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 nucleo-cytoplastic 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 variants of NIK, we identified a functional nuclear export signal within the COOH-terminal region 795–805, which counteracts the inherent NLS at amino acids 140–149. 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 divide the Rel family into three groups with different functions. p65 (NF-κB1), RelB, or c-Rel, which is absent in the smaller subunits p50 and p52, and the non-κB members c-Rel, p50, and p52. p65-κB signaling is important in the regulation of gene expression, cell proliferation, and cell survival, and proliferation (1–4). Their common characteristic is the high number of genes involved in inflammation, host defense, and cell differentiation. The NF-κB family has a central role in the inducible expression of a large number of genes. It is involved in the transcriptional activation of genes that are involved in inflammatory and immune responses, as well as in the regulation of cell proliferation and apoptosis.

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 a 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 NH2-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
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 IkB 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 IkB kinases phosphorylate IkB family members at conserved serine residues in the NH2-terminal signal-responsive domain. This signal-induced phosphorylation results in ubiquitylation 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 IkB proteins. IkB 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 IkB itself, located within the second ankyrin repeat, appears to be blocked when IkB 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 involved in the transcription of DNA encoding its own inhibitor (15). After translation in the cytosol, IkB is imported into the nucleus, where it is assumed to dissociate NF-κB from promoter regions. The newly formed NF-κB-IkB complex is then transported back to the cytosol by the means of a specific nuclear export sequence (NES) of IkB. Whereas a putative NES was originally postulated for the COOH terminus of IkB (16), it was recently shown that a NH2-terminal NES instead of the postulated COOH-terminal region is responsible for Crm1p-dependent nuclear export of IkB 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 IkB-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 IkB 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 IkB is apparently not able to prevent the nuclear import of NF-κB. This observation implies that the principal inhibitory role of IkBα 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 IkB has further important consequences. Since all macromolecular complexes have a certain equilibrium between dissociation and association, the existence of nuclear NF-κB-IkB complexes should also lead to the occurrence of a certain number of NF-κB-IkB molecules that are not bound to the inhibitor and therefore free for binding to promoter elements. The dynamic equilibrium between IkB-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 IkB 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 IkB dissociate from each other in the cytosol, followed by separate nuclear import, rather than a nuclear translocation of the whole NF-κB-IkB complexes as suggested based on the x-ray crystallographic data (21, 22).

The fact that both NF-κB and IkB 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 NH2-terminal region of NIK and a newly defined functional NES in the COOH-terminal domain. Moreover, the IkB 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-IkB 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 NheI and XbaI restriction sites, respectively, into pEYFP-C1 (CLONTECH). YFP-tagged NIK truncation mutants (pEYFP-NIK1–297, pEYFP-NIK1–794, pEYFP-NIK1–880) were generated by amplification of the corresponding cDNA by PCR using the sense-primer 5’-AAAAAATGCCCATAGGCAGTGGATGAGGAATGT-3’ and the following antisense primers: 5’-AAAGTCGACTGGCCGCTCCGGGAG-3’ (NIK1–297), 5’-AAAGTCGACACTTGTCGTCCTCCT-3’ (NIK1–794) and 5’-AAAGTCGACAGGGAGAGGTTGCTCGA-3’ (NIK1–880), respectively. The PCR products were cloned into the EcoRI and SalI sites of pEYFP-C1. For pEYFP-IKK1 and pEYFP-IKK1K44M expression plasmids, the corresponding cDNAs were amplified from vectors IKKI-HA and IKKIK44M-HA, which were a generous gift from M. Karin, using primers 5’-AAAAATGCCCATAGGCAGTGGATGAGGAATGT-3’ and the sense primer-5’-AAAGTCGACACTTGTCGTCCTCCT-3’, respectively, and cloned into the EcoRI and SalI sites of pEYFP-C1. pECFP-TRAF2 was generated by subcloning TRAF2 cDNA from pRK-F-TRAF2 (24) using BamHI and HindIII restriction sites into the BglII and HindIII restriction sites of pEYFP-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 XbaI and StuI restriction sites and cloned into XhoI and SalI sites of pEGFP-N1 (CLONTECH). Subsequently, pIkK2-EGFP was generated by replacing EGFP with YFP, which was excised out of pEYFP-C1 using AgeI and XbaI restriction sites.

NF-κB (p65) and IkBα 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 cm2) 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 cm2 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 IkBα-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 GFP constructs or both and
investigated 1 day after transfection on a Nikon Diaphot TMD microscope using XF104 and XF114 filter sets (Omega Optics 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 Wolf-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% CO2 between single time points. Alternatively, cells were imaged at 37 °C in a CO2-independent medium on a videomicroscope equipped with an inverted Leica microscope including computerized filter wheels and OpenLabTM Software (Improvement 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 the 485-nm emission 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 × 10⁶ cells per sample were washed with phosphate-buffered saline, scraped, and collected by centrifugation at 1,000 × 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 diithiothreitol, including the Complete™ protease inhibitor mixture, Roche Molecular Biochemicals), incubated on ice for 3 min and centrifuged at 1,500 × 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 suspension in 70 μl of NE buffer (20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 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 × 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 IκBa (Clone-C21), p65-NF-κB, IKK1, and IKK2. All antibodies were purchased from Santa Cruz.

RESULTS

NF-κB and IκBa 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, IκBa, 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 exist did not depend on protein synthesis, as it could not be influenced by addition of cycloheximide and it was also observed in stable 293 transfectants expressing both CFP-IκBa and YFP-p65 (data not shown). Strikingly, the nuclear accumulation of IκBa 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 IκBa 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 IκBa 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 YFP-p65 and about 8.0% of co-transfected IκBa are localized to the nucleus at steady state (Fig. 2C). Analysis of the molecular association of NF-κB and IκBa by FRET microscopy (as described in Ref. 26), showed that p65 and IκBa bind to each other not only in the cytosol, but also after LMB-induced nuclear accumulation within the nucleus (Fig. 2D).

To calculate the kinetics of the nuclear accumulation of p65 and IκBa, we performed time lapse morphological studies of
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. 4A). 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, whereas p65 remained mainly cytosolic for much longer times after addition of LMB (Fig. 4B).

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.
This model is in agreement with a recent report by Carlotti et al. (20) suggesting that a cytoplasmic dissociation of the NF-kB4IxB 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 IxBz after addition of LMB (data not shown) indicating that the nuclear accumulation of IxBz is based on a nonclassical nuclear import mechanism. This is in agreement with reports describing an ankyrin repeat-dependent unconventional nuclear import mechanism of IxBz, 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-kB and IxBz between cytosol and nucleus of quiescent cells we aimed to elucidate, whether other components of the NF-kB 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-kB inducing kinase NIK. In contrast, the other signaling molecules did not show significant accumulation in the nucleus within 90 min (Fig. 5A). This indicated nucleocytoplasmic shuttling of the upstream signaling kinase NIK in a time frame similar to NF-kB or IxBz. As with YFP-p65 and CFP-IxBz, 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. 5B).

p65-NF-kB and IxBz 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. 6A), 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. 6B).

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. 6B): YFP-NIK1–769 was found in the nucleus, supporting the presence of a NLS, but no NES in this mutant (Fig. 6B, second panel). The localization of the YFP-NIK1–794 variant was also restricted to the cell nucleus, indicating that a functional NES may lie further downstream (Fig. 6B, third panel). In striking contrast to this, YFP-NIK1–805 localized entirely to
the cytoplasm, showing the same distribution as full-length YFP-NIK (Fig. 6B, 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-NIK1–805 after addition of LMB. As with the full-length YFP-NIK, addition of LMB clearly resulted in nuclear accumulation of YFP-NIK1–805 (Fig. 6C). 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 NH2-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 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.

![Figure 6](image_url)
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<sup>1–794</sup>, which was confined to the nucleus, still retained significant ability to activate NF-κB (Fig. 6D). 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 IKK 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. 5A). 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. 7A). 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 an increase in nuclear localization, whereas the wild type protein and another mutant (IKK1-K237Q) showed a significant LMB-dependent nuclear accumulation (Fig. 7B). 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 TNFa for 15 min induced the expected substantial degradation of cytosolic IκBα. However, after LMB-induced nuclear accumulation of IκB, an addition of TNFa could not induce a comparable degradation of the nuclear IκBα, indicating that TNFa-mediated IκBα 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 TNFa-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-
protein amounts were subjected to SDS-PAGE and immunoblot analy-

sis of endogenous IkBα verified 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 IkBα.

FIG. 8. Immunofluorescence staining of endogenous IkBα 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 IkBα.

nuclear extracts

0 0.5 1 2 4 16 (h) LMB

- p65

- IkBα

- IKK1

- IKK2

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, IkBα, IKK1, and IKK2.

ning 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 IkBα 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-IKK1 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/IkBα 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 IkBα overexpression on this NF-κB reporter in the absence or presence of LMB. Co-expression of IkBα drastically inhibited the basal level of luciferase expres-

sion, 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/IkBα complex (Fig. 10). Thus nuclear NF-κB/IkB 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 IkBα 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 IkBα are present in the nucleus at low concentrations even in nonacti-

vated cells. This nuclear fraction of NF-κB and IkBα 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 IkBα, respectively. Thus we observed a significant difference in transport kinetics of the two molecules, with IkBα 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 IkBα 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 machinery (data not shown), whereas this was not the case for IkBα.

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

The dissociation of the NF-κB/IkBα 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 IkBα accumulated in the nucleus much faster. This observation can be explained by the fact that both p65 and IkBα contain inherent
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/IκB complexes would require the dissociation of the complex according to its inherent off-rate in addition. After re-association of NF-κB and IκB 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 shuttling between nucleus and cytosol. This observation demonstrates that the central NF-κB activating kinase IKK1 localizes to the nucleus are still able to activate NF-κB complexes. 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. Nonetheless, mutation of another lysine residue at position 237, which could belong to the second part of a bipartite NLS, did not show any significant consensus sequences with classical NLS. However, the striking observation that mutation of the lysine residue 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 Lys44 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 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 Lys44 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.

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

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 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 Lys44 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 for potential nuclear localization signals within IKK1 amino acid sequence for those hydrophobic domains revealed some potential export signals, as suggested previously (39, 40). By that means nuclear signaling molecules upstream of NF-κB might activate nuclear NF-κB IκBα complexes as illustrated in Fig. 11B.

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/IκB 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/IκB complexes shuttle constitutively between cytoplasm and nucleus extends our understanding of the regulation of this important signaling pathway from a rather static view, where IκB 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 IκB 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/IκB 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.

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