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Overexpression of RelA in Transgenic Mouse Thymocytes: Specific Increase in Levels of the Inhibitor Protein IκBα

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RelA (p65) is one of the strongest activators of the Rel/NF-κB family. As a first step to elucidate the mechanisms that regulate its activity in vivo, we have generated transgenic mice overexpressing RelA in the thymus. Although the levels of RelA were significantly increased in thymocytes of transgenic mice, the overall NF-κB-binding activity in unstimulated cells was not augmented compared with that in control thymocytes. This could be explained by the dramatic increase of endogenous IκBα levels observed in RelA-overexpressing cells in both cytoplasmic and nuclear compartments. The *ikba* mRNA levels were not augmented by overexpressed RelA, but IκBα inhibitor was found to be stabilized through association with RelA. Although a fraction of RelA was associated with cytoplasmic p105, no changes in the precursor levels were observed. Upon stimulation of RelA-overexpressing thymocytes with phorbol 12-myristate 13-acetate and lectin (phytohemagglutinin), different κB-binding complexes, including RelA homodimers, were partially released from IκBα. Association of RelA with IκBα prevented complete degradation of the inhibitor. No effect of phorbol 12-myristate 13-acetate–lectin treatment was detected on RelA associated with p105. Our data indicate that cytoplasmic retention of overexpressed RelA by IκBα is the major in vivo mechanism controlling the potential excess of NF-κB activity in long-term RelA-overexpressing thymocytes.

The Rel/NF-κB family of transcription factors plays a central role as an early mediator of the immune and inflammatory responses (2, 25, 43). All Rel/NF-κB proteins share a highly conserved region of approximately 300 amino acids which mediates dimerization and DNA binding. The mammalian members of this family can be divided in two classes: one class includes the NF-κB1 (p105-p50) and NF-κB2 (p100-p52) proteins that are synthesized as inactive cytoplasmic precursors (p105 and p100) which upon processing generate the active subunits p50 and p52, respectively. The other class comprises proteins containing transcriptional activation domains in their C termini, RelA (p65), RelB, and c-Rel (6, 7, 22, 25, 26, 43, 52). Proteins of both classes can form homodimers and heterodimers that bind to specific κB sites and differ in their transactivating capacities (8, 27, 28, 34–36, 46, 50, 55, 58, 61, 65, 71). The Rel/NF-κB members are differentially expressed in lymphoid tissues (10, 11, 72), and recent studies using knockout mice clearly demonstrate that p50 and RelB have distinct functions in hematopoietic cells (64, 73).

The different NF-κB activities are modulated by interaction with the IκB family of inhibitory proteins, which contain multiple copies of ankyrin repeats (3, 23, 52). The current model suggests that in unstimulated cells, NF-κB dimers remain in the cytoplasm as inactive complexes through association with IκBα that masks their nuclear localization signals. Upon stimulation by a variety of agents, IκBα is inactivated, allowing NF-κB dimers to translocate into the nucleus, where they bind to κB sites and regulate gene transcription. Recent evidence indicates that the inactivation of IκBα implies phosphorylation and degradation to produce NF-κB activation (4, 9, 12, 19, 20, 29, 41, 54, 56, 66, 70). The precursors p105 and p100 show significant homology to the IκB molecules in their C-terminal regions, and it has been suggested that they also trap NF-κB complexes in the cytoplasm (18, 30, 32, 47, 51, 57, 60, 67).

The RelA subunit is a strong transactivator, and it has been described to be subject to the control of the precursors p105 and p100 and the inhibitor IκBα (3, 23, 67). In this report we have examined in vivo the interactions between RelA and the NF-κB–IκB complexes occurring in the thymus, one of the primary lymphoid organs in adult mice. In addition, by using transgenic mice that overexpress RelA in the thymus, we have also investigated the regulatory mechanisms that exist in thymocytes to counteract the excess of NF-κB activity. Our data indicate that in long-term RelA-overexpressing thymocytes the potential excess of NF-κB activity is counteracted by a dramatic increase in IκBα, mainly due to its increased stability by the association with RelA.

MATERIALS AND METHODS

Transgene construction and generation of transgenic mice. Cloning procedures were performed essentially as described elsewhere (59). The mouse *rel*A cDNA (bp1 to 2166) was cloned into the *Bam*HI site of the p*lck*-GH vector. This vector contains a 3.2-kb fragment of the mouse *lck* promoter and a 2.1-kb fragment of the human growth hormone (GH) gene which provides introns and the polyadenylation signal sequence. A 2.1-kb fragment of the 3′ locus control region of the human CD2 gene was subsequently inserted downstream of the human GH sequence to obtain copy number-dependent and insertion site-independent levels of expression (24, 37). The construct was digested with *Sac*II, and separation of the transgene from vector sequences was accomplished by zonal sucrose gradient centrifugation as described previously (45).

The transgene (1 to 5 ng/μl) was microinjected into (C57BL/6J × DBA/2)F_1 (The Jackson Laboratory) eggs according to standard methodology. Microinjected eggs were transferred into oviducts of ICR (Sprague-Dawley) foster mothers as described elsewhere (31). Tail DNA from 10-day-old animals was prepared as previously described (42). DNA was amplified by PCR analysis (1 min at 95°C, 1.5 min at 55°C, and 1 min at 72°C) using the following primers recognizing human GH sequences: 5′-TTTGGGGTTCTGAATGTGAG-3′ and 5′-AGGC ACTGCCCTCTTGAAGC-3′. The endogenous β-TSH gene, primers 5′-GTA ACTCACTCATGCAAAGT-3′ and 5′TCCTCAAAGATGCTCATTAG-3′, was used as an internal control. The results shown in this report were obtained with transgenic line 27, and identical results were obtained for transgenic line 18 (data not shown).

Northern (RNA) blot assays. Total RNA from 4-week-old mouse thymocytes

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was isolated with the RNA isolation solvent RNAzol (Cinna/Biotecx). Poly(A)$^+$ mRNA was prepared with oligotex-dT resin (Qiagen). RNA samples [20 μg of total RNA or 3 μg of poly(A)$^+$ mRNA] were denatured in 50% formamide–2.2 M formaldehyde at 65°C for 10 min and electrophoresed through a 0.8% agarose gel containing 2.2 M formaldehyde. The gel was blotted onto Nytran membranes (Schleicher & Schuell) and hybridized with the following α-^{32}P-labeled probes: a 1.6-kb *Xba*I-*Eco*RI fragment from *rel*A cDNA (53) and a 0.9-kb *Xba*I-*Kpn*I fragment from *ikba* cDNA (69).

Western blot (immunoblot) assays. Whole-cell extracts from 4-week-old mouse thymuses were prepared as described elsewhere (39). Cytoplasmic and nuclear fractions from thymocytes were prepared according to the methods in reference 62. Aliquots of total-protein extract (60 μg) or of cytoplasmic and nuclear extracts (20 μg) were boiled in Laemmli buffer and run overnight on a 12.5% acrylamide–bisacrylamide (200:1) gel at 12 mA. The proteins were efficiently transferred onto nitrocellulose membranes as assessed by Ponceau S staining. The membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS)–0.1% Tween 20 for at least 2 h at room temperature, washed three times for 10 min each in PBS–0.1% Tween 20, and incubated with the specific antisera indicated in the figures (diluted 1:1,000 in PBS–0.1% Tween 20 containing 3% nonfat dry milk) for 1 to 2 h at room temperature. After the washes, the membranes were incubated with peroxidase-conjugated swine anti-rabbit immunoglobulin G (1:10,000 dilution in PBS–0.1% Tween 20 containing 1% nonfat dry milk) for 45 min at room temperature, washed again, and then analyzed by using the enhanced chemiluminescence system according to the manufacturer's recommendations (Amersham). For an antiserum description, see reference 72. To reprobe the membranes with different antisera, these were stripped in 62.5 mM Tris-HCl (pH 6.7)–2% sodium dodecyl sulfate (SDS)–100 mM β-mercaptoethanol at 55°C for 30 min. Possible contamination when preparing the nuclear and cytoplasmic extracts was checked by incubating the membranes with an antiserum specific for the cytosolic enzyme lactate dehydrogenase.

Cell culture. Thymic single-cell suspensions were prepared according to standard procedures (14) and then grown at 37°C in RPMI 1640 containing 10% heat-inactivated fetal calf serum.

Cell labeling, lysis, and immunoprecipitation. Isolated thymocytes were labeled for 2 h with 500 μCi of [^{35}S]methionine (1,000 Ci/mmol) in Dulbecco modified Eagle medium lacking methionine and containing 10% dialyzed fetal calf serum. The labeling medium was removed, and the cells were washed with cold PBS and lysed on ice by adding radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.5], 0.5% Nonidet P-40, 150 mM NaCl). Cell lysates were first cleared with preimmune serum (3 μl) for 3 h, then immunoprecipitated with specific antisera (3 μl) overnight, and incubated with 40 μl of protein A–Sepharose CL-4B (Pharmacia) for 2 to 3 h on a roller system at 4°C. The supernatant was kept for subsequent immunoprecipitation, and the beads were boiled for 10 min in denaturing buffer (50 mM Tris-HCl [pH 8.0], 0.5% SDS, and 70 mM β-mercaptoethanol), then fourfold diluted, and reprecipitated with specific antiserum. The immunocomplexes were washed twice with buffer A (0.2% Nonidet P-40, 10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 2 mM EDTA), once with buffer B (0.2% Nonidet P-40, 10 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 2 mM EDTA), and once with buffer C (10 mM Tris-HCl, pH 7.5). Samples were boiled in Laemmli buffer and run overnight on a 12.5% acrylamide–bisacrylamide (200:1) gel at 12 mA. Fixed gels were incubated with Entensify (DuPont-NEN). Gels were dried and exposed to Kodak X-Omat AR film at −70°C for 3 days.

EMSAs. The palindromic κB site used for electrophoretic mobility shift assays (EMSAs) has been previously described (17). Cytoplasmic and nuclear extracts (3 μg) were incubated with 20,000 cpm of labeled probe and 3 μg of poly (dI-dC) in buffer containing 20 mM *N*-2-hydroyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 17% glycerol in a 25-μl final volume for 15 min at 20°C. Complexes were separated on 5.5% native polyacrylamide gels run in 0.25× Tris-borate-EDTA buffer, dried, and exposed to Kodak X-Omat AR film at −70°C.

RESULTS

Generation of transgenic animals overexpressing RelA in the thymus. To study the posttranscriptional regulation of the NF-κB activity in T cells, we have generated a transgenic mouse model that places *rel*A under the control of the mouse *lck* proximal promoter (Fig. 1A). This promoter drives transcription of the transgene to all thymocyte subsets beginning at the earliest stages of T-cell development as previously described (1). The human GH gene sequences were included to provide introns and polyadenylation recognition sequences, and the 3′ locus control region of the human CD2 gene was included to confer copy number-dependent and position-independent expression to the transgene and extend its expression

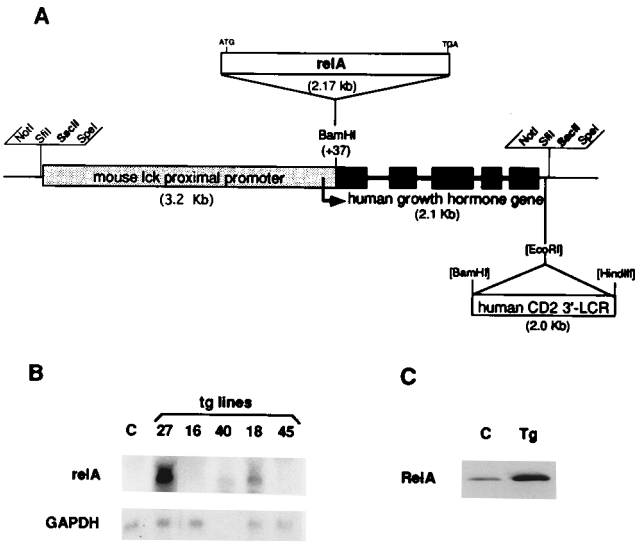


FIG. 1. Expression of the transgene in the thymuses of transgenic mice. (A) Scheme of the transgene. The complete mouse cDNA sequence of *rel*A (2.17 kb) was placed under control of the mouse *lck* proximal promoter (3.2 kb); the human GH gene sequences (2.1 kb) were added to confer stability to the transcripts, and the 3′-flanking sequences (3′ locus control region [3′-LCR]) of the human CD2 gene (2.0 kb) were added for copy number-dependent, position-independent expression of the transgene. (B) Northern blot analysis with total thymus RNA from control (C) and different transgenic (tg) lines hybridized with α-^{32}P-labeled specific probes for *rel*A and *GAPDH* genes. (C) Western blot analysis with thymic whole-cell protein extracts (60 μg) from control and transgenic mice, line 27. The membrane was incubated with specific antiserum against RelA.

to peripheral T cells (24). Several independent transgenic mouse lines were generated, and thymic expression of the *rel*A transgene was determined by Northern blot analysis (Fig. 1B). The highest level of transgene mRNA was detected in the thymuses of lines 27 and 18. This high *rel*A mRNA level correlated with the overexpression of a 65-kDa protein indistinguishable in size from the endogenous RelA, as shown by Western blot assays using specific antiserum (Fig. 1C).

Overexpression of RelA in the thymus does not result in gross alteration of T-cell development. In situ hybridization studies had previously shown that endogenous *rel*A mRNA is expressed in the thymic cortex of 17-day embryos and adult mice (72), suggesting that RelA plays an important role in T-cell development. Thymuses of RelA-transgenic mice were normal in size, and histological sections showed no gross morphological alterations of the thymic medulla and cortex. Analysis of thymocyte cells suspensions from 3-week-old transgenic and control mice by flow cytometry revealed no significant alterations in the T-cell subpopulations of transgenic animals as defined by CD4 and CD8 markers (data not shown). Thus, overexpression of RelA protein in thymocytes does not significantly affect their development. In addition, RelA-transgenic animals did not show an increased suceptibility to infections as compared with control littermates. These observations indicate that overexpression of RelA in thymocytes does not result in an aberrant maturation of T cells that takes place in secondary lymphoid tissues.

Overexpression of RelA increases endogenous IκBα levels in thymocytes. To investigate whether the κB-binding activity was altered in thymuses of transgenic mice as a consequence of RelA overexpression, we analyzed thymocyte protein extracts by EMSAs using a palindromic κB site (Fig. 2A). Cytoplasmic extracts from thymocytes of control and transgenic mice

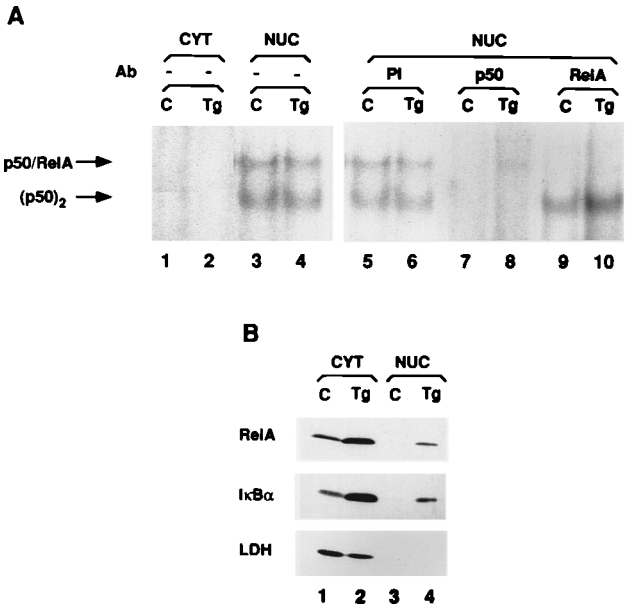


FIG. 2. Effects of RelA overexpression on κB-binding activity and IκBα protein levels. (A) Overexpression of RelA does not increase κB-binding activity in thymocytes. The EMSA shows the κB-binding activity of cytoplasmic (CYT) and nuclear (NUC) extracts from control (C) and transgenic (Tg) thymocytes to the palindromic κB-binding site. The addition of specific antisera (Ab) against p50 and RelA is indicated at the top. κB-binding complexes are indicated at the left. PI, preimmune serum. (B) The inhibitor protein IκBα is increased in vivo by overexpressed RelA. Western blot analysis of cytoplasmic and nuclear extracts (20 μg) from thymocytes of control and transgenic mice is shown. Specific antisera against RelA, IκBα, and lactate dehydrogenase (LDH) were used.

showed no binding activity (lanes 1 and 2), while two complexes were detected in the corresponding nuclear fractions (lanes 3 and 4). Anti-p50 antiserum eliminated both κB-binding activities (lanes 7 and 8), while anti-RelA antiserum removed only the more slowly migrating complex (lanes 9 and 10), indicating that it consisted of p50-RelA heterodimers while the faster-migrating complex consisted of p50 homodimers. The fact that the κB-binding activity in thymocytes from transgenic mice was not significantly altered compared with that in thymocytes from normal mice suggests that the DNA binding activity of the overexpressed RelA is inhibited.

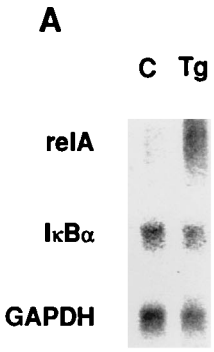
One of the major mechanisms to control the DNA binding activity of NF-κB complexes is to sequester them in the cytoplasm through the association with IκBα (5). Therefore, we determined the subcellular localization of RelA in thymocytes from control and transgenic mice by Western blot assays (Fig. 2B). In order to rule out a possible cross-contamination during the subcellular fractionation procedure, we used the cytoplasmic enzyme lactate dehydrogenase as a control. In thymocytes from both control and transgenic mice RelA was localized predominantly in the cytoplasm (lanes 1 and 2), but RelA was also readily detected in the nuclear fraction of thymocytes from transgenic mice (compare lanes 3 and 4). In control mice, the presence of nuclear RelA is evident only after a longer exposure of the blot (not shown).

It has been previously reported that RelA-containing complexes induce the expression of IκBα, creating an autoregulatory loop in which increased synthesis of the inhibitor serves to restore the uninduced state of the cell (9, 12, 16, 63, 66). Therefore, we investigated whether the levels of IκBα were altered in RelA-overexpressing thymocytes (Fig. 2B). In thymocytes of transgenic mice, the protein levels of IκBα were markedly increased with respect to those in thymocytes of control animals (compare lanes 1 and 2) and paralleled the subcellular localization of the overexpressed RelA (lanes 2 and 4). Interestingly, the relative increase of IκBα levels from control to transgenic mice is comparable to that of RelA. The fact that the κB-binding activity was not increased in nuclear fractions of RelA-overexpressing thymocytes compared to control cells, despite the higher levels of RelA in these fractions (Fig. 2B, compare lanes 3 and 4), could be due to the presence of nuclear IκBα. The fact that lactate dehydrogenase was undetectable in the nuclear fraction strongly argues against a cytoplasmic contamination. Immunoprecipitations of nuclear extracts of thymocytes from transgenic mice followed by Western blot analysis using specific antisera against RelA and IκBα demonstrated that indeed RelA was associated with IκBα (data not shown).

Recent results indicate that the transcriptional regulation of some *rel*/*nfkb* genes, similarly to that of *ikba*, is κB mediated (13, 44, 68). This prompted us to determine whether the overexpression of RelA in thymocytes affected the expression of other members of this family. Western blot analysis revealed that long-term overexpression of RelA in thymocytes was not accompanied by changes in the expression levels of c-Rel, RelB, NF-κB1, or NF-κB2 (see Fig. 4, left panel; also data not shown), underlining the specificity of the mechanisms resulting in increased IκBα levels.

***ikba* gene expression levels are not increased in thymocytes of RelA-transgenic mice.** Previous transfection studies performed in transformed cell lines have described two different mechanisms by which RelA regulates IκBα. One of them involves transcriptional induction of the *ikba* gene through the κB sites present in its promoter region (9, 12, 16, 63, 66), and the other involves a posttranslational mechanism that stabilizes the inhibitor through protein-protein interaction (56, 63). To determine if the *ikba* gene is induced by chronic RelA overexpression in vivo, poly(A)$^+$ mRNA from thymocytes of control and transgenic animals were prepared and Northern blot assays were performed using labeled probes specific for *rel*A and *ikba* (Fig. 3A). The *rel*A mRNA levels were 5- to 10-fold increased in the transgenic mouse compared with those in the control, while *ikba* mRNA levels were similar in both. These results indicate that in unstimulated thymocytes, long-term overexpression of RelA does not result in increased *ikba* mRNA levels.

Association of RelA and IκBα in thymocytes. Our results indicate that the observed increase in IκBα protein levels in RelA-overexpressing thymocytes is not caused by an induction of the *ikba* gene. Recent studies showed that the association of IκBα with NF-κB subunits significantly extends its half-life (56, 63). These findings prompted us to investigate whether the increased IκBα levels observed in these cells were due to stabilization of the protein through interaction with the overexpressed RelA. Isolated thymocytes of control and transgenic mice were labeled for 2 h in a medium containing [^{35}S]methionine and lysed under nondenaturing conditions, and cytoplasmic extracts were immunoprecipitated with RelA antiserum (Fig. 3B). The immunoprecipitate was denatured and then reprecipitated first with RelA (lanes 1, 2) and subsequently with IκBα (lanes 3 and 4) antisera to determine the amount of inhibitor present in RelA-containing immunocomplexes. To estimate the amount of inhibitor that was not associated with RelA, the supernatant of the first immunoprecipitation step was reprecipitated with IκBα antiserum (lanes 5 and 6). These data indicate that in unstimulated thymocytes of transgenic mice, more than 90% of the cytoplasmic IκBα is associated with RelA. These findings suggest a tightly regulated mecha-



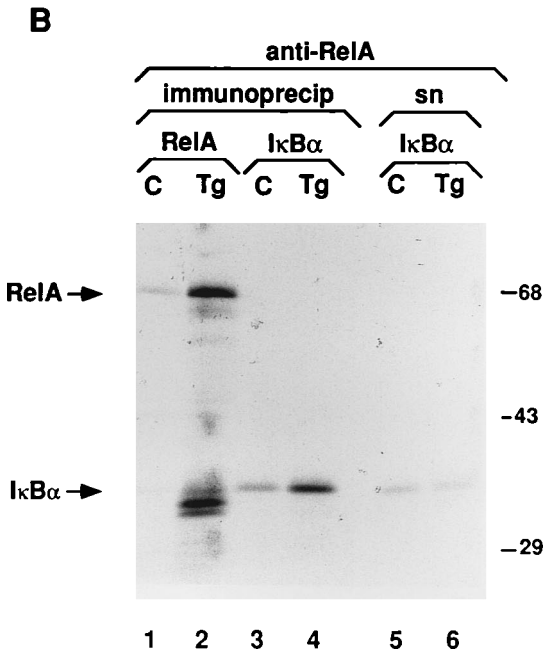


FIG. 3. (A) *ikba* mRNA levels are not increased in RelA-overexpressing thymocytes. Poly(A)$^+$ mRNA was isolated from thymocytes of control (C) and transgenic (Tg) mice, run in a 0.8% agarose gel (3 μg per lane), transferred to a Nytran membrane, and hybridized with α-^{32}P-labeled specific probes for *rel*A, *ikba*, and *GAPDH* genes. (B) Association of RelA and IκBα in thymocytes. Thymocytes from control and transgenic mice were labeled for 2 h in the presence of [^{35}S]methionine, lysed under nondenaturing conditions, and immunoprecipitated as described in Materials and Methods. Immunoprecipitation was done with specific RelA antiserum (anti-RelA). The immunoprecipitate (immunoprecip) was boiled in denaturing buffer, fourfold diluted, and then reprecipitated first with anti-RelA (lanes 1 and 2) and subsequently with anti-IκBα (lanes 3 and 4) antiserum to determine the amount of inhibitor present in RelA-containing immunocomplexes. The supernatant (sn) was reprecipitated with specific IκBα antiserum (IκBα) to estimate the amount of inhibitor that is not associated with RelA (lanes 5 and 6). The low-molecular-weight band observed in lane 2 might represent a degradation product of RelA. RelA and IκBα are indicated by arrows at the left, and the molecular weight markers (in thousands) are shown at the right.

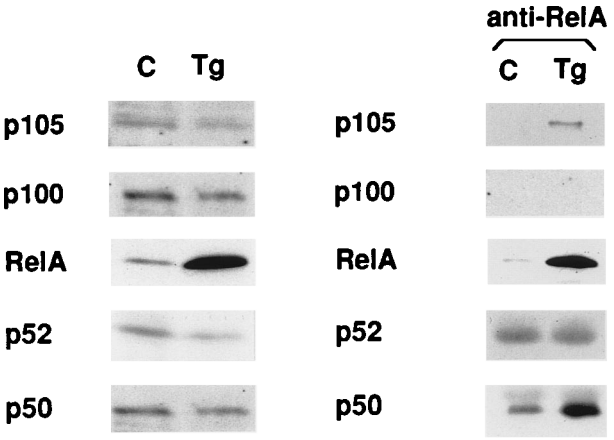


FIG. 4. (Left) RelA overexpression does not affect p105-p50 and p100-p52 levels in thymocytes. Western blots of cytoplasmic extracts from control (C) and transgenic (Tg) mice used p105-p50, p100-p52, and RelA antisera. (Right) RelA associates with p105 but not p100 in thymocytes. Cytoplasmic extracts from control and transgenic mice were immunoprecipitated under nondenaturing conditions with RelA antiserum (anti-RelA), and the immunocomplexes were analyzed by Western blotting.

nism by which the excess RelA is sequestered in the cytoplasm through association with IκBα, which is stabilized by this protein-protein interaction.

Overexpressed RelA interacts with p105 but does not increase p105 protein levels. Recent reports have indicated that RelA can associate with ankyrin motif-rich cytoplasmic proteins such as p105 and p100, precursors of the p50 and p52 NF-κB subunits, respectively. It has been postulated that the interactions with p100 and p105 are another important mechanism by which RelA activity is controlled (47, 51, 57, 67). Therefore, it was of interest to determine whether this interaction occurs in thymocytes and to examine the relevance of this association in the overall control of RelA activity in RelA-overexpressing cells. Cytoplasmic extracts from thymocytes of control and transgenic mice were prepared and immunoprecipitated under nondenaturing conditions with RelA antiserum. The immunocomplexes were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with specific antisera against NF-κB1 (p105-p50), NF-κB2 (p100-p52), and RelA (Fig. 4, right panel). RelA is shown as an internal control of the immunoprecipitated protein in transgenic and control mice. Although RelA was associated with p52, the precursor p100 was not detected under those conditions. Surprisingly, the amounts of p52 associated with RelA were similar in control and transgenic mice despite the increased levels of RelA present in transgenic mice. On the other hand, more p105 and p50 were associated with RelA in thymocytes from transgenic mice. However, the total amounts of p100-p52 as well as p105-p50 were comparable in control and transgenic mice as seen by Western blot assays using cytoplasmic (Fig. 4, left panel) and nuclear (not shown) extracts. Therefore, the increase in RelA-p105 and RelA-p50 complexes is most likely a direct consequence of the increased levels of RelA. This demonstrates that RelA in thymocytes is associated with the inhibitor p105 and this interaction, in contrast to that with IκBα, does not affect p105 protein levels.

RelA-IκBα association prevents complete degradation of the inhibitor upon stimulation of RelA-overexpressing thymocytes. In unstimulated thymocytes from transgenic mice, we have not observed an increase in the κB-binding activity despite RelA overexpression. As demonstrated above, this is due mainly to the retention of RelA-containing complexes in the cytoplasm by IκBα and to a lesser extent by p105. It has been previously shown that the combination of phorbol 12-myristate 13-acetate (PMA) and lectin (phytohemagglutinin [PHA]) strongly induces NF-κB activity in T cells (2, 25). Therefore, we examined by EMSA the nuclear κB-binding activity in control and RelA-overexpressing thymocytes stimulated for different

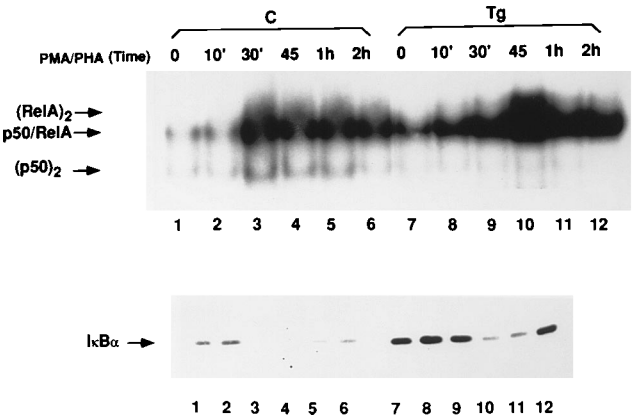


FIG. 5. Different NF-κB complexes are released from IκBα upon stimulation of control and transgenic thymocytes. Thymocytes were isolated and cultured for different periods of time (as indicated) in medium containing PMA (20 ng/ml) plus PHA (1 μg/ml). Cytoplasmic and nuclear extracts were prepared as described in Materials and Methods. (Top) The NF-κB-binding activity in the nuclear fraction of thymocytes from control (C) and transgenic (Tg) animals was tested by EMSA. The specific κB-binding complexes are indicated at the left. (Bottom) The fate of IκBα in the cytoplasmic fraction of thymocytes from control (lanes 1 to 6) and transgenic (lanes 7 to 12) mice was analyzed by Western blotting.

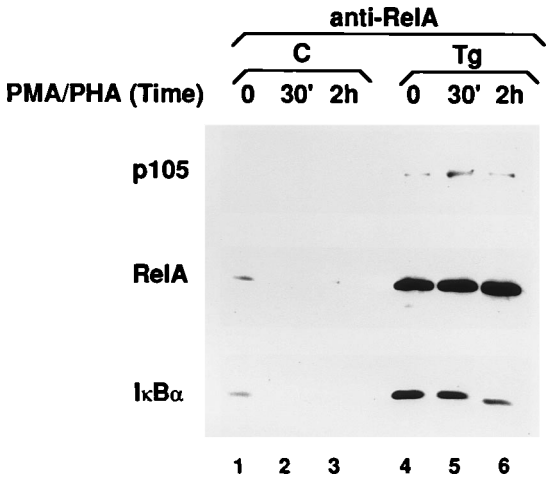


FIG. 6. In PMA-PHA-stimulated RelA-overexpressing thymocytes a large fraction of RelA remains in the cytoplasm associated with IκBα. Thymocytes from control (C) and transgenic (Tg) mice were incubated with PMA-PHA for different periods of time as indicated in the legend to Fig. 5. Cytoplasmic extracts were immunoprecipitated under nondenaturing conditions with RelA antiserum (anti-RelA), and the immunocomplexes were denatured and run in an SDS–12.5% PAGE. Western blot assays were performed using p105, RelA, and IκBα antisera as indicated.

periods of time with PMA-PHA (Fig. 5, top panel). In control thymocytes, a dramatic increase in κB-binding activity was observed after 30 min of stimulation; this activity was composed of p50 homodimers and p50-RelA heterodimers (lanes 1 to 6). In RelA-overexpressing thymocytes, a similar rapid induction of κB-binding activity was observed, but in addition, a new binding complex was detected (lanes 7 to 12). By supershift assays, we have identified this complex as RelA homodimer (not shown). It is important to note that the level of p50 DNA binding observed in RelA-overexpressing thymocytes following PMA-PHA treatment is lower than that in control thymocytes, reflecting a decrease in the amount of p50 homodimers due to the formation of p50-RelA heterodimers. Considering the amount of RelA present in thymocytes from transgenic mice, we had expected to observe a significantly higher increase in the overall κB-binding activity following PMA-PHA stimulation of the cells. A possible explanation is that not all RelA is dissociated from IκBα in RelA-overexpressing thymocytes. To investigate this, we studied the fate of IκBα in the cytoplasmic fraction of PMA-PHA-stimulated thymocytes (Fig. 5, bottom panel). After treatment of control thymocytes, IκBα was rapidly degraded and undetectable after 10 min (lanes 1–4); thereafter, IκBα levels increased and were detectable at significant amounts at 2 h (lanes 5 and 6). The decrease in the inhibitor protein levels correlated with the increased binding of p50-RelA heterodimers and p50 homodimers in the nuclear fraction (top panel, lanes 1 to 6). However, in stimulated thymocytes from transgenic mice the kinetic of IκBα degradation was delayed compared with that in thymocytes from control mice, and the inhibitor was not completely degraded even after long incubation periods with the PMA-PHA stimuli (Fig. 5, bottom panel, lanes 7 to 12). This observation suggests that a fraction of IκBα remains associated with RelA upon stimulation, thus explaining the partial release of RelA-containing complexes in RelA-transgenic mice. Immunoprecipitation of cytoplasmic fractions from control thymocytes by using RelA antiserum demonstrated that after 30 min of stimulation, RelA and the associated IκBα were undetectable in these fractions (Fig. 6, middle and bottom panels, lanes 1 to 3). In contrast, in RelA-overexpressing thymocytes a large proportion of RelA and associated IκBα remained in the cytoplasm following cellular stimulation (Fig. 6, middle and bottom panels, lanes 4 to 6). We also investigated whether the p105-RelA association was affected in stimulated thymocytes from control and transgenic mice. In both cases, no significant changes in the amount of p105 associated with RelA were observed within the first 2 h of stimulation (Fig. 6, top panel).

DISCUSSION

In order to examine in vivo the potential effects of RelA chronic expression, we have generated transgenic mice that overexpress RelA in the thymus. We believe that this system represents an advantage over using T-cell lines and transient-transfection studies, as it is in the more natural context of a living organism. This study focuses on the molecular mechanisms regulating RelA activity in thymocytes from control and RelA-transgenic mice. Our data demonstrate that overexpression of RelA in thymocytes does not produce an augmentation of NF-κB activity. This is due to an increase in the cellular levels of the inhibitory protein IκBα which ensure the retention of RelA complexes in the cytoplasm. The dramatic increase in IκBα is due mainly to its stabilization through its association with RelA and not to increased *ikba* mRNA levels.

Overexpression of RelA specifically increases IκBα levels. We have shown that the high levels of RelA overexpressed in thymocytes of transgenic mice did not significantly increase the nuclear κB-binding activity in these cells. Our data indicate that overexpressed RelA was found mainly in the cytoplasm of unstimulated thymocytes associated with the inhibitor IκBα and to a lesser extent with p105. A number of reports have shown that the genes encoding IκBα and p105 are transcriptionally upregulated by NF-κB complexes (9, 12, 13, 16, 38, 47, 48, 63, 66, 68). However, our studies demonstrate that in unstimulated thymocytes overexpressing RelA, the increased levels of IκBα protein were not accompanied by a transcriptional induction of the *ikba* gene (Fig. 3A). The observed increase of IκBα in RelA-overexpressing thymocytes is due to the associ-

ation of IκBα with RelA which extends the half-life of the inhibitor (data not shown). Therefore, the regulation of *ikba* by RelA complexes seems to play a minor role as a mechanism controlling NF-κB activity in thymocytes chronically expressing RelA. However, we cannot rule out the possibility that the *ikba* gene is transiently induced in thymocytes of transgenic mice at early developmental stages when the *lck* promoter becomes activated for the first time. Remarkably, other transactivator proteins of the Rel/NF-κB family, such as RelB, did not increase IκBα protein levels when overexpressed in thymocytes (71a). These results strongly argue in favor of a specific effect of RelA on the inhibitor IκBα.

In contrast to what was observed for IκBα, RelA overexpression in thymocytes did not affect the total protein levels of the cytoplasmic inhibitor p105. Since p105 was found associated with RelA, this result indicates that long-term overexpression of RelA in thymocytes affects neither the expression of the *nfkb1* gene nor the stability or processing of p105. We conclude that IκBα is the major regulatory inhibitor of RelA activity in thymocytes, while the cytoplasmic inhibitor p105 seems to play a secondary role by sequestering a fraction of the overexpressed RelA.

A large fraction of overexpressed RelA remains in the cytoplasm even after thymocyte stimulation. Our results demonstrated that PMA-PHA treatment of control thymocytes results in rapid degradation of IκBα, which coincides with NF-κB translocation to the nucleus. After 1 to 2 h IκBα becomes detectable again, probably because of its enhanced de novo synthesis (Fig. 5, lanes 5 and 6). In contrast, in RelA-overexpressing thymocytes, although the amount of inhibitor significantly decreased within 30 to 45 min of stimulation, it was still readily detectable (Fig. 5, lanes 9 and 10) and was comparable to the level observed in untreated control thymocytes (Fig. 5, compare lanes 1 and 10). This is in agreement with and would explain the large fraction of RelA-containing complexes remaining in the cytoplasm of stimulated thymocytes from transgenic mice (Fig. 6). The fact that IκBα is not rapidly degraded in RelA-overexpressing thymocytes upon stimulation may be explained in at least two ways: (i) The machinery involved in the phosphorylation and/or degradation of IκBα is limiting and therefore not able to rapidly process the large amount of IκBα present in RelA-overexpressing cells, or (ii) RelA complexes are initially dissociated from IκBα upon stimulation, but because of the vast excess of both proteins they quickly reassociate, thus exposing only a minor fraction of IκBα molecules to modification and degradation.

In our studies we did not observe significant changes in the amount of p105 associated with RelA following thymocyte stimulation, indicating that the association between p105 and RelA might be affected by signals different from those controlling the association of RelA with IκBα. These data point to the existence of different pools of RelA-containing complexes regulated by different stimuli.

IκBα is present in thymic nuclei of RelA-transgenic mice. It has been previously demonstrated that IκBα can localize to the nucleus (15, 33, 49, 74); however, the physiological significance of this remains controversial. We have detected IκBα in the nuclear fraction of thymocytes from RelA-transgenic mice. The nuclear localization of the inhibitor coincides with increased RelA levels in the same cellular compartment (Fig. 2B). If functional, nuclear IκBα should be able to associate with nuclear RelA and inhibit the latter's DNA binding activity. Immunoprecipitation and Western blot analysis demonstrated that IκBα was associated with RelA in nuclear extracts from RelA-overexpressing thymocytes. Accordingly, we did not observe any increase in the nuclear NF-κB-binding activity in unstimulated cells, in spite of increased RelA protein in the nucleus. However, when the same nuclear extracts were incubated in the presence of 0.8% deoxycholate, a detergent known to release NF-κB complexes from IκBα in vitro (2), a dramatic increase of κB-binding activity occurred (data not shown). These observations strongly suggest a direct role of the inhibitor IκBα in the nucleus, preventing uncontrolled NF-κB-binding activity.

RelA homodimers exist as inducible complexes in thymocytes from RelA-transgenic mice. We have identified the nuclear NF-κB-binding activity in stimulated thymocytes from control and RelA-transgenic mice as p50 homodimers and p50-RelA heterodimers. Recent evidence indicates that enhanced binding of p50 homodimers after T-cell stimulation might be due to postranslational modification of this protein (40). In RelA-transgenic mice, another binding complex identified as a RelA homodimer by supershift assays (data not shown) was detectable upon thymocyte stimulation (Fig. 5, top panel). This indicates that RelA homodimers represent an inducible κB-binding activity in the cytoplasm that translocates to the nucleus and binds DNA after cellular stimulation. Earlier studies have described the appearance of RelA-RelA κB-binding complexes in the human Jurkat T-cell line after stimulation (21). As in that report, we point out that detection of these complexes is largely influenced by the experimental conditions used. We observed that the use of lower salt concentrations in the binding reaction favors the binding of RelA homodimers while diminishing that of p50 homodimers. Although the RelA homodimers represent a relatively small fraction of the binding complexes, this κB-binding activity might be relevant, as prior transfection studies have indicated that RelA homodimers can specifically stimulate transcription from certain enhancer motifs (36).

The generation of transgenic mice overexpressing different members of the Rel/NF-κB family together with the generation of mice strains deficient in these proteins (64, 73) will be of great help for understanding the biological role of this family of transcription factors.

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