Critical Role of RelB Serine 368 for Dimerization and p100 Stabilization*

Received for publication, February 12, 2003, and in revised form, June 27, 2003
Published, JBC Papers in Press, July 21, 2003, DOI 10.1074/jbc.M301521200

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In mature B cells RelB-containing complexes are constitutively present in the nucleus, and they are less susceptible to inhibitory eB proteins. In most other cell types inhibitory eB proteins prevent nuclear translocation and activation of NFκB. We reasoned that this characteristic might be because of post-translational modifications of RelB. As a model system we used the murine S107 plasmacytoma cell line, which lacks endogenous RelB expression. Analysis of S107 cells expressing wild type RelB and serine 368 mutants reveals that serine 368 is not required for nuclear import but that it is critical for RelB dimerization with other members of the NFκB family. Similar effects were obtained when the conserved serine in RelA was mutated. We further demonstrate that expression of functional RelB, but not of serine 368 mutants, severely reduces p52 generation and strongly increases expression of the p52 precursor, p100. Wild type RelB, but not mutant RelB, prolonged p100 half-life. We therefore suggest an inhibitory effect of RelB on p100 processing, which is possibly regulated in a signal-dependent manner.

NFκB represents a family of dimeric transcription factors consisting of the mammalian members p50 (NFκB1), p52 (NFκB2), RelA (p65), RelB, and c-Rel. NFκB regulates the expression of a variety of genes, many of which are involved in immune and inflammatory responses. In addition, NFκB target genes play a role in cell growth and differentiation as well as in neoplastic transformation (reviewed in Refs. 1–4). Whereas the Rel proteins are directly synthesized as mature proteins, p50 and p52 are generated by proteolytical processing from their p105 and p100 precursors, respectively. A characteristic of all family members is the Rel homology domain, which comprises about 300 amino acids and is responsible for protein-protein interaction, DNA binding, and nuclear localization. In most cell types, NFκB proteins are retained in the cytoplasm by inhibitory eB proteins, the IκBs,1 which comprise IκBα, IκBβ, IκBγ, IκBε, and Bcl-3. Moreover, the precursors p100 and p105 are also known to display inhibitory functions and therefore are also classified as IκBs (5, 6). The IκB proteins are not restricted to the cytoplasm but have been shown to retrieve NFκB heterodimers from the nucleus and escort them back to the cytoplasm (7).

There are different pathways for induction of NFκB. In most cell types, stimulation with a variety of substances leads to activation of the IκB kinase complex (IKK). This complex consists of the catalytic subunits IKKα and IKKβ and the regulatory IKKγ subunit (also called NEMO, NFκB essential modulator). In the classical pathway, the activated IKK, predominantly IKKβ, catalyzes the phosphorylation of IκB. Subsequently, IκB is ubiquitinated and degraded, thereby releasing the NFκB proteins. As a consequence, NFκB (in this pathway, especially the heterodimer p50-RelA) can translocate to the nucleus, bind to DNA, and activate gene transcription (8).

Recently, a new pathway for NFκB activation has been proposed, and the IKK complex mediating this alternative pathway differs from the one in the classical pathway (9–11). This pathway is dependent on new protein synthesis, involves IKKα rather than IKKβ and IKKγ (NFκB essential modulator), and the signal is not propagated via IκB, but via p100, which is processed to p52. It has also been shown that NFκB-inducing kinase (NIK) positively regulates p100 processing, but it remains unclear whether NIK exerts its effect directly upon p100 (12) or whether it does so via IKKα activation (9). This alternative pathway can be triggered by lymphotoxin-β receptor and B cell-activating factor receptor ligation as well as CD40L and lipopolysaccharide signaling (13–18).

In mature B cells, NFκB is constitutively present in the nucleus. The exact mechanisms of this constitutive activity remain unclear; the alternative pathway just mentioned may be involved. Whereas predominantly p50-RelA heterodimers are responsible for inducible NFκB activity, RelB- and partially c-Rel-containing complexes are involved in this constitutive nuclear activity (19, 20). The finding that RelB expression is enhanced in tissues with constitutive activity and the observation that RelB-containing complexes are less susceptible to inhibition by IκBα further underline the important role of RelB in this context (21). However, it is not clear how RelB is protected from inhibition in mature B cells; one possibility could be some kind of post-translational modification that only occurs in lymphoid tissue. RelB itself cannot form homodimers, nor does it apparently dimerize with RelA or c-Rel; however, it can form heterodimers with p50 and p52 (20, 22). These heterodimers can induce transcription because of the carboxyl- and amino-terminal transactivation domains of RelB, both of which are...
required for full transactivation (23).

The NFκB/Rel protein family does not only exist in mammalian systems but is also found in Dro sophila, where the three known family members are called Dorsal, Dif, and Relish. Dorsal, a Rel homologue, is retained in the cytoplasm by the IκB protein Cactus, which can be phosphorylated and degraded in a signal-dependent manner. This process finally results in Dorsal release and nuclear translocation (24, 25). In the early embryo, however, Dorsal itself is phosphorylated and subsequently translocates to the nucleus, an event that is crucial for establishing a ventral to dorsal nuclear gradient and thus determining polarity of the embryo (26, 27).

The site of this phosphorylation event, serine 317, is evolutionarily conserved in all NFκB/Rel proteins, suggesting an important role also in the mammalian system (27). When we compared the amino acid sequence in the neighborhood of this serine, it became clear that the highest conservation is observed in the RelB protein. While phosphorylation of serine 317 regulates constitutive nuclear import of Dorsal, the constitutive presence of RelB in lymphoid tissue apparently also depends on a so-far unknown post-translational modification of RelB in mature B cells (21). Thus, serine 368 might be involved in the tissue-specific constitutive nuclear translocation of RelB. In this study, we therefore addressed the question whether nuclear targeting of RelB in mature B cells is regulated by phosphorylation at serine 368, the conserved residue of serine 317 in Dorsal. To examine this hypothesis, we generated serine to alanine and serine to glutamic acid mutations at position 368 of RelB and analyzed whether these mutations had an impact on nuclear translocation of RelB in mammalian cells.

We report here that serine 368 is not involved in a phosphorylation-dependent mechanism regulating the subcellular localization of RelB but is essential for RelB dimerization. In addition, our results also demonstrate that RelB dimerization with p100 protects p100 from processing to p52.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—For mutagenesis of the RelB MexNeo and pCDNA3 RelA plasmids, a kit from Stratagene (QuickChange XL site-directed mutagenesis kit) was used according to the manufacturer's protocol. The plasmids were denatured, and the mutagenic primers were annealed. After extension of the oligonucleotide primers, nonmutated parental DNA was removed by treatment with DpnI endonuclease. After transformation into competent bacteria, the received constructs were sequenced to confirm the point mutations and the integrity of the remaining protein.

Mutagenic primers used were RelB S368A, sense (GATGGG GTGT-GCGCCGAGCCGCCTGCC), antisense (GCCACCCGCTGCTTCGCCAAGCCCATC); RelA S281A, sense (CCGACCCGCTGCTTCGCCAAGCCCATC); RelA S281E, sense (GATGGG GTGT-GCGCCGAGCCGCCTGCC), antisense (GCCACCCGCTGCTTCGCCAAGCCCATC); RelA S281E, sense (GATGGG GTGT-GCGCCGAGCCGCCTGCC), antisense (GGAACTTGGGAATGGCTAGCGATCCAGTATTC); RelB S368E, sense (GATGGG GTGT-GCGCCGAGCCGCCTGCC), antisense (GCCACCCGCTGCTTCGCCAAGCCCATC); RelA S281E, sense (GATGGG GTGT-GCGCCGAGCCGCCTGCC), antisense (GGAACTTGGGAATGGCTAGCGATCCAGTATTC).

Cell Culture—S107, Jurkat T, and NIH 3T3 cells were grown in Dulbecco's modified Eagle medium (Invitrogen) containing 10% heat-inactivated fetal calf serum (PAN Systems, Aidenbach, Germany), 10 μg/mL penicillin, 100 μg/mL streptomycin, supplemented with 50 μM β-mercaptoethanol (final concentration).

Stable and Transient Transfection—For generation of stable transfectants, S107 cells were electroporated with 40 μg of wild type or mutant NFκB/Rel plasmid. Cells were transfected in 300 μL of culture medium by electroporation with a Bio-Rad gene pulser at 975 V and 250 F and immediately resuspended in 20 mL of medium (28). Cell clones with genomically integrated vectors were selected on medium containing 2 mg/mL G418 by limited dilution.

For transient transfections of stable S107 clones, cells were transfected with 20 μg of a NFκB-dependent luciferase reporter (3x κB luc, containing three copies of the κB motif immediately upstream of the β-globin TATA box) and 30 ng of a Renilla luciferase reporter under the control of the ubiquitin promoter (29). For transient cotransfections of stable S107 clones, cells were transfected with 20 μg of the respective plasmid (empty vector, p50 and p52 expression vectors together with 4 μg of the NFκB-dependent luciferase reporter and 30 ng of the ubiquitin-dependent Renilla luciferase reporter. For transient cotransfections of S107 cells and Jurkat T cells, cells were transfected with 20 μg of TexMex (empty vector) or 20 μg of the respective plasmid (RelA MexNeo, RelB S368A MexNeo, RelB S368E MexNeo), together with 4 μg of the NFκB-dependent luciferase reporter and 30 ng of the ubiquitin-dependent Renilla luciferase reporter. NIH 3T3 cells were transiently transfected with 20 μg of pCDNA3 (empty vector) or 20 μg of the respective plasmid (RelA, RelA S281A, RelA S281E), together with 4 μg of the NFκB-dependent luciferase reporter

Western Blotting—50–100 μg of protein extract were separated by SDS-PAGE and electrophoretically blotted to a polyvinylidene difluoride membrane. Membranes were incubated with specific antibodies against Rel proteins that are enhanced by enhanced chemiluminescence (ECL Western blotting detection kit; Amersham Biosciences). Specific antibodies were purchased from Santa Cruz Biotechnology (anti-RelA sc-372, anti-RelB sc-226, anti-p50/p105 sc-114X, anti-IkBa sc-371, anti-Bcl-3 sc-185, anti-IKK α scf-7607) and Upstate Biotechnology (anti-p52/p100; catalogue no. 6841).

Electrophoretic Mobility Shift Assay (EMSA)—Protein extracts (5 μg) were incubated for 30 min at room temperature with 3 μg of poly(dI/dC), 10 μg of bovine serum albumin in buffer containing 50 mM NaCl, 1 mM dithiothreitol, 10 mM Tris-HCl, 1 mM EDTA, 5% glycerol, and radiolabeled double-stranded oligonucleotides containing an Igκ enhancer consensus NFκB site or a Sp-1-specific site (5′-attcctagggcggggcggcgacgc-3′) in a total volume of 20 μL (28). The complexes formed were then separated on a 4% native polyacrylamide gel. For supershift experiments, 2.5 μg of protein extract were preincubated for 30 min with specific antibody before being treated as described before.

Cell Labeling—Cells were washed twice with PBS, once with methionine/cysteine-free medium supplemented with dialyzed, heat-inactivated fetal calf serum (10%), and incubated in this medium for 15 min at 37°C. The cells were resuspended in the same medium (4 × 10^6 cells/mL) containing [35S]methionine (100 μCi/mL; Amersham Biosciences) and incubated for 2 h at 37°C. Thereafter, the cells were washed once with prewarmed PBS, twice with complete medium, resuspended in an excess of this medium, and incubated at 37°C. Immunoprecipitation—Cells were washed twice with cold PBS and resuspended in lysis buffer (containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40). Following incubation on ice for 15 min, lysates were clarified by microcentrifugation for 15 min. To avoid specific binding, cell lysates containing 500 μg of protein were then precleared with protein A-agarose for one hour at 4°C on a rocking platform. After that, the specific antibody (cited under “Western Blotting”) together with protein A-agarose was added to the supernatant and incubated for four hours at 4°C. Following incubation on ice for 15 min, lysates were clarified by microcentrifugation for 15 min. To avoid specific binding, cell lysates containing 500 μg of protein were then precleared with protein A-agarose for one hour at 4°C on a rocking platform. After that, the specific antibody (cited under “Western Blotting”) together with protein A-agarose was added to the supernatant and incubated for four hours at 4°C. Following incubation on ice for 15 min, lysates were clarified by microcentrifugation for 15 min. To avoid specific binding, cell lysates containing 500 μg of protein were then precleared with protein A-agarose for one hour at 4°C on a rocking platform. After that, the specific antibody (cited under “Western Blotting”) together with protein A-agarose was added to the supernatant and incubated for four hours at 4°C. Following incubation on ice for 15 min, lysates were clarified by microcentrifugation for 15 min. To avoid specific binding, cell lysates containing 500 μg of protein were then precleared with protein A-agarose for one hour at 4°C on a rocking platform. After that, the specific antibody (cited under “Western Blotting”) together with protein A-agarose was added to the supernatant and incubated for four hours at 4°C. Following incubation on ice for 15 min, lysates were clarified by microcentrifugation for 15 min. To avoid specific binding, cell lysates containing 500 μg of protein were then precleared with protein A-agarose for one hour at 4°C on a rocking platform. After that, the specific antibody (cited under “Western Blotting”) together with protein A-agarose was added to the supernatant and...
RESULTS

Mutagenesis of RelB—The prominent role of serine 317 in phosphorylation-dependent nuclear translocation of Dorsal prompted us to mutate the homologous serine 368 of RelB. Interestingly, RelB shows a higher conservation in the amino acids surrounding Serine 368 than do the other NFκB/Rel proteins (Fig. 1). To abolish the potential phosphorylation site, serine 368 was mutated to alanine (RelB S368A). We also tried to mimic the effect of a potential serine phosphorylation by introducing a glutamic acid residue (RelB S368E). Each construct was completely sequenced to confirm the point mutation in a wild type context.

The murine S107 plasmacytoma cell line has previously been shown to be an appropriate model system for investigations of inducible and constitutive NFκB activity. The S107 cell line displays a specific defect in inducible NFκB activity and, more important in this context, completely lacks RelB and constitutive NFκB activity (31, 32). The other NFκB/Rel proteins are expressed but retained in the cytoplasm. Transfection of this cell line with a RelB expression vector results in the appearance of nuclear p50-RelB complexes and restores κB-dependent transcriptional activity. In contrast, transfection with a RelA expression vector does not have these consequences (32).

To test whether the mutations affect RelB function, we first performed transient transfection assays. S107 cells were transfected with wild type RelB (termed wt), RelB S368A (termed A), and RelB S368E (termed E), respectively, together with a κB-dependent luciferase reporter to determine κB-dependent transcriptional activity. The parental S107 cell line shows only weak constitutive κB-dependent transcriptional activity (Fig. 2A). This activity is strongly increased upon wild type RelB transfection. Cotransfection of RelB bearing a Ser to Ala mutation (RelB S368A) showed a significantly reduced activity. Surprisingly, the transcriptional activity of transfected RelB S368E was even lower than that of RelB S368A. From these results we concluded that introduction of the phosphomimetic mutation did not result in the proposed constitutive nuclear translocation, because we would have expected an enhanced transcriptional activity in this case.

To analyze whether these results were because of a S107 cell-specific effect, we performed similar transient transfection experiments with Jurkat T cells (Fig. 2B). Consistent with the data obtained in S107 cells, transcriptional activation is significantly lower in Jurkat T cells transfected with RelB S368A as compared with the wild type RelB transfected cells. Again, the cells transfected with RelB S368E showed the lowest activation. The results of the S368A mutation would be consistent with the initial hypothesis, whereas the behavior of the S368E mutation does not fit to the proposed model of constitutive nuclear translocation.

Subcellular Localization of RelB Mutants Is Not Altered in S107 Cells—To explain the defects displayed by RelB bearing the Ser to Ala and Ser to Glu mutations, several possibilities could be considered. The mutations could affect nuclear translocation of RelB. Alternatively, they could impair DNA binding and/or transactivation capabilities. Finally, the mutations could also alter RelB dimerization, which is a prerequisite for DNA binding and transactivation.

To address these questions, we generated S107 cell clones constitutively expressing the different RelB variants, which we designated S107 wt, A, and E clones, for RelB wild type, RelB S368A, and RelB S368E mutations, respectively. We first tested several of the S107 wt, A, and E clones for RelB expression in whole cell extracts to select clones with comparable RelB expression levels (Fig. 3 and data not shown). Several such clones were retrieved, and they behaved similarly; only
the results of one representative clone for each construct are shown in subsequent analyses. To examine subcellular localization, we prepared nuclear and cytoplasmic extracts of S107 wt, A, and E clones and monitored RelB distribution by Western blot assays (Fig. 3). Interestingly, the subcellular localization of RelB is not affected either by the A or by the E mutation; both RelB and the mutants are almost equally distributed between the cytoplasmic and the nuclear compartment. The mutations also do not interfere with protein stability. The quality of the nuclear and cytoplasmic fractions was controlled by the detection of the cytoplasmic proteins RelA and IKKβ (Fig. 3, lower panels). We conclude that despite the strong evolutionary conservation, serine 368 of RelB is not involved in the regulation of nuclear translocation of the protein, unlike the serine 317 residue in Dorsal. The same results were obtained by immunofluorescence (data not shown).

RelB Mutants Are Impaired in DNA Binding—To recapitulate the findings of the transient transfection experiments, S107 wt, A, and E clones were transfected in parallel with a κB-dependent firefly luciferase reporter (3×κB.luc) and the same reporter lacking the κB motifs. As expected, κB-dependent transcription is strongly activated in the wt clone, whereas this is not the case for the A and E clones (Fig. 4A). We had noted previously that the activity of wild type RelB can be augmented by cotransfection of a p50 expression vector (data not shown); we therefore asked whether this is also the case for the mutated forms of RelB. Upon cotransfection of wt and A clones with a p50 expression vector, there is a strong increase in NFκB-dependent transcription, with the wt and A cells reaching almost the same levels of activity (Fig. 4B). Thus, the defect in the A clone can be compensated to a large extent by transient overexpression of p50. Interestingly, the defect in the E clone cannot be reverted in that way. As compared with p50, cotransfection of p52 reveals a less efficient stimulation of both S107 wt and A cells, with the values for the E clone again remaining at a very low level (Fig. 4B). From control Western blots we conclude that we do not succeed in expressing p52 as efficiently as we do for p50 (data not shown); this could be responsible for the difference in co-stimulation of RelB-dependent transcription by p52.

We next addressed the question whether reduced DNA binding activity is responsible for the low activity in the A and E clones. Using EMSA with a κB-specific probe, we observed severe defects of the mutated RelB proteins. The κB binding activity is strongly reduced in the A clone and almost abolished in the E clone (Fig. 5). We detected four κB-specific complexes (a, b, c, and d), which represent complexes of different subunit composition. Previous experiments (32) suggest that complex d is composed of p50 homodimers that are found in comparable amounts in whole cell and nuclear extracts of all clones. In the cytoplasmic fraction this complex is absent, indicating that

**Fig. 3.** Subcellular localization of mutant RelB is not affected. Immunoblots with whole cell (wc), cytoplasmic (c), and nuclear extracts (n) from the S107 wt, A, and E clones were performed with the indicated antibodies. Upper panel, RelB expression of three representative clones. Both wild type RelB (wt) and the S368A (A) and S368E (E) mutants are equally distributed between cytoplasm and nucleus. Lower panels, the quality of nuclear extracts was confirmed by Western blots using antibodies specific for two cytoplasmic proteins (RelA and IKKβ).

**Fig. 4.** NFκB-dependent transcriptional activity is strongly reduced in S107 A and E clones. A, The stable wt, A, and E clones were transiently transfected with a κB-dependent reporter (3×κB.luc) as described under “Experimental Procedures.” The luciferase activity relative to the activity of the same reporter lacking the κB motifs is shown. B, upon co-transfection of p50 and p52, the A and E clones behave differently. The three different clones (S107 wt, A, and E) were transiently transfected with empty vector, with a p50, or with a p52 expression vector together with 3×κB.luc. The value for the wild type clone transfected with empty vector was arbitrarily set to 100; all other activities are given relative to this standard.

**Fig. 5.** DNA binding activity of the A and E mutants is severely impaired. EMSA with whole cell, nuclear, and cytoplasmic extracts from S107 A, E, and wt clones with a probe specific for κB or Sp-1, respectively. The κB shift (upper panel) shows that DNA binding activity is strongly reduced for the A mutant and nearly abolished for the E mutant, as compared with wild type RelB. The κB-specific complexes are indicated with a, b, c, and d. The EMSA with a probe specific for the transcription factor Sp-1 (lower panel) serves as a control; Sp-1-specific DNA binding is similar in all three clones.
FIG. 6. RelB is critically involved in complex formation. EMSA supershift assay of nuclear extracts from S107 A, E, and wt clones with a κB-specific probe and the indicated antibodies. As a control, EMSA was performed without antibody (left side) and with antibodies specific for IκBα and Bcl-3 (right side). The κB-specific complexes are indicated with a, b, c, and d.

There is little or no contamination with nuclear proteins. Complex c, representing the main part of DNA binding activity, is most probably composed of RelB-containing dimers. This complex is highly enriched in nuclear extracts and strongly reduced in the A and E clones. Complexes a and b were also decreased in the A and E clones. They are found at similar levels in the cytoplasmic and nuclear extracts. As a quality control, we performed an EMSA with the same extracts, using a SP-1-specific probe.

To further characterize the composition of the four different DNA binding complexes (a, b, c, and d) detected in Fig. 5, we performed supershift assays with specific antibodies. In these assays we used nuclear extracts from A, E, and wt clones and antibodies directed against different NFκB/Rel proteins (Fig. 6). Of the four DNA binding complexes, complex a is shifted to some extent with a RelA-specific antibody, whereas the other complexes do not react strongly with this antibody. In the presence of a RelB-specific antibody, complexes a, b, and c are almost completely supershifted, indicating that they all contain RelB. These three complexes are also affected to a different extent in the presence of p50/p105- and p52/p100-specific antibodies. Complex a is almost completely reduced by a p50/p105-specific antibody and less affected by a p52/p100-specific antibody, whereas complex b is completely supershifted by a p52/p100-specific antibody and to a lesser extent by a p50/p105-specific antibody. Complex c is strongly supershifted by a p50/p105-specific antibody but also affected by a p52/p100-specific antibody, suggesting that this DNA binding activity consists of mainly p50-RelB but also of p52-RelB heterodimers.

Complex d has previously been shown to consist of p50 homodimers (32). Apparently, the p50 antibody used in our experiments is less efficient in supershifting these complexes. Using antibodies for c-Rel, Bcl-3, and IκBα, we cannot observe any effect on the complexes (Fig. 6 and data not shown). Despite the data presented here, the composition of complexes a and b, which are also found in the cytoplasmic fraction, is not elucidated completely. We have shown that they both contain RelB; their slow migration could be because they contain p100 and/or p105. It is also conceivable that complexes a and b are composed of more than two NFκB proteins and thus represent trimeric or tetrameric complexes.

Additional experiments were performed to address these questions. Transient transfection of p50 into the wt and the A clone not only results in the appearance of p50 homodimers (Fig. 7d) but also in increasing amounts of p50-RelB heterodimers (c). In the E clone, however, no p50-RelB dimers can be detected at all. These observations further support the results of the cotransfection assays where p50 increased the ratio of nuclear RelB and thereby enhanced RelB-dependent transcription. The A mutation decreases p50-RelB dimerization, which can be forced again in the presence of abundant p50, whereas the E mutation completely represses this interaction. Interestingly, p50 expression shows only marginal effects on complexes a and b. This indicates a minor role of p50 in the formation of these complexes.

Mutagenesis of RelB Represses Dimerization with NFκB Family Members—The data presented up to now are consistent with a defect of the RelB mutants in both DNA binding and dimerization capability, because RelB cannot bind to DNA without a partner. RelB is known to interact primarily with p50, p52, and the p52 precursor, p100, whereas homodimerization of RelB and dimerization with RelA and c-Rel has not been described (20, 22, 33–35). However, our supershift assays suggest the existence of complexes containing RelA and RelB (Fig. 6). To address the dimerization capabilities of our mutants in detail, we performed a set of immunoprecipitations with extracts prepared from S107 wt, A, and E cells.

In Fig. 8A, we show that the single mutation generated at position 368 of RelB severely affects the dimerization capability of RelB. The interaction both with p50 and p52 is strongly reduced in S107 cells expressing RelB S368A and S368E, which is consistent with the data obtained in transfection and DNA binding experiments. Interestingly, we also find a co-precipitation of wild type RelB and RelA, which is again impaired in the case of RelB S368A and S368E (Fig. 8A, right panel). This observation might be a consequence of direct interaction of RelB with RelA; however, there could also be a different explanation. It is possible that RelB and RelA do not associate directly but interact indirectly in a complex consisting of more than two NFκB proteins. This hypothesis would help to explain the results of our supershift experiments and would also be consistent with previous experiments that failed to show a direct interaction between RelB and RelA (20).

To complete our immunoprecipitation studies, we also determined dimerization properties of our mutants toward the p100 and p105 precursor proteins (Fig. 8B). Recently, it has been stated that RelB preferentially dimerizes with p100 and that this interaction also plays an important role in the regulation of RelB subcellular localization, whereas dimerization with p105 is weak (36). We found similar results in our experimental system. We observe almost no immunoprecipitation of p105.
with a RelB-specific antibody, whereas there is a strong interaction between wild type RelB and p100. To our surprise, the p100 expression levels in S107 A and E cells were significantly lower compared with S107 wt cells. Thus, the conclusions we can draw concerning the dimerization properties of our mutants toward p100 are also affected.

**RelB Interferes with p100 Stability**—The above finding prompted us to further investigate the p52/p100 and the p50/p105 expression levels of the parental S107 cell line, S107 wt, A, and E cell clones, and, in addition, two S107 mass cultures previously transfected with wild type RelB and RelA, respectively (Fig. 9) (32). Indeed, it became clear that transfection of RelB-deficient S107 cells with functional RelB results in surprising changes in p52/p100 expression. Whereas parental, RelB-deficient S107 cells and S107 A and E clones, as well as RelA-expressing cells (S107 RelA), show a very low expression of p100 and a strong expression of p52, the S107 cells transfected with functional wild type RelB (S107 RelB mass culture and wt) exhibit a strong expression of p100 and reduced expression of p52. The individual clone expressing RelB (Fig. 9A, wt) shows slightly higher levels of p52 and slightly lower p100 levels compared with the mass culture (Fig. 9A, S107 RelB). Interestingly, at the same time, these two samples differ in their amount of RelB. This result is in line with our conclusion that the level of RelB expression directly correlates with the amount of stable p100 protein but inversely correlates with processed p52 levels.

Importantly, p50 and p105 expression levels are not influenced in the different S107 cell lines (Fig. 9A). This suggests a specific effect of RelB on p100 processing. In this context it is important to note that S107 cells stably transfected with RelA do not show this kind of effect. Taken together, these data imply that an intact dimerization function of RelB may play a critical role in p100 stabilization and/or regulation of p100 processing to p52 in B cells.

To test directly whether p100 processing and p52 generation is influenced by functional RelB, we investigated p100 levels in S107 RelB wt and RelB S368A cell clones by pulse-chase metabolic labeling. After a 2 h pulse-labeling phase the p100 expression level is strongly increased in RelB wt compared with RelB S368A cells (Fig. 9B). Subsequent chase for up to 12 h reveals that steady state levels of p100 decline differently. In RelB wt cells, p100 levels decrease moderately during the first hours of chase, resulting in a p100 half-life of approximately eight hours (Fig. 9C). In contrast, the half-life of p100 was only about two hours in RelB S368A cells (Fig. 9C). A comparable rapid turnover of p100 was seen in the parental S107 cells (data not shown). These results indicate that half-life of p100 is prolonged in the presence of functional RelB. Because S107 cells produce immunoglobulins (IgA) with a size of ~55 kDa, it is for technical reasons not possible to monitor p52 levels in these cell clones. However, the results of the Western blots (Fig. 9A) together with earlier findings (50) suggest that p52 generation is regulated cotranslationally.

**RelA Transactivation and DNA Binding Activity Is Blocked by Serine 281 Mutation**—We originally started with the hypothesis that subcellular localization of RelB is regulated in the same manner as Dorsal. This turned out not to be true. Given the striking evolutionary conservation of serine 317 in all NFκB/Rel proteins (Fig. 1), the question arises whether the observed effects were specific for RelB. Analogous to the RelB mutagenesis the conserved serine 281 of RelA was mutated to both alanine (RelA S281A) and glutamic acid (RelA S281E). Both constructs were sequenced to confirm the point mutation in wild type context. To analyze whether the mutations affect RelA function we performed transient transfection assays. NIH 3T3 cells were transiently transfected with wild type RelA, RelA S281A, and RelA S281E, respectively, together with a κB-dependent luciferase reporter gene. We observed that κB-dependent transcriptional activity is dramatically (300-fold) increased upon RelA transfection. In contrast, cotransfections of RelA bearing the Ser to Ala or Ser to Glu mutations do not result in any κB-dependent transcriptional activity (Fig. 10A). Although the expression level of the mutated RelA proteins is slightly lower than that of the wt protein (Fig. 10B), it is rather unlikely that these minor differences account for the total lack of transactivation. The DNA binding of NFκB/Rel proteins is necessary for the transcriptional activation of the NFκB-dependent reporter gene. We therefore asked whether the DNA binding activity of the mutated RelA proteins is affected. Indeed, our EMSA experiments show that κB binding activity is completely absent in NIH 3T3 cells transiently transfected with the mutated RelA proteins, whereas strong DNA binding was detected in those cells transfected with wild type RelA (Fig. 10C). These results demonstrate that mutation of the conserved serine 281 in RelA completely abolishes DNA binding activity and transactivation capability of RelA, similar to the serine 368 mutation of RelB. These conserved serines could be targets of a signal-dependent phosphorylation mechanism that could regulate the dimerization capacity of NFκB/Rel proteins.

**DISCUSSION**

Here, we demonstrate that serine 368 of RelB is essential for RelB dimerization with p100, p52, and p50. As a consequence, DNA binding and transactivation are affected in the mutated proteins. We also provide evidence that, unlike its *Drosophila* counterpart, serine 317 of Dorsal (27), serine 368 is not involved in the regulation of nuclear RelB translocation.

In *Drosophila*, a signaling pathway initiated by Toll receptor activation and finally leading to activation of Rel-related transcription factors (37) regulates dorsoventral patterning of the embryo and innate immune responses (reviewed in Ref. 38).

Upon activation of the transmembrane receptor Toll, the signal is relayed via the adaptor protein Tube and the protein kinase
Pelle onto the cytoplasmic Dorsal-Cactus complex, an event which involves phosphorylation and degradation of the IκB family protein Cactus and results in nuclear translocation of the Rel homologue Dorsal (39). It is important to note that phosphorylation not only occurs on Cactus but also on Dorsal itself (37, 40, 41). Dorsal is phosphorylated at several serine residues, but it has been shown that phosphorylation of serine 317 is decisive for nuclear translocation. A point mutation in serine 317 results in decreased nuclear import of Dorsal and in a strongly dorsalized phenotype of the embryo, whereas the mutated Dorsal is still able to interact with Cactus (27). In this study, we show that serine 368 of RelB is not required for

FIG. 9. RelB is necessary for p100 stabilization in S107 cells. A, Western blot analyses were performed with whole cell extracts from the S107 parental cell line and different stable transfectants. The S107 RelA and the S107 RelB cell lines are mass cultures stably transfected with RelA and RelB, respectively. The results of the three different clones (wt, A, and E) are shown on the right side. Immunoblots with antibodies against RelA (α-RelA, middle panel), and α-p50/p105 (lower panel) are shown. B, S107 RelB wt and RelB S368A cells were harvested after pulse radiolabeling for 2 h (0 h of chase) and the indicated chase time points and then subjected to immunoprecipitation (IP) with α-p52/p100 as described under “Experimental Procedures.” The positions of the p100 and IgA bands as well as bands of unidentified proteins are marked with an asterisk. C, the p100 levels at the indicated chase time points were quantified by PhosphorImager analysis. The p100 level after pulse radiolabeling (0 h of chase) was set at 100%.

Fig. 10. Serine 281 is necessary for RelA function. A, transient transfections of NIH 3T3 cells with empty vector, wild type RelA, RelA S281A, or RelA S281E together with a κB-dependent reporter (3×κB-luc) were performed as described under “Experimental Procedures.” The luciferase activity of the cells transfected with empty vector was arbitrarily set to 1; all other activities are given relative to this standard. B, Western blot analysis with whole cell extracts from the transiently transfected NIH 3T3 cells were performed in parallel. Immunoblots with antibodies against RelA (upper panel) and α-tubulin (α-Tub, lower panel, as a loading control) are shown. The position of ectopically (ect.) and endogenously expressed RelA is indicated. C, EMSAs with whole cell extracts prepared in parallel from the transiently transfected NIH 3T3 cells were performed with a probe specific for NFκB (upper panel) or Sp-1 (lower panel), respectively.

Pelle onto the cytoplasmic Dorsal-Cactus complex, an event which involves phosphorylation and degradation of the IκB family protein Cactus and results in nuclear translocation of the Rel homologue Dorsal (39). It is important to note that phosphorylation not only occurs on Cactus but also on Dorsal itself (37, 40, 41). Dorsal is phosphorylated at several serine residues, but it has been shown that phosphorylation of serine 317 is decisive for nuclear translocation. A point mutation in serine 317 results in decreased nuclear import of Dorsal and in a strongly dorsalized phenotype of the embryo, whereas the mutated Dorsal is still able to interact with Cactus (27). In this study, we show that serine 368 of RelB is not required for
nuclear import; it remains an unsolved question whether different post-translational modifications of RelB may be responsible for the constitutive nuclear translocation observed in mature B cells.

However, our finding that the serines 368 and 281 are critical for RelB and RelA dimerization properties raises further questions, especially with regard to protein structure and possible dimerization domains of these proteins. Up to now, no crystal structure of RelB heterodimers is available. Therefore, information about the structure of the RelB dimerization domain can only be drawn from mutagenesis studies and from crystal structures of homologous proteins. It has been shown that the carboxyl-terminal part of the Rel homology domain (between amino acids 264 and 379) is essential for effective RelB dimerization (33), which is consistent with the results obtained from crystal structure analysis of the dimerization domain of p50 and RelA (42, 43). Although the serine residues in p50 (Ser-340) and RelA (Ser-281) homologous to serine 368 (Fig. 1A) are located in α-helical domains of the proteins that were not reported to contribute directly to the dimer interface and DNA contact of the p50-RelA heterodimer, we hypothesize an important function of these serines for RelA and RelB dimer formation. This function cannot be replaced by either alanine or by glutamic acid. In the case of RelB we found that p50 co-expression can overcome the change of a polar to a hydrophobic amino acid side chain, whereas introduction of a negatively charged group cannot be compensated at all. Interestingly, introduction of a hydrophobic amino acid side chain in close vicinity to Ser-368 by mutation of cysteine 367 to alanine did not interfere with RelB dimerization properties (33).

It has been known for a long time that properties of Rel/NFκB proteins are regulated by phosphorylation (44, 45). Furthermore it has also been shown that phosphorylation plays a role in the regulation of RelB protein stability (46), which was not affected in our case. It is therefore an interesting question whether serine 368 of RelB and also serine 281 of RelA have just a structural function that was disrupted by our mutations or whether they are indeed a target for signal-dependent phosphorylation. In this respect, further investigations are required. However, the finding that the phosphomimetic E mutations of both RelB and RelA lead to loss of protein function could be a hint that the conserved serine, if it is a phosphorylation target, might regulate the release of dimerization partners of RelB and RelA.

An interesting outcome of this study is that in S107 cells, which lack RelB expression, p100 expression levels are low and p52 levels are high and that we can invert these levels by expressing functional RelB. It is known that the nfkβ2 gene is a target gene of NFκB (47), a fact that could simply explain the increase in p100 levels but not the decrease in p52 levels. In addition, we observe a prolonged half-life of p100 in the presence of functional RelB. p100 is known to inhibit transcriptional activity of p52-RelB heterodimers and therefore is classified as an 1xB protein (48). Recent investigations that also explain this function have defined an important role for p100 in the context of RelB subcellular regulation (36). According to this study, RelB is associated in the cytosol solely with p100, and this interaction represses RelB nuclear translocation. Moreover, the C-terminal part of p100 contains a nuclear export signal required for efficient retrieval of RelB from the nucleus. Our own studies further underline these findings, because we can show by immunoprecipitation that interaction between RelB and p105 is very weak in mature B cells, in contrast to the strong affinity that RelB displays toward p100. However, this difference is not observed in the case of p50 and p52. In addition, our results indicate that RelB stabilizes p100 and thereby may have a regulatory function for p100 processing. This function is unique to RelB because ectopic expression of RelA, which can dimerize with p100 (36), is not able to stabilize p100 in S107 cells. The effect RelB displays on p100 stability might be a direct consequence of the strong interaction between these proteins and the unique conformation of the dimer (36).

The processing of p100 to p52 itself, which occurs at the proteasome, is an event that is not fully understood. It is still not clear whether p52 is generated in a post-translational manner (12, 49) or whether it is also co-translationally synthesized (50), an event which would not require full-length p100. Recently, a modified mechanism involving continuous de novo protein synthesis was suggested; the authors (18) described an induced co-translational process of p52 generation in response to lymphotixin-β receptor ligation or lipopolysaccharide. From our results, we cannot answer the question whether p52 is exclusively produced by either of the proposed mechanisms. We would like to propose a model according to which p100 is constitutively processed in the absence of RelB in S107 cells. This constitutive processing may happen co-translationally. When RelB is present, it may associate with the nascent p100, thus preventing constitutive processing of p100 and forming a p100-RelB dimer pool that could be susceptible to NIK-IKKα signaling.

The novel mechanism of NFκB2 regulation discussed here is of special interest in the context of the new alternative NFκB signaling pathway (9). This pathway involves NIK and IKKα and plays a prominent role in the maturation of B cells and the formation of secondary lymphoid organs (14, 15, 51). Whereas the exact mechanism of this signaling pathway is not fully understood, it is clear that it eventually culminates in p100 phosphorylation and subsequent processing. According to our results this process could also depend on RelB expression levels, which increase during the differentiation of B lymphocytes (52). Interestingly, lipopolysaccharide-induced p100 processing and generation of p52 occurs only when RelB is expressed at the same time (18).

The role of NIK, whose overexpression enhances p100 phospho-

Acknowledgments—We thank B. Anic and U. Leschik for excellent technical assistance and A. Lasar for critical reading of the manuscript and useful comments.

REFERENCES

1. Denk, A., Wirth, T., and Baumann, B. (2000) Cytokine Growth Factor Rev. 11, 363–372
2. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
3. Karin, M., and Lin, A. (2002) Nat. Immunol. 3, 221–227
4. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) Nat. Rev. Cancer 2, 301–310
5. Whiteside, S. T., and Israel, A. (1997) Semin. Cancer Biol. 8, 75–82
6. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
7. Carlotti, F., Dower, S. K., and Qwarnstrom, E. E. (2000) J. Biol. Chem. 275, 41028–41034
8. Ghosh, S., and Karin, M. (2002) Cell 109 (suppl.) S81–96
9. Sendtner, U., Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonazzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C., and Karin, M. (2001) Science 293,
