, 0.1 M NaH₂PO₄, and 10 mM Tris, pH 8.0). The supernatant was applied to a charged nickel-agarose resin column (Novagen) and washed sequentially with 10 volumes of buffer A, 5 volumes of buffer B (8 mM urea, 0.1 mM NaH₂PO₄, 10 mM Tris) at pH 8.0, 5 volumes of buffer B at pH 6.3, and then eluted sequentially with 3 volumes of buffer B at pH 5.9, 5 volumes of buffer B at pH 4.5, and 5 volumes of buffer C (6 mM guanidine, 0.2 M acetic acid). Eluted proteins were dialyzed against retentation buffer (25 mM HEPES, pH 7.5, 80 mM KC1, 10 mM ZnCl₂, 3 mM β-mercaptoethanol, 2 mM reduced glutathione, 20 mM oxidized glutathione) sequentially with 8 mM urea, 4 mM urea, and 2 mM urea. GST fusion protein preparations were as described (38).

**RESULTS**

Serine/Thrreonine Phosphorylation Plays a Role in the Onset of β-Globin Expression—To begin our analyses, we addressed which amino acid might be the primary EKLF target(s) for the putative MEL kinase. Extracts were prepared from induced MEL cells and from cells at 24, 48, and 72 h post-HMBA induction and either probed with an anti-phosphotyrosine antibody or immunoprecipitated with the anti-phosphotyrosine antibody. The lack of an EKLF-reactive band in either case (data not shown) suggested that tyrosine may not be the amino acid that is the primary target for the MEL kinase.

The alternate target would be serine and/or threonine. If modification of these residues is prevented, and if they are involved in EKLF function, one prediction would be that globin expression would be directly affected, since EKLF is a necessary component for inducing high levels of β-globin (19, 20). To test this idea, MEL cells were treated at a non-toxic concentration with H7, which is a serine/threonine kinase inhibitor (39). These cells were then induced with HMBA, and hemoglobin synthesis was monitored at 24, 48, 72, and 96 h post-induction. The results (Fig. 1) show that hemoglobin synthesis is extensively delayed in the cells treated with H7, such that at 48 h, these cells still present no evidence of hemoglobin synthesis, whereas the non-H7-treated controls are 36% hemoglobin positive after HMBA induction. This data implicates serine/threonine kinases as important players in the onset of globin synthesis during terminal differentiation of MEL cells, consistent with the prediction above. However, these experiments do not directly implicate EKLF in this type of control.

**EKLF Is a Phosphoprotein—**To obtain more direct evidence for involvement of serine/threonine kinase(s) in EKLF function, we examined the status of the EKLF protein in MEL cells.
Although our anti-EKLF antibodies have been used for Western blot analysis (24), they have not proven to be useful reagents for immunoprecipitation. To circumvent this problem, we established a number of stable MEL cell lines that contain an inducible flag-tagged EKLF protein, named FLAG/EKLF. This protein contains the flag epitope linked at the amino terminus to the transactivation region of EKLF in the pSVneoHMT-IA vector (33). The vector contains a metallothionein promoter element, which makes it inducible with zinc. As shown in Fig. 2A, these cells synthesize an inducible protein product of the expected size that is recognized by anti-EKLF antibodies. Importantly, this protein can be immunoprecipitated by the M5 anti-flag antibody (data not shown).

Using this cell line we established that EKLF is a phosphoprotein in MEL cells by immunoprecipitating the induced FLAG/EKLF\(\Delta\)Zn protein after in vivo labeling with radioactive orthophosphate. Phosphoamino acid analysis of the precipitated protein (Fig. 2B) demonstrates that serine and threonine are phosphorylated, whereas there is no detectable tyrosine phosphorylation. This demonstrates that the EKLF transactivation domain is phosphorylated in MEL cells in a pattern consistent with our previous analyses.

A Consensus CKII Site within the EKLF Interaction Domain

Is Important for EKLF Activity—To localize potential target sites in EKLF of Ser/Thr kinase activities, we compared the murine and human EKLF amino acid sequences (25), particularly within the minimal transactivation domain, in search of conserved kinase target sequences. This uncovered a threonine within one consensus casein kinase II (CKII) site (TQ(E/D)D) located 23 residues from the amino terminus (Fig. 3A). The importance of this sequence was tested in two ways. Both tests relied on construction of a mutated derivative at this site within the mouse gene, converting Thr-Gln-Ala-Gly\(^{44}\) to Thr-Gln-Asp\(^{44}\) by site-directed mutagenesis (numbering of the mouse gene is based on the initiator methionine as amino acid 1). One test, already published (28), utilized an in vivo competition assay which demonstrated that increasing amounts of wild-type EKLF could compete with a positive-acting factor for transcription of a reporter in the 32DEpo1 erythropoietic cell line. This competitive effect was localized to the amino-terminal 40 amino acids of EKLF, a region containing the putative CKII site. Importantly, the competitive ability of this fragment was lost when the site-directed mutant described above (Thr-Gln-Ala-Gly\(^{44}\)) was used for these experiments. However, other site-directed mutants (e.g. Ser-Ala-Glu-Thr\(^{44}\) to Ser-Ala-Ala-Gly\(^{44}\)) still retained their competitive ability.

The second functional test was performed by co-transfection of the GAL/EKLF chimeric activator with the pG5BCAT reporter into 32DEpo1 cells. As shown in Fig. 3B, this reporter is extensively activated by GAL/EKLF, and only slightly affected by a derivative that contains the Ser-Ala-Ala-Gly\(^{44}\) mutation. However, the Thr-Gln-Ala-Gly\(^{44}\) mutation almost abolishes its transcriptional activity. We conclude from these two tests that the putative CKII site located 23 residues from the amino terminus is critically important both for protein-protein interactions and for efficient transcriptional activation. These data also more directly implicate an important role for serine/threonine kinase(s) in EKLF function, particularly within its minimal transcriptional activation domain.

CKII Phosphorylates the EKLF Interaction Domain in Vitro

And Stimulates EKLF Transactivation in Vivo—We next utilized a number of approaches to determine if CKII (or a related activity) might be responsible for modulating EKLF activity. Our substrate for the in vitro experiments was HIS-tagged EKLF that was affinity purified to homogeneity. The ability of MEL extracts to phosphorylate this substrate was tested in two ways. First, in solution we found that uninduced or induced MEL extracts contain kinase activities that are able to phosphorylate HIS/EKLF (Fig. 4A). Second, we used an in-gel kinase assay as a means to direct our attention to potential kinases that might be involved. In the in-gel kinase assay (40), the polyacrylamide gel is made in the presence of EKLF. MEL extracts were then electrophoresed through this gel, and kinase activities in situ were determined by incubation with labeled ATP after renaturation. During this process, kinases in the MEL extract are resolved in the gel, renatured, and act upon EKLF substrate, which remains interspersed throughout the gel matrix (41). The data in Fig. 4B demonstrate that there are a number of discrete activities of various molecular weights.

![Fig. 1. Effect of the H7 serine/threonine kinase inhibitor on induced MEL differentiation.](image1.png)

![Fig. 2. Phosphoamino acid analysis of radioactively labeled EKLF protein.](image2.png)
that are able to phosphorylate EKLF. These activities do not appear in the absence of EKLF substrate, excluding autophosphorylation signals from consideration (data not shown). Of particular interest is the activity at \(-40,000\) M, a size that corresponds to the catalytic subunit of CKII (30). Appearance of this band is selectively inhibited by heparin, a known specific inhibitor of CKII (42). In addition, the band comigrates with purified CKIIa. From this data we felt that MEL extracts contain CKII, and that this is one of the kinases that is able to modify EKLF.

Further characterization of this activity was performed in solution using purified HIS/EKLF and CKII (Fig. 5). First, we found that EKLF is a direct substrate for CKII \textit{in vitro}, and that serine and threonine are phosphorylated in the same amounts as observed \textit{in vivo}. Second, as expected, this activity is inhibited by heparin. By optimizing the reaction conditions, we found that a 5-min incubation with 25 units was sufficient for \textit{efficient in vitro} phosphorylation of EKLF by CKII.

Using these \textit{in vitro} conditions, we then directly tested our presumption that the Thr-Gln-Glu-Asp\textsuperscript{44} to Thr-Gln-Ala-Gly\textsuperscript{44} mutation described earlier could, in fact, disrupt threonine phosphorylation at Thr\textsuperscript{41}. For this purpose we created a chimera that contained the EKLF interaction domain (amino acids 20–60; see Fig. 3A) fused to GST, and a derivative that contained the Thr-Gln-Ala-Gly\textsuperscript{44} mutation. After phosphorylation in \textit{vitro} of these purified substrates by CKII, proteins were resolved on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Staining for total protein reveals similar amounts of substrate; but the autoradiograph reveals a significant drop in the ability of the mutant substrate to be phosphorylated (Fig. 6A). Furthermore, phosphoamino acid analysis indicates that threonine was the only amino acid phosphorylated within this substrate, and that this site was no longer a suitable substrate for casein kinase II after mutation of the adjacent amino acids (Fig. 6B).

Although these data are consistent with Thr\textsuperscript{41} status playing a role in EKLF function, we directly tested this notion by changing it to either alanine (to eliminate it as a substrate for phosphorylation) or aspartic acid (to mimic the negative charge seen in phosphothreonine (43, 44)) and testing their transactivation ability \textit{in vivo} (Fig. 7). Co-transfection of the GAL/GAL4/EKLF/CKII was performed with control and Thr\textsuperscript{41} mutated HIS/EKLF along with purified CKII and radioactive ATP prior to electrophoresis and autoradiography. Position of full-length HIS/EKLF is indicated. B, in-gel kinase assay was performed with HIS/EKLF in the gel and electrophoresis of pre- and post-induced MEL extracts as indicated (0, 1, 2, 3 days after induction). After renaturation in the absence or presence of heparin, gels were placed in kinase reaction buffer with radioactive ATP, followed by washing and autoradiography. Note that the fastest migrating band (\textit{left arrow}) is completely inhibited by heparin. The lanes on the right show data after electrophoresis of uninduced MEL extract along with purified CKII\textsubscript{a} subunit (as indicated) in the same gel. Note that the fastest migrating MEL-derived band co-migrates with purified CKII\textsubscript{a} (\textit{right arrow}). Molecular weight markers from top to bottom (indicated on the \textit{left} are: 199, 106, 69, and 44 kDa.

**FIG. 3.** Directed mutagenesis of the EKLF transactivation domain. A, homology between the murine and human EKLF orthologues is shown for the first 50 amino acids, which covers the EKLF interaction domain (28). The conserved CKII site is indicated, as are the site-directed mutants (Ser-Ala-Thr\textsuperscript{24} to Ser-Ala-Ala-Gly\textsuperscript{24} and Thr-Gln-Glu-Asp\textsuperscript{44} to Thr-Gln-Ala-Gly\textsuperscript{44}) used in B and in Fig. 6. B, transient co-transfection assays of 32DEpo1 cells were performed with pG5BCAT and the indicated expression vectors. Normalized results from multiple experiments are shown along with an autoradiograph of the thin layer plate from one experiment.

**FIG. 4.** Tests for the ability of MEL extracts to phosphorylate EKLF. A, uninduced (day 0) and HMBA-induced (day 2) MEL extracts were incubated in the presence (+) or absence (−) of purified recombinant HIS/EKLF and radioactive ATP prior to electrophoresis and autoradiography. Position of full-length HIS/EKLF is indicated. B, in-gel kinase assay was performed with HIS/EKLF in the gel and electrophoresis of pre- and post-induced MEL extracts as indicated (0, 1, 2, 3 days after induction). After renaturation in the absence or presence of heparin, gels were placed in kinase reaction buffer with radioactive ATP, followed by washing and autoradiography. Note that the fastest migrating band (\textit{left arrow}) is completely inhibited by heparin. The lanes on the right show data after electrophoresis of uninduced MEL extract along with purified CKII\textsubscript{a} subunit (as indicated) in the same gel. Note that the fastest migrating MEL-derived band co-migrates with purified CKII\textsubscript{a} (\textit{right arrow}). Molecular weight markers from top to bottom (indicated on the \textit{left} are: 199, 106, 69, and 44 kDa.

**FIG. 5.** \textit{In vitro} kinase assay. A, HIS/EKLF was incubated with increasing amounts of purified CKII in the absence or presence of heparin as indicated. Positions of EKLF and CKII\textsubscript{b} subunit are indicated on the \textit{left}. Molecular weight markers from top to bottom (indicated on the \textit{left} are: 199, 106, 69, and 44 kDa. B, \textit{in vitro} labeled HIS/EKLF protein was hydrolyzed and subjected to one-dimensional phosphoamino acid analysis. The positions of co-electrophoresed phosphoserine, phosphothreonine, and phosphotyrosine were visualized by staining with ninhydrin.
EKLF(20–124) chimeric activator and its derivatives indicates that the T41A alteration leads to a drastic drop in its transactivational activity, demonstrating the importance of threonine at position 41. However, the T41D compensatory acidic replacement works as well as wild-type, additionally suggesting that the threonine at this site must be phosphorylated for full in vivo activity. As a result, our previous EKLF competition analyses must have been disrupted by the inability of Thr 41 to be phosphorylated, rather than by an indirect effect resulting from the change in the adjacent amino acids. We conclude that Thr41 phosphorylation plays an important role in establishing proper EKLF interactions that result in transcriptional activation.

To further demonstrate a potential interaction between EKLF and CKII in MEL cells, a pull-down experiment, using the HIS-tag of HIS/EKLF, was performed by incubating purified HIS/EKLF with MEL extract. We found that CKII from the uninduced MEL extract could coprecipitate with HIS-EKLF (Fig. 8). These and the previous results demonstrate that EKLF interacts with and is a substrate for MEL-derived CKII. Finally, to garner evidence that CKII has a functional effect on EKLF, we co-transfected 32DEpo1 cells with pG5BCAT reporter, GAL/EKLF(20–124) which contains the minimal transcriptional activation subdomain (amino acids 20–124 (28)), and varying amounts of a CKII expressing plasmid (32). We found that reporter levels, which are already active in these cells in the presence of GAL/EKLF(20–124), were additionally increased up to 4-fold depending on the amount of co-transfected CKII (Fig. 9). From these data we conclude that the primary effect of CKII on EKLF is to increase its transcriptional activity, likely through its interaction with the minimal transactivation domain which contains the putative CKII site described earlier.

**DISCUSSION**

EKLF is a transcriptional activator whose function is crucial for generating optimal levels of β-globin transcription in the erythroid cell. Part of the specificity of this effect resides within its zinc fingers, which readily discriminate among various closely related CACCC elements (17, 18). However, the transactivation domain is another potential target for EKLF functional control. Our previous deletion and mutagenic analyses revealed that this 270-amino acid region contains stimulatory and inhibitory subdomains (28). The possibility that these opposite effects can be modulated post-transcriptionally is apparent since consensus phosphorylation sites reside within each subdomain.

**EKLF Activity Is Modulated by Its Phosphorylation Status**—Our present study suggests that the phosphorylation status of the EKLF minimal activation domain affects its transactivation and interactive functions. This raises intriguing possibilities concerning the control of EKLF activity during a variety of different processes. For example, even though EKLF protein levels remain unchanged after induction of the MEL cell line used in this study, changes in EKLF phosphorylation status may be part of the cellular response to HMBA and other induc-

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2 L. Ouyang, X. Chen, and J. J. Bieker, unpublished observations.
ers of MEL terminal differentiation. In addition, the modification status of EKLF may be different in cells which contain EKLF yet do not express β-globin, such as is the case in primitive erythroblasts (24), HEL cells (25), SKT6 cells (27), and FDCP-mix cells (26), when compared with committed, definitive erythroid cells. This control point would allow signals from extracellular effectors that lead to modification of EKLF to exert immediate effects at appropriate times during cellular differentiation.

Although our studies have focused attention on Thr41 phosphorylation and its relevance to transactivation and protein-protein interactions, it is readily apparent from the data that EKLF is primarily phosphorylated at serine residues. The significance of these other modifications have not been addressed by the present studies. However, these modifications may be more directly relevant to EKLF DNA binding, as other studies indicate that the phosphorylation status of EKLF affects its DNA binding ability. This effect might be predicted to localize to the EKLF inhibitory domain, which acts in cis (28). In this sense, EKLF may be behaving more like p53, whose conformation and DNA binding activity is modified by phosphorylation (45). In addition, there may be contacts between EKLF and other proteins at regions besides those detected by the in vitro competition assay that are affected by serine phosphorylation.

Role of Protein Kinase CKII in EKLF Regulation—The present studies have uncovered the importance of a putative casein kinase II site within the EKLF minimal activation domain. CKII is a ubiquitously expressed and highly conserved heterotetrameric enzyme that has been shown to modify the activity of a range of substrates that include transcription factors, oncogenes, cell cycle-dependent proteins, signal transducers, and structural proteins (reviewed in Refs. 30 and 31). More specifically, CKII is involved in modulating the DNA binding affinity of transcription factors such as c-Jun (46) and p53 (47). In addition, proteolysis of IκBα is dependent on CKII phosphorylation at a specific site (48). Nuclear transport can also be affected by CKII phosphorylation, as in the case of SV40 large T-antigen (49). Finally, transgenic expression of CKIIα in lymphocytes leads to an increased propensity of these cells to become malignant (50).

However, of more direct relevance to the present experiments are two recent sets of studies. First, CKII can modulate developmental processes. For example, overexpression of Dif2 (the Wingless (Wg) receptor) leads to increased association and phosphorylation of Dishevelled with CKII, suggesting that CKII plays a role in transduction of the Wg signaling pathway (51). In addition, inappropriate activity of the homeotic Antennapedia (ANTP) protein is prevented by its association with phosphorylation by CKII during Drosophila embryogenesis, which then allows normal suppression of ANTP by more posterior HOX genes to occur (52).

Second, CKII modification can alter protein-protein interactions. For example, complex formation between the viral VP16 protein and the cellular Oct-1 and HCF transcription factors is eliminated by mutation of a single CKII site in VP16 (53). In addition, association of TATA-binding protein with either the papilloma virus E7 protein or the adenovirus E1a protein is enhanced by CKII phosphorylation of the viral protein (54).

Biological Implications of EKLF Interaction Domain Phosphorylation—Together, these studies provide a strong precedent that CKII phosphorylation can alter protein-protein interactions which are critical for the correct modulation of normal developmental patterns of regulation. Since the EKLF minimal activation domain also overlaps with the interaction domain (as monitored by in vivo competition (28)), controlling the ability of EKLF to form its appropriate protein-protein partners can be postulated to affect regulation in at least two ways. First, interactions that are important for formation of the correct stereospecific arrangement locally at the promoter could be altered by changing the post-translational status of EKLF. For example, GATA1- and EKLF-binding sites placed in combination yield synergistic transactivation of a promoter as monitored by transient assays in vivo (55, 56). As in vitro analyses indicate that these two proteins can physically interact with each other, modification may affect the avidity of the interaction and thus the level of activity of the promoter.

Second, the absence of EKLF leads to complete lack of hypersensitive site formation at the β-globin promoter, and less complete but notable diminution of hypersensitive site 3 at the LCR (22). Based on current models of globin regulation, this suggests that EKLF is necessary for formation of the intact architecture of proteins that form at the β-like globin cluster, leading to an open chromatin configuration at the adult promoter that effectively competes over the fetal promoter for productive interaction with the LCR. As a result, absence of EKLF modification, and thus the lack of appropriate protein-protein contacts, could alter the structure and correct regulation of the globin cluster by decreasing the competitive edge of the adult globin promoter over that of the fetal globin promoter. Indeed, experiments with transgenic mice indicate that a lack of EKLF leads to increased levels of γ-globin expression that take longer than normal to shut off during murine development (22, 23). Similar effects on the murine embryonic β-like globin genes are observed during embryoid induction of EKLF-null ES cells.
Although CKII is constitutively expressed and active, it is of interest that in the aforementioned study (51), it was the association of Dsh with CKII, rather than changes in CKII activity itself, which provided the regulatory point. In our studies, both CKII activity and association with EKLF decrease after HMBA induction of MEL cells. At the same time, CKII is inhibited by 2,3-biphosphoglyceric acid, which accumulates to inhibitory levels only within red blood cells as they differentiate (57).

Future tests will focus on whether there are any differences in EKLF phosphorylation status during MEL differentiation or between primitive and definitive erythroid cell populations. In addition, the ability of mutated EKLF to rescue the β-thalassemia phenotype of EKLF-null ES cells will be a particularly stringent test of EKLF structure/function parameters. Identification of EKLF protein partners, and whether their interaction is modified by EKLF phosphorylation, will enable details of present models of globin regulation to be expanded. Resolution of these key issues will likely illuminate whether events that control the status of EKLF modification play any role in the control of β-globin gene switching during erythroid ontogeny.

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