Lymphotoxin and lipopolysaccharide induce NF- κ B-p52 generation by a co-translational mechanism

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The 'classical' NF- κ B activation pathway proceeds via I κ B kinase (IKK)- β / γ -mediated phosphorylation, induced ubiquitination and the degradation of small I κ Bs. An alternative, NF- κ B-inducing kinase and IKK- α -dependent pathway, which stimulates the processing of NF- κ B2/p100, has recently been suggested. However, no physiological stimulus has been shown to trigger the activation of this pathway. Here we demonstrate that persistent stimulation with lymphotoxin β (LT- β) receptor agonists or lipopolysaccharide (LPS), but not with interleukin-1 β , tumour necrosis factor- α or 12-*O*-tetradecanoylphorbol-13-acetate, induces the generation of p52 DNA-binding complexes by activating the processing of the p100 precursor. Induction of p52 DNA-binding activity is delayed in comparison with p50/p65 complexes and depends on *de novo* protein synthesis. p100 is constitutively and inducibly polyubiquitinated, and both ubiquitination and p52 generation are coupled to continuing p100 translation. Thus, both LT- β receptor agonists and LPS induce NF- κ B/p100 processing to p52 at the level of the ribosome.

EMBO reports 4, 82–87 (2003)

DOI: 10.1038/embor710

INTRODUCTION

Detailed knowledge has been gathered about the activation of NF- κ B sequestered by the small I κ Bs α , β and ϵ as well as the NF- κ B1/p105 precursor protein. In response to stimulation, these I κ Bs are phosphorylated by the I κ B kinase (IKK) complex, ubiquitinated by a process involving the SCF- β TRCP complex and completely degraded by the proteasome, resulting in the liberation and nuclear translocation of NF- κ B (Silverman & Maniatis, 2001; Ghosh & Karin, 2002). Of special interest has been the regulation of NF- κ B1/p105 and NF- κ B2/p100, because these precursor proteins act as I κ B molecules, whereas their p50 and p52 products are transcription factors. The generation of p50 or p52 from the precursors, and thus a change in function, requires a proteolytic processing step that is essential for

the homeostasis of NF- κ B dimers. Recently, overexpression of NF- κ B-inducing kinase (NIK) has been shown to trigger the processing of p100 to p52 by site-specific p100 phosphorylation and subsequent ubiquitination (Xiao *et al.*, 2001b). IKK- α -deficient mice exhibit defects in p100 processing in B-lymphocytes, and NIK-induced p100 processing requires IKK- α , indicating that IKK- α acts downstream of NIK under these conditions (Senftleben *et al.*, 2001). However, it is unknown which physiological stimuli induce p100 processing and the generation of p52 DNA-binding complexes.

RESULTS AND DISCUSSION

Previous studies have shown that lymphotoxin β (LT- β) receptor (LT- β R) signalling requires NIK, but controversial results have been obtained regarding the link between LT- β R, NIK, IKK and NF- κ B (Matsushima *et al.*, 2001; Yin *et al.*, 2001). We wished to determine whether LT- β R ligation is able to activate p100 processing and thus to generate DNA-binding complexes containing p52 (Fig. 1A, top panel). Tumour necrosis factor- α (TNF- α) rapidly stimulated NF- κ B DNA-binding complexes in HeLa cells, gradually declining within 28 h of stimulation. In contrast, rapid NF- κ B activation by LIGHT, a ligand for LT- β R (Bodmer *et al.*, 2002), was only modest (compare lanes 2 and 6 in Fig. 1A, top panel), but NF- κ B DNA-binding activity increased constantly over the duration of stimulation, resulting in persistent NF- κ B activation. Most TNF- α -induced NF- κ B consisted of p50/p65 heterodimers and contained only little p52 DNA-binding activity that gradually ceased during long-term stimulation. LIGHT induced p50/p65 complexes only weakly at early time points, whereas at later time points predominantly p52-containing complexes were generated, revealed by p52 antibody supershifting (Fig. 1A, middle panel). At the protein level, TNF- α caused an upregulation of p100 but not of p52. In contrast, LIGHT increased p52 production, but amounts of p100 were nearly unchanged (Fig. 1A, bottom panel). We tested a panel of different stimulators in various cell lines of epithelial and hepatic origin (HeLa, MCF-7, AKN-1 and Chang) for their ability to induce the generation of p52 DNA-binding complexes (Fig. 1B, and data not shown). As predicted, TNF- α , interleukin (IL)-1 β or 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which are all inducers of the canonical IKK- β / γ -dependent NF- κ B pathway, rapidly and transiently activated NF- κ B heterodimers, which are released after the degradation of small I κ Bs (see also Fig. 2B). In contrast, stimulation with LT- α 1 β 2 or LIGHT modestly activated NF- κ B heterodimers after 30 min, but

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Received 26 July 2002; revised 30 September 2002; accepted 4 November 2002

Fig. 1 | LT- β R ligation and LPS treatment induce p100 processing and generation of p52 DNA-binding complexes. (A) HeLa cells were stimulated with TNF- α or LT- β and extracts were tested for NF- κ B DNA-binding complexes by EMSA and for p100/p52 protein in western blots. In the middle panel, anti-p100/p52 antibody was added to each DNA binding reaction (p52 sup., supershift). (B) HeLa and MCF-7 cells were stimulated for 30 min or 4 h with TNF- α , IL-1 β , TPA, LT- α 1/ β 2 or LIGHT, and extracts were analysed for NF- κ B DNA-binding activity. Anti-p100/p52 antibody was added to each DNA binding reaction. (C) 70Z/3 cells were stimulated with LPS or TPA for the times indicated and extracts were analysed by EMSA for NF- κ B DNA-binding activity and for expression of NF- κ B/I κ B proteins by western blotting, as indicated. (D) Subunit composition of LPS-induced NF- κ B complexes (at 0, 2 and 24 h, as indicated) in 70Z/3 cells was determined by supershift analysis. (E) Immature dendritic cells treated with 1 μ g/ml LPS (1 and 24 h, as indicated) were analysed for NF- κ B activity and subunit composition. In D and E, supershifted NF- κ B complexes are indicated by open circles. Specificities of p50 and p52 antibodies were evaluated by supershifting of overexpressed or *in vitro*-translated proteins; no antibody that was used showed significant cross-reactivity with other NF- κ B family members (data not shown).

induced p52 DNA-binding activity more strongly after 4 h. p52 DNA binding coincided with an increase in the amount of p52, as detected by western blotting (data not shown).

The slow induction kinetics of p52 DNA binding led us to investigate whether lipopolysaccharide (LPS), which persistently activates NF- κ B in some cell types, is able to trigger the generation of p52 in 70Z/3 pre-B cells and primary dendritic cells (Fig. 1C, D, E). Whereas p65 protein levels were unchanged, RelB and p100 expression increased within 3 h of LPS treatment and stayed nearly constant thereafter. Furthermore, p52 generation was induced by LPS (Fig. 1C, lanes 1–5). This process was delayed compared with upregulation of p100. TPA only transiently activated NF- κ B and did not significantly induce p100/p52 expression (Fig. 1C, lanes 6–9). The rapidly LPS-induced NF- κ B activity consisted exclusively of p50 and p65, whereas the delayed persistent activation resulted in an accumulation of RelB, p52 and c-Rel in the complexes (Fig. 1D). LPS also induced p52 DNA binding after 24 h in human *in vitro*-differentiated myeloid immature dendritic cells, another cell type that responds to LPS with persistent NF- κ B activation (Fig. 1E). Thus, LT- β R ligation and LPS are both able to trigger p52 generation and DNA binding, probably through induced processing of p100. This process is considerably slower than induction by the rapid, canonical IKK- β / γ -dependent NF- κ B pathway.

We analysed the mechanism involved in p52 production and compared it with the classical IKK/NF- κ B pathway. Protein synthesis was required for LIGHT-induced generation of p52 protein and DNA-binding activity in HeLa cells (Fig. 2A, compare lanes 2 and 3 with 6 and 7), indicating that p52 could be derived from newly synthesized p100. In contrast, rapidly activated p52-containing complexes after stimulation with TNF- α were not sensitive to cycloheximide (CHX) (Fig. 2B), indicating that those complexes are most probably released after the degradation of small I κ Bs and not a result of precursor processing. *De novo* protein synthesis was also required for p52 generation in 70Z/3 cells in response to LPS (Fig. 2C, compare lanes 3 and 4 with 6 and 7). Induced p52 production and DNA-binding activity were also blocked by ALLN (Fig. 2A, and data not shown), as expected for proteasome-dependent processing. However, this could also indicate that, in addition, an obligatory initial NF- κ B activation step was blocked. A possible requirement of initial NF- κ B activation through the canonical IKK- β / γ pathway was analysed for LPS-induced processing in 70Z/3 derivative 1.3E2 cells, lacking IKK- γ , in 70Z/3 cells after retroviral expression of the NF- κ B super-repressor I κ B α Δ N, and in immature dendritic cells after transient transfection of I κ B α Δ N (Fig. 2C, D). In fact, in all cases upregulation of p100 expression and induced p52 generation was lost. Importantly, neither IKK- γ deficiency nor I κ B α Δ N overexpression had an effect on the precursor-to-product ratio in the absence of any stimulus, indicating that constitutive processing was not affected. Taken together, enhanced generation of p52 in response to LIGHT or LPS requires *de novo* protein synthesis. In addition, LPS-induced processing depends on initial activation of the canonical IKK/NF- κ B signalling pathway.

It is difficult to determine the proteasome dependence for induced processing of p100, because proteasomal degradation is also required for initial NF- κ B activation. Therefore, p100 polyubiquitination was investigated as indirect evidence for ubiquitin/proteasome-mediated processing (Fig. 3). Indeed, polyubiquitination of p100 could be detected in MCF-7, 70Z/3 and 1.3E2 cells after blockade of the proteasome by ALLN (Fig. 3A, B). Ubiquitination was enhanced by stimulation with LIGHT or LPS, respectively. LPS-induced polyubiquitination was seen in 70Z/3 cells and was not visible in 1.3E2 cells, which

lacked enhanced processing after stimulation (see Fig. 2C). Importantly, ubiquitination induced by LIGHT and LPS was completely blocked when the cells were treated with CHX, suggesting that polyubiquitination, just like enhanced processing, requires continuing protein synthesis. The same was true of constitutive ubiquitination of p100, which was also inhibited in the presence of CHX (Fig. 3A, B). In contrast, LPS-induced ubiquitination and degradation of I κ B α were not affected by treatment with CHX (Fig. 3C, and data not shown), excluding the possibility that inhibition of p100 ubiquitination was merely due to the depletion of some basic component of the ubiquitination/degradation machinery. Enhanced polyubiquitination was not observed with IKK- α in the presence of LPS and ALLN (Fig. 3D), showing that p100 ubiquitination was specific. Thus, p100 polyubiquitination coincides with processing of p100 to p52, and both constitutive and stimulated ubiquitination depend on *de novo* protein synthesis.

To test directly whether induced p52 generation is a post-translational or co-translational process, the p100/p52 ratio was investigated by pulse-chase analysis. When the [35 S]methionine pulse was added during stimulation with LIGHT, generation of p52 in MCF-7 and HeLa cells was significantly increased (Fig. 4A, lanes 4 and 6). TNF- α induced an increase in p100, but not in p52 (Fig. 4A, lane 7), as expected from the long-term accumulation of p100 amounts (see Fig. 1A). In contrast, p100 levels in HeLa cells were reduced after 3 h but no p52 was generated when the pulse was performed first and the stimulus (LIGHT or TNF- α) was added during the chase (Fig. 4B). Perhaps owing to turnover, p100 levels slightly decreased at the 3 h time points. To determine whether LPS-induced processing also requires continuing p100 synthesis, we treated 70Z/3 cells with LPS and afterwards inhibited translation with CHX (Fig. 4C). p52 production after LPS treatment for 8 h was blocked when *de novo* protein synthesis was inhibited for the last 4 h by CHX (Fig. 4C, compare lanes 2 and 4). The same result was observed in a pulse-chase analysis in 70Z/3 cells (Fig. 4D). p100 and p52 amounts were strongly increased by the addition of LPS during the pulse with [35 S]methionine, but the ratio of both proteins stayed constant during the time of the chase despite the continuous presence of LPS (Fig. 4D, upper panel). Under the same condition, the half-life of I κ B α was drastically reduced, as expected for post-translational degradation (Fig. 4D, lower panel). Thus, signal-induced processing of p100 and the generation of p52 occur either during translation, before full-length p100 is released from the ribosome, or immediately after p100 translation is completed, but it still requires signals from the translation machinery.

Constitutive processing of p105 was first described as a post-translational process (Palombella *et al.*, 1994; Orian *et al.*, 1995). Later studies proposed that constitutive processing of both p105 and p100 occurs during translation (Lin *et al.*, 1998, 2000; Heusch *et al.*, 1999). After induction of the IKK pathway, p105, just like the other small I κ Bs, is degraded post-translationally (Heissmeyer *et al.*, 1999, 2001) (Fig. 5). We can now show that p100 is inducibly processed in response to LT- β R ligation or LPS, an event that requires continuous *de novo* protein synthesis of p100. Thus, an induced co-translational mechanism is giving rise to p52 after stimulation. To our knowledge, this is the first evidence of a stimulation-dependent conversion of an inhibitor to an activator at the level of the ribosome. In comparison with the post-translational destruction of an inhibitor, such as the small I κ Bs and p105, or the new synthesis of an activator, such as RelB, induced co-translational processing offers an alternative strategy for the induction of NF- κ B activity. Importantly, the upregulation of p100 expression alone is not sufficient to induce co-translational

Fig. 2 | LT- β receptor ligation- and LPS-induced p100 processing depends on IKK/NF- κ B activation and protein synthesis. (A) HeLa cells were stimulated with LIGHT for 4 and 8 h alone (lanes 2 and 3), or in the presence of the protein synthesis inhibitor CHX (lanes 4–7) or the proteasomal inhibitor ALLN (lanes 8–11). Extracts were analysed for p52 DNA-binding activity by EMSA, including anti-p52 antibodies (top panel). p100/p52 protein amounts were determined by western blotting (middle panel), and Oct1 DNA-binding activity was determined as an internal control. (B) HeLa cells were activated by either TNF- α (30 min) or LIGHT (6 h) in the presence or absence of CHX, and p52 DNA-binding activity was analysed by the addition of supershifting anti-p52 antibodies to the NF- κ B EMSA reaction. (C) 70Z/3 and 1.3E2 cells, or 70Z/3 cells infected with control retrovirus (CV) or with FlagI κ B α Δ N-expressing retrovirus (FlkBa Δ N), were stimulated with LPS or LPS/CHX as indicated, and tested for NF- κ B DNA-binding activity (top panel) and for p100/p52, RelB, p65 and I κ B α /I κ B α Δ N proteins (lower panels). As judged by trypan blue exclusion, CHX did not strongly affect the viability of cells during the time of incubation. In every case we observed more than 90% viable cells (data not shown). ns, non-specific. (D) Mock-transfected and I κ B α Δ N-transfected dendritic cells (DC) were treated with LPS and NF- κ B DNA-binding activity; p100, p52 and I κ B α protein levels were determined, as indicated.

Fig. 3 | Translation-dependent constitutive and induced p100 polyubiquitination. (A) MCF-7 cells were treated with LIGHT for 4 h in the absence or presence of CHX and proteasomal degradation was blocked by ALLN for the last hour of incubation. (B) 70Z/3 and 1.3E2 cells were treated with LPS for 5 h. CHX was added, where indicated, 2 h before lysis; ALLN was included for the last 1 h. (C) 70Z/3 cells were treated with LPS for 20 min after preincubation with CHX and/or ALLN, as indicated. (D) 70Z/3 cells were treated with LPS (5 h) and ALLN (1 h) before extraction. Extracts were immunoprecipitated (IP) either with anti-p100/p52, anti-IκBα or anti-IKK-α antibodies and western blotting (WB) of the input material and after immunoprecipitation was performed as indicated on both sides of the panels. ns, non-specific.

processing, as is shown by the outcome of stimulation with TNF- α (Fig. 1A and 4A), indicating that an additional signal is required to induce the NF- κ B2 activation pathway. Whereas TNF- α fails to activate this pathway, LT- β R ligation seems to be a stronger activator than LPS, even though LPS induces p100 expression more strongly. It might well be that LPS actually requires initial NF- κ B activation and enhanced transcription and translation of the *nfb2* gene to exert its effect on processing, whereas LIGHT could also enhance processing in the absence of, or with lower increases in, classical NF- κ B activa-

tion. The different precursor-to-product ratios favour this hypothesis. Nevertheless, induced processing by both LIGHT and LPS is delayed and requires protein synthesis, indicating that a second stimulus must be generated to promote co-translational p52 generation.

What could be the nature of the second stimulus that is required for induced co-translational processing of p100? Possible candidates that regulate this pathway are NIK and IKK- α (Senftleben *et al.*, 2001; Xiao *et al.*, 2001a). Overexpression of NIK has been proposed to induce post-translational processing of p100 (Xiao *et al.*, 2001b).

Fig. 4 | LT- β receptor ligation- and LPS-induced processing of p100 requires continuing protein synthesis. (A) Left panel, MCF-7 cells were treated with solvent or LIGHT for 3 h in the presence of [35 S]methionine to pulse-label the cells. Right panel, HeLa cells were treated with solvent, LIGHT or TNF- α for 4 h. [35 S]methionine was added to the cells during the last 1 h of stimulation (pulse). Samples were immunoprecipitated with an isotype control (lanes 1 and 2) or anti-p100/p52 antibody (lanes 3 and 4), and precipitates were analysed by SDS-PAGE followed by autoradiography. The band corresponding to p52 in lanes 4 and 6 is marked with an open circle. (B) HeLa cells were labelled with [35 S]methionine for 1 h before stimulation and the chase was performed in the presence of cytokines, as indicated. Immunoprecipitation was performed as in A and analysed by SDS-PAGE followed by autoradiography. The migration of p100 and p52 is indicated. (C) 70Z/3 cells were treated with LPS for 4 or 8 h, as indicated (lanes 2–4). CHX was added during the last 4 h of stimulation with LPS or for 4 h alone (lanes 4 and 5). NF- κ B DNA-binding activity was analysed by EMSA and NF- κ B/I κ B protein amounts were determined by western blotting. Enhanced p52 protein amounts in lane 2 are indicated by an open circle. ns, non-specific. (D) 70Z/3 cells were either left untreated or were stimulated with LPS for 4 h. During the last 1.5 h, cells were labelled with [35 S]methionine. Cells were washed and incubated in complete medium again in the absence or presence of LPS for up to 2 h. After immunoprecipitation, p100/p52 and I κ B α were analysed by SDS-PAGE followed by autoradiography. The positions of p100, p52 and I κ B α are indicated.

Pulse-chase analysis in the reported settings does indicate an involvement of overexpressed NIK in p52 generation. However, it is not appropriate to discriminate between co-translational and post-translational mechanisms, because the transfection is performed before labelling. It is therefore perfectly possible that NIK and IKK- α could be involved in LT- β R- or LPS-stimulated co-translational processing of p100. It has recently been demonstrated that NIK induces

Fig. 5 | Model for production of p50 and p52 from the NF- κ B precursor molecules NF- κ B1/p105 and NF- κ B2/p100 after stimulation. On activation with TNF- α or LPS, NF- κ B1/p105 is completely degraded post-translationally, leading to a release of p105-bound Rel molecules. LT- β R ligation and LPS rapidly activate canonical IKK/NF- κ B signalling, which leads to upregulation of the *nf- κ B2/p100* gene. Both pathways also induce the delayed co-translational processing of p100 and thereby the generation of homodimeric or heterodimeric p52 complexes.

the recruitment of β -transducin repeat-containing protein (β -TrCP), a component of the SCF ubiquitin ligase complex, to p100 (Fong & Sun, 2002). Thus, activated IKK- α and/or NIK could interact with the nascent p100 polypeptide at the ribosome and recruit the SCF ubiquitin ligase complex during translation or immediately after translation has been completed, resulting in processing of p100 by the 26S proteasome. Interestingly, the death domains of p105 and p100 interact avidly with IKK- α and IKK- β , and overexpression of kinase-inactive IKK- β severely interferes with constitutive processing of p105 (Heissmeyer et al., 2001).

It will be important to determine whether persistent NF- κ B activation through co-translational generation of p52 is needed to activate a different network of NF- κ B2-dependent target genes by the binding of specific DNA sites or the recruitment of different cofactors on target promoters. *In vivo*, deletion of the *nfkb2* gene has been shown to result in impaired lymphoid organ structure and immune responses (Caamano et al., 1998; Franzoso et al., 1998), a phenotype that is partly shared with LT- β R-deficient mice (Matsumoto, 1999). The long-lasting induced p52 activation, as opposed to the short-term activation of p50/p65 complexes, might be crucial for the maintenance and propagation of an effective immune response. Similarly, long-term exposure to bacterial LPS might trigger the activation of a distinct p52-dependent target gene repertoire that might have a role in differentiation processes such as the maturation of dendritic cells. The explanation of these possibilities awaits future analysis.

MATERIALS AND METHODS

Cell culture. 70Z/3, 1.3E2 and primary human dendritic cells were cultured in RPMI medium with 10% FCS, 2 mM glutamine, penicillin/streptomycin and 50 μ M 2-mercaptoethanol. All cell culture

reagents were purchased from Invitrogen. Stimulations were performed with either 1 μ g ml⁻¹ (dendritic cells) or 10 μ g ml⁻¹ (70Z/3) LPS (Sigma). Stably transfected 70Z/3 cells expressing I κ B Δ N (amino-acid residues 71–317 of hI κ B α) were generated by retroviral transfection and subsequent selection in medium containing geniticin (8 μ g ml⁻¹). Both pools and individual clones were used, and showed similar effects. Dendritic cells were generated from CD14⁺ human peripheral blood mononuclear cells by the addition of granulocyte-macrophage colony-stimulating factor and IL-4 to the medium in accordance with standard procedures. I κ B Δ N-expressing dendritic cells were obtained by electroporation with a commercially available kit in accordance with the manufacturer's specifications (human dendritic cell nucleofactor kit I; amaxa). HeLa and MCF-7 cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin. Where indicated, cells were stimulated with soluble human LIGHT (100 ng ml⁻¹; Alexis), LT- α 1 β 2 (100 ng ml⁻¹; R&D Systems), human TNF- α (25 ng ml⁻¹; Biomol), TPA (200 ng ml⁻¹; Sigma) and human IL-1 β (10 ng ml⁻¹; Biomol). Protein synthesis was inhibited by co-incubation with 25 μ g ml⁻¹ CHX (Sigma), and proteasome degradation/processing was inhibited with 50 μ g ml⁻¹ ALLN (Biomol).

Plasmids and antibodies. Human FlagI κ B Δ N (amino-acid residues 71–317) cDNA was cloned into pFBneo or pcDNA3 (both from Invitrogen) by standard polymerase chain reaction procedures. Antibodies used were as follows: anti-p100/p52 antibody (human, Upstate; mouse, D-32, Santa Cruz), IgG1 isotype control antibody (BDBioscience), anti-p65 antibodies (A, Santa Cruz for human and Biomol for mouse p65), anti-I κ B α antibody (C-21; Santa Cruz), anti-RelB antibody (C-19; Santa Cruz), anti-c-Rel antibody (C; Santa Cruz), anti-IKK- α antibody (H744; Santa Cruz) and anti-ubiquitin antibody (Babco).

Electrophoretic mobility-shift assays (EMSA) and western blot. Cells were lysed in whole cell extraction buffer containing 20 mM HEPES pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 1% Nonidet P40, 1 mM dithiothreitol (DTT) and protease inhibitors (Boehringer). Protein concentrations were determined. EMSA and western blots were performed in accordance with standard procedures.

Pulse-chase experiments. MCF-7 and HeLa cells were labelled in DMEM medium, 70Z/3 cells in RPMI medium without methionine, with 100 μ Ci ml⁻¹ [³⁵S]methionine. Cellular lysis and immunoprecipitations were performed overnight in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors (Boehringer) and 1 mM DTT) at 4 °C. Precipitates were collected with protein A-Sepharose and after extensive washing with RIPA buffer were eluted with SDS loading buffer and subjected to SDS-PAGE.

Ubiquitination assays. Cells were treated with LIGHT or LPS in the absence or presence of CHX. ALLN was added, where indicated, for the last 1 h. Cells were lysed in 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 30 mM N-ethylmaleimide, 1 mM DTT and protease inhibitors (Boehringer). p100/p52, I κ B α or IKK- α was

immunoprecipitated overnight. Precipitates were collected with protein G-Sepharose, washed and resolved by SDS-PAGE, then blotted and probed with either anti-ubiquitin or the respective antibodies used in the immunoprecipitation.

ACKNOWLEDGEMENTS

We thank E. Scharschmidt and S. Jungmann for technical assistance. This work was supported in part by DFG grant Sche 277/6–1 to C.S.

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