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Peptide-Induced Negative Selection of Thymocytes Activates Transcription of an NF-κB Inhibitor

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Summary

Negative selection eliminates thymocytes bearing autoreactive T cell receptors (TCR) via an apoptotic mechanism. We have cloned an inhibitor of NF-κB, IκBNS, which is rapidly expressed upon TCR-triggered but not dexamethasone- or γ irradiation-stimulated thymocyte death. The predicted protein contains seven ankyrin repeats and is homologous to IκB family members. In class I and class II MHC-restricted TCR transgenic mice, transcription of IκBNS is stimulated by peptides that trigger negative selection but not by those inducing positive selection (i.e., survival) or nonselecting peptides. IκBNS blocks transcription from NF-κB reporters, alters NF-κB electrophoretic mobility shifts, and interacts with NF-κB proteins in thymic nuclear lysates following TCR stimulation. Retroviral transduction of IκBNS in fetal thymic organ culture enhances TCR-triggered cell death consistent with its function in selection.

Introduction

Apoptosis is a morphologically stereotyped form of programmed cell death utilized by metazoan organisms during normal development as well as for homeostasis (Kerr et al., 1972; Horvitz et al., 1982; Vaux and Korsmeyer, 1999). Excess cells at each stage of organogenesis are eliminated by this mechanism. Within the hematopoietic system, this phenomenon is well described (Cohen et al., 1992).

In T lymphoid development, αβ T cell receptors (TCR) are generated by a stochastic process of rearrangement of variable gene segments. Through combinatorial diversity, billions of different TCRs are generated, ensuring protection against a myriad of infectious agents without a requirement to encode all TCR specificities in the germline (Davis and Bjorkman, 1988). One potential hazard of this diversity-generating mechanism is the creation of self-reactive TCRs. Thus, T cell repertoire generation must be carefully regulated through a developmental selection program termed negative selection (von Boehmer, 1991). Those TCRs that recognize self-MHC molecules and their associated peptides (pMHC) too strongly are eliminated by negative selection, whereas those thymocytes with low affinity for self-MHC undergo maturation and are exported to the periphery in a process termed positive selection (Goldrath and Bevan, 1999). These selection processes act predominantly at the level of immature $CD4^+CD8^+$ double-positive (DP) thymocytes and are complex, being dependent not only on the monomeric affinity of the TCR-pMHC interaction (Alam et al., 1996) but also on the interplay between costimulation, coreceptor ligation, and TCR/pMHC affinity (Sebzda et al., 1999).

In mammalian cells, certain apoptotic pathways require gene transcription whereas others do not. Examples of the former are dexamethasone- or superantigen-induced lymphocyte death, while apoptosis triggered by CD95 is an example of the latter (Vaux and Korsmeyer, 1999). Since TCR triggering leads to the induction of gene expression, transcription factors would also be expected to play a role in thymocyte development and selection. The transcription factor IRF-1 appears to be involved in both positive and negative selection (Penninger et al., 1997), while nur77, another transcription factor, is involved in negative selection processes (Liu et al., 1994; Amsen et al., 1999). Deletion of the *nur77* gene in vivo (Lee et al., 1995) does not result in impaired thymic selection, implying that other pathways may be operative in negative selection. In this regard, NF-κB transcription factors have been implicated in providing both survival and death signals (Ghosh et al., 1998) and are expressed at high levels in the thymus in a constitutively active state (Voll et al., 2000).

To identify additional key genes expressed in thymocytes undergoing peptide-triggered selection, we have utilized a TCR transgenic mouse system in which antigen-induced negative selection could be induced (Ghendler et al., 1998) to perform representational difference analysis (RDA) (Lisitsyn et al., 1993; Hubank and Schatz, 1994). A subtracted library was prepared, and a cDNA which encodes a functional inhibitor of NF-κB, termed IκBNS, was cloned.

Results

cDNA Cloning of IκBNS

Homozygous N15 TCRtg RAG-$2^{-/-}$ H-2^b mice (hereafter referred to as N15 TCRtg) bearing a TCR specific for the VSV8 peptide bound to the H-2 K^b MHC class I molecule were previously established to investigate processes of negative selection (Clayton et al., 1997; Ghendler et al., 1998; Sasada et al., 2000). These studies showed that DP thymocytes underwent apoptosis within 3 hr of a single 24 μg VSV8 administration (Clayton et al., 1997). DP thymocytes were isolated by fluorescence-activated cell sorting from N15 TCRtg RAG-$2^{-/-}$ TCR transgenic mice on an H-2^b or H-2^d MHC background.

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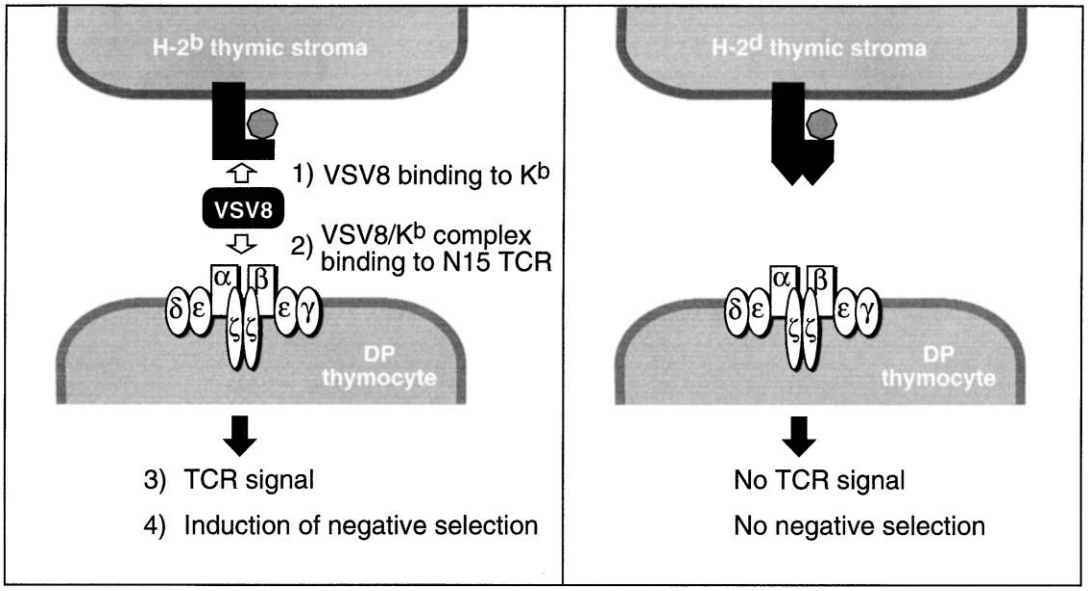

Figure 1. Scheme of N15 TCRtg RAG-2$^{-/-}$ DP Thymocyte Interactions with Selecting versus Nonselecting Thymic Stromal Cells
Details are described in the text.

The N15 TCRtg mice were injected 1 hr previously with VSV8 peptide to induce negative selection. The resulting RNAs from DP thymocytes were used to perform RDA (Lisitsyn et al., 1993; Hubank and Schatz, 1994) with the VSV8-stimulated N15 TCRtg RAG-2$^{-/-}$ H-2^{b} RNA as the tester and the unstimulated N15 TCRtg RAG-2$^{-/-}$ H-2^{d} RNA as the driver. The mRNA pool from the N15 TCRtg RAG-2$^{-/-}$ H-2^{d} mice is representative of DP thymocyte transcripts but, importantly, lacks those mRNAs induced via TCR activation since the N15 TCR cannot be triggered on the H-2^{d} background (Figure 1). The mRNA pool of the N15 TCRtg mice 1 hr after VSV8 peptide injection contains the same DP thymocyte mRNAs plus mRNAs induced during TCR-triggered negative selection. Thus, RDA using these two DP thymocyte populations will preferentially amplify cDNA copies of mRNAs induced during negative selection.

Resultant PCR products were size fractionated and subcloned to create a subtracted library. Of these inserts, 80% hybridized with a nur77 probe and were excluded from further analysis. Plasmid DNAs were prepared from randomly chosen colonies of the remaining 20% and inserts used to probe Northern blots of total thymus RNA from N15 TCRtg RAG-2$^{-/-}$ H-2^{d} and VSV8-injected N15 TCRtg mice. One insert showed strong induction in the VSV8-injected N15 TCRtg RNA but did not hybridize to the N15 TCRtg RAG-2$^{-/-}$ H-2^{d} RNA (see below). Reanalysis of the nur77-unrelated clones in the subtracted library showed that half hybridized with this cDNA. The 176 bp insert of this clone was used to isolate a full-length clone from a thymic cDNA library.

Comparison with Known IκB Proteins

The predicted protein sequence of this cDNA is highly homologous to members of the IκB family of proteins as aligned in Figure 2A. Therefore, we have termed this gene product IκBNS. The clone encodes a 327 amino acid protein of approximately 35 kDa containing seven ankyrin domains (labeled A–G, Figure 2), four potential protein kinase C phosphorylation sites ([S/T]-X-[R/K] at aa 20–22, 43–45, 220–222, and 289–291), and one potential casein kinase II phosphorylation site ([S/T]-X(2)-[D/E] at aa 214–217). The IκBNS protein sequence has highest similarity (43%) with "molecule-possessing ankyrin repeats induced by lipopolysaccharide" (MAIL, or IκBζ or Interleukin-1-inducible nuclear ankyrin-repeat protein [INAP]), an inducible IκB protein (Kitamura et al., 2000; Yamazaki et al., 2001; Haruta et al., 2001). Other IκB family members have 29%–39% similarity by BLAST search. The Figure 2B dendrogram shows that after MAIL, human Bcl-3 is most similar.

A comparison of the ankyrin domains of six IκB family members is presented in Figure 2A. Ankyrin repeats span over 33 residues and are highly divergent (Bork, 1993) but contain a basic core motif of two antiparallel α helices connected by a tight-hairpin loop. When an alignment using the entire IκBNS protein sequence is performed, the first six of seven ankyrin domains in IκBNS align with ankyrin domains 1 through 6 of the structurally characterized IκB protein, IκBα (1nfi) (Jacobs and Harrison, 1998). Repeats B, C, E, and F are the most conserved whereas the N-terminal (A) and C-terminal (G) are much less conserved. Ankyrin repeats do not behave independently but associate in tandem to provide stabilizing interactions (McDonald and Peters, 1998; Zhang and Peng, 2000). The C-terminal and N-terminal ankyrin repeats interact only with one neighboring repeat and therefore are less evolutionarily constrained.

Ankyrin repeat D of IκBNS deserves special attention. IκBNS and MAIL have an insertion in ankyrin repeat D within the tight β-turn of the ankyrin repeats of p100,

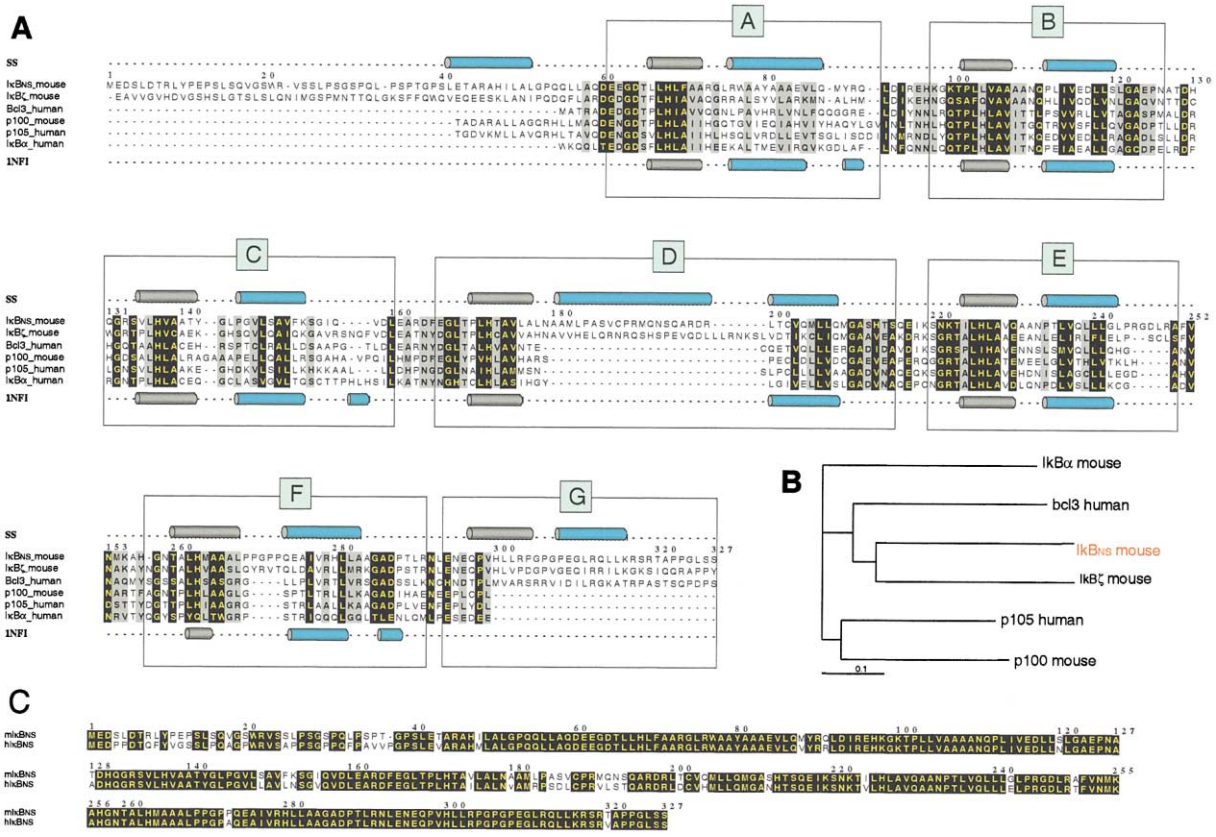

Figure 2. Multiple Sequence Alignment of IκBNS

(A) The full sequence of IκBNS is shown. Numbering refers to IκBNS. The ankyrin domains of IκBNS are boxed and labeled from A to G. Secondary structure (ss) predictions for IκBNS are shown above the alignment with the inner helix of the ankyrin repeat core shown in gray and the outer in blue. Secondary structure motifs of IκBα were obtained from pdb 1NFI (Jacobs and Harrison, 1998) and are shown below the alignment. Sequences were aligned using the program ClustalX (Thompson et al., 1997), and the secondary structure prediction of IκBNS was determined using PSI-PRED (Jones, 1999). Amino acid positions with identities or similarities in five or more of the six proteins aligned are highlighted in black with yellow letters. Amino acid positions with identities or similarities in four or more of the six proteins are highlighted in gray. For this analysis, V/L/I, S/T, N/Q, D/E, K/R, and W/F are considered equivalent. Residues shown are for human IκBα aa 66–287, for human Bcl-3 aa 31–278, for murine IκBζ aa 292–629, for human p105 aa 522–756, and for murine p100 aa 467–705.

(B) A dendrogram of the figure is displayed with IκBα as the root and was derived using the Neighbor-joining method (Saitou and Nei, 1987).

(C) Comparison of mouse (top) and hypothetical human (bottom) IκBNS protein sequences. The human sequence is derived from a tBLAST search against the human genome database using mIκBNS as the query sequence and shows 91% identity between murine and human sequences.

p105, Bcl-3, and IκBα (Figure 2). Secondary structure predictions indicate, however, that IκBNS has an extended outer helix rather than a long loop insertion between the inner and outer helices of the ankyrin repeat. If this is the case, the packing of the ankyrin repeats would be interrupted at this point, and a second tandem of ankyrin repeats would start at ankyrin repeat E and terminate with ankyrin repeat G.

Human Homolog

A BLAST search revealed high similarity with a human genomic sequence in Genbank consisting of 39,163 bp from human chromosome 19. IκBNS matches this human chromosome 19 sequence in discontinuous stretches over a large area (from bp 23,000 to bp 33,500), suggesting the presence of a homologous human gene with seven or more exons. Comparison of the murine IκBNS protein sequence with that of the hypothetical human protein shows 91% identity (Figure 2C). Hence, a human *IκBNS* gene exists and likely serves a comparable function to the mouse counterpart.

Analysis of IκBNS mRNA Expression

Using the 176 bp RDA product as a probe, we examined the tissue distribution of this gene as shown in Figure 3A. While faint hybridization to spleen mRNA was noted, heart, brain, lung, liver, skeletal muscle, kidney, and testis were negative. Thus, this gene appears to have a narrow tissue distribution relative to the β-Actin positive control. In addition, very low levels can be detected in RNA from control C57BL/6 thymus (Figure 3B, right panel, Ctl lane), perhaps as a consequence of ongoing negative selection. However, in N15 TCRtg RAG-2$^{-/-}$ H-2^{d} or N15 TCRtg (data not shown) animals, IκBNS message is not detected in the thymus of control animals but is apparent within 1 hr of i.v. injection of VSV8 peptide into N15 TCRtg mice (Figure 3B, left panel). Similarly, IκBNS message is strongly induced within 1 hr of pigeon

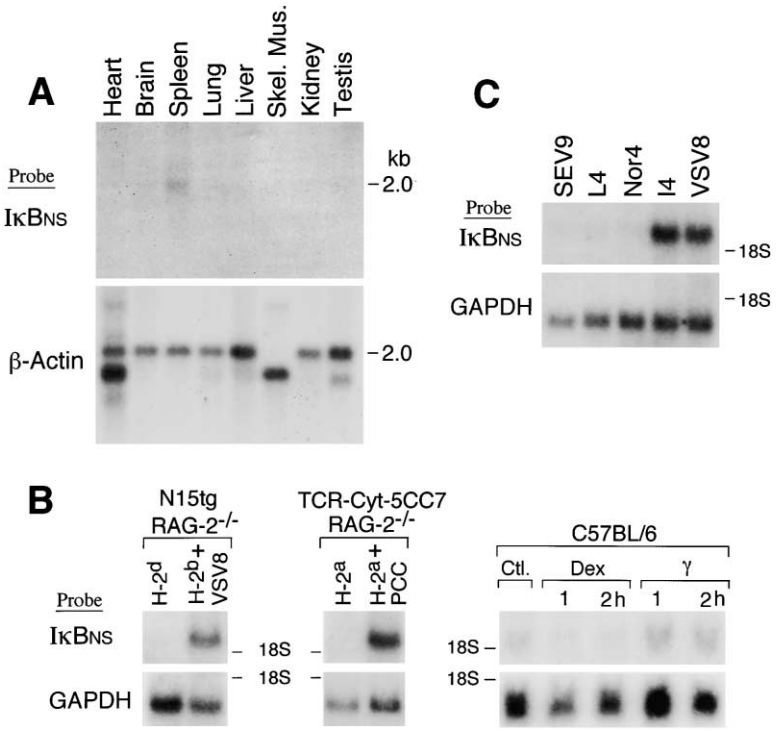

Figure 3. RNA Blot Analysis of IκBNS Expression

(A) A mouse multiple tissue Northern was probed with IκBNS (upper panel) or β-Actin control probe (lower panel). Each lane contains 2 μg of polyA+ mRNA. The IκBNS blot was exposed for 4 days; the β-Actin blot was exposed for 4 hr. The position of a 2.0 kb marker is indicated.

(B) Ten micrograms of total thymic RNA was run in each lane. RNA was isolated from the thymus of untreated N15 TCRtg RAG-2$^{-/-}$ H-2^{d} animals, N15 TCRtg animals 1 hr after i.v. injection of 24 μg VSV8, untreated TCRCyt5CC7-I-RAG-2$^{-/-}$ H-2^{a} animals, TCRCyt5CC7-I-RAG-2$^{-/-}$ H-2^{a} animals 1 hr after i.v. injection of 24 μg PCC peptide, untreated C57BL/6 animals, and C57BL/6 animals 1 and 2 hr after injection of 0.5 mg dexamethasone or 500 rads whole body γ irradiation.

(C) Four micrograms of total thymic RNA isolated from N15 TCRtg FTOC was run in each lane. RNAs were prepared from FTOC after 2 hr incubation with 10 μM of the indicated peptides. The position of the 18S ribosomal RNA band is indicated.

cytochrome c (PCC) peptide (aa 88–104) injection into TCRCyt5CC7-I-RAG-2$^{-/-}$ H-2^{a} mice which bear a TCR-recognizing PCC bound to the I-E^{k} class II MHC molecule (Seder et al., 1992) (Figure 3B, middle panel). Under these conditions, PCC peptide administration induces negative selection of DP thymocytes in TCRCyt5CC7-I-RAG-2$^{-/-}$ H-2^{a} mice (our unpublished data). Note the absence of IκBNS message in the basal state. These results demonstrate that induction of negative selection in both class I- and class II-restricted TCR transgenic animals results in increased levels of IκBNS mRNA. This message is also induced in thymic RNA isolated from C57BL/6 mice treated in vivo with anti-CD3ε monoclonal antibody, a treatment known to induce death in DP thymocytes (data not shown) (Smith et al., 1989). While apoptotic death of immature thymocytes is also induced by glucocorticoids (Wyllie, 1980) and γ irradiation (Sellins and Cohen, 1987), thymic RNAs from mice treated with 0.5 mg dexamethasone or 500 rads show essentially no induction of IκBNS when mRNA levels are normalized with GAPDH message (Figure 3B). Thus, IκBNS is induced only by TCR-triggered events in immature thymocytes. Analyses of the N15 TCRtg mice using variant peptide ligands have demonstrated that the VSV8 peptide p4 position is critical in determining thymic selection outcomes (Ghendler et al., 1998; Sasada et al., 2000). This residue is centrally located in the VSV8/K^{b} pMHC complex and contacted by the CDR3 loop regions of the N15 TCR Vα and Vβ domains (Teng et al., 1998). Hence, small changes in the peptide there can be sensed by the TCR. While VSV8 and the isoleucine variant (I4) induce negative selection in FTOC using N15 TCRtg mice, leucine (L4) or norvaline (Nor4) substitutions result in positive selection (Clayton et al., 1997; Ghendler et al., 1998; Sasada et al., 2000). SEV9, a Sendai virus-derived peptide that binds K^{b} with an affinity equal to that of VSV8 but is not recognized by the N15 TCR, serves as a control peptide. As shown in Figure 3C, the negatively selecting peptides, VSV8 and I4, result in IκBNS mRNA induction within 2 hr of addition to N15 TCRtg FTOC. In contrast, the L4 and Nor4 peptides that induce positive selection and the control nonselecting SEV9 peptide do not upregulate steady-state expression of the IκBNS mRNA. Induction of IκBNS mRNA therefore correlates with the process of negative selection in thymocytes.

IκBNS Protein Expression and Localization

Thymic lysates were prepared from N15 TCRtg mice 0.5, 1, and 2 hr after injection of VSV8 peptide and uninjected mice and analyzed for expression of IκBNS by Western blot using a mAb produced for this purpose. Figure 4A shows that IκBNS protein (~35 kDa) is induced within 30 min of VSV8 treatment. The amount of protein continues to increase up to 2 hr. IκBNS protein peaks at about 2 hr postinjection and then begins to decrease (data not shown). For comparison, the same Western blot was examined for IκBα expression and for β-Actin as a loading control. Regulation of IκBα protein is mediated through phosphorylation-ubiquitination processes resulting in its degradation and activation of NF-κB (reviewed in Karin and Ben-Neriah, 2000). The DSGL[D/G/E]S motif targeting phosphorylation of serines 32 and 36 found in IκBα, β, and ε is not present in IκBNS (Figure 2). Furthermore, there are no lysines equivalent to lysines 21 and 22 in IκBα, which can serve as targets for ubiquitination (Karin and Ben-Neriah, 2000), implying that IκBNS is insensitive to this degradation pathway. In addition, control of the basal IκBα protein level is through a PEST sequence located in the carboxy-termi-

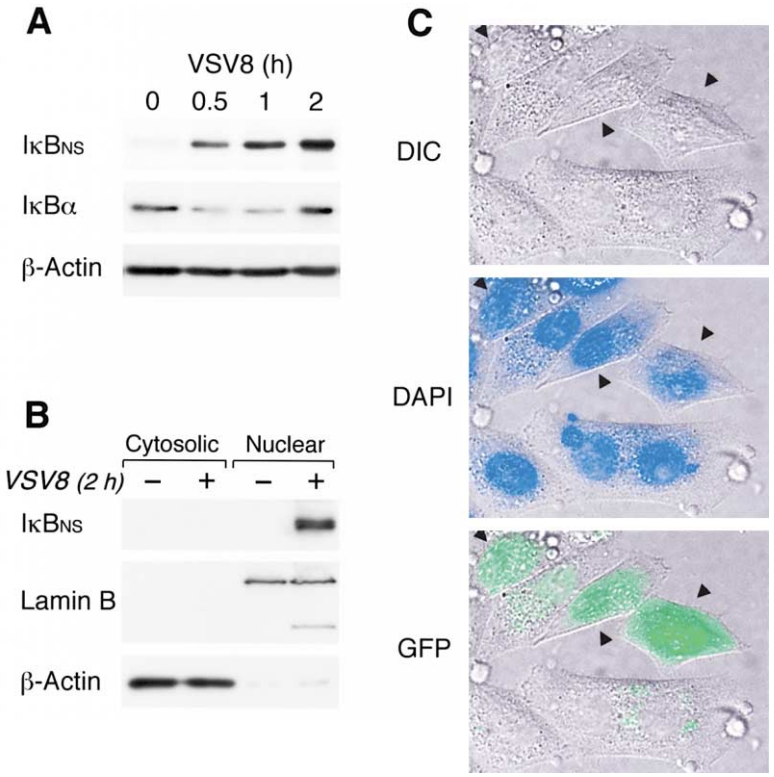

Figure 4. Analysis of IκBNS Protein Expression and Subcellular Localization

(A) Western blot analysis of IκBNS and IκBα in thymic lysates from N15 TCRtg mice uninjected (0) or 0.5, 1, and 2 hr after in vivo VSV8 treatment. Twenty micrograms of thymic lysate proteins was run per lane on SDS/PAGE and transferred to PVDF membranes. The filter was Western blotted with an anti-IκBNS mAb, 138, and then stripped and reprobed with anti-IkBα and then anti-β-Actin.

(B) Western blot analysis of nuclear and cytoplasmic subcellular fractions of thymic lysates isolated from N15 TCRtg mice uninjected or 2 hr after in vivo VSV8 treatment. For SDS/PAGE analysis, each lane was loaded with proteins equivalent to 2×10^6 cells, transferred to PVDF membranes and blotted with an anti-IκBNS mAb, and then sequentially stripped and reprobed with anti-Lamin B as a nuclear marker followed by anti-β-Actin as a cytoplasmic marker.

(C) Fluorescence microscopic analysis of subcellular localization of eGFP-IκBNS fusion protein. HeLa cells were transfected with a construct encoding an eGFP-IκBNS fusion protein and examined by differential interference contrast (DIC) microscopy, DAPI staining, and eGFP fluorescence for the subcellular location of eGFP-IκBNS fusion protein. Arrowheads indicate transfected cells.

nal portion (reviewed in Karin and Ben-Neriah, 2000) but not found in IκBNS. Western blot analysis of IκBα in thymic lysates demonstrates expression of IκBα in thymocytes of uninjected N15 TCRtg mice (Figure 4A); TCR triggering by VSV8 results in substantial degradation of this protein at 0.5 and 1 hr poststimulation (to 1/3 the basal level at each time point). By 2 hr, the IκBα level has returned to that seen in the uninjected animals as judged by densitometry scanning. In contrast, IκBNS levels in N15 TCRtg thymocytes are minimally detected in the basal state, increase 6-fold by 0.5 hr after VSV8 injection, and continue to increase up to 2 hr postinjection (~8-fold at 1 hr and ~10-fold at 2 hr). Both the absence of IκBα-like degradation signals in the IκBNS protein and the rapid kinetics of IκBNS RNA induction suggest that IκBNS is likely under tight transcriptional control.

To determine the cellular localization of IκBNS, we performed Western blot analysis on nuclear and cytosolic fractions from N15 TCRtg thymocytes isolated from untreated mice and from mice 2 hr after VSV8 injection (Figure 4B). As controls, the same blot was probed for Lamin B, a marker for the nucleus, and β-Actin as a cytoplasmic marker. Figure 4B shows that IκBNS is selectively found in the nuclear fraction of thymocytes from VSV8-injected mice. Interestingly, Lamin B, a known 70 kDa substrate for caspases (Rao et al., 1996), is cleaved to generate a 50 kDa product in the VSV8-treated thymocytes indicative of caspase activation by VSV8-triggered negative selection as was previously observed (Clayton et al., 1997). Furthermore, a chimeric protein containing eGFP fused to the amino terminus of IκBNS localizes predominantly to the nucleus when expressed in HeLa cells (Figure 4C), whereas the GFP protein alone is not nuclear, instead revealing a diffuse cellular distribution (data not shown). These results are consistent with a nuclear location for IκBNS.

NF-κB Inhibition by IκBNS

The major function of IκBα is to bind NF-κB in the cytoplasm and prevent its nuclear translocation and subsequent DNA binding by blocking these sites on NF-κB. To determine if IκBNS has a similar inhibitory function, we assayed an NF-κB-sensitive luciferase reporter in Cos-7 cells cotransfected with IκBNS. NF-κB activity was induced by a 7 hr treatment of transfected Cos-7 cells with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) prior to analysis of luciferase activity. PMA-treated Cos-7 cells showed an increase of approximately 5- to 10-fold in NF-κB luciferase activity relative to untreated cells (Figure 5A). Cotransfection with an IκBNS construct or with an IκBα construct reduced PMA-induced NF-κB-driven luciferase activity in Cos cells. IκBNS also reduced the activity of NF-κB-sensitive reporter constructs in transiently transfected Jurkat T cells (data not shown). To further assay IκBNS function, we tested the effects of a chimeric GST-IκBNS fusion protein on NF-κB electrophoretic mobility shift assays (EMSAs). NF-κB gel shift analysis was performed on nuclear extracts from N15 TCRtg mice 1 hr after VSV8 injection (Figure 5B). Addition of GST-IκBNS and GST-IκBα but not GST blocks the gel shift band in N15 TCRtg thymic nuclear extracts. Although not shown, GST-IκBNS also blocks NF-κB binding in nuclear lysates of anti-CD3ε mAb-treated C57BL/6 animals. Addition of 100× cold NF-κB probe blocks formation of the NF-κB band, but 100× cold AP-1 probe has no effect, demonstrating the specificity of this gel shift. The GST-fusion proteins have no

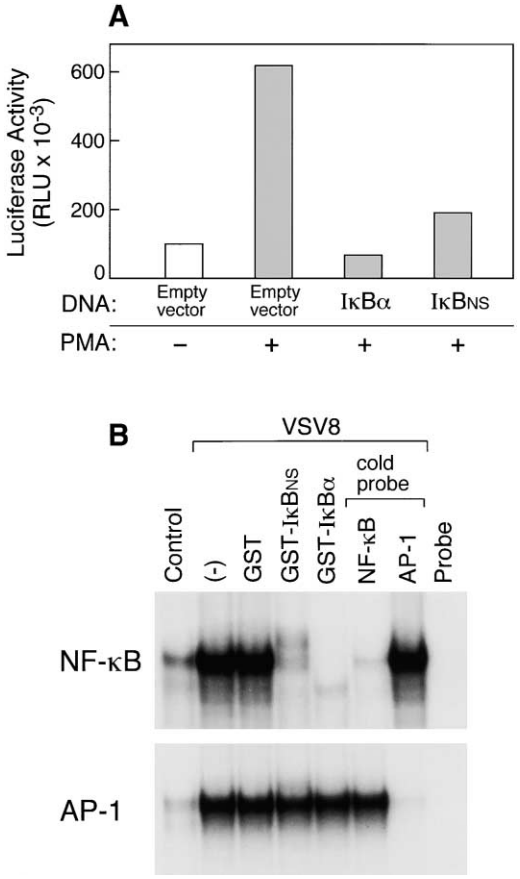

Figure 5. IκBNS Inhibits NF-κB-Induced Expression of Luciferase in Transfected Cos-7 Cells and NF-κB EMSAs

(A) Cos-7 cells were cotransfected with pRL null and (kB_3) NF-κB/luciferase constructs plus empty vector or, alternatively, cotransfected with pRL null and (kB_3) NF-κB/luciferase constructs plus IκBNS or IκBα constructs. Twenty-four hours after transfection, PMA was added to induce NF-κB activity. After 7 hr, Cos-7 cells were lysed and luciferase activity determined. The relative light units (RLU) are shown $\times 10^{-3}$ on the ordinate. One representative experiment out of five is shown.

(B) Nuclear thymic lysates were prepared from N15 TCRtg animals untreated (Control) or 1 hr after i.v. injection of 24 μg VSV8 (VSV8) and assayed by EMSA with no additions (Control and −), 1 μg of GST protein, 1 μg of GST-IκBNS, or 1 μg of GST-IκBα. Also added were a 100-fold excess of cold NF-κB probe or cold AP-1 probe. The upper panel uses an NF-κB probe and the lower panel an AP-1 probe. A probe-only lane (Probe) without lysate is shown.

effect on the AP-1 gel shift, but the 100× cold AP-1 probe inhibits formation of this band. Collectively, these data suggest that IκBNS and IκBα can bind NF-κB components and prevent subsequent DNA binding.

Interaction of IκBNS with NF-κB Family Members

To examine the specificity of IκBNS binding in vivo, thymic cytosolic and nuclear extracts were prepared from N15 TCRtg mice 10, 30, or 60 min after i.v. VSV8 injection or from uninjected animals and used for Western blot analysis of IκBNS- and IκBα-interacting proteins. The total cellular levels of p50, p65, and RelB proteins are shown in Figure 6A. There is little change in their cytosolic level upon VSV8 injection; in contrast, p50, p65, and RelB proteins are barely detectable in the nuclear fraction in control animals. Within 10 min of TCR triggering, however, all are readily observed in the nucleus. Presumably, triggering through the TCR has induced degradation of cytosolic IκBα and allowed translocation of these proteins to the nucleus. Both c-Rel and p52 show similar patterns of expression to those in Figure 6A, although p52 is detectable in the nuclear fraction of the 0 time point (data not shown).

GST, GST-IκBNS, and GST-IκBα were incubated with the thymic lysates and interacting proteins identified by Western blot analysis (Figure 6B). GST protein (Ctl) does not interact with any of the proteins analyzed. Aside from some basal p50-IκBNS interaction, the interaction of GST-IκBNS and GST-IκBα with p50, p65, and RelB proteins is largely dependent on TCR triggering. The presence of endogenous cytosolic IκBα at the 0 time point likely blocks interaction with the GST-IκBα and GST-IκBNS fusion proteins. In the cytosolic fraction, interaction of p50 and RelB are readily detected in the IκBNS pull-down experiment at 10, 30, and 60 min. However, no p65 association is detected at any time point tested even though p65 is present in this fraction (Figure 6A) and can interact with IκBα (Figure 6B). The preference of IκBNS for p50 and IκBα for p65 is borne out in interaction analysis on in vitro-translated proteins (Figure 6C). Moreover, GST-IκBα binds all three proteins in the cytosol consistent with its ability to interact with various NF-κB heterodimers. The amount of cytosolic p50 bound to GST-IκBNS and GST-IκBα is approximately equivalent, while more cytosolic RelB is bound by GST-IκBα than by GST-IκBNS. Although not shown, the pattern of interaction with cytosolic c-Rel is exactly like that of RelB for both GST-IκBNS and GST-IκBα.

Within the nuclear fraction, however, GST-IκBNS displays surprisingly similar NF-κB binding compared to that of GST-IκBα, interacting with p50, p65, and RelB (although binding more p50). While the amounts of total p50, p65, and RelB proteins are approximately equal in both nuclear and cytoplasmic compartments (Figure 6A) (aside from the 0 time point where no p65 or RelB is detected in the nuclear fraction of control mice), IκBNS binding is more apparent in the nuclear than cytosolic fractions. GST-IκBNS binds to p52 only in the nuclear fraction, and the pattern of interaction with c-Rel is exactly like that of RelB (data not shown). That the GST-IκBNS interaction differs between the nuclear and cytosolic fractions is noteworthy. In the nucleus, NF-κB proteins may be bound to DNA and, hence, the conformation in the presence of DNA altered to allow interaction with IκBNS in the nuclear fraction. A second possibility is that there may be TCR triggering-induced modification of NF-κB proteins that allows an interaction with GST-IκBNS in the nucleus. A third possibility might involve a distinct protein(s) within the nucleus that facilitates the interaction of IκBNS with p65 and RelB (and c-Rel) in this compartment.

Retroviral Transduction of IκBNS Disrupts Thymocyte Development and Increases Anti-TCR-Induced Death in FTOC

To determine the functional consequence of IκBNS expression on thymocyte development, we retrovirally

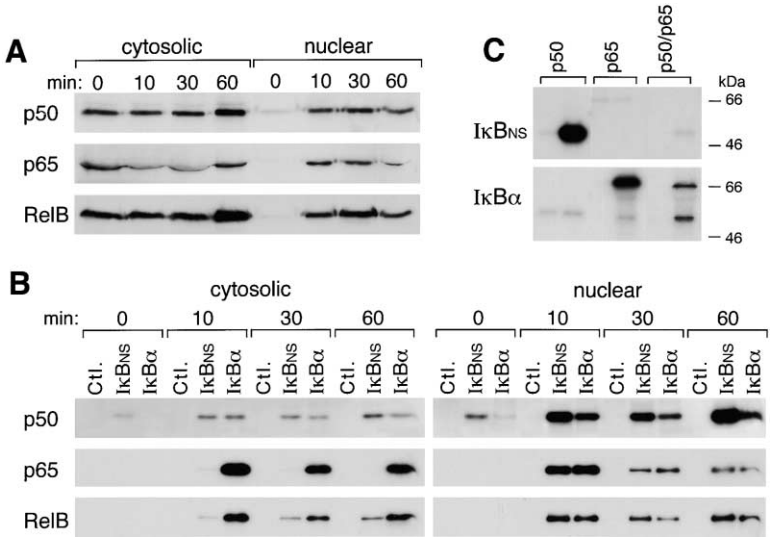

Figure 6. Analysis of the Interaction of GST-IκBNS or GST-IκBα with NF-κB Components In Vivo and In Vitro

Nuclear and cytosolic thymic lysates were prepared from uninjected N15 TCRtg mice or mice 10, 30, or 60 min after VSV8 peptide injection.

(A) Western blot analysis of cytosolic and nuclear lysates for p50, p65, and RelB NF-κB proteins. The same amount of protein was added to each lane.

(B) Cytosolic and nuclear fractions of thymic lysates were incubated with GST (Ctl), GST-IκBNS, or GST-IκBα proteins bound to beads and the interacting proteins identified by Western blot analysis with the indicated subunit-specific antibodies following SDS-PAGE.

(C) Analysis of the interaction of IκBNS and IκBα with in vitro-translated p50 and p65 proteins. [^{35}S]Radiolabeled murine NF-κB proteins were produced by in vitro translation and incubated individually or in combination with 5 μg GST, GST-IκBNS, or GST-IκBα coupled to beads. Associated proteins were identified by autoradiography after SDS-PAGE. In each pair of lanes incubated with an NF-κB protein(s), the left lane shows binding to the GST control beads, and the right lane shows interaction with GST-IκBNS (upper panel) or GST-IκBα (lower panel) beads.

transduced DN fetal thymocytes with a bicistronic construct encoding IκBNS and eGFP (Res et al., 1996; Heemskerk et al., 1997) and used these progenitors to repopulate FTOC. As shown in Figure 7, transduction of day 14 fetal thymocytes by either LZRS-eGFP (control vector) or LZRS-IκBNS-eGFP results in eGFP$^+$ thymocytes in all four CD4/8 thymocyte subsets after repopulation of fetal thymic lobes. The same cultures contain non-transduced eGFP$^-$ thymocytes serving as internal controls. FTOC from LZRS-eGFP-transduced thymocytes contain 16% DN, 58% DP, 18% CD8SP, and 8% CD4SP thymocytes in the eGFP$^+$ fraction; these percentages are almost identical to those of the eGFP$^-$ fraction from the same transduction as well as to the eGFP$^-$ cells from the LZRS-IκBNS-eGFP transduction. In contrast, the eGFP$^+$ cells from the LZRS-IκBNS-eGFP transduction (i.e., the IκBNS-expressing thymocytes) consistently contain lower percentages of DP and SP thymocytes (20%–40% overall reduction) and an increased percentage (>2-fold) of DN thymocytes. Thus, expression of IκBNS alters the development of thymocytes in FTOC. As retroviral transduction of DN fetal thymocytes in hanging drops does not alter the number of viable cells in the initial suspension culture or the absolute number of DN cells in subsequent FTOC, this perturbation cannot be a consequence of developmental arrest at the DN stage. Equivalence of the four DN subpopulations based on CD44/CD25 profiling are consistent with this view (data not shown). In contrast, the absolute number of transduced DP thymocytes as marked by eGFP in LZRS-IκBNS-eGFP- versus LZRS-eGFP-transduced thymocytes is reduced by more than 1 order of magnitude. These data suggest that IκBNS expression primarily arrests thymocyte development at the DP stage. In addition, treatment of the LZRS-IκBNS-eGFP-transduced FTOC with anti-CD3ε antibody results in a markedly greater loss in the eGFP$^+$ DP thymocytes than in the eGFP$^-$ DP thymocytes (Figure 7). Treatment of LZRS-eGFP-transduced thymocytes with anti-CD3ε results in no such differential loss (data not shown). Thus, IκBNS expression increases the sensitivity of DP thymocytes to anti-CD3ε-induced death. As the loss of IκBNS-expressing thymocytes is even more pronounced after anti-CD3ε treatment of IκBNS-transduced cells, additional TCR-triggered cellular factors most likely contribute to the apoptotic process.

Discussion

NF-κB transcription factors are a highly conserved family of dimeric proteins found in many cell types. These inducible transcription factors activate various genes in response to proinflammatory and noxious stimuli (reviewed in Ghosh et al., 1998; Karin and Ben-Neriah, 2000). The remarkable ability of NF-κB proteins to respond quickly to cell surface perturbations is a function of the regulation of NF-κB transcriptional activity by inhibitors called IκB proteins. NF-κB exists in the cytoplasm complexed with an IκB protein; upon the cell receiving a signal to activate NF-κB, IκB is phosphorylated and degraded via the proteasome. The free NF-κB then enters the nucleus and activates gene transcription by binding particular DNA sites. Six IκB proteins termed IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and MAIL (reviewed in Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Kitamura et al., 2000; Yamazaki et al., 2001; Haruta et al., 2001) have been identified. IκBα is the best characterized member of this family and functions by blocking the nuclear localization signal and DNA binding of NF-κB proteins, thus preventing nuclear entry and NF-κB-induced gene transcription in the absence of appropriate cell stimulation (Ghosh et al., 1998; Karin and Ben-Neriah, 2000). The IκB family members may function by different mechanisms. For example, IκBα, IκBβ, and IκBε bind strongly to p65- or c-Rel-containing dimers, and Bcl-3 more strongly interacts with p52 and p50 homodimers (Ghosh et al., 1998). Whereas IκBα maintains the balance of cytoplasmic versus nuclear NF-κB

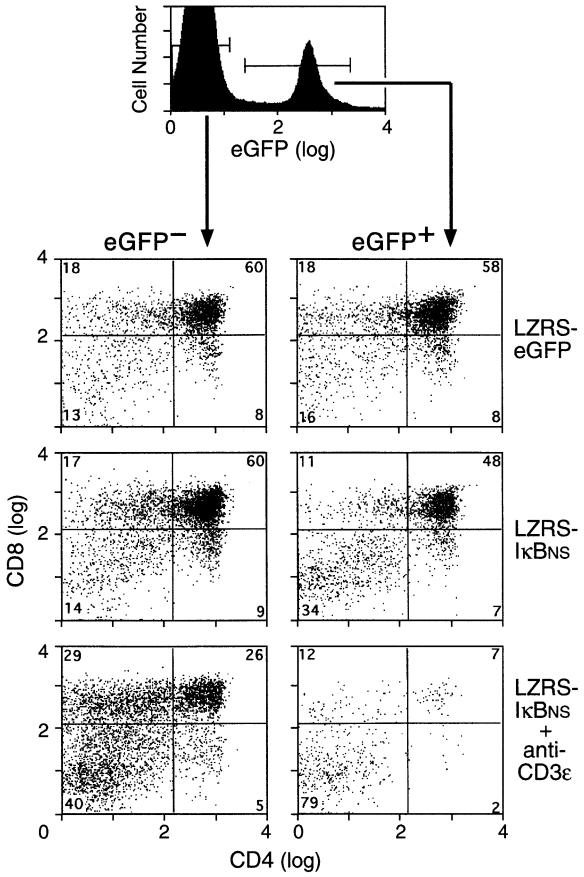

Figure 7. Retrovirally Transduced IκBNS Impairs Thymocyte Development in FTOC and Increases the Sensitivity of Thymocytes to Anti-CD3ε-Induced Cell Death

Day 14 DN fetal thymocytes were treated with IL-7 and SCF and transduced with LZRS-eGFP or LZRS-IκBNS retroviral constructs. Transduced thymocytes were placed in a hanging drop culture with a fetal thymic lobe from $\gamma c^{-/-}$/RAG-$2^{-/-}$ mice for 2 days and then transferred to gelfoam cultures and incubated for 7 days prior to FACS analysis. The GFP^+ and GFP^- thymocytes from LZRS-eGFP (upper panel) were analyzed separately for CD4 and CD8. Similar analysis was done for thymocytes transduced with LZRS-IκBNS (middle panel) and for thymocytes transduced with LZRS-IκBNS and then treated for 18 hr with 200 μg/ml anti-CD3ε (bottom panel). The percentages of cells in each quadrant are shown. The total numbers of thymocytes were 2.4×10^6 for the LZRS-eGFP culture, 1.9×10^6 for the LZRS-IκBNS culture, and 4×10^5 for the LZRS-IκBNS plus anti-CD3ε culture. The percentage of GFP^+ cells was 20% in the LZRS-eGFP-transduced FTOC, 0.5% in the LZRS-IκBNS-transduced FTOC, and 0.66% in the LZRS-IκBNS culture treated with anti-CD3ε. Note that the LZRS-eGFP and LZRS-IκBNS viral titres are similar based on quantitation of GFP^+ thymocytes in suspension 72 hr after transduction. Results are representative of three independent transduction experiments.

by a dynamic shuttling process (Johnson et al., 1999), Bcl-3 is instead a nuclear protein that acts to increase transcription, perhaps by removing the inhibitory p50 or p52 homodimers from DNA. Furthermore, Bcl-3 contains transactivation domains and may form activating complexes with p50 and p52 (Bours et al., 1993; Fujita et al., 1993). Other nuclear coregulators bind Bcl-3, suggesting that Bcl-3 may act as a bridging factor between these proteins and p50 or p52 (Dechend et al., 1999). IκBγ has a limited distribution and is not well characterized (Ghosh et al., 1998).

The role of the NF-κB/IκB transcriptional regulatory pathways in T cell development is currently unclear. Targeted gene disruptions of individual NF-κB family members do not demonstrate marked defects in lymphocyte development, but this lack of a thymic phenotype may result from redundancy in this important gene family (Ghosh et al., 1998). Double knockouts demonstrate more severe phenotypes, suggesting that this is the case (Gerondakis et al., 1999). The current consensus is that NF-κB proteins play a role in lymphocyte development and affect survival of developing thymocytes through effects on proliferation, protection from TNF-induced apoptosis, and regulation of antiapoptotic genes such as bcl-xL (Hettmann et al., 1999; Senftleben et al., 2001; Voll et al., 2000).

The distribution of IκBNS expression suggests a more restricted role for this protein in NF-κB regulation during T cell development. Consistent with this notion is the inability of anti-CD3ε mAb to stimulate IκBNS mRNA in spleen T cells (data not shown). Furthermore, the correlation of IκBNS induction with TCR-induced negative selection signals and the ability of retroviral transduced IκBNS to enhance TCR-triggered thymocyte death implies a key function in determining death/survival of immature thymocytes. What is the function of IκBNS and how does this protein affect NF-κB activity during negative selection? If NF-κB provides survival signals to developing thymocytes, then induction of an inhibitor of this activity would clearly result in cell death. However, an IκBα super-repressor transgene prevented anti-CD3ε-triggered cell death (Hettmann et al., 1999). Thus, simply inhibiting NF-κB activation does not appear to result in TCR-triggered thymocyte apoptosis, at least as stimulated by anti-CD3ε mAb crosslinking. To the contrary, these findings suggest that NF-κB activation is required for TCR-triggered thymocyte apoptosis. One explanation is that IκBNS may behave in a different manner than IκBα. IκBNS, like Bcl-3, with which it is most structurally homologous (Figure 2), binds to p50 and p52 homodimers. As described above, these NF-κB complexes act as inhibitors of transcription by blocking NF-κB sites on DNA. IκBNS may remove these inactive complexes from DNA and redirect TCR-triggered gene transcription (Franzoso et al., 1992; Bours et al., 1993; Fujita et al., 1993). Alternatively, IκBNS may also act as a bridging component between NF-κB dimers and other proteins. The interaction of the IκBNS-GST protein with p50 and p65 selectively within the nucleus implies that this is the case (Figure 6).

With respect to peptides tested, a similar pattern of mRNA induction occurs for IκBNS and nur77, a transcription factor also linked to negative selection processes (Liu et al., 1994; Amsen et al., 1999). Thus, the same TCR signal may induce nur77 and IκBNS either as a consequence of activation of a very early upstream gene that regulates both or by TCR-triggered posttranslational modification of a protein(s) that then activates these two genes directly. Nur77 and IκBNS account for the vast majority of differentially expressed clones among our RDA products.

A strength of the TCR transgenic system used herein is the detailed knowledge available concerning the ef-

fects of structural alterations of peptide sidechains on the signal transmitted to thymocytes (Ghendler et al., 1998; Sasada et al., 2000). This information has allowed us to correlate the induction of IκBNS with those peptide stimuli that trigger negative selection in N15 TCRtg transgenic mice. Similarly, in the class II system, TCRCyt5CC7-I-RAG-2$^{-/-}$ H-2^{a} mice injected with the negatively selecting PCC peptide also express IκBNS message in the thymus within 1 hr of treatment. Thus, IκBNS serves as a molecular marker of negative selection in both class I and class II TCR transgenic mice. In nontransgenic mice, IκBNS is induced by anti-CD3ε mAb injection, and retroviral transduction of IκBNS in FTOC enhances such TCR-triggered apoptosis, indicating a role for IκBNS in negative selection of wild-type mice as well. That IκBNS gene expression is strongly linked to TCR-triggered negative selection and not other forms of apoptosis is clear from the observation that γ irradiation and dexamethasone fail to induce IκBNS message in thymocytes.

That TCR triggering differentially induces transcription of genes and biochemical signaling events predicated on the extent of TCR-crosslinking or ligand affinity is well documented (Meuer et al., 1984; Fujita et al., 1986). The TCR signal required to induce positive selection necessitates a weaker TCR-pMHC interaction than that required for negative selection (Alam et al., 1996). IκBNS appears to be a qualitative transducer of divergent TCR ligation signals: those signals resulting in negative selection induce transcription of this gene while nonselecting or positively selecting peptides for which the TCR has less affinity do not. Although not directly studied here, differences in the number of pMHC complexes presented to the thymocytes (i.e., VSV8 K^{b} versus L4 K^{b}) on stromal cells cannot be the basis of the observed differential IκBNS transcription, since all altered peptide ligands tested had identical anchor residues and K^{b} binding affinity (Ghendler et al., 1998). The basis of thymocyte fate determination stimulated by peptides can now be addressed in explicit molecular terms and the ability to manipulate repertoire generation through modulation of this NF-κB inhibitory pathway investigated.

Experimental Procedures

Animals

N15 TCRtg RAG-2$^{-/-}$ H-2^{b} and N15 TCRtg RAG-2$^{-/-}$ H-2^{d} mice were generated as previously described (Ghendler et al., 1998). C57BL/6 and TCRCyt5CC7-I-RAG-2$^{-/-}$ mice were obtained from Taconic (Germantown, NY). Mice were maintained and bred under sterile barrier conditions at the animal facility of the Dana-Farber Cancer Institute. The OLA129 × Fvb mice used for retroviral transduction and FTOC were maintained at the Netherlands Cancer Institute.

Peptides

SEV9, PCC, VSV8, and VSV8 variant peptides were synthesized by standard solid phase methods on an Applied Biosystems 430A synthesizer (Foster City, CA) and authenticity verified as described (Sasada et al., 2000). For in vivo injections, 24 μg peptide in PBS was injected intravenously into mice.

RDA

Thymii were dissected from 3-week-old N15 TCRtg mice 1 hr after i.v. injection of 24 μg VSV8 peptide or from untreated 3-week-old N15 TCRtg RAG-2$^{-/-}$ H-2^{d} mice. Dissociated thymocytes were stained with anti-CD4 mAb (BD Pharmingen, San Diego, CA). In these class I-restricted transgenic animals, all CD4^{+} cells are DP. DP thymocytes were sorted on a MoFlo (Cytomation, Fort Collins, CO), and RNA was prepared as previously described (Lerner et al., 1996). The RNA was polyA selected using the polyA Spin mRNA Isolation Kit (New England Biolabs, Beverly, MA) and used for RDA (Lisitsyn et al., 1993; Hubank and Schatz, 1994). Due to limiting amounts of mRNA, we did not proceed to difference product 3 and instead prepared a subtracted library using difference product 2. For this purpose, difference product 2 was size fractionated and DNA from one gel slice containing an obvious PCR band ligated to pZErO-1 (Invitrogen, San Diego, CA), transformed, and colonies screened with a full-length nur77 probe (our unpublished data). DNA was prepared from nur77-negative clones, sequenced, and used as probes in Northern blot analysis.

Constructs

Full-length IκBNS was obtained by screening a thymus cDNA library in λZAP Express (Stratagene, La Jolla, CA) prepared from anti-CD3ε-treated C57Bl/6 mice (our unpublished data). To produce an eGFP-IκBNS fusion protein or a retroviral construct, IκBNS was subcloned into pEGFP-C1 (Clontech, Palo Alto, CA) or pLZRS-eGFP, respectively. For protein detection, a Flag tag was added to IκBNS by PCR and the product subcloned into pcDNA3.1 (Invitrogen). To produce a fusion protein with GST, Flag-tagged IκBNS was subcloned into pGEX-4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden). GST-IκBNS was purified following the procedures recommended by Amersham Pharmacia Biotech and used as an immunogen to produce a Western blotting anti-IκBNS monoclonal antibody (termed 138) in Balb/c mice (Li et al., 1998). The superinhibitor IκBα from CD2 MAD (Hettmann et al., 1999) was PCR'd and subcloned into pcDNA3.1 and pGEX-4T-1, respectively. GST-IκBα protein was purified as described above.

Production of Retroviral Supernatant

The retroviral Phoenix-E ecotropic packaging cell line (1 × 10^{6} cells) was transfected with LZRS plasmid DNA (20 μg) using the calcium phosphate method (GIBCO-BRL). Two days posttransfection, cells were trypsinized and grown under puromycin selection in Iscove's medium to 80% confluency. Medium was replaced with FTOC medium (Iscove's medium with 20% FCS/nonessential aa/50 μM β-mercaptoethanol/4 mM L-glutamine, penicillin, and streptomycin), and cells were incubated 15 hr more at 37°C. FTOC medium was collected, incubated on ice for 10 min, and centrifuged for 5 min at 1500 rpm. Viral supernatants were aliquoted and stored at −80°C.

FTOC and Retroviral Transduction

For N15 TCRtg FTOC, fetal thymii were removed at day 15.5 (observation of vaginal plug is day 1) and cultured in AIM-V media (Invitrogen, Carlsbad, CA) in Transwell dishes (Costar, Cambridge MA) in a humidified atmosphere with 5% CO_2 for 5 days. On day 5, peptides were added to 10 μM. Thymocytes were harvested 2 hr after peptide addition and RNA prepared (Qiagen RNeasy Mini Kit, Qiagen, Valencia, CA).

For OLA129 × Fvb FTOC and retroviral transduction, fetal thymii were removed at day 17, dissociated, and placed in FTOC medium at 100,000 cells/well (volume of 100 μl) with 50 ng/ml IL-7 and SCF in a 96-well plate. 100 μl of viral supernatant containing 20 μg/ml Lipofectamine (GIBCO-BRL) was added and the plate centrifuged at 1800 rpm for 45 min at 30°C, then incubated at 37°C overnight. Cells were collected by centrifugation and 30 μl/well placed in a Terasaki plate. Another 30 μl of cells was grown in 200 μl FTOC medium with 50 ng/ml Il-7 and SCF and analyzed by FACS after 2 days for eGFP expression to determine transduction efficiency. One freshly isolated day 14 γc$^{-/-}$ RAG-2$^{-/-}$ fetal thymic lobe was placed in each well and the plate inverted and incubated at 37°C for 2 days. The lobes were then transferred to ATTP 0.8 μM filters (Millipore) on gelfoam (Pharmacia and Upjohn Company) preincubated 2 days in FTOC medium. After 7 days, thymocytes were analyzed by FACS.

Northern Analysis

Total thymic RNA was prepared and Northern blots performed as previously described (Lerner et al., 1996; Clayton et al., 1997).

Transfections, Fluorescence Microscopy, and Luciferase Assays

Cos-7 cells were transfected using calcium phosphate (Turner et al., 1990) in six-well dishes with 0.2 μg pRL null (Promega, Madison, WI) plus 5 μg $(kB)_3$ luciferase plasmid (Plaisance et al., 1997). 10 μg of pcDNA3.1 or pcDNA3.1-IκBNS or pcDNA3.1-IκBα was cotransfected with the two luciferase plasmids. 24 hr later, cells were left untreated or activated with 50 ng/ml PMA for 7 hr. Cell lysates were prepared using the Dual-Luciferase Reporter Assay System (Promega) and luciferase activity measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The pRL null vector served as an internal control for transfection (Behre et al., 1999).

For nuclear localization studies, HeLa cells were plated on sterile glass coverslips and transfected as above with 10 μg of pEGFP-C1 (Clontech) or pEGFP-C1-IκBNS. 24 hr later, cells were fixed in 1% paraformaldehyde in PBS for 15 min at 4°C. The coverslips were washed with PBS and incubated with 300 nM DAPI (4′,6-diaminidino-2-phenylindole, dihydrochloride, Molecular Probes, Eugene, OR) in PBS for 30 min at RT in the dark. After extensive washes with PBS, the coverslips were mounted on microscope slides and observed under a Nikon Diaphot 300 fluoromicroscope (MVI, Inc., Avon, MA). Differential interference contrast (DIC) as well as fluorescent images were recorded using a digital SPOT camera (Diagnostic Instruments, Sterling Heights, MI) and IPLab software (Scanalytics, Inc., Fairfax, VA).

Electrophoretic Mobility Shift Assays

Nuclear and cytoplasmic extracts were prepared as previously described (Schreiber et al., 1989) from the thymus of C57Bl/6 mice or N15 TCRtg mice treated as described in the text. Protein concentrations were determined by Coomassie Protein Assay Reagent (Pierce, Rockford, IL). NF-κB (MWG-Biotech, Inc, High Point, NC), and AP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) double-stranded probes were kinased and used to determine binding activity. 4 μg nuclear extracts were incubated for 30 min at RT with 1 × 10^4 counts of probe in 20 μl binding buffer (20 mM Tris [pH 7.5], 0.1 M NaCl, 2 mM DTT, 1 mM EDTA, 1 mg/ml BSA, 0.1% NP-40, and 4% glycerol) containing 1 μg poly(dIdC) (Amersham Pharmacia Biotech). DNA-protein complexes were electrophoresed on 5% polyacrylamide gels in 0.5× TBE. The gels were then dried and autoradiographed. Where indicated, 1 μg GST, GST-IκBNS, or GST-IκBα was added to the thymic lysates and incubated 30 min on ice prior to addition of the probe.

Western Blot Analysis

Mice were untreated or injected i.v. with 24 μg VSV8 peptide for the times indicated in the figures. Total thymic lysates were prepared from N15 TCRtg thymocytes by lysing for 15 min in PBS/1 mM EDTA/0.5% NP40/1 mM PMSF/10 μg/ml aprotinin/5 μg/ml leupeptin/5 μg/ml pepstatin A. Nuclei were spun out and extracted in the same lysis buffer adjusted to 300 mM NaCl for 20 min. After spinning 15 min at maximum speed in an Eppendorf microfuge, the lysates were combined, protein concentration determined using a BCA assay (Pierce), and 20 μg of total protein run on 10% SDS/PAGE and analyzed by Western blot. For subcellular fractionation, thymocyte suspensions (5 × 10^7 cells per sample) were washed, resuspended in 250 μl NB (10 mM PIPES [pH 7.4]/10 mM KCl/2 mM $MgCl_2$/1 mM dithiothreitol/1 mM PMSF), allowed to swell 20 min, and Dounced (50 strokes). The suspension was layered over 30% (w/v) sucrose in NB and centrifuged at 800 g in a swinging bucket rotor for 30 min at 4°C. The nuclei were washed once in NB, boiled in 100 μl of 1× RSB and sonicated. 2 × 10^6 cell equivalents were applied to 10% SDS-PAGE and analyzed by Western blot. The anti-Lamin B antibody was from Santa Cruz, the anti-β-Actin antibody was from Sigma (St. Louis, MO), and the anti-IκBα antibody was from Abcam Ltd. (Cambridge, UK).

Protein Interaction Assays

Murine NF-κB family members p50 and p65, separately or together, were in vitro-translated in the presence of [^{35}S]cysteine/methionine (NEN Life Science Products, Boston, MA) using TNT-coupled reticulocyte lysate systems (Promega). In vitro-translated proteins were incubated with 5 μg of GST fusion proteins coupled to Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) for 2 hr at 4°C in PBS/0.1% Triton X-100/1 mM PMSF. After washing, associated proteins were analyzed by 10% SDS-PAGE followed by autoradiography.

Mice were injected i.v. with VSV8 peptide or left untreated and sacrificed at various time points. Single-cell suspensions and extracts of thymocytes were prepared as described above under RDA and EMSA, respectively. Protein concentrations were determined using the Micro BCA Protein Assay Reagent Kit (Pierce). Extracts were adjusted to 20 mM HEPES (pH 7.9)/150 mM NaCl/0.1% Triton X-100/1 mM PMSF (final volume 750 μl) and precleared with Glutathione Sepharose 4B beads for 1 hr at 4°C. Subsequently, extracts were incubated with GST, GST-IκBNS, and GST-IκBα beads for 1 hr at 4°C. Finally, beads were washed with 20 mM HEPES (pH 7.9)/150 mM NaCl/0.1% Triton X-100/1 mM PMSF, boiled in standard RSB, and analyzed by Western blot with the indicated antibodies (p52, p65, c-Rel, and RelB antibodies from Santa Cruz; p50 antibody from Stressgen, Victoria, BC).

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