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Transcriptional Repression of the IL-2 Gene in Th Cells by ZEB

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Th1- and Th2-type cells mediate distinct effector functions via cytokine secretion in response to immunologic challenge. Precursor Th cells transcribe IFN-γ, IL-2, and IL-4 upon activation. Repeated stimulation of Th precursor cells in the presence of IL-4 leads to terminally differentiated Th2 cells that have lost the ability to transcribe the IL-2 gene. We provide evidence that repression of IL-2 gene expression in Th2 cells and partial repression in Th1 cells are mediated by ZEB, a zinc finger E box-binding transcription factor. This factor binds to a negative regulatory element, NRE-A, in the IL-2 promoter, thereby acting as a potent repressor of IL-2 transcription. The Journal of Immunology, 1998, 160: 4433–4440.
syngeneic APC and Ag. Briefly, splenocytes were prepared from I-A\(^d\) or I-A\(^b\) mice, irradiated with a dose of 2000 rad, and added to Th cells at a ratio of 10 to 1. APC and Th cells were resuspended in complete RPMI at 10\(^6\) cells/ml. The mixture of Th and APC was then added to 24-well plates at 1 ml/well. Twenty-four hours after plating, the culture media were supplemented with either 10 U/ml rIL-2 or Con A-stimulated rat spleen cell supernatant to 1000 U/ml Volume.

Polarized effector Th cells were generated by culturing splenocytes from transgenic mice bearing a Bv3BVa11 TCR specific for pigeon cytochrome c peptide 88–104 with APCs and cytokines (24, 25). Briefly, spleen cells from 3- to 6-mo-old mice were prepared and passed over nylon wool columns. The nonadherent cells were treated with anti-C3D and anti-J11D Abs plus complement. Cells purified by this protocol are 90% naive, resting CD\(^4^+\) (26). The naïve CD\(^4^+\) cells were cultured with mito-

Electrophoretic mobility shift assays (EMSA)

The cultured Th cells were harvested by centrifugation and resuspended at 5 \times 10^5 cells/ml. Cells were stimulated by the addition of PMA (10 ng/ml) plus ionomycin (500 ng/ml) and returned to culture. Three hours after stimulation, CHX was added to the appropriate cultures at a final concen-

The NRE-A, NRE-A mutant, and reporter mutant sequences were synthesized as separate, single-stranded complimentary oligos, and then annealed by

Results

The NRE-A site has been defined in the IL-2 promoter as a po-

tential binding site for a negative transcription factor that dampens IL-2 production in the human Jurkat tumor (18). Furthermore, in vivo treatment of stimulated murine Th2 and Th1 cells with CHX suggests the activity of an IL-2 transcriptional repressor (16, 17). To determine whether a connection exists between these two de-

Figure 1, nuclear extracts from PMA plus ionomycin-stimulated IL-2-nonproducing Th2, and IL-2-producing Th1 and EL-4 thymo-

The addition of a 100-fold molar excess of unlabeled NRE-A sequence to the binding reaction completely interfered with the formation of both the upper and lower complexes (Fig. 1A, lane 3, and Fig. 1, B and C, respectively, lane 1). As an additional control, a second NRE-A mutant sequence that is

The reaction mixtures were then electrophoresed on a non-denaturating acrylamide gel, and two retarded DNA/protein complexes were observed (Fig. 1A, lane 4, and Fig. 1, B and C, respectively, lane 1). As an additional control, a second NRE-A mutant sequence that is

Transfection of reporter gene constructs and reporter gene assays

All constructs used in this study have been described previously: pIL-2 (110–101)/Luc, pIL-2 (548)/Luc (18), and the ZEB expression and antisenes plasmids (19). EL-4 and D10G4.1 Th cells were transfected by electroporation. Cells were removed from long term culture, washed twice, and resuspended at a concentration of 5 \times 10^5 cells/ml in RPMI. A quan-

as an unrelated DNA sequence containing the Oct-1 binding site (Fig. 1A, lane 1; Fig. 1B, lane 4; and Fig. 1C, lane 5) was unable to block the formation of the upper complex, indicating that protein from the nucleus of the Th cells binds specifically to the NRE-A sequence. As an additional control, a second NRE-A mutant sequence that is present in a mutant IL-2 promoter construct described later was unable to compete for the binding of radioactively labeled NRE-A sequence to the Th cell nuclear protein (Fig. 1C, lane 4). These results demonstrate that the nuclei of activated EL-4, Th1, and Th2 cells contain DNA-binding proteins specific for the NRE-A site.

ZEB and the Th cell nuclear factor have identical NRE-A-binding specificity

To establish that the DNA-binding specificity of the T cell nuclear protein is consistent with that of ZEB, the Th cell nuclear extracts were replaced in the binding mixture by rZEB, expressed in bac-

The binding pattern with rZEB correlates with that obtained using Th cell nuclear proteins. Mobility of the radioactively labeled NRE-A site was retarded by the binding of ZEB protein (lane 1), and a 100-fold molar excess of unlabeled NRE-A sequence competed away this binding (lane 2), while an equal amount of unlabeled mutant NRE-A sequence (lane 3) or an unrelated DNA sequence (lane 4) could not. These data demonstrate that both rZEB and the NRE-A-binding protein from Th cells display an identical
NRE-A sequence-specific binding in EMSA, suggesting that the nuclear protein in the T cell extracts may in fact be ZEB or a highly related protein.

The NRE-A-binding activity is ZEB or a highly related protein

To confirm that the nuclear NRE-A-binding protein in Th cells is indeed ZEB, we used ZEB-specific Abs in the EMSA experiments to see if they would react with the Th cell protein and block its interaction with the NRE-A element. As expected, addition of polyclonal Abs raised against the chicken homologue of ZEB, βEF1 (29), to the reaction mixture containing GST-ZEB completely prevented the formation of the GST-ZEB-labeled NRE-A site complex (Fig. 2, lane 9). In contrast, an equal amount of control polyclonal anti-NF-AT Ab did not affect the formation of the GST-ZEB-labeled NRE-A site complex (Fig. 2, lane 10). Most importantly, the addition of anti-ZEB Ab to the reaction mixtures containing Th cell nuclear protein similarly abolished the formation of the specific protein-labeled NRE-A site complex (Fig. 2, lane 4). Thus, it was of interest to test whether CHX treatment had any influence on the level of nuclear NRE-A-binding activity. As clone D10G4.1 (16), and enhanced IL-2 transcription in EL-4 cells (17). Thus, it was of interest to test whether CHX treatment had any influence on the level of nuclear NRE-A-binding activity. As

The binding of ZEB to the IL-2 promoter NRE-A site in Th cells is reduced by CHX treatment

The results presented to date indicate that nuclear extracts from activated Th2, EL-4, and Th1 cells contain the NRE-A-binding protein, ZEB. Figure 3 demonstrates that the binding of nuclear ZEB to the NRE-A site is reduced by CHX treatment of the Th cells. We had shown previously that treatment of stimulated cells with CHX 3 h postactivation led to IL-2 transcription in the Th2 cell type.
can be seen in Figure 3, ZEB binding to NRE-A is almost totally abolished in the nuclei of activated EL-4 cells that have been treated with CHX (lane 2), compared with nuclear extracts from activated EL-4 cells without CHX treatment (lane 4). CHX treatment of resting EL-4 cells also sharply reduces the binding activity of ZEB for NRE-A (Fig. 3, lane 1 compared with lane 3). These results establish that CHX-sensitive ZEB binding to the NRE-A site is correlated with IL-2 transcriptional silencing. As CHX is known to inhibit protein synthesis, we conclude that this compound is interfering with the production of ZEB after cellular activation. This could account for the derepression of IL-2 gene transcription observed in D10G4.1 Th2 cells (16) and an increase in transcription observed in EL-4 cells (17).

NRE-A-binding activity in polarized effector Th1 cells and Th2 cells

To confirm our findings in long-term Th cell clones, we examined ZEB NRE-A binding in Th cells that are directly derived from a common naive precursor. Purified naïve CD4+ cells isolated from AND TCR transgenic mice were polarized into effector Th1 and Th2 cells by culture with IFN-γ and IL-12 or IL-4, respectively (24, 25). As can be seen in Figure 4A, unstimulated polarized effector Th1 and Th2 cells (lanes 1 and 3, respectively) contain NRE-A-binding activity. Upon treatment with PMA plus ionomycin for 6 h, NRE-A-binding activity is reduced in IL-2-producing Th1 cells by 40% (lane 2), compared with resting Th1 cells, as determined by densitometry reading. On the other hand, NRE-A-binding activity remains elevated in Th2 cells compared with resting Th2 cells (23% reduction) (lane 4). Figure 4B depicts the NRE-A-binding specificity of the nuclear factor in these cells. As previously shown in Th clones (Fig. 1), the binding of 32P-labeled NRE-A sequence to Th nuclear protein (lane 1) is fully competed away by a 100-fold molar excess of unlabeled NRE-A (lane 2), but not by the same amount of a mutant NRE-A sequence (lane 3). This EMSA analysis of NRE-A binding shows that activity is reduced in polarized Th1 cells that produce large amounts of IL-2 and maintained in Th2 cells that make little IL-2. These results are significant for two reasons: 1) They are consistent with the role of ZEB as an IL-2 transcriptional repressor shown in Th clones; and 2) they suggest that Th1 and Th2 cells derived from the same precursors have different levels of ZEB activity following activation.

Kinetics of ZEB-binding activity upon stimulation of Th cells

Once we had established that ZEB binds to the IL-2 NRE-A element in activated Th cells, we wanted to determine a time course of ZEB-binding activity in Th2 cells. If ZEB is a transcriptional repressor of IL-2, then one would expect that ZEB NRE-A-binding activity would be highest when IL-2 transcription is initiated. This EMSA analysis of NRE-A binding shows that activity is reduced in polarized Th1 cells that produce large amounts of IL-2 and maintained in Th2 cells that make little IL-2. These results are significant for two reasons: 1) They are consistent with the role of ZEB as an IL-2 transcriptional repressor shown in Th clones; and 2) they suggest that Th1 and Th2 cells derived from the same precursors have different levels of ZEB activity following activation.
An IL-2 promoter bearing a mutant ZEB binding NRE-A site has activity in Th2 cells and enhances activity in EL-4 cells, compared with a wild-type IL-2 promoter

When the wild-type IL-2 promoter construct, pIL-2(-548)Luc, was transiently transfected into the non-IL-2-transcribing Th2 clone, D10G4.1, an insignificant level of reporter activity was observed after activation with PMA plus ionomycin (Fig. 6A, top bar compared with the bar directly below). This result is consistent with the findings reported by others using the D10G4.1 clone (14, 15). Thus, the wild-type IL-2 promoter construct accurately reflects the activity of the endogenous IL-2 gene in Th2 cells, which is transcriptionally silent upon activation. On the other hand, an IL-2 promoter construct, pIL-2(-110/101)Luc, lacking a functional ZEB binding NRE-A site, produced a significant amount of luciferase activity in PMA plus ionomycin-stimulated D10G4.1 Th2 cells (Fig. 6A, third row), compared with the wild-type construct (top row). As expected, neither construct exhibited significant luciferase activity in resting D10G4.1 Th2 cells (Fig. 6A, second and fourth rows). This pattern of promoter activity was also observed in another Th2 clone, CDC25 (data not shown), indicating that binding of ZEB to the NRE-A site is a potential mechanism by which activated Th2 cells repress IL-2 production in vivo.

When the same promoter constructs were tested in IL-2-producing EL-4 tumor cells, the NRE-A mutant IL-2 promoter construct produced typically fivefold higher luciferase activity after cellular activation (Fig. 6B, third row), compared with the wild-type IL-2 promoter (Fig. 6B, top row). The key difference between these results and those observed in the Th2 cells is the significant inducibility of the wild-type IL-2 promoter construct in activated EL-4 cells (top row) (note that the scale of units used in Fig. 6B is 10 times higher than in Fig. 6A). This indicates that ZEB binding to the IL-2 promoter NRE-A site can
block IL-2 transcription in both Th2 and Th1 cells. Interestingly, similar results were obtained in the B cell line Bal7 (data not shown), and it is known that B cells express high levels of ZEB (19). The observation that the wild-type IL-2 promoter construct in EL-4 cells produces fivefold less luciferase activity than the mutant construct, along with data obtained in the EMSA, suggests that the NRE-A-binding factor is a negative transcriptional regulator of the IL-2 gene in Th1 cells.

Cotransfection of ZEB antisense constructs increases the inducible transcription of the IL-2 promoter

To confirm that ZEB binding to NRE-A represses IL-2 transcription, ZEB antisense constructs were used to disrupt the activity of the putative repressor (Fig. 7, A and B). Vectors containing antisense sequences to the carboxyl- and amino-terminal halves of ZEB were cotransfected with the IL-2 reporter constructs into EL-4 cells. These antisense vectors are under control of the CMV promoter, which yields strong constitutive expression in lymphocytes (31). As controls, vectors containing noncoding, partial ZEB sequences in the sense orientation or pBluescript plasmid DNA lacking ZEB sequences were cotransfected with the IL-2 promoter constructs. When the ZEB antisense vectors were cotransfected with the wild-type IL-2 promoter construct, a threefold increase in the activity of the reporter gene was seen in response to PMA plus ionomycin (P + I) was added to the groups of cells, as indicated. Relative light units were normalized by the protein concentration of each extract. Representative data from three independent experiments are shown.

**FIGURE 7.** Transcription of the wild-type IL-2 promoter in EL-4 cells is increased by disruption of ZEB production by antisense. A wild-type IL-2 promoter construct (A) or an NRE-A mutant IL-2 promoter construct (B) was transfected into EL-4 cells in combination with plasmids containing either sequences for ZEB in the antisense (antisense) orientation, partial ZEB sequences in the sense orientation (control), or pBluescript (pBluescript) plasmid. PMA plus ionomycin (P + I) was added to the groups of cells, as indicated. Relative light units were normalized by the protein concentration of each extract. Representative data from three independent experiments are shown.
Discussion

The findings shown in this study identify a negative regulatory element of IL-2 transcription

We had reported earlier that repression of IL-2 transcription in activated Th2 cells is controlled by a CHX-sensitive element (16). These studies extend initial observations by Efrat and Kaempfer (32), who had also concluded that IL-2 transcription is controlled by a CHX-sensitive transcriptional repressor. Furthermore, we had demonstrated that IL-2 transcription is increased in Th1 cells by CHX treatment after activation, and that this enhanced response was not solely due to increased NF-κB activity, resulting from the block in inhibitory IkB production by CHX (17). These observations led us to propose that the activity of an IL-2-specific transcriptional silencer was responsible for the silencing of IL-2 expression in activated Th2 cells and dampening of the IL-2 response in Th1 cells.

The present results identify ZEB as the repressor factor. We show by EMSA that nuclear extracts from Th cells contain specific NRE-A-binding activity. The binding specificity of rZEB for the NRE-A site is identical to that of the Th cell nuclear factor. Furthermore, the ability of anti-ZEB Abs to ablate the NRE-A-binding complex in the gel retardation studies demonstrates that at least one component in this complex shares an epitope with ZEB. Therefore, we conclude that the NRE-A-binding complex in the Th cell clones contains ZEB or a highly related protein. It was also found that a CHX treatment protocol that induces IL-2 transcription in activated Th2 cells and increases transcription in EL-4 cells, decreases the binding activity of ZEB to the NRE-A in the nucleus of these cells. In addition, EMSA analyses of NRE-A-binding proteins in polarized Th cells demonstrate that activated Th1 cells have reduced levels of IL-2 promoter NRE-A binding, while in Th2 cells NRE-A-binding activity remains high. We believe that activated Th2 cells are unable to reduce ZEB binding to the IL-2 promoter NRE-A site, despite induction of IL-2 transcriptional activators.

These in vitro data correlate well with the results obtained in functional assays, involving transient transfection of wild-type and NRE-A mutant IL-2 promoter constructs. We show that a construct lacking a ZEB binding NRE-A site produced significant reporter gene expression in activated Th2 cells, compared with a wild-type promoter construct that showed only negligible activity. These results indicate that the interaction of ZEB with the NRE-A site can repress IL-2 transcription in a cell type that normally does not produce IL-2 upon activation. This is especially interesting in light of the fact that nuclear extracts from D10G4.1 Th2 cells have been shown to contain NF-AT proteins (14, 15, 33) that regulate the transcriptional activation of multiple cytokine genes, including IL-2 (7) and IL-4 (14). In addition, we have observed that in stimulated Th1-like EL-4 cells, the NRE-A mutant IL-2 construct is at least five times more active than the wild-type IL-2 construct. These results are consistent with our observation that CHX treatment 3 h postactivation enhances transcriptional inducibility of the wild-type, but not the NRE-A mutant IL-2 promoter construct (data not shown).

The cotransfection of EL-4 cells with ZEB antisense vectors, along with wild-type or mutant IL-2 reporter constructs, provides further evidence that identifies ZEB as the IL-2 silencer in Th cells. The presumed disruption of ZEB mRNA translation by these vectors led to a significant increase in the inducibility of the IL-2 wild-type promoter construct, while the activity of an IL-2 NRE-A mutant promoter was much less affected. Furthermore, we have evidence that the ZEB antisense treatment that we use does in fact reduce the nuclear concentration of ZEB in EL-4 cells (data not shown). These independently derived experimental results indicate that ZEB is an inducible, CHX-sensitive repressor of IL-2 gene transcription in Th2 and, to a lesser extent, Th1 cells. Again, based on our results, it appears that ZEB binding to the promoter NRE-A site disrupts the formation of a stable transcription complex, thereby preventing IL-2 expression. These findings are all the more remarkable in that ZEB binding to the NRE-A site appears to overcome the effect of multiple activators that are known to bind to IL-2 promoter elements (7).

Analysis of transcription factor binding sites in the IL-2 promoter by other investigators also suggests the existence of a repressor element in a sequence that overlaps the NRE-A site (34). Furthermore, our studies agree with results obtained in anergized Th cells, showing that NRE-A-binding factors mediate the IL-2 transcriptional silencing observed in these cells (35). In addition, it has been reported that the NRE-A site can confer repression on a heterologous AP-1 promoter construct in an in vitro transcription assay (35).

A key difference between Th1 and Th2 subsets is the extinction of IL-2 production in Th2 cells. While there are few differences in IL-2 transcriptional activators between Th1 and Th2 cells (9, 15, 36, 37), elevated cAMP activity (38–40) has been suggested to cause IL-2 transcriptional silencing in Th2 cells. In this regard, it should be noted that not only do Th2 cells tolerate a significantly higher level of cAMP (41), they contain more cAMP activity than Th1 cells (42). Other studies indicate that activation-induced cAMP inhibition of IL-2 transcription in Th cells occurs by blocking tyrosine phosphorylation of a 100-kDa protein (38). This phosphoprotein might then affect ZEB activity in Th2 cells.

More recently, it was shown that fused Th1/Th2 cell hybrids produce both IL-2 and IL-4, leading the authors to conclude that a Th2-specific repressor of IL-2 transcription is unlikely (43). However, these results can also be explained by an increase in transcriptional activators provided by the Th1 cell, overriding the effect of an IL-2 repressor in the fused cells. In addition, Th2 cells from NF-AT1 knockout mice produce normal amounts of IL-2 protein (44, 45), contradicting the hypothesis that combined NF-AT/AP-1 activity fully accounts for the regulation of IL-2 transcription. However, targeted disruption of the other known NF-AT gene family members may yet prove the necessity of this factor in IL-2 transcription. Together, these preliminary findings illustrate the complexity of IL-2 transcriptional regulation and demonstrate that a mechanism that fully defines its expression in Th cells has yet to be described.

Our data extend the findings of these studies and show that ZEB is present in the nuclei of resting Th2 and Th1 cells. In Th1 cells, ligation of the TCR/CD3 complex and CD28 by Ag/APC interaction induces the production of AP-1 and translocation of NF-AT and NF-κB proteins to the nucleus. We propose that the binding of these and other factors to the IL-2 promoter overrides ZEB-mediated repression and results in the transcription of IL-2 in Th1 cells, while in Th2 cells these activating factors are unable to overcome the repressive activity of ZEB binding to the IL-2 promoter NRE-A site.

There are many potential mechanisms that could link activation of Th2 cells to transcriptional repression of IL-2 by ZEB. Our results indicate that activation increases ZEB activity in these cells. It is important to note that ZEB and its homologues contain at least one potential protein-protein interaction site in a proline-rich domain (19, 46). Thus, it is possible that ZEB NRE-A-binding activity can be regulated in Th cells by protein-protein interactions.
EXPERIMENTS are underway to further define the regulation of IL-2 by ZEB in Th cells and to determine how the expression of ZEB is controlled.

ZEB has been shown to be a transcriptional repressor in diverse systems. In vitro studies suggest that ZEB binding to the μE5 E box element in the IgH promoter silences transcription of this gene (19). This conclusion is supported by data obtained in transgenic mice bearing a mutant IgH μE5 site. These mice produce inappropriate IgH mRNA transcripts in muscle, heart, and lung (47). In addition, it has been reported that δEFl, the chicken homologue of ZEB, represses transcription of δ-crystallin, a component of the eye, and muscle-specific genes in the developing chicken embryo (48). ZEB has also been suggested as a potential transcriptional silencer of the CD4 gene (49). Thus, ZEB, which is highly conserved in evolution (46, 50), may act as a repressor of multiple genes, including IL-2.

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References


