THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 13, Issue of March 29, pp. 10842–10851, 2002

Printed in U.S.A.

Signaling Molecules of the NF-κB Pathway Shuttle Constitutively between Cytoplasm and Nucleus*

Received for publication, December 31, 2001
Published, JBC Papers in Press, January 18, 2002, DOI 10.1074/jbc.M112475200

Andreas Birbach‡, Peter Gold, Bernd R. Binder, Erhard Hofer, Rainer de Martin, and Johannes A. Schmid§

From the Department of Vascular Biology and Thrombosis Research, University of Vienna, and the Competence Center for Bio-Molecular Therapeutics, A-1235 Vienna, Austria

We aimed to investigate the dynamics of the NF-κB signaling pathway in living cells using GFP variants of p65-NF-κB, IκBα, tumor necrosis factor-receptor associated factor 2 (TRAF2), the NF-κB inducing kinase (NIK) and IκB kinases (IKK1 and IKK2). Detailed kinetic analysis of constitutive nucleocytoplasmic shuttling processes revealed that IκBα enters the nucleus faster than p65. Examination of signaling molecules upstream of NF-κB and IκBα revealed a predominant cytoplasmic localization at steady state. However, after addition of leptomycin B, NIK rapidly accumulated in the nucleus, whereas we could not detect any significant effect on TRAF2 or IKK2. Using various truncation mutants of NIK, we identified a functional nuclear export signal within the COOH-terminal region 795–805, which counteracts the inherent NLS at amino acids 143–149. Prolonged incubation in the presence of LMB also leads to nuclear accumulation of IKK1, which was dependent on a lysine residue at position 44, which is also essential for kinase activity. Investigation of endogenous protein levels by immunofluorescence staining and Western blots verified the results obtained with GFP chimeras. We conclude that NF-κB·IκB complexes and the upstream signaling kinases NIK and IKK1 shuttle between cytoplasm and nucleus of nonactivated cells and that this process leads to a basal transcriptional activity of NF-κB.

Transcriptional activators of the nuclear factor-κB (NF-κB)[1]/Rel family have a central role in the inducible expression of a high number of genes involved in inflammation, host defense, cell survival, and proliferation (1–4). Their common characteristic is a so-called Rel homology domain of about 300 amino acids, which is responsible both for binding to a consensus DNA sequence and for homo- or heterodimerization. One of the most important distinctions between various family members is the presence of a transactivation domain (*e.g.* in p65, RelB, or c-Rel), which is absent in the smaller subunits p50 and p52.

The most abundant forms of NF-κB are p65/p50 heterodimers and p65/p65 homodimers, while other complexes were reported to have specific roles in certain cell types. In most cases, NF-κB dimers are kept inactive by binding to an inhibitory molecule of the IκB family, with IκBα being the most important one. Other members of this inhibitory family such as IκBβ or IκBε have been shown to differ from IκBα by their constitutive turnover and their signal-induced degradation (1–4).

An intriguing feature of NF-κB is that it can be activated by a vast variety of distinct stimuli, including not only inflammatory cytokines such as TNFα or interleukin-1, but also physical stress such as UV- or γ-irradiation, reactive oxygen intermediates, as well as virus-derived dsDNAs or RNAs (5, 6). Given its importance in chronic and acute inflammation and its potential as target in a great variety of diseases (7–10), much effort was focused on the elucidation of signaling cascades leading to the activation of NF-κB. For the central inflammatory pathway of TNFα-mediated activation, it was shown that binding of the trimeric ligand results in a trimerization of corresponding receptors (TNFR1 and TNFR2) (11), followed by the association of intracellular adapter proteins to the cytoplasmic domain of the receptors. These adapter molecules comprise proteins with death domains such as TRADD (TNFR associated death domain protein), which bind to TNFR1, or proteins with a TRAF (TNFR associated factor) domain, which is also included in the cytoplasmic tail of TNFR2. Members of the latter family such as TRAF1 or TRAF2 can directly bind to TNFR2 or indirectly via TRADD to TNFR1. Since overexpression of TRAF2 activates NF-κB, and mutants lacking the NH_2-terminal RING finger domain act as dominant negative inhibitors of this pathway, it is assumed that TRAF2 plays an important role in the signaling from the receptor to downstream kinases. However, studies with cells derived from $TRAF2^{-/-}$ mice indicated that its function can be taken over by other signaling proteins. It has been hypothesized that receptor-induced oligomerization of adapter proteins leads to binding, autophosphorylation, and activation of downstream kinases. Candidate enzymes are certain members of the MAP (mitogen-activated protein) 3 kinase (MAP3K) family. One of these is the NF-κB inducing kinase NIK, which interacts with various members of the TRAF family. Another kinase that has been assumed to be involved in NF-κB activation is MEKK1, which has been shown to interact specifically with TRAF2 via its RING finger domain. However, recent knockout studies suggest that NIK or MEKK1 alone are dispensable for activation of NF-κB by certain stimuli such as interleukin-1 or TNFα (12, 13), whereas NIK is essential for signaling via the lymphotoxin-β receptor (12). This indicates a

* This work was supported in part by Austrian Science Foundation Grant SFB5-12 and by the Competence Center Bio-Molecular Therapeutics, Vienna. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Friedrich Miescher Institut, Maulbeerstrasse 66, CH-4058 Basel, Switzerland.

§ Supported by the EMBL, Heidelberg, in the European Advanced Light Microscopy Facility during a short term fellowship funded by the EMBL/EurALMF Program. To whom correspondence should be addressed: Brunnerstr. 59, A-1235 Vienna, Austria. Tel.: 43-1-4277-62555; Fax: 43-1-4277-62550; E-mail: Johannes.Schmid@univie.ac.at.

[1] The abbreviations used are: NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor-α; NLS, nuclear localization signal; IKK, IκB kinase; NES, nuclear export sequence; HUVEC, human umbilical vein endothelial cells; GFP, green fluorescent protein; LMB, leptomycin B.

receptor- and cell type-specific action of different MAP 3-kinases.

Although a great variety of stimuli activate NF-κB, apparently all the respective signaling pathways converge at the level of the IκB kinase (IKK) complex consisting of the kinases IKK1 and -2 and the essential adapter molecule NEMO/IKKγ. The IKKs are activated by phosphorylation at serine residues within the activation loop, and it was postulated that upstream activators might have certain specificity for one or the other kinase, such as NIK for IKK1 (14). Upon activation IκB kinases phosphorylate IκB family members at conserved serine residues in the NH_2-terminal signal-responsive domain. This signal-induced phosphorylation results in ubiquitinylation and subsequent degradation of the inhibitor by 26 S proteasomes and release of NF-κB.

The classical view of the regulation of NF-κB is that it is kept inactive by cytoplasmic retention due to binding to IκB proteins. IκB is thought to mask the nuclear localization signal (NLS) of NF-κB, thereby preventing the interaction of NF-κB with the nuclear import machinery. Moreover, the nonconventional NLS of IκB itself, located within the second ankyrin repeat, appears to be blocked when IκB is bound to NF-κB. Thus, the NLS of NF-κB is unmasked upon degradation of its inhibitor and the transcription factor can be imported into the nucleus, where it binds to specific promoter elements. Besides a number of other genes, NF-κB is also inducing the transcription of DNA encoding its own inhibitor (15). After translation in the cytosol, IκB is imported into the nucleus, where it is assumed to dissociate NF-κB from promoter regions. The newly formed NF-κB·IκB complex is then transported back to the cytosol by the means of a specific nuclear export sequence (NES) of IκB. Whereas a putative NES was originally postulated for the COOH terminus of IκB (16), it was recently shown that a NH_2-terminal NES instead of the postulated COOH-terminal region is responsible for Crm1p-dependent nuclear export of IκB and the bound NF-κB (17, 18). Moreover, it was also postulated that NF-κB contains a NES by itself (19), which would allow an IκB-independent nuclear export of this transcription factor.

The finding that both molecules apparently contain NLS, as well as NES domains raises the question how the intracellular distribution of these proteins is regulated functionally in the course of activation and de-activation of the signaling pathway, as well as in quiescent cells. Recent reports could demonstrate that both NF-κB and IκB accumulate in the nucleus after addition of the nuclear export blocking agent leptomycin B in the absence of any additional signaling event (17, 18). Hence IκB is apparently not able to prevent the nuclear import of NF-κB. This observation implies that the principal inhibitory role of IκBα is not the cytosolic sequestration of the transcription factor, but rather to inhibit binding of NF-κB to specific DNA sequences and to shift its steady state localization to the cytosol. This view of NF-κB and IκB has further important consequences. Since all macromolecular complexes have a certain equilibrium between dissociation and association, the existence of nuclear NF-κB·IκB complexes should also lead to the occurrence of a certain number of NF-κB molecules that are not bound to the inhibitor and therefore free for binding to promoter elements. The dynamic equilibrium between IκB-bound and unbound NF-κB has to be postulated not only for the nuclear, but also for cytosolic complexes. Thus, association and dissociation of NF-κB and IκB in the cytosol would be accompanied by nuclear translocation of both molecules separate from each other and a corresponding binding equilibrium in the nucleus, counteracted by association of NF-κB with specific DNA sequences. The recent report by Carlotti *et al.* (20) demonstrates that NF-κB and IκB dissociate from each other in the cytosol, followed by separate nuclear import, rather than a nuclear translocation of the whole NF-κB·IκB complexes as suggested based on the x-ray crystallographic data (21, 22).

The fact that both NF-κB and IκB accumulate in the nucleus after inhibition of nuclear export prompted us to test whether upstream signaling molecules of the NF-κB pathway shuttle between cytosol and nucleus as well. We observed a rapid nucleocytoplasmic distribution of the NF-κB inducing kinase NIK that was dependent both on an apparent classical NLS in the NH_2-terminal region of NIK and a newly defined functional NES in the COOH-terminal domain. Moreover, the IκB kinase 1 (IKK1) was also shown to translocate to the nucleus dependent on a critical lysine residue at position 44. These observations indicate not only that NF-κB and its inhibitor are dynamically shuttling between cytosol and nucleus, but also signaling molecules upstream of NF-κB. Thus, nuclear NF-κB·IκB complexes might be activated by kinases, which exhibit nucleocytoplasmic shuttling, themselves.

MATERIALS AND METHODS

Plasmids—pEYFP-NIK was generated by subcloning NIK cDNA from the expression vector pcDNA-HA-NIK (23) using *Nhe*I and *Xba*I restriction sites, respectively, into pEYFP-C1 (CLONTECH). YFP-tagged NIK truncation mutants (pEYFP-NIK^{1-769}, pEYFP-NIK^{1-794}, pEYFP-NIK^{1-805}) were generated by amplification of the corresponding cDNAs by PCR using the sense-primer 5′-AAAGAATTCCACCATGGCAGTGATGGAAATG-3′ and the following antisense primers: 5′-AAAGTCGACTTCCTGCTCCGGGACG-3′ (NIK^{1-769}), 5′-AAAGTCGACAATTTGCTCCTGCTCCTC-3′ (NIK^{1-794}) and 5′-AAAGTCGACCAGGGAGAGGCTGTCGA-3′ (NIK^{1-805}), respectively. The PCR products were cloned into the *Eco*RI and *Sal*I sites of pEYFP-C1. For pEYFP-IKK1 and pEYFP-IKK1K44M expression plasmids, the corresponding cDNAs were amplified from vectors IKK1-HA and IKK1K44M-HA, which were a generous gift from M. Karin, using primers 5′-AAAGAATTCCACCATGGAGCGGCCCCCG-3′ and 5′-AAAGTCGACTCATTCTGTTAACCTACTC-3′, respectively, and cloned into the *Eco*RI and *Sal*I sites of pEYFP-C1. pECFP-TRAF2 was generated by subcloning TRAF2 cDNA from pRK-F-TRAF2 (24) using *Bam*HI and *Hin*dIII restriction sites into the *Bgl*I and *Hin*dIII restriction sites of pECFP-C1 (CLONTECH). The expression vector for pIKK2-EGFP was designed by excising IKK2 cDNA out of the plasmid wt-IKK2-FL described in Ref. 25 using *Xba*I and *Stu*I restriction sites and cloned into *Nhe*I and *Sma*I sites of pEGFP-N1 (CLONTECH). Subsequently, pIKK2-EYFP was generated by replacing EGFP with EYFP, which was excised out of pEYFP-C1 using *Age*I and *Xba*I restriction sites.

NF-κB (p65) and IκBα fusion proteins with fluorescent proteins have been described previously (26). All constructs were verified by restriction and sequence analysis.

Cell Culture and Transfections—Isolation and culturing of human umbilical vein endothelial cells (HUVEC) was described previously (27), as was culturing of 293 and HeLa cells (26). Transient transfections were performed using the LipofectAMINE Plus system (Invitrogen) according to the manufacturer's protocol. In brief, 293 cells (one well of a six-well plate, 10 cm^2) were transfected with 1 μg of DNA, 4 μl of Plus reagent, and 3 μl of LipofectAMINE for 4 h, HeLa cells with 1.5 μg of DNA, 5 μl of Plus reagent, and 3 μl of LipofectAMINE for 6 h and HUVEC with 1.5 μg of DNA, 8 μl of Plus reagent, and 4 μl of LipofectAMINE for 2.5 h. For transfection in Lab-Tek chamber slides (Nunc), HUVEC were transfected with 300 ng of DNA, 1.6 μl of Plus reagent, and 0.8 μl of LipofectAMINE for 2.5 h (values for 2 chambers of 0.25 cm^2 size each).

Reporter Gene Assays—HUVEC, 293, or HeLa cells were transiently transfected with a luciferase reporter construct under the control of the NF-κB-dependent IκBα-promoter (15) or 5 tandem repeats of the NF-κB-binding site. A β-galactosidase vector containing an Rous sarcoma virus promoter served as internal control. The tested GFP constructs, reporter and control constructs were co-transfected in equivalent amounts as described. Cell extracts were prepared by repeated freeze-thawing in 0.1 M potassium phosphate buffer (pH 7.8). Luciferase activity was determined as described (15) and related to β-galactosidase activity determined with chlorophenol red-β-D-galactopyranoside as substrate and colorimetric detection at 570 nm.

Fluorescence Microscopy and Image Analysis—HUVEC or HeLa cells were transiently transfected with YFP or CFP constructs or both and

investigated 1 day after transfection on a Nikon Diaphot TMD microscope using XF104 and XF114 filter sets (Omega Opticals Inc., Brattleboro, VT) specific for YFP and CFP, respectively. Images were taken by means of a cooled charge-coupled device camera (Kappa DX30, Kappa GmbH, Gleichen, Germany) using the manufacturer's software (Kappa Image Base). For nuclear transport studies, leptomycin B (LMB, a gift from Barbara Wolff-Winiski, Novartis Research Institute, Vienna, Austria) was directly added to the serum-containing medium at a concentration of 20 nM. For time-lapse experiments, cells were incubated at 37 °C, 5% CO_2 between single time points. Alternatively, cells were imaged at 37 °C in a CO_2-independent medium on a video-enhanced inverted Leica microscope including computerized filter wheels and OpenLab™ Software (Improvision Ltd., United Kingdom) and images were taken at frequent time intervals both with CFP and YFP filter sets. The fluorescence intensities in the cytosol and the nucleus were quantified using NIH Image Software (or the Windows™-based equivalent software "ScionImage" provided by Scion Corporation Inc.), the background was subtracted and ratios of cytosolic to nuclear fluorescence were calculated for each individual cell. Mean ratios and standard deviations were determined and the kinetics of nuclear accumulation after addition of LMB were fitted by a single exponential decay algorithm (using Graphpad Prism™ 3.0, Graphpad Software, Inc.). Confocal laser scanning microscopy was performed on Zeiss LSM510 equipment using the 514-nm line of the Ar-laser for excitation of YFP and the 458-nm line for excitation of CFP, combined with appropriate dichroic mirrors and emission band filters to discriminate between CFP and YFP fluorescence. The optical sectioning was set to a thickness of 0.8 μm and images were taken approximately at the center of the nucleus.

Preparation of Nuclear Extracts and Western Blot Analysis—HeLa cells were incubated for different periods of time in the presence of LMB, followed by preparation of nuclear extracts. In brief, about 3×10^6 cells per sample were washed with phosphate-buffered saline, scraped, and collected by centrifugation at $1,000 \times g$ for 5 min. The pellet was suspended in 200 μl of CE buffer (10 mM Hepes pH 7.6, 60 mM KCl, 1 mM EDTA, 0.075% Nonidet P-40, 1 mM dithiothreitol, including the Complete™ protease inhibitor mixture, Roche Molecular Biochemicals), incubated on ice for 3 min and centrifuged at $1,500 \times g$ for 4 min at 4 °C. The supernatant (cytosolic extract) was removed and the pellet (nuclei) was washed with 1 ml of CE buffer without Nonidet P-40. The nuclei were pelleted as above, followed by suspending in 70 μl of NE buffer (20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol and protease inhibitors as in the CE buffer) and incubation on ice for 10 min with frequent mixing. Finally the suspension was centrifuged at $14,000 \times g$ for 10 min at 4 °C and the supernatant (nuclear extract) was combined with concentrated SDS-loading buffer and heated to 95 °C for 5 min. Nuclear proteins were separated by SDS-PAGE and Western blots were performed using antibodies against IκBα (Clone-C21), p65-NF-κB, IKK1, and IKK2. All antibodies were purchased from Santa Cruz.

RESULTS

NF-κB and IκBα Shuttle Rapidly between Cytosol and Nucleus—To investigate the intracellular distribution of various components of the NF-κB signaling pathway in living cells, we generated CFP, GFP, and YFP chimeras of p65-NF-κB, IκBα, TRAF1, TRAF2, NIK, IKK1, and IKK2 for transient or stable transfections and morphological analysis. The advantage of this approach is that two different signaling components expressed as CFP and YFP chimeras, respectively, can be investigated simultaneously *in vivo* with appropriate filter sets. Furthermore, the cells can be analyzed without any background, which is often observed in immunofluorescence staining. However, for a valid interpretation of the results, it is necessary to confirm that the CFP and YFP fusion proteins are still functional as compared with the wild type protein. We have demonstrated previously that both p65-NF-κB and IκBα are still functional as GFP chimeras and that they are still binding to each other even if both of them are tagged with a CFP, GFP, or YFP moiety (26). Analysis of other components of the NF-κB signaling cascade by reporter gene analysis using NF-κB-dependent luciferase reporter constructs verified that the GFP fusion proteins were functional with activities similar to those of the wild type proteins (Fig. 1). The difference in case

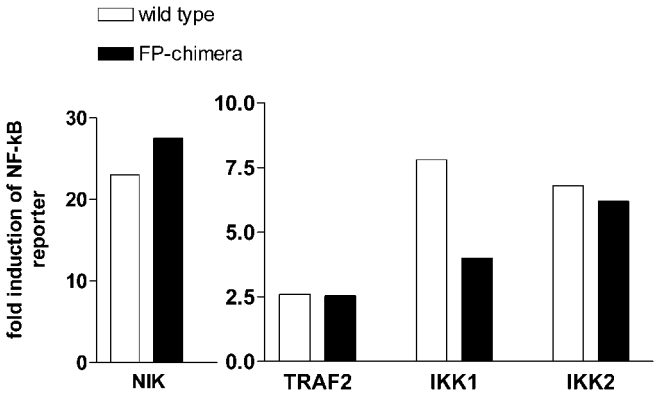

FIG. 1. **Functional integrity of GFP fusion proteins.** HeLa cells were transiently transfected with an NF-κB-dependent luciferase reporter construct together with an NF-κB-independent β-galactosidase control vector and either wild type or fluorescent protein-chimeric signaling molecules (NIK, TRAF2, IKK1, or IKK2) 1 day after transfection, cell extracts were prepared and luciferase activity was determined and normalized to the NF-κB-independent β-galactosidase activity. The induction of the luciferase reporter activity compared with the vector control (without signal molecule construct) is indicated. All samples were transfected with equal amounts of DNA. A representative experiment is shown.

of IKK1 is apparently due to the use of different promoters for this construct and its GFP counterpart. Western blot analysis of transfected GFP constructs showed the expected molecular weights and verified the integrity of the expressed GFP chimeras (data not shown).

To investigate a potential dynamic distribution of NF-κB and IκBα between cytosol and nucleus, we transfected HUVEC or HeLa cells with equal amounts of YFP-p65 and CFP-IκBα, respectively. After addition of leptomycin B to block Crm1p-dependent nuclear export processes (28), the cells were imaged at different time points with appropriate CFP and YFP filter sets. These experiments could demonstrate a rapid nuclear accumulation of both proteins after inhibition of nuclear export, indicating continuous nucleocytoplasmic shuttling independent from any known NF-κB activating stimulus (Fig. 2). This process did not depend on *de novo* protein synthesis, as it could not be influenced by addition of cycloheximide and it was also observed in stable 293 transfectants expressing both CFP-IκBα and YFP-p65 (data not shown). Strikingly, the nuclear accumulation of IκBα appeared to be even faster than that of p65. In many of the cells that have been examined, it was evident that starting from an equal cytoplasmic steady state localization of IκBα and p65, the nuclear IκB concentration increased more rapidly than that of p65. Finally, both proteins reached a nearly complete nuclear localization about 4 h after addition of LMB.

To quantify the steady state distribution of YFP-p65 and CFP-tagged IκBα between cytosol and nucleus in living cells, we applied confocal laser scanning microscopy using optical sectioning parameters that eliminate an out-of-focus fluorescence. By that means the cytosolic fluorescence above or below the nucleus cannot contribute to the fluorescence signal measured in the nuclear region. These studies indicated that about 5.6% of transfected NF-κB and about 8.0% of co-transfected IκBα are localized to the nucleus at steady state (Fig. 2*C*). Analysis of the molecular association of NF-κB and IκBα by FRET microscopy (as described in Ref. 26), showed that p65 and IκBα bind to each other not only in the cytosol, but also after LMB-induced nuclear accumulation within the nucleus (Fig. 2*D*).

To calculate the kinetics of the nuclear accumulation of p65 and IκBα, we performed time lapse morphological studies of

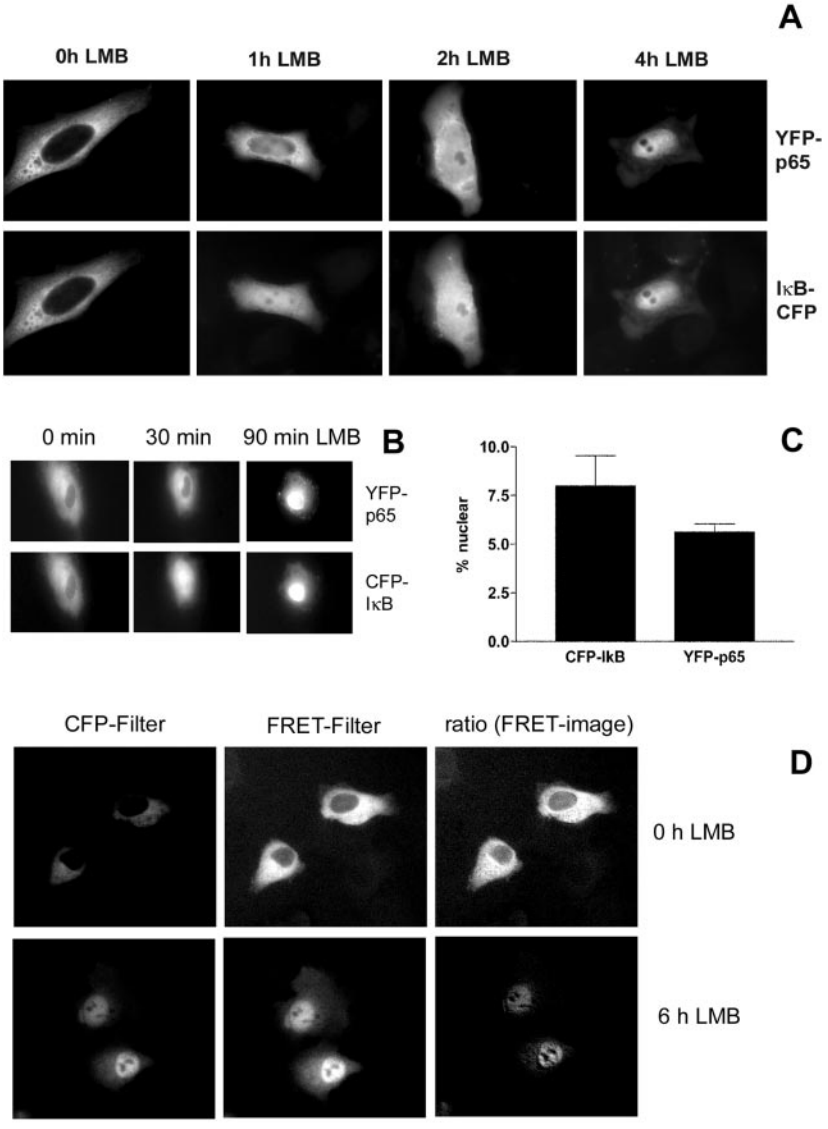

FIG. 2. **Nucleocytoplasmic shuttling of p65-NF-κB and IκBα.** *A*, HeLa cells were co-transfected with YFP-p65 and CFP-IκBα. 1 day after transfection, living cells were analyzed by fluorescence microscopy using specific filters for YFP and CFP-fluorescence, respectively, followed by addition of LMB (20 nM) and subsequent imaging with a ×100 objective for a good resolution of cytoplasm and nucleus. Representative cells are shown. The experiment was performed several times with essentially the same result (as also quantified in Fig. 3). *B*, LMB-dependent nuclear accumulation of YFP-p65 and CFP-IκBα in living endothelial cells 1 day after transient transfection. The same cell is shown for the indicated time points. *C*, HeLa cells transfected with YFP-p65 and CFP-IκBα were analyzed 1 day after transfection by confocal laser scanning microscopy using an optical sectioning of 0.8 μm. YFP and CFP images were captured with different excitation wavelengths and appropriate filter sets at 12 bit intensity resolution. Images were imported into ScionImage™ with the 4096 gray level resolution and nuclear and cytosolic fluorescence was quantified. The nuclear fluorescence is expressed in percent of the cytosolic intensity ($n = 8$ for CFP-IκBα and $n = 29$ for YFP-p65, *error bars* represent S.E.M.). *D*, the interaction of YFP-p65 and IκBα-CFP was visualized by FRET microscopy as described in Ref. 26 before and after LMB-dependent nuclear accumulation. In brief, images were taken with the CFP filter set (CFP-excitation and CFP-emission) and a FRET filter set (CFP-excitation and YFP-emission) using the same camera settings. A higher intensity of the FRET filter image compared with the CFP image as reflected by a positive ratio image indicates FRET and thus a molecular association of both molecules. Under these conditions a negative control of CFP and YFP expressing cells shows a clearly negative, black ratio image as described in Ref. 26.

cells incubated at 37 °C in the presence of LMB. Images of CFP and YFP fluorescence were taken at frequent intervals and the ratio of cytosolic to nuclear fluorescence was calculated for individual cells as described under "Materials and Methods." Mean ratio values were fitted with a single exponential decay algorithm indicating a half-time of the cytosol-nucleus redistribution of about 14 min for p65 and of about 7 min for IκBα (Fig. 3).

To investigate whether the kinetics of the nucleocytoplasmic shuttling is affected by the relative amounts of NF-κB and IκBα, we performed similar studies after transient transfection with either YFP-p65 or CFP-IκBα in excess. After transfection with p65 and IκBα at a ratio of 9:1, many of the cells had already a predominant nuclear localization of p65. However, some of the cells still exhibited a major cytosolic localization of both p65 and IκBα. Morphological analysis of these cells after addition of LMB revealed a striking difference between YFP-p65 and CFP-IκBα in the kinetics of nuclear accumulation. While p65 accumulated in the nucleus already after about 5 to 10 min, the kinetics of the nuclear import of IκBα was significantly slower (Fig. 4*A*). Conversely, transfection of YFP-p65 and CFP-IκBα at a ratio of 1:9 and selection of cells with predominant cytosolic localization of IκBα resulted in a very fast nuclear accumulation of IκBα alone within 5–10 min,

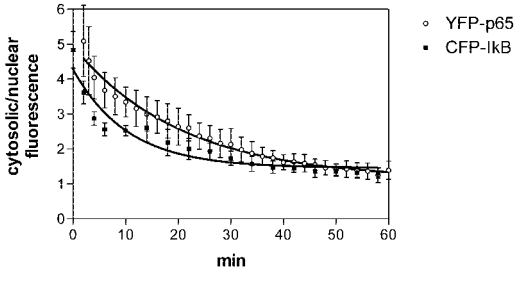

FIG. 3. **Quantification of the kinetics of LMB-dependent nuclear accumulation of YFP-p65 and CFP-IκBα.** HeLa cells were transfected with YFP-p65 and CFP-IκBα at equal concentrations, followed by time lapse fluorescence microscopy of living cells at 37 °C 1 day after transfection. The accumulation of fluorescent molecules in the nucleus was analyzed as described under "Materials and Methods" and the ratio of cytosolic to nuclear fluorescence is specified together with the single exponential fit of the data points ($n = 5$ for CFP-IκBα and $n = 3$ for YFP-p65, *error bars* represent S.E.M.).

whereas p65 remained mainly cytosolic for much longer times after addition of LMB (Fig. 4*B*).

Taken together, these data indicate that p65 and IκBα can enter the nucleus independently of each other and that a dissociation of the p65·IκB complex might be the rate-limiting step in the nucleocytoplasmic shuttling process of these proteins.

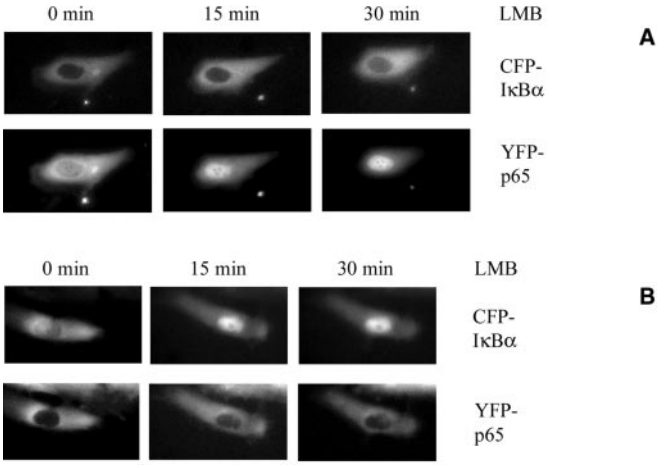

FIG. 4. **Distinct kinetics of nuclear accumulation of YFP-NF-κB and IκB-CFP at different expression ratios.** *A*, primary HUVEC cultures were transfected with a 9:1 ratio of YFP-p65:IκB-CFP and cells were observed which showed a strong overexpression of the p65 fusion protein, yet still little nuclear localization. LMB was applied as described under "Materials and Methods" and images were taken every 5 min. *B*, HUVEC were treated as in *A*, but the transfection mixture was YFP-p65:IκB-CFP (1:9) and IκBα overexpressing cells were observed.

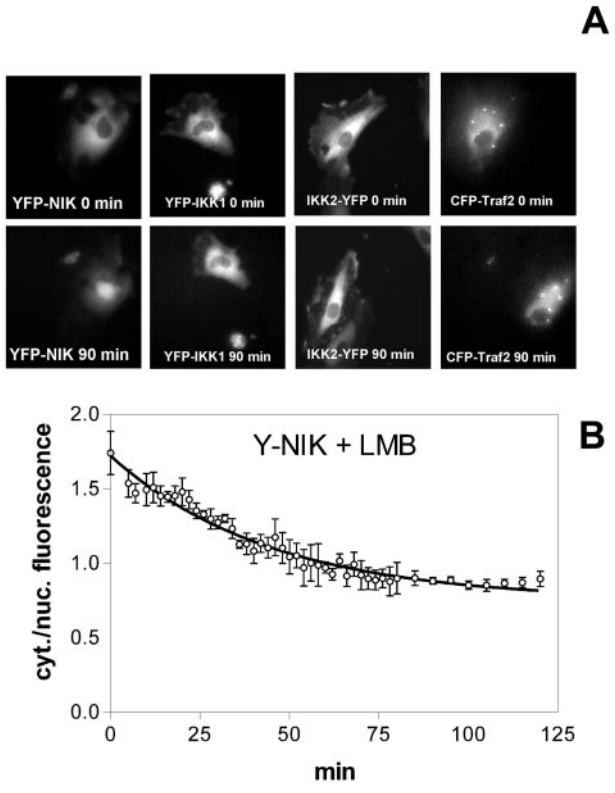

FIG. 5. **Analysis of nucleocytoplasmic shuttling of NF-κB signaling molecules.** *A*, YFP or CFP chimera of NIK, IKK1, IKK2, or TRAF2 were expressed in primary HUVEC cultures and LMB (20 nM) was applied to the culture medium. Time lapse fluorescence microscopy of individual cells was performed over a period of 2 h. Images show typical cells of the different transfected cultures before and 90 min after application of the drug. *B*, kinetics of nuclear accumulation of YFP-NIK: LMB was applied to HeLa cells transfected with YFP-NIK fusion protein and images were taken at frequent time intervals. Calculation of cytosolic *versus* nuclear fluorescence was performed as described under "Materials and Methods" and fitted by a single exponential decay algorithm ($n = 3$, *error bars* represent S.E.M.).

This model is in agreement with a recent report by Carlotti *et al.* (20) suggesting that a cytoplasmic dissociation of the NF-κB·IκB complex is followed by nuclear import of the single subunits rather than the complex as a whole. A further support for this notion is the observation that an incubation of cells with the cell permeant NLS-peptide SN50, which inhibits classical nuclear import pathways, was able to delay the nuclear accumulation of p65, but not that of IκBα after addition of LMB (data not shown) indicating that the nuclear accumulation of IκBα is based on a nonclassical nuclear import mechanism. This is in agreement with reports describing an ankyrin repeat-dependent unconventional nuclear import mechanism of IκBα, which cannot be inhibited *in vitro* by an excess of a protein containing a classical NLS (29).

NIK Exhibits Nucleocytoplasmic Shuttling and Contains Functional Nuclear Import and Export Signals—Given the rapid and effective shuttling of NF-κB and IκB between cytosol and nucleus of quiescent cells we aimed to elucidate, whether other components of the NF-κB pathway are subject to a similar dynamic intracellular distribution.

Transfection of HUVEC or HeLa cells with fusion constructs of GFP variants and NIK, TRAF2, IKK1, or IKK2, followed by addition of LMB and imaging of the cells revealed a rapid nuclear accumulation of the NF-κB inducing kinase NIK. In contrast, the other signaling molecules did not show significant accumulation in the nucleus within 90 min (Fig. 5*A*). This indicated nucleocytoplasmic shuttling of the upstream signaling kinase NIK in a time frame similar to NF-κB or IκB. As with YFP-p65 and CFP-IκBα, we performed a more detailed kinetic analysis of the nuclear accumulation of NIK, which revealed a half-time of about 30 min for the nucleocytoplasmic re-distribution (Fig. 5*B*).

p65-NF-κB and IκBα are proteins, for which both cytosolic and nuclear functions are well known and these molecules contain both nuclear localization and nuclear export signals (4, 17, 18), which prime them for nucleocytoplasmic shuttling. However, for the signaling kinase NIK neither the shuttling between cytosol and nucleus has been described, nor domains of the protein that might be responsible for such an intracellular distribution. Thus, we performed a comprehensive computer analysis of the NIK amino acid sequence searching for putative NLS and nuclear export signals. A consensus sequence for a conventional nuclear localization signal was found in the amino-terminal region of NIK comprising seven basic amino acids (143–149, Fig. 6*A*), which appeared even stronger than the SV40 large T antigen consensus sequence. Crm1p-dependent nuclear export domains are not as clearly defined and contain a stretch of hydrophobic residues, predominantly leucine or isoleucine, separated by short spacer domains of 1–4 amino acids (30).

A search for putative nuclear export sequences revealed two potential domains in the COOH-terminal region (amino acids 770–779 and 795–805). To verify whether these domains contribute to the intracellular localization, we generated truncation mutants of NIK comprising region 1–769, 1–794, and 1–805, thus containing the consensus NLS and either none of the putative NES or the first or both export signals (Fig. 6*A*, *bottom*).

All truncated proteins were fused to YFP and their expression in different cell lines was confirmed by Western blot (data not shown). Transient transfection and fluorescence microscopy of these truncated YFP chimeras in HUVEC revealed clearly different steady-state localizations of the fusion proteins (Fig. 6*B*): YFP-NIK$^{1-769}$ was found in the nucleus, supporting the presence of a NLS, but no NES in this mutant (Fig. 6*B*, *second panel*). The localization of the YFP-NIK$^{1-794}$ variant was also restricted to the cell nucleus, indicating that a functional NES may lie further downstream (Fig. 6*B*, *third panel*). In striking contrast to this, YFP-NIK$^{1-805}$ localized entirely to

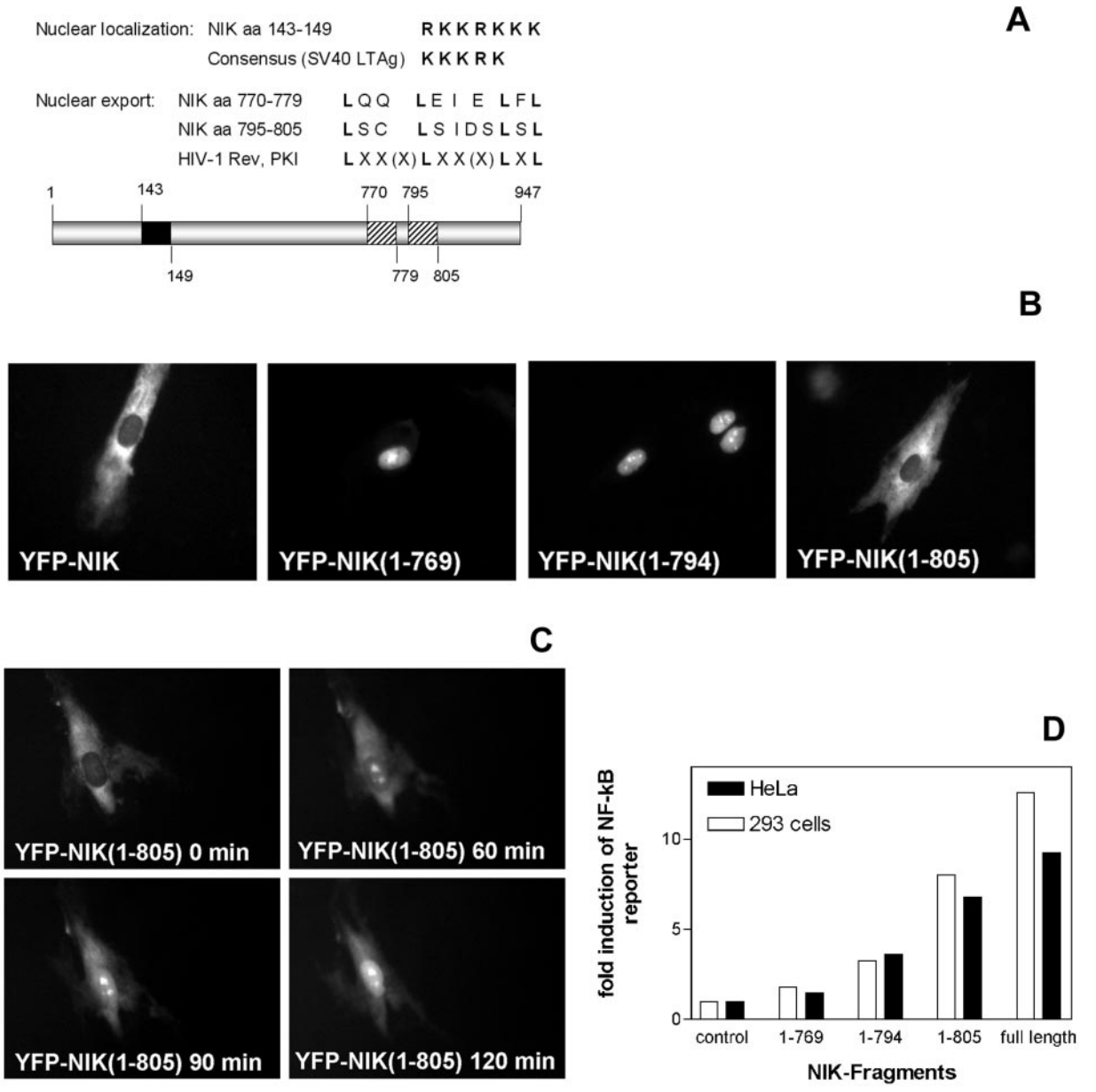

FIG. 6. **Identification of shuttling domains in NF-κB inducing kinase.** *A*, amino acid sequence analysis of NIK. *Top*, comparison of consensus NLS with NIK amino acids (*aa*) 143–149. *Middle*, previously published leucine-rich NES of the HIV-1 Rev and the PKI protein and putative export signals in NIK. *Bottom*, schematic presentation of possible import (*filled box*) and export (*hatched boxes*) signals within the NIK protein. *B*, steady-state localization of YFP-NIK truncation mutants in HUVEC, transfected with the denoted plasmids as described under "Materials and Methods." *C*, nucleocytoplasmic shuttling of NIK^{1-805}. HUVEC were transfected with YFP-NIK^{1-805} and 20 nM LMB applied to the culture medium. Individual cells were observed over time (2 h) and images taken every 30 min. *D*, reporter gene assay of NIK mutants (a representative experiment is shown): HeLa cells or 293 cells were transiently transfected with expression plasmids of the respective NIK-truncation mutants, an NF-κB-dependent luciferase reporter construct and an Rous sarcoma virus-β-galactosidase expression vector as internal control. Luciferase activity was measured in cell lysates prepared 24 h after transfection and normalized to β−galactosidase activity (see "Materials and Methods"). Protein levels of the NIK variants were tested by immunoblotting in a control experiment and were approximately equal for all 4 variants.

the cytoplasm, showing the same distribution as full-length YFP-NIK (Fig. 6*B*, *outer panels*). This demonstrates the presence of a leucine-rich nuclear export signal within amino acids 795–805 of NIK. The question, whether this NES is actually responsible for the Crm1p-dependent nuclear export was further addressed by imaging the intracellular distribution of YFP-NIK^{1-805} after addition of LMB. As with the full-length YFP-NIK, addition of LMB clearly resulted in nuclear accumulation of YFP-NIK^{1-805} (Fig. 6*C*). The export triggered by the NES at position 795–805 obviously exceeds the nuclear import induced by the NLS leading to a predominant cytosolic localization at steady state. Altogether, the NH_2-terminal NLS and the COOH-terminal NES lead to the continuous nucleocytoplasmic shuttling of the NF-κB inducing kinase NIK with rather fast kinetics.

The nucleocytoplasmic shuttling of NIK raises the important question, whether NIK that is localized to the nucleus is able to contribute to the activation of the NF-κB signaling pathway. To test for this, various NIK truncation mutants that show either cytosolic or nuclear localization were co-transfected together with NF-κB-dependent luciferase reporter constructs. Full-length NIK is capable of activating NF-κB when overexpressed (31) and retained this ability when fused to YFP (Figs. 1 and 6*D*). Stepwise truncation of the COOH terminus resulted in

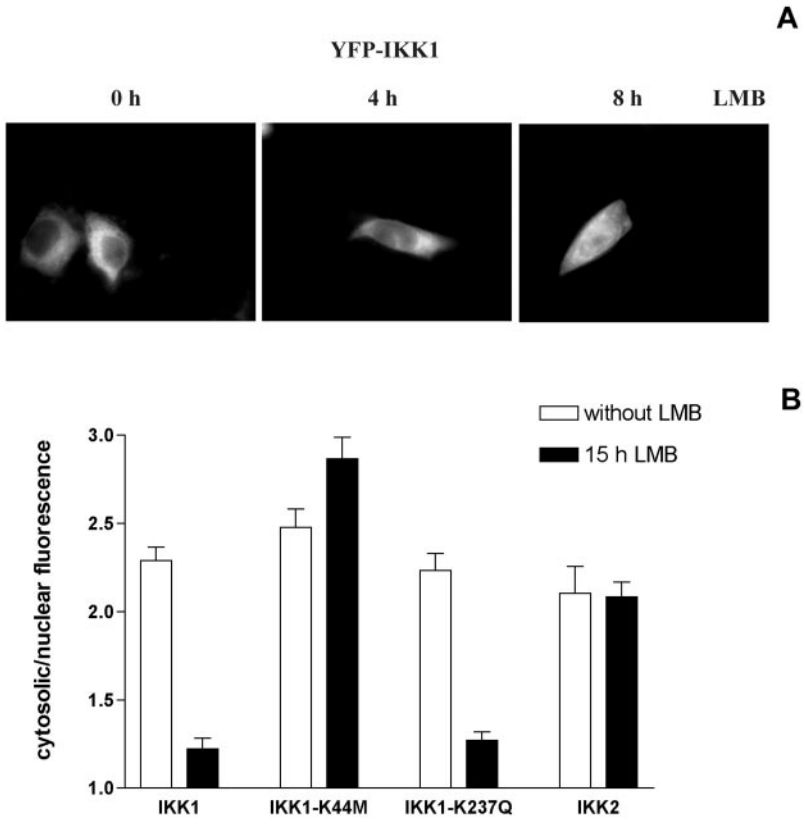

FIG. 7. **Nuclear accumulation of IKK1 after prolonged incubation in the presence of LMB.** *A*, HeLa cells were transfected with YFP-IKK1 and imaged 1 day after transfection before addition of LMB, as well as 4 and 8 h after the addition. *B*, cytosolic and nuclear fluorescence of HeLa cells expressing YFP fusion proteins of IKK1, IKK1(K44M), IKK1 (237Q), or IKK2 was quantified in the absence of LMB and 15 h after addition of LMB (20 nM). 50 cells per sample were quantified (*error bars* represent S.E.M.).

concomitant decrease of NF-κB reporter activity. This is consistent with the presence of interaction domains with downstream IKK effectors in the COOH-terminal region of NIK (32). Interestingly, YFP-NIK$^{1-794}$, which was confined to the nucleus, still retained significant ability to activate NF-κB (Fig. 6*D*). This indicates that nuclear NF-κB·IκB complexes can be activated by NIK. Since NIK has previously been shown to act through the IκB kinase complex and activate IKK1 preferentially over IKK2 (14) this would imply the presence of a nuclear IκB kinase.

Nucleocytoplasmic Shuttling of YFP-IKK1—Neither IKK1 nor IKK2, which would represent potential downstream substrates of NIK, showed a clear nuclear accumulation within about 1 h after addition of LMB (Fig. 5*A*). To test whether IKK1 or IKK2 show at least a certain degree of nucleocytoplasmic shuttling, we performed fluorescence microscopy studies with YFP fusion proteins with longer incubations in the presence of LMB. Interestingly, a noticeable increase of nuclear YFP-IKK1 could be observed about 4 h after addition of LMB indicating slow but significant nucleocytoplasmic shuttling of YFP-IKK1 (Fig. 7*A*). Moreover, in endothelial cells, both YFP-IKK1 and YFP-IKK2 showed some nuclear localization at least in a certain percentage of the cells at steady state, which was much more pronounced for IKK1 (data not shown). Since, the dominant negative variant of IKK1 (IKK1-K44M) appeared always cytosolic, we compared the YFP-K44M mutant with wild type YFP-IKK1 after prolonged incubation in the presence of LMB. Strikingly, the YFP-IKK1-K44M protein did not exhibit any increase in nuclear localization, whereas the wild type protein and another mutant (IKK1-K237Q) showed a significant LMB-dependent nuclear accumulation (Fig. 7*B*). Thus, IKK1 is able to translocate into the nucleus by a nuclear import mechanism that depends on a lysine residue at position 44 that is also essential for the kinase activity of IKK1. Remarkably, YFP-IKK2 did not accumulate in the nucleus under the same conditions, indicating a functional difference between these two related signaling kinases with respect to the intracellular distribution.

Nucleocytoplasmic Shuttling Can Be Verified for Endogenous Proteins—We aimed to investigate whether the nucleocytoplasmic shuttling that we observed for various CFP- or YFP-tagged fusion proteins of signaling molecules in living cells reflects the behavior of endogenous proteins. For that purpose, HeLa cells were incubated in the presence of LMB for different periods of time, followed by immunofluorescence studies and Western blot analysis. Immunofluorescence staining of IκBα in HeLa cells verified the results obtained with the CFP fusion protein. Furthermore, analysis of the kinetics of nuclear accumulation after addition of LMB revealed very similar results. Incubation of HeLa cells in the presence of TNFα for 15 min induced the expected substantial degradation of cytosolic IκBα. However, after LMB-induced nuclear accumulation of IκB, an addition of TNFα could not induce a comparable degradation of the nuclear IκBα, indicating that TNFα-mediated IκB degradation occurs predominantly in the cytosol (Fig. 8). This is in agreement with a recent report describing that nuclear IκBα is at least in part protected from TNFα-induced degradation (33). Nevertheless, some basal proteolytic degradation was recently demonstrated also for nuclear IκBα (34).

In addition to immunofluorescence staining experiments of endogenous signaling molecules, we performed immunoblot analysis of nuclear extracts obtained after different times of incubation in the presence of LMB. Western blots of nuclear p65 and IκBα verified that both proteins accumulate in the nucleus, with IκBα occurring somewhat earlier than p65 NF-κB. This is in line with the fluorescence microscopy data obtained with transient expression of CFP- and YFP-tagged molecules (Fig. 9). Moreover, a LMB-dependent nuclear accumulation could be observed for endogenous IKK1 as well. In this case, the nuclear occurrence as assessed by immunoblot-

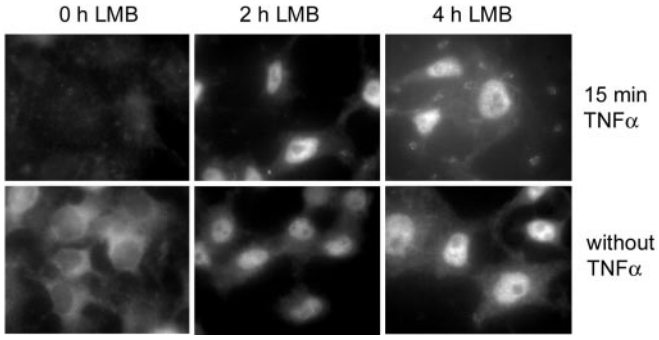

FIG. 8. **Immunofluorescence staining of endogenous IκBα verifies the nucleocytoplasmic shuttling.** HeLa cells were incubated in the presence of LMB (20 nM) for different periods of time. Half of the samples were treated with TNFα (200 units/ml) for 15 min at the end of the LMB incubation, followed by fixation and immunofluorescence staining of endogenous IκBα.

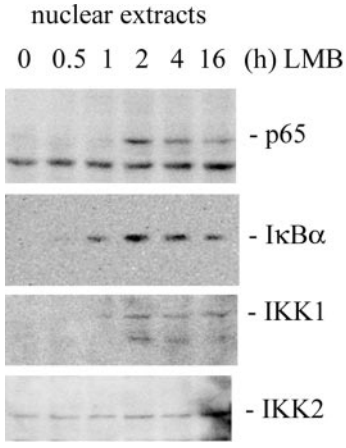

FIG. 9. **Western blot analysis of endogenous nuclear proteins after addition of LMB.** HeLa cells were incubated in the presence of LMB for the indicated periods of time, followed by preparation of nuclear extracts as described under "Materials and Methods." Equal protein amounts were subjected to SDS-PAGE and immunoblot analysis was performed for p65, IκBα, IKK1, and IKK2.

ting of endogenous IKK1 appeared faster than that observed by fluorescence microscopy of YFP-IKK1, which might be due to the YFP-tag. In contrast to IKK1, IKK2 did not show any significant increase in the nucleus upon incubation in the presence of LMB. However, a small, but significant amount of IKK2 was present in the nucleus even before addition of LMB and remained constant.

The observation of a constitutive nucleocytoplasmic shuttling process of NF-κB and IκBα implies that a small amount of the transcription factor is always present in the nucleus together with its inhibitor, as also detected by confocal laser scanning microscopy of YFP- and CFP-tagged fusion proteins. This raises the question, whether this also leads to a small constitutive transcriptional activity of NF-κB. Such a basal activity of NF-κB might be the result of a certain dissociation of nuclear NF-κB·IκBα complexes. In addition, it might be caused by some basal activity of the upstream signaling kinases that are shuttling between cytosol and nucleus as well. We intended to test for a potential constitutive NF-κB activity in the absence of known NF-κB inducers and to assess whether this might be caused by nuclear NF-κB. For this purpose, we performed reporter gene assays using a luciferase reporter construct under the control of a 5-fold repeat of the NF-κB-binding site and examined the effect of IκBα overexpression on this NF-κB reporter in the absence or presence of LMB. Co-expression of IκBα drastically inhibited the basal level of luciferase expression, both in the absence of LMB, and after incubation with LMB for periods of time that are sufficient to induce a nearly complete nuclear localization of the NF-κB·IκBα complex (Fig. 10). Thus nuclear NF-κB·IκBα complexes are the basis of a basal transcriptional activity of NF-κB.

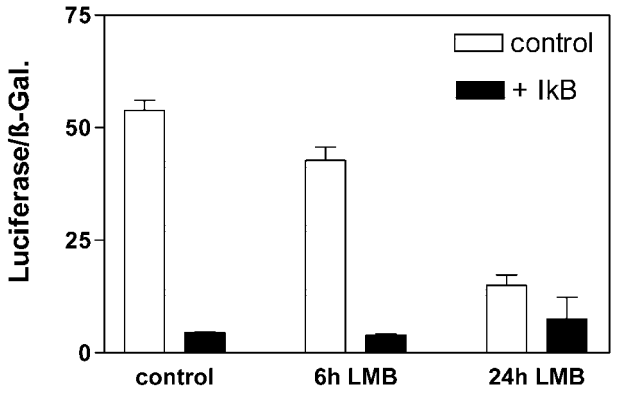

FIG. 10. **Basal activity of NF-κB as assessed by reporter gene assays.** 293 cells were transfected with a luciferase reporter construct containing 5 tandem repeats of an NF-κB-binding site and an NF-κB-independent β-galactosidase vector for normalization. Either an IκBα expression plasmid or a control vector were co-transfected, followed by incubation in the presence or absence of LMB for the indicated time periods. Extracts were prepared and assayed for luciferase and β-galactosidase activity ($n = 3$, *error bars* represent the standard deviation).

DISCUSSION

The results of our study show that NF-κB and IκBα are present in the nucleus at low concentrations even in nonactivated cells. This nuclear fraction of NF-κB and IκBα is most likely caused by a continuous shuttling between cytosol and nucleus, since addition of the nuclear export inhibitor LMB results in the accumulation of both proteins in the nucleus. This is in agreement with recent publications by Johnson *et al.* (18) and Huang *et al.* (17). We aimed at further characterizing this phenomenon by analyzing the kinetics of the shuttling process using time-lapse microscopy at 37 °C. These experiments could demonstrate that the LMB-induced re-distribution is a rapid process. For the initial 60 min after addition of LMB we calculated half-time values of 14 and 7 min for NF-κB and IκBα, respectively. Thus we observed a significant difference in transport kinetics of the two molecules, with IκBα being somewhat faster than NF-κB, which was further verified for endogenous protein levels using Western blot analysis of nuclear extracts. This difference in kinetics is apparently due to different transport mechanisms, as import of IκBα depends on other factors than those of the classical nuclear import machinery (29, 35). Moreover, we found a delay of the LMB-dependent nuclear accumulation of p65 after application of a peptide blocking the classical nuclear import pathway (data not shown), whereas this was not the case for IκBα.

Taken together, these data indicate a dynamic model of NF-κB·IκB complexes (Fig. 11*A*): cytoplasmic dissociation of NF-κB and IκBα is followed by nuclear import of the single subunits rather than the complex as a whole, with IκBα being imported faster. The proteins then re-associate in the nucleus, as shown by FRET microscopy (Fig. 2*D*). A similar model was also suggested by Carlotti *et al.* (20), where dissociation of IκBα from p65 is thought to occur prior to nuclear import.

The dissociation of the NF-κB·IκBα complex in the cytoplasm appears to be the rate-limiting step of the LMB-induced nuclear accumulation of both proteins at equal concentrations, based on the observation that an excess of p65 or IκBα accumulated in the nucleus much faster. This observation can be explained by the fact that both p65 and IκBα contain inherent

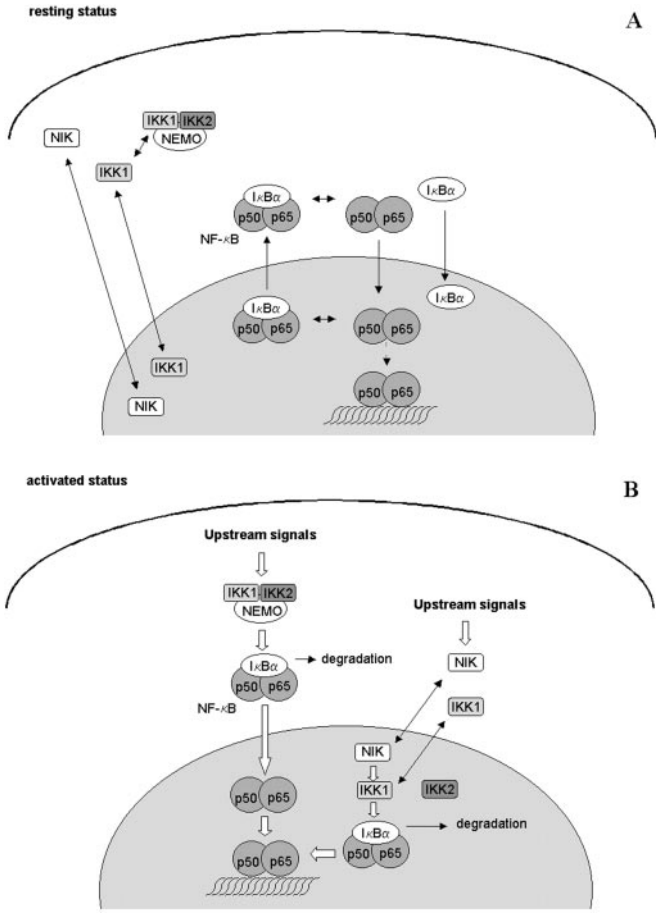

FIG. 11. **Dynamic model of NF-κB activity.** *A*, dissociation of NF-κB·IκBα complexes and nucleocytoplasmic shuttling of the subunits leads to basal transcriptional activity of NF-κB, possibly enforced by the shuttling of upstream signaling kinases. *B*, activation of NF-κB via the signaling kinases NIK and IKK1 may lead to activation of nuclear NF-κB·IκBα complexes.

nuclear import and export signals leading to a dynamic distribution of the single molecules between nucleus and cytosol. Addition of LMB would result in a rapid nuclear accumulation of the unbound protein, which is only dependent on the kinetics and the capacity of the import machinery. In contrast to this, the nuclear import of NF-κB·IκBα complexes would require the dissociation of the complex according to its inherent off-rate in addition. After re-association of NF-κB and IκBα in the nucleus, the complex is then shuttled back to the cytoplasm either mediated by IκBα, as suggested previously (16–18), or possibly by the inherent NES of p65 (19).

In principle, the observed nucleocytoplasmic shuttling of NF-κB and IκBα might simply reflect a leakiness of the localization machinery given that both molecules have cytosolic and nuclear functions and contain both nuclear localization and nuclear export signals. However, it was intriguing to observe that upstream signaling kinases such as IKK1 and NIK, which were thought to have primarily cytosolic roles, exhibit a significant nucleocytoplasmic shuttling, as well. This observation was particularly striking for the NF-κB inducing kinase NIK, having a strong consensus sequence for nuclear localization, which is counteracted by a very efficient nuclear export signal that we mapped to amino acids 795–805.

Although it was reported that NIK is dispensable for TNFα-mediated activation of NF-κB, it was recently also shown to have an essential role in NF-κB activation mediated by lymphotoxin-β (12, 36). Furthermore, it was postulated that NIK has an important role in processing of the NF-κB2 subunit p100 (37). The p100 NF-κB protein contains IκB-like ankyrin repeats that are cleaved by proteasomes after NIK and IKK1-dependent phosphorylation leading to the processed p52 form of NF-κB (37, 38). Thus, the nucleocytoplasmic shuttling of NIK and also IKK1 might have important biological consequences in these signaling processes. Consistent with a potential role of nuclear NIK in NF-κB activation, we found that NIK truncation mutants missing the nuclear export signal and thereby localizing to the nucleus are still able to activate NF-κB-dependent reporter genes. However, this induction of NF-κB transcriptional activity was weaker than with full-length NIK, which might be due to a reduced interaction with downstream IKKs.

NIK has been shown to preferentially activate IKK1, the other kinase found to undergo shuttling (14). In contrast to NIK, which exhibited a rapid and efficient nucleocytoplasmic shuttling similar to that of p65, YFP fusion proteins of IKK1 showed rather slow LMB-dependent nuclear accumulation. In biochemical assays, the LMB-induced occurrence of endogenous IKK1 in the nucleus appeared faster than that of YFP chimera, which might be the result of the YFP moiety. In general, the kinetics of accumulation in the nucleus will also depend on binding to interaction partners that influence nuclear import or export and the dissociation kinetics of these macromolecular interactions. From this point of view, the nucleocytoplasmic shuttling of IKK1 will probably be affected by association with IKK2 and the signalosome. Alternatively, it might also be influenced by binding to NIK, which exhibits an efficient and rapid nucleocytoplasmic shuttling. A computer search for potential nuclear localization signals within IKK1 did not show any significant consensus sequences with classical NLS. However, the striking observation that mutation of the lysine residue at position 44 prevents LMB-dependent nuclear accumulation indicates that this basic residue might be part of a bipartite NLS. Nonetheless, mutation of another lysine residue at position 237, which could belong to the second part of a putative bipartite NLS, did not show any effect. This implies that it is not an essential moiety of the mechanism inducing nuclear import of IKK1. However, it is interesting to notice that the residue Lys^{44} that we defined as being important for nuclear import, is also essential for the kinase activity of IKK1. Thus, binding of IKK1 to its substrates might also influence its nucleocytoplasmic partitioning.

In contrast to classical unipartite nuclear localization signals, nuclear export signals exhibit a much higher degree of variability, being characterized by a stretch of hydrophobic residues with more or less defined spacer regions between. A computer search of the IKK1 amino acid sequence for those hydrophobic domains revealed some potential export signals in the middle and COOH-terminal part of the protein. Taken together, the fact that both NF-κB·IκBα complexes and upstream signaling kinases shuttle between the cytosol and the nucleus by active, energy-dependent processes implies that this might have important biological roles. Moreover, we could demonstrate that the central NF-κB activating kinase IKK2 localizes to the nucleus, as well, although without exhibiting a significant shuttling between nucleus and cytosol. This nuclear portion of IKK2 could have a role in ensuring a basal activity of NF-κB in nonactivated cells and in amplifying signals entering the nucleus by the shuttling kinases. In this context it could be phosphorylated and hence activated by IKK1, as has been suggested previously (39, 40). By that means nuclear signaling kinases upstream of NF-κB might activate nuclear NF-κB·IκBα complexes as illustrated in Fig. 11*B*.

What might be the roles of nuclear NF-κB complexes and upstream kinases? The constant presence of NF-κB in the

nucleus could, for instance, drive transcription of genes essential for certain basic processes, such as the cell cycle, in which NF-κB was recently implicated, *e.g.* as transcription factor driving the expression of cyclin D1 (41, 42). A thermodynamic consideration suggests that even high affinity complexes exhibit a certain degree of dissociation and re-association, which implicates that some NF-κB molecules might be free from its inhibitor in the nucleus and are probably able to bind to promoter regions. Besides dissociation of nuclear NF-κB·IκBα complexes, the presence of upstream signaling kinases in the nucleus could also explain such a basal low activity of NF-κB, given that these kinases are thought to exhibit some basal constitutive enzymatic activity (43, 44). Moreover, other components for IκB degradation, such as the ubiquitinylation machinery and proteasomes, are present in the nucleus too (18, 45). Consistent with this, a basal constitutive activity of NF-κB under nonstimulated conditions was observed in a reporter gene assay (Fig. 10). Such a low but constant activity could possibly be sufficient for induction of certain genes, while being too low for inducing the expression of other genes like those triggering inflammatory responses.

In conclusion, the fact that NF-κB·IκBα complexes shuttle constitutively between cytoplasm and nucleus extends our understanding of the regulation of this important signaling pathway from a rather static view, where IκBα was thought to inhibit the activity of NF-κB by preventing its nuclear import, to a more dynamic view. These new aspects of NF-κB regulation suggest that the primary role of IκBα is to inhibit the binding of p65 to DNA and to shift the predominant steady state distribution to the cytosol. Furthermore, the dynamic distribution of NF-κB·IκBα complexes, as well as upstream signaling kinases implies another level of regulation, which would allow both a constitutive activity of NF-κB and the possibility of further activation by signaling events in the nucleus.

Acknowledgments—We are grateful for the helpful support of Rainer Pepperkok, Jens Rietdorf, and Andreas Girod at the EMBL. We thank Michaela Kind and Sonja Novak for expert technical assistance, Barbara Wolff-Winiski, Novartis Research Institute, Vienna, for providing leptomycin B, and Wilfried Ellmeier as well as our colleagues for critically reading the manuscript. Moreover, we are grateful to many colleagues for providing us with plasmids.

REFERENCES

1. Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455
2. De Martin, R., Hoeth, M., Hofer-Warbinek, R., and Schmid, J. A. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** E83–88
3. de Martin, R., Schmid, J. A., and Hofer-Warbinek, R. (1999) *Mutat. Res.* **437,** 231–243
4. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* **14,** 649–683
5. Mercurio, F., and Manning, A. M. (1999) *Curr. Opin. Cell Biol.* **11,** 226–232
6. Pahl, H. L. (1999) *Oncogene* **18,** 6853–6866
7. Baldwin, A. S. (2001) *J. Clin. Invest.* **107,** 241–246
8. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274,** 784–787
9. Wang, C. Y., Cusack, J. C., Jr., Liu, R., and Baldwin, A. S., Jr. (1999) *Nat. Med.* **5,** 412–417
10. Yamamoto, Y., and Gaynor, R. B. (2001) *J. Clin. Invest.* **107,** 135–142
11. Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995) *Trends Cell Biol.* **5,** 392–399
12. Yin, L., Wu, L., Wesche, H., Arthur, C. D., White, J. M., Goeddel, D. V., and Schreiber, R. D. (2001) *Science* **291,** 2162–2165
13. Yujiri, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsu, Y., Clarke, P., Tyler, K., Oka, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7272–7277
14. Ling, L., Cao, Z., and Goeddel, D. V. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3792–3797
15. de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H., and Bach, F. H. (1993) *EMBO J.* **12,** 2773–2779
16. Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R. T., Virelizier, J. L., and Dargemont, C. (1997) *J. Cell Sci.* **110,** 369–378
17. Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1014–1019
18. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) *EMBO J.* **18,** 6682–6693
19. Harhaj, E. W., and Sun, S. C. (1999) *Mol. Cell. Biol.* **19,** 7088–7095
20. Carlotti, F., Dower, S. K., and Qwarnstrom, E. E. (2000) *J. Biol. Chem.* **275,** 41028–41034
21. Jacobs, M. D., and Harrison, S. C. (1998) *Cell* **95,** 749–758
22. Huxford, T., Huang, D. B., Malek, S., and Ghosh, G. (1998) *Cell* **95,** 759–770
23. Natoli, G., Costanzo, A., Moretti, F., Fulco, M., Balsano, C., and Levrero, M. (1997) *J. Biol. Chem.* **272,** 26079–26082
24. Takeuchi, M., Rothe, M., and Goeddel, D. V. (1996) *J. Biol. Chem.* **271,** 19935–19942
25. Oitzinger, W., Hofer-Warbinek, R., Schmid, J. A., Koshelnick, Y., Binder, B. R., and de Martin, R. (2001) *Blood* **97,** 1611–1617
26. Schmid, J. A., Birbach, A., Hofer-Warbinek, R., Pengg, M., Burner, U., Furtmuller, P. G., Binder, B. R., and de Martin, R. (2000) *J. Biol. Chem.* **275,** 17035–17042
27. Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J. A., Binder, B. R., and Lipp, J. (1998) *J. Exp. Med.* **188,** 211–216
28. Wolff, B., Sanglier, J. J., and Wang, Y. (1997) *Chem. Biol.* **4,** 139–147
29. Sachdev, S., Bagchi, S., Zhang, D. D., Mings, A. C., and Hannink, M. (2000) *Mol. Cell. Biol.* **20,** 1571–1582
30. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) *Cell* **90,** 1051–1060
31. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) *Nature* **385,** 540–544
32. Lin, X., Mu, Y., Cunningham, E. T., Jr., Marcu, K. B., Geleziunas, R., and Greene, W. C. (1998) *Mol. Cell. Biol.* **18,** 5899–5907
33. Rodriguez, M. S., Thompson, J., Hay, R. T., and Dargemont, C. (1999) *J. Biol. Chem.* **274,** 9108–9115
34. Renard, P., Percherancier, Y., Kroll, M., Thomas, D., Virelizier, J. L., Arenzana-Seisdedos, F., and Bachelerie, F. (2000) *J. Biol. Chem.* **275,** 15193–15199
35. Sachdev, S., Hoffmann, A., and Hannink, M. (1998) *Mol. Cell. Biol.* **18,** 2524–2534
36. Matsushima, A., Kaisho, T., Rennert, P. D., Nakano, H., Kurosawa, K., Uchida, D., Takeda, K., Akira, S., and Matsumoto, M. (2001) *J. Exp. Med.* **193,** 631–636
37. Xiao, G., Harhaj, E. W., and Sun, S. C. (2001) *Mol. Cell.* **7,** 401–409
38. Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C., and Karin, M. (2001) *Science* **293,** 1495–1499
39. O'Mahony, A., Lin, X., Geleziunas, R., and Greene, W. C. (2000) *Mol. Cell. Biol.* **20,** 1170–1178
40. Yamamoto, Y., Yin, M. J., and Gaynor, R. B. (2000) *Mol. Cell. Biol.* **20,** 3655–3666
41. Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G., and Baldwin, A. S., Jr. (1999) *Mol. Cell. Biol.* **19,** 5785–5799
42. Hinz, M., Krappmann, D., Eichten, A., Heder, A., Scheidereit, C., and Strauss, M. (1999) *Mol. Cell. Biol.* **19,** 2690–2698
43. Yang, J., and Richmond, A. (2001) *Cancer Res.* **61,** 4901–4909
44. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866
45. Reits, E. A., Benham, A. M., Plougastel, B., Neefjes, J., and Trowsdale, J. (1997) *EMBO J.* **16,** 6087–6094