

# RelB/p50 Dimers Are Differentially Regulated by Tumor Necrosis Factor- $\alpha$ and Lymphotoxin- $\beta$ Receptor Activation

CRITICAL ROLES FOR p100\*

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**Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and lymphotoxin- $\beta$  receptor (LT $\beta$ R) signaling both play important roles in inflammatory and immune responses through activation of NF- $\kappa$ B. Using various deficient mouse embryonic fibroblast cells, we have compared the signaling pathways leading to NF- $\kappa$ B induction in response to TNF- $\alpha$  and LT $\beta$ R activation. We demonstrate that LT $\beta$ R ligation induces not only RelA/p50 dimers but also RelB/p50 dimers, whereas TNF- $\alpha$  induces only RelA/p50 dimers. LT $\beta$ R-induced binding of RelB/p50 requires processing of p100 that is mediated by IKK $\alpha$  but is independent of IKK $\beta$ , NEMO/IKK $\gamma$ , and RelA. Moreover, we show that RelB, p50, and p100 can associate in the same complex and that TNF- $\alpha$  but not LT $\beta$  signaling increases the association of p100 with RelB/p50 dimers in the nucleus, leading to the specific inhibition of RelB DNA binding. These results suggest that the alternative NF- $\kappa$ B pathway based on p100 processing may account not only for the activation of RelB/p52 dimers but also for that of RelB/p50 dimers and that p100 regulates the binding activity of RelB/p50 dimers via at least two distinct mechanisms depending on the signaling pathway involved.**

NF- $\kappa$ B transcription factors are key regulators of transcription of a variety of genes involved in inflammatory and immune responses and in the control of cell proliferation, differentiation, and apoptosis (1–7). In mammalian cells, the NF- $\kappa$ B family is composed of five members, RelA (p65), RelB, c-Rel (Rel), NF- $\kappa$ B1 (p50 and its precursor p105), and NF- $\kappa$ B2 (p52 and its precursor p100), and exists as a heterogeneous collection of homodimers and heterodimers (3, 8).

In resting cells, NF- $\kappa$ B activity is tightly controlled by I $\kappa$ B family members, which include I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , Bcl-3, p100, and p105 (9, 10). Phosphorylation of a NF- $\kappa$ B inhibitor protein at specific serine residues by the IKK<sup>1</sup> complex targets it for

ubiquitination and subsequent degradation by the proteasome, thus enabling NF- $\kappa$ B dimers to translocate into the nucleus (11). The IKK complex is composed of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, IKK $\gamma$  (also known as NEMO) (11). The disruption of genes encoding individual subunits have demonstrated that IKK $\beta$  and IKK $\gamma$  are required for mediating the canonical NF- $\kappa$ B activity (*i.e.* RelA/p50 dimers) induced by inflammatory signals (12–15), whereas IKK $\alpha$  participates in other physiological processes. In particular, IKK $\alpha$  has been shown to be essential for the regulation of keratinocyte differentiation (16, 17), receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) induced I $\kappa$ B $\alpha$  degradation in mammary epithelial cells (18), and appropriate basal and inducible processing of NF- $\kappa$ B2 p100 precursor to p52 in B cells and lymphotoxin- $\beta$  receptor (LT $\beta$ R)-expressing cells (19–21).

RelB is the only NF- $\kappa$ B member that cannot homodimerize and only triggers potent transcriptional activation when coupled to p50 or p52 (22–25). Analyses of RelB-deficient mice have shown that RelB is essential to the development of medullary epithelium, mature dendritic cell function, and secondary lymphoid tissue organization (26–29). *Relb*<sup>-/-</sup> mice also spontaneously develop a generalized persistent non-infectious multi-organ inflammatory syndrome (30).

Until recently, the canonical NF- $\kappa$ B (RelA/p50) was considered to be the predominant inducible  $\kappa$ B DNA binding activity in most cell types in response to a broad range of stimuli, whereas RelB represented the major constitutive  $\kappa$ B activity in lymphoid cells (31, 32). However, in the past few months, an alternative mechanism for inducing NF- $\kappa$ B activity has emerged based on the observation that inducible IKK $\alpha$ -dependent p100 processing allows the resultant p52 to function as transcriptional activator (20, 21, 33, 34). Remarkably, a pathway-dependent specificity in p52 binding partner was demonstrated. Whereas RelA/p52 dimers are the targets of the canonical pathway, nuclear translocation of RelB/p52 is regulated via the alternative NF- $\kappa$ B pathway and leads to the transcription of a specific pool of genes (21). Most importantly, all of these studies point to a crucial role for the alternative NF- $\kappa$ B pathway in controlling the development, organization, and function of lymphoid tissue.

The participation of RelB in non-lymphoid function is much less well documented. Although RelB was initially identified as an immediate-early gene in fibroblasts (24), it has now been shown to play an essential role in limiting the expression of

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<sup>1</sup> The abbreviations used are: IKK, I $\kappa$ B kinase; LT $\beta$ R, lymphotoxin- $\beta$  receptor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TNFR, TNF receptor; mAb, monoclonal antibody; Ab, antibody; MEF, mouse embryonic fibroblasts;

EMSA, electrophoretic mobility shift assays; RT, reverse transcription; WT, wild type; NIK, NF- $\kappa$ B-inducing kinase; MCP-1, monocytic chemoattractant protein-1.

proinflammatory mediators in lipopolysaccharide-induced fibroblasts (35, 36), thereby playing an important role in the resolution of acute inflammation. Interestingly, in the non-lymphoid cells examined so far (*e.g.* NIH 3T3, smooth muscle cells), RelB was found mainly in association with p50 but not p52 in the inducible  $\kappa$ B DNA binding complexes (24, 37, 38). In contrast to recent progress in understanding the regulation of RelB/p52 dimers, the mechanisms controlling the inducible RelB/p50 DNA binding activity are still poorly understood.

In this study, we have investigated the regulation of RelB/p50 dimers in fibroblasts in response to ligation of TNFR and LT $\beta$ R, two members of the TNFR superfamily involved in the regulation of inflammatory and immune responses (39–43). We demonstrate that RelB/p50 activation triggered by LT $\beta$ R ligation requires processing of p100 that is mediated through IKK $\alpha$  but not IKK $\beta$ , IKK $\gamma$ , or RelA. Moreover, we show that RelB, p50, and p100 can associate in the same complex and that TNF- $\alpha$  signaling leads to the inhibition of RelB DNA binding via an increased association of p100 with RelB/p50 dimers in the nucleus.

#### EXPERIMENTAL PROCEDURES

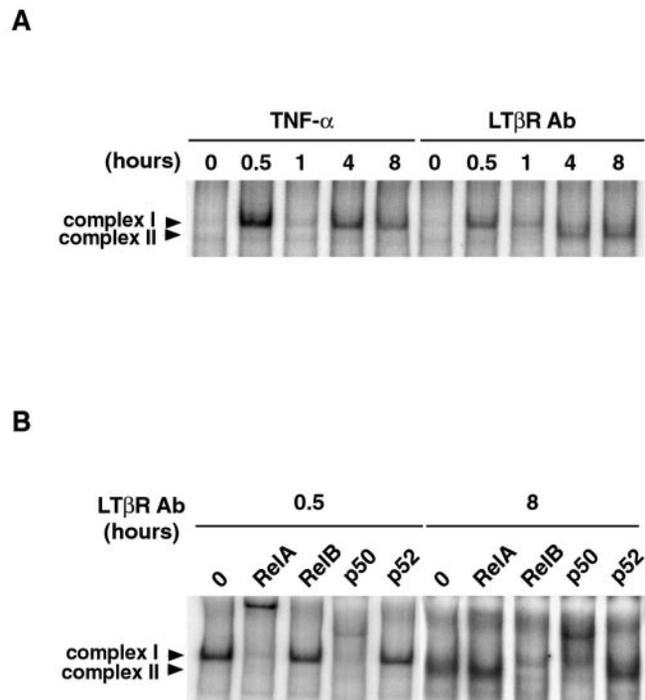
**Reagents and Antibodies**—Murine recombinant TNF- $\alpha$  was purchased from Sigma, and agonistic anti-LT $\beta$ R mAb AC.H6 was a kind gift from J. Browning. J. Hiscott and N. Rice generously provided anti-p52/p100 and anti-p50/p105 polyclonal antibodies. The remaining antibodies were purchased from Santa Cruz Biotechnology (IKK $\alpha$ , RelA, RelB, p105/p50, cRel, and phospholipase C $\gamma$ -1), Upstate Biotechnology (IKK $\beta$ , p100/p52, and p105/p50), and BD Biosciences (IKK $\gamma$ ).

**Cell Culture and Cell Lines**—IKK $\alpha$ -, IKK $\beta$ -, NEMO/IKK $\gamma$ -deficient mouse embryonic fibroblasts (MEFs) were described previously (12, 14, 16). RelA-, RelB-, and NF- $\kappa$ B2-deficient MEFs were a kind gift from A. Beg, F. Weih, and J. Caamano, respectively. MEFs were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100  $\mu$ g/ml streptavidin. HT29 (ATCC) were cultured in McCoy's 5A medium with the same supplements.

**Cell Extract Preparation**—Whole cell extracts were prepared as reported previously (44). For cytosolic and nuclear proteins, cells were lysed for 5 min on ice in hypotonic buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol, 1  $\mu$ M leupeptin, 1  $\mu$ M aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cytosolic fraction was harvested after centrifuging the lysate for 5 min at 4500  $\times$  g. The nuclear pellet was washed once with hypotonic buffer and lysed for 30 min on ice in extraction buffer (20 mM Hepes, 500 mM NaCl, 1.5 mM MgCl $_2$ , 0.5 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, 1  $\mu$ M leupeptin, 1  $\mu$ M aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Anti-phospholipase C- $\gamma$  was used as control for cytoplasmic contamination in nuclear fractions.

**Coimmunoprecipitation and Immunoblotting**—For coimmunoprecipitation experiments, 500  $\mu$ g of nuclear or whole cell extracts were immunoprecipitated for 2 h or overnight at 4  $^{\circ}$ C using specific antibodies, after which protein A/G-agarose beads (Amersham Biosciences) were added and incubation continued for 90 min at 4  $^{\circ}$ C. After four washes in lysis buffer, the beads were heat-denatured to release the proteins. Immunoprecipitated proteins were resolved on 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore). Immunoblotting was performed with specific antibodies and visualized using the ECL Western blotting detection kit (Amersham Biosciences). For double immunoprecipitation, nuclear or whole cell extracts were incubated with anti-p50 antibody and protein A/G-agarose beads. After five washes in lysis buffer, the antigen-antibody complexes were eluted with a 15-fold excess (w/w) of the specific peptide (Santa Cruz Biotechnology) overnight at 4  $^{\circ}$ C. The resulting supernatants were immunoprecipitated with anti-RelB antibody and protein A/G-agarose beads. The immune complexes obtained were separated on 8% SDS-polyacrylamide gel and detected by immunoblotting with anti-p100 antibody.

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts were prepared and analyzed as previously described using the human immunodeficiency virus long terminal repeat tandem  $\kappa$ B oligonucleotide as  $\kappa$ B probe (45). For supershift assays, nuclear extracts were



**FIG. 1. LT $\beta$ R but not TNFR stimulation activates RelB/p50 DNA binding.** A, nuclear extracts from wild type MEFs treated with TNF- $\alpha$  or agonistic LT $\beta$ R antibody (Ab) for the indicated periods of time were analyzed by EMSA using a  $^{32}$ P-labeled human immunodeficiency virus-long terminal repeat tandem  $\kappa$ B oligonucleotide as probe. B, for supershift, nuclear extracts from WT MEFs treated with anti-LT $\beta$ R Ab for 30 min or 8 h were incubated with the indicated antibodies prior to incubation with the labeled probe. Complex I, RelA/p50; complex II, RelB/p50.

incubated with specific antibodies for 30 min on ice before incubation with the labeled probe.

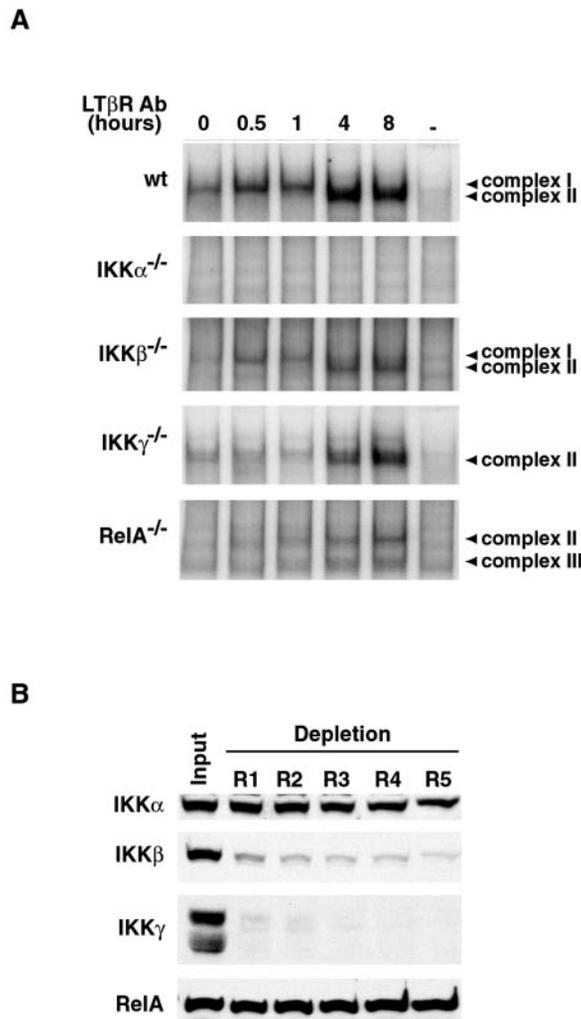
**RT-PCR**—RT-PCR were performed as described previously (46). Linear response ranges were determined for each gene to semiquantify their expression levels. Primer sequences are available upon request.

#### RESULTS

**Ligation of LT $\beta$ R but Not TNFR Activates RelB/p50 DNA Binding**—We used EMSA to evaluate the nuclear NF- $\kappa$ B DNA binding activity induced by ligation of LT $\beta$ R and TNFR. As shown in Fig. 1A, whereas nuclear extracts from untreated WT MEFs contained only low levels of NF- $\kappa$ B DNA binding activity, treatment with either TNF- $\alpha$  or agonistic anti-LT $\beta$ R mAb AC.H6 both resulted in two phases of NF- $\kappa$ B activation. TNF- $\alpha$ -induced NF- $\kappa$ B binding activity was detected after 30 min of treatment (complex I), decreased to basal levels after 60 min, returned to near maximal levels after 4 h of treatment, and persisted for at least 8 h. Complex I was also induced after 30 min of treatment with anti-LT $\beta$ R antibody, but a faster migrating  $\kappa$ B complex (complex II) was detected after 4–8 h of treatment.

The subunit composition of the NF- $\kappa$ B DNA binding complexes was then examined by supershift assays (Fig. 1B). Incubation of the agonistic LT $\beta$ R Ab-stimulated protein extracts with anti-RelA and anti-p50 antibodies supershifted complex I almost completely, whereas complex II was effectively supershifted with anti-RelB and anti-p50 antibodies. Antibodies to p52 (Fig. 1B) and c-Rel (data not shown) had very little effect on either complex.

**LT $\beta$ R-induced Binding of RelB/p50 Dimers Requires IKK $\alpha$  but Not IKK $\beta$  nor IKK $\gamma$** —To determine which subunit of the IKK complex controls the binding of RelB/p50 dimers in response to LT $\beta$ R ligation, we analyzed the DNA binding activity of the nuclear NF- $\kappa$ B complexes in IKK $\alpha$ -, IKK $\beta$ -, and IKK $\gamma$ -



**FIG. 2. LTβR-induced binding of RelB/p50 dimers requires IKKα but not IKKβ, IKKγ, or RelA.** *A*, WT (*wt*), IKKα<sup>-/-</sup>, β<sup>-/-</sup>, γ<sup>-/-</sup>, or RelA-deficient MEFs were treated with agonistic LTβR Ab for the indicated periods of time, and EMSA analyses were carried out on nuclear extracts as in Fig. 1A. -, probe alone; *complex III*, p50/p50. *B*, IKKα-containing complex exists independently of IKKβ and IKKγ. Whole cell extracts from untreated WT MEFs were subjected to five rounds of immunodepletion (R1–R5) using anti-IKKγ antibody. IKKα, β, γ, and RelA levels were determined by immunoblotting before depletion (*Input*) and after each round.

deficient fibroblasts (Fig. 2A) and found that a weak constitutive binding of RelB/p50 (complex II) was present only in MEFs lacking IKKγ. Most importantly, we found that IKKα is absolutely required for the induction of RelB/p50 DNA binding (complex II), whereas IKKβ and IKKγ are not. In contrast, RelA/p50 binding (complex I) was strongly reduced in IKKβ-deficient MEFs and abolished in IKKα- and IKKγ-deficient MEFs. During the preparation of this paper, LTβR-ligation-induced RelB/p50 activation was also reported by others to be independent of IKKγ (47). Because RelB transcription has been reported to be regulated by RelA (48), we also analyzed LTβR-mediated NF-κB DNA binding activity in MEFs lacking RelA. Complex II was clearly induced in the absence of RelA, albeit at somewhat lower levels. A strong constitutive binding of a third complex (complex III) was also observed in these cells. Super-shift assays revealed that complex III corresponds to a p50-containing complex (data not shown).

Given that IKKα exerts a specific function that is not controlled by the two other subunits of the IKK complex, it is possible that some of the IKKα present in cells is not incorpo-

rated into the large IKKα/IKKβ/IKKγ-containing complex (49). To test this possibility, we carried out immunodepletion experiments on whole cell extracts from fibroblasts. Five rounds of depletion were performed using an anti-IKKγ antibody, and the IKK subunit content was analyzed after each round by immunoblotting for IKKα, IKKβ, and IKKγ. IKKβ was almost entirely depleted from the protein extracts after one round of IKKγ depletion (Fig. 2B), showing that most of the IKKβ binds to IKKγ. In contrast, a considerable fraction of IKKα was still detectable after five rounds of IKKγ depletion. This observation suggests the existence of an IKKα-containing complex independent of IKKβ and IKKγ. Whether this alternative complex represents IKKα homodimers or IKKα associated with a different protein(s) remains to be determined.

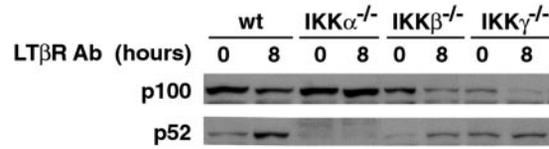
*IKKα but Not IKKβ and IKKγ Regulates LTβR-induced p100 Processing and RelB Nuclear Translocation in Mouse Embryonic Fibroblasts*—We have previously reported that LTβR-induced processing of p100 generates RelB/p52 dimers that translocate to the nucleus to activate a set of specific target genes (21). To determine whether a control mechanism based on p100 processing could also apply to RelB/p50 dimers, we examined p100 and p52 protein levels in WT, IKKα<sup>-/-</sup>, IKKβ<sup>-/-</sup>, and IKKγ<sup>-/-</sup> fibroblasts (Fig. 3A). Treatment with anti-LTβR antibody strongly enhanced processing of p100 to p52 in WT fibroblasts but failed to do so in IKKα-deficient MEFs (Fig. 3A). Importantly, LTβR ligation led to p100 processing with kinetics parallel to those of RelB/p50 binding. Although the steady state level of expression of p100 is low in IKKβ- and even lower in IKKγ-deficient fibroblasts compared with WT fibroblasts, p100 processing still occurred in these cells in response to treatment with the agonistic LTβR Ab. Thus, coincident with the induction of RelB/p50 DNA binding (Fig. 2A), IKKα but not IKKβ and IKKγ is also absolutely required for LTβR-induced processing of p100 (Fig. 3A). These results strongly suggest that the IKKα-dependent p100 processing plays a critical role in the regulation of LTβR-induced activation of RelB/p50 dimers. To further investigate underlying mechanisms, we compared intracellular distributions of RelB in LTβR-stimulated IKKα<sup>-/-</sup>, IKKβ<sup>-/-</sup>, and IKKγ<sup>-/-</sup> MEFs (Fig. 3B). We observed that LTβR ligation-induced RelB nuclear localization was abolished in IKKα- but not β- or γ-deficient MEFs (Fig. 3B). In addition, although similar constitutive RelB protein levels were observed in the cytoplasm of the three IKK-deficient cell lines, constitutive RelB levels in the nucleus were markedly greater in IKKγ-deficient cells, which may explain the constitutive RelB/p50 DNA binding activity observed in these cells (Fig. 2A). Taken together, our data demonstrate that IKKα is required for the LTβR-induced activation of RelB/p50 dimers. Most probably, the processing of p100 and thus the removal of this main inhibitory partner of RelB allows RelB nuclear translocation and DNA binding.

*Nuclear p100 Inhibits RelB/p50 DNA Binding in Response to TNF-α*—To determine whether the failure of TNF-α to induce RelB DNA binding is the result of a lack of nuclear translocation of RelB, we compared RelB protein levels and cellular distributions in response to TNF-α versus agonistic LTβR activation in WT MEFs (Fig. 4). TNF-α induction resulted in a strong increase of RelB in both cytoplasm and nucleus, whereas LTβR stimulation markedly increased only nuclear RelB. Importantly, the levels of induced nuclear RelB were similar in response to TNFR and LTβR ligation. Thus, the increased nuclear RelB expression level observed in TNF-α-induced fibroblasts does not lead to an increased binding activity, suggesting a nuclear control of RelB/p50 activity.

The absence of TNF-α-induced RelB DNA binding, despite its accumulation in the nucleus, could also be attributed to a

A

FIG. 3. **IKK $\alpha$  is required for LT $\beta$ R-induced p100 processing and RelB nuclear translocation.** A, IKK $\alpha$  but not  $\beta$  and  $\gamma$  is required for LT $\beta$ R-induced p100 processing. WT (*wt*), IKK $\alpha$ -,  $\beta$ -, or  $\gamma$ -deficient MEFs were treated or not with TNF- $\alpha$  for 8 h, and p100 and p52 protein expression was determined by immunoblotting in whole cell extracts. B, RelB nuclear translocation in response to LT $\beta$ R ligation requires IKK $\alpha$  but not  $\beta$  and  $\gamma$ . MEFs lacking IKK $\alpha$ ,  $\beta$ , or  $\gamma$  were treated with agonistic LT $\beta$ R Ab for the indicated periods of time, and RelB expression was determined by immunoblotting in cytoplasmic and nuclear extracts.



B

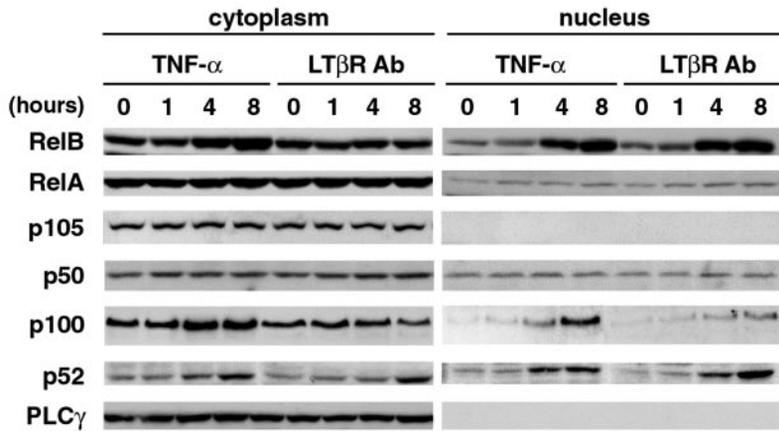
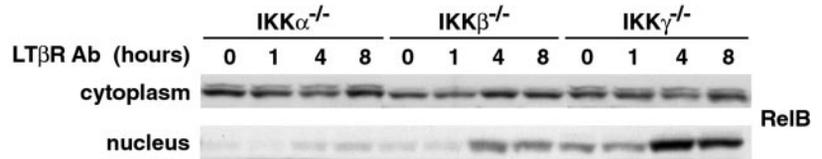


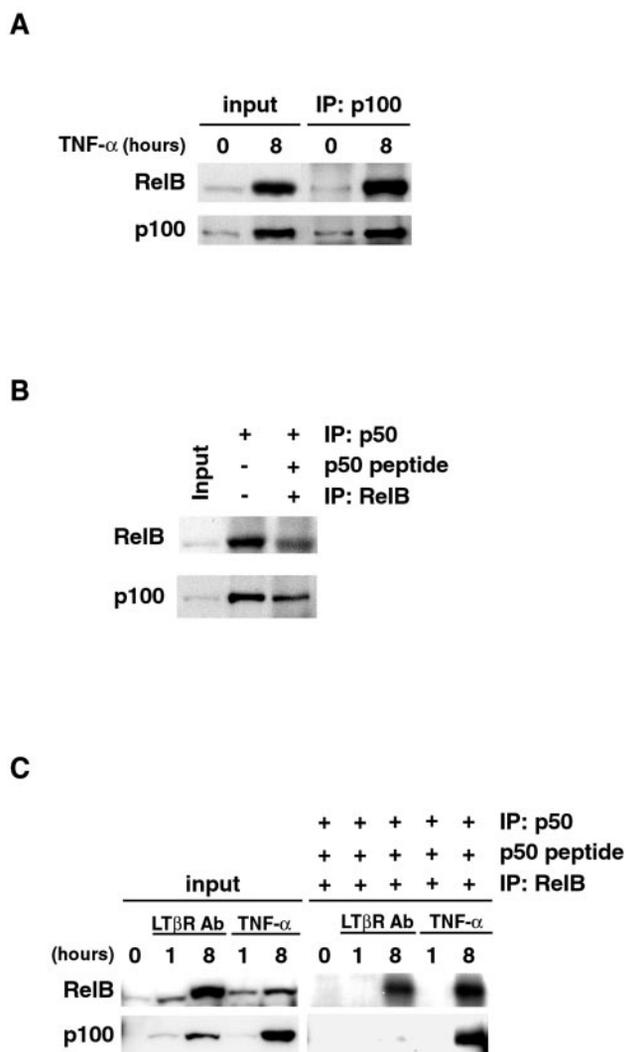
FIG. 4. **TNFR ligation induces both cytoplasmic and nuclear accumulation of p100 in WT MEFs.** Cytoplasmic and nuclear extracts of WT MEFs treated with TNF- $\alpha$  or agonistic LT $\beta$ R Ab for the indicated periods of time were analyzed by immunoblotting for the indicated proteins. Phospholipase C- $\gamma$ 1 (PLC $\gamma$ ) was used as a quality control to verify the absence of cytoplasmic contamination in the nuclear extract.

lack of production of its heterodimerization partners p50 and p52 and/or to the absence of p100 degradation. Therefore, we also compared the protein levels and cellular distributions of p105/p50 and p100/p52 in TNF- $\alpha$ - and anti-LT $\beta$ R-treated WT MEFs (Fig. 4). TNFR and LT $\beta$ R ligation both had very little effect on p105 and p50 protein levels and cellular distribution. Although no nuclear p105 was detected, a fraction of p50 was constitutively present in the nucleus. Within 4 h after TNFR ligation, p100 levels increased slightly in the cytoplasm but strongly in the nucleus. In contrast, LT $\beta$ R ligation led to p100 processing accompanied by nuclear accumulation of p52 (Fig. 4) with kinetics parallel to those of RelB/p50 binding. Because the availability of RelB for its DNA binding heterodimerization partner p50 is similar in TNF- $\alpha$  and anti-LT $\beta$ R mAb AC.H6-stimulated fibroblasts, we hypothesized that the TNF- $\alpha$ -induced increase of nuclear p100 might block RelB/p50 DNA binding.

To test this hypothesis, we first examined whether RelB associates with p100 in the nucleus of TNF- $\alpha$ -treated fibroblasts *in vivo*. p100 was immunoprecipitated using antibody directed against its C-terminal domain to avoid immunoprecipitation of p52. As shown in Fig. 5A, endogenous RelB coimmunoprecipitates with p100 in the nucleus of TNF- $\alpha$ -activated WT fibroblasts. Importantly, the increase of RelB protein levels parallels the increase of p100 protein levels. In contrast, using whole cell extracts as well as nuclear fractions, no association was detected between RelB and p105 (data not shown). We next

investigated whether p100 was able to sequester RelB/p50 dimers within the nucleus of TNF- $\alpha$ -treated cells. To first confirm the existence of such an association, we performed double immunoprecipitation using whole cell extracts from untreated fibroblasts. A first immunoprecipitation was performed with an anti-peptide antibody directed against p50 followed by incubation with the corresponding p50 peptide to elute the precipitated proteins. The eluate was then immunoprecipitated with an anti-RelB antibody. An analysis of this second eluate by immunoblotting with an anti-p100 antibody revealed that p100 participates in a multi-protein complex that contains both p50 and RelB (Fig. 5B). The existence of such a p100/RelB/p50 complex is not restricted to fibroblasts. Indeed, we have also observed the association of p100 with RelB/p50 dimers in HT29 cells (see below). To further explore underlying mechanisms, we then used nuclear fractions instead of whole cell extracts in double immunoprecipitation as described above. Most importantly, 8 h of TNF- $\alpha$  stimulation resulted in a strong increase in the association of nuclear RelB/p50 dimers with p100, whereas such an association was not detected in the nucleus of LT $\beta$ R-activated cells (Fig. 5C).

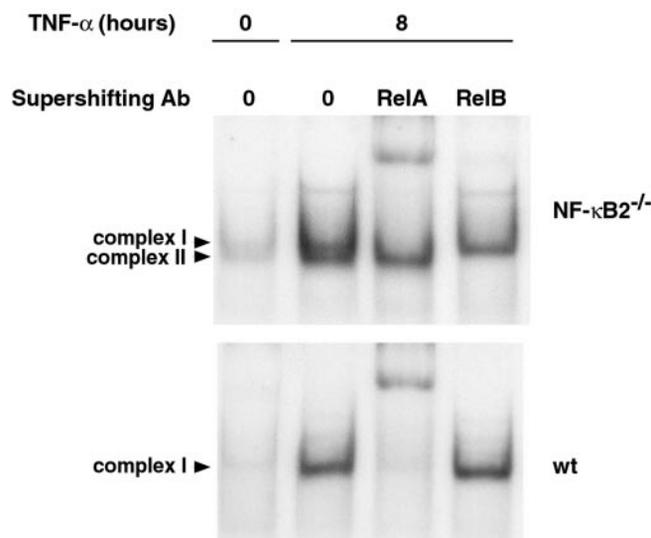
To further elucidate the inhibitory role of p100 on RelB/p50 DNA binding activity downstream of TNFR, we analyzed  $\kappa$ B DNA binding activity in NF- $\kappa$ B2-deficient fibroblasts, *i.e.* lacking p100 (Fig. 6). A constitutive binding of RelB/p50 dimers (complex II) was observed in these cells. Importantly, TNF- $\alpha$  treatment resulted in dramatic increase of RelB/p50 DNA bind-



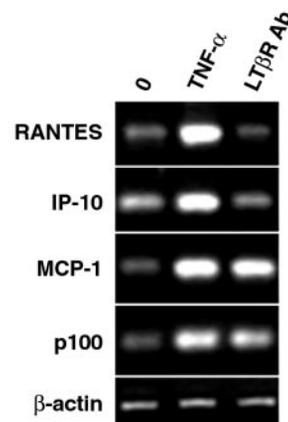
**FIG. 5. Increased association of p100 with RelB/p50 dimers in the nucleus of TNF- $\alpha$ -treated cells.** *A*, RelB associates with p100 in the nucleus of TNF- $\alpha$ -treated WT fibroblasts. Nuclear extracts prepared from either untreated or TNF- $\alpha$ -treated (8 h) WT MEFs were subjected to immunoprecipitation (IP) with anti-p100 antibody and analyzed for associated RelB. *B*, RelB, p50, and p100 are present in the same multimeric complex. The double immunoprecipitation was performed using whole cell extracts from WT MEFs as described under "Experimental Procedures." *C*, TNF- $\alpha$  but not agonistic LT $\beta$ R Ab induces the formation of the RelB/p50/p100 multimeric complex in the nucleus of HT29 cells. Nuclear extracts from HT29 cells treated with TNF- $\alpha$  or agonistic LT $\beta$ R Ab for the indicated periods of time were subjected to double immunoprecipitation as above and analyzed for associated p100.

ing (complex II) in the absence of p100, whereas only RelA-containing dimers (complex I) were induced by TNF- $\alpha$  in WT fibroblasts (Fig. 6). Together, these results demonstrate that p100 inhibits TNF- $\alpha$ -induced RelB/p50 DNA binding, most probably via the "trapping" of nuclear RelB/p50 dimers by p100.

**Ligation of LT $\beta$ R and TNFR Differentially Regulate Gene Expression**—We have shown that LT $\beta$ R ligation induces both the canonical NF- $\kappa$ B pathway, leading to a rapid and transient activation of RelA/p50 dimers, and the alternative NF- $\kappa$ B pathway, leading to a more delayed and sustained activation of RelB-containing dimers (Ref. 21 and this report). In contrast, TNF- $\alpha$  only induces the canonical NF- $\kappa$ B pathway, leading primarily to the activation of RelA-containing dimers. To address the physiological relevance of the alternative pathway in the activation of gene expression, WT MEFs were either left



**FIG. 6. TNF- $\alpha$  induces RelB/p50 DNA binding in NF- $\kappa$ B2-deficient fibroblasts.** Nuclear extracts from WT (*wt*) and NF- $\kappa$ B2-deficient MEFs, either untreated or treated with TNF- $\alpha$  for 8 h, were analyzed for NF- $\kappa$ B activity by EMSA. For supershift, nuclear extracts were incubated with the indicated antibodies prior to incubation with the labeled probe.



**FIG. 7. TNFR and LT $\beta$ R ligation do not induce the same NF- $\kappa$ B-responsive genes.** RT-PCR was performed with PCR primer pairs of the indicated genes using total RNAs prepared from WT fibroblasts, either untreated or treated with TNF- $\alpha$  or agonistic LT $\beta$ R Ab for 8 h. All of the RT-PCRs were performed in the linear range for each transcript compared with  $\beta$ -actin as a reference control. All of the RT-PCRs were performed three times with the same results.

untreated or treated with agonistic LT $\beta$ R mAb or TNF- $\alpha$  for 8 h and expression of several NF- $\kappa$ B-responsive genes with roles in inflammation was monitored by semiquantitative RT-PCR (Fig. 7). LT $\beta$ R mAb and TNF- $\alpha$  both induced the genes encoding monocyte chemoattractant protein-1 (MCP-1) and p100, but expression of other target genes including those for the chemokines RANTES (regulated on activation normal T cell expressed and secreted) and interferon-inducible protein-10 was clearly specifically induced by TNF- $\alpha$  (Fig. 7). None of the genes tested thus far was specifically induced in response to LT $\beta$ R ligation. Nevertheless, our results indicate that there is only partial overlap in the set of genes induced by LT $\beta$ R mAb and TNF- $\alpha$ , suggesting that the LT $\beta$ R-induced activation of p100 processing may control a set of specific target genes that remain to be identified.

#### DISCUSSION

In the study presented here, we have explored the TNFR- and LT $\beta$ R-mediated signaling events that control NF- $\kappa$ B activ-

ity in fibroblasts. We observe that different IKK subunits are required for RelA and RelB regulation. Consistent with previous observations, the data also show that TNF- $\alpha$ -induced RelA/p50 activation requires IKK $\beta$  and IKK $\gamma$ , whereas IKK $\alpha$  is dispensable (12, 15, 17, 50). In contrast, no RelB/p50 DNA binding is induced by TNFR ligation, whereas LT $\beta$ R ligation activates both RelA/p50 and RelB/p50 complexes. In addition, we demonstrate that LT $\beta$ R-induced binding of RelA/p50 requires IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ , whereas LT $\beta$ R-induced binding of RelB/p50 absolutely requires IKK $\alpha$  but not IKK $\beta$  and IKK $\gamma$ . Recently, it has been reported that IKK $\alpha$  may function as an essential component of the classical IKK complex, being specifically required for RANKL-mediated activation of this complex in mammary epithelial cells (18). The data presented here thus provide a second body of evidence for a crucial role of IKK $\alpha$  in the induction of canonical NF- $\kappa$ B DNA binding activity.

In contrast to the canonical NF- $\kappa$ B (RelA/p50), we and others have observed that p100 is the only I $\kappa$ B family member that strongly inhibits RelB activity (48, 51, 52).<sup>2</sup> Recently, we have shown that LT $\beta$ R-induced IKK $\alpha$ -dependent p100 processing controls RelB/p52 dimer nuclear translocation and gene regulation (21). In this report, we show that the control of p100 processing also plays a critical role in the regulation of LT $\beta$ R-induced activation of RelB/p50 dimers. Therefore, the newly discovered alternative NF- $\kappa$ B pathway based on p100 processing seems to account not only for the regulation of RelB/p52 dimers but also for that of RelB/p50 dimers. Although RelB/p52 dimers might be expected to result from the processing of RelB/p100 dimers, it was less clear *a priori* how p100 processing could control RelB/p50 binding activity. Interestingly, we have found that endogenous p100, p50, and RelB can associate in a single multi-protein complex in fibroblasts as well as in HT29 cells. Thus, our data suggest that LT $\beta$ R ligation releases RelB/p50 dimers from their interaction with full-length p100, allowing RelB nuclear translocation and subsequent DNA binding. Endogenous complexes containing p100 together with RelA/p50 (53, 54) or RelB/p50 (55) have also been found in human breast and lymphoid cancer cells, suggesting that the release of NF- $\kappa$ B dimers from p100 inhibition could represent a more general mechanism for regulation of NF- $\kappa$ B activity.

In IKK $\beta$ -deficient fibroblasts, we have observed a clear reduction of LT $\beta$ R-induced binding of RelB/p50 dimers that does not correlate with an impaired processing of p100 or decreased RelB nuclear translocation. Most probably, the diminished RelB/p50 activity is related to the markedly reduced RelB protein expression in these cells (Fig. 3B). Interestingly, a weak constitutive RelB/p50 DNA binding was detected in MEFs lacking IKK $\gamma$ , correlating with a high constitutive level of nuclear RelB and a very low level of p100 expression in these cells (Fig. 3). A constitutive RelB/p50 DNA binding was also detected in NF- $\kappa$ B2-deficient fibroblasts (Fig. 6). These observations suggest that there are at least two levels of complexity in the regulation of RelB/p50 activity: 1) the overall expression level of RelB and p100 proteins; and 2) the control of p100 processing.

Although TNF- $\alpha$  signaling did not induce RelB/p50 DNA binding in WT fibroblasts, a marked increase of RelB protein level was observed in the nucleus of these cells. This absence of a direct correlation between the nuclear localization of RelB and its DNA binding activity clearly suggested that an additional negative control of RelB activity existed in the nucleus of TNF- $\alpha$ -treated fibroblasts. Here again, p100 was a good candidate, because the level of nuclear p100 was also strongly increased in response to TNF- $\alpha$ . Indeed, we observe that TNF- $\alpha$

signaling strongly induces RelB/p50 activity in NF- $\kappa$ B2-deficient cells, suggesting that it is not the processing of p100 *per se* but rather the “removal” of p100 that allows RelB/p50 dimers to bind to the DNA. In addition, we demonstrate that the association of p100 with RelB/p50 dimers is dramatically increased in the nucleus of TNF- $\alpha$ -treated cells. In conclusion, TNF- $\alpha$ -induced assembly of the p100/RelB/p50 multimeric complex in the nucleus seems to account for the inhibition of RelB/p50 DNA binding, implying that p100 controls RelB/p50 dimers not only in the cytoplasm but also in the nucleus.

How might full-length p100 interact with NF- $\kappa$ B dimers? NF- $\kappa$ B members all contain an N-terminal Rel homology domain responsible for DNA binding, dimerization, and association with the I $\kappa$ Bs (3, 56). The C-terminal domain of p100, like the other I $\kappa$ Bs, is characterized by an ankyrin-rich domain that interacts with NF- $\kappa$ B via the Rel homology domain. Structures of co-crystals of NF- $\kappa$ B proteins in association with I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  have been determined previously (56), and it emerges that the dimerization domain of the NF- $\kappa$ B dimers is the primary region of interaction with I $\kappa$ Bs. It seems plausible that p100 might self-associate through its dimerization domain and that its C-terminal ankyrin domain could then serve as a platform for the binding of RelB/p50 dimers.

Analyses of NF- $\kappa$ B knock-out mice have revealed that mice lacking p100/p52 have marked defects in splenic microarchitecture very similar to those observed in LT $\beta$ R-, NIK-, and RelB-deficient mice (29, 57, 58). Interestingly, during the revisions of this paper, mice lacking RelB were also reported to be deficient in Peyer's patch organogenesis (59), a phenotype also observed in NF- $\kappa$ B2-, NIK-, and LT $\beta$ R-deficient animals. Animals lacking p50 do not show those dramatic developmental defects. Nevertheless, their Peyer's patches are reduced in number and size (60). Therefore, although p50-containing dimers are not absolutely required, they seem to contribute to the Peyer's patch developmental program. Therefore, it is tempting to conclude that the processing of p100 downstream of LT $\beta$ R is critically involved in the functions of stromal cells during secondary lymphoid organ development, most probably through the control of RelB/p52 and, perhaps to a lesser extent, RelB/p50-responsive genes.

In addition to the lymphoid organ defects, RelB-deficient mice display a multi-organ inflammatory syndrome that contributes significantly to premature mortality in these mice (28). In an effort to better elucidate the physiological relevance of the LT $\beta$ R-induced alternative NF- $\kappa$ B pathway, we have performed RT-PCR on several known NF- $\kappa$ B target genes with roles in inflammation. We have observed that in WT fibroblasts, p100 and monocyte chemoattractant protein-1 are induced by ligation of both TNFR and LT $\beta$ R. Interestingly, MCP-1 was previously found to be specifically regulated by NIK in response to LT $\beta$ R but not TNFR activation (61). Because NIK is required for LT $\beta$ R-induced p100 processing (21), the loss of MCP-1 induction observed in NIK-deficient cells could reflect the lack of activation of RelB-containing dimers. These findings suggest that RelB/p50 dimers control the transcription of inflammatory genes downstream of LT $\beta$ R. Chromatin immunoprecipitation experiments and microarray analyses designed to determine which genes are specifically regulated by RelB heterodimers will provide a direct test of this hypothesis.

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