Discrimination between RelA and RelB Transcriptional Regulation by a Dominant Negative Mutant of IκBα*

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RelA and RelB belong to the nuclear factor-κB (NF-κB-Rel) transcription factor family. Both proteins are structurally and functionally related, but their intracellular and tissue distributions are different. In resting cells, RelB is found mostly in the nucleus, whereas RelA is sequestered in the cytosol by protein inhibitors, among which IκBα is the dominant form in lymphocytes. Upon cellular activation IκBα is proteolyzed, allowing RelA dimers to enter the nucleus and activate target genes. To study the selectivity of gene regulation by RelA and RelB, we generated T cell lines stably expressing a dominant negative mutant of IκBα. We show that selective inhibition of RelA-NF-κB decreased induction of NFκBI, interleukin-2, and interleukin-2R, whereas wild type IκBα was expressed in the transgenic IκBα; however, wild type IκBα was expressed in the transgenic cell clones but with much slower kinetics than that in control cells. Wild type IκBα expression was concomitant with RelB up-regulation, suggesting that RelB could be involved in transcription of IκBα through binding to an alternative site. These results indicate that RelB and RelA have both distinct and overlapping effects on gene expression.

Nuclear factor κB (NF-κB)† is part of the Rel family of eukaryotic transcription factors which share structural and functional properties. Although ubiquitously expressed in higher eukaryotes NF-κB has been intensively studied mostly in cells belonging to the immune system where it was first discovered (for review, see Refs. 1 and 2). NF-κB-Rel factors were shown to participate in the expression of genes essential for the immune responses and to regulate gene transcription during inflammatory reactions. The prototypical NF-κB is a homodimer or heterodimer composed of 50-kDa (p50) and/or 65-kDa (p65 or RelA) polypeptides. In vertebrates other members of the family are c-Rel, RelB, and p52. The tissue and cellular distribution of the three last members is more restrained than that of the prototypical NF-κB. For example, the expression of RelB was described as being predominant in dendritic cells from primary and secondary lymphoid organs (3–6). RelB has also been detected in other cells and tissue but in lower amounts or after specific activation. c-Rel and p52 are also expressed mainly in cells from the hematopoietic lineages. P50 and p52 are generated by proteolytic processing of precursor polypeptides (p105 (NFκB1 gene) and p100 (NFκB2 gene), respectively) (1, 7). Each member of the NF-κB-Rel family contains a 300-amino acid sequence called the Rel homology domain, which is critical for nuclear translocation, protein-protein interactions, and sequence-specific DNA binding. All members of the NF-κB-Rel family form dimers. The dimers can be classified into two pools on the basis of their intracellular localization, which is critical in regulating their activity. One pool of NF-κB-Rel dimers is cytosolic in the absence of cellular activators, whereas the second pool is constitutively nuclear. The intracellular location of the dimers depends on the capacity of the NF-κB-Rel family members to interact with ankyrin repeat-containing proteins, collectively called IκB. The cytoplasmic IκBs inhibit NF-κB-Rel complexes by preventing both NF-κB-Rel nuclear translocation, and their interaction with specific decameric DNA sequences called κB (8, 9). Thus IκBs represent intracellular regulators of NF-κB activity. Several members of IκB regulatory family have been characterized, including IκBα, IκBβ, IκBγ, the two NF-κB protein precursors p105 and p100, and bcl-3. Except for bcl-3, IκB molecules are mostly cytosolic, although nuclear IκBα has been reported in cultured cells (10–12) and in vivo.‡ p50 and p52 homodimers as well as RelB-p50 and RelB-p52 heterodimers do not interact efficiently with cytosolic IκBs. Consequently they are found in nuclei of cells that produce these complexes (13). Therefore their regulation should be distinct from the cytosolic forms of NF-κB. The p50 and p52 homodimers were reported to interact with the nuclear bcl-3. The resulting trimers seem to constitute transcriptional activators, whereas p50 and p52 homodimers are unable to enhance RNA polymerase II-driven transcription (14). In contrast to other members of the NF-κB family, RelB contains in its N-terminal domain a leucine zipper-like structure that is essential for transactivation of target genes (15). However, the regulation of RelB activity is still poorly understood.

Studies of T lymphocytes, isolated from IκBα-deficient mice, demonstrated that the dominant IκB regulator of NF-κB-Rel is IκBα, the product of the MAD3 gene (16, 17). Activation of cells with adequate signals such as T cell receptor triggering, phorbol esters, interleukin 1 (IL-1), tumor necrosis factor (TNF-α), and others results in IκBα degradation by 26 S proteasomes (for review, see Ref. 7). This renders dimers, which contain RelA and c-Rel proteins, free to translocate into nuclei where they activate transcription of target genes. The molecular mechanism resulting in IκBα proteolysis is complex and not completely elucidated. However, at least two post-translational

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† The abbreviations used are: NF-κB, nuclear factor κB; IL, interleukin; TNF, tumor necrosis factor; ICAM-1, intercellular adhesion molecule-1; HIV, human immunodeficiency virus; CMV, cytomegalovirus; EMSA, electrophoretic shift assay; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; Luc, luciferase; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay; PHA, phytohemagglutinin.

‡ J. Feuillard and M. Körner, unpublished observation.
covalent modifications have been reported to be essential for its degradation. The first critical event is phosphorylation of serines 32 and 36 in the NH2-terminal region of IxBα, carried out by Ser/Thr kinase(s) including a multienzyme complex of 700 kDa (18). This double phosphorylation of IxBα does not lead to dissociation from NF-kB, but it is prerequisite for the second modification step, which is the ubiquitination of two NH2-terminal lysines at positions 21 and 22 (19). Subsequently the phosphorylated and ubiquitinated IxBα is proteolyzed by the 26 S-proteasome complex (20, 21).

Once released from IxBα, NF-kB-Rel proteins translocate rapidly to the nucleus where they exert their regulatory functions by interacting with specific deacemeric kB sequences and the general transcription factor TFIIB (22). A plethora of genes transcriptionally regulated by NF-kB have been shown to contain kB sequences in their promoters (for review, see Ref. 23). In T cells, gene products involved in cell adhesion (intercellular cell adhesion molecule-1; ICAM-1), cell growth control (IL-2, its receptor IL-2Rα, and c-myc), and proinflammatory mediators (IL-6, TNF-α) are suspected of being transcriptionally regulated by NF-kB. Furthermore, viruses with T cell tropism, such as HIV, are also thought to be infected by the usual NF-kB promoter domain and the 0.4SK promoter and the 0.2SK promoter. Whereas the 0.4SK contains all three kB sites from the MAD3 promoter domain, whereas the 0.2SK contains only the proximal kB1 site, and the 0.45KxaκB contains only the kB2 and kB3 sites. To monitor the transcription of the three constructs of MAD3 promoter, the pβgal-promoter vector (CLONTECH), which contains a functional LacZ gene downstream of the SV40 early promoter, was cotransfected, and the β-galactosidase activity was measured by spectrophotometry in the presence of 100 mM o-nitrophenol-β-D-galactoside. The ICAM-1 promoter-Luc construct (pGL1.3) was described by Ledebur and Parks (40) and was provided by Dr. R. R. Rector (Rush, Chicago). The c-myc promoter (−3252 to +56) and c-fos promoter (−711 to +42) CAT constructs are described in Ref. 41. The LTR3 CAT-218 construct containing the 218 base pairs upstream from the transcription initiation start of the HIV 5′-LTR (42) was provided by Dr. R. B. Gaynor (UCLA, Los Angeles). Finally, Dr. G. R. Crabtree (HHMI, Stanford, CA) provided us with the IL-2 promoter-Luc construct (pGL1S3CAT) (∼326 to +45). Transient transfections of the cells were performed by electroporation at 200 V, 500 microfarads (Bio-Rad electroporation system) with 20 μg of plasmid DNA/5×10⁶ cells. 2 h after transfection, cells were split into two pools. One pool of cells was incubated in RPMI (untreated cells), and the other pool was incubated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1 μg/ml phytomagglutinin (PHA) for 24 h (activated cells). The cells were then collected, washed once with phosphate-buffered saline, and lysed by three cycles of freezing/thawing in 150 mM Tris-HCl, pH 8. Cell extracts, normalized for total protein content (43), were assayed for CAT activity using [14C]chloramphenicol (NEN Life Science Products) according to Gorman et al. (44). The chloramphenicol conversion was quantified using a Betalager 1200 apparatus (Biospace, France). The results were expressed as percent of chloramphenicol conversion/mg of protein (relative CAT units). Transfection experiments were repeated at least three times, using two independent plasmid preparations. Lucifer assays were performed using the Promega luciferase assay system. The cells were lysed with 25 mM Tris phosphate, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100, pH 7.8. The light emission was measured in a luminometer (Bio-Rad). The results were calculated as relative light units (light emission/background/mg of protein).

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**MATERIALS AND METHODS**

**Cells**—The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum. The parental HPB-ALL cell line was cultured in RPMI 1640 medium containing glutamine, antibiotics, and 10% fetal calf serum.
RNA (10–20 μg) was fractionated by electrophoresis on 0.7% agarose gels containing 2.2 M formaldehyde. Gels were blotted on Hybond N membranes (Amersham) according to the indications of the manufacturer. Membranes were hybridized with "32P-labeled probes in Quickhyb solution (Stratagene) according to the protocol supplied by the manufacturer, at 65 °C. Membranes were autoradiographed for 1–12 h at –70 °C with intensifying screens. Membranes were stripped by boiling in H2O and rehybridized with the β-actin probe to normalize loading of RNA samples.

Measurement of IL-2 Production by Enzyme-linked Immunosorbent Assay (ELISA)—Control, A3, and D7 clone cells (10⁶ cells/condition) were activated by PMA (10 ng/ml) plus PHA (1 μg/ml) for 12 h at 37 °C. Cell supernatants were tested for IL-2 by ELISA using the Immunotech human IL-2 ELISA kit (Immunotech, France). All assays were performed in quadruplicate.

Measurement of CD25 Expression by Flow Cytometry—Control, A3, F10, and D7 cells were activated for 24 h by PMA (10 ng/ml) and PHA (1 μg/ml). Unstimulated cells and PMA plus PHA-treated cells were tested for CD25 by flow cytometry using a phycocerythrin-conjugated human CD25-specific monoclonal antibody from Caltag Laboratories and fluorescence-activated cell sorter apparatus from Becton-Dickinson.

RESULTS

Characterization of Stable HPB-ALL Clones Transfected with the 32/36A Mutant IκBα—The parental HPB-ALL cell line is a T cell tumor producing IL-2 and IL-6 in response to T cell activators, such as phorbol esters, in the presence of a Ca²⁺ influx activators (PHA, ionomycin, CD3-specific antibodies, etc.). Its phenotype is close to a double positive thymocyte (CD4⁺/CD8⁺, CD1a⁺, CD3⁺). We chose this clone as a model for studying the inhibition of the inducible NF-κB by a dominant negative form of IκB (IκBα32/36A). The stability of the integration of the mutant IκBα was verified by Southern blotting of DNA extracted from several clones isolated by limiting dilution and cultured for 1 month in the presence of the selective antibiotic (not shown). Three clones, A3, D7, and F10, were identified as stably transfected with IκBα32/36A. To verify that the IκBα cDNA was expressed in these clones, we performed Western blot analysis of the cytosolic fractions of the control and the “mutant” clones, using a monoclonal antibody specific for the NH₂-terminal domain of IκBα (36). The wild type and the mutant IκBα are distinguishable on the basis of their electrophoretic migration because the 32/36A mutant migrates slightly slower in SDS gels (32). In the three clones that integrated IκBα32/36A, a slower migrating protein was specifically detected by the antibody in addition to the wild type IκBα (Fig. 1A). Judging by the immunoblot results, the mutant and wild type IκBα were expressed at comparable levels in clone A3 and F10, whereas in clone D7, the mutant IκBα was more highly expressed relative to the wild type IκBα. In control cells, only the faster migrating 36-kDa IκBα was detected. In none of the mutant clones did expression of the transgenic IκBα prevent constitutive production of the wild type IκBα.

To determine whether the mutant IκBα could inhibit translocation of NF-κB, we performed gel shift experiments with nuclear extracts from resting and PMA plus PHA-treated cell clones (Fig. 1B). In control cells, a 1-b PMA plus PHA activation generated a nuclear translocation of κB oligonucleotide-binding proteins, visible as a doublet. In contrast, in the three stable clones, neither constitutive nor inducible κB oligonucleotide binding activities were detected, suggesting that the mutant IκBα prevented NF-κB translocation.

IκBα32/36A Blocks Nuclear Translocation of RelA-NF-κB but Not of RelB—To investigate the duration of NF-κB inhibition by the mutant IκBα, we analyzed NF-κB nuclear translocation during a time course of PMA plus PHA treatment. In the control cells, κB binding activities were clearly detectable at the 3 h time point and increased in intensity up to 24 h of treatment (Fig. 2A). In the A3 clone, no significant κB binding activity was detected until 7 h of activation. However, by the 7 h time point, a κB binding activity, migrating as a doublet, was clearly detected and reached levels similar to the control by 24 h of activation (Fig. 2A). To identify the proteins in the complexes bound to κB oligonucleotide, we tested the abilities of antibodies specific for RelA, c-Rel, p50, and RelB to affect the EMSA patterns of control and A3 cells after 7 and 24 h of PMA plus PHA stimulation. In the absence of specific antibodies, several complexes were detectable in the control cells. The discrimination of these complexes was difficult in this type of gels; but clearly, in the A3 clone, only two bands were detectable after 7 h of cell stimulation, whereas in control cells additional, slower migrating bands, existed (see Fig. 2B and photographically enlarged view in Fig. 2C). Antibodies specific for RelA and RelB demonstrated that the two upper bands from the control cells contained RelA, whereas one of the lower bands contained RelB (Fig. 2B). p50-specific antibodies removed the two lower bands from both A3 and control cells. Thus, the upper band in A3 clone was composed of RelB-p50 dimers, whereas the lower band was the p50 homodimer. Antibodies specific for c-Rel had no effect on the κB-binding proteins in control or A3 cells (Fig. 2), whereas they inhibited efficiently c-Rel-p50 binding in control cells (not shown). Thus, whereas in control cells both RelA and RelB dimers were detected, in the A3 clone only RelB-p50 and p50 dimers were detected. After 24 h of PMA plus PHA activation, both control and A3 nuclear extracts contained only the two faster migrating complexes (p50-p50 and RelB-p50) (Fig. 2B). These results demonstrated that in control HPB-ALL cells, the initial effect of PMA plus PHA activation led to nuclear translocation of cytosolic NF-κB proteins (RelA homo- and heterodimers). As expected, in the IκBα32/36A-transfected A3 clone, the translocation of these proteins was inhibited. Prolonged stimulation led to RelB activation in both cell clones. Further-
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more, after 24 h of PMA plus PHA treatment activation of the RelA-containing complexes was also inhibited. It was not surprising to observe comparable levels of RelB in both control and A3 cell clones since RelB activation was reported to be independent of IκBα. Similar results were obtained with the two other IκBα<sub>32/36A</sub> transfected F10 and D7 cell clones (not shown). Thus, we have generated a cell system in which the prototypical NF-κB is inhibited selectively by IκBα<sub>32/36A</sub>, but activation of RelB remains potentially intact. Western blotting analysis of nuclear extracts from A3 and HPB-ALL cells further assessed the presence of RelB. RelB nuclear amounts were increased upon PMA plus PHA stimulation (Fig. 3). In addition, immunochemical analysis with RelB-specific antibodies confirmed the increase of RelB in nuclei of control cells and A3 cells after 24 h of stimulation (not shown). This is suggestive of a transcriptional, or at least, pretranslational, regulation of the RelB in PMA plus PHA-activated HPB-ALL T cells.

**IκBα<sub>32/36A</sub> Expression Is Increased in PMA-stimulated Cells**—In the absence of stimulation, wild type IκBα has a rapid turnover that is independent of serines 32 and 36 phosphorylation and of ubiquitination (46). After stimulation with PMA or TNF-α, IκBα is modified by phosphorylation and ubiquitination, and the balance between degraded and newly synthesized IκBα turns transiently in favor of the degradation (47). As a result, IκBα is detected in lower amounts in cytosol from short term activated cells. However, the resulting activation of NF-κB induces newly synthesized IκBα that is detectable within 1–2 h after activation. This neosynthesized IκBα is, in turn, probably responsible for the inhibition of the RelA-containing NF-κB at later time points of PMA plus PHA treatment (see Fig. 2B). This cycle of activation-induced proteolysis/resynthesis of IκBα is initiated by the phosphorylation of serines 32 and 36. To examine the fate of the IκBα<sub>32/36A</sub> versus the wild type IκBα in activated cells, we performed kinetic experiments in which the A3 and the control clones were treated with PMA plus PHA for increasing lengths of time. Western blot analysis of IκBα after up to 2 h (Fig. A4A) and 24 h (Fig. A4B) of activation by PMA plus PHA showed that the wild type IκBα was degraded almost completely within 30 min in control cells. After 1 h of PMA plus PHA treatment it was resynthesized progressively, reaching initial levels after as soon as 5 h of activation (Fig. A4B). In the A3 clone, the wild type IκBα was also degraded rapidly in response to cell activation, but neosynthesized IκBα was detectable only after 7 h of PMA plus PHA treatment, reaching initial levels after as 9 h time point (Fig. 4A and B). The expression of the wild type IκBα, the IκBα<sub>32/36A</sub> was not degraded in response to cell activation. In fact, levels of IκBα<sub>32/36A</sub> increased from the 30 min time point to reach a steady maximum at 2 h (Fig. A4A) probably because the CMV promoter is activated independently of NF-κB activation. These experiments clearly demonstrated the stability of the mutant IκBα in activation conditions that lead to wild type IκBα proteolysis.

**Fig. 2. Identification of κB-binding complexes in control and IκBα<sub>32/36A</sub>-expressing clones.** Panel A, kinetic analysis of κB oligonucleotide binding activities performed by EMSA. Equal amounts of nuclear proteins from untreated (0) or PMA plus PHA activated (from 3 to 24 h) cells were used. Control cells are pCDNA3 transfected HPB-ALL cells. Panel B, inhibition of κB-binding complexes with specific antibodies. Antisera specific for RelA, c-Rel, RelB, and p50 were added to the reaction mixture 45 min before the addition of the 32P-labeled PRE oligonucleotide. The nuclear proteins used were extracted from control cells (C) and the A3 cell clone activated by PMA plus PHA for 7 h (upper panel) and 24 h (lower panel). Positions of the major κB-binding complexes in the absence of competing antibodies are indicated on the left side of each panel. Panel C, enlarged view of the κB-binding complexes from control cells (C) and the A3 cells after 7 h of PMA plus PHA activation.
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Table I
Effect of NF-κB inhibition on PMA plus PHA activation of various promoters

| Promoters | Control | A3 | D7 | F10 |
|-----------|---------|----|----|-----|
| ICAM-1    | 3.35    | 1.7 | 1.3 | 2   |
| p105      | 189.0   | 19.5| 81.7| 8   |
| MAD3      | 3.3     | 0.9 | 0.9 | 2.5 |
| HIV LTR   | 42.5    | 3.8 | 8.1 | 8.2 |
| IL-6      | 14      | 1.3 | 2.9 | 2.3 |
| IL-2      | 76      | 2.3 | 2.5 | 4.3 |
| c-Myc     | 2.2     | 2.4 | 1.9 | 2.3 |
| c-Fos     | 2.6     | 3   | 2   | 2.3 |

Table II
Percentage of residual Luc and CAT activities in IκBα32/36A transfected cell clones

| Promoters | Control | A3 | D7 | F10 |
|-----------|---------|----|----|-----|
| ICAM-1    | 100     | 50.7| 38.8| 59.7|
| p105      | 100.0   | 10.3| 43.2| 4.2 |
| MAD3      | 100     | 0   | 0   | 75.7|
| HIV LTR   | 100     | 8.9 | 19  | 19.3|
| IL-6      | 100     | 9.2 | 20.7| 16.4|
| IL-2      | 100     | 3   | 3.3 | 5.6 |
| c-Myc     | 100     | 109 | 86  | 104.5|
| c-Fos     | 100     | 115 | 77  | 88  |

Discussion

Differential Effect of IκBα32/36A on Several κB-dependent Promoters—To test the functional consequences of the selective inhibition of RelA containing NF-κB, we performed transient transfections of A3, D7, F10, and control cells, with a series of reporter gene constructs (CAT or Luc) linked to promoter regulatory regions of seven genes suspected to constitute targets for NF-κB. In each of these promoters, at least one κB consensi

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cells, but no induction by PMA plus PHA was observed. In the
control cells, MAD3 (IκBa) mRNA reached a steady state after
as little as 30 min of PMA plus PHA treatment. In A3 cells, two
transcripts were detected with the MAD3 probe. The higher
mobility transcript, corresponding to the mutant IκBa, was
strongly augmented in the early time points of activation, peak-
ing at 1 h, whereas the slower migrating wild type messenger
was detected at later time points (6–24 h). Thus, whereas
transcription of IL-2 and IL-6 genes was inhibited completely
in the A3 clone, induction of the wild type IκBa messenger RNA
was delayed in the A3 clone compared with the control cells.
Finally, the c-myc messenger was produced constitutively in
these cells, and no induction by PMA plus PHA was detected in
either control or A3 cells.

Both IL-2 and IL-2Ra Productions Are Inhibited by the
IκBa32/36A Mutant—We further investigated the regulation of
IL-2, an important T cell proliferation regulator, by measuring
its production at the protein level in two of the stable clones (A3
and D7) by ELISA (Fig. 7). In both transgenic clones, IL-2
production was 10% of the control after 12 h of PMA and PHA
activation. Thus, the result obtained with the Northern blot
analysis of IL-2 induction was confirmed at the protein level.

The α subunit of the IL-2 receptor (CD25) is another poten-
tial target for NF-κB regulation (48, 49). To analyze further the
effect of IκBa32/36A on the IL-2-regulated growth control we
measured the expression of the CD25 by flow cytometry. In the
absence of activation, no CD25 was detected on the surface of
the clones. After 24 h of PMA plus PHA treatment, 68% of the
control cells expressed CD25. In the A3 clone, a strong inhibi-
tion was observed because only 7% of cells were labeled with
the CD25-specific antibody. In the F10 and D7 clones, the
inhibition was less potent; 40% and 28%, respectively, of these
cells were found to be CD25 positive (summarized in Table III).
Despite the variability among the three clones, together these
results indicated that the transcription of IL-2Ra requires
RelA-NF-κB activation.

DISCUSSION

In the present paper, we have reported the effect of a selec-
tive inhibition of the RelA-containing NF-κB on gene expression
in T cell clones. We have generated this cell system by stable transfection of a mutant form of IκBa which has been
shown previously to block inducible RelA and c-Rel nuclear translocation (32). The particular T cell line that we used also naturally expresses RelB. Three independent cell clones, A3, D7, and F10, were selected by limiting dilution. In agreement with previous reports (30–32), no proteolysis or ubiquitination of the mutant IxBα was detected under conditions in which signal-induced degradation of the wild type IxBα occurred. In all three of the clones, the stability of the transgene led to an efficient inhibition of the RelA-containing NF-κB DNA binding, whereas RelB-p50 DNA binding capacity was unchanged, as expected. Therefore the signal-activable NF-κB was inhibited selectively, whereas the inducible IxBα-independent RelB-p50 complex remained potentially active.

Expression of RelB is unusual in T cells. RelB is dominantly expressed in dendritic cells from both primary and secondary lymphoid organs (3, 5, 6) and in B cells at later stages of development. Its involvement in dendritic cells development was demonstrated clearly in RelB knockout mice (4, 50), but the role of RelB in gene expression remains obscure. Thus, our cell system is a convenient model for discriminating between gene transcription regulated selectively by the RelA-NF-κB and genes that may be regulated by RelB. In this respect, among the genes that we have studied the regulation of IxBα expression is of particular interest. It has been shown, indeed, that among the three κB consensus sites found in the human MADS promoter region, it is the most proximal site, κB1, that mediates PMA and TNFα activation through binding of RelA complexes (39). The κB2 site is recognized by RelA complexes, but it is not able to mediate efficient activation of the MADS promoter in cells producing only RelA and c-Rel complexes. The κB3 site was unable to bind NF-κB proteins extracted from myeloid cells (39). In RelA cells all three κB sites were reported to contribute efficiently to TNFα activation of IxBα promoter (51). In addition, transfection of Jurkat cells with RelB vectors led to increased levels of IxBα, suggesting that RelB is capable of enhancing IxBα expression (13). We used three-luciferase reporter constructs that contain, respectively, all three (0.4SK), the two upstream (0.45ΔxB), or only the κB1 (0.2SK) sites of the IxBα promoter. With these constructs the induced transcription of Luc was inhibited potently in all of the IxBα clones. These results demonstrate that in the MADS promoter, the three κB sites mediate IxBα transcription by selectively binding forms of NF-κB which are themselves regulated by IxBα. RelB was unable to compensate for the lack of NF-κB activation in this assay. However, expression of IxBα did occur but at later time points of PMA stimulation than in control cells. The expression of the wild type IxBα mirrored the increase of RelB DNA binding activity and protein. In addition, reexpression of IxBα did not occur in the MCF7 cells stably transfected with IxBα2/36A cells that do not express RelB (52). These results strongly suggest that not only does RelB regulate IxBα transcription through a site different from that which binds the prototypical NF-κB, but that NF-κB is not required for full IxBα expression when RelB is produced in sufficient amounts. This effect might be specific for human IxBα since the porcine IxBα promoter domain, which contains six κB consensus sites, was not activated by overexpression of RelB (53). The alternative RelB-specific regulation of IxBα could have functional consequences in cells that produce high levels of RelB such as dendritic cells. In such cells, NF-κB activity could be regulated negatively by IxBα overexpression due to its RelB. However, the correlation between MADS expression and RelB activation remains to be established.

The potential compensatory effect of RelB was not observed with all of the genes that we examined. For example, expression of IL-2 was inhibited dramatically at both RNA and protein levels. IL-2 promoters possess multiple regulatory sequences among which are a single κB consensus site and multiple sites capable of interacting with κB-driven protein complexes. Expression of a dominant negative mutant of κB abolishes IL-2 expression (54) probably because it coordinately blocks the IL-2 transcriptional regulation at multiple sites. In activated T cells, the major forms of NF-κB which bind to the κB site in the IL-2 promoter were shown to be p50 homodimers (55) and RelA homo- and heterodimers (56). Paradoxically, disruption of the c-rel gene in mice also inhibited induced IL-2 production despite the presence of RelA and p50 (57). A possible explanation of this effect was reported recently by Smith Shapiro et al. (58), who show that c-Rel regulation of the IL-2 promoter might be mediated by AP1 rather than directly through binding to κB sites. In contrast to c-Rel, RelA was not able to activate AP1-dependent luciferase expression (58). Here we show that in the absence of RelA dimers, RelB-p50 cannot rescue IL-2 expression. Further, the degree of IL-2 inhibition by IxBα2/36A transfection brings additional strong evidence that activation of RelA dimers is a limiting step for IL-2 transcriptional initiation.

Contrary to c-Rel disruption, inhibition of RelA dimers also diminished expression of the IL-2 receptor (CD25). Therefore, the classical NF-κB dimers seem to be involved in regulating the whole IL-2 growth control system.

In contrast to MADS, RelB was not able to enhance the signal induced expression of NFKB1 (p105), indicating that selective activation of RelA dimers is required for the signal-induced expression of p105. However, the p50 protein (the processed, functional product of the NFKB1 gene) and the p105 mRNA were produced in both parental and the IxBα transgenic cells, independent of cell activation. This suggests that the constitutive expression of NFKB1 is independent of κB enhancers.

Interestingly, c-myc expression was not inhibited by the inhibition of NF-κB. The c-myc promoter upstream κB site was shown to bind to RelA and c-Rel dimers and to be a positive regulator of the c-myc promoter in CAT assays in B lymphoma cells (59). Here we show that c-myc is expressed constitutively, not only in the parental HPB-ALL cells, but also in the IxBα2/36A transgenic cell clones. The c-myc promoter activity was only feebly enhanced by PMA plus PHA, and it was not decreased by the inhibition of RelA-NF-κB. It is therefore possible that RelB is able to activate c-myc expression constitutively. Alternatively, c-myc expression could be independent of the κB sites in HPB-ALL cells.

HIV LTR contains two direct repeats of the κB site in tandem. These κB sites are critical for the initial steps of HIV replication (24, 60, 61). It has been shown that although RelA-p50 up-regulates the HIV promoter through binding to the κB tandem sequence, c-Rel behaves as a repressor of the RelA-p50 in the context of HIV LTR and the CD25 promoter (62). In control HPB-ALL cells, PMA plus PHA activated the transcription from the HIV LTR by 42-fold over the basal level. This activation was inhibited by 90% in the IxBα2/36A transgenic cell clones. Thus RelB was unable to substitute for RelA dimers. IxBα2/36A could be a powerful tool for repressing HIV
replication in infected cells. However, since HIV replication is independent of NF-κB in the presence of the HIV Tat regulatory factor (61), we are currently investigating by infection experiments whether the effect seen in the CAT assay can be extrapolated to the viral replicative cycle.

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