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The Role of the Ets2 Transcription Factor in the Proliferation, Maturation, and Survival of Mouse Thymocytes

Arnaud Zaldumbide, Françoise Carlotti, Philippe Pognonec, and Kim E. Boulukos

In this study, we investigated the effects of Ets2 expression on the proliferation, maturation, and survival of thymocytes by establishing transgenic mice that specifically express Ets2 or a dominant negative form of Ets2, Δets2, in the thymus. We show that, in young animals, there are fewer T cells in Δets2 transgenic thymi and that the maturation of these T cells is affected at the CD4⁺CD8⁻ double-negative to CD4⁺CD8⁺ double-positive transition compared with wild-type littermate mice. Partial recovery in the number of thymocytes and full T cell maturation are restored with increasing age of Δets2 transgenic animals. However, thymocytes from adult Δets2 transgenic mice cultured ex vivo are more sensitive to cell death and to glucocorticoid-induced apoptosis than are T cells from control littermate mice. We also show that T cells from adult ets2 transgenic mice proliferate faster than their wild-type littermates. The proliferation and survival of these T cells are clearly affected upon apoptotic signals: glucocorticoid-induced apoptosis induces T cells from ets2 transgenic mice to continue to proliferate in vivo and to survive better ex vivo than T cells from control littermates. It has been shown that c-Myc expression is required for thymic proliferation and improves thymocyte survival of dexamethasone-treated animals. We show that the expression of c-Myc, an Ets2 target, is elevated in T cells freshly isolated from thymi of ets2 transgenic mice pretreated with dexamethasone. Together, these results show that Ets2 plays a role in the proliferation and survival of thymocytes, implicating a Myc-dependent pathway. The Journal of Immunology, 2002, 169: 4873–4881.

The transcription factor Ets2 was first identified by its homology to v-Ets (1, 2) of the erythroid-myeloid transforming E26 avian retrovirus (3, 4). Ets2 is a member of the ets family of transcription factors with ∼30 members described to date (reviewed in Ref. 5). Based on amino acid sequences, several Ets subgroups have been identified and Ets2 is classed with it most closely related member, Ets1, the progenitor to v-Ets. Both Ets1 and Ets2 are implicated in regulating genes involved in proliferation, differentiation, apoptosis (reviewed in Refs. 5 and 6), and senescence processes (7), suggesting the importance of these two proteins in cells. Indeed, ets2 gene disruption studies resulted in early embryonic lethality (8), and disruption of the ets1 gene leads to 50% mortality around birth and the absence of NK and NK T cells (9, 10). Because gene knockouts can often be compensated by redundant family members or redundant signaling pathways, transgenic studies expressing proteins in specific tissues help elucidate functions of different proteins.

During T cell development, precursor cells exit the bone marrow and migrate to the thymus where these cells differentiate into mature thymocytes through a highly ordered series of proliferation and maturation events. These events are characterized by the expression of CD4 and CD8 coreceptors. Immature T cells that are CD4⁻CD8⁻ double-negative (DN)³ mature to an intermediate CD4⁺CD8⁻ double-positive (DP) stage and terminate into mature CD4⁺CD8⁺ or CD4⁻CD8⁺ single-positive (SP) T cells. Thymocytes bearing functional TCRs are positively selected and differentiate into fully functional T cells. The remaining cells undergo negative selection and die by apoptosis. Therefore, the thymus is an excellent in vivo model for studying cellular processes of proliferation, differentiation, and apoptosis.

Several articles showed that Ets2 and Ets1 are expressed during T cell development (11–13). By RT-PCR, it was shown that both ets2 and ets1 mRNAs are expressed at constant levels throughout T cell development with increasing expression of both transcripts at the pre-T DP stage (13). However, Western analysis indicated that Ets1 protein is abundantly expressed in DN and SP T cell subpopulations, whereas expression of the Ets2 protein is at the DP stage (12). This reciprocal expression suggests that Ets1 and Ets2 proteins have distinct roles during T cell development. Mouse model studies have addressed only the role of Ets1, not that of Ets2, in thymic development. Ets1 was shown to influence cell development by affecting both cellularity and maturation at the DN to DP transition in recombination-activating gene (RAG)²−/− complementation studies (14, 15). In addition, T cell defects were observed for TCR-mediated proliferation (15). Furthermore, gene disruption experiments used to generate ets1 knockout mice indicate that the loss of Ets1 results in T cell activation defects and that Ets1 is clearly required for NK and NK T cells (9, 10).

In this study, we investigated the effects of Ets2 expression on cell proliferation, maturation, and survival in the thymus. We established transgenic mice that specifically express Ets2 or a dominant negative form of Ets2 (Δets2), which competes with endogenous Ets2 at the DP stage. We show that the Δets2 transgene...
affects the number and maturation of thymic cells in young animals. As Δets2 transgenic mice reach adulthood, normal T cell development is observed, although cell numbers are only partially recovered. However, thymocytes from adult Δets2 transgenic mice cultured ex vivo are more sensitive to cell death and glucocorticoid-induced apoptosis than are T cells from control littermate mice. In addition, we find that Ets2 expression allows thymocytes to proliferate and survive better upon the induction of apoptotic signals. We show that c-Myc, an Ets2 target gene, is up-regulated in rapidly proliferating Ets2-expressing thymocytes pretreated with dexamethasone, demonstrating that Ets2 plays a role in proliferation and survival of thymocytes probably via a Myc-dependent pathway.

Materials and Methods

Generation of transgenic mice

We cloned 2.1 kb of the human full length ets2 cDNA into p1017 vector (16) consisting of the lck proximal promoter, the human growth hormone genomic sequence, and a poly(A) + site to generate lckH9262-ets2 transgenic mice. A second construct of 1.1 kb corresponding to the DNA binding domain of Ets2 and functioning as a dominant negative mutant (17) was also cloned into p1017 to generate lckH9262-Δets2 transgenic mice. After microinjection of each construct into C57BL/6 DBA eggs and reimplantation into foster females, several founder lines were obtained by Service de’Experimenteration Animale et de Transgénese (Villejuif, France). Two families for each construct were further analyzed and characterized. lckH9262-ets2 and lckH9262-Δets2 mice were backcrossed at least four times with C57BL/6 mice to generate over 96.8% C57BL/6 background. Mice were maintained in a pathogen-free animal facility at the Université de Nice (Nice, France). We used the MICE software (pognonec@unicre.fr) to follow the different transgenic families and their generations (18).

Cell analysis

Single cell suspensions were obtained by washing thymi in sterile PBS and disrupting the tissue on nylon meshes with tips of plungers of sterile 1-ml syringes. Cells were washed and centrifuged in PBS, and single cell suspensions were counted on a hemocytometer by trypan blue exclusion. R-PE-conjugated rat anti-mouse CD4 (L3T4b), and FITC-conjugated CD8α (Ly-2) mAbs were purchased from BD PharMingen (San Diego, CA). Cells (1 × 10⁶) were washed in PBS containing 0.1% BSA; cells were incubated in 100 μl of PBS/0.1% BSA with 5 μg/ml Abs for 30 min at 4°C in the dark. Cells were then washed in PBS containing 0.1% BSA, fixed in PBS plus 1% formaldehyde, and 10,000–20,000 events were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA).

Cell viability was determined by plating cells at 10⁵ cells/ml in RPMI 1640 supplemented with 10% FCS. For in vitro induction of apoptosis, dexamethasone at 0.1 μM was added to freshly isolated cells. The vehicle, DMSO, was added to controls. Cell numbers were determined by counting on a hemocytometer by trypan blue exclusion at each time point indicated in Results.

In vivo proliferation was determined by injecting i.p. 1 mg of 5-bromo-2’-deoxyuridine (BrdU) diluted in PBS. Animals were sacrificed 4 h later and thymocytes were obtained as described above. Thymocytes (5 × 10⁶) were washed in PBS containing 0.1% BSA, and then permeabilized and fixed overnight at 4°C in 2% paraformaldehyde 0.01% Tween. Cells were washed in PBS with 0.1% BSA, resuspended in DNsase buffer (150 mM NaCl, 4.2 mM MgCl₂, and 10 μl 1 M HCl), and incubated with 50 μl of DNase I for 30 min at 37°C. Cells were again washed in PBS, incubated with 20 μl of anti-BrdU-FITC-conjugated Ab (no. 347583; BD Biosciences) in 80 μl of PBS/Tween for 30 min at room temperature, and then diluted in 1 ml PBS for analysis by FACS.

Expression of transgenes

For slot blot analysis, DNA was obtained from mouse tails, following proteinase K/SDS phenol chloroform extraction and ammonium acetate precipitation, and loaded in triplicate onto nitrocellulose membranes. Membranes were then hybridized to a 32P-labeling ets2 gel-purified fragment.

For Southern analysis, 10 μg of DNA was digested with the EcoRI restriction enzyme, and then the digested DNA was fractionated by electrophoresis on 0.8% agarose gels. After transfer, hybridization was performed as described by the manufacturer. Copy numbers were determined by comparing dilutions of 0.78–50 pg of the original pEκH9262-ets2 plasmid digested with EcoRI. The values from 6.25 to 50 pg were graphed to calculate the numbers of copies using the MacBAS program (data not shown).

For Western blot analysis, freshly prepared thymocytes from untreated or dexamethasone-treated animals were lysed, and 40 μg of total protein from each lysate in Laemmli buffer was electrophoresed on either 10 or 15% polyacrylamide-bisacrylamide gels. Migrated proteins were then transferred to polyvinylidene difluoride membranes as described by the manufacturer (DuPont-NEN, Boston, MA), immunoblotted with Ets2 (Santa Cruz (sc-351), Ets1 (sc-350), c-Myc (sc-764), Bcl-x (sc-634), Bcl-2 (sc-492-G) and β-tubulin (tub2.1; Sigma-Aldrich, St. Louis, MO) Abs, and revealed by ECL as described by the manufacturer.

Morphology

Histological staining was performed by fixing thymi with 10% paraformaldehyde overnight at 4°C. A series of 60-min dehydration steps was performed by increasing ethanol concentrations from 30, 50, 70, to 90%, followed by three incubations with 100% ethanol. The thymi were then placed in a mixture of active resine (50%) and ethanol (50%) at 4°C overnight. Then thymi were placed in 100% active resine to which the hardener was added. Slices (5–10 μm) were obtained using a microtome. After transfer to glass slides, slices were stained with hematoxylin, rinsed in water, dried at 70°C, and mounted with Eukitt (EMS, Fort Washington, PA).

Results

Recent studies by RT-PCR have shown that both ets1 and ets2 are detected in all stages of T cell development from pro-T cells that have not yet been committed to the T cell lineage to mature peripheral CD4+CD8− helper or CD4−CD8+ killer cells in peripheral tissues (13). The highest expression of both ets1 and ets2 mRNA is found in the DP pre-T cell population where TCRβ has already been rearranged and TCRα is undergoing rearrangement (summarized in Fig. 1). Although both transcripts are similarly expressed throughout T cell development in the thymus, Ets1 and Ets2 proteins are differentially expressed. Ets1 is found in CD4−CD8− DN cells and CD4−CD8+ CD8+ SP cells, whereas Ets2 protein is specifically expressed in CD4+CD8+ DP T cells (12).

To determine the role of Ets2 during thymic development, transgenic mice were generated. Visualized schematically in Fig. 2A are

![FIGURE 1. Summary of the expression of ets2 and ets1 during T cell development. Schematically represented are the different maturation steps of T cells in the thymus and secondary lymphoid tissues. These results of ets2 and ets1 mRNA expression are summarized based on semiquantitative RT-PCR studies from Anderson (13).](http://www.jimmunol.org/)
mice. An
ets2 transgenic families were performed, and DNAs were migrated on
EcoRI gives a doublet of 2.2 and 2.1 kb corresponding to the full length ets2 and hGH, respectively, using a probe containing ets2 and hGH sequences (data not shown), or a band at 2.2 kb using an ets2-specific probe (Fig. 2B). Sizes of 1.5 and 2.1 kb corresponding to ∆ets2 and hGH, respectively, were obtained using a probe containing both ets2 and hGH sequences (data not shown) or a band of 1.5 kb using an ets2-specific probe (Fig. 2B) following EcoRI digestions of tail DNAs from lckPΔ-∆ets2 transgenic mice. Two families expressing either ets2 or ∆ets2 transgenes were used for further studies. Western analysis confirmed the expression of Ets2 with an apparent molecular mass of 60 kDa and of ∆Ets2 with an apparent molecular mass of 16 kDa (Fig. 2C) in two different families of lckPΔ-ets2 and of lckPΔ-∆ets2 transgenic mice, respectively.

To determine whether thymic cellularity or T cell maturation is affected in the different lckPΔ-ets2 or lckPΔ-∆ets2 transgenic mice families, thymi were obtained from both young and adult animals. The morphology of 1-wk-old wild-type (WT) and transgenic thymi was similar (Fig. 3A) with the cortex and medulla clearly organized and distinguishable (Fig. 3B). However, the expression of ∆ets2 in thymi from 1-wk-old animals resulted in reduced cellularity (5-fold decreases in cell numbers compared with WT littermates; Fig. 3C) and a partial blockage in maturation specifically at the DN to DP transition (24.3% ± 9.5% for lckPΔ-∆ets2 vs 3.6% ± 1.1% for WT; Fig. 3D). Fewer T cell numbers were observed in thymi from young lckPΔ-ets2 transgenic mice compared with those from WT littermates (Fig. 3C). The expression of Ets2 in the thymi of young animals had no effect on the maturation of T cells (Fig. 3D).

Thymic cellularity and maturation were determined for animals as they reached adulthood. Six- to 8-wk-old animals from the four transgenic families were sacrificed, and total numbers of viable T cells from isolated thymi were determined. Although cellularity of lckPΔ-ets2 transgenic animals remained lower when compared with control littermates (2-fold), the differences were considerably reduced with increasing age (compare Fig. 4A, 2-fold, with Fig. 3C, 5-fold). The partial default in the maturation of DN cells from young lckPΔ-ets2 transgenic animals appeared to be corrected with age, because no differences in T cell maturation was observed in 6- to 8-wk-old lckPΔ-∆ets2 animals when compared with WT controls (Fig. 4B). In addition, the morphology of thymi from adult lckPΔ-ets2 and lckPΔ-∆ets2 transgenic mice and WT littermates was similar, with the cortex and medulla regions clearly defined (Fig. 4C). Surprisingly, the number of viable T cells from adult lckPΔ-ets2 transgenic mice was lower than the number of T cells from control littermate thymi (Fig. 4A). We hypothesized that the lower number of thymocytes from lckPΔ-ets2 transgenic mice could be the result of lower proliferative rates, decreased survival, or increased apoptosis of these T cells.

To address this question, we first evaluated the effects of Ets2 on in vivo thymocyte proliferation. BrdU was injected i.p. in adult lckPΔ-ets2 and lckPΔ-∆ets2 transgenic mice families and control littermates. Animals were sacrificed 4 h later, and thymocytes were obtained, incubated with BrdU Abs, and analyzed by FACS. No difference in proliferation was observed in thymocytes obtained from lckPΔ-∆ets2 transgenic mice and those obtained from control littermates. Much to our surprise, thymocytes from lckPΔ-ets2 mice proliferated two times faster than those from WT siblings (Fig. 5).
Therefore, the reduced thymic cellularity of lck<sup>Pr</sup>–∆ets2 transgenic mice does not appear to be due to a lower proliferative rate.

To determine the survival of thymocytes from the different transgenic lines compared with their control siblings, the following experiments were performed. The same number of T cells from the four adult lck<sup>Pr</sup>–ets2 and lck<sup>Pr</sup>–∆ets2 transgenic mice families and control littermates were cultured ex vivo in RPMI medium containing 10% FCS, and their viability was assessed by trypan blue exclusion over 72 h (Fig. 6A). There was no significant difference in the number of viable thymocytes from lck<sup>Pr</sup>–ets2 transgenic mice compared with thymocytes from control littermates; however, the number of viable thymocytes from lck<sup>Pr</sup>–∆ets2 transgenic mice was significantly lower (Fig. 6A). These results indicate that the expression of the dominant negative form of Ets2 does affect the survival of these thymocytes ex vivo. In addition, these results show that the decrease in cellularity of thymus from two different families of adult lck<sup>Pr</sup>–ets2 transgenic mice is not due to decreased survival or increased apoptosis.

Ets1 and Ets2 proteins are reciprocally expressed in different thymic subpopulations (12). To exclude the possibility that this mutually exclusive expression is due to Ets2 directly or indirectly down-regulating the expression of Ets1, we investigated the level of Ets1 expression in thymi from lck<sup>Pr</sup>–ets2 and lck<sup>Pr</sup>–∆ets2 transgenic mice and control littermates by Western analysis. As can be seen in Fig. 6B, the level of Ets1 remains constant independent of the expression of the ets2 and ∆ets2 transgenes.

We previously showed that Ets2 expression protects macrophages from cell death by apoptosis when deprived of a growth factor required for the survival of these cells (6, 20). Glucocorticoid hormones cause rapid depletion of thymocytes by apoptosis (21). Glucocorticoid treatment of normal mice leads to the rapid elimination of cortical CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes, while mature CD4<sup>+</sup> SP or CD8<sup>+</sup> SP thymocytes are less sensitive (22). Because endogenous ets2 expression is most abundant in DP thymocytes, while mature CD4<sup>+</sup> SP or CD8<sup>+</sup> SP thymocytes are less sensitive (22). Because endogenous ets2 expression is most abundant in DP thymocytes, while mature CD4<sup>+</sup> SP or CD8<sup>+</sup> SP thymocytes are less sensitive (22). Because endogenous ets2 expression is most abundant in DP thymocytes, while mature CD4<sup>+</sup> SP or CD8<sup>+</sup> SP thymocytes are less sensitive (22). 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among lckPr-ets2 and lckPr-Δets2 transgenic mice and control siblings (data not shown). However, dexamethasone treatment of lckPr-ets2 and lckPr-Δets2 transgenic mice and WT siblings resulted in inhibited thymic proliferation (Fig. 7A compared with Fig. 5) and depletion of cells of the CD4+CD8+ DP thymocyte subpopulations (Fig. 7B).

It was shown that 50% of thymocytes isolated from mice 6 h after injections with dexamethasone were viable yet programmed to die, as evidenced by annexin V-labeling (Boehringer Ingelheim, Ingelheim, Germany) (23). The result of these experiments led us to investigate the effects of dexamethasone on lckPr-Δets2 and lckPr-ets2 transgenic mice and their control siblings at this earlier time point. Dexamethasone or PBS was injected i.p. Two hours postinjection, animals were injected with BrdU for 4 h. Animals were then sacrificed, and thymocytes were obtained and labeled with anti-BrdU or with CD4 and CD8 Abs. Although the proliferative rates were observed. The expression of the ets2 transgene allows a higher proliferative rate of 6-h dexamethasone-treated thymocytes (10- to 20-fold, respectively) compared with the proliferation of those thymocytes from dexamethasone-treated control or lckPr-Δets2 transgenic mice (Fig. 8A).

Western analysis was performed to identify genes downstream of Ets2 that allow thymocyte proliferation and permit a greater resistance to dexamethasone-induced apoptosis. Two well-characterized anti-apoptotic proteins of the Bcl-2 family have been extensively studied in different thymic populations. Bcl-2 is expressed throughout thymic development except as immature DN thymocytes mature to intermediate DP thymocytes just before positive selection (24, 25) when Bcl-xL becomes abundantly expressed (26). No variation in the level of Bcl-2 expression was observed in thymocytes from different transgenic families and WT siblings (data not shown). We previously showed that the bcl-x gene can be transcriptionally regulated by Ets2, suggesting that bcl-x would be a good candidate as a target gene (6, 20). Unexpectedly, the level of Bcl-xL remained high and unchanged in lysates obtained from WT and lckPr-ets2 and lckPr-Δets2 transgenic mice and continued to remain constant even after dexamethasone treatment of the transgenic mice (Fig. 8A). Therefore, although bcl-x is a direct target of Ets2 in macrophages, this does not appear to be the case in thymocytes.

The c-Myc protein has been implicated in survival, proliferation and apoptotic processes. Recent studies showed that c-Myc−/− cells fail to proliferate normally at the late DN stage and mature into DP thymocytes (27). In addition, c-Myc was shown to be down-regulated by dexamethasone treatment, and DP thymocytes from c-myc transgenic mice were less susceptible to dexamethasone-induced death (23). Furthermore, c-myc was shown to be a direct target gene of Ets2 (28). Therefore, we asked whether Ets2
expression in lckP-ets2 transgenic mice resulted in the up-regulation of c-Myc expression. As can be seen in Fig. 8A, although in vivo dexamethasone treatment of WT siblings leads to decreases in the levels of c-Myc expression, c-Myc is still abundantly detected in dexamethasone-treated Ets2-expressing thymocytes. These results suggest that the role of Ets2 in proliferation and perhaps a higher resistance to apoptotic signals by corticosteroids may, at least in part, function via a Myc-dependent mechanism. It is worth noting that c-Myc is not down-regulated in dexamethasone-treated lckP-ets2 transgenic animals (1.6 vs 0.2 arbitrary units for WT thymocytes (Fig. 8A)) but is up-regulated (1.6 vs 0.3 arbitrary units for PBS-treated thymocytes (Fig. 8A)). This suggests that the Ets2 DNA binding domain, perhaps by interacting with coactivators, is sufficient to induce c-Myc expression to a level comparable to that seen in untreated control and lckP-ets2 transgenic mice. However, this level of expression remains much lower than that observed in thymocytes from dexamethasone-treated lckP-ets2 transgenic animals (1.6 vs 4 arbitrary units for ets2; Fig. 8A). It could be that other Myc-independent pathways are required to induce proliferation of dexamethasone-treated lckP-ets2 transgenic mice. Another possibility, which may not be exclusive of the first, is that c-Myc expression needs to reach a certain level, i.e., comparable to the level of c-Myc in dexamethasone-treated lckP-ets2 transgenic animals, for the maintenance and/or activation of the proliferation pathway. In any case, although c-Myc is detected in dexamethasone-treated lckP-ets2 transgenic thymocytes, these cells clearly stop proliferating (10.4% BrdU incorporation in thymocytes from PBS-injected lckP-ets2 transgenic mice (Fig. 5) vs 1% BrdU incorporation in thymocytes from dexamethasone-injected lckP-ets2 transgenic mice (Fig. 8A)).

To determine whether Ets2 expression may be important for thymic cell survival upon apoptotic signals, 1 × 10⁶ thymocytes cultured ex vivo from the four different lckP-ets2 and lckP-Δets2 transgenic mice families and control littermates were treated with dexamethasone during a 48-h period and cell viability was determined by trypan blue exclusion at various time points (Fig. 9). Cell viability was greatly affected in lckP-Δets2 transgenic thymocytes. Interestingly, the expression of Ets2 appeared to protect thymocytes from glucocorticoid-induced apoptosis as compared with WT littermates. By 21 h, differences in viability were apparent. lckP-ets2 thymocytes resisted dexamethasone-induced apoptosis better than the T cells from control littermates. Dexamethasone treatment led to a rapid decrease in the number of viable thymocytes from lckP-Δets2 transgenic mice. By 48 h, these differences were accentuated. The sensitivity to dexamethasone treatment was determined by calculating the ratio of viability in dexamethasone-treated (Fig.
insensitivity to dexamethasone, thymocytes from lck and its expression parallels protein transfer following coloration, Bcl-x L

To investigate the function of Ets2 in the thymus, we es-

ern analysis indicates that c-Myc expression is up-regulated in thymocytes fi
cations were performed using the MacBAS, version 2.2, program. West-
/H11001
mRNA (13) and protein (12) expression at the CD4
Ets2 is found throughout thymic development with increased
expression of the et s2 transgene following a 6-h in vivo dexamethasone
treatment. Dexamethasone (2.5 mg) was injected into animals for 6 h. Two
two hours postinjection, animals were injected with 1 mg of BrdU as described
in Fig. 5. Animals were sacrificed, and thymocytes were isolated and la-
abeled with BrdU or CD4 and CD8 Abs or prepared for Western analysis.
A, The percentage of BrdU incorporation in thymocytes of lckP^ets2 transgenic lines was shown to be 20-fold and 10-fold higher than that for
ets2 transgenic mice and WT control siblings, respectively. Forty
micrograms of lysates, as determined by the BCA Protein Assay kit (Per-
bio-Pierce, Rockford, IL) (data not shown), were subjected to 10–15% SDS-PAGE. Following transfer, incubation with anti-c-Myc Abs was per-
formed. Because the Bcl-xL is not regulated upon dexamethasone treatment
and its expression parallels protein transfer following coloration, Bcl-xL
was used as a control. We arbitrarily set the level of c-Myc to one in lysates
from PBS-injected WT animals. The average of fold inductions of c-Myc
for all other values are corrected according to Bcl-xL expression. Quanti-
fications were performed using the MacBAS, version 2.2, program. West-
ern analysis indicates that c-Myc expression is up-regulated in thymocytes
from lckP^ets2 transgenic lines. These experiments were repeated four
times showing similar results (data not shown). B, The different CD4CD8
compartments were shown by FACS analysis and are graphically repre-

Discussion

Ets2 is found throughout thymic development with increased
mRNA (13) and protein (12) expression at the CD4^+CD8^+ DP
stage. To investigate the function of Ets2 in the thymus, we es-

tablished transgenic mice expressing Ets2 or a dominant negative
mutant, ΔEts2, specifically in the thymus. We showed that Ets2 expression in the thymus results in a lower cellularity but a higher
proliferative rate. These seemingly contradictory observations
could be explained by a faster T cell migration from the thymus to
secondary organs. The lower cellularity most likely cannot be ex-
plained by greater apoptosis, because Ets2-expressing thymocytes
cultured ex vivo survive in a similar fashion to WT control litters-
mates. Moreover, cells from the thymus of lckP^ets2 transgenic mice survive much better ex vivo after stimulation with apoptosis-
inducing dexamethasone. However, we cannot exclude the possi-
bility that lower cellularity of et s2 transgenic thymus may be the
result of TCR-mediated apoptosis, because apoptosis of T lymph-
cytes mediated by TCR is distinct from that induced by dexa-
methasone (29), and rapidly proliferating cells, as in the case of
ets2 transgenic thymocytes, are more sensitive to TCR-mediated
death. Further studies will be needed to clarify this. Therefore,
although Ets2 does not appear to be required for thymocyte dif-
derentiation, we observe that Ets2 expression allows proliferation
and better survival of thymocytes. Finally, we identified that c-
Myc, a previously described Ets2 target gene, is activated in Ets2-
expressing thymocytes upon dexamethasone treatment. Because
c-Myc^−/− studies as well as c-Myc transgenic studies show that
c-Myc is required for both thymic cell proliferation and for inhib-
iting dexamethasone-induced apoptosis (23, 27), a likely mechani-
sm is that Ets2 permits T cell proliferation and/or survival via a
Myc-dependent pathway.

Thymic development is affected at the DN to DP in young, but
not adult, lckP^ets2 transgenic mice. The lower cellularity of
thymus from lckP^ets2 is marked in young animals and remains
significantly lower as animals reached adulthood. In addition, thymo-
cytes from lckP^ets2 transgenic mice are highly sensitive to
cell death by apoptosis simply by cultivating thymocytes ex vivo
or by ex vivo treatments with the glucocorticoid, dexamethasone.

Ets1, the founding member of the et s family, is also expressed
during T cell development (11, 12). The first studies addressing the

FIGURE 8. Expression of c-Myc correlates with proliferation and the
expression of the et s2 transgene following a 6-h in vivo dexamethasone
treatment. Dexamethasone (2.5 mg) was injected into animals for 6 h. Two
hours postinjection, animals were injected with 1 mg of BrdU as described
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abeled with BrdU or CD4 and CD8 Abs or prepared for Western analysis.
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from lckP^ets2 transgenic lines. These experiments were repeated four
times showing similar results (data not shown). B, The different CD4CD8
compartments were shown by FACS analysis and are graphically repre-

FIGURE 9. The et s2 transgene protects against dexamethasone-induced
apoptosis ex vivo. A, Cells (1 × 10^6) isolated from thymi of lckP^ets2
transgenic or control littermate mice were cultured in triplicate ex vivo
with 0.1 μM dexamethasone for 48 h. Viable cell numbers were deter-
mined using trypan blue exclusion at 3, 6, 21, 30, and 48 h. The total
number of viable cells were plotted against time, and standard errors are
indicated. Values of p = 0.0035 are very significant, and p values of 0.0001
are extremely significant. B, The ratio of viable cells from dexamethasone-
treated thymocytes over nontreated thymocytes (Fig. 6) is plotted. Thymo-
cytes from lckP^ets2 transgenic are the most resistant (values of ~1), and
thymocytes from lckP^Δets2 transgenic mice are the most susceptible to
cell death (values >2).

9A) vs nontreated cells (Fig. 6). Taking the value of one to indicate
insensitivity to dexamethasone, thymocytes from lckP^ets2 trans-
genic animals were almost completely resistant to glucocorticoid
treatment compared with control littersmates (Fig. 9B). The greatest
sensitivity to dexamethasone was observed in thymocytes from transgenic mice expressing the dominant negative mutant of et s2,
Δets2.

9B)
role of Ets1 in the thymus used the RAG2−/− complementation assay (14, 15). ES cells containing homozygous mutations of the ets1 gene were produced and injected into RAG2-deficient blastocysts to generate chimeric mice. The absence of Ets1 in these chimeric animals resulted in T cell maturation and T cell activation defects. However, once the Ets1−/− mouse was generated, although cellularity of the thymus was often reduced, no differences in the relative proportions of DP CD4+CD8− or SP CD8+CD4− were observed (9) suggesting that maturation defects were either a result of the RAG2−/− complementation system or somehow compensated in Ets1−/− mice. We observe a default in maturation in young animals expressing a ets2 transgene. Not only has ΔEts2 been shown to functionally compete with endogenous Ets1 and Ets2 proteins in binding to their specific Ets binding sites, ΔEts2 binds more efficiently to its binding sites than full-length Ets2 or Ets1 proteins (reviewed in Ref. 5). Although both ets1 and ets2 transcripts are detected in a similar manner throughout T cell development, the expression of Ets1 and Ets2 proteins has been shown to be mutually exclusive. Ets1 is specifically expressed in DN and in resting CD4+ and CD8+ SP thymic subsets, whereas Ets2 expression is limited to DP T cells (12, 30). Because the expression of the Δets2 transgene is under the control of the lck promoter, which becomes active at the DN to DP transition, ΔEts2 specifically interferes with endogenous Ets2, but not Ets1, in the CD4+CD8− DP population although ΔEts2 could compete with Ets1 in CD4+CD8− DN T cells. Because the maturation defect of lckPr−/Δets2 transgenic mice at the DN to DP transition is corrected with the age of the animal, most likely other redundant pathways compensate for the loss of activation by Ets2 and Ets1.

Similarly cultivating thymocytes from RAG2−/− Ets1−/− chimeric mice ex vivo led to increased susceptibility to cell death of these cells (14, 15). Interestingly, we observe that thymocytes from lckPr−/Δets2 transgenic mice cultivated ex vivo are highly sensitive to cell death and thymocytes from dexamethasone-treated lckPr−/Δets2 transgenic mice are more resistant to apoptosis. These results show that both Ets proteins function in cell survival rather than being required for T cell maturation.

Transgenic studies have shown that the antiapoptotic protein Bcl-xL inhibited death after dexamethasone, gamma-irradiation, and anti-CD3 treatment (31). Several transcription factor families have been described as regulating the transcription of the bcl-x gene (32) including Ets transcription factors (6, 20). Surprisingly, Ets2 expression in transgenic mice had no effect on Bcl-xL levels. Two possibilities that could explain this apparent discrepancy exist. First, we showed (20) that a clear synergy exists between Ets2 and another Ets family member, PU.1, in transactivating the bcl-x promoter first described by Grillot et al. (33). Because PU.1 is not expressed in thymic cells (13), no such synergy could exist to regulate this region of the bcl-x gene. Second, other bcl-x promoter regions have recently been identified and shown to be active with different tissue specificities (34, 35). It is possible that, although the promoter region originally identified by Grillot is active in a T cell line, it may not be active in primary thymocytes. Furthermore, although Bcl-xL may inhibit dexamethasone-induced apoptosis in transgenic mice (31), endogenous expression of Bcl-xL remains constant even after dexamethasone treatment of WT and lckPr−/Δets2 and lckPr−Δets2 transgenic mice, in agreement with similar results obtained by Wang et al. (23) using dexamethasone-treated BALB/c mice.

The c-Myc transcription factor is associated with cell proliferation and its deregulation is associated with different types of cancers including leukemias and lymphomas. Recent studies showed that c-Myc−/− cells fail to proliferate normally at the late DN stage and mature into DP thymocytes (27). Interestingly, c-Myc was shown to be a direct target of Ets2 (28). c-Myc can induce apoptosis upon certain apoptotic signals, but c-Myc may also inhibit apoptosis. In the murine WEHI-231 B cells, c-Myc expression renders the cells resistant to anti-ig-mediated apoptosis (36). c-Myc is down-regulated upon dexamethasone treatment of BALB/c animals, and c-Myc renders transgenic animals less sensitive to glucocorticoid-induced cell death (23). These studies, which used transgenic mice expressing c-Myc under the control of a mouse mammary tumor virus dexamethasone-inducible promoter/enhancer, showed that, although Myc prolongs survival, c-Myc is not sufficiently to completely abrogate the effects of dexamethasone-induced cell death. We observe that Ets2 expression in lckPr−/ets2 transgenic mice allows greater resistance to dexamethasone as evidenced by a high proliferative rate and that c-Myc expression remains high when compared with that of lckPr−/Δets2 transgenic or control littermates where c-Myc expression is considerably lower.

Ets2 is expressed in a variety of tissues and cell types, and, although many potential target genes have been identified (reviewed in Ref. 37), little is known about the function of Ets2 in vivo. Disruption of the ets2 gene is early-embryonic lethal due to a trophoblast defect demonstrating that Ets2 is essential for placenta function (8). Transgenic animal studies have implicated Ets2 in bone formation (38) and in macrophage development (39). The presence of Ets2 throughout thymic development suggested a role of this protein in this organ. Using transgenic approaches, we showed that, although Ets2 expression may participate in the maturation of thymocytes, Ets expression clearly permits T cells to proliferate and survive, and we propose that Ets2, via activation of c-Myc, may be required for permitting proliferation and reducing cell death upon apoptotic signals.

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