Lymphotoxin β Receptor Induces Sequential Activation of Distinct NF-κB Factors via Separate Signaling Pathways*

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Lymphotoxin β receptor (LTβR)-induced activation of NF-κB in mouse embryo fibroblasts was mediated by the classical pathway and by an alternative or second pathway. The classical pathway involved the IκB kinase (IKK)β- and IKKγ-dependent degradation of IκBo and resulted in the rapid but transient activation of primarily RelA-containing NF-κB dimers. The alternative or second pathway proceeded via NF-κB-inducing kinase (NIK)-, IKKα-, and protein synthesis-dependent processing of the inhibitory NF-κB2 p100 precursor protein to the p52 form and resulted in a delayed but sustained activation of primarily RelB-containing NF-κB dimers. This second pathway was independent of the classical IKK complex, which is governed by its central IKKγ regulatory subunit. The sequential engagement of two distinct pathways, coupled with the negative feedback inhibition of RelA complexes by NF-κB2-induced resynthesis of IκBo, resulted in a pronounced temporal change in the nature of the NF-κB activity during the course of stimulation. Initially dominant RelA complexes were replaced with time by RelB complexes. Therefore, the alternative activation path mediated by processing of p100 was necessary for sustained NF-κB activity in mouse embryo fibroblasts in response to LTβR stimulation. Based on the phenotype of mice deficient in various components of the LTβR-induced activation of p100 processing, we conclude that this pathway is critically involved in the function of stromal cells during the generation of secondary lymphoid organ microarchitectures.

NF-κB1 transcription factors are critical mediators in the fight of the host against invading pathogens (reviewed in Refs. 1–5). These factors are integral parts of the innate machinery that translates initial detection of foreign pathogens, for example by epithelial cells, into activation of these cells, including production of chemokines and cytokines to in turn attract and activate professional immune cells. The innate system further involves NF-κB factors to produce antipathogenic effectors as well as chemokines and cytokines to mediate evolving cell-cell communications needed to coordinate responses. Depending on the exact nature of the initial innate response, NF-κB factors then help to develop the appropriate adaptive responses by lymphocytes. In the final phase of the immune response, NF-κB factors have important roles during the expansion and differentiation of lymphocytes involved in the adaptive response. Beyond the innate and adaptive antipathogenic responses, NF-κB factors are also essential during development and maintenance of lymphoid organ structures (6, 7), and they make important contributions during the development of hematopoietic cells, including B cells and osteoclasts (8, 9). To carry out its diverse physiologic roles, NF-κB factors not only help to induce expression of various factors and effectors, but depending on the cellular context, they also transcriptionally induce proteins that function to protect cells from apoptosis and that help to stimulate proliferation (1, 2, 5, 10).

NF-κB is a collective term for a family of dimeric complexes comprised of combinations of five polypeptides, RelA, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2. p50 and p52 are the N-terminal parts of the longer p105/NF-κB1 and p100/NF-κB2 proteins, respectively, and they are generated by proteolytic processing (1, 2, 4, 5). High levels of p50 are produced constitutively by a cotranslational mechanism. In contrast, usually only small amounts of p52 exist in cells, but higher amounts may be induced by select signals.

To activate NF-κB, appropriate environmental signals must bring about the release of NF-κB dimers from their bound cytoplasmic inhibitors, in particular from the prototypical inhibitor IκBo and its close relatives, IκBβ and IκBox (1, 2, 4, 5). NF-κB factors are in addition subject to various direct and indirect mechanisms that modulate their ability to stimulate transcription, dependent also on promoter context (1, 2, 5), but the release from the inhibitors is a first and necessary step in the activation process. Most of the NF-κB activation signals, and in particular inflammatory cytokines, such as TNFα and IL-1, induce the phosphorylation of the IκBs followed by the rapid ubiquitin- and proteasome-mediated degradation of the inhibitors, thus freeing NF-κB dimers to migrate to the nucleus to initiate gene transcription (1, 2, 4, 5). IκBs are phosphorylated on two conserved serines by the IκB kinase (IKK) complex. IKKs consist of the catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ (also known as Nemo).

Most signals have been shown to activate NF-κB by the classical, IKK-dependent pathway and, in particular, to be dependent on the IKKβ catalytic and IKKγ/Nemo regulatory subunit to bring about the degradation of small IκB inhibitors (1, 2, 5, 11). In addition to the small IκBs, the long forms of the NF-κB1 and NF-κB2 proteins, p105 and p100, can also act as cytoplasmic inhibitors of bound Rel proteins due to the presence of IκB-like inhibitory ankyrin domains in their C-terminal halves (1, 2, 4, 5). p105 may be completely degraded in response to some signals in a manner similar to that of small IκBs, including IKKβ/IKKγ-induced phosphorylation of two serines

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‡ The abbreviations used are: NF-κB, nuclear factor-κB; IκB, inhibitor of κB; IKK, IκB kinase complex; NIK, NF-κB-inducing kinase; LT, lymphotoxin; LTβR, lymphotoxin β receptor; aly, alymphoplasia; TNF, tumor necrosis factor; MEF, mouse embryo fibroblast; EMSA, electrophoretic mobility shift assay; BAFF, B cell-activating factor belonging to the TNF family.
embedded in a small IκB-like phosphorylation motif (12). Recent-  
ly, a second or alternative signaling pathway has been reported to  
liberate NF-κB activity via induced processing of p100 inhibitor (13, 14). Although physiologic signals for this pathway are not reported, processing was mediated by the NF-κB-inducing kinase (NIK) and IKKα in the present report, we demonstrate that physiologic signaling via the lymphotixin receptor (LTβR) in stromal cells induced the degradation of IκBα via the classical pathway, and it induced processing of p100 via an alternative pathway. p100 processing was shown to be dependent on NIK and IKKα in independent of IKKβ and IKKγ/Nemo. Therefore, the p100 processing pathway was entirely independent of the IKK complex, not just of the IKKβ kinase subunit. We also demonstrate that transient activation of the classical pathway caused the transient activation of the p50-RelA dimers, whereas the delayed and sustained liberation of p50-RelB and p52-RelB complexes. We also provide an explanation and supporting evidence for how p100 processing liberated p50-RelB complexes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**IKKα−/− and IKKβ−/− mouse embryonic fibroblasts (MEFs) were kindly provided by Drs. Q. Li and I. M. Verma, and IKKα−/− and IKKβ−/− MEFs were kindly provided by Drs. M. Pasparakis and K. Rajewsky, NF-κB1−/− and NF-κB2−/− MEFs were kindly provided by Dr. E. Claudio. To prepare embryonic fibroblasts from wild-type and aly/aly mice, 12-day-old embryos were dissected, heads and inner organs were removed, and remaining parts were minced, filtered, and subjected to trypsin (0.25%) digestion for 10 min at 37 °C. The resulting cells were filtered and washed in Dulbecco’s modified Eagle’s medium (Invitrogen). Fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

**Transfections and Western Analyses—**Fibroblasts were plated into 6-well plates at 105 cells/well 24 h prior to stimulation. Stimulation was done under serum-free conditions. Following stimulation, nuclear and cytoplasmic extracts were prepared essentially as described (17). Briefly, fibroblasts were mechanically removed, washed twice in phosphate-buffered saline, and resuspended in 400 μl of low salt buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, supplemented with protease and phosphatase inhibitors) and incubated for an additional 15 min on ice. Triton-X-100 was added to a final concentration of 0.6%, and the suspension was vigorously vortexed for 10 s. The nuclei were pelleted, and the supernatant served as cytoplasmic extract. The pelleted nuclei were resuspended in 50 μl of high salt buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, supplemented with protease and phosphatase inhibitors) and incubated for an additional 15 min on ice. 2.5 μl of this preparation were used in DNA binding reactions. An NF-κB-binding site from the κ light chain enhancer was used as a probe: 5′-AGTGGAGGGACCTTTCCAGGC-3′ (Promega, Madison, WI). Oct1 oligonucleotides were used in controls: 5′-TGTTCGAAATGTACATGAAA-3′. Complementary and annealed oligonucleotides were end-labeled with [32P]ATP. For super-shift analyses, nuclear extracts were preincubated with antibodies for 30 min. For Western blot analysis, 20,000 cpm of probe were used per assay. The binding reaction was carried out at room temperature for 15 min in a total volume of 25 μl containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 0.5 mM dithiothreitol, 50 μg/ml poly(dI-dC)poly(dI-dC), 4% glycerol. For super-shift analyses, nuclear extracts were preincubated with antibodies for 30 min. For Western blot analysis, 20,000 cpm of probe were used per assay. The efficiency of the nuclear-cytoplasmic fractionation was confirmed in several ways, including by the fact that entry of factors was dependent on stimulation and the fact that RelA did not enter nuclei in NEMO-deficient cells, whereas RelB did (see Fig. 6, A and C).

For immunoprecipitation, cytoplasmic and nuclear preparations were generated from 1.5 × 106 cells/condition. These extracts were adjusted to 150 mM NaCl and mixed with anti-RelB antibodies (SC-848) or anti-NEMO antibodies (SC-8380) (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control that had been conjugated to agarose beads (Pierce). Following a 2-h incubation at 4 °C, the agarose beads were washed four times in 150 mM NaCl, 25 mM Hepes, pH 7.3, 10% glycerol, 1% Triton-X-100, and the immunoprecipitated preparations were subjected to SDS-PAGE.

**Antibodies and Reagents—**The agonistic monoclonal anti-murine LTβR antibodies were kindly provided by Dr. J. Browning and used at 10 μg/ml. For EMSA supershift experiments, the following antibodies to detect murine proteins were used: anti-RelA (SA-171) (Biomol, Plymouth Meeting, PA); anti-RelB (SC-290X), anti-c-Rel (SC-71X), anti-NF-κB1 (SC-114X), anti-NF-κB2 (SC-848X) (Santa Cruz Biotechnology). For Western analyses, the following antibodies were used: anti-IκBα (SC-945), anti-IκBα (SC-371), anti-RelB (SC-226), anti-c-Rel (SC-71) (Santa Cruz Biotechnology). Polyclonal anti-murine RelA, anti-murine p105, and anti-human p100 antibodies (also detects murine p100) were raised against the 13 C-terminal amino acids (RelA), the 15 N-terminal amino acids (p105), and the 398 N-terminal amino acids (p100), respectively. TNFα was purchased from PeproTech (Rocky Hill, NJ); Light and platelet-derived growth factor (PDGF-BB) were purchased from R&D Systems (Minneapolis, MN). Light was used at 20 ng/ml. The IKKβ-specific inhibitor PS1145 was kindly provided by Dr. J. Adams, Millennium Pharmaceuticals (Cambridge, MA). To inhibit protein synthesis, cells were pretreated with 50 μM cycloheximide (Sigma) for 30 min prior to stimulation.

**RESULTS**

**LTβR Induces Processing of p100 and Degradation of IκBα in Mouse Embryo Fibroblasts—**NF-κB2-deficient mice are impaired in their splenic microarchitecture, they lack Peyer’s patches, and they are severely impaired in lymph node formation, primarily due to defects within the stromal compartment (6, 7, 18, 19). These deficiencies in secondary lymphoid organs, which also include loss of follicular dendritic cell networks, in turn contribute to defective immune responses in these mutant mice. Similar deficiencies have been noted in aly/aly mice (20, 21) and in mice lacking lymphotixin β (LTβR) receptor or its ligands (LTα/β and Light) (20, 22–25), members of the TNF receptor/ligand family. aly/aly mice are mutated in NIK (26). Overexpression of the wild-type form of NIK induces processing of the p100 protein of NF-κB2 to p52, but its mutant form (aly) does not (13). Furthermore, splenocytes from aly/aly mice contain much less of the p52 protein of NF-κB2 than aly/aly mice while maintaining normal levels of p100 (21). NIK-induced processing in B cells depends on IKKα (14), and thus IKKα-deficient B cells contain much less p52 protein (14). Finally, although IKKα-deficient animals die perinatally, which limits their analysis, it could nevertheless be shown that these mutant mice are deficient in Peyer’s patches organogenesis (27). Based on these data, we hypothesized that critical functions of the LTβR receptor on stromal cells depend on signaling via NIK, IKKα, and NF-κB2 and thus may involve processing of p100.

To test for this possibility, we subjected MEFs to an agonistic antibody directed against the LTβR. We investigated with Western analyses for the expression of the NF-κB2 proteins p100 and p52, as well as the NF-κB1 proteins p105 and p50, the inhibitor of NF-κB1 (IκBα), IκBβ, RelA, RelB, and c-Rel at six time points during an 8-h stimulation with anti-LTβR antibodies (Fig. 1). We also tested for expression of these proteins at 15 min and 8 h of stimulation with TNFα. The experiments revealed a marked decrease in p100 and a concomitant increase in p52, beginning just before 4 h and maximal by 8 h of stimulation via the LTβR. No such changes in p100 and p52 levels were seen with TNFα stimulation. TNFα instead caused the nearly complete degradation of IκBα and the partial degradation of IκBβ by 15 min of stimulation; IκBα levels were partly restored by 8 h of stimulation. LTβR stimulation induced only a partial degradation of IκBα, which showed a delayed onset.
TNFα/

| TNFα | anti-LTβR | Time (h) | Stimulus |
|------|-----------|----------|----------|
| 8    | .25       | 0        | p100     |
| 0    | .25       | .5       | p105     |
| 0    | .25       | .5       | p50      |
|      |           | 1        | ixβA     |
|      |           | 2        | ixβB     |
|      |           | 4        | RelA     |
|      |           | 8        | RelB     |
|      |           |          | c-Rel    |

**Fig. 1.** LTβ receptor engagement induces processing of p100/NF-κB2 to p52 as well as IκBα degradation in wild-type MEFs. Wild-type MEFs were stimulated with TNFα or with agonistic anti-LTβ receptor antibodies for the times shown. Total extracts were prepared by SDS lysis and subjected to SDS-PAGE followed by Western analysis with antibodies against NF-κB and IκB proteins as indicated.

when compared with that induced by TNFα. The amounts of IκBα began to increase again after 2 h of stimulation via the LTβR and were above starting levels by 8 h. Most likely this was due to increased synthesis in response to activated NF-κB, in the absence of continued degradation of this inhibitor (see below). We failed to observe consistent changes in the amounts of the other proteins analyzed in Fig. 1, with the exceptions of RelB, whose amounts were increased, and p105, whose amounts were modestly decreased after 8 h of stimulation with TNFα. In contrast to p52, the amounts of the p50 form of NF-κB1 did not increase after LTβR stimulation. These data indicated that LTβ receptor stimulation in MEFs caused processing of p100 to generate p52 and a more modest degradation of the IκBα inhibitory protein, implying engagement of two pathways to activate NF-κB.

**LTβR-induced Processing of p100 Depends on Protein Synthesis, NIK, and IKKα but Not IKKβ or IKKγ—Light and the membrane-bound LTβR are natural ligands of the LTβ receptor. In addition to the agonistic antibody, Light could also be shown to induce processing of p100 to p52 in MEFs, whereas platelet-derived growth factor did not (Fig. 2). Given the delayed onset of processing, we asked whether the underlying mechanisms might involve intermediate steps requiring protein synthesis. Light-induced processing of p100 was sensitive to the protein synthesis inhibitor cycloheximide (Fig. 2), and this result was confirmed when cells were stimulated with the agonistic antibody to the LTβ receptor (data not shown). Thus, signal-induced processing of p100 required the new or continued synthesis of a protein, which could explain the slow onset of processing upon stimulation.

Next, we investigated the mechanism underlying LTβR-induced processing by taking advantage of mutant mice impaired or lacking in various signaling components. We generated MEFs from alyaly mice, which carry a mutation in NIK. Agonistic antibodies to the LTβR failed to induce processing of p100 to p52 in alyaly MEFs (Fig. 3A). Therefore, NIK was required for LTβR-mediated processing of p100, consistent with the ability of NIK to induce processing in transfected cells and the impaired LTβR-induced NF-κB transcriptional activity in NIK-mutated and NIK-deficient MEFs (27, 28).

NIK was shown previously to depend on IKKα to induce processing in B cells, and consistent with this, LTβR signaling also failed to induce processing in MEFs from IKKα-deficient mice (Fig. 3A; also known as Nemo) were permissive for LTβR-induced processing of p100, as were MEFs deficient in NF-κB2 (Fig. 3A). The regulatory subunit IKKγ and the two catalytic subunits IKKα and IKKβ together constitute the classical IKK core complex. Therefore, although LTβR-induced processing did require the IKKα subunit, it was nevertheless independent of the classical, IKKγ/Nemo-containing IKK complex that controls degradation of the IκBα inhibitors in response to many signals.

By comparison with wild-type MEFs, the amounts of p100 appeared to be somewhat reduced in NF-κB1- and IKKβ-deficient MEFs but were especially reduced in IKKγ-deficient MEFs. Nevertheless, processing still occurred in response to LTβR stimulation, resulting in a more substantial depletion of p100 in the mutant versus wild-type cells (a longer exposure of the IKKγ-deficient MEFs is shown in Fig. 3A, lower panel). Basal and LTβR-induced activation of NF-κB via the classical
IKK to IκB degradation path may be required for optimal p100 expression. MEFs deficient in IKKγ may have contained especially low levels of p100 because the classical activation pathway was completely blocked in these mutant cells, whereas residual activity may have persisted in the IKKβ-deficient mutants due to the presence of IKKα. We also used an IKKβ-specific inhibitor, PS1145, to provide further support for the suggested role of the classical activation route in maintaining p100 levels while having no role in processing. To control for the activity of this inhibitor, we confirmed a dose-dependent inhibition of TNFα-induced degradation of IκBα after 15 min of stimulation (Fig. 3B). Increasing amounts of PS1145 also decreased the amounts of p100 after 8 h of stimulation via the LTβ receptor, presumably due to reduced new synthesis of p100, whereas processing to p52 was essentially unaffected (Fig. 3B). Therefore, optimal expression of p100 depended on basal and induced activation of the classical, IKK-mediated pathway for NF-κB, but processing of p100 did not and instead only depended on the IKKα subunit.

It remained theoretically possible that the inability to process p100 in IKKα-deficient and NIK-impaired MEFs in response to LTβ stimulation was not directly related to loss of IKKα or NIK function. We therefore tested whether transfection of these mutant and wild-type MEFs with p100 together with IKKα, IKKβ, or NIK could confirm the conclusions reached with Fig. 2. Overexpression of IKKα and especially of NIK in wild-type MEFs induced processing of p100 to generate p52, whereas IKKβ did not (Fig. 4A). IKKα and NIK were similarly able to induce processing in IKKγ (Nemo)-deficient (Fig. 4B), IKKβ-deficient (Fig. 4D), and NIK-impaired (aly/aly) (Fig. 4E) MEFs. This confirmed that the classical Nemo/IKKβ pathway was irrelevant for processing and that the inability of aly/aly MEFs to allow processing could be overcome simply by supplying wild-type NIK or IKKα. This latter result also placed IKKα downstream of NIK, which was confirmed by the fact that overexpression of IKKα in IKKα-deficient MEFs resulted in processing, whereas overexpression of wild-type IKKα did not (Fig. 4C). Therefore, we concluded that processing of p100 to p52 as induced by LTβR stimulation depended on and proceeded via NIK and then IKKα but was independent of classical IKKβ (Nemo) and IKKβ-dependent NF-κB activation; classical IKK activity did, however, help maintain p100 levels.

**LTβR Induces Sequential Activation of RelB and RelA by Distinct Pathways**—Next, we investigated LTβR-initiated NF-κB activation in EMASas designed to determine the composition of activated NF-κB. Wild-type MEFs contained some basal κB DNA binding activity composed primarily of p50 homodimers and p50-RelA heterodimers (faster and slower migrating shifted bands, respectively), as assessed in EMSA supershift experiments with antibodies to the various NF-κB subunits (Fig. 5A). Approximately equal amounts of extracts were loaded, and this was confirmed in separate EMASas in which DNA binding activity to the cognate site for the Octamer-1 transcription factor was assessed (data not shown). LTβR stimulation for 2 h resulted in increased amounts of DNA binding activity composed primarily of p50-RelA and, to a lesser degree, p50-RelB (Fig. 5B; RelA supershifts marked). After 8 h of stimulation, the binding activity of p50-RelA dimers had decreased, whereas p50-RelB activity had increased further, and p52-RelB activity could be detected as well (Fig. 5C; RelB and p52 supershifts marked). In addition, p50 homodimer binding activity appeared to have increased somewhat. No significant c-Rel DNA binding activity was noted in these MEFs, although this antibody was able to detect c-Rel binding in lymphoid cells (data not shown).

The EMSA supershifts with 8-h-stimulated extracts revealed predominantly p50-containing complexes with an apparently much smaller contribution of p52-containing complexes. One must consider, however, that the EMSA assays are not quantitative and do not necessarily correlate with the degree to which a given NF-κB dimer has been released from cytoplasmic inhibition. Different dimers bind standard κB DNA elements with varying strength, and p52-containing dimers in particular have not been carefully tested for preferred binding sites. Furthermore, the various antibodies used differ in strength of binding and may in addition be differentially affected in the supershift assay. It is possible, therefore, that these EMSA assays underestimated the amounts of p52-containing complexes in particular. This notion was supported by nuclear-cytoplasmic fractionation experiments, which revealed considerable migration of p52 and RelB into nuclei starting by 2 h of LTβR stimulation and increasing thereafter, whereas the amount of RelA in nuclei was highest after 2 h of stimulation, declining thereafter (Fig. 6A; RelB migrated as two closely spaced bands with the upper band preferentially translocating to nuclei). In addition, RelB coimmunoprecipitation experiments confirmed that RelB was associated with p52 in the nucleus (Fig. 6B; coimmunoprecipitated p52 is marked by an asterisk). Together the data revealed an early activation of p50-RelA dimers, which decreased after 2 h, whereas activation of RelB dimers continued to increase past 2 h.

We speculated that the partial and transient degradation of IκBs seen early after LTβR exposure (see above) might be responsible for the early and transient increase in the p50-RelA binding activity, whereas the processing of p100 might be responsible for the late rise in p52-RelB binding activity and possibly also in p50-RelB binding activity. To test this theory, we investigated the activation of NF-κB with EMSA assays in the mutant MEFs. LTβR-mediated stimulation of IKKβ- and
IKKγ (Nemo)-deficient MEFs for 8 h resulted in strong activation of p50-RelB and, to an apparently lesser degree, p52-RelB, similar to what was observed in wild-type MEFs (Fig. 7, A and B), not even after 2 h of stimulation (data not shown; see also Fig. 6C) These results indicated that the classical activation pathway via IKKβ/IKKγ (Nemo) was not required for DNA binding activation of p50-RelB or p52-RelB but was required for activation of p50-RelA.

To confirm that LTβR-mediated stimulation of IKKγ (Nemo)-deficient MEFs caused translocation of both p52 and RelB into nuclei, we also performed nuclear-cytoplasmic fractionation experiments. As shown in Fig. 6C, RelB and p52 entered nuclei of these mutant cells and continued to do so during the course of stimulation, similar to what was seen with wild-type cells (Fig. 6A), whereas RelA failed to be translocated into nuclei, as expected for these mutant cells, which also served as a control for the experiments.

We next tested the relevance of the alternative pathway in the activation of NF-κB binding activity in response to LTβR stimulation. IKKα-deficient and NIK-impaired (aly/aly) MEFs failed to activate p50-RelB or p52-RelB after 8 h of stimulation (Fig. 7, C and D, respectively). As in wild-type MEFs, p50-RelA was activated after 2 h of stimulation in both mutant MEFs (data not shown) and was still clearly detected after 8 h (Fig. 7, C and D; RelA supershifts marked). Together these results demonstrated that activation of p50-RelA required IKKβ and IKKγ (Nemo) but not IKKa or NIK. On the other hand, activation of p50-RelB and p52-RelB required IKKa and NIK but not IKKβ or IKKγ. We also note that activation of p50-RelA appeared to be somewhat prolonged in the absence of IKKa or NIK, whereas activation of p50-RelB and p52-RelB seemed to be slightly enhanced in the absence of IKKβ and IKKγ at early times (data not shown).

p100 Processin Activates p52-RelB and p50-RelB Complexes—The data described indicated that the alternative pathway of activation via NIK and IKKa and, by extension, processing of p100 were responsible for the activation of not only p52-RelB but also for the activation of p50-RelB. Although p52-RelB dimers could result from processing of p100-RelB complexes, it was less obvious how p50-RelB dimers might be activated. To investigate underlying mechanisms further, we analyzed LTβR-induced activation in NF-κB-deficient MEFs since these cells lack the p100 inhibitor to begin with. In these mutant MEFs, p50-RelB was already basally activated in the absence of any added stimulus, and this binding activity was increased slightly further with stimulation via the LTβR (Fig. 8A; RelB supershifts marked). The basal activity suggested that it was not the act of processing of p100 per se but the absence of the p100 inhibitor that led to p50-RelB binding activity. RelB is reported to preferentially associate with p100 (32), and in the absence of p100, RelB is presumably free to associate with p50. We speculated that LTβR stimulation of NF-κB-deficient MEFs may have led to a further increase above basal levels of p50-RelB DNA binding activity as a result of new synthesis of both RelB and NF-κB1 induced via the classical NF-κB activation route. In support of the theory that RelB complexes were easily activated in the absence of p100, we demonstrated that TNFα stimulation activated p50-RelB DNA binding activity to extremely high levels in NF-κB-deficient MEFs (Fig. 8B, lower panel). By comparison, TNFα-stimulation of wild-type MEFs, which contain p100, did not result in such high activation of RelB complexes (Fig. 8B, upper panel; RelB supershifts in wild-type and NF-κB-deficient MEFs are marked). The RelB activity observed after long-term stimulation of wild-type with TNFα (Fig. 8B, upper panel) was most likely due to the high amounts of RelB protein induced via the classical NF-κB activation pathway, some of which may have escaped sequestration by p100 (Fig. 1). These results supported the notion that the absence or presence of p100 was the key to whether p50-RelB dimers were readily formed or not.
The study presented here shows that LTβR-mediated stimulation of mouse embryo fibroblasts resulted in the engagement of two separate signaling pathways, leading to activation of distinct NF-κB complexes. We established that the initial or classical activation was mediated by IKKβ- and IKKγ-dependent degradation of IκBα and subsequent liberation of primarily p50-RelA complexes, independent of NIK and IKKα. We further demonstrated that the second or alternative activation was mediated by NIK- and IKKα-dependent processing of the inhibitory p100 protein of NF-κB2, independent of IKKβ and IKKγ/Nemo. This latter pathway led to activation of primarily p50-RelB and p50-RelA dimers. These conclusions were derived from analyses of LTβR-stimulated wild-type and mutant MEFs impaired in NIK or lacking IKKα, IKKβ, IKKγ, or NF-κB2. LTβR-induced p50-RelA activation was modest and transient, consistent with an only partial and transient loss of IκBα induced by the classical pathway. With time of stimulation, amounts of IκBα increased to above starting levels to again inhibit RelA complexes. In contrast, DNA binding activity of RelB complexes increased and began to dominate the NF-κB binding activity. Stimulation of the LTβR on fibroblasts, therefore, initiated two separate pathways to sequentially activate different NF-κB complexes. The change in the types of NF-κB complexes activated is likely to result in a corresponding change in the genes targeted with time during the course of stimulation via LTβR. The data presented here identify the LTβR as a physiologic inducer of the second pathway of activation. The results furthermore clarify the importance of this pathway since long term κB binding activity in response to LTβR stimulation was entirely dependent on p100 processing.

A prior analysis of NIK-deficient MEFs discovered gene induction defects specific to stimulation via the LTβR but not the TNFα-receptor (28). Since the LTβR-induced κB binding activity analyzed at early times after stimulation was not impaired in the NIK-deficient MEFs (it is dominated by the classical activation path as shown here), it was speculated that LTβR-induced changes in transactivation potential might have been defective in the absence of NIK. Although it remains possible that NIK is also required to enhance transactivation in response to LTβR stimulation, our data suggest that the lack of processing of p100 in NIK-deficient MEFs and thus the lack of sustained activation of RelB complexes in particular could also explain the loss of gene induction observed.

The alternative activation pathway involving NIK, IKKα, and p100 processing activated not only p52-RelB complexes, which could be generated directly from p100-RelB, but also activated p50-RelB. Activation of this latter complex was a consequence of removal of p100 via processing, the preferred and inhibitory binding partner for RelB.

The experiments of Fig. 8B also demonstrated that LTβR-induced activation of RelB DNA binding activity in wild-type MEFs was dependent on protein synthesis (upper panel), consistent with the dependence of p100 processing on protein synthesis. We observed some RelB binding activation even in the presence of the protein synthesis inhibitor cycloheximide when NF-κB2-deficient MEFs were stimulated with the LTβR (Fig. 8B, lower panel). In the absence of p100 (NF-κB2-deficient MEFs), RelB proteins are likely to be associated with p105 and p50. It is possible that basal or induced turnover of the inhibitory p105 might have led to a further release and thus activation of RelB complexes, as p105 would not be replaced in this situation. LTβR-induced activation of RelA complexes was noticeably enhanced in the presence of cycloheximide in wild-type (Fig. 8B, upper panel) and especially in NF-κB2-deficient MEFs (lower panel), presumably due to the complete loss of relevant inhibitors in the absence of resynthesis.
activation of p50-RelB complexes without the need of any additional NIK- and IKKα-mediated signals in the process.

The observed sensitivity of p100 processing to protein synthesis inhibitors is intriguing. It suggested that induced or continued synthesis of one or more proteins was required. Such a mechanism is consistent with the observed long delay in p100 processing, in all experiments requiring 2 or more h of LTβR stimulation before a clear increase in p52 was apparent. Continuous synthesis of p100 could be required, especially if processing is somehow linked to translation. Also, continuous or induced synthesis of NIK could be required, especially since cells may express only small amounts of NIK normally. Mechanisms that increase the amounts of NIK might be sufficient to induce processing, based on the fact that transfected NIK is highly active even in the absence of any added signals (Fig. 4). Nevertheless, induced or continued synthesis of other proteins cannot be ruled out.

MEFs in which the alternative pathway was impaired appeared to show a more sustained activation of RelA complexes, whereas MEFs in which the classical pathway was impaired appeared to have activated RelB complexes more rapidly. Although these quantitative assessments need to be independently confirmed, they do raise the interesting possibility that the two pathways may compete in wild-type cells. IKKα might be limiting and thus may not have been immediately available to the second pathway.

The results project a dynamic interplay of the pathways and complexes activated with time of stimulation. Loss of any of the components of the classical activation pathway (especially loss of IKKγ) appeared to reduce the amounts of p100/NF-κB2 present in MEFs. In addition to NF-κB2, RelB, c-Rel, and NF-B1 are also known targets of NF-κB. Activation via the classical route may be needed to maintain NF-κB components, including NF-κB2 and RelB, to allow maximal effects of the alternative activation pathway. Different signals may involve the activation paths to different degrees and to different effects when viewed over the course of prolonged stimulation, depending on cell type.

Recently, we discovered that ligation of the B cell-activating factor (BAFF)-receptor by BAFF on B cells induces processing of the NF-κB2 protein p100 to generate p52 (33). Although the requirements and effects of processing in B cells could not be investigated as thoroughly as was possible with MEFs here, p100 processing in B cells could be shown to depend on NIK and protein synthesis, to be independent of IKKα processing in B cells could be shown to depend on NIK, and NF-κB2 are severely impaired in formation of B cell follicles (6, 7, 18–25).

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