

Cytosolic, nuclear and nucleolar localization signals determine subcellular distribution and activity of the NF- κ B inducing kinase NIK

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Summary

It has been shown previously that the transcription factor NF- κ B and its inhibitor I κ B α shuttle constitutively between cytosol and nucleus. Moreover, we have recently demonstrated nucleocytoplasmic shuttling of the NF- κ B-inducing kinase NIK, a component of the NF- κ B pathway, which is essential for lymph node development and B-cell function. Here we show that nuclear NIK also occurs in nucleoli and that this localization is mediated by a stretch of basic amino acids in the N-terminal part of the protein (R¹⁴³-K-K-R-K-K-K¹⁴⁹). This motif is necessary and sufficient for nucleolar localization of NIK, as judged by nuclear localization of mutant versions of the full-length protein and the fact that coupling of these seven amino acids to GFP also leads to accumulation in nucleoli. Using fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP)

approaches, we demonstrate a dynamic distribution between nucleoli and nucleoplasm and a high mobility of NIK in both compartments. Together with the nuclear export signal in the C-terminal portion of NIK that we have also characterized in detail, the nuclear/nucleolar targeting signals of NIK mediate dynamic circulation of the protein between the cytoplasmic, nucleoplasmic and nucleolar compartments. We demonstrate that nuclear NIK is capable of activating NF- κ B and that this effect is diminished by nucleolar localization. Thus, subcellular distribution of NIK to different compartments might be a means of regulating the function of this kinase.

Key words: NF- κ B inducing kinase, NF- κ B pathway, Nucleolus, FRAP, FLIP

Introduction

Transcriptional activators of the nuclear factor- κ B (NF- κ B)/Rel family have a central role in the inducible expression of a large number of genes involved in inflammation, host defense, cell survival and proliferation (de Martin et al., 2000; de Martin et al., 1999; Ghosh and Karin, 2002). NF- κ B can be activated by many distinct stimuli, including pro-inflammatory cytokines such as TNF α or IL-1, but also physical stress such as UV- or γ -irradiation, reactive oxygen intermediates and virus-derived dsDNAs or RNAs. The activating signals are transduced by signaling cascades that converge on the I κ B kinases (IKKs) that phosphorylate the NF- κ B inhibitory molecules, including members of the I κ B family and inhibitory domains of NF- κ B precursors, leading to their ubiquitination and subsequent degradation by the proteasome. NF- κ B is then freed and its steady state localization is shifted to the nucleus where it binds to specific sequences on chromosomal DNA. Upstream of the IKKs, several proteins have been identified that are thought to convey signals from receptor complexes. Amongst these are several members of the MAP 3kinase (MAP3K) family of kinases, including MEKK1 and the NF- κ B-inducing kinase, NIK. Originally NIK was identified as a binding partner for the tumor necrosis factor (TNF) receptor associated factor 2 (TRAF2) and therefore thought to be the

kinase transducing signals from this central inflammatory receptor (Malinin et al., 1997). However, genetic data from mutant mice showed NIK to be involved in alternative NF- κ B signaling pathways: naturally occurring mutant mice with the alymphoplasia (*aly/aly*) mutation, which lack lymph nodes and Peyer's patches, were shown to contain a point mutation in the C-terminal part of NIK (Shinkura et al., 1999). This mutation (G855R) is thought to disrupt interaction of NIK with an upstream member of the TRAF family. A complete disruption of the NIK gene (*Nik*^{-/-}) leads to the same phenotype, which is reminiscent of lymphotoxin β receptor knockout mice [*Lt β r*^{-/-} (Futterer et al., 1998)]. Indeed, cells from *Nik*^{-/-} mice showed normal response to TNF α or IL1 treatment, while NF- κ B activation in reaction to LT β was impaired (Yin et al., 2001). Since *aly/aly* mice, show a cell-autonomous defect in B-cell function in addition to the LT β R^{-/-} phenotype (Karrer et al., 2000; Yamada et al., 2000), at least one other ligand/receptor pair is involved in NIK signaling and was identified to be the TNF receptor family member CD40 (Coope et al., 2002). Moreover, similar defects as in *aly/aly* and *Nik*^{-/-} mutants have been observed in *Nf- κ b2*^{-/-} mice. This member of the NF- κ B family of transcriptional activators is generated by proteolytical processing of the p100 precursor by the proteasome, giving rise

to the mature NF- κ B/p52 protein. Indeed, introduction of the *aly* mutation into NIK blocks its ability to promote p100 processing in transfected cells (Xiao et al., 2001), an effect which seems to be mediated by the IKK1 subunit of the IKK complex. IKK1 is the IKK subunit that is preferentially phosphorylated by NIK (Ling et al., 1998) and triggers proteasome-mediated processing of p100 to p52, which can then undergo nuclear translocation (Senfleben et al., 2001). Thus, NIK, IKK1 and p100/p52 seem to be components of a NF- κ B activation pathway which relies on regulated NF- κ B2 processing rather than I κ B degradation and is necessary for secondary lymphoid organ development and B cell maturation.

Recently, components of the NF- κ B signaling pathway, in particular NF- κ B (p65/RelA) itself, I κ B α , NIK and IKK1 have been shown to undergo nucleocytoplasmic shuttling in non-activated cells (Birbach et al., 2002; Huang et al., 2000; Johnson et al., 1999; Tam et al., 2000). Thus, these signaling molecules that cycle between the cytosol and the nucleus may provide a means to ensure basal transcriptional activity under resting conditions, but could also facilitate initiation of the activation cascade upon stimulation inside the nucleus.

The nucleus is now known to be a highly dynamic organelle containing distinct subcompartments (Carmo-Fonseca, 2002; Dundr and Misteli, 2001). Although these substructures are not defined by membranes, they have to be regarded as distinct compartments as they can be morphologically identified by light and electron microscopy, or they can be biochemically isolated and enriched, and contain a defining subset of resident proteins. The nucleolus is probably the best characterized of these nuclear subcompartments: it forms around the nucleolar organizer regions that contain the genes coding for ribosomal DNA. Its main function is the complex process of ribosome biogenesis, yet there is increasing evidence that it is also involved in other processes such as maturation of some tRNAs, assembly of ribonucleoprotein machines and regulatory functions by nucleolar retention of proteins [reviewed in (Dundr and Misteli, 2001)]. Localization of proteins to the nucleolus can be viewed in contrast to nuclear targeting, which involves transport across the nuclear pore complex. In case of a subnuclear structure such as the nucleolus, proteins would probably accumulate in a steady-state compartment by interaction with local high-affinity binding sites (Misteli, 2001). Thus, domains necessary for proper localization inside the nucleolus may be seen as retention signals rather than as classical transport signals.

In this study we have examined nucleocytoplasmic shuttling of the NF- κ B-inducing kinase NIK. We find that inhibition of nuclear export leads to accumulation of NIK in nuclei and nucleoli. Partial mutation of a putative nuclear localization signal (NLS) in the N-terminal part of the protein results in exclusion of NIK from the nucleoli, yet the protein can still enter the nucleus. However, a complete mutation of the putative NLS confirms its identity as a nuclear targeting signal and shows that the motifs for nuclear and nucleolar localization of NIK are directly linked. This amino acid stretch is necessary and sufficient for nucleolar targeting and together with the C-terminal nuclear export signal (NES), which we define by further point mutation analysis, is responsible for the dynamic exchange of NIK between subcellular compartments. Furthermore, we demonstrate rapid kinetics of this shuttling process by fluorescence loss in photobleaching (FLIP)

techniques and the dynamics of the protein in all three compartments by fluorescence recovery after photobleaching (FRAP) approaches.

Materials and Methods

Plasmids

Constructs used in this study were based on the YFP-NIK or YFP-NIK(1-794) and YFP-NIK(1-769) plasmids described previously (Birbach et al., 2002). Point mutations were introduced using the QuikChange kit (Stratagene) and the following sense primers: 5'-GGA AAG CGT CGC AGC AAA GCC GCT GCA GCT GCA AAG AAG AAG AGC TCA AAG TCC C-3' (143-6A), 5'-GGA AAG CGT CGC AGC AAA GCC GCT GCA GCT GCA GCT GCA GCT AGC TCA AAG TCC C-3' (143-9A), 5'-CTC TCG TGC CTC AGC ATC GAC AGC GCT TCC GCT TCG GAT GAC AGT GAG-3' (L803A_L805A), 5'-CTC TCG TGC CTC AGC GCT GAC AGC GCT TCC GCT TCG GAT GAC AGT GAG-3' (I800A_L803A_L805A), 5'-GAG CAG GAG CAA ATT GCC TCG TGC GCA AGC GCT GAC AGC GCT-3' (NESmut, based on prior mutation of 1800, L803, L805). Antisense primers had respective reverse complementary sequences. C- and N-terminal fusions of the nucleolar targeting signal to GFP, were cloned by inserting a double stranded oligonucleotide coding for the amino acids R-K-K-R-K-K-K into *Bgl*III and *Hind*III sites of the vectors pEGFP-C1 and pEGFP-N1, respectively (NIK-NLS-for: AGA TCT AGA ATG AGA AAG AAG CGA AAA AAG A, containing *Bgl*III, *Xba*I sites and a start codon and NIK-NLS-rev: AAG CTT CTT TTT TCG CTT CTT TCT CAT TCT A containing a *Hind*III site).

CFP-L23 was constructed by replacing the GFP-tag of a GFP-L23 construct obtained from Nabel Yaseen, group G. Blobel (Yaseen and Blobel, 1997) with CFP from the vector pECFP-C1 (Clontech).

Cell culture and transfections

Cell culture of 293 and HeLa cells was described previously (Schmid et al., 2000) as was culturing of endothelial cells (Stehlik et al., 1998) and transient transfections with Lipofectamine-Plus™ (Invitrogen) (Birbach et al., 2002). The breast cancer cell line MB-MDA-231 was cultured in RPMI supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C in a humidified, 5% CO₂ atmosphere. Transfections with calcium-DNA precipitates were done essentially according to a well established protocol (Webster and Perkins, 1999) with some minor modifications. In brief, cells were seeded at a density of about 40 000 per cm² one day before transfection. DNA (2-4 μ g/10 cm²) was diluted in a total of 62 μ l distilled sterile water, 9 μ l of sterile 2 M calcium chloride solution were added and the solution was gently mixed. 71 μ l of sterile Hepes-buffered saline solution (280 mM NaCl, 1.5 mM Na₂HPO₄·7H₂O, 50 mM Hepes, titrated exactly to pH 7.05) was added dropwise with gentle mixing in between and incubated for 2 minutes to allow formation of the DNA-calcium precipitate. The precipitate was then added dropwise to the cells spreading the whole surface of the cell culture. The volumes are given for 10 cm² of cell culture and were adjusted accordingly for smaller surface areas. For live cell microscopy, cells were seeded on 15 mm round glass coverslips before transfection. In comparison to Lipofectamine-Plus™, transfections with calcium-DNA precipitates often resulted in more moderate, physiological expression levels.

Fluorescence microscopy, fluorescence recovery after photobleaching and fluorescence loss in photobleaching

Immunofluorescence staining of endogenous NIK was done with paraformaldehyde-fixed cells that were permeabilized with 0.5% Triton X-100 in PBS using anti-NIK antibodies (clone H-248 from

Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and counterstaining with DAPI to stain DNA.

Conventional epifluorescence microscopy with a Nikon Diaphot inverted microscope equipped with a CCD camera and digital image analysis was as described previously (Birbach et al., 2002). Confocal laser scanning microscopy was performed with Zeiss LSM510 equipment. CFP and YFP images were captured sequentially in the multitrack mode using the 458 nm and the 514 nm line of the Argon laser for CFP and YFP, respectively, and a 458/514 nm beamsplitter. CFP was detected with a bandpass 475-525 nm filter and YFP with a longpass 530 nm filter.

FRAP analysis was carried out with living cells grown on round coverslips. Cells were transfected with various YFP fusion proteins and mounted on an incubation chamber filled with medium 1 day after transfection. Cells were examined with a 100 \times oil immersion objective on a Zeiss LSM510 confocal microscope and circular regions of interest were specified for bleaching and scanning (bleach regions). Time series were taken with one scan before bleaching, about 70 iterations of bleaching with 100% laser power of the 514 nm line followed by 50-100 scans of the bleach region (with about 4% of laser power). To obtain a good time resolution just the bleach region was scanned instead of the whole cell and an averaging of two or four scans was applied to reduce the electronic noise and improve the quantification. An image of the whole cell was taken before and after the FRAP time series in order to calculate the loss in total fluorescence of the compartment investigated (which was in the range of 5-10% for cytosolic or nuclear proteins, but sometimes higher than 50% for nucleolar proteins). This overall loss in fluorescence was taken into consideration when calculating the actual fluorescence recovery and correct plateau values defining the fraction of mobile versus immobile molecules (Reits and Neeffjes, 2001; White and Stelzer, 1999). Mean fluorescence intensities of the bleach region over time were measured using ScionImageTM 4.0.2 (the Microsoft Windows equivalent to NIH Image) and the values were exported to Microsoft ExcelTM to calculate the FRAP curves (with correction for total fluorescence loss). These corrected values were copied to GraphPad PrismTM 3.02 for non-linear regression analysis using the single exponential association algorithm: $y = span \times (1 - e^{-kx}) + bottom$ - with 'bottom' being the relative fluorescence intensity immediately after the bleaching (before recovery), 'span' representing the extent of fluorescence recovery and k defining the rate of recovery. Data were weighted by $1/y^2$ to minimize the relative distances squared (which improves the curve fitting for the initial phase of the fluorescence recovery). The half-life of fluorescence recovery $t_{1/2}$ is defined by $0.69/k$ and the plateau, which represents the percentage of mobile molecules is given by $span + bottom$. For a valid comparison of different FRAP experiments, the calculated half-lives of fluorescence recovery were normalized to the bleached area.

FLIP (fluorescence loss in photobleaching) was applied to determine whether YFP-fusion proteins of NIK variants distribute dynamically between two different compartments, such as nucleus and cytosol or nucleoli and nucleoplasm. For this purpose, a rather large bleaching area was specified for one compartment (e.g. the cytosol) - an image was taken of the whole cell before the bleaching, followed by bleaching with full laser power at 514 nm for about 30 seconds and capturing of another image. This sequence was repeated for about 10 minutes. A loss in nuclear fluorescence upon bleaching of a region in the cytosol indicates export of fluorescent proteins from the nucleus or shuttling between nucleus and cytosol. Similarly, a loss in nucleolar fluorescence upon bleaching of a nucleoplasmic region indicates a dynamic distribution between nucleoli and nucleoplasm.

Reporter gene assay

293 cells were transfected in 12-well plates by the calcium DNA precipitation method as described above. Different variants of NIK (0.5 μ g each) were co-transfected with a luciferase reporter construct

containing 5 NF- κ B binding sites (0.5 μ g) and an NF- κ B independent β -galactosidase construct (0.5 μ g) under the control of a constitutive ubiquitin-promoter for normalization. Cells were lysed with the reporter lysis buffer of a Luciferase Assay SystemTM (Promega) and luciferase activity was measured according to the manufacturer's protocol using a Berthold single tube luminometer.

β -Galactosidase activity for normalization was measured with chlorophenol red- β -D-galactopyranoside as substrate and photometric detection at 570 nm using a microtiter plate reader.

Results

Nuclear localization of NIK and accumulation in nucleoli

In a previous report we demonstrated that NF- κ B inducing kinase, NIK, and one of its major substrates, I κ B kinase 1 (IKK1), continuously shuttle between the cytosol and the nucleus. This was demonstrated with YFP-tagged fusion proteins which acted very similarly or identically to the wild-type proteins in NF- κ B reporter gene assays indicating functional integrity of YFP-NIK (Birbach et al., 2002).

While NIK is shuttling between cytosol and nucleus, its steady state localization is in many cell types in the cytosol. However, investigating certain breast cancer cells we found that endogenous NIK localizes primarily to the nucleus emphasizing that nuclear NIK might have important functional roles (Fig. 1A).

To further investigate nuclear NIK and the regulation and function of the nucleocytoplasmic shuttling, we aimed to characterize the nuclear localization sequence and the role of nuclear NIK in NF- κ B activation in more detail. Interestingly, blockade of nuclear export with leptomycin B promoted not only nuclear localization of YFP-NIK, but also an accumulation in large subcompartments within the nucleus. This was also observed for truncation mutants lacking the C-terminal nuclear export signal that we identified previously (Birbach et al., 2002) (Fig. 1B). These subnuclear structures were thought to be nucleoli, from their appearance in light microscopy, size and number. To confirm this assumption, we carried out cotransfections of YFP-NIK plasmids with a CFP-L23 construct, because L23 is a ribosomal protein that localizes to nucleoli upon overexpression (Gleizes et al., 2001). Fluorescence microscopy using distinct filter sets for CFP and YFP showed clear colocalization of YFP-NIK truncations and CFP-L23 (Fig. 1C). Thus, YFP-NIK lacking its nuclear export sequence or accumulating in the nucleus after blockade of nuclear export by leptomycin B is concentrated in nucleoli, with another fraction being present in the nucleoplasm. Interestingly, nucleolar localization is sometimes also observed for endogenous NIK in the breast cancer cells that exhibited a predominant nuclear localization (Fig. 1D).

The putative N-terminal NLS contains a nucleolar retention signal

We had previously reported the identification of a classical nuclear localization signal in NIK based on sequence analysis (Birbach et al., 2002). To demonstrate the functionality of the NLS motif, we introduced point mutations into this stretch of basic amino acids. First, we mutated the initial four basic residues, which most closely resembled the classical nuclear localization sequence of SV40 large T antigen to neutral

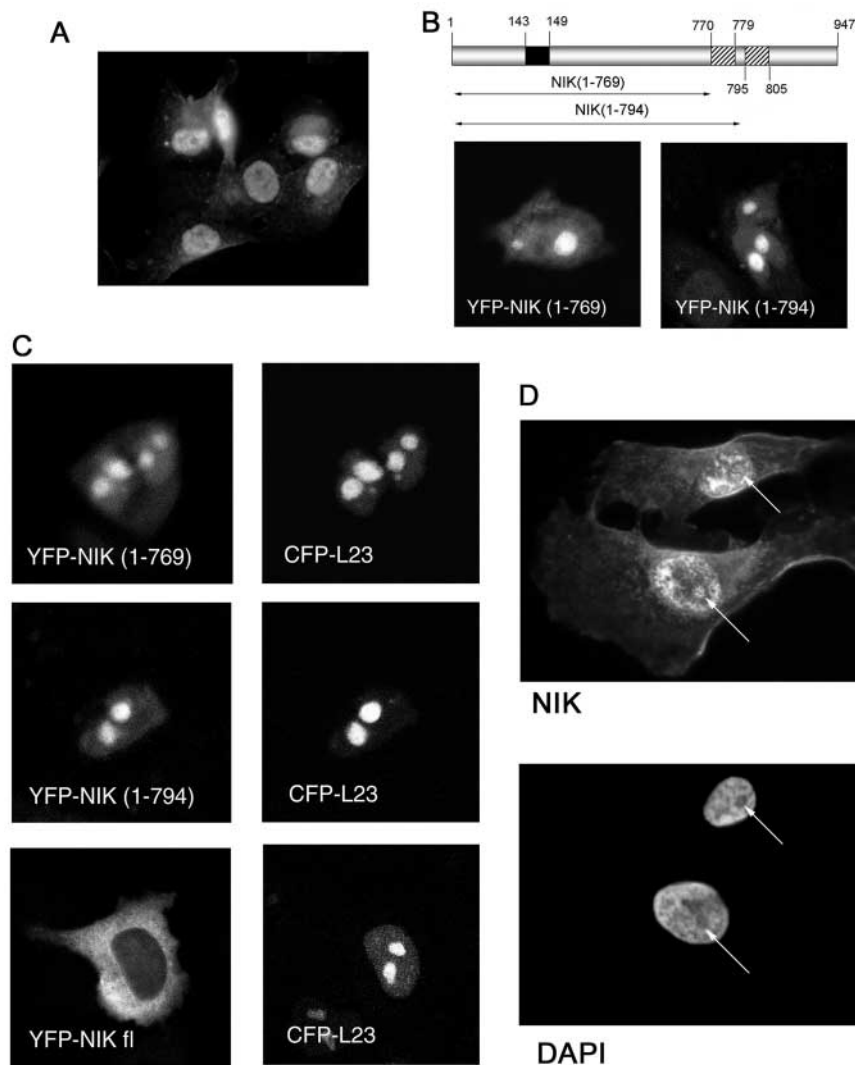


Fig. 1. Nuclear and nucleolar localization of NIK. (A) Immunofluorescent staining of endogenous NIK in the breast cancer cell line MB-MDA-231 indicates predominant nuclear localization of NIK in these cells. (B) Top: schematic drawing of NIK showing the predicted nuclear localization sequence (amino acids 143-149, black box) and two potential nuclear export sequences, as predicted by computer search (hatched boxes). Bottom: truncation mutants of YFP-NIK lacking either the first or both of the putative nuclear export sequences accumulate in the nucleus and nucleolar-like structures as assessed by confocal laser scanning microscopy. 293 cells were transiently transfected with the fusion constructs using the calcium phosphate method and moderately expressing cells were assessed by confocal laser scanning microscopy. (C) Verification of the nucleolar localization of YFP-NIK truncation mutants. 293 cells were transfected as described before using YFP-NIK(1-769), YFP-NIK(1-794) or full length YFP-NIK together with CFP-L23, a ribosomal protein accumulating in nucleoli. (D) Immunofluorescent staining of endogenous NIK in the breast cancer cell line MB-MDA-231 and counterstaining with DAPI. Nucleolar structures are indicated by arrows.

alanines. Unexpectedly, microscopy of YFP-fusion proteins of NIK 1-794 truncations carrying this mutation (denoted 143-6A), still showed the protein to be almost exclusively present in the nucleus. However, staining was restricted to the nucleoplasm, with nucleoli devoid of fluorescence (Fig. 2A). This shows that the basic stretch between amino acids 143 and 146 of NIK is essential for nucleolar localization of the protein.

We then wanted to know whether the identified motif of seven basic amino acids in NIK is sufficient to target any protein to nuclei and nucleoli. We therefore fused the sequence R-K-K-R-K-K-K to GFP, either at the N terminus or the C terminus. As shown in Fig. 2B, both of these fusion proteins accumulated in nucleoli, with the C-terminal fusion being somewhat more effective. GFP molecules containing the basic stretch fused to the N terminus, although also localizing to nucleoli, had a slightly higher fluorescence in the cytosol than in the nucleoplasm, indicating that the N-terminal motif is a weaker signal for the nuclear import machinery. The nucleolar accumulation of these GFP-mutants is in striking contrast to GFP alone, which is always diffusely spread throughout the cell (data not shown). Thus, the nuclear/nucleolar retention signal that we have identified in NIK is sufficient to target a

protein without an inherent localization sequence such as GFP to nucleoli.

The leucine-rich C-terminal NES at position 795-805 counteracts nuclear localization by the N-terminal NLS

We have previously demonstrated the presence of a leucine-rich nuclear export signal between amino acids 795 and 805 in NIK using truncation mutants of the kinase (Birbach et al., 2002). Another putative NES is present between amino acids 770 and 779 and may theoretically further contribute to the nuclear export of the kinase. Truncation mutants of NIK containing the first putative NES, but not the second localize predominantly to the nucleus indicating that this sequence is not strong enough to counteract the NLS and is not able to shift the steady state localization to the cytosol. However, a subtle contribution to nuclear export cannot be ruled out from these data. To test whether the first putative NES contributes to nuclear export, we applied a confocal microscopy based method, in which one compartment of the cell (the cytosol) is repetitively bleached, and the overall fluorescence is monitored between the bleaching cycles. A loss in fluorescence in another,

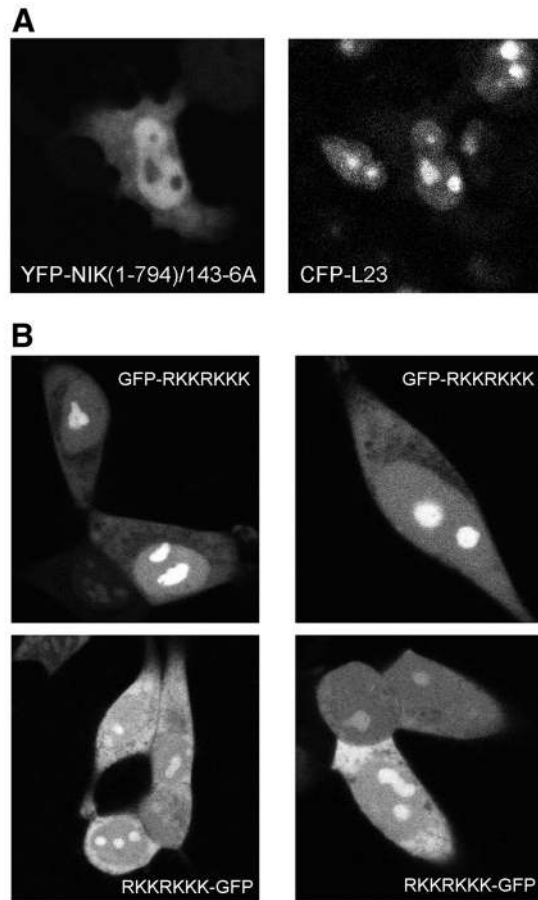


Fig. 2. Identification of a nucleolar localization signal. (A) Mutation of the basic amino acids in the region 143-146 (RKKR) to alanine residues prevents localization of YFP-NIK1-794 in nucleoli while still retaining its nuclear accumulation. Moderately expressing cells were analyzed by confocal microscopy. (B) Fusion of the seven basic amino acids 143-149 of NIK to either the C terminus of GFP (GFP-RKKRKKK) or the N terminus of GFP (RKKRKKK-GFP) induces accumulation of the modified GFP in nucleoli. An additional accumulation in the nucleoplasm is observed for the C-terminally tagged GFP-RKKRKKK.

non-bleached compartment (e.g. the nucleus) means a dynamic shuttling of fluorescent molecules between the bleached and the non-bleached regions. We applied this method (fluorescence loss in photobleaching, FLIP) to YFP-NIK(1-794), which contains the first potential NES, and to a truncation mutant YFP-NIK(1-769), which does not contain this putative NES. Both mutants localize predominantly to the nucleus with little fluorescence in the cytoplasm. FLIP experiments with bleaching of the pool of cytoplasmic fluorophores showed that nuclear fluorescence intensity remained constant over several minutes for both truncation mutants while cytosolic fluorescence decreased steadily (Fig. 3). This illustrates that these fusion proteins do not shuttle out of the nucleus, as nuclear fluorescence would constantly decrease in case of a dynamic equilibrium with the cytosol. Thus, the putative first NES that was predicted by a computer search for positions 770-779 present in YFP-NIK(1-794) is not active and does not contribute to nuclear export of the protein. To also verify the

NES at position 795-805 for full length NIK, we created point mutation constructs of NIK in which leucines and isoleucines in the NES were replaced by alanines. Mutation of leucines 803 and 805 (L803A_L805A) shifted a significant amount of YFP-NIK fluorescence to the nucleus, while additional alteration of isoleucine 800 (I800A_L803A_L805A) did not have a significant supplementary effect. Mutation of all leucines and isoleucines between positions 795 and 805 (L795A_L798A_I800A_L803A_L805A, also called NESmut) resulted in most of the YFP-NIK present localizing within the nucleus (Fig. 4A). Cotransfection studies with CFP-L23 confirmed that most of the fusion proteins carrying the NES mutation accumulated in nucleoli (Fig. 4B). Consequently, additional mutation of the nucleolar retention signal (amino acids 143-146), as described above, resulted in staining of the nucleoplasm with nucleoli being omitted (Fig. 4C). In order to verify that the remaining three basic amino acids at position 147-149 promote nuclear localization, we went on to exchange all seven consecutive basic amino acids between positions 143 and 149 with neutral alanines. Introduction of these mutations into a nuclear YFP-NIK chimera with mutated NES resulted in cytoplasmic localization of the fusion protein (Fig. 4D). This shows that the NLS of NIK is located at position 147-149 and

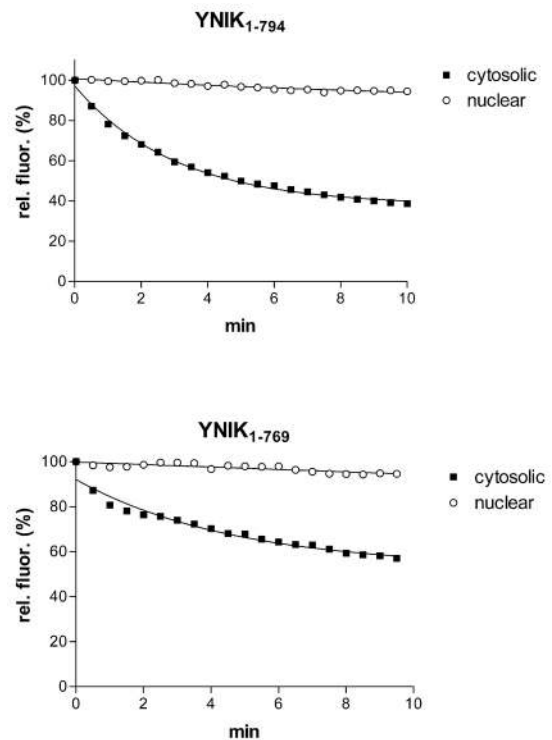


Fig. 3. Fluorescence loss in photobleaching (FLIP) proves that the putative first NES is not active. 293 cells were grown on coverslips and transfected (top) with YFP-NIK(1-794), which contains the first predicted NES, or with (bottom) YFP-NIK(1-769) lacking this domain. The coverslips were mounted onto an incubation chamber filled with medium and live cells were examined by laser scanning microscopy. The cytosol was bleached with high laser power and cytosolic as well as nuclear fluorescence intensities were recorded over time as described in the Materials and Methods section. Fluorescence decreased significantly in the cytosol whereas it remained constant in the nucleus for both constructs indicating that nuclear NIK did not shuttle into the cytosol.

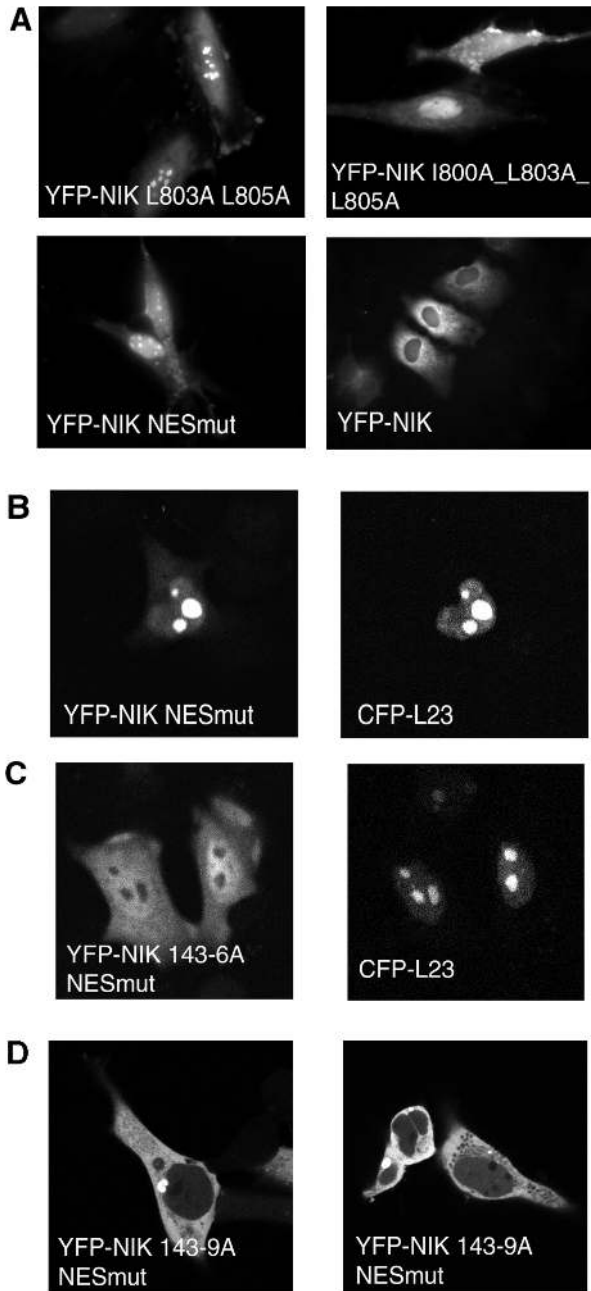


Fig. 4. Characterization and verification of nuclear export and nucleolar localization sequences by point mutations in full-length NIK. (A) To verify the functionality of the NES at position 795-805 that was previously identified with truncation mutants, point mutations of the conserved leucine or isoleucine residues were generated. Mutation of leucines at positions 803 and 805 to alanine induced a significant shift from the cytosol into the nucleus and accumulation in nucleoli. The same pattern was observed for variants in which isoleucine-800 or all hydrophobic amino acids of the predicted NES (NESmut) were replaced by alanine. (B) The NES mutant of full-length NIK accumulates in nucleoli and colocalizes with nucleolar CFP-L23 chimera. (C) Replacement of the basic amino acids RKKR at positions 143-146 with alanines in the NES-mutant form of NIK leads to nuclear localization with exclusion of nucleoli. (D) Mutation of all seven basic amino acids RKKRKKK at positions 143-149 of the NES mutant results in a predominant cytosolic localization.

is directly attached to the nucleolar retention signal at position 143-146.

Taken together, these data confirm the identity of the C-terminal NES and the N-terminal NLS of NIK and demonstrate the distribution of the protein between the cytoplasmic, nucleoplasmic and nucleolar compartment.

Dynamic redistribution of NIK between nucleoli and nucleoplasm

Based on our observation of the nucleolar accumulation of NIK, we wanted to investigate whether this nucleolar pool is in a dynamic equilibrium with NIK in the nucleoplasm. To answer this question we performed FLIP experiments similar to those described above. In this case we bleached inside the nucleus in regions not covered by nucleoli. As shown in Fig. 5, FLIP analysis revealed a decrease in the fluorescence of both the nucleoplasm and the nucleolus. The decline of YFP signal in the nucleolar compartment showed some delay to the fluorescence loss in the nucleoplasm and exhibited a longer half-life as compared to the nucleus. For NIK(1-769) the half-life of fluorescence loss in the nucleus was about 2.9 minutes with the half-life of nucleolar NIK fluorescence in the range of 6.9 minutes. For NIK(1-794) the data were very similar with nuclear and nucleolar half-lives of 2.1 and 6.1 minutes, respectively. These data indicate a rapid exchange of protein between nucleolar and nucleoplasmic compartments.

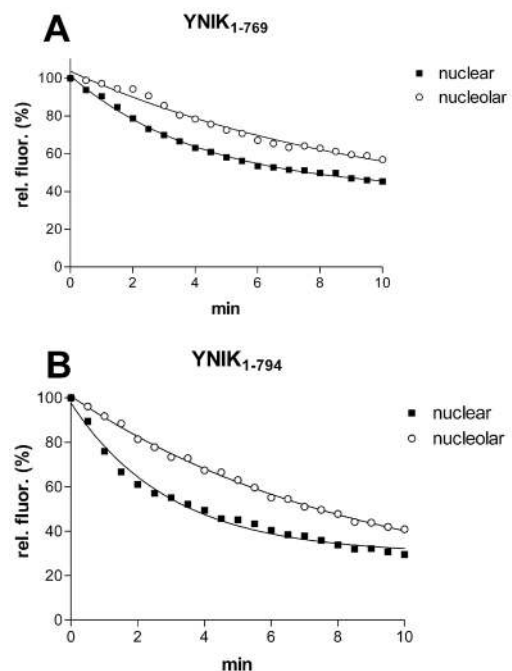


Fig. 5. Fluorescence loss in nucleoli upon bleaching in the nucleoplasm reveals dynamic distribution of NIK between nucleoli and nucleoplasm. FLIP experiments were performed with (A) YFP-NIK(1-769) and (B) YFP-NIK(1-794) as described in the Materials and Methods section. A bleach region was defined in the nucleoplasmic area, distant from nucleoli, and fluorescence intensities were measured in the nucleoplasm and in nucleoli over time. The decrease in nucleolar fluorescence indicates rapid exchange of nucleolar proteins with the nucleoplasm.

Dynamics and mobility of NIK in cytosol, nucleus and nucleoli

To gain further insight into diffusion kinetics and mobility of NIK in the different cellular compartments, we performed studies on fluorescence recovery after photobleaching (FRAP) for cytosolic, nuclear or nucleolar variants of YFP-NIK. This kind of assay was done with living cells grown on coverslips and mounted on a chamber filled with medium. The cells were imaged by confocal laser scanning microscopy usually at about 4% laser intensity with the 514 nm line of the Argon laser. Bleaching and scanning of a region in the compartment of interest was done as described in the Materials and Methods. Fluorescence recovery in the bleaching region resulting from diffusion of molecules from outside was monitored and quantified with appropriate control and compensation algorithms that account for the total loss in fluorescence in the course of bleaching and scanning. Half-life values of fluorescence recovery were normalized to the area of the bleached circle in order to compare the values for the different compartments. Interestingly, these analyses indicated that cytosolic NIK exhibits the lowest proportion of mobile molecules ($54.1 \pm 2.4\%$) as compared to nuclear NIK with ($82.4 \pm 7.2\%$) and nucleolar NIK with ($80.7 \pm 13.1\%$) (Fig. 6A). These data indicate that cytosolic NIK binds to some immobile elements such as cytoskeletal structures in the cytosol, whereas a significantly higher percentage is mobile in nucleoplasm and nucleoli. The half-time of fluorescence recovery was in the range of 1.6 seconds for cytosolic NIK, while nuclear and nucleolar NIK had half-times of recovery in the range of 0.8-0.9 seconds. Normalizing the halftimes of recovery with the area bleached indicated that NIK is most mobile in the nucleoplasm (with $t_{1/2}/\mu\text{m}^2$ of 0.129 ± 0.046 seconds), while in the cytosol the normalized half-time of recovery was 0.424 ± 0.094 seconds. Nucleolar NIK seemed to be present in two different states with respect to mobility, with one population exhibiting a normalized half-time of 1.14 ± 0.15 seconds and another fraction with a value of 6.75 ± 0.5 seconds, indicating association with elements of low mobility. We aimed to compare the mobility of NIK in the nucleolus with other GFP-tagged molecules localizing to nucleoli. Therefore, we performed FRAP experiments with GFP containing the 7 amino acid nuclear/nucleolar localization sequence of NIK (RKKRKKK-GFP) – as a rather inert marker localizing to nucleoli, and we also did FRAP experiments with the GFP-chimera of the ribosomal protein L23, a molecule that accumulates in nucleoli in the course of ribosome biogenesis. The nucleolar GFP version showed very fast diffusion kinetics with a half time of about 0.3 seconds and a mobile fraction of nearly 100% (Fig. 6B). However, normalization of the half-time with the bleached area revealed a value of 0.818 ± 0.174 seconds/ μm^2 , which is significantly slower than wild-type GFP in the cytosol, indicating that the basic residues mediate an association with molecules of low mobility. Nucleolar GFP-L23 showed slower diffusion with a half-time of fluorescence recovery in the range of 2.4 seconds and an area-normalized half-time of 2.37 ± 0.27 seconds/ μm^2 .

Localization of NIK in nucleoli results in a reduced NF- κ B activation potential

An important question is whether the different localizations of NIK – as a molecule that shuttles between cytosol,

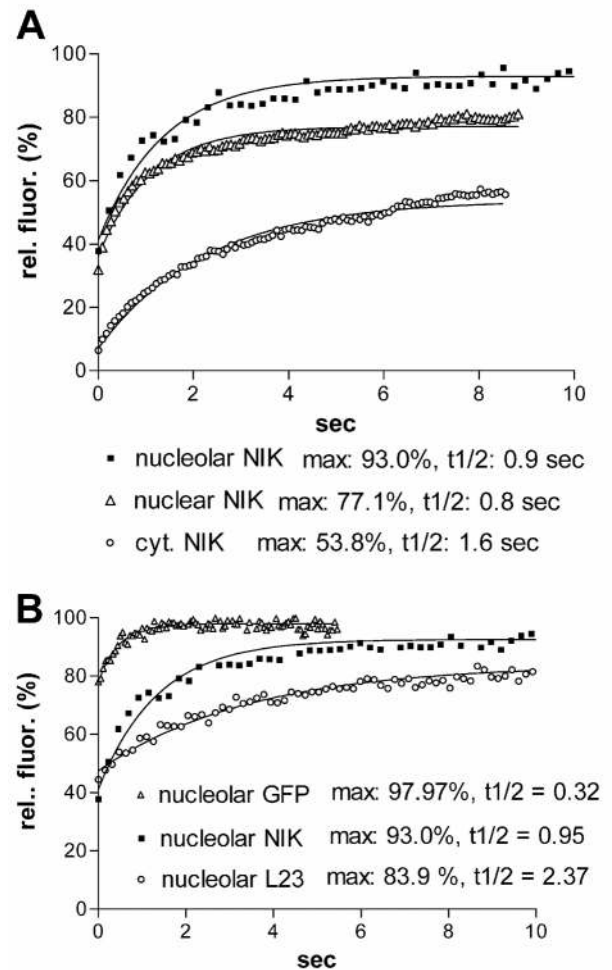


Fig. 6. FRAP analysis to determine diffusion and mobility of NIK variants within a compartment. (A) Mobility of NIK in cytosol (cyt.), nucleoplasm (nuclear) and nucleoli (nucleolar) as determined by FRAP. Variants of YFP-NIK with the respective major localization were analyzed for fluorescence recovery after photobleaching as described in the Materials and Methods section. The values for the calculated maximum recovery (max) indicating the fraction of mobile molecules and the calculated half-time ($t_{1/2}$) of diffusion are specified. Representative curves are shown. Mean values of halftimes normalized for the bleaching area and standard deviations are specified in the text. (B) Mobility of nucleolar YFP-NIK compared to the GFP-chimera of the ribosomal protein L23 and nucleolar GFP. Halftimes of diffusion and maximum values are given.

nucleoplasm and nucleoli, is relevant for its function in the NF- κ B signaling pathway. We addressed this question by using reporter gene assays with a NF- κ B-dependent luciferase reporter that was co-transfected with NIK variants that exhibit different steady state localizations. The highest NF- κ B activation potential was observed for the NIK variant that had both the nuclear localization sequence and the functional nuclear export sequence mutated – therefore a variant of NIK that is localized primarily in the cytosol without being able to shuttle efficiently between cytosol and nucleus (Fig. 7). Wild-type NIK had an NF- κ B activation potential, which was similar to the variant of NIK that localized predominantly to the

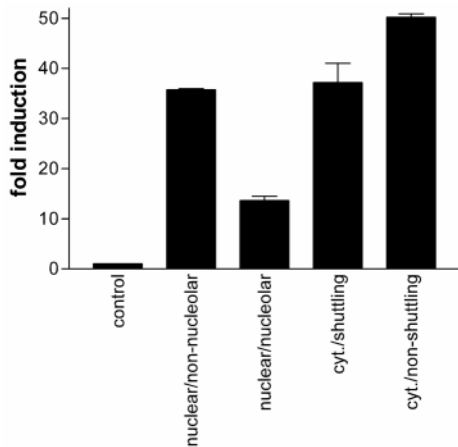


Fig. 7. Effect of NIK variants on NF- κ B activation in reporter gene assays. 293 cells were transfected with a NF- κ B-dependent luciferase reporter (containing 5 NF- κ B binding sites) and a constitutively expressed β -galactosidase construct for normalization together with NIK variants exhibiting distinct intracellular localizations. The fold induction over the vector control is given for nuclear/non-nucleolar NIK (NES mutant, 143-146A), nuclear/nucleolar NIK (NES mutant), for cytoplasmic NIK that shuttles between cytosol and nucleus (cyt./shuttling; YFP-NIK wild type) and for cytoplasmic NIK that has both NES and NLS mutated (NES mutant, 143-149 A; cyt./non-shuttling).

nucleus but not to nucleoli (the form of NIK in which both the NES and the nucleolar targeting sequence were mutated). Strikingly, this nuclear/non-nucleolar form of NIK had a much higher tendency to activate NF- κ B than the nuclear form of NIK, which also shows nucleolar accumulation. This form was actually the variant of NIK with the least NF- κ B induction potential amongst the different NIK constructs tested. It is worth noting that interaction domains of NIK with its upstream activator, Traf2, and its downstream effector, IKK1, both lie in the C-terminal domain of the protein (Malinin et al., 1997; Lin et al., 1998). Thus, differences in the activation potential between nucleolar and non-nucleolar variants of NIK described here are not due to a change in binding affinity to these partners. These data indicate that nucleolar NIK is sequestered from the other components of the NF- κ B signaling pathway and suggest an inhibitory role for the nucleolar localization of NIK with respect to NF- κ B activation.

Discussion

We have investigated the dynamic subcellular localization of the NF- κ B-inducing kinase NIK, which shuttles constitutively between cytoplasm, nucleoplasm and nucleoli. Nucleolar retention depends on a stretch of four basic amino acids (RKKR) in the N-terminal half of the protein, which is directly linked to a nuclear targeting sequence (KKK) (Fig. 8). The nucleolus is a structure assembled around clusters of repeated ribosomal genes (rDNA) coding for ribosomal RNA. In addition to nucleic acids, the nucleolus also contains a large number of proteins, which have recently been characterized in HeLa cells by a proteomic approach (Andersen et al., 2002).

Consequently, a protein found in the nucleolus can be targeted there by contact with either DNA, RNA or protein. One may assume that a stretch of basic residues offers the opportunity for an interaction with phosphate groups in nucleic acids. In fact, the nucleolar retention signal of human T cell leukemia virus type I protein p27^{x-III} and the HIV-Tat protein are also highly positively charged (Siomi et al., 1990; Siomi et al., 1988) and it was shown that arginine-rich basic domains bind to RNA in nucleoli (Calnan et al., 1991; Gustafson et al., 1998). Thus one possibility for nucleolar accumulation of NIK is an interaction with structural motifs in rRNA. Interestingly, our FRAP data of normalized halftimes indicated two different states of NIK in the nucleolus, with one having a mobility similar to the RKKRKKK-tagged GFP, and another one being significantly less mobile. Given the known characteristics of basic amino acid stretches to bind to RNA, it appears plausible that nucleolar GFP, as well as the more mobile fraction of nucleolar NIK bind to rRNA, while the less mobile fraction of nucleolar NIK additionally binds to other nucleolar structures.

It is an interesting phenomenon that a significant number of signaling molecules including p65 NF- κ B, I κ B α , Smad proteins and many others contain both nuclear localization and nuclear export sequences that counteract each other (Arenzana-Seisdedos et al., 1997; Gama-Carvalho and Carmo-Fonseca, 2001; Harhaj and Sun, 1999; Huang et al., 2000; Johnson et al., 1999; Reguly and Wrana, 2003). Signaling molecules such as transcription factors, which have roles both in the nucleus and in the cytosol, clearly require nuclear import and nuclear export mechanisms. However, more and more proteins without an obvious nuclear function are being found to shuttle between cytosol and nucleus (Gama-Carvalho and Carmo-Fonseca, 2001). Most of these proteins contain both NLS and NES domains, raising the question of how these opposing localization mechanisms are balanced and dynamically regulated. Several possibilities appear significant for regulating the equilibrium between counteracting localization signals. First, one signal can be structurally exposed and more easily accessible to the transport machinery, e.g. the NES in NIK in contrast to its NLS. Second, modifications such as phosphorylations can change this balance. Such a mechanism seems likely for IKK1, the signaling kinase downstream of NIK, which shuttles between cytosol and nucleus, as it was shown recently that its steady state localization shifts to the nucleus after addition of TNF α , i.e. after phosphorylation of its activation loop (Anest et al., 2003). The NLS of IKK1 has not yet been identified, although our own data (Birbach et al., 2002) suggest that the lysine residue at position 44, which is at the



Fig. 8. Schematic illustration of the localization signals of NIK. From the results obtained with various mutants of YFP-NIK, a scheme of the targeting domains is depicted, showing the nucleolar localization sequence (NoLS, amino acids RKKR at position 143-146), the nuclear localization sequence (NLS, amino acids KKK at 146-149), and the functional nuclear export sequence (NES, amino acids 795-805).

active site of the enzyme, appears to be part of a non-classical NLS. Assuming that a phosphorylation at the activation loop would expose the active site of the enzyme, it seems plausible that activation via TNF α would then also expose a domain that supports nuclear import and shifts the steady state towards the nucleus. In principle, a protein modification such as phosphorylation can facilitate nuclear import, but might also have the opposite effect. Computer analysis (Blom et al., 1999) of the human NIK sequence indicates that serines in close proximity to the nuclear/nucleolar retention signals at position 143-149 (including serines 150, 151) are likely to be phosphorylation sites. In this case negative charges introduced by phosphorylation events might interfere with nuclear targeting or nucleolar sequestration mediated by basic amino acids. Thus phosphorylation events are potential mechanisms controlling subcellular or subnuclear localizations of NIK by other kinases. It is important to note that a predominant localization in a specific compartment at steady state does not mean that the protein is acting exclusively there. Since there is a dynamic distribution between the different compartments, those molecules that are at a different location than the predominant one might still have important biological functions. Thus various cellular signaling events might not only alter the steady state distribution of molecules, but also affect transport rates into or out of specific compartments. In this work, we have examined kinetic properties of protein exchange between compartments by means of photobleaching techniques using GFP chimeras. Similar approaches have been undertaken by other groups (Cole et al., 1996; Kimura and Cook, 2001; Phair and Misteli, 2000), underlining the usefulness and applicability of this method. Our measurements on fluorescence loss in nucleoli upon photobleaching of the nucleoplasm imply a rather fast and efficient distribution of NIK between these two compartments. This is in line with other reports demonstrating rapid and significant cycling of various proteins involved in rRNA transcription and processing between nucleoli and the nucleoplasm (Chen and Huang, 2001). Besides the kinetics of distribution between distinct compartments, the mobility and diffusion dynamics of signaling molecules within a given compartment is an important parameter influencing their biological activity. In that respect, the high mobility of NIK in the nucleoplasm and the high fraction of mobile molecules in nucleoli are quite remarkable. The lowest percentage of mobile molecules as judged from the plateau of the fluorescence recovery was observed in the cytosol, which might indicate that NIK has a tendency to bind to cytoskeletal elements probably via bridging molecules, or it might be part of signal transduction scaffolding complexes. Similar associations with the cytoskeleton were also reported for other signaling kinases, such as MEKK1 (Christerson et al., 1999).

Obviously, an important question that remains to be clarified is what is the function of NIK in the nucleus and moreover in the nucleolus? Apparently a central role of nuclear NIK is to activate IKK1, which is also shuttling between cytosol and nucleus (Birbach et al., 2002) – and which seems to be important in activating non-classical NF- κ B pathways (NIK-IKK1-I κ B α -NF- κ B or NIK-IKK1-NF- κ B2). It is striking that these pathways seem to be crucial in B-cells in which the nucleus constitutes the major part of the cell body. In this case, localization or sequestration of NIK in nucleoli might have important regulatory roles for NF- κ B signaling and B-cell

biology. Our findings that endogenous NIK is primarily nuclear in certain breast cancer cells where it also sometimes accumulates in nucleoli might suggest a special role of nuclear NIK for an elevated constitutive NF- κ B activity in these cells, which might causally contribute to the malignancy. Another potential role for nuclear NIK might be related to the recently identified function of IKK1 in histone phosphorylation. It was shown that nuclear IKK1 can upregulate NF- κ B target genes also by binding to promoter elements, direct phosphorylation of histone H3 and subsequent facilitation of the transcriptional process (Anest et al., 2003; Yamamoto et al., 2003). Very recently, another nuclear function was defined for NIK and IKK1, that is direct activation of p65 NF- κ B via phosphorylation of serine-536 on the transactivation domain (Jiang et al., 2003). All these nuclear functions of IKK1 and NIK might be regulated at least in part by the distribution of NIK between nucleoli and nucleoplasm. A well-known example of a signaling pathway regulated by nucleolar sequestration is that of the p53 tumor-suppressor gene product, as its main regulator, Mdm2, is controlled by nucleolar retention (Lohrum et al., 2000; Weber et al., 1999). Similarly, our data indicate that nucleolar sequestration might influence the NF- κ B-inducing activity of NIK. In reporter gene assays, we found that nuclear NIK variants can upregulate NF- κ B-dependent gene expression and that this ability is negatively regulated by nucleolar localization, as mutation of the nucleolar targeting sequence enhanced the NF- κ B activation potential. This is in line with a report showing that deletion of the N-terminal part of NIK, containing the basic amino acids that we identified as a nuclear/nucleolar localization signal, augments NF- κ B activation, which led to the concept of a 'negative-regulatory domain' in the N-terminal region of NIK (Xiao and Sun, 2000). However, spatial sequestration alone is probably not sufficient to explain the reduction of NF- κ B activation by nucleolar localization, because nucleolar NIK shuttled rather efficiently to the nucleoplasm. Thus additional mechanisms such as interactions with other signaling molecules or phosphorylation events might be effective in regulating the activity of nuclear NIK by increasing or decreasing the fraction of NIK present in nucleoli.

In conclusion, we consider nuclear NIK crucial for various nuclear functions of IKK1 in the course of cytokine-induced gene expression and NF- κ B activation in a cell type-specific manner and we suggest that nucleolar localization of NIK might be a mechanism for regulating these activities. Certainly further studies will be necessary to elucidate the different roles of NIK in cytosol, nucleus and nucleoli and the mechanisms that balance the functions in different compartments.

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