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Negative regulation of CD4 expression in T cells by the transcriptional repressor ZEB

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Abstract

ZEB, an E-box binding transcriptional repressor, is an important regulator of T cell and muscle development. Targeted disruption of ZEB in mice resulted in a strong reduction of thymocytes and the few T cells that reached the mature stage were predominantly CD4$^+$. CD4 expression during the various stages of T cell differentiation is controlled at the transcriptional level by a complex array of regulatory elements in the CD4 gene locus, consisting of at least three enhancers, one promoter and one silencer. Here we present evidence that CD4 gene expression is negatively regulated by ZEB. We show that ZEB binds to the 5′E-box in the CD4-3 element of the proximal CD4 enhancer in competition with the transcriptional activators E12 and HEB, thereby reducing CD4 expression on CD4 single-positive but not CD4/CD8 double-positive T cells. The conversion of the CD4 proximal enhancer into a potential silencer element by the transcriptional repressor ZEB offers an additional concept of CD4 gene regulation in T cells.

Introduction

CD4 is an important glycoprotein not only for mature T cell activation but also for T cell development. CD4 is expressed in the T_h cell subset and via its intracellular domain it binds to p56^{lck}, thereby contributing to signal transduction into the nucleus (1). In contrast, most cytotoxic T cells are characterized by expression of the CD8 molecule. In addition to its role in mature T cell activation, CD4 is expressed differentially during T cell development. Targeted disruption of CD4 in mice leads to a severe defect in T_h cell differentiation, indicating that CD4 plays an important role in this process (2,3). CD4 is expressed first at low levels in pluripotent hematopoietic stem cells (4) and this expression level is kept on early T cell precursors (5), arriving in the thymus from the bone marrow. Then CD4 is down-regulated in CD4$^-$CD8$^-$ double-negative thymocytes. After low expression of CD4, both molecules are up-regulated in CD4$^+$CD8$^+$ double-positive thymocytes. Further contact of their TCR with thymic epithelial cells induces positive and negative selection resulting in mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ single-positive T cells (6). This different expression pattern of CD4 during T cell development is controlled at the transcriptional level by a complex array of regulatory elements in the CD4 gene locus (7). These elements consist of at least three enhancers (8–11), one promoter (12) and one silencer (13,14), and the expression of CD4 in mature and immature T cell subsets is controlled by the different elements in a developmentally coordinated manner (see Discussion).

ZEB, a zinc finger/homeodomain transcription factor also termed δ-EF1 (15), ZFH-1 (16) or AREB6 (17), was originally described as a transcriptional repressor binding to the negative regulatory element (NRE) of the IL-2 gene promoter (18), thereby counteracting IL-2 expression, e.g. in anergic T cells (19). However, various studies have shown that ZEB also is a developmental regulator for the differentiation of mesoderm-derived tissues (15,20). The factor is involved in the coordinated expression of skeleton muscle-specific genes and targeted disruption of the ZEB gene in mice results in defects in skeleton muscle development (21). Moreover, such mice also have severe defects in T cell differentiation at various developmental stages without affecting B cells (22). The number of thymocytes is strongly reduced and the few T cells that reach the mature stage are predominantly CD4$^+$. This indicates that without ZEB either the silencer-mediated suppression of the CD4 gene during the differentiation of double-

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positive thymocytes to mature $CD8^+$ T cells is incomplete or the positive selection is forced towards $CD4^+$ T cells. One target gene of ZEB during T cell development described is the integrin VLA-4. VLA-4 is an essential molecule for the migration of precursor T cells into the thymus (23,24) and is negatively regulated by the transcriptional repressor ZEB (25).

ZEB binds to the DNA sequence CAGGTG/A, which can represent a subset of E-boxes (CANNTG) (17). ZEB competes with basic helix-loop-helix (bHLH) transcriptional activators for DNA binding to the same element and is able to actively repress transcription of its target genes (26). Since E-boxes are involved in the transcriptional control of developmentally regulated genes, we looked for T cell-specific genes activated through E-boxes as potential target genes of the transcriptional repressor ZEB. One is the CD4 gene, whose important proximal enhancer contains a binding site for TCF1/LEF1 transcription factors and three E-boxes (11). Two of these E-boxes, located in the so-called CD4-3 element, are absolutely necessary for the enhancer's function, and are bound by the bHLH proteins E12 and HEB (27).

Here we show that ZEB binds to the 5′E-Box in the CD4-3 element of the proximal CD4 enhancer, and competes with the transcriptional activators E12 and HEB for DNA binding. Furthermore we found that overexpression of ZEB in T cells converts the CD4 proximal enhancer into a silencer element, leading to a reduction of CD4 expression. Our data show that the CD4 gene is a target of the transcriptional repressor ZEB, which could explain the increased proportion of $CD4^+$ mature T cells in ZEB-deficient mice.

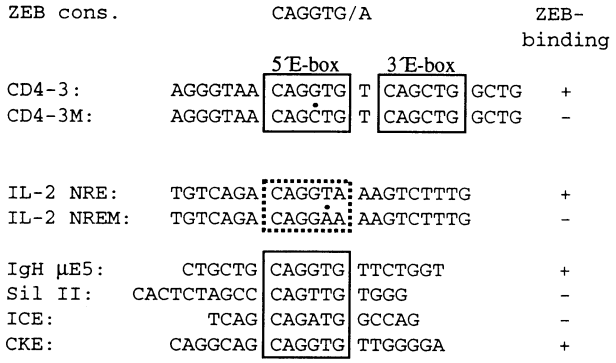

Fig. 1. Sequences of the oligonucleotides used as probes and for competition experiments. E-boxes are indicated by solid boxes, the ZEB recognition region of the IL-2 NRE by open boxes, and point mutations in CD4-3M and IL-2 NRE-M by a dot. Also summarized is the binding of ZEB to the different oligonucleotides (see Fig. 2B and C). CD4-3, proximal enhancer CD4-3 element; IgH μE5, E-Box from the IgH enhancer; Sil II, second element from CD4 silencer; ICE, E-box from insulin promoter; CKE, E-box from muscle creatin kinase promoter.

Methods

Antibodies

Rabbit anti-human ZEB antiserum was generated by immunization with a peptide of the 15 C-terminal amino acids of human ZEB coupled to the carrier keyhole limpet hemacyanin. Antiserum was diluted 1:20 and used without further purification. Anti-E12, anti-HEB and anti-MyoD1 were used for supershifting according to the manufacturer's protocols (Santa Cruz Biotechnology, Santa Cruz, CA). For FACS analysis, phycoerythrin (PE)-coupled anti-CD4, anti-VLA-4 antibodies and anti-TNP mIgG1 isotype control (PharMingen, Heidelberg, Germany) were used.

Plasmids

pCD4E/P-Luc was made by cloning a blunted *Hin*DIII–*Xho*I fragment of pBLCD4/0.35CAT (gift from Dr D. Littman, UCSF, San Francisco, CA) into the *Sma*I site of pGL3-Basic (Promega, Mannheim, Germany). The point mutation in pCD4E/P-M was induced by site-directed mutagenesis of pCD4E/P according to the manufacturer's protocol (Quick Change kit; Stratagene, La Jolla, CA). p2xCD4-3-Luc and p2xCD4-3M-Luc were constructed by cloning double-stranded DNA oligonucleotides encoding 2xCD4-3 or 2xCD4-3M (see Fig. 1) into the *Xho*I–*Hin*DIII sites of pGL3-B. pSV-β-Gal (Promega) was used to normalize transfection levels. pGSThZEB-cterm was made by cloning a PCR product encoding amino acids 893–1125 of human ZEB into the *Bam*HI–*Xho*I sites of pGEX-5X-2 (Pharmacia, Freiburg, Germany). The following plasmids were kindly provided by various researchers: pCI-neo-ZEB by Dr A. Postigo (Washington University School of Medicine, St Louis, MO), pCDM8A-REB6 by Dr K. Kawakami (Jichi Medical School, Tokyo, Japan), pSCD/MT (dominant negative c-Myb) and pMCEF-c-Myb (c-Myb expression vector) by Dr K. Weston (Institute of Cancer Research, London, UK), and pIL-2pLuc by Dr T. Wirth (MSZ, Würzburg, Germany), pEBCKEts1 by Dr J. Ghysdael (Curie Institute, Orsay, France).

Electrophorectic mobility shift assays (EMSA)

Nuclear extracts were prepared as described by Schreiber *et al*. (28). However, a mixture of various protease inhibitors (complete; Boehringer Mannheim, Mannheim, Germany) was used to avoid fast degradation of ZEB protein. For bacterial expression of recombinant proteins the GST system (Pharmacia) was used. For binding competition experiments (Fig. 2D) all recombinant proteins (E12 and c-terminal ZEB) were mixed before addition to the binding reaction (amounts of bacterially expressed proteins were equalized with bacterially expressed GST). Binding reactions were performed in a final volume of 15 μl binding buffer optimized for ZEB [20 mM HEPES, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 100 μM $ZnSO_4$, 1 mM DTT, 4% Ficoll and 1 μg poly(dA–dT)] containing 2 μg of nuclear extract or indicated amounts of purified recombinant protein and 1 μg BSA. Finally, 1×10^4 c.p.m. of ^{32}P end-labeled double-stranded DNA oligonucleotide as probe was added, which turned out to be important to detect ZEB binding. Reactions were incubated at room temperature for 20 min and separated on a 5% non-denaturing polyacrylamide gel in 0.25% TBE. For competition experiments, unlabeled oligonucleotides were premixed to the radiolabeled probe. For supershift experiments antibodies were added to the reaction mixtures after a 10 min incubation and the reaction was then incubated for an additional 20 min at room temperature.

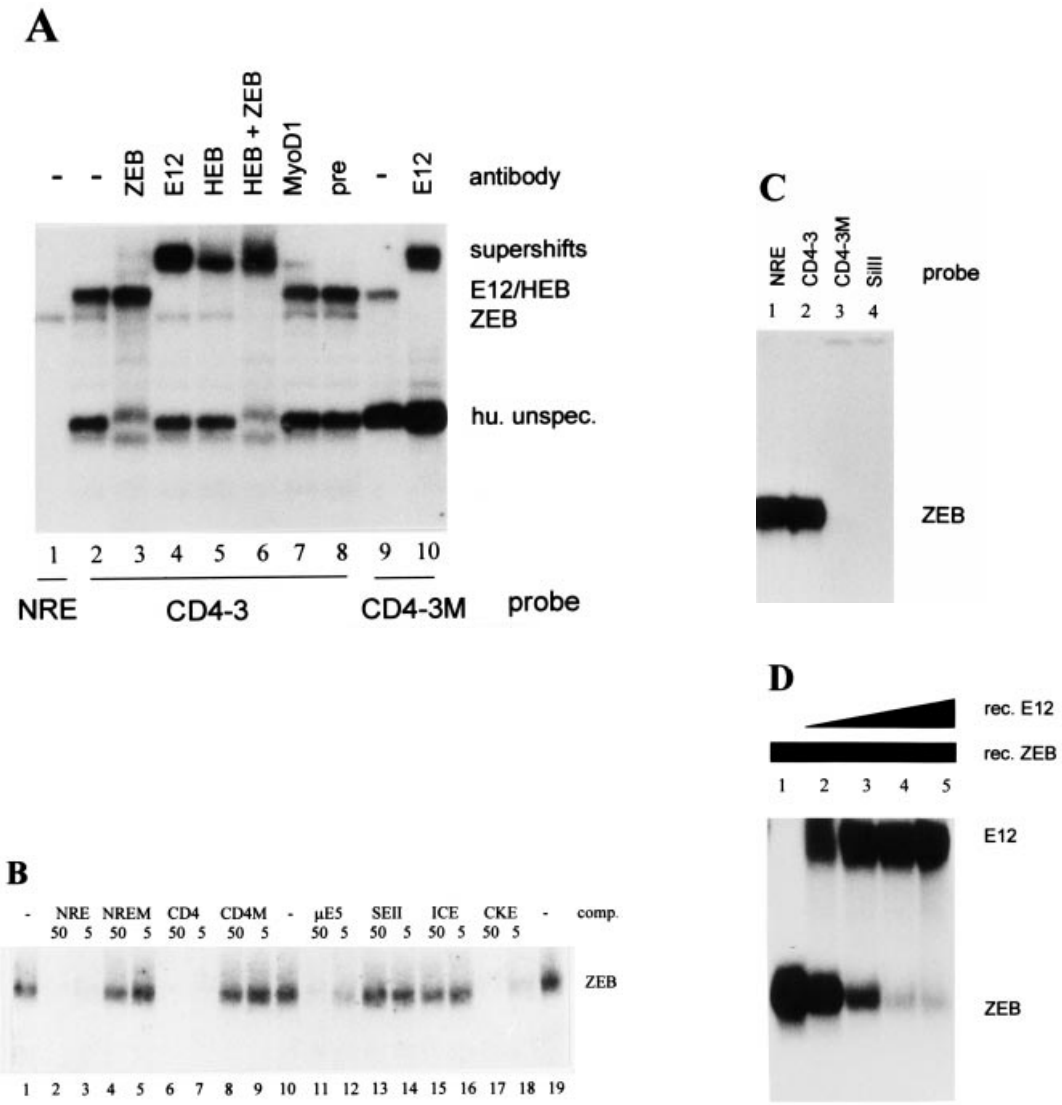

Fig. 2. ZEB and E12/HEB bind to the CD4-3 element of the CD4 proximal enhancer. (A) EMSA using Jurkat T cell nuclear extracts and IL-2 NRE (lane 1), CD4-3 (lanes 2–8) and CD4-3M (lanes 9 and 10) as probes. Antibodies used for supershifting are indicated above the lanes (pre, preimmune serum). IL-2 NRE is only bound by ZEB (lane 1), and CD4-3 by two specific complexes and a human unspecific complex (lane 2). Treatment with anti-ZEB antiserum (lane 3) eliminates the lower complex (which runs exactly like the IL-2 NRE binding complex) and leads to a weak supershift. Antibodies against E12 or HEB supershift the upper complex (lanes 4 and 5). Both specific complexes are eliminated by anti-ZEB and anti-HEB (lane 6). Mutated CD4-3 is only bound by E12/HEB (lanes 9 and 10). (B) Competition of ZEB binding to IL-2 NRE by various unlabeled oligonucleotides (50 or 5 ng) (for exact sequences see Fig. 1). Introduction of a point mutation into the IL-2 NRE (lanes 4 and 5) and the CD4-3 5′E-box (lanes 8 and 9) disturbs ZEB binding. Note that also the Sil II E-box of the CD4 silencer (SEII, lanes 13 and 14) cannot compete ZEB binding. (C) ZEB binds to IL-2 NRE and CD4-3, but not to CD4-3M and Sil II. Bacterially expressed ZEB C-terminal DNA binding domain (20 ng) was incubated with the indicated probes. (D) E12 displaces ZEB from binding to CD4-3. Constant amounts of bacterially expressed ZEB C-terminal DNA binding domain (20 ng) and increasing amounts of bacterially expressed E12 (2.5, 5, 10 and 20 ng; lanes 2–5) were used.

Transient transfections and reporter assays

CsCl gradient-purified plasmid DNAs were introduced into 2×10^6 Jurkat or A.301 human T cells by electroporation (280 V, 1050 µF) using a gene pulser (EquiBio, Angleur, Belgium). Transfection efficiency was >60% with electroporation. When indicated, Jurkat cells were stimulated with 1 µM ionomycin and 10 nM PMA (Sigma, St Louis, MO). Transfection of 1×10^6 COS-1 cells (African green monkey kidney cells) was performed in a 6-cm well using the standard DEAE–dextran technique (30% transfection efficiency). At 24 h after transfection cells were lysed by sonification, and cell extracts were assayed for luciferase and β-galactosidase activity following the manufacturer's protocol (Dual-Light system; Tropix, Bedford, MA). Transfection efficiencies were normalized using pSV-β-Gal as transfection control. All transfections were performed at least 3 times.

FACS analysis

After transfection for luciferase reporter assays, 10^6 cells were separated, washed in PBS and 5×10^5 cells used per staining according to the manufacturer's protocol. Stained cells were analyzed on a FACSTrak (Becton Dickinson, Heidelberg, Germany) using R-PE-conjugated antibodies directed against CD4/RPA-T4 (30155X; PharMingen), CD49d (VLA-4)/9F10 (31474X; PharMingen) or TNP-KLH/107.3 (03005A, PharMingen) as an isotype control (IgG1,κ). The cytometer was calibrated by the use of CaliBRITE beads (349502; Becton Dickinson) and the program AUTOCOMP, which is part of the cytometer's standard software. During collection a polygonal gate for viable cells based on FSC/SSC [linear scale (>450)/log scale ($>10^1$)] had been set by eye. The resulting data were analyzed with the help of WinMDI version 2.7 (Windows Multiple Document Interface), which can be obtained as freeware from the Scripps Research Institute (http://facs.scripps.edu/software.html). Overlays were generated and smoothed by five iterations.

Results

ZEB binds to the CD4-3 site of the CD4 proximal enhancer, and is able to displace the transcriptional activators E12 and HEB

Comparison of the consensus binding sequence of ZEB and regulatory sequences of genes involved in T cell development revealed a potential binding site for ZEB in the CD4-3 element of the proximal enhancer of the CD4 gene (11). The CD4-3 element contains two E-boxes, but only the 5′E-box is a potential binding site for ZEB (Fig. 1). Therefore we performed different binding studies with the CD4-3 element. By EMSA with nuclear extract from Jurkat T lymphoma cells we could show that the CD4-3 site is bound by multiple protein complexes (Fig. 2A, lane 2). The fastest migrating complex (termed human unspecific) was only detected in human cell extracts and showed an unspecific competition pattern (data not shown). In contrast, the NRE of the IL-2 promoter, which is not an E-box [because of a G to A nucleotide change in the last position (see Fig. 1)], binds only one factor (Fig. 2A, lane 1), which is known to be the repressor ZEB (18). To prove the identity of the two specific CD4-3 binding complexes, we performed supershift experiments. Addition of a ZEB-specific antiserum eliminated the lower specific complex, which ran at the same position as the IL-2 NRE binding complex (Fig. 2A, lane 3), indicating that the binding protein is ZEB. Moreover, a weak supershift was detectable. Antibodies against the bHLH proteins E12 and HEB supershifted the slowest migrating complex, leaving the lower specific complex (Fig. 2A, lanes 4 and 5). A combination of anti-ZEB and anti-HEB antibodies eliminated and supershifted both complexes (Fig. 2A, lane 6). An unrelated antibody and pre-immune serum did not eliminate the complex formations. We further used a CD4-3 probe with a point mutation in the 5′E-Box (CD4-3M; see Fig. 1), known to inhibit ZEB binding, but not the binding of bHLH factors. This probe was only bound by the upper complex (Fig. 2A, lane 9) and again supershifted by anti-E12 antibody (Fig. 2A, lane 10). These data indicate that CD4-3 is bound by the bHLH proteins E12 and HEB, and the repressor ZEB.

In order to further characterize the binding behavior of ZEB to CD4-3, we performed competition experiments using unlabeled oligonucleotides. Unlabeled CD4-3 element was able to efficiently compete for ZEB binding to the IL-2 NRE (Fig. 2B, lanes 6 and 7). After mutating one of the two important central G residues in the 5′E-box (Fig. 1, CD4-3M), CD4-3 could no longer compete with NRE, indicating that ZEB binds, as predicted, to the 5′E-box of CD4-3 (Fig. 2B, lanes 8 and 9). Note that the E-box of the CD4 silencer element Sil II (29) also cannot compete for ZEB binding (Fig. 2B, lanes 13 and 14) (see Discussion). These results were further confirmed by using recombinant ZEB (C-terminal DNA binding domain, amino acids 893–1125) and various probes. ZEB bound efficiently to IL-2 NRE and CD4-3, but not to CD4-3M and Sil II (Fig. 2C).

We next investigated if bHLH factors could compete with ZEB for binding to the CD4-3 element. Increasing amounts of recombinant E12 were able to efficiently displace the ZEB C-terminal DNA binding domain from the CD4-3 probe (Fig. 2D). Thus the transcriptional repressor ZEB binds to the 5′E-box in CD4-3 site of the CD4 proximal enhancer in competition with the transcriptional activators E12 and HEB.

Overexpression of ZEB represses the CD4 proximal enhancer/promoter

We further wanted to know if ZEB binding has an effect on the function of the CD4 proximal enhancer. That could be relevant, since during the last differentiation step to mature single-positive T cells, CD4 expression is mainly activated by the proximal enhancer, regulating CD4 promoter activity (8). The promoter has binding sites for Myb (30), Ets (12) and the bHLH-Zip factor MAZ (31). Therefore we tested if ZEB is able to repress a luciferase reporter construct driven by the CD4 proximal enhancer and promoter (pCD4E/P-Luc). ZEB repressed the activity of this constructs in both Jurkat ($CD4^+CD8^-$) and A.301 ($CD4^+CD8^+$) T cells to 48 and 76% activity respectively (Fig. 3A). The introduction of a point mutation into the 5′E-box of the CD4-3 enhancer element (pCD4E/P-M) (Fig. 1), which inhibits the binding of ZEB but not of the activators E12/HEB to the proximal promoter, reduced the suppressive effect of ZEB. This indicates again the specificity of ZEB binding and in particular its pivotal role in the regulatory function of the CD4-3 5′E-box. However, in both cell lines the repression of the CD4 enhancer/promoter construct was only about half as effective as the ZEB-induced repression of the TPA/ionomycin-activated IL-2 enhancer/promoter (Fig. 3A). Thus ZEB binding to the 5′E-box the CD4 proximal enhancer is able to suppress CD4 enhancer/promoter activity. However, compared to the IL-2 promoter the inhibition by ZEB was less effective. Increasing amounts of co-transfected ZEB expression vector led to a dose-dependent repression of the CD4 proximal enhancer/promoter construct (Fig. 3B). This is consistent with the model of a competing binding of ZEB and the activators E12/HEB. To show that the repression is mediated through the CD4-3 element we used a reporter clone driven by two copies of this element (Fig. 3C). p2xCD4-3-Luc was efficiently suppressed dose dependently in Jurkat cells by increasing amounts of

ZEB. ZEB had no effect on p2xCD4-3M-Luc, which is consistent with a lack of ZEB binding to CD4-3M (see Fig. 2C).

Myb and Ets counteract transcriptional repression by ZEB.

In contrast to the IL-2 promoter, the CD4 promoter is activated by Myb and Ets transcription factors, which are known to exert a strong cooperative activity (32). It was shown for the VLA-4 promoter that ZEB was not able to repress the cooperative effect of both factors (25). We therefore tried to weaken this cooperation on the CD4 promoter by co-transfecting Jurkat and A.301 T cells with a dominant-negative expression construct of c-Myb (dnMyb), ZEB and the reporter pCD4E/P-Luc. dnMyb alone only had a weak inhibitory effect (repression was always stronger in Jurkat than in A.301 cells) (Fig. 4A). However, a combination of ZEB and dnMyb led to a strong synergistic inhibition of transcription, indicating that the activity of one of both promoter activating factors alone, here Ets, can be repressed more efficiently by ZEB. In order to support this hypothesis we activated the pCD4E/P reporter construct in Cos cells, which do not express endogenous Myb and Ets, by co-transfecting Myb and Ets-1 expression vectors alone or in combination. Activation by Ets-1 or Myb alone could be repressed by ZEB to 63 and 53% respectively (Fig. 4B). However, a combination of both activators, leading to only a weak additional stimulation, was almost resistant to suppression by ZEB. These results provide evidence that a combined effect of Myb and Ets factors on the CD4 promoter is able to partially overcome repression by ZEB.

Overexpression of ZEB reduces the CD4 expression on the cell surface of CD4 single-positive T cells

In order to investigate if ZEB also inhibits CD4 protein expression, reflecting the effect of ZEB on all interacting CD4 regulatory elements in a chromatin context, we performed FACS analysis. Overexpression of ZEB in CD4 single-positive Jurkat cells reduced the surface expression of CD4, but did not alter levels of VLA-4 (Fig. 4). In contrast the CD4 expression of the $CD4^+CD8^+$ double-positive A.301 T cells, which probably represent an earlier differentiation stage, was not influenced by ZEB.

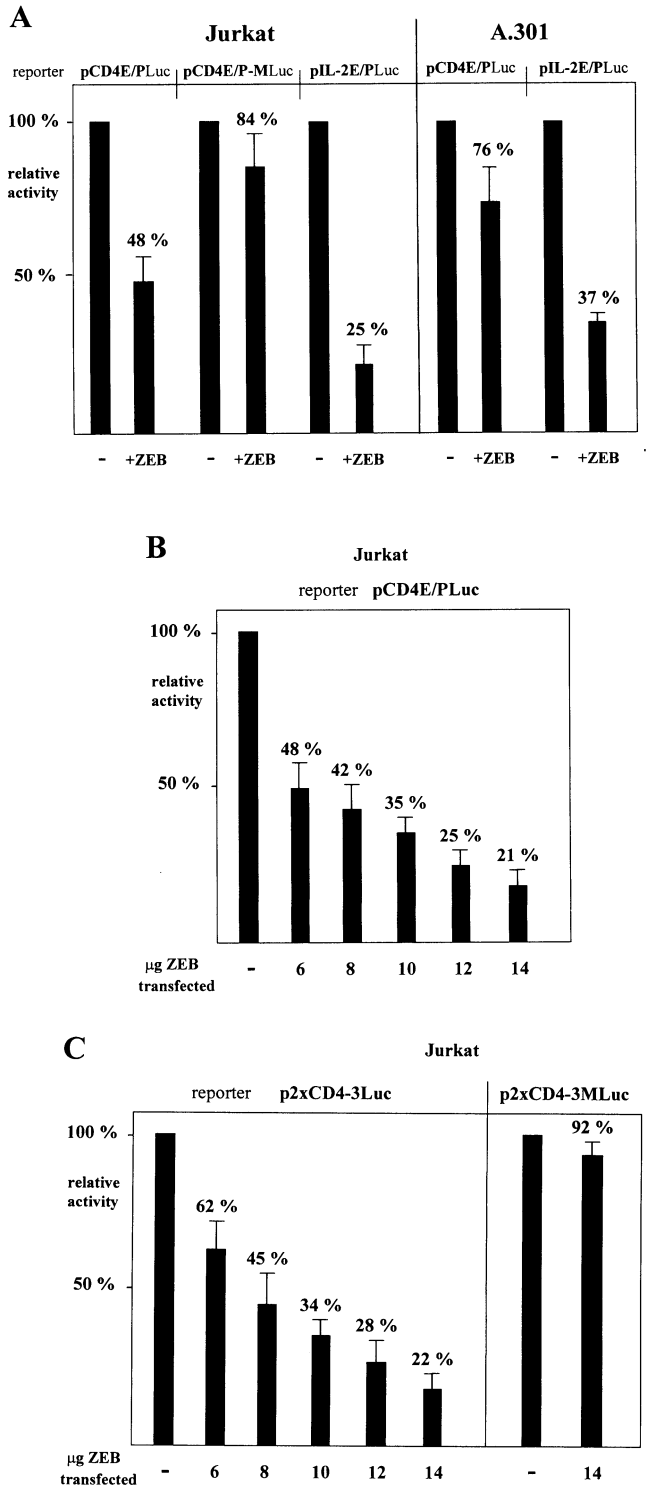

Fig. 3. ZEB inhibits the activity of the CD4 proximal enhancer and promoter. (A) Jurkat and A.301 T cells were co-transfected with 6 µg of the indicated reporter constructs and 8 µg of pCI-neoZEB (ZEB) or control vector (–). ZEB inhibits IL-2-(pIL-2E/PLuc) and to a lesser extend CD4 enhancer/promoter (pCD4E/PLuc). A point mutation in the CD4-3 element (pCD4E/P-MLuc) blocks ZEB repression. (B) Increasing amounts of transfected ZEB in Jurkat cells lead to a dose-dependent repression. (C) Jurkat cells were co-transfected with the indicated amounts of ZEB expression vector and the indicated reporters. ZEB inhibits 2xCD4-3-Luc, but not 2xCD4-3M-Luc. Always shown is the percentage of remaining activity after repression. SEM is indicated.

Discussion

In this study we showed that the transcriptional repressor ZEB binds to the CD4-3 element of the proximal CD4 enhancer, thereby competing with the bHLH activators E12 and HEB for binding to the same element. Overexpression of ZEB in T cells results in an active repression of a transfected reporter construct driven by the CD4 enhancer/promoter. The ZEB inhibitory effect can be enhanced by elimination of Myb activity on the CD4 promoter. Furthermore ZEB can reduce the expression of CD4 protein on the surface of CD4 single-

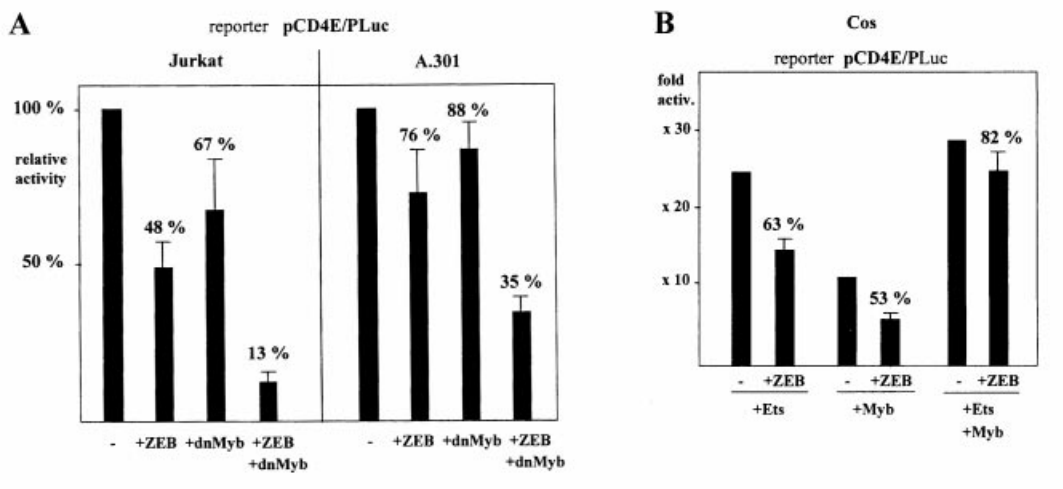

Fig. 4. Inhibition of the CD4 proximal enhancer and the promoter by ZEB interferes with the transcriptional activators Ets and Myb. (A) Jurkat and A.301 T cells were co-transfected with 6 μg of the pCD4E/PLuc reporter construct and 8 μg each of the indicated repressor plasmids. A combination of ZEB and dnMyb expression constructs showed the strongest, synergistic inhibition. (B) Cos cells were co-transfected with 6 μg of the pCD4/EPLuc reporter construct, 6 μg of the indicated transcriptional activators (Ets, Myb) and 8 μg ZEB expression vector or control plasmid. Activation by Ets and Myb together can hardly be repressed by ZEB. All cells were co-transfected with pSV-β-Gal to normalize transfection efficiency. Amounts of transfected DNA were equalized with the appropriate control vectors. For (A) the percentage of remaining activity after repression is shown. For (B) the inhibition is shown in relation to stimulation by the activators. SEM is indicated.

positive T cells. These data indicate that ZEB cannot only compete with a transcriptional activator for binding to the CD4 enhancer, but can also actively repress the CD4 promoter, thereby converting the function of the CD4 proximal enhancer to a potential silencer element. Our results are supported by a very recent publication of Grégoire and Roméo (33). They identified the GATA-3 gene as another gene repressed by ZEB. GATA-3 is a transcription factor required for the early development of the T cell lineage. They showed that GATA-3 expression is controlled by a silencer element through an identical CAGGTG E-box which we defined as a potential repressor element in the proximal CD4 enhancer. Moreover, as we demonstrated for this CD4-3 5′E-Box, the GATA-3 E-box is also competitively bound by the repressor ZEB and the activators E12/HEB.

ZEB is known to control the exact temporal and spatial expression of specific genes during muscle differentiation (21). Targeted disruption of the C-terminal ZEB region in mice indicates that ZEB is also regulating such processes during T cell differentiation (22). These mice show a severe defect in T cell differentiation resulting in up to 500-fold reduction of thymocytes. This developmental block is not absolute, since few T cells are found in the periphery. However, most of the mature T cells are $CD4^+$, indicating that in the absence of ZEB either the silencer-mediated suppression of the CD4 gene normally resulting in mature $CD8^+$ T cells is affected or the positive selection is forced towards $CD4^+$ T cells. During the differentiation of double-positive thymocytes to mature single-positive T cells the CD4 gene is activated by the distal and the T cell-specific proximal enhancer. Additionally it is suppressed by the late intronic silencer in $CD8^+$ T cells (8). This silencer consists of three binding elements (Sil I, II and III). Sil II is absolutely necessary for silencer function. Since Sil II also contains an E-Box (CAGTTG), ZEB was postulated to be a potential repressor binding to this site (25,29), but the missing central two G-residues and our data that the Sil II E-Box cannot be bound by ZEB (Fig. 2B, lanes 13 and 14, and Fig. 2C, lane 4) rule out this possibility. Probably Sil II is bound by a similar factor as the 3′E-Box of the proximal enhancer CD4-3 element (29). Since the T cell-specific Sil II binding factor, essential for silencer function, is also present in $CD4^+$ T cells as is the factor binding to Sil I, CD4 silencing in $CD8^+$ T cells cannot be explained by the silencer alone. Duncan *et al*. (29) postulated a functional interaction of the factor binding to Sil II and factors binding to the CD4-3 element of the proximal CD4 enhancer. Our data showing that the transcriptional repressor ZEB binds exactly to this element and the fact that in ZEB-deficient mice CD4 silencing seems to be affected, support the hypothesis that ZEB can mediate a functional interaction of the proximal enhancer (or then co-silencer) and the silencer in suppressing the CD4 gene expression. The latest results from Kim and Siu (34) show that activation of the Notch signal pathway is able to suppress CD4 gene transcription independently of the CD4 silencer through interference with the CD4 enhancers and the CD4 promoter. Since it is not known how ZEB function is regulated in thymocytes, it is obvious that Notch can also activate ZEB. Further experiments will address this point.

As indicated by our transient transfection assays (Fig. 3), ZEB alone cannot absolutely suppress the strong promoter activity mediated by Myb and Ets transcription factors in mature $CD4^+$ T cells. This makes sense in the context of a recent publication: constitutive expression of ZEB is used by T_h2 cells to silence IL-2 gene expression through the IL-2 NRE motif (35). Since T_h2 cells also need to express CD4, parallel suppression of CD4 by ZEB alone would counteract activation and cytokine production of these cells. However, functional interaction of ZEB with a silencer factor, which by

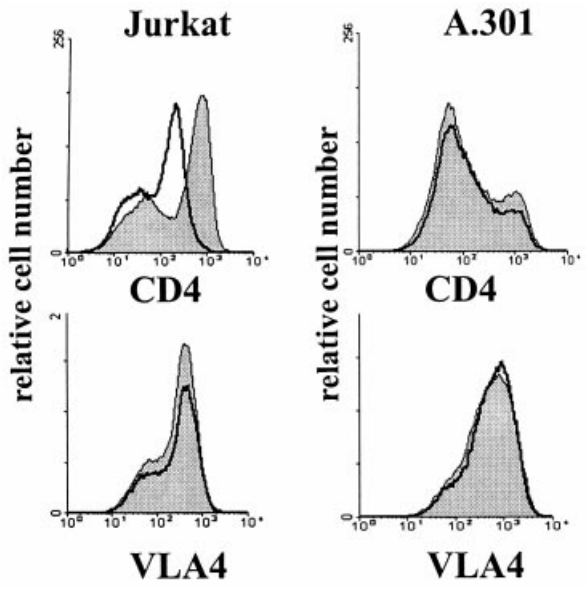

Fig. 5. ZEB suppresses the surface expression of CD4 in Jurkat but not in A.301 T cells. A $CD4^+$ subclone of Jurkat T cells or CD4/8 double-positive A.301 T cells were transfected with a ZEB expression construct (thick line) or control vector (shaded area). Cells were then stained with antibodies directed against CD4 or CD49d (VLA-4). Whereas ZEB decreased the amount of CD4 on the surface of Jurkat T cells, no change was seen in the surface expression of CD49d (VLA-4). Fluorescence intensity (*x*-axis) is denoted on a logarithmic scale, cell number (*y*-axis) on a linear scale. All maxima of cell numbers are the same (256 relative units), so that the curves are directly comparable.

itself is also not able to inhibit promoter activity, could then be able to completely switch off CD4 expression in $CD8^+$ T cells. Additional experiments are necessary to prove the existence of such a potential interaction between the CD4 proximal enhancer and silencer mediated by the transcriptional repressor ZEB.

One result was the reduction of CD4 expression in single $CD4^+$ Jurkat cells by ZEB, but not in CD4/8 double-positive A.301 T cells (Fig. 5). Since A.301 represents an earlier differentiation stage, these data are consistent with a recent publication: CD4 expression on CD4/8 double-positive thymocytes is activated by a newly discovered 'double-positive' enhancer (8), which may not be a target of ZEB. During positive selection to single-positive T cells the proximal and distal enhancer are reactivated, and are now responsible for CD4 expression. As indicated by our FACS data from Jurkat cells, in these cells the CD4 gene activation by the proximal enhancer is then a potential target of ZEB. However, we cannot exclude additional indirect effects of ZEB on CD4 gene transcription. These could include transcriptional suppression of T cell transcription factors, necessary for CD4 gene activation by ZEB, as previously described for GATA-3 (33). VLA-4 protein expression in Jurkat and A.301 was not affected, which can be explained by the even stronger restrictive effect of the Myb/Ets cooperation on ZEB function, as already shown by Postigo *et al*. (25). The mechanism how ZEB actively suppresses transcription and how cooperation of Myb and Ets can overcome this effect is not known. Probably ZEB acts directly on the basic transcription machinery by recruiting a co-repressor protein (36).

The severe defect in thymocyte differentiation of ZEB-deficient mice shows that ZEB also plays a role in early T cell development, probably by inhibiting additional still undefined target genes. Recently defined genes suppressed by ZEB are the transcription factor GATA-3 (33) and the integrin VLA-4, important for the migration of hematopoietic precursor cells into the thymus (25). ZEB binds to an E-Box in the VLA-4 promoter and is able to suppress its activity until binding of both Myb and Ets factors is able to overcome the function of ZEB. The importance of CD4 in T cell development and the striking similarity of regulatory elements in the CD4 proximal enhancer and the promoter with those of the VLA-4 gene (both genes are controlled by E-boxes and a cooperation of Myb and Ets factors) imply that CD4 may also be a target of ZEB repression in early T cell development. This is supported by our results that the CD4 proximal enhancer can function as a silencer if bound by ZEB, and that this effect can be counteracted by Myb and Ets binding to the CD4 promoter. However, because of the limited number of cells in the different stages of very early T cell development, there is little EMSA data about the binding of the relevant factors E12, HEB, Myb, Ets and ZEB to their DNA elements.

Our findings demonstrate that the transcriptional repressor ZEB binds to the proximal enhancer of the CD4 gene and subsequently can convert its function from an enhancer to a potential silencer, resulting in reduced CD4 expression. This offers an additional concept of CD4 gene regulation during late stages of T cell development. A fine regulated activity in a developmentally coordinated manner of ZEB and the Sil binding proteins as repressors and activators like Myb, Ets and E12/HEB may be responsible for the correct expression of CD4. Moreover, other genes important in T cell development, like $p56^{lck}$ (37), are regulated by the same activators and are therefore also potential targets of ZEB. Further work will address the question about a differential activity and interaction of these activators and repressors in various developmental stages of T cells.

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Abbreviations

bHLH	basic helix loop helix
EMSA	electrophoretic mobility shift assay
Luc	luciferase
NRE	negative regulatory element
PE	phycoerythrin
Sil	Silencer

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