IκBα Overexpression Delays Tumor Formation in v-rel Transgenic Mice
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Summary
We have previously shown that transgenic mice expressing the oncoprotein v-R el under the control of a T cell–specific promoter develop T cell lymphomas. Tumor formation was correlated with the presence of p50/v-R el and v-R el/v-R el nuclear κB-binding activity. Since experimental evidence has led to the suggestion of a potential tumor suppressor activity for IκBα, we have studied the role of IκBα in the transforming activity of v-R el by overexpressing IκBα in v-rel transgenic mice. Overexpression of IκBα in v-rel transgenic mice resulted in an extended survival, and the development of cutaneous T cell lymphomas of CD8+CD4− phenotype. These phenotypic alterations were associated with a dramatic reduction of p50/v-R el, but not v-R el/v-R el nuclear DNA binding activity and an increased expression of the intercellular adhesion molecule 1. Our results indicate that v-R el homodimers are active in transformation and that the capacity of v-R el-containing complexes to escape the inhibitory effect of IκBα may be a key element in its transforming capability.

The oncogene v-rel was originally identified as the transforming component of the avian retrovirus reticuloendotheliosis virus. v-R el induces neoplastic disease in birds, and compared to c-R el, is missing 2 NH2-terminal and 118 COOH-terminal amino acids, and has several internal changes (1–5). v-R el is a member of the Rel/NF-κB family of eukaryotic transcription factors, which includes c-R el, R elA (p65), R elB, NF-κB1 (p50/p105), NF-κB2 (p52/p100), and the Drosophila proteins Dorsal, dorsal-related immunity factor, and R elish (6–12).

The finding that v-R el and the corresponding protooncogene c-R el are members of the Rel/NF-κB transcription factor family led to the suggestion that transformation resulted from v-R el–induced changes in gene expression (1–5). R el/NF-κB proteins are related through an ~300-amino acid NH2-terminal region known as the Rel homology domain (R HD)1, which contains sequences important for dimerization, DNA binding, inhibitor binding, and nuclear localization. The activity of R el/NF-κB complexes is modulated by their interaction with the IκB family of inhibitors, which contain ankyrin repeats. In unstimulated cells, R el/NF-κB dimers remain in the cytoplasm as inactive complexes through association with IκB molecules that mask their nuclear localization signals. A wide variety of stimuli result in the rapid phosphorylation and degradation of IκB molecules and nuclear translocation of Rel/NF-κB complexes (6–16).

The mechanism by which v-R el induces oncogenic transformation is not clear, and it was originally believed that v-R el could only transform avian cells (1–5). Recently, we demonstrated that v-R el also has the capacity of transforming mammalian cells in vivo. Transgenic mice expressing v-R el in thymocytes develop T cell lymphomas with very poor prognosis. In tumor cells from v-R el transgenic mice, there are two major DNA-binding complexes containing v-R el homodimers and p50/v-R el heterodimers. However, when v-rel transgenic mice were crossed with p50-deficient animals, T cell leukemia appeared at an earlier stage, suggesting that the v-R el homodimer is the essential transforming complex (17).

In this report we address the question of whether overexpression of the inhibitory IκBα protein, which has been suggested to have tumor suppressor activity (18), can reverse the transforming activity of v-R el in v-rel transgenic mice. Overexpression of IκBα extended the survival of v-rel transgenic mice and reduced the severity of lymphomas. Surprisingly, IκBα overexpression resulted in a change in the clinical expression of the disease with an expansion of CD8+CD4− T cells in peripheral tissues. These T cell changes were associated with increased levels in the expression of the intercellular adhesion molecule 1 (ICAM-1),...
increased dermoptosis and the development of cutaneous lymphoma. T cells from v-rel/ikba double transgenic mice presented a dramatic reduction of p50/v-Rel but not of v-R el/v-R el nuclear DNA-binding activity. Our results indicate that v-R el homodimers are active in transformation and that v-R el containing complexes have an intrinsic capability to escape the inhibitory effect of IkBα. We postulate that variations in the clinical expression of related lymphoid malignancies may reflect subtle changes in the nuclear composition and interplay among different REL/ NF-κB and IκB molecules.

Materials and Methods

Plasmid Construction and Generation of Transgenic Mice. The generation of v-rel transgenic mice has been previously described (17). A detailed description of the generation and characterization of ikba transgenic mice will be reported elsewhere (Perez, P., unpublished observations). Screening of IκBα transgenic mice was performed as described (19), and line 1 was selected for its high level of expression as determined by immunoblot analysis using a mouse monoclonal IκBα antibody. To generate double v-rel/ikba transgenic mice, ikba transgenic mice were bred to homozygocity and crossed with heterozygote v-rel transgenic animals. The mice obtained from these intercrosses were screened by PCR using a pair of specific v-rel oligonucleotides: 5′-TTCTCCACCACTCCTCGATTCACTG-3′ and 5′-ATCTCTGACGCTTTCTTCACAATAGA-3′.

Histopathology and Immunofluorescence. Mice were killed and tissues were immersion fixed in 10% buffered formalin. Tissues were embedded in paraffin blocks and processed by routine methods, sectioned at 5–10-μm thickness, stained with hematoxylin and eosin, and examined by light microscopy. Single cell suspensions from tumor-bearing spleens were prepared according to standard procedures and spun onto slides as previously described (20). For immunofluorescence, the cytospins were incubated at 4°C overnight with anti-v-Rel or anti-IκBα polyclonal antibodies. Anti-v-R el and anti-IκBα antibodies were visualized with a donkey anti-rabbit immunoglobulin conjugated with Texas red and with Alexa-594-labeled goat anti-rabbit IgG antibodies, respectively. The slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and imaged using an epifluorescence microscope (Axiovert 100; Carl Zeiss, Jena, Germany). Coverslips were mounted in Prolong Gold Antifade Reagent (Invitrogen, Carlsbad, CA) or with Vectorshield mounting medium (Vector Labs, Burlingame, CA).

Production of Anti-IκBα Antibodies. Rabbit polyclonal antibodies to IκBα were generated by immunizing New Zealand White rabbits with IκBα expressed in E. coli and purified from cell extracts using 1 ml of cell extract per ml of 0.5% sodium deoxycholate. The immunization protocol included 10 subcutaneous injections of 2 mg of IκBα emulsified in complete Freund's adjuvant (CFA) and 10 subcutaneous injections of 5 mg of IκBα emulsified in incomplete Freund's adjuvant (IFA) at 3-week intervals. The animals were bled before and after each injection. The IκBα-specific antibodies were affinity-purified using 0.5 ml of IκBα immobilized on 1 ml of cyanogen bromide-activated Sepharose (Pharmacia, Piscataway, NJ) for 1 h at room temperature, washed, and eluted with 0.1 M glycine (pH 2.5). The purity and specificity of the antibodies were assessed by Western blot analysis.

Flow Cytometric Analysis. Single cell suspensions of spleens bearing tumors were placed in RPMI 1640 medium supplemented with 10% FCS, 4 mM glutamine, 50 mM β-mercaptoethanol, 50 U/ml penicillin, and 50 mg/ml streptomycin. Flow cytometry was performed with a flow cytometer and cell sorter (Epics Profile II; Coulter Corp., Hialeah, FL). Single cell suspensions from ikba and v-rel/ikba transgenic thymi and spleens were prepared and analyzed for surface expression of CD4 (clone H129.19), CD8 (clone 7D4), TCR-α/β (clone H57-597), B220 (clone RA3-6B2), HSA (clone J11d), M-α-1 (clone M17/70HL), and Gr-1 (clone R B6-8C5) as previously described (21). Monoclonal antibodies were obtained from GIBCO BRL (Gaithersburg, MD) and Pharmingen.

Western Blot Assays. Cytoplasmic and nuclear fractions from thymocytes were prepared as previously described (22). Aliquots 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. Western blotting procedures and antibodies have been previously described (23). The proteins were transferred onto nitrocellulose membranes and transfer efficiency assessed by Ponceau S staining. Purity of the nuclear extracts was checked by incubating the membranes with an antisera specific for the cytosolic enzyme lactate dehydrogenase.

Cell Labeling, Lysis, and Immunoprecipitation. Isolated thymocytes were labeled for 2 h with 500 μCi/ml of [35S]methionine (1,000 Ci/mmol) in DMEM lacking methionine and containing 10% heat inactivated and dialyzed FCS. The labeling medium was removed and the cells were washed with cold PBS and lysed on ice by adding radio immunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl). Cell lysates were first cleared with a preimmune serum (3 μl) for 3 h and then immunoprecipitated with specific antisera as previously described (23).

Electrophoretic Mobility Shift Assays. The palindromic κB site used for these assays has been previously described (23). Nuclear extracts (3 μg) were incubated with 20,000 cpm 32P-labeled probe, 3 μg poly (dI/dC) in buffer containing 20 mM HEPES, pH 7.9, 60 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM PM SF, and 17% glycerol for 15 min on ice. Protein loading was checked by Oct 1 DNA-binding activity. Complexes were separated on 5.5% native polyacrylamide gels run in 0.25 × Tris base, borate acid, EDTA buffer, dried, and exposed to X-Omat AR film (Kodak, Rochester, NY) at −70°C.

Semiquantitative Reverse Transcription Analysis. Semiquantitative PCR was performed as described previously (17). The PCR conditions were established such that amplification of the cDNAs was linearly dependent on the concentration of the corresponding messenger RNA (mRNA). This was achieved by performing the reactions in the presence of [32P]dCTP; due to the greater sensitivity of autoradiography as opposed to ethidium bromide staining, a relatively low number of PCR cycles was sufficient to detect the amplified product. In this way, the cDNA concentration remained the rate-limiting factor throughout the amplification procedure. Lymph node T cells derived from ikba, v-rel, and v-rel/ikba transgenic mice were isolated on murine T cell enrichment columns (R&D Sys. Inc, Minneapolis, MN) according to manufacturer’s recommendation from v-rel and v-rel/ikba transgenic mice. T cells were spun onto slides and incubated at 4°C overnight with anti-ICAM-1 FITC-labeled monoclonal antibody (Pharmingen, San Diego, CA).

Results

Generation of v-rel Transgenic Mice Overexpressing IκBα in the Thymus. The transcriptional activity of Rel/NF-κB family of proteins is regulated, in part, by their association with inhibitory molecules (IκBα) that sequester them as inactive complexes in the cytoplasm. IκBα is the predominant inhibitory protein in most cell types (5, 13–16). We recently generated transgenic mice specifically expressing v-R el in thymocytes (17). These animals develop T cell multicentric aggressive lymphomas. To study the ef-
effect of IκBα on the transforming capability of v-Rel, we produced double transgenic v-rel/ikba mice by crossing transgenic mice expressing v-Rel with transgenic mice overexpressing IκBα. Both transgenes are under the control of the mouse T cell–specific Id proximal promoter.

The expression of v-Rel and IκBα in thymocytes from the double transgenic mice was assessed by immunoprecipitation (Fig. 1A). These analyses demonstrate the association of v-Rel complexes with IκBα (Fig. 1, lanes 1 and 3) and show that there is an excess of IκBα protein that is not associated with v-Rel (Fig. 1, lane 4). In addition, these data show that under nondenaturing conditions, and total protein extract was immunoprecipitated with anti-v-Rel antibodies. The immunoprecipitated was denatured to dissociate the v-Rel-containing immune complex and then reprecipitated, first with IκBα and subsequently with IκBβ and v-Rel antibodies (left). The supernatant of the total protein extract treated with anti-v-Rel antibody was subsequently reprecipitated with anti-IκBα and anti-IκBβ antibodies (right). B The levels of IκBα protein expression were analyzed by Western blot in total protein extracts from control (w.t.), ikba transgenic, v-rel transgenic and v-rel/ikba double transgenic thymocytes.

Figure 1. v-Rel/IκBα complexes (A) and IκBα protein expression (B) in v-rel/ikba double transgenic thymocytes. To generate v-rel/ikba double transgenic mice, ikba homozygous transgenic mice were crossed with v-rel heterozygous transgenic mice. Both transgenes were under the control of the Id promoter. Screening of double transgenic mice was done by PCR analysis using v-rel-specific oligonucleotides. (A) Immunoprecipitations were performed as previously described (31). [35S]methionine-labeled thymocytes were lysed under nondenaturing conditions, and total protein extract was immunoprecipitated with anti-v-Rel antibodies. The immunoprecipitated was denatured to dissociate the v-Rel-containing immune complex and then reprecipitated, first with IκBα and subsequently with IκBβ and v-Rel antibodies (left). The supernatant of the total protein extract treated with anti-v-Rel antibody was subsequently reprecipitated with anti-IκBα and anti-IκBβ antibodies (right). (B) The levels of IκBα protein expression were analyzed by Western blot in total protein extracts from control (w.t.), ikba transgenic, v-rel transgenic and v-rel/ikba double transgenic thymocytes.

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The levels of IκBα protein expression in v-rel/ikba double transgenic mice was determined by Western blot with whole protein extract prepared from transgenic thymocytes and compared with the levels of IκBα protein expression in wild-type, ikba transgenic, and v-rel transgenic thymocytes (Fig. 1B). The highest levels of IκBα protein expression were detected in v-rel/ikba double transgenic thymocytes (lane 4). This represents almost two times the amount of IκBα present in ikba transgenic (lane 2) and v-rel transgenic thymocytes (lane 3). As described previously, there is an increase in the levels of IκBα in v-rel transgenic thymocytes when compared with control thymocytes (lane 1) due to IκBα protein stabilization by v-Rel el-containing complexes (17).

IκBα Overexpression Cancers v-Rel in Nucleus cytoplasmic Distribution. To examine whether the overexpression of IκBα in v-rel transgenic mice alters the nuclear–cytoplasmic distribution of v-Rel, we performed Western blot analyses using thymocyte extracts from wild-type, v-rel transgenic, and v-rel/ikba double transgenic mice (Fig. 2A). In v-rel transgenic thymocytes, a significant amount of v-Rel, ~30% of the total protein, was localized in the nucleus (lanes 3 and 4). In contrast, in v-rel/ikba double transgenic thymocytes, >90% of the v-Rel protein is retained in the cytoplasm, and only a small amount translates to the nucleus (lanes 5 and 6). The blot containing lanes 5 and 6 was overexposed to detect the min amounts of v-Rel protein in the nucleus. Incubation of the same membrane with antisera specific for the cytosolic enzyme lactate dehydrogenase demonstrated no cross-contamination of nuclear and cytoplasmic extracts (data not shown).

The changes in the nuclear/cytoplasmic distribution of v-Rel protein in the double transgenics were further confirmed by immunofluorescence (Fig. 2B). v-Rel el protein in v-rel transgenic thymocytes is detected in both nucleus and cytoplasm; in contrast, in v-rel/ikba double transgenic thymocytes, v-Rel is mainly detected in the cytoplasm (a and b, respectively). In addition, the distribution of v-Rel and IκBα proteins in transformed T cells from v-rel/ikba double transgenic mice is also mainly in the cytoplasm (c and d, respectively).

IκBα Overexpression Decreases p50/v-Rel Heterodimer, but Not v-Rel Homodimer DNA-binding Activity. To investigate whether the kB-binding activity was altered in thymocytes of v-rel/ikba double transgenic mice compared to v-rel transgenic mice, thymocyte protein extracts were analyzed by electrophoretic mobility shift assays (EMSA) using a palindromic kB site (Fig. 2C).

The p50/v-Rel el DNA binding activity in nuclear extracts from v-rel/ikba double transgenic thymocytes presents a dramatic reduction compared to nuclear protein extracts from v-rel transgenic thymocytes (compare lanes 1 and 4). However, the v-Rel/v-Rel el DNA binding activity remains almost unchanged (compare lanes 2 and 5). The amount of nuclear protein extract added to the DNA binding reaction was controlled by measuring Oct 1 DNA-binding activity. Oct 1 DNA-binding activity was comparable between the different protein samples (data not shown).

These results indicate that v-Rel homodimers are significantly less sensitive than p50/v-Rel heterodimers to the inhibitory effect of IκBα. The reduced affinity of v-Rel el for IκBα may be one of the key molecular basis for v-Rel transformation.

IκBα Overexpression Extends Survival and Changes the Cellular Expression of v-rel Transgenic Mice. To investigate the consequences of the changes in kB binding activity in v-rel/ikba double transgenic mice, a colony of these animals was bred and their health status monitored over time in a nongerm-free environment (Fig. 3). The v-rel/ikba double transgenic mice presented a longer survival and appeared completely healthy for a longer period of time when compared with v-rel transgenic mice. The mortality curve demonstrates that high levels of IκBα extended the survival of v-rel transgenic mice (75% of v-rel transgenic mice succumbed before 25 wk of age, whereas 100% of the v-rel/
Figure 2. Change of nucleus/cytoplasm distribution of v-Rel in v-rel/ikba double transgenic thymocytes. (A) Western blot analysis. Cytoplasmic (C) and nuclear (N) fractions from thymocytes and Western blot assays were performed as previously described (31). (B) Immunofluorescence. Thymocytes from 6-wk-old v-rel (a) and v-rel/ikba (b) transgenic mice were spun onto slides, incubated with anti-v-Rel antibodies, and visualized with donkey anti-rabbit Texas red-labeled antibodies. T cells from spleen-bearing tumors of v-rel/ikba double transgenic mice were spun onto slides, incubated with anti-v-Rel (c) or anti-IκBα (d) antibodies, and visualized with donkey anti-rabbit Texas red-labeled antibodies. (C) Decreased p50/v-Rel-binding activity in v-rel/ikba double transgenic thymocytes. Electrophoretic mobility shift assays were performed as previously described (23) using nuclear protein extracts from v-rel and v-rel/ikba double transgenic thymocytes. Lanes 1 and 4 were treated with preimmune sera (p.i.), lanes 2 and 3 were treated with anti-p50 antibody (α-p50), and lanes 3 and 6 were treated with anti-v-Rel antibody (α-v-Rel).

Figure 3. Overexpression of IκBα increases survival of v-rel transgenic mice and changes the clinical expression. (A) Mortality curve. Deaths in v-rel/ikba transgenic animals occurred at later times and were the result of secondary opportunistic infection according to pathologic analysis and microbiology. Autopsy, and histologic examination of v-rel/ikba transgenic mice revealed a less severe lymphomatous infiltrate than in v-rel transgenic mice, n = 29 for v-rel 35, and n = 13 for v-rel/ikba. (B) T-cell cutaneous lymphoma in v-rel/ikba double transgenic mice. All v-rel/ikba transgenic mice developed skin lesions characterized by thickening and exfoliative plaques. Arrow indicates a v-rel/ikba transgenic mouse; the control mouse is an ikba transgenic littermate. (The lesions were never observed in v-rel transgenic mice.)
Young v-rel/ikba double transgenic mice, between 3 and 10 wk old, appeared normal as assessed by habits, weight, posture, and histopathology. However, all v-rel/ikba double transgenic mice developed skin lesion in face, ears, tail, and feet after 15 wk of age. These lesions, characterized by loss of hair, thickening of the skin, and exfoliative plaques (Fig. 3 B) progressed slowly with no major compromise in the health status of the mice. Evaluation for a longer period of time revealed that v-rel/ikba double transgenic mice succumbed due to secondary infections by pathogens that colonize the skin; antibiotic prophylactic treatment extended their survival for an additional 2–4 wk (data not shown). Autopsies of v-rel/ikba double transgenic mice did not reveal massive tumoral cell burden in lung, liver, lymph nodes, and spleen, indicating that in contrast to v-rel transgenic mice, massive tumor cellular infiltration was not the primary reason for organ failure in the double transgenic animals (17).

**T Cell Cutaneous Lymphoid Infiltrates in v-rel/ikba Double Transgenic Mice.** The earliest manifestation of disease in most of the v-rel/ikba double transgenic mice was the appearance of lymphoid infiltrates in skin, which were first noticed at 15 wk of age. Histological lesions were characterized by epidermal hyperplasia, infiltration of lymphocytes into the dermis, and proliferation of epidermis (Fig. 4 A–D). Histological sections of skin, lung, liver, spleen, and lymph node showed lymphoid infiltrates in the dermis, epidermis, and paracortical areas (Fig. 4 E–H). Photomicrographs were taken at ×100 (A, B, E, and F), ×250 (C and D), ×125 (G and H).
pearance of facial skin lesions that progressed slowly (Fig. 3 B). Light microscopy revealed cellular infiltration of the dermis by large lymphocytes of irregular nuclei (Fig. 4, a–d). Isolated foci of epidermal infiltration by tumor cells were occasionally seen, but typical Pautrier's microabcesses and massive epidermal infiltration were not observed (Fig. 4 e).

The histologic appearance of this lymphoid infiltration resembles one seen in cutaneous T cell lymphomas (24–27). In contrast to v-rel/ikba transgenic mice, dermal compromise was rarely seen in v-rel transgenic mice (17). Histopathology of v-rel/ikba transgenic animals revealed much less severe tumoral cellular infiltration in other tissues beside skin (17). For instance, in the lung of v-rel transgenic mice, tumoral cellular infiltration around bronchi and alveoli was severe enough to cause organ failure. In v-rel/ikba transgenic mice, lung compromise was much less severe with only tumoral cell infiltration around the bronchi, but not in the parenchyma (17: Fig. 4 e). The liver in v-rel transgenic mice was always enlarged and massively infiltrated by tumor cells that frequently plugged the vessels and produced extensive areas of ischemia and tissue necrosis. In contrast, in v-rel/ikba double transgenic mice, the liver was not enlarged, and lymphoid infiltration was seen in hepatic sinusoids and around the portal triad, but areas of necrosis were not observed (Fig. 4 f). The spleen in v-rel transgenic mice was dramatically enlarged with complete distortion of the normal architecture and massive areas of tissue necrosis. On the other hand, in v-rel/ikba double transgenic mice, the spleen was moderately enlarged and remnants of the normal splenic architecture with demarcation of white and red pulp areas were always present (Fig. 4 g).

Lymph nodes in v-rel/ikba double transgenic mice were not severely compromised, even at very late stages of disease, as was the case in v-rel transgenic mice (Fig. 4 h, and data not shown). Thymic atrophy was a constant observation in both v-rel and v-rel/ikba transgenic mice (data not shown). These histopathological studies demonstrate that v-rel/ikba double transgenic mice developed lymphoma, but the magnitude of the visceral compromise was much less than in v-rel transgenic mice of the same age. These results indicate that IkBα overexpression does not prevent tumor formation, but does delay the appearance of tumors, the progression of the disease, and decreases the severity of the lymphomatous state observed in v-rel transgenic mice. In addition, these results indicate that IkBα overexpression induces transcriptional changes in v-R el–transformed T cells that resulted in their increased tropism for the skin.

Flow Cytometric Analysis of Lymphoid Cells from v-rel/ikba Double Transgenic Mice. To understand the changes in the clinical expression of v-rel/ikba double transgenics compared to v-rel transgenic mice, we analyzed lymphoid cell populations by flow cytometry (Fig. 5). Flow cytometric analysis of lymphoid cells in v-rel transgenic mice have been previously described (17). Analyses of thymuses and spleens from young v-rel/ikba double transgenic animals (3–8 wk) did not reveal any alterations when compared to ikba transgenic littermates (Fig. 5 A, a–d, and data not shown). Ikba transgenic littermates were used as a control, since no differences in lymphoid populations have been detected in ikba transgenic mice when compared to wild-type animals (Perez, P., unpublished results and data not shown).

Analysis of spleens from moribund and euthanized v-rel/ikba double transgenic animals showed an expanded T cell population characterized by intermediate and low levels of the TCR α and β chains (Fig. 5 B, a and b). In addition, a striking difference between v-rel transgenic and v-rel/ikba double transgenic mice was a significant increase in CD8+ single positive T cells in v-rel/ikba transgenic mice, a population of cells that was not always increased in v-rel transgenic mice. These results indicate that IkBα overexpression in v-rel transgenic mice alters the developmental profile of the v-rel transformed T cells, allowing the T cells to reach a more mature phenotype. Only a moderate increase of T cells coexpressing markers associated with an immature phenotype (HSA) was detected in the double transgenic (Fig. 5 B, e and f), in contrast to the dramatic changes that we previously observed in v-rel transgenic mice. An increased population of granulocytes as a result of infection was detected in v-rel/ikba double transgenic mice (Fig. 5 B, g and h).

v-R el–containing Complexes Have Lower Affinity for IkBα than c-R el–containing Complexes. To understand why higher levels of IkBα are required to reduce nuclear translocation of v-R el and p50/v-R el cB-binding activity in transgenic thymocytes, we compared the inhibitory effect of IkBα on cB binding activity in nuclear protein extract derived from
Increased Levels in the Expression of a Cell Adhesion Molecule

v-rel transgenic (Fig. 6 A) and Δc-rel transgenic thymocytes. Δc-rel transgenic animals express a truncated version of the mouse c-R el protein (Δc-R el) under the control of the Ick promoter, and, like the v-R el oncogenic protein, lacks part of the COOH-terminal transcriptional activation domain keeping the RHD intact. The generation of Δc-rel transgenic mice has been previously described (17). Increasing levels of purified baculovirus expressed IκBα protein were added to identical amounts of nuclear protein extracts from Δc-rel transgenic thymocytes (Fig. 6 A a) and v-rel transgenic thymocytes (Fig. 6 A b) before starting the κB-binding reaction. The total amount of protein extract used in the reaction was tested by O ct 1 DNA-binding activity and the identification of the complexes by antibody supershifts (data not shown). In the absence of added IκBα, similar amounts of total κB binding activities were detected in Δc-rel transgenic (Fig. 6 A a, lane 1) and in v-rel transgenic thymocytes (Fig. 6 A b, lane 1). However, when increased amounts of IκBα protein were added to the binding reaction, the binding of p50/Δc-R el was dramatically reduced (Fig. 6 A a, lanes 2 and 3), whereas the binding of v-R el containing complexes was slightly affected (Fig. 6 A b, lanes 2 and 3). At 100 ng of IκBα, the binding of v-R el-containing complexes is significantly reduced (Fig. 6 A b, lane 4), and only when the highest amounts of IκBα were used (1 μg) and when endogenous p50/p50 κB binding also began to be affected (Fig. 6 A a, lane 5), was the binding of v-R el-containing complexes completely reduced (Fig. 6 A b, lane 5). These results demonstrate that the binding of v-R el-containing complexes is more resistant than the binding of complexes containing the cellular homologue Δc-R el to the inhibitory effect of IκBα, and offer an attractive explanation for the molecular mechanisms involved in the transforming activity of v-R el. This is in agreement with previous in vitro studies that demonstrated a reduced IκBα inhibition of DNA binding by the oncogenic v-R el protein when compared to the nononcogenic c-R el protein (28).

Discussion

In this work we have demonstrated that overexpression of the R el/N F-κB inhibitory protein, IκBα, delays the development of T cell lymphomas in a transgenic mouse model that expresses the oncogenic v-R el protein under the control of the lck T cell–specific promoter (17). Previous studies indicate that v-R el transformation requires its...
translocation to the nucleus and binding to a set of specific R el/N F-κB responsive genes (1–5).

Role of IκBα in v-Rel Transformation. Previous studies have suggested a potential tumor suppressor activity for IκBα (4). IκBα-deficient mice displayed elevated levels of nuclear R el/NF-κB activity in hemopoietic tissues. Unfortunately, these animals do not survive long enough for evaluation of appearance of lymphoid malignancies (31, 32).

In this work, we have demonstrated that although v-rel/ikba double transgenic thymocytes expressed IκBα protein at very high levels, there was still some nuclear v-R el protein in the double transgenic cells, suggesting an intrinsic ability of v-R el to escape the inhibitory effect of IκBα. This observation was correlated with an increased resistance of v-R el DNA-binding activity to the inhibitory effect of IκBα when compared to a truncated version of c-R el that lacks the transcriptional activation domain. The reduced affinity of v-R el for IκBα may be associated with some of the internal mutations present in the R el homology domain of v-R el (1–5). It is noteworthy to mention that transgenic mice overexpressing either R elA or R elB in T cells under the control of the lck did not develop lymphomas. In the case of rela transgenic mice, the absence of tumor formation was correlated with the fact that there was no increased DNA-binding activity in T cells, because R elA protein was efficiently retained in the cytoplasm by IκBα (19). However, relb transgenic thymocytes presented a dramatic increase in DNA-binding activity due to the fact that R elB complexes were not efficiently retained in the cytoplasm because of their low affinity for IκBα (33), indicating that increased nuclear levels of R el/NF-κB activity appear to be insufficient for transformation. Therefore, together with the low affinity for IκBα, it appears that v-R el possesses additional and unique properties that render a potential transforming potential in mammalian cells.

The fact that IκBα overexpression extended the survival but did not prevent tumor formation in v-rel transgenic mice indicates that it would be impossible to achieve in vivo the levels of IκBα required to compensate for the reduced affinity of v-R el for IκBα to prevent v-R el transformation.

ICAM-1 Overexpression in Lymphoproliferative Disorders. When nuclear κB-binding activity was assessed by EMSA in v-rel/ikba double transgenic thymocytes, the most striking observation was the dramatic reduction in the p50/v-R el DNA-binding activity with almost no change in v-R el/ v-R el DNA-binding activity. The reduction in the p50/v-R el DNA binding correlated with the changes observed in the clinical expression of v-rel/ikba double transgenic mice, and supports our previous indications that v-Rel may be active as a p50/v-R el heterodimer and as a homodimer (17), with the p50/v-R el heterodimeric form being more susceptible to IκBα inhibition. Because of the changes in the clinical expression and the differences observed in p50/v-R el and v-R el/v-R el DNA-binding activity in v-rel/ikba double transgenic T cells, it is possible to speculate that v-Rel either as a heterodimeric complex with p50 or as a homodimeric complex may target different sets of κB-regulated genes. An example of this differential regulation is the observation of increased levels of ICAM-1 in T cells derived from v-rel/ikba double transgenic mice, but not in T cells derived from v-rel transgenic mice. Interestingly, strong induction of ICAM-1 has been observed in human lymphoproliferative disorders (29, 34), and ICAM-1 overexpression in v-rel/ikba double transgenic mice may be responsible for the increased tropism of T cells for the skin observed in these animals (25, 22).

R el/N F-κB Activity in Cutaneous T Cell Lymphomas. The lymphomatous infiltration observed in v-rel/ikba transgenic mice resembles that seen in T cell cutaneous lymphomas, the Sezary syndrome, mycosis fungoides, or other related disorders (35). Our observation that v-rel/ikba double transgenic mice developed cutaneous T cell lymphomas is particularly interesting in light of the observation that chromosomal translocations associated with structural alterations of the R el/NF-κB family of proteins have been documented in several cases of T cell cutaneous lymphomas in human patients (26, 35–40). In particular, rearrangement and altered expression of the NFKB-2 gene have been identified in the HUT 78 human cutaneous T cell leukemia line (39, 40). In addition, structural alterations of the NFKB-2 gene have also been identified very commonly (14%) in neoplasms derived from mature T cells such as mycosis fungoides and Sezary syndrome (24, 38). These structural alterations may contribute to lymphomagenesis by determining a constitutive activation of the NF-κB system and, in particular, of NFKB-2 target genes (36, 37). This data, in addition to our observations, strongly supports the notion that structural alterations of other R el/NF-κB family members may play a role in lymphomagenesis. Furthermore, our results indicate that variations in the clinical expression of lymphoid malignancies such as mycosis fungoides and Sezary syndrome may be based on subtle changes in the nuclear composition and interplay among the different R el/NF-κB and IκB molecules. In this sense, v-rel and v-rel/ikba transgenic mice constitute a potential model to study the molecular mechanisms involved in the generation of lymphoid malignancies.

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