Immunity, Vol. 16, 9–21, January, 2002, Copyright ©2002 by Cell Press

Helix-Loop-Helix Proteins Regulate Pre-TCR and TCR Signaling through Modulation of Rel/NF-κB Activities

Dongsoo Kim,[1] Min Xu,[1] Lei Nie,[1] Xiao-Cong Peng,[1] Eijiro Jimi,[2] Reinhard E. Voll,[2] Thuan Nguyen,[1] Sankar Ghosh,[2] and Xiao-Hong Sun[1,3]

[1] Immunobiology and Cancer Program
Oklahoma Medical Research Foundation
825 NE 13th Street
Oklahoma City, Oklahoma 73104

[2] Section of Immunobiology
Department of Molecular Biophysics and Biochemistry
Howard Hughes Medical Institute
Yale University School of Medicine
New Haven, Connecticut 06520

Summary

E2A and HEB are basic helix-loop-helix transcription factors essential for T cell development. Complete inhibition of their activities through transgenic overexpression of their inhibitors Id1 and Tal1 leads to a dramatic loss of thymocytes. Here, we suggest that bHLH proteins play important roles in establishing thresholds for pre-TCR and TCR signaling. Inhibition of their function allows double-negative cells to differentiate without a functional pre-TCR, while anti-CD3 stimulation downregulates bHLH activities. We also find that the transcription factor NF-κB becomes activated in transgenic thymocytes. Further activation of NF-κB exacerbates the loss of thymocytes, whereas inhibition of NF-κB leads to the rescue of double-positive thymocytes. Therefore, we propose that E2A and HEB negatively regulate pre-TCR and TCR signaling and their removal causes hyperactivation and apoptosis of thymocytes.

Introduction

Stages of T cell development in the thymus are characterized by the expression of cell surface molecules such as the CD4 and CD8 coreceptors. The most immature thymocytes express neither CD4 nor CD8 (double-negative stage, DN), and further development leads to the expression of both CD4 and CD8 (double-positive stage, DP). Finally, the DP cells mature into fully differentiated cells that express either CD4 or CD8 (CD4 or CD8 single-positive cells, SP) and exit the thymus (Haks et al., 1999; von Boehmer et al., 1999). In addition, the expression of two other cell surface proteins, CD44 and CD25, has been used to further subdivide DN cells into four additional developmental stages: $CD44^+CD25^-$ (DN-I), $CD44^+CD25^+$ (DN-II), $CD44^-CD25^+$ (DN-III), and $CD44^-CD25^-$ (DN-IV) (Godfrey et al., 1993; Hoffman et al., 1996). Successful rearrangement of the β chain of the T cell receptor (TCR) gene at the DN-III stage leads to the assembly and expression of a pre-TCR that contains the rearranged β chain and the pTα protein. Further development of αβ T cells to the DP stage is driven by signals from the pre-T cell receptor (pre-TCR) along with signals from cytokine receptors (Haks et al., 1999). The pre-T cell receptor signals through the Src family of protein tyrosine kinases (PTK) including lck and Fyn. They phosphorylate ITAM motifs in the pre-TCR, thus providing docking sites for cytosolic PTK including Syk and ZAP70. Through subsequent phosphorylation of the adaptor molecules, SPL-76 and LAT, these kinases trigger the ras-MAP kinase (MAPK) and protein kinase C (PKC) pathways, which play a critical role in the survival, proliferation, and differentiation of β-selected double-negative thymocytes, as well as in allelic exclusion of the β locus (Kruisbeek et al., 2000). However, major questions related to how developing thymocytes interpret signals delivered via pre-TCR and TCR are not entirely understood.

Knockout mice lacking components of the pre-TCR signaling pathway, such as pTα, TCRβ, CD3γ, CD3ε, lck and Fyn, ZAP70 and syk, SLP76, or LAT, display a shared phenotype—namely, a developmental block at the DN-III stage (Aifantis et al., 1997; Cheng et al., 1997; Clements et al., 1998; Fehling et al., 1995; Groves et al., 1996; Haks et al., 1998; Malissen et al., 1995; Pivniouk et al., 1998; Sugawara et al., 1998; van Oers et al., 1996; Zhang et al., 1999). These results suggest that the DN-III to DN-IV transition represents a critical step for pre-TCR-mediated development of T cells. The recombination-activating genes, RAG1 and RAG2, are essential for the rearrangement of *TCR*α and β genes, and therefore disruption of RAG1 or RAG2 also leads to a complete block of thymocyte differentiation at the $CD44^-CD25^+$ DN-III stage (Mombaerts et al., 1992; Shinkai et al., 1992). Because the absence of RAG proteins only abolishes rearrangement of the TCR genes, RAG-deficient mice are useful models for testing the role of different factors that influence the pre-TCR signaling pathway. For example, it has been shown that the introduction of activated lck, Ras, or Raf, as well as antibodies against CD3ε, can overcome the developmental block at the DN-III stage in RAG-deficient mice (Aifantis et al., 1997; Gartner et al., 1999; Iritani et al., 1999; Shinkai and Alt, 1994; Swat et al., 1996).

The nuclear effectors of the pre-TCR signaling pathway include transcription factors activated by the Ras-MAPK and PKC pathways. Activation of MAP kinases (Erks, JNKs, and p38) leads to their translocation into the nucleus, where they phosphorylate and activate transcription factors of the Fos, Jun, Est, and Egr families (Jacobs, 2000). The AP-1 DNA binding complex, which consists of Fos and Jun proteins, is known to be involved in cell proliferation and transformation, but its role in thymocyte development is not clear. Overexpression of the Egr1 transcription factor, however, leads to the differentiation of RAG2-deficient cells to the immature CD8 single-positive stage, which lies between the DN and DP stages (Miyazaki, 1997). Pre-TCR signaling also leads to the activation of transcription factors that belong to the Rel/NF-κB family (Aifantis et al., 2001; Sen et al., 1995; Voll et al., 2000; Zuniga-Pflucker et al., 1993).

[3] Correspondence: sunx@omrf.ouhsc.edu

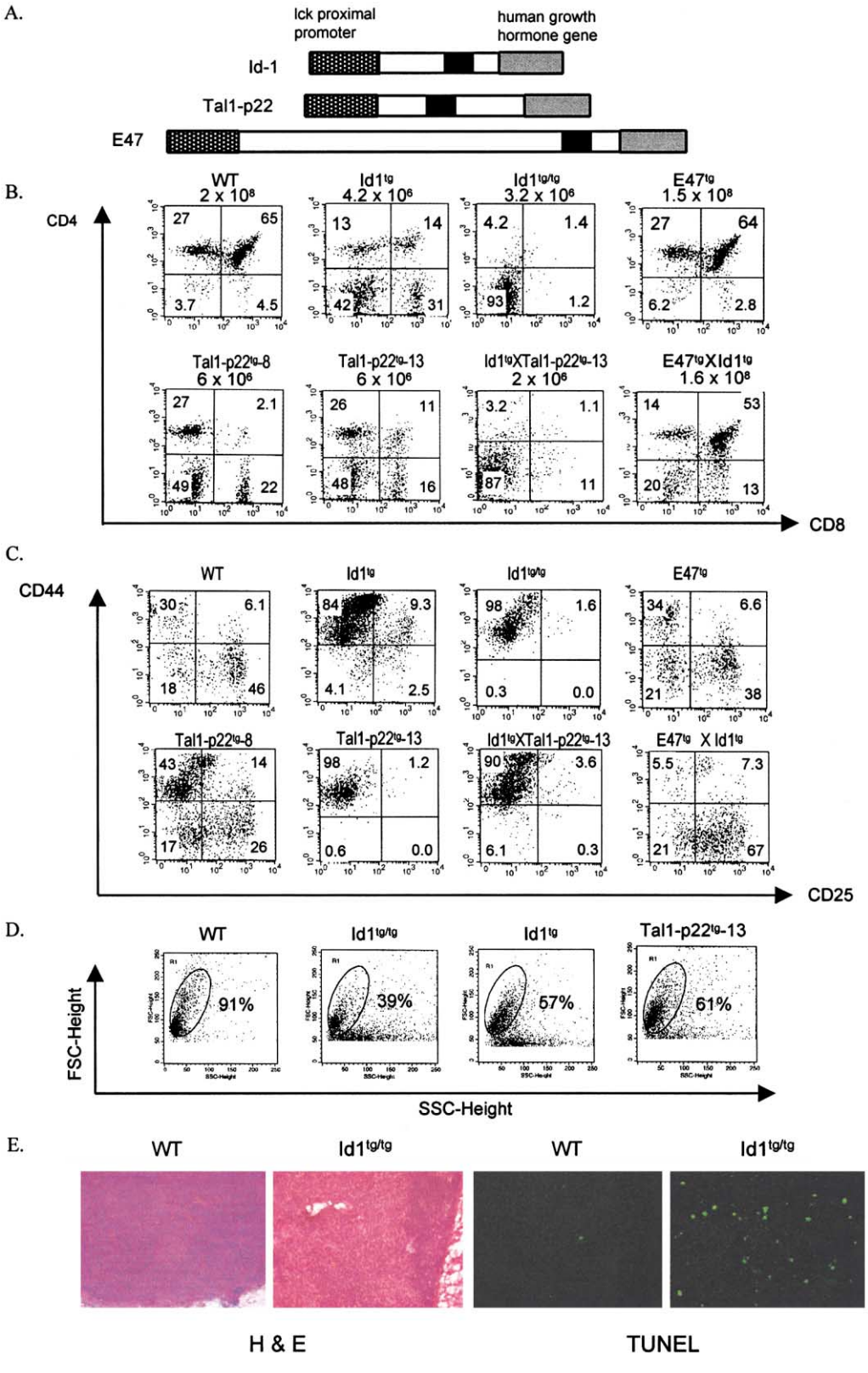

Figure 1. Dose-Dependent Inhibition of T Cell Development by Id1 and Tal1 Transgenes

(A) Schematic diagrams of transgenic constructs expressing Id1, Tal1-p22, and E47 cDNAs. Expression of the transgenes was driven by the proximal promoter of the lck gene as represented by a dark shaded box. The human growth hormone gene provided 3′ untranslated sequences as shown by the light shaded box. Various cDNA inserts are depicted as open boxes with bHLH domains marked as filled boxes.

(B) CD4 and CD8 staining of total thymocytes from mice with the indicated genotypes. The Id1 homozygous and heterozygous transgenic

Recently, the importance of NF-κB in pre-TCR signaling was demonstrated by using a transgene expressing a constitutively active form of IκB kinase β (IKKβ), which led to the development of thymocytes to the DP stage in a RAG1-deficient background (Voll et al., 2000).

E2A (including the alternative splicing products E12 and E47) and HEB are the bHLH transcription factors expressed in the thymus and have been shown to be involved in early stages of T cell development (Bain et al., 1997; Barndt et al., 1999; Zhuang et al., 1996). Because of the functional redundancy of *E2A* and *HEB* genes, mice deficient for either the *E2A* or *HEB* gene exhibit only a partial block in thymocyte development. E2A- or HEB-deficient mice have reduced numbers of total thymocytes. In particular, the percentages of DP cells are lower and the percentages of DN cells are higher than in wild-type mice. The combined biological function of both E2A and HEB proteins could only be elucidated by the examination of transgenic mice expressing transinhibitors for both E2A and HEB proteins. Thus, we generated transgenic mice expressing the HLH inhibitor, Id1, which forms heterodimers with both E2A and HEB and inhibits the DNA binding activities of these proteins (Kim et al., 1999). Additionally, Barndt et al. (2000) created knockin mice bearing a dominant-negative mutation of the HEB gene, which encodes a mutant HEB protein that fails to bind to DNA and also interferes with the activity of E2A proteins. Thymocyte development was severely blocked in both types of mice, and there was evidence of massive apoptosis in the Id1-transgenic mice. Detection of rearranged *TCR*α and *TCR*β genes in the apoptotic cells led to the conclusion that they were derived from more mature thymocytes, most likely DP cells. The ability of Id1 to induce apoptosis of developing thymocytes suggests that bHLH proteins may function to ensure the survival of T cells as they differentiate, although the underlying mechanism by which they regulate this process remains to be understood.

Another family of inhibitors of E2A and HEB proteins are products of the *Tal1* gene, also called SCL (Begley et al., 1989; Chen et al., 1990). The *Tal1* gene encodes two polypeptides, p42 and p22, through differential usage of the translation initiation sites in its transcript (Begley et al., 1991). *Tal1* is normally not expressed in T cells but is activated aberrantly in ~70% of human acute lymphoblastic leukemias (Bash et al., 1995; Brown et al., 1990). Both forms of the Tal1 protein contain HLH motifs that are capable of mediating dimerization with E2A and HEB proteins (Hsu et al., 1991). Although these heterodimers of Tal1 with E2A or HEB can bind to DNA, they are unable to activate transcription (Hsu et al., 1994; Park and Sun, 1998). Except for the target bHLH proteins, Id1 and Tal1 neither share any common interaction partners nor dimerize with each other (Sun et al., 1991). We have now generated transgenic mice in which the p22 form of Tal1 is expressed under the control of the proximal promoter of the lck gene. Similar to Id1, the transgenic expression of Tal1 inhibited T cell development. Coexpression of Id1 with Tal1 led to a synergistic suppression of thymocyte development, suggesting that these inhibitory molecules were probably affecting similar targets, most likely the bHLH proteins, E2A and HEB.

To further investigate the role of bHLH proteins in thymocyte development, we crossed Id1 transgenic mice with RAG1-deficient mice and found that Id1 expression enabled the differentiation of DN cells to the DP stage. Surprisingly, we found that the transcription factor NF-κB was strongly activated in thymocytes from Id1 and Tal1 transgenic mice. Further activation of NF-κB in these mice resulted in more severe impairment of T cell development while inhibition of NF-κB alleviated the T cell deficiency. These data suggest that the role of bHLH proteins in T cell development may be to control the threshold of stimulation through the pre-TCR and TCR signaling pathways. Inhibition of their function leads to hyperactivation of the pre-TCR signaling pathway and results in the differentiation of RAG1-deficient DN cells to the DP stage in the absence of pre-TCR. However, the hyperactivation of these signaling pathways also causes massive apoptosis of DP thymocytes because it mimics signaling that leads to negative selection. Therefore, the bHLH proteins play multiple roles in different stages of thymocyte development.

Results

Dose-Dependent Inhibition of T Cell Development by Id1 and Tal1

We previously found that T cell development in Id1 transgenic mice is severely impaired, and massive apoptosis occurs in the thymus (Kim et al., 1999). Since Id1 acts as a dominant, competitive inhibitor of E2A and HEB proteins in vitro, its effect on T cell development might be expected to be dose dependent. Indeed, mice homozygous for the Id1 transgene exhibited more complete inhibition of T cell development than heterozygous transgenic mice (Figures 1B and 1C). Over 90% of thymocytes in the homozygous mice belonged to the $CD44^+CD25^-$ DN-I population. This population can be further divided into two groups based on their expression of the CD24 marker (Kim et al., 1999). Increasing the concentration of one of the E2A proteins, E47, neutralized the effect of Id1 and rescued T cell development in the Id1 and E47 transheterozygous mice (Figures 1B and 1C). The developmental profile and total cellularity of the transheterozygous mice are similar to those of the wild-type mice.

mice are labeled as $Id1^{tg/tg}$ and $Id1^{tg}$, respectively. Transheterozygous mice are designated by separating the names of the two transgenes with an "X." The percentages of cells in each quadrant are indicated and the numbers of total thymocytes are listed under each genotype.
(C) CD44 and CD25 staining of DN cells in the same mice as shown in (B).
(D) Forward and side scatter analysis of mice with the indicated genotypes. Viable thymocytes were counted with the same gate settings, and the percentages in total thymocytes are shown next to the gate.
(E) TUNEL analysis of wild-type and Id1 transgenic mice. Frozen thymus sections were used for TUNEL labeling with fluorescein isothiocyanate conjugated dUTP. After fluorescence microscopy, the same sections were stained with H&E and photographed using light microscopy with the same magnification.

We have now generated transgenic mice expressing Tal1-p22, another inhibitor of the E2A and HEB proteins. Like Id1, Tal1 has a high affinity for E2A and HEB proteins. However, Tal1 heterodimers are incapable of driving transcription because Tal1 lacks an activation domain that is compatible with E2A (Park and Sun, 1998). Two independent lines of Tal1 transgenic mice, Tal1-p22-8 and -13, displayed phenotypes similar to Id1 transgenic mice, namely a dramatic reduction in total thymic cellularity and an accumulation of DN cells along with a concomitant loss of DP cells (Figures 1B and 1C). More interestingly, when Tal1 transgenic mice were crossed with Id1 transgenic mice, a more severe impairment of T cell development was observed in the transheterozygous Id^{tg}X$Tal1\text{-}p22^{tg}$-13 animals (Figures 1B and 1C). This phenotype is similar to that seen in the Id1 homozygous mice, suggesting that the Id1 and Tal1 proteins most likely act through a shared mechanism in a dose-dependent manner. Since E2A and HEB are the only known targets for Id1 and Tal1, it is reasonable to assume that the effects of Id1 and Tal1 are mediated through inhibition of these bHLH proteins.

As shown in Figure 1D, massive apoptosis occurred in Id1 and Tal1 transgenic mice, as revealed by forward and side scatter analysis. Percentages of viable cells ranged from 39%–61% in Id1 and Tal1 transgenic mice. Apoptosis in Id1 transgenic mice was further evaluated by TdT-mediated dUTP nick end-labeling analysis (TUNEL) (Figure 1E). Id1 transgenic thymi were much smaller in size, with only a thin rim of cortex and a low density of thymocytes compared to those from wild-type mice. However, numerous apoptotic cells labeled with fluorescent dUTP were found throughout the transgenic thymus, whereas very few labeled cells were detected in the wild-type thymus. Together with our previous observations of DNA fragmentation by gel electrophoresis and TCR gene rearrangement in the fragmented DNA (Kim et al., 1999), we conclude that developing T cells undergo an increased rate of apoptosis in Id1 and Tal1 transgenic mice. This apoptotic phenomenon is consistent with a striking characteristic of the developmental profiles of homozygous or heterozygous Id1 and Tal1 transgenic mice, namely a dramatic loss of DP cells (Figure 1B). Because DP cells constitute about 70% of wild-type thymocytes, depletion of this population could account for the dramatic reduction of total thymic cellularity observed in these mice. Since we have previously shown that the apoptosing cells have undergone TCRα and/or β gene rearrangement, we hypothesize that one of the causes for apoptosis of the DP cells may be that these DP cells receive excessive stimulatory signals from the pre-TCR or TCR in Id1 or Tal1 transgenic mice.

Induction of RAG1-Deficient Cell Differentiation by Id1

To explore the possibility that Id1 overexpression or loss of E2A/HEB function may impact pre-TCR signaling, we crossed Id1 transgenic mice with RAG1-deficient mice. Due to the lack of functional TCRβ chains to form pre-TCR complexes, T cell development is arrested at the DN-III stage in RAG1-deficient mice. Interestingly, moderate levels of Id1 expression in heterozygous transgenic mice forced differentiation of RAG1-deficient cells from the DN-III stage to the DP stage, although the total number of thymocytes in the thymus did not increase (Figure 2). This dramatic result suggests that inhibition of bHLH proteins by Id1 allows developing thymocytes to bypass a differentiation checkpoint at the DN-III to DP transition, which normally requires signals from pre-TCR complexes. Although the cells can differentiate to the DP stage, the numbers of DP cells did not increase, probably because Id1 expression only removed the checkpoint for differentiation but could not mimic the proliferative effect of pre-TCR stimulation, which results in the expansion in the numbers of DP cells. Nevertheless, the data in this experiment suggest that bHLH proteins play important roles during β selection in T cell development, perhaps by providing a checkpoint to regulate the level of pre-TCR signaling.

The situation was quite different for homozygous Id1 transgenic mice on the RAG1-deficient background. These animals completely lacked DP cells, and most thymocytes were at the DN-I stage (Figure 2). The developmental profiles of these mice were similar to those of Id1 homozygous or Id1/Tal1 transheterozygous mice shown in Figure 1. Thus, it appears that in the presence of high levels of E2A/HEB inhibitors, thymocyte development may also be impaired earlier at the DN-I to DN-III stages, suggesting that the function of bHLH proteins may be required at multiple stages during thymocyte development. It is possible, however, that complete abolishment of E2A and HEB function may directly lead to enhanced cell death, thereby contributing to the increased loss of cells seen in the homozygous mice or in the Id1 and Tal1 transheterozygous mice.

Downregulation of E2A/HEB Function by CD3 Stimulation

Since inhibition of E2A/HEB function by Id1 overexpression allowed DN cells to differentiate to the DP stage without functional pre-TCR complexes (Figure 2), E2A and HEB may provide a checkpoint for pre-TCR signals. Therefore, it is possible that pre-TCR signals normally downregulate E2A/HEB function when DN cells differentiate to the DP stage. To test this hypothesis, we injected RAG1-deficient mice with antibodies against CD3ϵ to mimic pre-TCR signaling and assayed the DNA binding activities of E2A and HEB. As shown in Figure 3, E2A/HEB binding activities decreased dramatically 8 or 12 hr after an intraperitoneal injection with 50 μg of the anti-CD3ϵ antibody, 145-2C11, but recovered 7 days later. These data suggest that pre-TCR signaling can transiently reduce the activity of E2A and HEB proteins.

Activation of NF-κB by Id1 and Tal1

To understand the molecular mechanisms by which E2A and HEB might influence pre-TCR (and also the TCR) signaling, we examined the function of nuclear effectors known to be downstream of pre-TCR signaling in Id1 transgenic mice. AP-1 DNA binding activities of the Fos and Jun proteins are activated by pre-TCR stimulation (Jacobs, 2000). However, amounts of AP-1 binding complexes detected by electrophoretic mobility shift assays (EMSA) increase only slightly in Id1 transgenic as compared to wild-type and RAG1-deficient thymocytes (Figure 4A). Amounts of nuclear extracts in the binding reac-

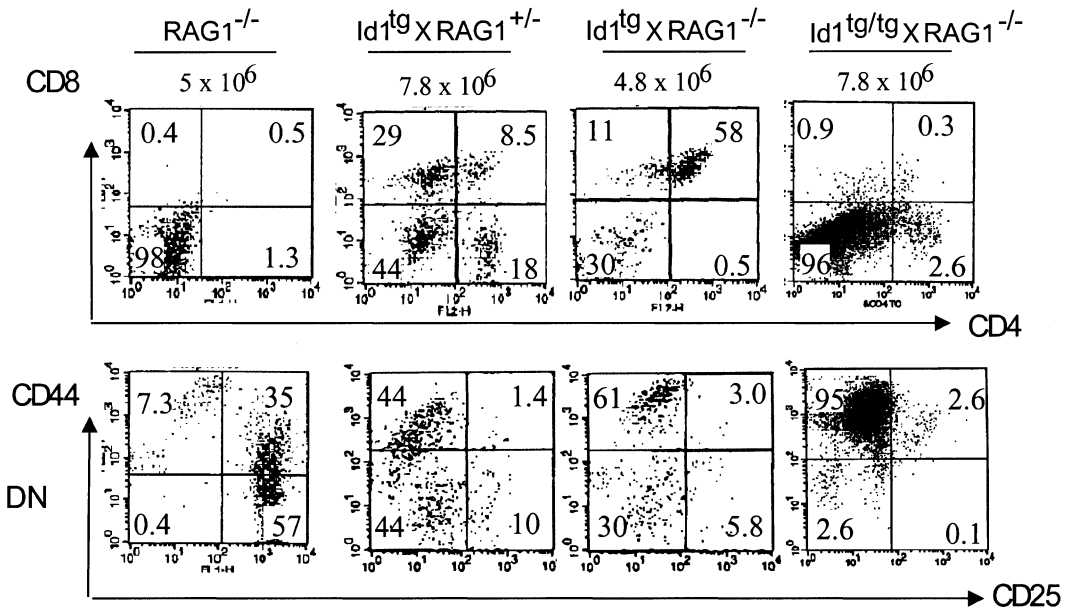

Figure 2. Id1 Expression Enables RAG1-Deficient Cells to Differentiate to the DP Stage

Total thymocytes from mice with the indicated genotypes were stained for CD4 and CD8. Cells gated as DN were further analyzed for CD44 and CD25 expression. Percentages of cells in each quadrant are indicated. Numbers of total thymocytes are listed under each genotype.

tions were normalized with Oct-1 binding activities. Constitutive signaling through pre-TCR has recently been shown to stimulate the DNA binding activities of NFAT complexes (Aifantis et al., 2001); however, NFAT activities were not enhanced in Id1 transgenic mice (Figure 4A).

In contrast, NF-κB, which is also a downstream effector of pre-TCR signaling, was dramatically activated in Id1 transgenic mice as measured by EMSA (Figure 4A). Activation of both MAPK and PKC pathways by pre-TCR signals leads to the degradation of IκB and nuclear translocation of NF-κB complexes, which bind to NF-κB recognition sequences in the promoters of NF-κB-responsive genes (Karin and Ben Neriah, 2000). The NF-κB family of transcription factors consists of multiple members that share a rel-homology domain, including p50, p52, relB, p65, and c-Rel (Ghosh et al., 1998). These proteins form homo- or heterodimers that bind to DNA. However, only complexes containing relB, p65, and c-Rel are transcriptionally active. Supershift analyses with antibodies against p50, p65, and c-Rel revealed that the enhanced DNA binding activity in thymocytes from Id1 transgenic mice could primarily be attributed to complexes containing c-Rel (Figure 4B).

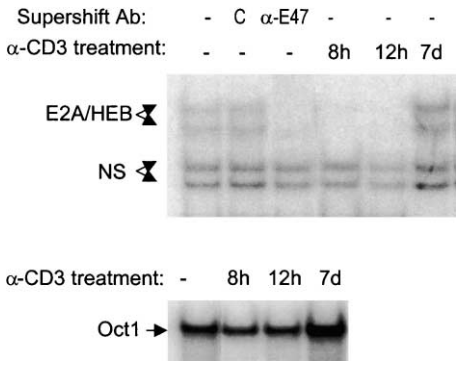

Figure 3. Downregulation of E2A/HEB Binding Activities by Anti-CD3ε Treatment

EMSA was performed using nuclear extracts from RAG$^{-/-}$ mice with no treatment or with anti-CD3ε antibodies for 8 hr, 12 hr, or 7 days. Two complexes specifically binding to the E box-containing probe are probably mixtures of E2A/HEB homo- and heterodimers. They are thus labeled E2A/HEB. Nonspecific binding complexes are indicated as "NS." Antibodies specific to E2A proteins (α-E47) were used for the supershift assay and may potentially react to HEB proteins. Nonspecific control antibodies (C) for the supershift assay were directed against C/EBP-β. Amounts of Oct-1 binding complexes were used as a control.

Since Id1 homozygous transgenic mice have predominantly DN-I cells in the thymus, it is conceivable that the enhanced NF-κB binding activity was intrinsic to that subset of cells. To directly test this possibility, we analyzed the NF-κB activities in Id1 and Tal1 heterozygous transgenic mice, which have sufficient numbers of cells beyond the DN-I stage (Figures 1B and 1C). In this experiment, nuclear extracts were prepared from thymocytes of Id1 and Tal1 heterozygous mice, in which DN-I cells comprised only 57% and 13% of total thymocytes, respectively. As shown in Figure 4C, NF-κB activities in these heterozygous mice were elevated as in Id1 homozygous mice. This result therefore suggests that NF-κB activation is likely to be the consequence of a loss of E2A/HEB function rather than being intrinsic to the DN-I cells enriched in thymocytes of Id1 homozygous transgenic mice. Moreover, it has been shown that the DN-I and DN-II cells do not have high levels of NF-κB activity (Voll et al., 2000). Supershift experiments with antibodies specific to c-Rel revealed that the majority

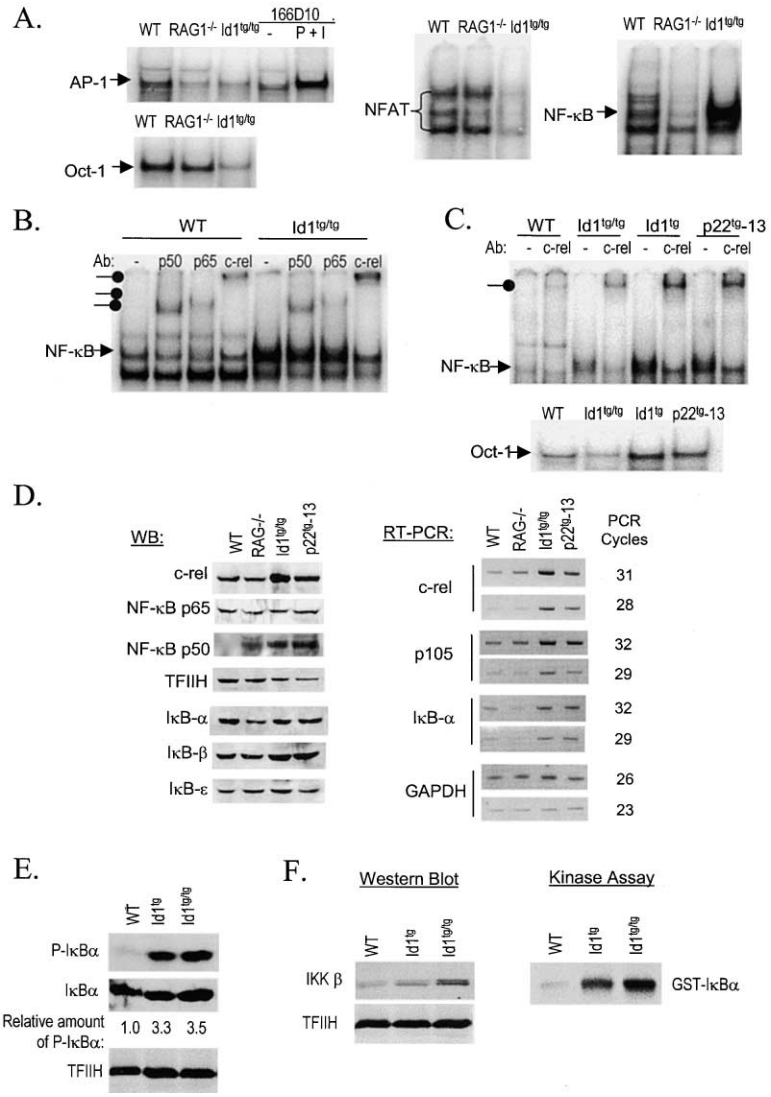

Figure 4. Activation of NF-κB in Id1 and Tal1 Transgenic Mice

(A) EMSA using nuclear extract from wild-type, RAG1$^{-/-}$, and Id1$^{tg/tg}$ mice. DNA binding complexes for each probe are indicated on the left side of the gels. Amounts of Oct-1 binding complexes were used to estimate the quantities and qualities of nuclear extracts used for each binding reaction. EMSA with nuclear extracts from 166D10 T cells cultured with or without stimulation with PMA and Ionomycin (P+I) were performed as positive controls for AP-1 activities.

(B) Supershift of NF-κB complexes in wild-type and Id$^{1tg/tg}$ mice (marked with —●) with antibodies specifically against different NF-κB proteins as indicated on the top of the lanes.

(C) Comparison of NF-κB DNA binding activities in different transgenic mice. EMSA and supershift analyses with anti-c-rel antibodies were performed with nuclear extracts from mice with the indicated genotype. Oct-1 binding complexes served as controls.

(D) Expression of NF-κB and IκB proteins. Western blots were performed using whole-cell extracts from mice with the indicated genotypes. Proteins specifically reacting to the antibodies are indicated on the side of the gels. Amounts of TFIIH protein were used as a control for the amounts of proteins loaded in each lane. RT-PCR assays were carried out using cDNAs prepared from total RNA from mice with the indicated genotype. PCR amplification was performed with primers specific to each transcript as labeled and in two different numbers of cycles as indicated. Amounts of GAPDH product were used as an internal control.

(E) IκBα phosphorylation. Western blots with antibodies against IκBα with and without phosphorylation (P-IκBα and IκBα) were performed with whole-cell extracts from thymocytes of the indicated genotypes. The amount of proteins detected in each lane was quantified with a LuminImager. The amount of phospho-IκBα was normalized against that of total IκBα, and the relative amount of phospho-IκBα in each sample is listed below the lane. Amounts of TFIIH served as an internal control for the amount of protein loaded.

(F) Levels and activities of the IκB kinase. Whole-cell extracts from indicated thymocytes were used in Western blots with antibodies against IKKβ and TFIIH. For kinase assays, the extracts were immunoprecipitated with antibodies against NEMO (IKKγ), and the precipitates were used in kinase assays as previously described (Voll et al., 2000). GST-IκBα was used as a substrate.

of NF-κB complexes contained c-Rel. The presence of c-Rel normally suggests constitutive activation of NF-κB (Miyamoto et al., 1994), so inhibition of E2A/HEB function most likely leads to the prolonged activation of NF-κB activity in these thymocytes.

Levels of NF-κB and IκB proteins in the wild-type, RAG1-deficient, Id1 and Tal1 transgenic mice were then compared using immunoblot analysis (Figure 4D). Among the NF-κB proteins, levels of p65 were similar, while those of c-Rel and p50 were greatly increased in Id1 and Tal1 transgenic mice. Levels of IκBα, IκBβ, and IκBε remained relatively unchanged compared to wild-type mice, probably reflecting their continuous degradation and resynthesis. Taken together, these observations are consistent with our finding that NF-κB activities are significantly enhanced in Id1 and Tal1 transgenic mice. Since expression of c-Rel and p105 (which gives rise to p50) has previously been shown to be induced by NF-κB (Chiao et al., 1994; Lin et al., 1995), the increased steady-state levels of these proteins may have resulted from increased gene expression mediated by active NF-κB in these cells. Indeed, the levels of mRNAs encoding these proteins, as well as IκBα which is also regulated by NF-κB (Miyamoto et al., 1994), were significantly higher in Id1 and Tal1 transgenic mice than in wild-type or RAG1-deficient mice (Figure 4D). These results suggest that constitutive activation of NF-κB occurs in the transgenic mice, which in turn leads to increased production of p50 and c-Rel proteins.

To investigate the mechanism through which Rel/NF-κB proteins are activated in Id1-expressing thymocytes, we examined the status of phosphorylation of IκBα by immunoblotting with antibodies against phospho-IκBα. Interestingly, the amount of phospho-IκBα in thymo-

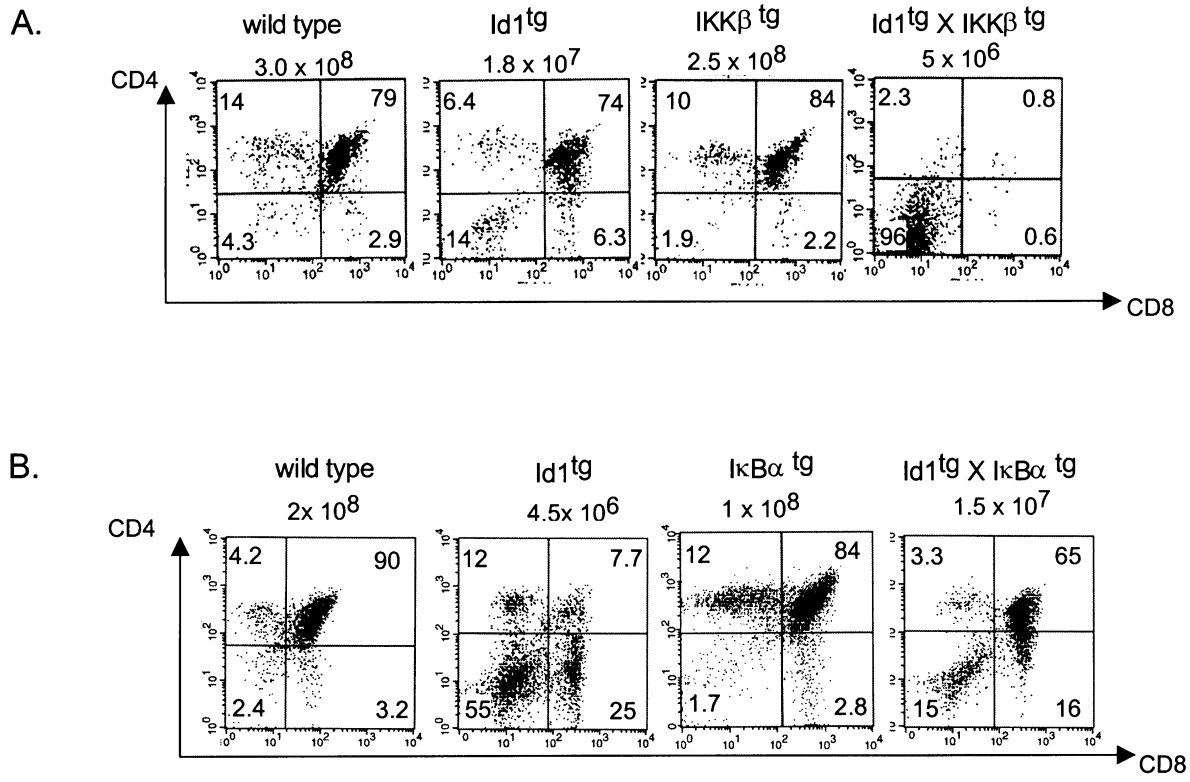

Figure 5. Crosstalks between bHLH and NF-κB Proteins in T Cell Development

(A) Synergistic effects of Id1 and IKKβ transgenes.

(B) Rescue of Id1 transgenic DP cells by inhibition of NF-κB. Total thymocytes from littermates with the indicated genotypes were stained for CD4 and CD8. The total number of thymocytes is listed under each genotype. Data shown are examples of at least five (for [A]) and ten (for [B]) similar experiments.

cytes from Id1 homo- or heterozygous transgenic mice was significantly greater compared to wild-type mice (Figure 5E), suggesting that NF-κB activation in these thymocytes probably occurred through activation of the IκB kinase. To determine whether the classical IκB kinase complex was activated in these cells, we examined the level of kinase activity in immunoprecipitates using antibodies against NEMO/IKKγ, the regulatory subunit of IKK complexes (Karin and Ben Neriah, 2000). Although the amount of IKKβ, the catalytic subunit mainly responsible for inducible phosphorylation of IκBα, was elevated slightly in Id1-expressing thymocytes, the IKK kinase activity was markedly increased in the thymocytes from Id1 transgenic mice compared to wild-type mice (Figure 4F). Therefore, these results suggest that Id1 expression leads to activation of the classical NF-κB signaling pathway involving the IKK complex, resulting in the subsequent phosphorylation of IκBα.

Synergistic Effects on T Cell Development through Activation of NF-κB and Inhibition of E2A/HEB Function

If activation of NF-κB in Id1 and Tal1 transgenic mice contributes to the dramatic loss of T cells, it would be expected that further activation of NF-κB in these mice would exacerbate the situation. To test this hypothesis, we crossed Id1 or Tal1 transgenic mice with IKKβ transgenic mice, in which a constitutively active form of IKKβ is expressed under the control of the lck proximal promoter (Voll et al., 2000). Expression of this form of IKKβ in transgenic mice has been shown to facilitate the survival of DN-IV cells in the wild-type background and in the progression of RAG1-deficient DN-III cells to the DP stage. As shown in Figure 5A, heterozygous Id1 transgenic mice in this litter had a higher percentage of DP cells and slightly larger numbers of thymocytes compared to those shown in Figure 1. This is probably due to variations in the levels of Id1 transgene expression in a mixed genetic background. Transgenic mice carrying the IKKβ allele exhibited a relatively normal developmental profile. However, T cell development was completely arrested in the Id1 and IKKβ transheterozygous littermate, a phenotype equivalent to that in Id1 homozygous mice or Id1 and Tal1 transheterozygous mice (Figure 1). A similar observation was also obtained by crossing Tal1 ($p22^{tg}$-8) with IKKβ transgenic animals (data not shown). These results thus support the hypothesis that the dramatic reduction in T cell numbers observed in the Id1 and Tal1 transgenic mice may, at least in part, be due to aberrant hyperactivation of NF-κB.

Inhibition of NF-κB Partially Rescues DP Cells in Id1 Transgenic Mice

If activation of NF-κB upon removal of E2A and HEB contributes to a loss of DP cells, then inhibition of NF-κB should facilitate the survival of DP cells in Id1 transgenic

Table 1. Summary of Key Phenotypes of Transgenic or Knockout Mice

Mice	DN (%)	DP (%)	Total Number	Data Source
WT	3.7	65	2×10^8	Figure 1
Id1^{tg}	42	14	4×10^6	Figure 1
Id1$^{tg/tg}$	93	1.4	3×10^6	Figure 1
p22^{tg}-8	49	2.1	6×10^6	Figure 1
p22^{tg}-13	48	11	6×10^6	Figure 1
RAG1$^{-/-}$	98	0.5	5×10^6	Figure 2
Id1^{tg} × RAG1$^{-/-}$	30	58	5×10^6	Figure 2
[1]Id1^{tg}	14	74	1.8×10^7	Figure 5
IKKβ^{tg}	1.9	84	2.5×10^8	Figure 5
Id1^{tg} × IKKβ^{tg}	96	0.8	5×10^6	Figure 5
[2]Id1^{tg}	55	7.7	4.5×10^6	Figure 5
IκBα^{tg}	1.7	84	1×10^8	Figure 5
Id1^{tg} × IκBα^{tg}	15	65	1.5×10^7	Figure 5

[1] A littermate of the Id xIKK transheterozygous mouse.
[2] A littermate of the Id xIκBα transheterozygous mouse.

animals (Figure 5B). To test this hypothesis, Id1 transgenic mice were crossed with transgenic mice expressing a superinhibitor of NF-κB, an IκBα protein with mutations that render the protein resistant to signal-mediated degradation (Voll et al., 2000). Although heterozygous mutant IκBα transgenic mice displayed a relatively similar developmental profile to wild-type mice, Id1 and IκBα transheterozygous mice had significantly higher percentages of DP cells as compared to Id1 transgenic mice (65% versus 7.7%), accompanied by a reduction in the percentage of DN cells. Furthermore, the total numbers of thymocytes in the transheterozygotes were increased by at least 3-fold relative to the Id1 heterozygotes. Therefore, these data suggest that inhibition of NF-κB by the superinhibitor IκBα can partly overcome the death of DP cells. The partial rescue observed here might be due to an insufficient amount of the superinhibitor expressed in the heterozygous mice, as the superinhibitor has been shown to act in a dose-dependent manner (Voll et al., 2000). Alternatively, hyperactivation of NF-κB may be only one of the factors involved in the apoptosis of DP cells in Id1 transgenic mice, and other defects cannot be corrected by the inhibition of NF-κB.

Taken together, these results are compatible with the notion that the function of bHLH and NF-κB families of proteins must be closely modulated during T cell development, and cooperation between these molecules is critical for appropriate interpretation of signals originating from pre-TCR and TCR.

Discussion

This study revealed that two families of transcription factors appear to cooperate to regulate critical steps in T lymphocyte differentiation. All functional bHLH activity could be experimentally reduced in a dose-dependent manner with combinations of Id1 and Tal1 transgenes. This led to a severe impairment of thymocyte development in transgenic mice, which are characterized by partial or complete loss of DP cells and a dramatic reduction in the total numbers of thymocytes (summarized in Table 1). Inhibition of bHLH activity also allowed the progression of thymocytes to the DP stage in the absence of pre-TCR signaling, whereas mimicking pre-TCR signals by anti-CD3 treatment reduced bHLH activity. Additionally, we found that NF-κB activity was augmented in thymocytes lacking bHLH function. Although Rel/NF-κB family proteins normally provide survival signals to maturing T lineage lymphocytes (Voll et al., 2000), in the absence of bHLH function, the activation of NF-κB exacerbated cell death while the inhibition of NF-κB rescued DP cells (Table 1). Collectively, these observations indicate that HLH proteins serve as modulators of pre-TCR and TCR signaling, in part by regulating activities of NF-κB proteins.

The Role of E2A and HEB Proteins in Pre-TCR-Mediated T Cell Development

T cell differentiation, expansion, and survival are largely dependent upon signals transmitted through pre-TCR and TCR. In the absence of a functional pre-TCR, T cell development is arrested at the DN-III stage, where a developmental checkpoint exists. Our data suggest that E2A/HEB proteins play an important role in modulating this checkpoint by providing a block for the differentiation of DN cells, which is normally overcome by signaling from the pre-TCR complexes. Therefore, overexpression of the inhibitors Id1 and Tal1 probably mimics pre-TCR signaling by reducing the activity of E2A/HEB proteins at the DN-III to DN-IV developmental transition (Figure 6). As shown in this report, the lowering of E2A/HEB function also leads to the activation of some of the nuclear effectors that are downstream of pre-TCR signaling pathways such as NF-κB.

If bHLH proteins such as E2A and HEB play an important role in setting up a checkpoint for pre-TCR signaling, then elimination of the function of these proteins might be an integral step in normal pre-TCR-mediated differentiation of DN cells. Indeed, signaling through the pre-TCR pathway induced by anti-CD3ε treatment transiently decreased bHLH DNA binding activity in RAG1-deficient mice. Interestingly, it has been shown that activation of the MAP kinase pathway in T cells, either by anti-CD3 treatment or by expression of the constitutively active lck or Ras protein, enhances the expression of the *Id3* gene, a member of the Id class of bHLH inhibitors (Bain et al., 2001). Therefore, it is not surprising that E2A/HEB activities are inhibited after treatment with the

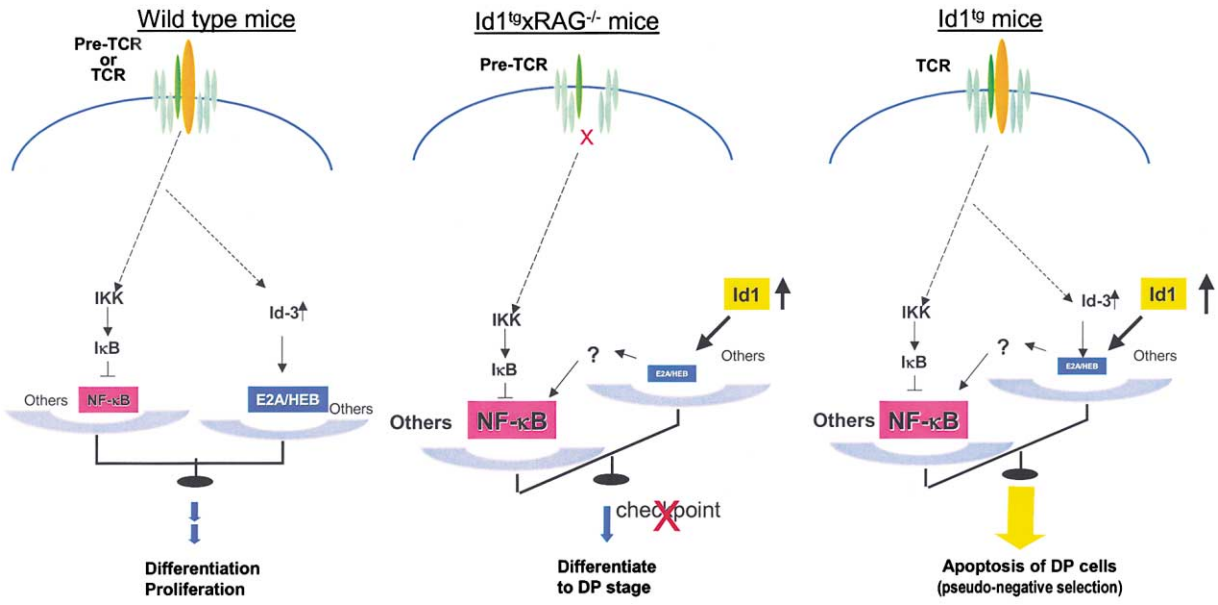

Figure 6. Models of E2A and HEB Function

(Left) During normal T cell development, both the pre-TCR and TCR signals through similar pathways that lead to activation of NF-κB transcription factors as well as other transcription factors such as AP-1 and NF-AT. E2A and HEB may balance signals from pre-TCR or TCR by negatively influencing the function of the downstream transcription factors. This could prevent overstimulation, ensuring proper differentiation and proliferation.

(Center) Id1 expression inhibits E2A and HEB and thus removes the DN to DP transition checkpoint, allowing cells to differentiate in the absence of pre-TCR signals in RAG1-deficient mice.

(Right) Once the cells reach the DP stage, inhibition of E2A and HEB by Id1 causes overstimulation of the cells by TCR engagement and leads to consequent apoptosis.

anti-CD3 antibody. Furthermore, treatment of RAG1-deficient mice with the anti-CD3ε antibody also leads to the activation of NF-κB in the DN-III cells (Voll et al., 2000). These findings would suggest that inhibition of E2A/HEB function by Id1 and Tal1 is accompanied by the activation of NF-κB (Figure 4).

It is important to note that other transcription factors, such as the Ikaros family of proteins, also play a role in this checkpoint because disruption of the Ikaros gene allows thymocytes to differentiate to the DP stage in mice lacking RAG1 (Winandy et al., 1999). Also, similar to the effect reported in this paper, the forced differentiation of thymocytes to the DP stage through removal of Ikaros occurs without a significant expansion in the numbers of thymocytes. Unlike E2A/HEB and Ikaros, whose removal appears to push differentiation of thymocytes to the DP stage in RAG1-deficient mice, overexpression of a constitutively active form of IKKβ leads to the stimulation of endogenous NF-κB activity in the thymus and also promotes the progression of RAG1-deficient cells to the DP stage without increases in the numbers of thymocytes (Voll et al., 2000). Expression of the Egr-1 immediate-early gene, which is normally turned on during β selection (Shao et al., 1997), enables RAG2-deficient cells to differentiate to the immature CD8 single-positive stage (Miyazaki, 1997). However, it remains unclear how the loss or gain of function of these transcription factors with distinct target genes can all influence this same checkpoint in pre-TCR-mediated thymocyte development.

The Role of E2A/HEB in TCR-Mediated T Cell Development

T cells that develop in the thymus have to first pass β selection for pre-TCR function and then undergo both positive and negative selection before moving on to the periphery. While the purpose of positive selection is to favor the expansion of cells carrying functional TCRs with moderate affinities to self-peptide and self-MHC complexes, the purpose of negative selection is to remove cells expressing TCR with high affinities to self-peptide and self-MHC complexes (Nossal, 1994; von Boehmer, 1994). Two hypotheses have been proposed to explain why signals from TCR engagement with MHC complexes can result in dramatically different outcomes during positive and negative selection: cell survival and expansion or cell death. The differential signal hypothesis suggests that distinct signals are transmitted during positive selection and negative selection and therefore the consequences of such signaling are different (Alberola-Ila et al., 1996). The alternative avidity hypothesis states that the positive and negative outcomes of TCR engagement with the MHC complexes depend on the strength of the signals delivered by the receptors, which is proportional to the affinities of the receptors to the MHC complexes and the numbers of the receptors on the cells (Ashton-Rickardt et al., 1994).

One of the most remarkable effects seen in the Id1 or Tal1 transgenic mice is the nearly complete depletion of DP cells. Since Id1 overexpression promotes the differentiation of RAG1-deficient cells to the DP stage, we

would suggest that inhibition of E2A/HEB function by Id1 might have lowered pre-TCR signal thresholds required for cells to differentiate to the DP stage. Without E2A/HEB function, cells can reach the DP stage without pre-TCR signals. We suspect that the lack of E2A/HEB function also reduces the threshold for TCR-mediated stimulation at the DP stage. As a consequence, DP cells might overreact to signals normally meant for positive selection and trigger apoptosis through the mechanisms analogous to those normally occurring during negative selection, a process that we refer to as "pseudo-negative selection" (Figure 6). This hypothesis is consistent with our findings that DP cells are depleted in Id1 transgenic mice and the apoptotic cells appear to have undergone rearrangement in the TCRα and/or β chain loci. Therefore, it appears that E2A and HEB help to determine the appropriate level of response to TCR-mediated stimulation.

Further support for this hypothesis comes from examination of Id3-deficient mice (Rivera et al., 2000). The lack of Id3 diminished class II-mediated positive selection and impaired differentiation of CD4 SP cells when tested by crossing Id3-deficient mice with the AND transgenic mice, which express a class II-restricted TCR. Also, when the H-Y transgenes encoding class I-restricted TCR against a male-specific antigen were introduced into Id3-deficient mice, class I-mediated positive selection was somewhat impaired in female mice, but negative selection was severely perturbed in the male animals. Reciprocally, disruption of the E47 exon in the *E2A* gene caused a partial reduction in the activities of bHLH proteins and an acceleration of both class I- and II-mediated positive selection without any effect on negative selection (Bain et al., 1999). These results suggest that E2A proteins may normally have a negative effect on TCR signaling and are inhibited by regulated expression of the *Id3* gene during positive and negative selection. Failure to suppress E2A function at appropriate stages by Id3 could impair both positive and negative selection. However, constitutive and complete inhibition of all bHLH activities, as seen in Id1 and Tal1 transgenic mice, would result in hyperstimulation and consequent cell death due to pseudo-negative selection.

The Relationship between E2A/HEB and NF-κB

One of the most striking and surprising observations in this report was the dramatic increase in NF-κB activity in Id1 and Tal1 transgenic mice. Although the Rel/NF-κB transcription factors normally act as survival factors and have been shown to promote the survival of β-selected DN-IV cells (Voll et al., 2000), overactivation of NF-κB in Id1 and Tal1 transgenic mice appears to lead to massive apoptosis. There could be several explanations for these results. First, the biological outcome, apoptosis or survival, may depend on the level of NF-κB activation. A moderate level of activation might facilitate survival of thymocytes, as probably occurs during β selection. However, an extremely high level of activation of NF-κB, as seen in Id1 and Tal1 transgenic mice, could lead to cell death (Senftleben et al., 2001). Second, cells at different developmental stages may react differently to NF-κB activation. While activation of NF-κB promotes the survival of DN-III and DN-IV cells as they differentiate, it could result in apoptosis at the DP stage through a pseudo-negative selection mechanism. This hypothesis would be consistent with the depletion of DP cells in Id1 and Tal1 transgenic mice. Furthermore, expression at a higher level of the constitutively active form of IKKβ in the homozygous transgenic mice resulted in a dramatic activation of NF-κB and also caused apoptosis of the DP cells (E.J. and S.G., unpublished data). A third explanation could be that differences in the components of NF-κB complexes in wild-type and Id1 or Tal1 transgenic mice may yield different outcomes in cellular response, possibly as a result of different target genes being expressed. Although NF-κB complexes normally activated during pre-TCR stimulation in wild-type mice consisted predominantly of p50 and p65 subunits (Voll et al., 2000), complexes detected in Id1 and Tal1 transgenic mice were highly enriched in c-Rel, which has been shown to cause apoptosis when overexpressed in avian embryos and bone marrow cells (Abbadie et al., 1993). Finally, the inhibition of the E2A and HEB function by Id1 and Tal1 may have a broader effect than the activation of NF-κB alone. Therefore, a combination of these effects may lead to the massive apoptosis observed in these transgenic mice. In keeping with this idea, the Id1 and Tal1 transgenic mice develop T cell lymphomas at about 3 months of age (Kim et al., 1999), but IKKβ transgenic mice do not have this phenotype, which would suggest that the loss of E2A/HEB function might influence other cellular processes such as growth control.

How the loss of E2A/HEB function results in NF-κB activation is not understood and needs to be further investigated. As shown in Figure 4F, we have found that the IKK complex is activated in Id1-expressing thymocytes. Under normal circumstances in thymocytes, the IKK complex is activated as a consequence of signaling from the pre-TCR or the TCR by a pathway involving protein kinase C θ and MAP kinases. However, neither the activity of PKCθ nor MAP kinases is significantly increased in the Id1 transgenic thymocytes (data not shown), suggesting that NF-κB activation in these cells is due to signaling through some other pathway. Inhibition of E2A and HEB function by Id1 and Tal1 probably leads to a reduction in the expression of target genes of E2A and HEB, and some of these genes could directly or indirectly control NF-κB activation by this alternate pathway. A better understanding of the mechanisms by which NF-κB is activated in Id1 and Tal1 transgenic mice will therefore be aided by a greater knowledge of E2A and HEB target genes.

In summary, the studies reported in this paper describe a novel role for E2A and HEB proteins in regulating thymocyte differentiation. These proteins appear to serve as modulators of pre-TCR and TCR signaling by establishing thresholds for stimulation. In the presence of weak or no pre-TCR signals, E2A and HEB block further differentiation of the DN cells. In the presence of strong signals, they prevent overstimulation, which may cause apoptosis. E2A and HEB appear to carry out these functions, at least in part, by modulating the activities of NF-κB proteins, which are important downstream effectors of both pre-TCR and TCR signaling pathways.

Experimental Procedures

Mice

The Id1 transgenic mouse line was previously described as Id1-28 (Kim et al., 1999). Homozygous Id1 transgenic mice were generated by intercrossing Id1-28 male and female mice. The Tal1 transgenic construct was generated by inserting human Tal1 cDNA encoding the 22 kDa polypeptide into the BamHI site of the vector, plck-hGH (Garvin et al., 1990), which contains the T cell-specific proximal promoter of the lck gene and the human growth hormone gene with introns and polyadenylation signals. The IKKβ and IκBα transgenic mice were described previously by Voll et al. (2000). The RAG1-deficient mice are gifts from E. Spanopoulou (Spanopoulou et al., 1994). Genotyping of the mice was carried out by using PCR assays with primers specific for each of transgenes and knockout alleles as previously described. Genotyping to the Tal1 transgenic mice was performed using primers binding to Tal1 cDNA: GTGAA CGGGGCCTTTGCC and ATTGAGCAGCTTGGCCAAGAAG.

Flow Cytometry

Cell suspensions were prepared from the thymus and stained with antibodies for two-color or three-color FACS analysis on a FACScan-II (Becton-Dickinson, Franklin Lakes, NJ). Tricolor (TC) conjugated-α-CD4, fluorescein isothrocyanate (FITC)-α-CD8, and TC-α-CD8 antibodies were purchased from CALTAG Laboratories (Burlingame, CA), while FITC-α-CD25 and phycoerythrin (PE)-α-CD44 were from Pharmingen (San Diego, CA). Analysis of DN cells was carried out by staining total thymocytes with TC-α-CD4 and TC-α-CD8 together with PE-α-CD44 and FITC-α-CD25. TC-negative cells were gated for analysis of CD44 and CD25 markers.

Electrophoretic Mobility Shift Assays

Total thymocytes from wild-type, RAG1-deficient, Id1 or Tal1 transgenic mice were purified by centrifugation for 20 min at 1500 rpm on a layer of ficoll-plaque cushion to remove dead cells. For RAG1-deficient mice treated with anti-CD3 antibodies for 0–12 hr, thymocytes were used for nuclear extract preparation without purification. Nuclear extracts were prepared from these cells by using an NP-40 lysis method. In brief, 10^7 cells were resuspended in 200 μl buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, and 1 mM DTT) and incubated on ice for 25 min before adding 5 μl of 10% NP-40. The nuclei were collected by centrifugation and extracted by vortexing vigorously in 50 μl of buffer C (10 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, and 1 mM DTT). The nuclear extracts were obtained by collecting the supernatants after centrifugation. Protein concentration of each extract was measured using the BCA reagents from Pierce (Rockford, IL). Five micrograms of each nuclear extract was used in each EMSA. EMSA with an Oct-1 probe was used to further normalize the amount and quality of nuclear extract in each binding reaction. To generate probes for EMSAs, double-stranded oligonucleotides containing the binding sites for AP-1, NF-κB, NFAT, and E2A/HEB were purchased from Promega (Madison, WI) or Santa Cruz Biotechnologies (Santa Cruz, CA), and end-labeled with polynucleotide kinase and ^{32}P-γ-ATP. The probes were purified by electrophoresis on a 10% nondenaturing polyacrylamide gel. Oligonucleotides containing the Oct-1 binding site, TGTCGAATC GAAATCCTCACCTT, were synthesized and labeled similarly. Approximately 20,000 cpm of each probe was used for each binding reaction. The 20 μl binding reactions were carried out by mixing each nuclear extract with an appropriate probe and 1 μg of polydI-dC in a buffer containing 20 mM HEPES (pH 7.9), 40 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. The reaction mixtures were incubated at room temperature for 15 min, then loaded on a 5% nondenaturing gel, and electrophoresed in 0.5× TBE buffer. For supershift experiments, antibodies were added to the mixtures at the end of the binding reactions and incubated for an additional 5 min. The antibodies used for supershift assays were all purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Immunoblot Analysis

Ficoll-purified thymocytes from wild-type, RAG1-deficient, and Id1 or Tal1 transgenic mice were lysed in RIPA buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.4 mM KH_2PO_4, 1% NP40, 0.5% Na deoxycholate, and 0.1% SDS), and the supernatants were collected after centrifugation. Protein concentrations were determined by using the BCA reagents (Pierce, Rockford, IL). Fifty micrograms of each protein extract was analyzed using SDS-polyacrylamide gels. Antibodies against c-rel, p65, p50, IκBα, IκBβ, and IκBε were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies against IKKβ- and phospho-IκBα were from Cell Signaling Technology (Beverly, MA).

Acknowledgments

We would like to thank Drs. Paul Kincade and Linda Thompson for critical reading of the manuscript. We are grateful to Dr. John Hirst and Viji Dandapani for assistance in flow cytometry. The work was supported by grants from the National Institute of Health (to X.-H.S, AI33597, CA77553, and RR15577 [COBRE AWARD], and to S.G., AI33443) and additional funds from the Oklahoma Medical Research Foundation to X.-H.S. and the Howard Hughes Medical Research Institute to S.G.

Received May 22, 2001; revised October 30, 2001.

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