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Nuclear Retention of IκBα Protects It from Signal-induced Degradation and Inhibits Nuclear Factor κB Transcriptional Activation*

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Transcriptional activation of nuclear factor κB (NF-κB) is mediated by signal-induced phosphorylation and degradation of its inhibitor, IκBα. However, NF-κB activation induces rapid resynthesis of IκBα, which is responsible for post-induction repression of transcription. Newly synthesized IκBα translocates to the nucleus, where it dissociates NF-κB from DNA and transports NF-κB from the nucleus to the cytoplasm in a nuclear export sequence-dependent process that is sensitive to leptomycin B (LMB). In the present study, LMB was used as a tool to inhibit nuclear export sequence-mediated nuclear protein export and evaluate the consequences for regulation of NF-κB-dependent transcriptional activity. Pretreatment of cells with LMB inhibits NF-κB-dependent transcriptional activation mediated by interleukin 1β or tumor necrosis factor α. This is a consequence of the inhibition of signal-induced degradation of IκBα. Although LMB treatment does not affect the signal transduction pathway leading to IκBα degradation, it blocks IκBα nuclear export. IκBα is thus accumulated in the nucleus, and in this compartment it is resistant to signal-induced degradation. These results indicate that the signal-induced degradation of IκBα is mainly, if not exclusively, a cytoplasmic process. An efficient nuclear export of IκBα is therefore essential for maintaining a low level of IκBα in the nucleus and allowing NF-κB to be transcriptionally active upon cell stimulation.

The NF-κB[1]/Rel family of transcription factors is implicated in regulation of the expression of a number of cellular genes involved in immune responses, inflammation ,and apoptosis (for recent reviews, see Refs. 1–3). In vertebrates, the NF-κB family of proteins is composed of transcriptionally active p65/Rel A (4, 5), c-Rel (6), or Rel B (7) and transcriptionally silent p50/NF-κB1 (8, 9) or p52/NF-κB2 (10–12). All NF-κB proteins share a conserved region known as the Rel homology domain that contains the nuclear localization signal as well as the dimerization and DNA binding functions. The NF-κB form activated by extracellular signals is composed of p50 and p65. NF-κB transcriptional activity is controlled by inhibitor IκB proteins that contain ankyrin repeat domains. Association of p50/p65 with IκB not only occludes the nuclear localization sequence of p50 and p65, leading to cytoplasmic sequestration, but also prevents NF-κB DNA binding activity. Several IκBs have been described including IκBα (13), IκBβ (14), IκBϵ (15), and Bcl-3 (16). Additionally, the precursors of p50 (p105) and p52 (p100) possess inhibitory ankyrin repeat domains that in isolation are known as IκBγ (17–19) and IκBδ (20, 21), respectively.

IκBα is organized in three domains: (*a*) an unstructured amino-terminal (aa 1–72) signal response domain, (*b*) a central region (aa 73–242) consisting of five ankyrin repeat domains, and (*c*) a carboxyl-terminal region (aa 243–317) containing a highly acidic domain (aa 276–317) that is bound to the ankyrin repeat domain by a protease-sensitive linker (aa 243–275) and is protected by bound p65 (22). Both amino- and carboxyl-terminal domains are required for the signal-induced degradation of IκBα (23, 24). After signal induction, IκBα is phosphorylated on Ser-32 and Ser-36 (25–29) by the recently described dimeric IκB kinase (IKK) (30–34). After phosphorylation, IκBα is ubiquitinated on Lys-21 and Lys-22 (35–37), which targets the molecule for degradation by the proteasome. Once IκBα is degraded, NF-κB can translocate to the nucleus, where it induces the transcription of several genes including that of its inhibitor, IκBα.

Newly synthesized IκBα is accumulated in the cytoplasm but also in the nucleus, where it terminates NF-κB-dependent transcription. This is accomplished by inhibition of the NF-κB/DNA interaction and export of NF-κB back to the cytoplasm (38). The latter function of IκBα is conferred by a leucine-rich nuclear export sequence (NES) present in its carboxyl-terminal region (aa 265–277) (39) and homologous to the NES found in many proteins including the human immunodeficiency virus-type 1 Rev protein and the protein kinase A inhibitor (40–42). Such NESs constitute transferable transport signals that are necessary and sufficient to mediate rapid and active export from the nucleus to the cytoplasm. The nuclear protein CRM1 (also known as exportin 1) has been recently identified as the NES receptor (43–46). CRM1 belongs to the karyopherin β family and, in particular, it shares sequence homology in the Ran-GTP binding domain with members from this family (47). The formation of CRM1/NES complex is facilitated by the presence of Ran in its GTP-bound form. It has been proposed that this ternary complex is transported through the nuclear pore complex and dissociates in the cytoplasm due to GTP hydrolysis by Ran-GAP (43). In addition, CRM1 has been shown to be the cellular target of the drug leptomycin B (LMB) that inhibits NES-mediated protein export both *in vivo* and *in vitro* (43–45, 48).

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[1] The abbreviations used are: NF-κB, nuclear factor κB; TNF, tumor necrosis factor; IL-1β, interleukin 1β; LMB, leptomycin B; CX, cycloheximide; NES, nuclear export sequence; aa, amino acid(s); IKK, IκB kinase; SLO, streptolysin O; SUMO-1, small ubiquitin-like modifier 1.

In the present study, LMB was used as a tool to inhibit NES-mediated nuclear protein export and evaluate the consequences for regulation of NF-κB-dependent transcriptional activity. Pretreatment of cells with LMB inhibits NF-κB-dependent transcriptional activation mediated by IL-1β or TNFα. This is a consequence of the inhibition of signal-induced degradation of IκBα. Although LMB treatment does not affect the signal transduction pathway leading to IκBα degradation, it blocks IκBα nuclear export. IκBα is thus accumulated in the nucleus, and in this compartment it is resistant to signal-induced degradation. These results indicate that the signal-induced degradation of IκBα is mainly, if not exclusively, a cytoplasmic process. An efficient nuclear export of IκBα is therefore essential for maintaining a low level of IκBα in the nucleus and allowing NF-κB to be transcriptionally active upon cell stimulation.

EXPERIMENTAL PROCEDURES

Reagents—Leptomycin B was a gift from B. Wolff-Winiski (Novartis) and was used at 20 nM. TNFα, which was obtained from the MRC Reagent Project, and IL-1β (Sigma) were used at 10 ng/ml for the indicated time. Cycloheximide (Sigma) was used at 100 μg/ml. Z-LLL-H (MG132) was a gift from F. Baleux (Institut Pasteur) and was used at 20 μM.

Plasmid Construction—The pSVB (CLONTECH) and pRC/RSV (Invitrogen) vectors were digested with *Not*I. Corresponding DNA fragments were ligated to obtain the pRC/RSV reporter plasmid in which β-galactosidase transcription is driven by the RSV promoter.

Cell Culture and Transfections—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Before treatment with IL-1β or TNFα, the medium was changed to Dulbecco's modified Eagle medium without serum. HeLa cells were transfected by electroporation as described previously (39). A total of 10 μg of plasmid DNA encoding the chimeric protein 4NBC (24) was transfected in 5×10^6 HeLa cells. After transfection, cells were seeded in four wells of 6-well plates, and incubation continued for 24 h.

The expression of both the 3Enhancer-kB-conA-Luc (49) and the pRC/RSV-β-galalactosidase plasmids was stabilized in HeLa cells using neomycin selection. Single cell clones were obtained by limiting dilution of the neomycin-resistant cells. The 57A cell line was selected on the basis of TNFα-induced luciferase activity and constitutive β-galactosidase activity.

Western Blot Analysis—Cells grown in 6-well plates were harvested in 150 μl of lysis buffer (50) for Western blot analysis. 15 μl of whole cell extracts were resolved in 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidine difluoride membranes (Sigma) by electroblotting, and processed for Western blotting as described previously (38). When indicated, membranes were stripped and processed as reported previously (29). Primary polyclonal antibodies anti-IκBα or anti-p65 (C-21 and C-20) were from Santa Cruz Biotechnology. The previously described anti-SV5 tag monoclonal antibody (51) to detect the chimeric 4NBC protein was obtained from Dr. R. E. Randall. Monoclonal anti-α-tubulin and horseradish peroxidase-labeled anti-rabbit or anti-mouse antibodies were purchased from Amersham. An enhanced chemiluminescence detection system was used to detect specific antigen-antibody interactions (POD; Boehringer Mannheim).

Cell Fractionation and Immunoprecipitation—Cells were washed twice with Dulbecco's modified Eagle medium and once with Buffer S (115 mM potassium acetate, pH 7.3, 25 mM Hepes, pH 7.4, and 2.5 mM $MgCl_2$) at 37 °C and then treated with 2 μg/ml streptolysin O (SLO) (52) in Buffer S for 3 min at 37 °C. SLO supernatant (cytosolic extracts) was kept on ice, cells were washed once with Buffer S, and the resulting washing volume was mixed with SLO supernatant (cytosolic extracts). Cells were then washed three times with Buffer S and solubilized in Buffer T (30 mM Tris, pH 8.6, 150 mM NaCl, 2 mM EDTA, and 2% Triton X-100). This extract corresponds to the nuclear fraction. The quality of fractionation was controlled by Western blotting using monoclonal antibodies against heterogeneous nuclear ribonucleoprotein C (nuclear protein) and γ-adaptin (cytosolic protein). Nuclear and cytosolic extracts were incubated for 15 min at 4 °C and centrifuged at $10{,}000 \times g$ for 10 min. Anti-p65 polyclonal antibodies and protein G-agarose beads were added to the supernatants and incubated for 4 h at 4 °C. Beads were then washed, boiled for 5 min in Laemmli sample buffer, and analyzed by 10% SDS-polyacrylamide gel electrophoresis and Western blotting with anti-IκBα polyclonal antibodies.

Indirect Immunofluorescence Analysis—For indirect immunofluorescence analysis, HeLa cells grown on coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 10 min. Monoclonal antibodies to IκBα (10B) and polyclonal antibodies to NF-κB p65 (C-20; Santa Cruz Biotechnology) were applied for 30 min, followed by a 30-min incubation with fluorescein isothiocyanate or Texas Red (TR)-conjugated donkey anti-mouse or anti-rabbit IgG (Jackson). Coverslips were mounted in Mowiol (Hoechst, Frankfurt, Germany). Confocal laser scanning microscopy and immunofluorescence analysis were performed with a TCS4D confocal microscope based on a DM microscope interfaced with a mixed-gas argon-krypton laser (Leica Laser Technik). Fluorescence acquisitions were performed with the 488 nm and 568 nm laser lines to excite fluorescein isothiocyanate and Texas Red dyes, respectively, with a ×100 oil immersion PL APO objective. Data presented on the same figure were registered at the same laser and multipliers settings.

Measurement of Luciferase and β-Galactosidase Activities—75×10^3 HeLa 57A cells for each time point were stimulated with 10 ng/ml TNFα or 10 ng/ml IL-1β as described previously and incubated for an additional 7 h. Cells were lysed as reported previously (38). β-Galactosidase activity was measured using the Galacto-Light Plus kit (Tropix) according to the manufacturer's instructions. Both β-galactosidase and luciferase activities were measured in a bioluminometer (Berthold). Values for fold activation of the luciferase reporter are the average of four separate determinations and are compared with the uninduced value. In each case, β-galactosidase activity was used as an internal control.

RESULTS

Transcriptional Activation of NF-κB Is Inhibited by Leptomycin B—It has been previously reported that IκBα that is newly synthesized in response to NF-κB activation translocates to the nucleus, where it dissociates NF-κB from DNA and transports NF-κB from the nucleus to the cytoplasm in a NES-dependent process that is sensitive to LMB. To explore the role of nuclear export in the regulation of NF-κB-dependent transcriptional activity, LMB was used to inhibit NES-mediated nuclear protein export. To monitor transcriptional activity, a cloned HeLa cell line (57A) was derived that contains NF-κB-dependent luciferase and NF-κB-independent β-galactosidase reporters stably integrated into the genome. To activate NF-κB-dependent transcription, HeLa 57A cells were stimulated with TNFα or IL-1β for 7 h (Fig. 1; TNF or IL-1 conditions) or for 30 or 40 min, respectively, and further incubated for 7 h at 37 °C in the absence of stimulus (Fig. 1; *TNF+chase* or *IL-1+chase*). Unstimulated cells were exposed to control medium lacking activators (Fig. 1; *NS*). After the indicated time, cells were lysed, and reporter activity was measured. Because β-galactosidase reporter activity does not change in response to TNFα or IL-1β, this was used to normalize the NF-κB-dependent luciferase activity.

Continuous exposure of cells to TNFα and IL-1β for 7 h induced a 196- and 358-fold stimulation, respectively, compared with the basal noninduced activity. After 30 min of TNFα or IL-1β incubation followed by a 7-h incubation in the absence of stimulus, the transcriptional activity was 71- and 213-fold activation, respectively. This results in a 64% and 41% reduction in activity compared with the continuously activated cells. In contrast, when cells were treated with 20 nM LMB for 30 min before cell stimulation, only 51- and 102-fold activation was obtained by continuous stimulation with TNFα and IL-1β, respectively. LMB treatment thus led to a 74% and 72% inhibition of the NF-κB transcriptional activity induced by TNFα or IL-1β, respectively. Moreover, remaining NF-κB activities were not modified when LMB-treated cells were transiently exposed to TNFα or IL-1β and incubation continued in the absence of stimulus. These results indicate that an inhibition of NES-mediated nuclear protein export not only prevents the post-induction repression of NF-κB-dependent transcription but also strongly represses the initial activation of NF-κB upon cell stimulation. Similar results were obtained with an independent lung-derived cell clone (A549) that contains NF-κB-depend-

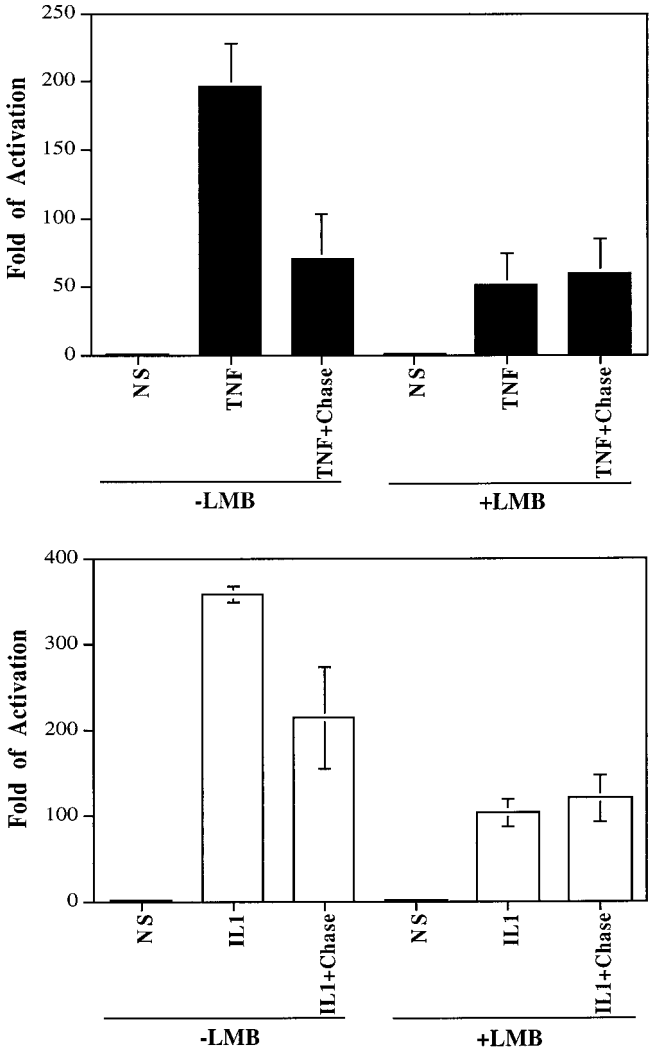

FIG. 1. **Transcriptional activation of NF-κB is inhibited by LMB.** HeLa 57A cells containing NF-κB-dependent luciferase and NF-κB-independent β-galactosidase integrated reporters were untreated or pretreated for 30 min with LMB before incubation for 7 h with TNFα or IL-1β (*TNF* and *IL-1*) or control medium (*NS*). When indicated (*Chase*), TNFα- and IL-1β-treated cells were extensively washed after a 30-min incubation and were maintained in culture for an additional 7 h in the absence of stimulation. At the end of the incubation period, total cell extracts were analyzed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity and reported as fold activation relative to the enzymatic activity in unstimulated cells. The values represent an average of four independent experiments. *Bars*, S.D.

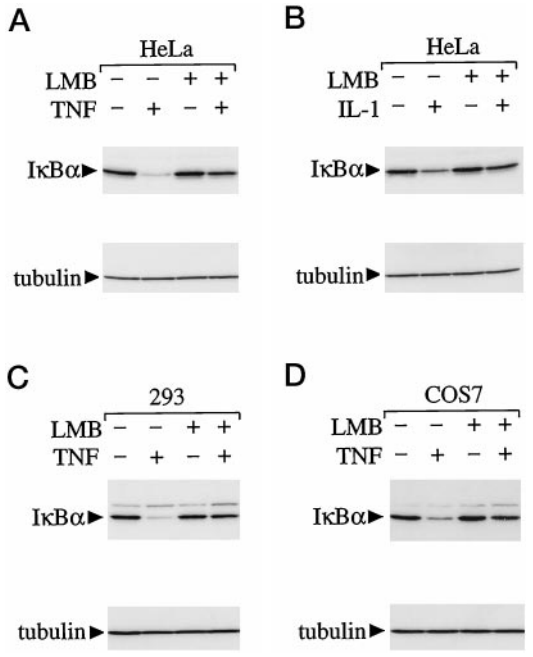

FIG. 2. **LMB inhibits signal-induced degradation of IκBα.** HeLa (*A* and *B*), COS7 (*D*), and 293 (*C*) cells were untreated or pretreated with LMB for 90 min, as indicated. Where indicated, cells were further stimulated for 30 min with TNFα (*A, C,* and *D*) or for 40 min with IL-1β (*B*). Whole cell extracts were analyzed by Western blotting with a polyclonal antibody specific for IκBα and a monoclonal antibody to α-tubulin.

ent luciferase and NF-κB-independent β-galactosidase reporters stably integrated into the genome (data not shown).

Leptomycin B Inhibits Signal-induced Degradation of IκBα—Activation of NF-κB transcriptional activity is mediated by signal-induced degradation of IκBα, which allows the released NF-κB to translocate to the nucleus. The effect of LMB on signal-induced degradation of IκBα expression was therefore examined. Thus, untreated HeLa or LMB-pretreated cells were exposed to TNFα for 30 min or to IL-1β for 40 min (Fig. 2, *A* and *B*). Whole cell extracts were analyzed by Western blotting using an anti-IκBα antibody. As expected, IκBα was rapidly degraded after TNFα or IL-1β treatments, but pretreatment of the cells with LMB substantially inhibited signal-induced degradation of IκBα. This effect was not restricted to HeLa cells because TNFα-induced degradation of IκBα was also inhibited in 293 or COS7 cells pretreated with LMB (Fig. 2, *C* and *D*). These data suggest that LMB inhibits NF-κB transcriptional activity by reducing the signal-induced degradation of IκBα.

Nuclear Accumulation of IκBα Mediated by Leptomycin B Protects It from Signal-induced Degradation—To understand how LMB, a drug that blocks NES-mediated nuclear protein export, inhibits IκBα degradation, the subcellular localization of both IκBα and NF-κB p65 was analyzed by cell fractionation and biochemical analysis as well as indirect immunofluorescence. HeLa cells were either untreated or pretreated with LMB 30 min before stimulation with TNFα. After 30 min, TNFα was removed, and the cells were either analyzed directly or incubated for an additional 60 min in the absence of TNFα (chase). IκBα levels were determined by Western blotting of whole cell extracts. In the absence of LMB, TNFα induced IκBα degradation, followed by resynthesis during the chase period (Fig. 3*A, lanes 1–3*). LMB treatment inhibited the TNFα-induced reduction in IκBα level but had no effect on the amount of IκBα present after the 60-min chase period (Fig. 3*A, lanes 4–6*).

To establish the subcellular localization of IκBα and the p65 subunit of NF-κB under these experimental conditions, cells were fractionated using Streptolysin O, a bacterial toxin that permeabilizes cells without affecting the integrity of the nuclear envelope (52–54). Cytoplasmic and nuclear extracts were analyzed either by Western blotting with an anti-p65 antibody or by immunoprecipitation with an anti-p65 antibody and Western blotting with an anti-IκBα antibody (Fig. 3*B*). In parallel, intact cells treated under the same experimental conditions were processed for immunofluorescence and analyzed using anti-p65 and anti-IκBα antibodies (Fig. 3*C*).

In the absence of any cell stimulation, p65 was predominantly cytoplasmic and was found to interact with IκBα, whereas IκBα was localized in both the cytoplasm and the nucleus (Fig. 3*B, lane 1*; Fig. 3*C, left panel*). Treatment with LMB led to the nuclear accumulation of IκBα and p65, although to a lesser extent (Fig. 3*B, lane 4*; Fig. 3*C, right panel*).

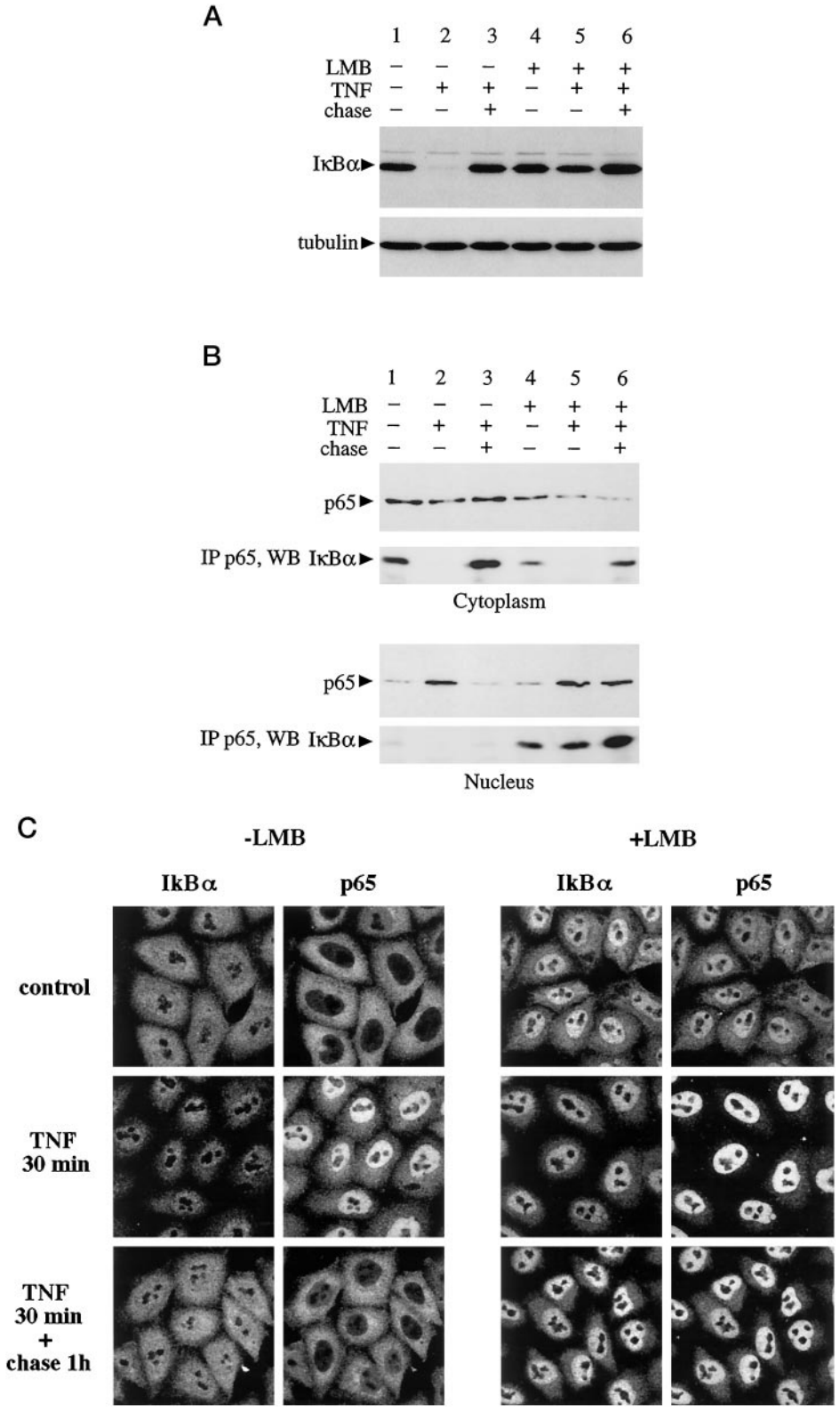

FIG. 3. **IκBα, which was retained in the nucleus by LMB, does not undergo signal-induced degradation.** *A*, HeLa cells were untreated or pretreated for 30 min with LMB. When indicated, cells were further incubated for 30 min with TNFα (*lanes 2* and *5*) or for 30 min with TNFα plus a 60-min chase (*lanes 3* and *6*). Whole cell extracts were analyzed by Western blotting with a polyclonal antibody specific for IκBα and a monoclonal antibody to α-tubulin. *B*, HeLa cells were treated as indicated in *A* and fractionated into cytoplasmic and nuclear fraction using SLO. Cytoplasmic and nuclear extracts were either analyzed directly by Western blotting with an anti-p65 antibody (*upper panel*) or immunoprecipitated with an anti-p65 polyclonal antibody before analysis by Western blotting with an IκBα polyclonal antibody (*lower panel*). *C*, HeLa cells treated as indicated in *A* were processed for indirect immunofluorescence and double stained with a mouse monoclonal antibody to IκBα and a rabbit polyclonal antibody to Rel A. Primary antibodies were detected with a fluorescein isothiocyanate-conjugated anti-mouse IgG and a Texas Red-conjugated anti-rabbit IgG. Cells were visualized by confocal laser scanning microscopy, and photographs correspond to the accumulation of four optical sections in one projection.

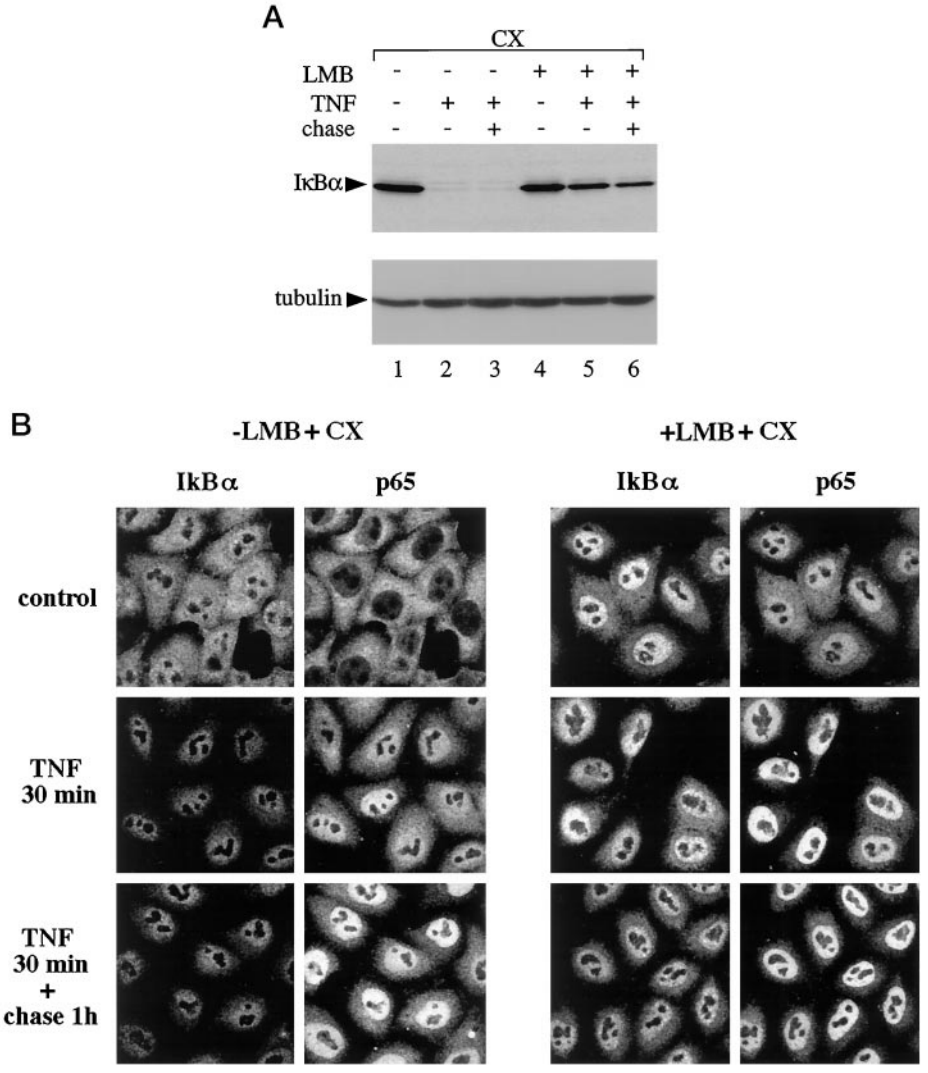

FIG. 4. **LMB does not increases IκBα synthesis.** *A*, HeLa cells were treated as described in the Fig. 3 legend, but cycloheximide was added together with TNFα or for the last 30 min in unstimulated cells. Whole cell extracts were analyzed by Western blotting with a polyclonal antibody specific for IκBα and a monoclonal antibody to α-tubulin. *B*, HeLa cells treated as indicated in *A* were processed for indirect immunofluorescence and double stained with a mouse monoclonal antibody to IκBα and a rabbit polyclonal antibody specific for p65. Primary antibodies were detected with a fluorescein isothiocyanate-conjugated anti-mouse IgG and a Texas Red-conjugated anti-rabbit IgG. Cells were visualized by confocal laser scanning microscopy, and photographs correspond to the accumulation of four optical sections in one projection.

This result suggests that IκBα and a fraction of NF-κB are continuously shuttling between the nucleus and the cytoplasm, even in the absence of cell stimulation. Upon TNFα stimulation of cells that were not treated with LMB, IκBα was degraded in the cytoplasm with a small fraction still present in the nucleus, and p65 was partially translocated to the nucleus (Fig. 3*B, lane 2*; Fig. 3*C, left panel*). The addition of TNFα to LMB-treated cells led to the loss of IκBα from the cytoplasm without affecting the nuclear content of IκBα. Thus, the remaining IκBα after TNFα stimulation of LMB-treated cells was exclusively nuclear. Nuclear translocation of p65 was increased by LMB treatment but mainly resulted in the nuclear accumulation of a transcriptionally inactive IκBα-bound form of Rel A (Fig. 1; Fig. 3*B*, *lane 5*; Fig. 3*C, right panel*). In cells that were not treated with LMB that had been exposed to TNFα but incubated for an additional 60 min in the absence of TNFα, IκBα returned to prestimulation levels as a result of the *de novo* synthesis of the protein. Binding of the newly synthesized IκBα to p65 allowed the IκBα/p65 complexes to relocalize to the cytoplasm (Fig. 3*B, lane 3*; Fig. 3*C, left panel*). Under identical conditions, IκBα levels in LMB-pretreated cells returned to those observed before TNFα stimulation. However, LMB treatment inhibited the relocalization of both IκBα and Rel A to the cytoplasm (Fig. 3*B, lane 6*; Fig. 3*C, right panel*).

In conclusion, inhibition of NES-mediated nuclear protein export leads to the accumulation of IκBα in the nucleus, where it is resistant to signal-induced degradation. Nuclear IκBα can interact with NF-κB and therefore prevent the DNA binding of the transcription factor. Moreover, LMB-mediated inhibition of nuclear export blocks the transport of NF-κB/IκBα complexes back to the cytoplasm. An efficient nuclear export of IκBα is thus required to maintain a low level of IκBα in the nucleus and allow NF-κB to be efficiently activated upon cell stimulation.

Leptomycin B Does Not Increase IκBα Synthesis—The data presented in Figs. 1–3 indicated that LMB inhibits signal-induced activation of NF-κB by partitioning IκBα in the nucleus, where it is resistant to degradation. However, it is a formal possibility that LMB has no effect on IκBα degradation but rather stimulates the synthesis of IκBα. To distinguish between these possibilities, cells pretreated with LMB or untreated cells were exposed to TNFα in the presence of cycloheximide. The expression and subcellular localization of IκBα and p65 were analyzed by Western blotting (Fig. 4*A*) and indirect immunofluorescence using anti-p65 and anti-IκBα antibodies (Fig. 4*B*).

Simultaneous TNFα and CX treatments did not affect signal-induced IκBα degradation and nuclear translocation of p65 (Fig. 4*A, lane 2*; Fig. 4*B, left panel*). However, CX abolished the *de novo* synthesis of IκBα and relocalization of p65 to the cytoplasm that occurs after TNFα treatment and an additional 1-h incubation in the absence of TNFα (Fig. 4*A lane 3*; Fig. 4*B, left panel*). These data confirm that the expression of newly synthesized IκBα in the nucleus is responsible for the nuclear

export of NF-κB to the cytoplasm. Treatment of cells with LMB and CX in the presence or absence of TNFα stimulation did not substantially modify the subcellular distribution of IκBα and p65 observed previously in cells treated only with LMB (Fig. 4*B, right panel*). IκBα was still detected in LMB- and CX-treated cells stimulated with TNFα, and when the incubation was continued in the absence of TNFα, the amount of IκBα in LMB- and CX-treated cells was only slightly decreased (Fig. 4*A, lanes 4–6*). Thus, LMB treatment does not induce IκBα synthesis but rather protects IκBα from signal-induced degradation as a consequence of its nuclear sequestration.

Nuclear IκBα Is Not Accessible to Signal-induced Modification—To confirm that LMB treatment protects IκBα from signal-induced degradation through its accumulation in the nucleus and not through an inhibition of the TNFα signal transduction cascade, a fusion protein containing the amino- and carboxyl-terminal regions of IκBα fused to *Escherichia coli* β-galactosidase (24) was used. Because this protein contains the necessary sequence information from IκBα, it undergoes signal-induced degradation in response to TNFα. However, because it does not contain the ankyrin repeats from IκBα, it does not translocate to the nucleus and is localized exclusively in the cytoplasm, even in the presence of LMB (data not shown). Thus, in LMB-treated cells transfected with a plasmid expressing 4NBC, the endogenous IκBα will be located in the nucleus, whereas 4NBC will be located in the cytoplasm. HeLa cells were transiently transfected with the 4NBC-encoding plasmid, treated with LMB or control medium, and then exposed to TNFα and CX or control medium. After 60 min, the levels of 4NBC and endogenous IκBα were determined by Western blotting. The combined action of TNFα and CX induced the degradation of both endogenous IκBα and exogenous 4NBC, although, as expected, to varying degrees (Fig. 5*A*, IκBα, 90% degraded; 4NBC, 58% degraded). In the presence of LMB, the extent of TNFα- and CX-induced degradation of 4NBC was unaltered (60% degraded), whereas IκBα was inhibited (46% degraded, Fig. 5*A*), indicating that LMB does not directly affect the TNFα signal transduction cascade. Moreover, *in vitro* kinase assays using immunopurified IKKα and IKKβ from HeLa cell extracts showed that the TNFα-induced IKK activity was not affected by the LMB treatments (data not shown).

To determine the step of the signal-induced degradation of IκBα that is prevented by the localization of IκBα in the nucleus, untreated HeLa cells or HeLa cells treated with the proteasome inhibitor MG132 were exposed to TNFα or control medium for 15 min. This experimental condition allows the accumulation of a more slowly migrating phosphorylated form of IκBα (Fig. 5*B, lanes 1–4*). To confirm that this form corresponded to phosphorylated IκBα, the same blot was stripped and reprobed with an antibody specifically recognizing IκBα phosphorylated at Ser-32 (data not shown). In contrast, IκBα degradation was not observed when cells were pretreated with LMB, and the TNFα plus MG132-mediated accumulation of the phosphorylated form of IκBα was severely reduced (Fig. 5*B, lanes 5–8*). These data indicate that nuclear IκBα is not accessible to signal-induced phosphorylation.

However, this observation does not exclude the possibility that other downstream steps leading to signal-induced degradation of IκBα could also be affected; thus, any effect of LMB on IκBα ubiquitination was evaluated. HeLa cells were pretreated with the proteasome inhibitor MG132 and then treated with either LMB, TNFα, or a combination of LMB and TNFα. As expected, MG132 treatment prevented TNFα-induced degradation and allowed the accumulation of slowly migrating multi-ubiquitinated forms of IκBα (29) (Fig. 5*C, lane 2*). In the presence of MG132, LMB, and TNFα, the amount of ubiquitinated IκBα was strongly reduced but not abolished (Fig. 5*C, lane 4*) and probably corresponds to modification of the remaining cytoplasmic IκBα. Thus, nuclear sequestration of IκBα by LMB prevents its proper phosphorylation and subsequent ubiquitination after signal induction. The conditions described above did not alter the levels of the SUMO-1 modified form of IκBα (50).

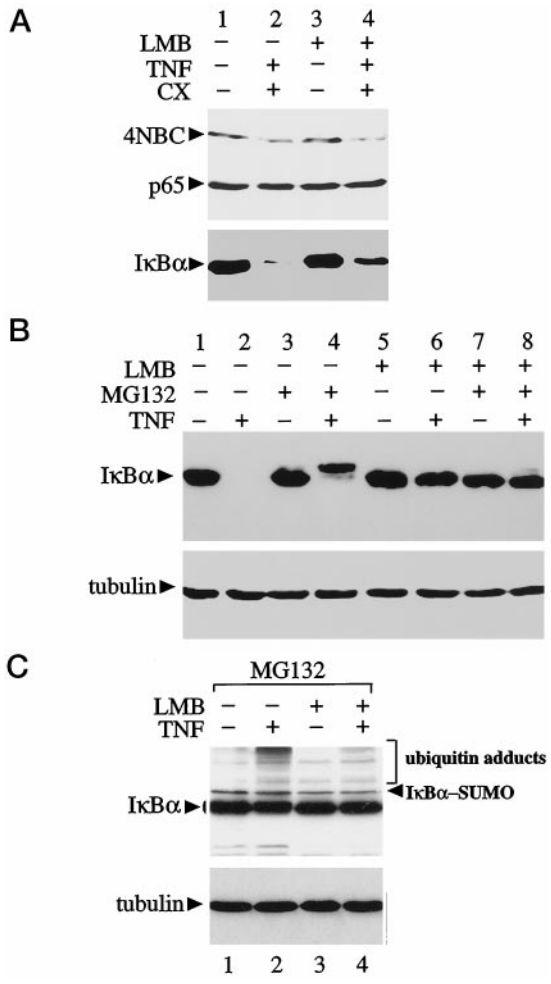

FIG. 5. **Nuclear IκBα is not a substrate for signal-induced modifications required for degradation.** *A*, HeLa cells transfected with the plasmid encoding the 4NBC chimera were preincubated in control medium (*lanes 1* and *2*) or with LMB (*lanes 3* and *4*) for 60 min and further treated with TNFα plus CX (*lanes 2* and *4*) for 60 min. Total cell extracts were analyzed by Western blotting with the SV5 Pk tag monoclonal antibody and a polyclonal antibody specific for p65. The same membrane was stripped and reprobed with an anti-IκBα antibody. Levels of IκBα and 4NBC were determined by densitometry. *B*, HeLa cells were untreated (*lanes 1* and *2*) or pretreated for 60 min with MG132 (*lanes 3* and *4*), LMB (*lanes 5* and *6*), or both MG132 and LMB (*lanes 7* and *8*). After a 15-min incubation with TNFα (*lanes 2, 4, 6,* and *8*), whole cell extracts were analyzed by Western blotting with a polyclonal antibody specific for IκBα and a monoclonal antibody to α-tubulin. *C*, HeLa cells were pretreated with MG132 (*lanes 1–4*) and LMB (*lanes 3* and *4*) for 90 min and further incubated for 30 min with TNFα (*lanes 2* and *4*). Whole cell extracts were analyzed by Western blotting with a polyclonal antibody specific for IκBα and a monoclonal antibody to α-tubulin. SUMO-1- and ubiquitin-conjugated forms of IκBα are indicated (*B*).

DISCUSSION

The experiments reported here indicate that inhibition of IκBα nuclear export not only prevents the post-induction repression of NF-κB-dependent transcription but also strongly represses the initial activation of NF-κB upon cell stimulation.

Indeed, nuclear IκBα appears to be resistant to signal-induced phosphorylation and degradation, and this results in nuclear accumulation of transcriptionally inactive IκBα/NF-κB complexes. Nuclear export of IκBα and many other proteins that shuttle between the nucleus and the cytoplasm is mediated by a leucine-rich NES that is recognized by CRM1/exportin 1 (43–46). In our experiments, nuclear export of IκBα was inhibited by the drug LMB. LMB specifically targets CRM1 by blocking its interaction with the NES (45). Although the inhibition of CRM1 by LMB is highly specific, it was important to rule out the possibility that LMB might be interfering with the signal transduction pathways that lead to NF-κB activation. To address this point, we used a lacZ fusion protein (4NBC) containing the amino and carboxyl termini of IκBα. When this protein is expressed in cells, it is unable to translocate to the nucleus but undergoes signal-induced degradation in response to agents such as TNFα and IL-1β (24). Signal-induced degradation of the 4NBC protein was unaffected by LMB (Fig. 5*A*), indicating that LMB does not inhibit the signal transduction pathway that leads to IκBα degradation. It is also clear that LMB does not inhibit transcription in a nonspecific fashion because the activity of the integrated RSV-driven lacZ reporter was unaffected by the presence of LMB (Fig. 1). The conclusion from these experiments is that in HeLa cells, signal-induced phosphorylation and degradation of IκBα occurs exclusively in the cytoplasm. One possibility to explain this restriction is that an essential component of the signal transduction pathway that leads to IκBα phosphorylation cannot gain access to the nucleus. It appears that the IκBα kinases (IKKα and IKKβ) are present in a large signaling complex that may contain upstream kinases such as NF-κB inducing kinase (55, 56) and scaffolding proteins such as NEMO (57). It has yet to be determined if this large complex can be imported into the nucleus. The alternative argument would be that the signal modification machinery has access to the nucleus, but that the nuclear IκBα is in some way refractile to modification. Mechanisms to achieve this could include prior covalent modification of IκBα to a form that is no longer recognized by the IKK signaling complex. It has recently been demonstrated that IκBα is modified by the small ubiquitin-like protein SUMO-1 (50). Because this protein is linked to the same lysine residues that are used for ubiquitination of IκBα, this renders the SUMO-1 modified form of IκBα resistant to signal-induced degradation. Although the known proteins that are substrates for SUMO-1 modification have been detected in the nucleus or are involved in nuclear transport (58), we have no evidence to support the notion that nuclear IκBα detected in the presence of LMB is resistant to signal-induced phosphorylation because it is modified by SUMO-1. It is also possible that IκBα could interact with a nuclear protein that occludes the region in IκBα containing residues Ser-32 and Ser-36, thus protecting it from signal-induced phosphorylation.

In unstimulated cells, there is clearly a requirement for the transcription of essential NF-κB-dependent genes. Low-level transcription of these genes does not take place simply as a consequence of NF-κB-independent transcription, because IκBα overexpression effectively abolishes the activity of a NF-κB-dependent reporter in unstimulated cells.[2] Thus, it appears that the cell has evolved a highly dynamic system to provide for continued low-level transcription of NF-κB-dependent genes. This homeostatic mechanism requires the continuous proteasome-mediated breakdown of IκBα, which generates a stream of free NF-κB that can translocate to the nucleus. Once in the nucleus, NF-κB activates NF-κB-dependent genes, including that of IκBα. After transport to the cytoplasm, IκBα mRNAs are translated, and the free IκBα is directed to the nucleus, where it interacts with DNA-bound NF-κB and dissociates the DNA-protein complex. By virtue of the presence of a NES in IκBα (39), NF-κB/IκBα complexes are recognized by CRM1, which mediates nuclear export (45). At this point, equilibrium is reestablished. Thus, rather than having a simple on-off switch, the cell can delicately alter the NF-κB transcriptional response by varying the rate at which IκBα is turned over. The most extreme perturbation of this equilibrium comes after exposure of the cells to agents such as TNFα or IL-1β. In this situation, cytoplasmic IκBα is completely degraded, and a massive pulse of NF-κB is released into the nucleus to initiate high-level transcription of NF-κB-dependent genes. However, the same mechanism is used to bring the system back into homeostasis (38, 39). A remarkably similar homeostatic mechanism seems to operate to control the level of p53 within the cell. In this case, the product of the *hdm2* gene targets p53 for ubiquitin-mediated proteasomal degradation, and disruption of this interaction during the damage response leads to the accumulation of p53. Nuclear translocation of p53 activates transcription of the *hdm2* gene, and the newly synthesized protein enters the nucleus, where it terminates p53-dependent transcriptional activation. hdm2 also contains a NES, and this is used to export the p53/hdm2 complex to the cytoplasm using the same pathway that is used for nuclear export of IκBα. Inhibition of hdm2-mediated export revealed that nuclear export of hdm2 is required to accelerate the degradation of p53 (59). In the case of both IκBα and p53, ubiquitin-mediated proteasomal degradation occurs in the cytoplasm, even though proteasomes are found in both compartments. However, proteasomal components are distributed differentially between the nucleus and the cytoplasm (60, 61), suggesting that nuclear and cytoplasmic proteasomes may have unique properties. The advantage to the cell of these homeostatic mechanisms to control NF-κB- and p53-dependent transcription is that they are both highly sensitive to perturbation, and they can provide a finely tuned response to external signals.

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