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Differential interactions of Rel–NF-κB complexes with IκBα determine pools of constitutive and inducible NF-κB activity

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The Rel–NF-κB family of transcription factors plays a crucial role in the regulation of genes involved in inflammatory and immune responses. We demonstrate that *in vivo*, in contrast to the other members of the family, RelB associates efficiently only with NF-κB1 (p105–p50) and NF-κB2 (p100–p52), but not with cRel or p65. The RelB–p52 heterodimers display a much lower affinity for IκBα than RelB–p50 heterodimers or p65 complexes. However, similarly to the other Rel–NF-κB complexes, RelB–p52 can upregulate the synthesis of IκBα leading to the cytoplasmic trapping of dimers which have a higher affinity for the inhibitor. We suggest that a hierarchy of interactions between IκBα and the different Rel–NF-κB complexes governs their cellular distribution. This results in the presence of two distinct pools of NF-κB activity which differ in their composition: one a constitutive nuclear and the other an inducible cytoplasmic activity.

Key words: gene regulation/IκBα interaction/Rel–NF-κB complex/transcription factor

Introduction

One of the central problems of molecular biology is to understand the mechanisms underlying temporal and tissue-specific gene expression and activation of specific genetic programs in response to extracellular stimuli. Accumulated evidence indicates that transcription initiation represents the major regulatory event controlling specificity of gene expression. This is carried out by the combined action of transcription factors interacting with the regulatory elements of genes. Rapid responses to external signals are often mediated by the inducible activation of transcription factors by post-translational mechanisms, as opposed to their *de novo* synthesis. Examples of post-translational mechanisms serving this purpose are the ligand-induced DNA binding of nuclear receptors (reviewed in Evans, 1988; Green and Chambon, 1988) and phosphorylation (reviewed in Hunter and Karin, 1992). Interactions with inhibitory molecules represent yet another mechanism to control the activity of transcription factors which would be amenable to rapid induction. Associations of NF-κB proteins with the IκB family of inhibitors is the best studied example of such a controlling mechanism.

The Rel–NF-κB family of transcription factors is involved in the regulation of immune and acute-phase responses at the transcriptional level (reviewed in Grilli *et al.*, 1993; Liou and Baltimore, 1993). The mammalian members of this family include p105–p50 (NF-κB1, also known as KBF1 and H2TF1), p100–p52 (NF-κB2, also known as p50B, p49 and Lyt-10), cRel, p65 (RelA) and RelB. All members of the Rel–NF-κB family share a 300 amino acid region of homology called the Rel homology domain (RHD) which mediates dimerization and DNA binding (for a review see Baeuerle, 1991; Blank *et al.*, 1992; Bose, 1992; Gilmore, 1992; Nolan and Baltimore, 1992; Grilli *et al.*, 1993; Grimm and Baeuerle, 1993). The activity of Rel–NF-κB complexes is regulated by their interactions with the various IκB molecules (reviewed in Nolan and Baltimore, 1992; Beg and Baldwin, 1993; Gilmore and Morin, 1993). Association with IκBα results in the cytoplasmic retention of NF-κB dimers which form an inactive, inducible cytoplasmic pool of NF-κB. NF-κB activity, while constitutive in mature B cells, can be rapidly induced in other cells by a variety of agents including cytokines, pathogen infections or reactive oxygen intermediates (Grilli *et al.*, 1993; Grimm and Baeuerle, 1993). The induction of Rel–NF-κB activity involves the release of IκBα from the Rel–NF-κB complexes, allowing their rapid translocation to the nucleus (reviewed in Beg and Baldwin, 1993; Gilmore and Morin, 1993). The biochemical mechanism underlying this dissociation has not been fully elucidated yet, but available evidence indicates that it involves IκBα phosphorylation (Cordle *et al.*, 1993; Devary *et al.*, 1993; Diaz-Meco *et al.*, 1993; Finco and Baldwin, 1993; Li and Sedivy, 1993) and degradation (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Henkel *et al.*, 1993; Rice and Ernst, 1993; Sun *et al.*, 1993; Chiao *et al.*, 1994; Frantz *et al.*, 1994). The precursors of p50 and p52, p105 and p100 respectively, show significant homology to the IκB molecules in their C-terminal region, and it has been suggested that they also trap NF-κB complexes in the cytoplasm (Inoue *et al.*, 1992; Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann *et al.*, 1993; Scheinman *et al.*, 1993; Sun *et al.*, 1994).

It has been demonstrated recently that p65- and cRel-containing complexes induce expression of IκBα, creating an autoregulatory loop in which increased synthesis of inhibitor serves to restore the uninduced state of the cell (Brown *et al.*, 1993; de Martin *et al.*, 1993; Scott *et al.*, 1993; Sun *et al.*, 1993). Such an autoregulatory mechanism would ensure the transient nature of the NF-κB-mediated gene activation. Thus, one way to regulate the NF-κB-mediated genetic response is by controlling the cellular localization of the dimers. Rapidly accumulating evidence

suggests that the various Rel−NF-κB complexes have different target specificities and that levels of transcriptional activation elicited by these transactivators differ depending on the particular κB binding site, despite comparable levels of DNA binding (Schmid *et al.*, 1991; Fujita *et al.*, 1992; Hansen *et al.*, 1992; Kretzschmar *et al.*, 1992; Kunsch *et al.*, 1992; Nakayama *et al.*, 1992; Perkins *et al.*, 1992; Tan *et al.*, 1992; Sica *et al.*, 1992; Kunsch and Rosen, 1993; Narayanan *et al.*, 1993; Shu *et al.*, 1993; Hansen *et al.*, 1994). These data thus underscore the importance of individual complexes and indicate that combinatorial associations of Rel−NF-κB proteins provide another way of controlling the specificity of the NF-κB-mediated responses.

We have been interested in understanding the physiological role of RelB (Ryseck *et al.*, 1992; Carrasco *et al.*, 1993; Dobrzanski *et al.*, 1993). In this report we analyse the capacity of RelB to form complexes with other members of the family and the interactions of RelB heterodimers with IκBα—the prototype of an IκB inhibitor. We have found that *in vivo*, RelB, in contrast to p65 and cRel, forms dimers only with p50, p52 and their respective precursors p105 and p100. Interestingly, the RelB−p52 heterodimer does not associate efficiently with IκBα; however, similarly to the other Rel−NF-κB complexes, RelB heterodimers can upregulate the levels of endogenous IκBα. Our data indicate that this leads to the cytoplasmic trapping of Rel−NF-κB complexes with a higher affinity for the inhibitor, like the p65-containing complexes, thus changing the composition of the NF-κB activity in the cell. We demonstrate further that IκBα has a differential affinity for the various Rel−NF-κB complexes *in vivo*, suggesting a hierarchy of interactions which determines the cellular distribution of Rel−NF-κB dimers. Based on these results we propose a model suggesting how the specificity of action of the NF-κB activity can be achieved given the large number of possible combinations present in the cell.

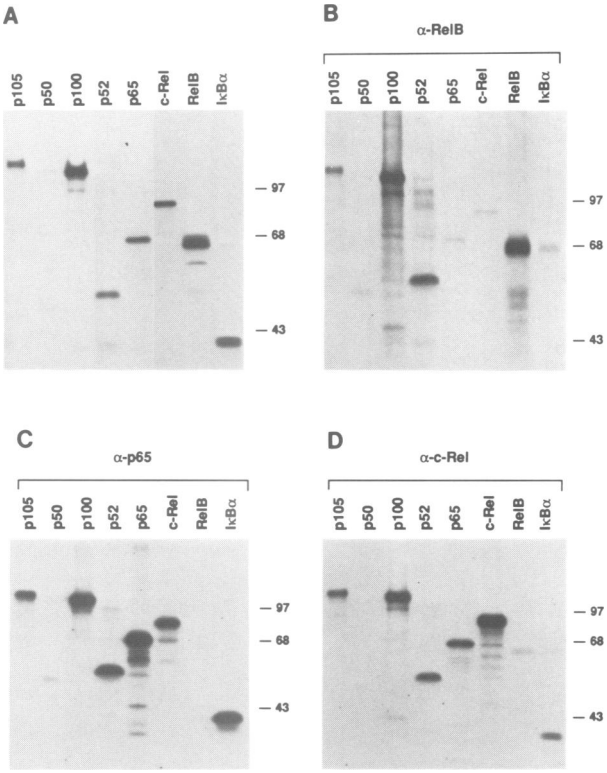

Fig. 1. Analysis of Rel−NF-κB complexes in Daudi cells. Daudi cells were labelled with [^{35}S]methionine and lysed under denaturing (**A**) or native (**B**−**D**) conditions. To compare the ability of RelB, p65 and cRel to form complexes *in vivo*, native cell lysates were incubated with the corresponding antibody. Immunocomplexes were precipitated with protein A−Sepharose. The immunoprecipitated proteins were released, sequentially reprecipitated with the indicated antibody and subjected to SDS−PAGE, followed by fluorography. All immunoprecipitations were performed as described in Materials and methods. (A) Total amounts of Rel−NF-κB proteins present in Daudi cells. Each protein was immunoprecipitated twice with the specific antibody. (B) Analysis of RelB-containing complexes. (C) Analysis of p65-containing complexes. (D) Analysis of cRel-containing complexes.

Results

RelB is involved in a limited number of interactions in vivo

To determine the capacity of RelB to form complexes with other Rel−NF-κB proteins *in vivo*, T and B cell lines were screened for the expression of RelB. Daudi cells (a human B cell line) were chosen for further analysis because they express high levels of RelB and other family members (Figure 1A). To identify which members of the Rel−NF-κB family interact with RelB, Daudi cells were labeled with [^{35}S]methionine, and proteins associated with RelB were screened using specific antibodies. The results indicate that RelB forms heterodimers with p52 and p50, and with their respective precursors p100 and p105 (Figure 1B). The amount of p100−p52 and p105−p50 associated with RelB is similar to that found with p65 and cRel (Figure 1C and D). Since RelB, p65 and cRel are expressed at comparable levels, the results suggest that their capacities to interact with p100−p52 and p105−p50 are similar. However, in contrast to p65 which readily forms complexes with cRel (Figure 1C and D; Hansen *et al.*, 1992, 1994), RelB interacts with cRel only weakly, if at all (Figure 1B and D). No significant interaction between RelB and p65 was detected *in vivo* (Figure 1B and C), in accordance with our *in vitro* results (R.-P.Ryseck and P.Dobrzanski, unpublished results). These data demonstrate that RelB has a more restricted ability to form dimers compared with the other members of the family.

RelB−p52 heterodimer is not inhibited by any of the known IκB molecules

Since the activity of the members of the Rel−NF-κB family of transcription factors is regulated by their interaction with the IκB family of molecules (for a review see Nolan and Baltimore, 1992; Beg and Baldwin, 1993; Gilmore and Morin, 1993), we analyzed whether RelB-containing heterodimers are associated with IκBα *in vivo*. Surprisingly, we found very little if any IκBα co-immunoprecipitated with RelB complexes (Figure 1B), in contrast to those containing p65 or cRel which are associated with significant amounts of IκBα (Figure 1C and D). This result suggests that RelB−p52, which as shown above represents the major RelB-containing heterodimer in Daudi cells, does not interact efficiently with IκBα. Our previous *in vitro* observation that the DNA binding of RelB−p50 complexes can be blocked by IκBα (Tewari *et al.*, 1992),

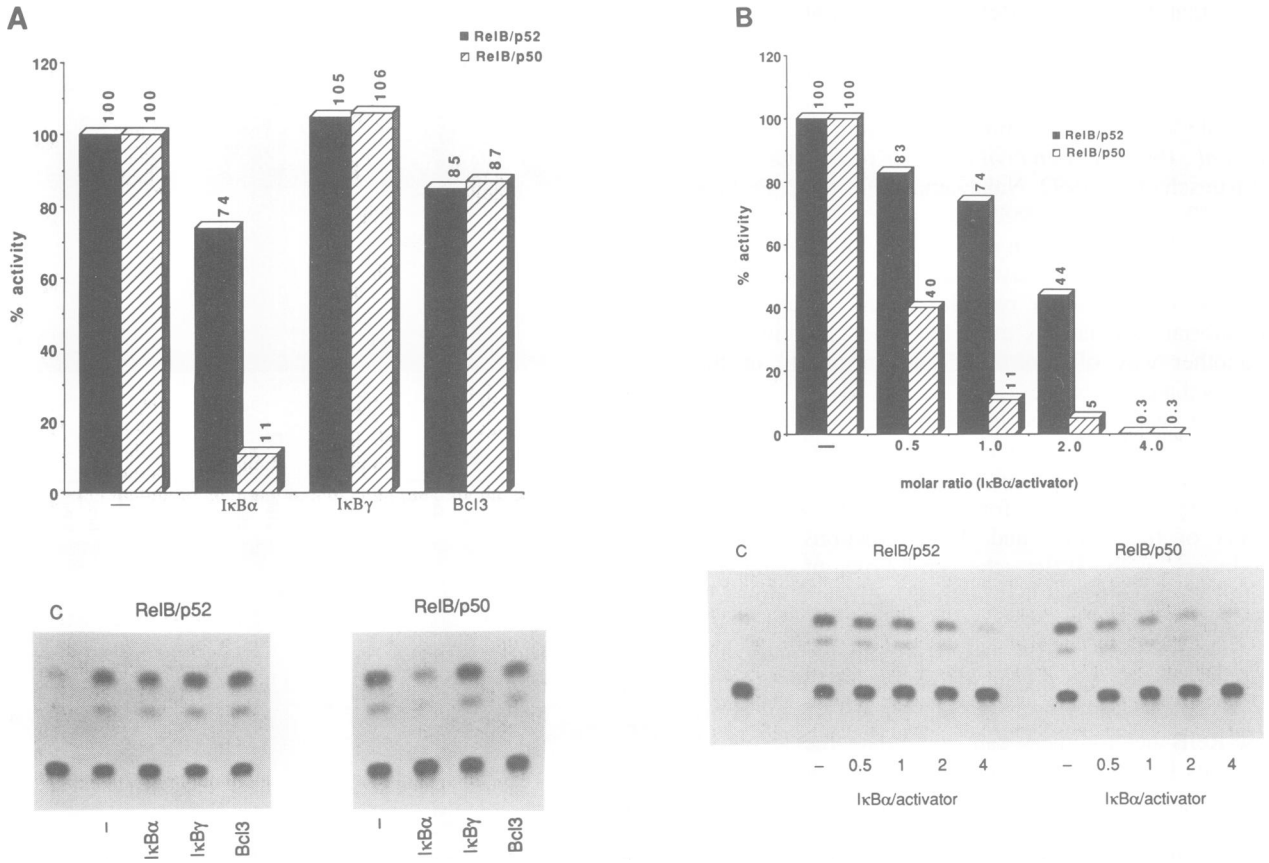

Fig. 2. Effect of different IκB molecules on the transcriptional activity of RelB−p52 and RelB−p50 heterodimers. Jurkat cells were cotransfected with expression vectors encoding RelB and p52, or RelB and p50, together with vectors expressing IκBα, IκBγ or Bcl3, as indicated, and the 8× κB−tk-CAT reporter plasmid. Cells were harvested 48 h after transfection. Transfections and CAT assays were performed as described in Materials and methods. (**A**) Effect of different IκB proteins on RelB−p52 and RelB−p50 activity. Equal amounts (3 μg) of each of the expression vectors were cotransfected with 2 μg of the reporter plasmid. The total amount of DNA transfected was adjusted to 18 μg with the expression vector pMexneo. Upper panel: transcriptional activity was calculated as an average of three independent experiments quantitated on a PhosphorImager. The expression generated by the cotransfection of vectors expressing RelB and p50 or RelB and p52 represents 100% activity. Lower panel: representative CAT assay of a transfection experiment. Transfection of pMexneo alone serves as a control (C). (**B**) RelB−p52 and RelB−p50 are differentially inhibited by IκBα. Constant amounts (2.5 μg) of each of RelB-, p52- and p50-expressing vectors were cotransfected with increasing amounts, as indicated, of vector expressing IκBα and 2 μg of the reporter plasmid. Details of upper and lower panels are as described in (A).

prompted us to test whether RelB−p52 DNA binding can also be inhibited by IκBα. In agreement with the weak interactions observed *in vivo*, *in vitro*-translated RelB−p52 heterodimers consistently required 2- to 4-fold more IκBα than RelB−p50 heterodimers for the full inhibition of DNA binding (not shown).

To determine whether the observed differential inhibition by IκBα of the DNA binding of RelB complexes *in vitro* is reflected in a differential repression of their transcriptional activity, transient transfection assays were performed. Jurkat cells were cotransfected with equimolar amounts of expression vectors coding for RelB, IκBα and either p52 or p50, together with a reporter construct carrying the chloramphenicol acetyltransferase (CAT) gene under the control of κB binding sites. The cotransfection of equimolar amounts of vector encoding IκBα results in a strong inhibition of the RelB−p50 transcriptional activity (down to 11% of the control without inhibitor), whereas the activity of RelB−p52 is only slightly reduced (Figure 2A). Therefore, it was of interest to determine whether the transcriptional activity of RelB−p52 heterodimers could be efficiently inhibited by other members of the IκB family, namely IκBγ (Hatada *et al.*, 1992; Inoue *et al.*, 1992; Liou *et al.*, 1992) and Bcl3 (Ohno *et al.*, 1990; Kerr *et al.*, 1991; Nolan *et al.*, 1993). To this end, RelB and p52 or RelB and p50, were cotransfected together with equimolar amounts of vectors coding for IκBγ or Bcl3. As demonstrated in Figure 2A, IκBγ showed no effect and Bcl3 only minimal inhibition of the transcriptional activity of both RelB heterodimers. Western blot analysis verified that all vectors expressed comparable amounts of protein (not shown).

To analyse further the differential effect of IκBα on the transcriptional activity of RelB−p52 and RelB−p50 heterodimers, constant amounts of activators were cotransfected with increasing amounts of IκBα. As shown in Figure 2B, four times more IκBα was required to significantly inhibit RelB−p52 activity (56%) compared with RelB−p50 (60%). The activity of RelB−p50 was strongly inhibited by equimolar amounts of IκBα expression vector, whereas a 2-fold molar excess basically eliminated all transcriptional activity. In contrast, a 4-fold excess of the IκBα expression vector was required to completely inhibit the transcriptional activity of

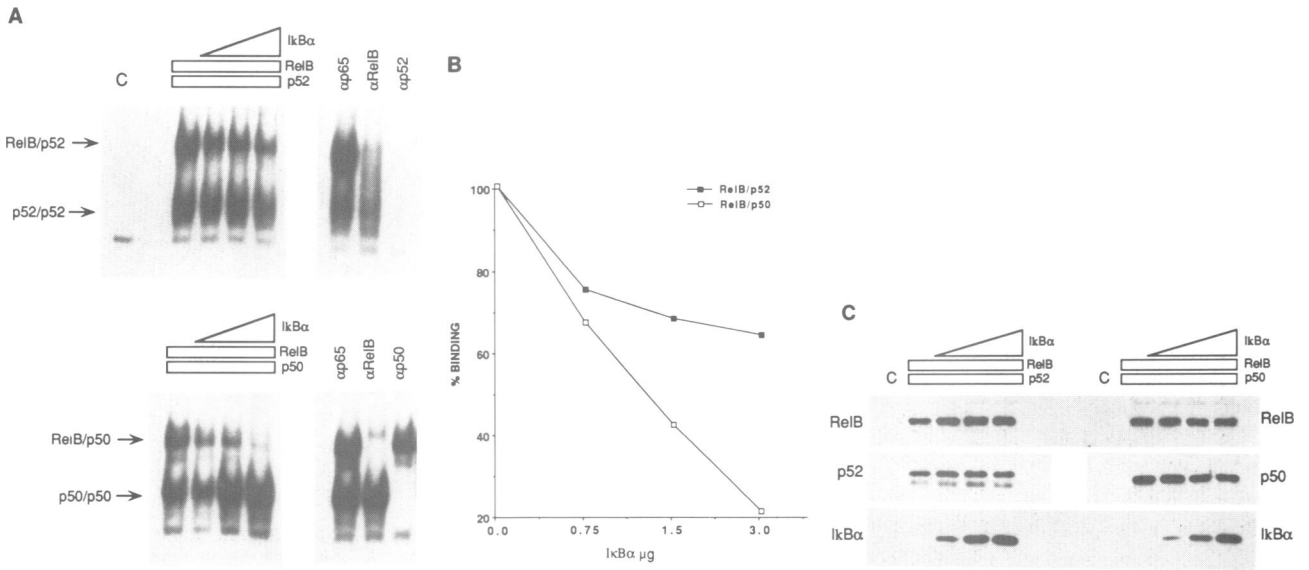

Fig. 3. Differential effect of IκBα on RelB−p52 and RelB−p50 DNA binding activity *in vivo*. COS cells were transfected with constant amounts (2 μg each) of vectors expressing RelB and p52 or RelB and p50, together with increasing amounts of IκBα expressing vector (as indicated). The total amount of DNA transfected was adjusted to 10 μg with expression vector. Whole-cell extracts were prepared 48 h after transfection. (**A**) EMSA with extracts prepared from cells transfected with the indicated expression vectors. To confirm the identity of the extract-generated bands, specific antibodies were included in binding reactions, as shown. Extract from cells transfected with the expression vector alone served as a control (C). (**B**) Quantitation of EMSA results. An average of two independent experiments quantitated with a PhosphorImager is shown. The values obtained for heterodimers were normalized to the amount of p52 or p50 homodimers binding, and serve as an internal control. Binding of the heterodimer without cotransfected IκBα represents 100% activity. (**C**) Expression of RelB, p52, p50 and IκBα in transfected COS cells. Parallel whole-cell extracts used for EMSA (A) were subjected to SDS−PAGE, followed by Western blotting analysis using specific antibodies, as indicated. Proteins were detected using the ECL system.

RelB−p52, further confirming that IκBα has a lower affinity for RelB−p52 than for RelB−p50. These results indicate that *in vivo* RelB−p52 is not efficiently inhibited by any of the known inhibitors (IκBα, IκBγ and Bcl3), whereas the activity of RelB−p50 can be modulated only by IκBα.

To investigate whether the observed differential inhibition of transcriptional activity of RelB−p52 versus RelB−p50 could be correlated with the inhibition of DNA binding, COS cells were transfected with vectors that express high levels of RelB, p52, p50 and IκBα. Constant amounts of RelB and p52 or RelB and p50, were cotransfected together with increasing amounts of IκBα. Whole-cell extracts were prepared from transfected cells and κB binding activity was determined in electrophoretic mobility shift assays (EMSA; Figure 3A). The control lane showed very little binding activity, whereas extracts from transfected cells generated two major bands. As determined by specific antibodies, the upper band corresponds to either the RelB−p52 or RelB−p50 heterodimer, and the lower band represents either the p52 or p50 homodimer. The anti-p50 antibody completely abolished binding of the p50 homodimer and reduced, but did not eliminate, binding of the RelB−p50 heterodimer, in accordance with previous reports demonstrating that the p50-specific antiserum reacts better with homodimers (Kieran *et al.*, 1990; Lernbecher *et al.*, 1993). Even at the lowest amount of transfected IκBα, the binding of the RelB−p50 complex is strongly reduced. The highest amount of inhibitor transfected almost abolished binding of RelB−p50, whereas RelB−p52 still displayed significant binding. The binding activity of the homodimers was, as expected, not affected by the increasing amounts of IκBα expressed and served as an internal control. Figure 3B shows the quantitation of two independent experiments. The data demonstrate that *in vivo* the DNA binding activity of RelB−p52 is much more resistant to inhibition by IκBα than is the binding of RelB−p50. Western blot analysis verified that comparable amounts of protein were expressed in transfected cells (Figure 3C). These data validate our interpretation of the EMSA results which are consistent with those obtained in the CAT assays (Figure 2B).

RelB heterodimers increase levels of endogenous IκBα

The lack of efficient inhibition of RelB−p52 activity by any of the known IκB molecules *in vivo* was particularly intriguing in view of the recent observations that other members of the Rel−NF-κB family, namely p65 and cRel, induce expression of IκBα, generating an autoregulatory feedback mechanism (Brown *et al.*, 1993; de Martin *et al.*, 1993; Scott *et al.*, 1993; Sun *et al.*, 1993). To test whether the RelB heterodimers can increase the level of endogenous IκBα, COS cells were transfected with vectors expressing RelB and p52, RelB and p50 or p65 alone. After 48 h the level of endogenous IκBα was analyzed by Western blot (Figure 4A). RelB−p52 and RelB−p50 strongly increase the cellular level of IκBα. We consistently observed higher levels of IκBα protein following transfection of p65 than of RelB heterodimers. This is probably due to the increased stability of IκBα resulting from its stronger interaction with p65 than with RelB−p52 or RelB−p50. Nevertheless, the results indicate that RelB−p52 and RelB−p50 strongly induce expression of IκBα.

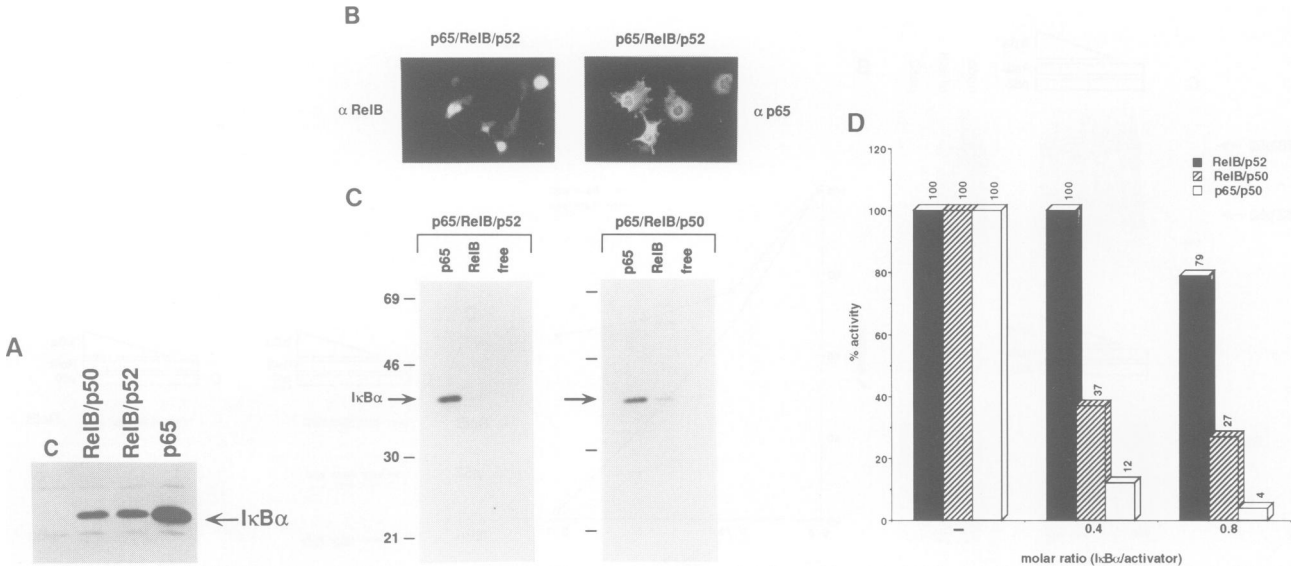

Fig. 4. (**A**) RelB heterodimers increase the levels of endogenous IκBα. COS cells were transfected with expression vectors encoding the indicated Rel–NF-κB proteins. As a control, the expression vector alone was transfected (C). Whole-cell extracts prepared 48 h after transfection were subjected to SDS–PAGE, followed by Western blot analysis using the ECL system. (**B**) Immunofluorescence analysis of the cellular localization of p65 and RelB following cotransfection of vectors expressing p65, RelB and p52 into COS cells. Fixed cells were subjected to immunofluorescence using anti-RelB- (left panel) or anti-p65 (right panel)-specific antibodies. FITC-conjugated donkey antibody against rabbit IgG was used as a secondary antibody. Similar results were obtained for p65, RelB and p50 cotransfections (not shown). (**C**) IκBα differentially interacts with p65 and RelB complexes *in vivo*. COS cells were transfected with p65, RelB and p52 (left panel) or p65, RelB and p50 (right panel). Forty-eight h later, cells were labeled with [^{35}S]methionine and lysed under native conditions. Extracts were incubated sequentially with antibodies against p65, RelB and IκBα. The immunoprecipitated complexes were released and tested for the presence of IκBα using specific antibodies. Lanes p65 and RelB indicate the IκBα found associated with p65 and RelB, respectively. IκBα not associated with p65 or RelB, i.e. what remains in the extract following depletion of p65 and RelB complexes by the corresponding antibodies, is referred to as 'free'. All incubations with antibodies and protein A–Sepharose were performed for 2 h at 4°C. (**D**) Effects of IκBα on the transcriptional activity of RelB–p52, RelB–p50 and p65–p50. Jurkat cells were cotransfected with a constant amount (3 μg) of each of RelB-, p65-, p52- and p50-expressing vectors, together with the indicated amount of IκBα expressing vector and 2 μg of the reporter plasmid. The total amount of transfected DNA was adjusted to 12 μg with expression vector. All other details are as in the legend to Figure 1.

RelB heterodimers represent a constitutive NF-κB activity

The induction of IκBα by RelB–p52, together with its low affinity for the inhibitor, suggested that RelB–p52 might be part of a constitutive nuclear NF-κB activity. Its ability to upregulate IκBα synthesis would ensure cytoplasmic trapping of p65-containing complexes. This assumption was tested in triple cotransfection experiments in which COS cells were cotransfected with vectors expressing p65, RelB and p52 or p65, RelB and p50. After 48 h the cellular localization of the transfected proteins was determined by immunofluorescence and Western blot analyses of the nuclear and cytoplasmic extracts. Immunofluorescence assays demonstrate (Figure 4B) that in ~90% of expressing cells p65 was exclusively cytoplasmic, whereas 10% of cells showed staining in both cytoplasm and nucleus. In contrast, RelB staining was found mainly in the nucleus. By Western blot analysis we further verified that RelB, p52 and p50 were predominantly nuclear, whereas the majority of p65 was found in the cytoplasmic fraction (not shown). In control experiments RelB–p52 and RelB–p50 alone were transfected into COS cells and their cellular localization was analysed by immunofluorescence and Western blotting of nuclear and cytoplasmic fractions. We found that both heterodimers were predominantly nuclear, suggesting that weak interactions of RelB complexes with IκBα are not limited to lymphoid cells (not shown). To ensure that the majority of the RelB molecules are complexed, cotransfections were performed with a relative excess of p52 or p50, so that the observed nuclear localization of RelB is not due to free, uncomplexed RelB migrating to the nucleus, but reflects the true cellular distribution of RelB–p52 and RelB–p50. The difference in the cellular localization of p65- and RelB-containing complexes suggests that RelB–p52 represents the constitutive NF-κB activity, in contrast to p65 complexes which remain in the cytoplasm, probably due to their association with the endogenous IκBα. Interestingly, under these conditions RelB–p50 behaves like RelB–p52 and is found predominantly in the nucleus (not shown). This indicates that the affinity of RelB–p50 for IκBα, although higher than that of RelB–p52, is still lower than that of p65 complexes, suggesting that *in vivo* in the presence of limiting amounts of IκBα, RelB–p50 might in part contribute to the constitutive NF-κB activity.

To test whether the cytoplasmic localization of p65 in transfected COS cells was due to its association with endogenous IκBα, the amount of IκBα associated with p65 and RelB was estimated by immunoprecipitation following [^{35}S]methionine labeling of the cells. Figure 4C demonstrates that ~90% of the total cellular IκBα is found associated with p65, whereas only a minor fraction interacts with RelB–p52 or RelB–p50 heterodimers. The absence of free IκBα indicates that the inhibitor is present in limiting quantities. Since p65 and RelB were expressed at similar levels (not shown), the observed differences in the amount of IκBα associated with these complexes

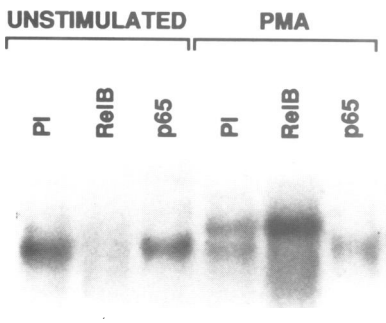

Fig. 5. Analysis of Rel–NF-κB binding activity in Daudi cells. Whole-cell extracts were prepared from Daudi cells which were either unstimulated or incubated for 45 min with 100 ng/ml of PMA. κB binding activity was tested in EMSA using a ^{32}P-labeled palindromic κB probe. To analyze the identity of DNA binding complexes, specific antibodies were included in the binding assays as indicated. Equal amounts of a preimmune serum were included in control reactions (PI).

reflects the real differences in their affinity for IκBα. These results suggest a hierarchy of interactions between IκBα and different RelB and p65 complexes. To compare more directly the affinity of IκBα for RelB–p52, RelB–p50 and p65–p50, transient transfection assays were performed. Jurkat cells were cotransfected with constant amounts of RelB–p52, RelB–p50 and p65–p50 together with increasing amounts of inhibitor. Figure 4D demonstrates that transcriptional activity of p65–p50 heterodimer is indeed the most sensitive to the IκBα-mediated inhibition, followed by that of RelB–p50. These data further confirm that IκBα can differentially interact with various Rel–NF-κB complexes. Thus, *in vivo*, IκBα has much higher affinity for p65 complexes than for any of the RelB heterodimers, providing an explanation for the cytoplasmic localization of p65 in cotransfection assays.

Our results further suggest that in unstimulated cells expressing RelB, p50, p52 and p65, RelB complexes will constitute the major nuclear κB binding activity, whereas the p65 complexes would be associated with IκBα and therefore be retained in the cytoplasm. To verify these predictions, the NF-κB binding activity present in Daudi cells was analyzed by EMSA. The extract from unstimulated cells generated a major band which contained RelB but not p65, as demonstrated by its sensitivity to the corresponding antibodies (Figure 5), indicating that in unstimulated cells there is very limited, if any, κB binding activity which could be attributed to p65 complexes. Activation of cells with phorbol 12-myristate 13-acetate (PMA) induces a new binding activity which contains p65, as demonstrated by its abolition by the corresponding antibody. In contrast, DNA binding of RelB heterodimers is not increased by treatment with PMA, suggesting that they are constitutively active in the cell. PMA treatment has been shown to induce κB binding activity by releasing Rel–NF-κB dimers from their association with IκBα (Henkel *et al.*, 1993; Sun *et al.*, 1993). The data thus demonstrate that in cells expressing RelB and p65 the latter remains complexed with IκBα and as a consequence represents the inducible NF-κB activity, while RelB complexes represent the constitutive activity.

Discussion

Interactions of RelB with other members of the Rel–NF-κB family

We have determined the capacity of RelB to interact with the other members of the NF-κB family *in vivo*. Our studies demonstrate that RelB forms heterodimers only with p50 and p52 and their respective precursors p105 and p100. The amounts of p50–p105 and p52–p100 associated with RelB reflect the relative abundance of these proteins in the cell, suggesting that RelB has similar affinity for these molecules. We also found that p65 and cRel associate with p52–p100 and p50–p105 with similar affinities to RelB. These results led us to conclude that the interactions of RelB and p65 with p52–100 and p50–105 are governed by the concentrations of the relevant proteins in the cell. However, in contrast to p65 which readily forms complexes with cRel (Hansen *et al.*, 1992, 1994), we found no evidence of RelB–cRel interactions *in vivo*, in accordance with our *in vitro* observations. We also failed to detect any significant interactions between RelB and p65. These data, together with the inability of RelB to form homodimers (Ryseck *et al.*, 1994), indicate that RelB, compared with the other members of the family, is involved in a limited number of interactions *in vivo*. Such a restricted ability to form dimers might suggest that RelB plays a specific function in regulating the expression of a particular set of genes *in vivo*.

Regulation of the activity of RelB heterodimers by the IκB molecules

Since RelB–p50 and RelB–p52 heterodimers are strong transactivators (Bours *et al.*, 1992; Ryseck *et al.*, 1992), we analyzed if this activity can be modulated by members of the IκB family. We found that IκBα displayed a differential inhibition on both complexes. Several lines of evidence, including EMSA of proteins generated *in vitro* and *in vivo*, and transient transfection assays demonstrated that RelB–p52 has much lower affinity for IκBα than RelB–p50. However, transcriptional activity of RelB–p52 can be fully inhibited by an excess of IκBα (Figure 2). In view of the recent findings that the uncomplexed IκBα is degraded rapidly in the cells (Beg *et al.*, 1993; Brown *et al.*, 1993; Henkel *et al.*, 1993; Rice and Ernst, 1993; Sun *et al.*, 1993; Chiao *et al.*, 1994; Frantz *et al.*, 1994), it seems unlikely that such an excess of IκBα can be achieved *in vivo*. The lack of interaction between RelB–p52 and IκBα in Daudi cells, despite the abundance of the inhibitor, supports the notion that IκBα is a weak modulator, if at all, of RelB–p52 activity *in vivo*. Interestingly, we have also found that none of the other known IκB molecules, IκBγ or Bcl3 substantially inhibit the activity of RelB–p52 or RelB–p50. The lack of efficient *in vivo* inhibition of the RelB–p52 heterodimer by any of the known IκB molecules suggests that this complex might function as a constitutively active transcription factor. These data emphasize the importance of the processing of the precursors p100 and p105 as a regulatory event controlling NF-κB activity (Inoue *et al.*, 1992; Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann *et al.*, 1993; Scheinman *et al.*, 1993; Sun *et al.*, 1994).

One might expect, however, that such a highly regulated system as NF-κB–IκB should have the ability to modulate

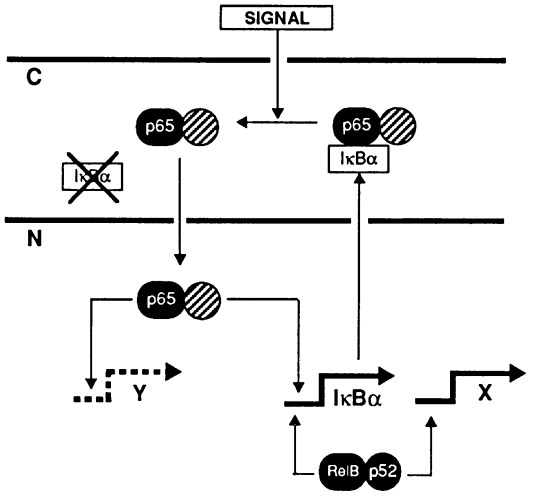

Fig. 6. Model explaining how the differential interactions with IκBα determine the constitutive versus inducible character of Rel–NF-κB activity leading to the existence of two pools of complexes which differ in their composition and cellular localization. 'X' represents a subset of NF-κB-regulated genes driven by the constitutively active RelB–p52 dimers. In response to the external signal the inducible pool, represented here by p65 complexes, triggers a specific genetic program (Y). The dashed circle is any member of the Rel–NF-κB family which combines with p65 to form dimers and with high affinity for IκBα. Cytoplasm (C) and nucleus (N) are indicated. See text for a discussion.

the activity of RelB–p52. It is then plausible that in certain cells RelB–p52 activity could be regulated by an as yet unknown inhibitor. One might also consider other possible regulatory mechanisms such as post-transcriptional modification or modulatory interactions with other proteins.

Differential interactions of Rel–NF-κB complexes with IκBα and their functional consequences

The lack of efficient inhibition of the RelB–p52 activity by any of the known IκB molecules *in vivo* was of particular interest considering the recent reports which show that other members of the Rel–NF-κB family, p65 and cRel, induce expression of IκBα, generating an autoregulatory feedback mechanism (Brown *et al.*, 1993; de Martin *et al.*, 1993; Scott *et al.*, 1993; Sun *et al.*, 1993). The findings that various Rel–NF-κB complexes, including both RelB heterodimers, are able to upregulate the synthesis of IκBα, together with our observation that these complexes exhibit differential affinity for IκBα (Figure 4D), have an important implication for the regulation of NF-κB activity. It suggests a model (Figure 6) which predicts that RelB–p52 would function as a constitutive nuclear NF-κB activity, regulating expression of specific genes, including IκBα. This would ensure a constant supply of IκBα to the cytoplasm, trapping NF-κB molecules with high affinity for IκBα, like p65–p50 or p65–p52. These complexes would then represent the inactive cytoplasmic reservoir of the inducible NF-κB activity. The results of the triple transfection experiments (Figure 4B) and the analysis of the constitutive and PMA-inducible NF-κB activity in extracts from Daudi cells (Figure 5) fully support this model. In addition, RelB, cRel and p65 complexed with precursors p100 and p105 are also part of the cytoplasmic fraction. However, since the proteolytic processing, even after activation, is rather slow (Mercurio *et al.*, 1993), these complexes would not contribute to the rapid alteration of the NF-κB activity in response to the changing environment.

Our model predicts that the constitutive nuclear and the inducible cytoplasmic pools would consist of different Rel–NF-κB complexes, e.g. RelB–p52 versus p65-containing complexes. This is important in view of the rapidly accumulating evidence indicating that different Rel–NF-κB complexes exhibit vastly different target specificities (Schmid *et al.*, 1991; Fujita *et al.*, 1992; Hansen *et al.*, 1992; Kretzschmar *et al.*, 1992; Kunsch *et al.*, 1992; Nakayama *et al.*, 1992; Perkins *et al.*, 1992; Sica *et al.*, 1992; Tan *et al.*, 1992; Kunsch and Rosen, 1993; Narayanan *et al.*, 1993; Shu *et al.*, 1993). Therefore, the differential regulation of the various complexes would enable the cell to turn on specific genetic programs in response to external stimuli by activating a specific set of Rel–NF-κB complexes. One could further predict that the inducible activity would activate genes involved in rapid responses like the acute-phase response and inflammation. The constitutive activity would regulate those genes whose protein products are required for a prolonged period of time, like immunoglobulin G or major histocompatibility complex. According to the model, the predicted cellular distribution of the complexes would be determined by their relative affinity for IκBα and by the relative abundance of the inhibitor and of NF-κB complexes. Those with the highest affinity for IκBα, like p65 heterodimers, would be mainly cytoplasmic and would represent the inducible pool. On the other hand, complexes with the lowest affinity for IκBα, like RelB–p52, would be predominantly nuclear and provide the constitutive activity. Furthermore, one could envisage that the constitutive versus inducible nature of certain complexes, like RelB–p50, would depend on the context of the Rel–NF-κB complexes expressed in the cell. In the presence of only RelB–p52 complexes, RelB–p50 would be a primary target for the cytoplasmic trapping by IκBα. However, in the presence of p65–p50 heterodimers the latter would mainly associate with the inhibitor, allowing RelB–p50 complexes to move to the nucleus. This would provide the system with the needed flexibility and would assure its dynamic nature. In a recent report, Lernbecher *et al.* (1993), using transgenic mice, correlated the expression of a reporter construct driven by κB binding sites with the presence of constitutively active RelB complexes in lymphoid tissues, supporting our model in an animal system. Although initially this concept was developed based on the experiments involving RelB and p65 heterodimers only, it can easily incorporate the cRel-containing complexes. It remains to be tested how the affinity of cRel complexes for IκBα compares *in vivo* with that of other Rel–NF-κB complexes. However, the recent observation by Liou and Baltimore (1993), who found that in certain B cell lines cRel–p50 is constitutively active, whereas p65–p50 is inducible, further supports our model and indicates that p65 heterodimers might indeed represent the 'ultimate' NF-κB-inducible activity. The very tight regulation of the p65–p50 activity suggests the importance of this heterodimer in cellular responses. Our model does not take into account the possible contribution of homodimers like p50–p50 or complexes between

p50 and Bcl3, since there is some controversy as to what the role of these complexes might be (Franzoso *et al.*, 1992; Fujita *et al.*, 1992). The existence of the nuclear and cytoplasmic pools of NF-κB complexes, which differ in composition, explains at least in part the specificity of action that can be achieved given the large number of possible NF-κB complexes.

Materials and methods

Cell culture and transfections

Jurkat cells were grown, electroporated and processed as described previously (Dobrzanski *et al.*, 1993). An 8× κB–tk-CAT reporter vector was used in all experiments. CAT assays were performed as described (Gorman *et al.*, 1982). Reactions were normalized for β-galactosidase and/or protein concentrations. All CAT assays were quantitated in a PhosphorImager (Molecular Dynamics).

COS cells were transfected by DEAE–dextran methods as described (Seed and Aruffo, 1987). Typically, a 10 cm dish was transfected with 1–5 μg of each expression vector, as indicated in the figure legends. The total amount of DNA was adjusted with expression vector alone.

Antibodies

Specific antibodies used in these studies have been described previously (Ryseck *et al.*, 1992; Tewari *et al.*, 1992). The regions used for generating the fusion proteins were: for the murine cRel, amino acids 1–185 and 366–588; for RelB, amino acids 348–538; for p65, amino acids 399–489; for the N-terminal region of NF-κB1, amino acids 1–120 (p50 subunit); for the C-terminal region of NF-κB1, amino acids 575–972 (p105); for the N-terminal region of the human NF-κB2, amino acids 1–82 (p52); for the C-terminal region of NF-κB2, amino acids 775–901 (p100); and for the rat IκBα, the complete coding sequence.

Labeling and immunoprecipitations

COS cells were rinsed twice with DMEM lacking methionine 48 h after transfection and then labeled for 2 h with [^{35}S]methionine (0.4 mCi/ml) in methionine-free medium.

For the labeling of Daudi cells, 2×10^6 cells were rinsed twice with DMEM lacking methionine and then labeled for 24 h with 1 mCi of [^{35}S]methionine/ml in DMEM containing 1 mg/l methionine supplemented with 20% dialyzed fetal calf serum. Immunoprecipitations under denaturing and native conditions were performed as described previously (Kovary and Bravo, 1991).

Immunofluorescence

COS cells were transfected with the indicated expression vectors. A total of 14–16 h after transfection, cells were trypsinized and plated onto coverslips. After a further 24–36 h, cells were fixed with methanol (2 min, room temperature) and incubated with the indicated antibody. FITC-conjugated donkey antibody against rabbit IgG was used as a secondary antibody.

Cell extracts and electrophoretic mobility shift assay

Whole-cell extracts from transfected COS cells were prepared 42–48 h after transfections, as described (Ganchi *et al.*, 1992). Whole-cell extracts from Daudi cells were prepared as described (Lernbecher *et al.*, 1993). κB binding activity was analyzed in EMSA using a ^{32}P-labeled oligonucleotide containing a palindromic κB site (Ballard *et al.*, 1990) as described (Tewari *et al.*, 1992), except that NP-40 was omitted in the binding reactions. Where indicated, specific antibodies were included.

Constructs

The CAT reporter construct 8× κB–tk-CAT containing eight copies of the murine immunoglobulin enhancer κB site was generated as described (Ryseck *et al.*, 1992). The pMexneo (Martin-Zanca *et al.*, 1989)-based expression vectors for RelB and p50 were described (Ryseck *et al.*, 1992). Constructs expressing rat IκBα (Tewari *et al.*, 1992), human p52 (Bours *et al.*, 1992), IκBγ (Inoue *et al.*, 1992) and Bcl3 (Ohno *et al.*, 1990) were generated by inserting the corresponding coding sequences into pMexneo.

For experiments in COS cells the corresponding coding sequences were recloned into the expression vector pcDNAI/Amp (Invitrogen), except for the p50-expressing construct, Rc/CMV FLAGp50, which was a kind gift from Dr M.Karin.

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