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An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IκBα

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The potent transcriptional activities of Rel/NF-κB proteins are regulated in the cytoplasm and nucleus by the inhibitor, IκBα. The mechanism, by which IκBα can either sequester NF-κB in the cytoplasm or act as a nuclear post-induction repressor of NF-κB, is uncertain. We find that IκBα shuttles continuously between the nucleus and cytoplasm. This shuttling requires a previously unidentified CRM1-dependent nuclear export signal (NES) located within the N-terminal domain of IκBα at amino acids 45–55. Deletion or mutation of the N-terminal NES results in nuclear localization of IκBα. NF-κB (p65) association with IκBα affects steady-state localization but does not inhibit its shuttling. Endogenous complexes of IκBα–NF-κB shuttle and will accumulate in the nucleus when CRM1 export is blocked. We find TNFα can activate the nuclear IκBα–NF-κB complexes by the classical mechanism of proteasome-mediated degradation of IκBα. These studies reveal a more dynamic nucleocytoplasmic distribution for IκBα and NF-κB suggesting previously unknown strategies for regulating this ubiquitous family of transcription activators.

Keywords: IκBα–NF-κ/nuclear export/nucleocytoplasmic shuttling/Rel A

Introduction

The NF-κB/Rel family of inducible transcription factors is involved in the highly regulated expression of numerous genes involved in disparate processes such as growth, development, inflammatory/immune response, auto-regulation, and transcription of viral genomes (reviewed in Ghosh *et al*., 1998). Members of the Rel family include p65 (RelA), p105/p50, p100/p52, RelB, c-Rel and the viral oncoprotein (v-Rel). These factors associate as homo- or heterodimers to form transcriptionally competent complexes known as nuclear factor kappa B (NF-κB). The Rel polypeptides are structurally related and share a highly conserved 300+ amino acid region known as the Rel homology domain (RHD), which contains the sequences necessary for subunit dimerization, DNA binding/transcription activation, nuclear localization and complex formation with inhibitors.

The biological activities of these transcription factors are regulated by their concentration and subcellular localization. A family of structurally related proteins known as the inhibitors of NF-κB (IκB) is responsible for the regulation of the DNA-binding activity and nucleo/cytoplasmic distribution of NF-κB (reviewed in Ghosh *et al*., 1998). Members of the IκB family share an ankyrin repeat domain (ARD) of five or more ankyrin repeats of ~33 amino acids that interface with the RHD of NF-κB homo/heterodimers, forming a stable inhibited complex.

The best characterized IκB protein is IκBα. IκBα binds to a heterodimer of p65/p50, the most ubiquitous and biologically active NF-κB. IκBα association with NF-κB disrupts DNA binding and masks the nuclear localization signals (NLSs) located in the C-terminal region of the RHD (Beg *et al*., 1992; Ganchi *et al*., 1992). Masking of the NLS is believed to impede nuclear translocation of NF-κB resulting in its retention in the cytoplasm where it can not mediate any transcriptional effects. Any one of several extracellular stimuli can activate NF-κB by initiating a signal transduction pathway that leads to phosphorylation, ubiquitination and ultimately degradation of IκBα. Once IκBα is degraded, the NLSs within the RHD are unmasked, allowing NF-κB to enter the nucleus and initiate transcription of target genes (Chen *et al*., 1995; Scherer *et al*., 1995; Traenckner *et al*., 1995). One consequence of NF-κB activation is the induction of IκBα expression caused by an NF-κB consensus binding site within the IκBα promoter (Sun *et al*., 1993; Cheng *et al*., 1994; Chiao *et al*., 1994). The increased synthesis of IκBα leads to quenching of NF-κB activity, thereby establishing an auto-regulatory loop. This ensures rapid, controlled, transient NF-κB-mediated transcription in response to specific signals.

Several lines of evidence suggest that the newly synthesized IκBα goes into the nucleus, strips NF-κB from its DNA binding sites and mediates its export from the nucleus to re-establish a cytoplasmic pool of inhibited complexes (Zabel and Baeuerle, 1990; Arenzana *et al*., 1995; Arenzana-Seisdedos *et al*., 1997). Pulse–chase studies of cells activated with TNFα have demonstrated a transient accumulation of *de novo* IκBα in the nucleus. These studies showed that the subsequent depletion of nuclear IκBα was correlated with the suppression of NF-κB-dependent transcription (Arenzana *et al*., 1995). This model is supported by the recent identification of sequences in IκBα that have been shown to mediate both import and export.

NLS activity contained within the IκBα ARD has been reported recently (Sachdev *et al*., 1998; Turpin *et al*., 1999). Hannink and coworkers found that the IκBα ARD can mediate nuclear import when fused to a cytoplasmic protein (Sachdev *et al*., 1998). They showed that mutating a hydrophobic region of the second ankyrin repeat prevented

ectopically expressed myc-IκBα from appearing in the nucleus. A nuclear export function of IκBα is strongly suggested by the ability of Leptomycin B (LMB), a specific inhibitor of the nuclear export signal receptor CRM1 (Fornerod *et al*., 1997; Fukuda *et al*., 1997; Wolff *et al*., 1997), to cause the relocalization of IκBα from the cytoplasm to the nucleus (Rodriguez *et al*., 1999). A putative nuclear export signal (NES) was recently identified in the C-terminal region of IκBα. Multiple mutations within the proposed NES-like sequence caused a decrease in the rate of IκBα export when injected into nuclei of *Xenopus* oocytes (Arenzana-Seisdedos *et al*., 1997). The presence of both an NLS and an NES suggests that IκBα has the potential to shuttle between the cytoplasm and the nucleus.

In our study of the localization of IκBα, we find that it shuttles continuously between the nucleus and cytoplasm. Moreover, this shuttling is mediated by an NES that is located in the N-terminal region of IκBα rather than the previously reported C-terminal NES. Formation of IκBα–NF-κB complexes alters the dynamics of IκBα localization but does not disrupt shuttling. Finally, we find that the nuclear complexes of IκBα–NF-κB can be activated by the classical mechanism of proteasome-dependent degradation of IκBα. This reveals a complex profile for the nucleocytoplasmic distribution of IκBα–NF-κB complexes, which may provide additional mechanisms for regulating this significant and ubiquitous family of transcriptional activators.

Results

IκBα contains an NES at position 45–55

To initiate our study of the nuclear export of IκBα, we examined the protein for potential NESs using an HIV-1 Rev NES deletion complementation assay (Hope *et al*., 1991). The mutant Rev derivative (RevΔNES), truncated at amino acid (aa) 78, has normal RNA binding and multimerization activity but lacks the Rev effector domain, a leucine-rich NES; consequently, it can be complemented by the fusion of a heterologous NES (Kim *et al*., 1996). To quantitate Rev function, we used the pDM128 reporter system (Hope *et al*., 1990), which contains the chloramphenical transferase (CAT) gene and the Rev binding sequence (RRE) within an intron (Figure 1A). Normally, RNA is exported after splicing is completed, thereby deleting the CAT sequence. In the presence of Rev, unspliced CAT RNA is exported to the cytoplasm. Hence, CAT activity is an indirect measure of Rev-mediated export.

To identify likely NESs in IκBα, we fused the entire wild-type (wt) coding sequence (1–316) and several IκBα fragments (Figure 1B) in-frame to the C-terminal region of RevΔNES. The RevΔNES derivatives were transfected into 293T cells and assayed for CAT activity. As a positive control, we used the previously characterized NES of human T-cell leukemia virus type I (HTLV-1), Rex aa 80–96, which restores greater than wild-type activity to RevΔNES (Kim *et al*., 1996). The results (Figure 1C) revealed that fragments encompassing the N-terminus of IκBα could restore Rev function, whereas fragments derived from the ARD or C-terminus could not. Full-length IκBα did not complement RevΔNES. However, the 1-316 fusion protein could not be detected by Western blot analysis (Figure 1D), suggesting that it was not expressed or was unstable. Region 186–316 did not complement RevΔNES although it includes the previously reported NES at aa 265–275 (Arenzana-Seisdedos *et al*., 1997). Western analysis showed that 186–316 was expressed at levels similar to those seen in the functional N-terminal derivatives (Figure 1D). Additionally, two smaller IκBα C-terminal fragments, 248–316 and 186–225, were unable to complement RevΔNES. In contrast, 1–120 restored Rev function, suggesting that it contained an NES. To characterize the export function of this sequence, we tested several derivatives between aa 1 and 120. IκBα sequences, 1–72, 22–72 and 1–66 complemented RevΔNES, whereas 1–49 and 22–49 did not. This suggests an NES is located in the N-terminal region of IκBα between aa 22 and 72.

Scrutiny of N-terminal sequences encompassing aa 22–72 revealed a putative CRM1-interacting NES motif (Bogerd *et al*., 1996; Kim *et al*., 1996; Fornerod *et al*., 1997) (Figure 1E) located at aa 45–55. Within this putative NES, the large hydrophobic amino acids required for function are conserved in IκBα in many species. The previously proposed C-terminal NES sequence, however, is not conserved in the chicken IκBα homolog pp40 (Figure 1F). We mutated the putative core of the IκBα N-terminal NES, isoleucine 52 and leucine 54 to alanines (I52A,L54A) in the context of IκBα 1–72 (Figure 1E). Previously, this type of mutation was shown to abrogate export function in CRM1 dependent NESs (Malim *et al*., 1991; Kim *et al*., 1996). As shown in Figure 1C, mutation of these two hydrophobic amino acids abolished export function of RevΔNES-IκBα 1–72. These results reveal that aa 45–55 of IκBα can function as an NES complementing RevΔNES function.

The N-terminal NES is sufficient and necessary for the nuclear export of GST–IκBα fusion proteins

To assess the export ability of IκBα under different experimental conditions, we tested several IκBα fragments in a nuclear injection assay. By utilizing multi-nucleated cells, we could distinguish between export, import and shuttling. Import is indicated by a cytoplasmic injected protein accumulating in the nucleus, whereas relocalization of a nuclear injected protein to the cytoplasm suggests export and shuttling is indicated by the appearance of a nuclear injected protein in uninjected nuclei within a polykaryon.

We generated several glutathione-*S*-transferase (GST) IκBα fusion proteins, which were mixed with rhodamine-labeled dextran, an injection site marker too large to diffuse through nuclear pores, and injected into a single nucleus or the cytoplasm of an NIH 3T3 polykaryon. After a 60 min incubation at 37°C, cells were fixed and GST–IκBα fusion proteins were visualized by indirect immunofluorescence. GST–IκBα wt injected into a nucleus relocalized to the cytoplasm (Figure 2A). By adding 5 nM LMB to the culture media 30 min prior to injection, we found that export of GST–IκBα wt was blocked (Figure 2B). This demonstrates that IκBα is actively exported via the CRM1 pathway.

Next, we tested 1–72 and 73–316. Figure 2C shows that 1–72 exported from the nucleus of injection and

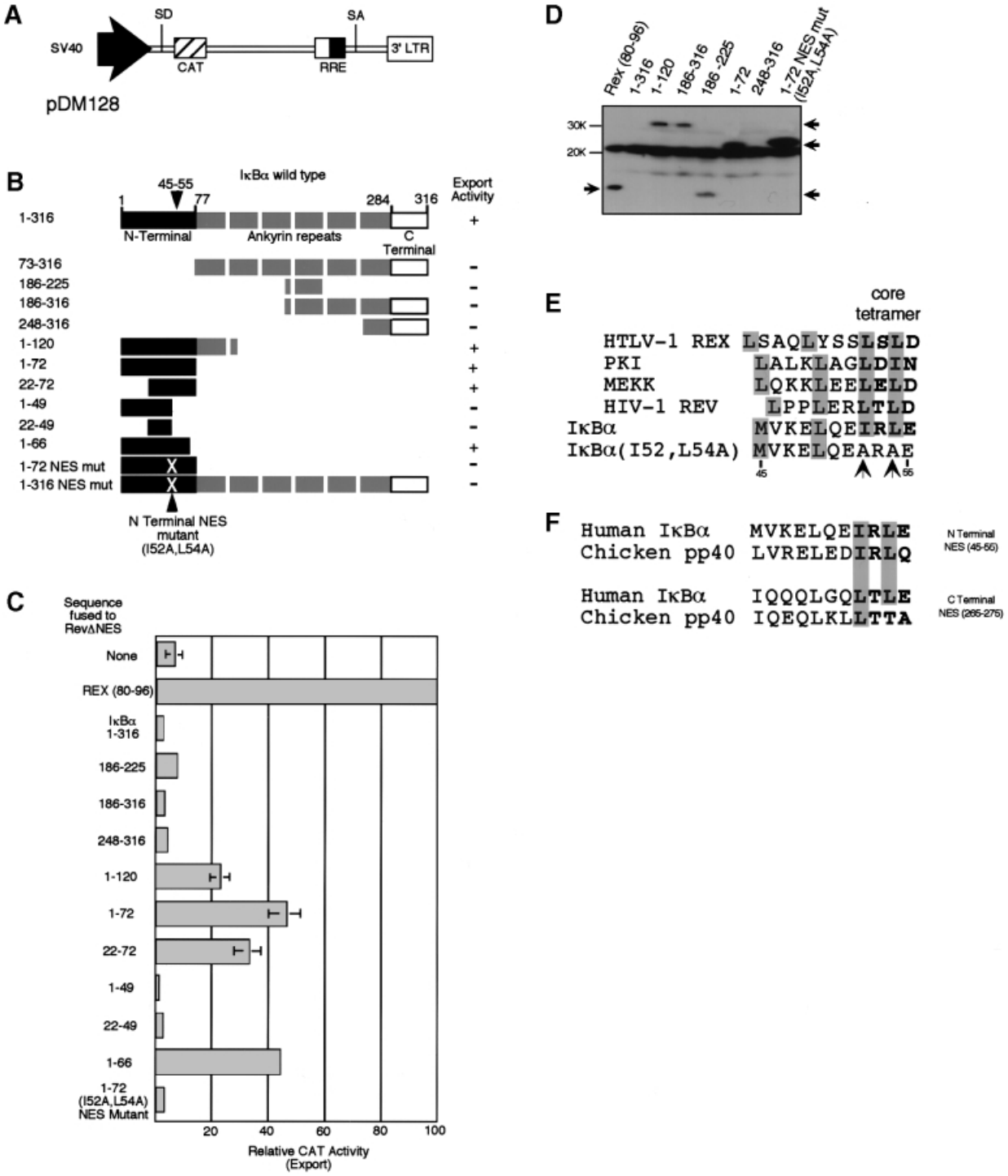


Fig. 1. IκBα contains an NES at position 45–55. (**A**) Schematic of pDM128. RNAs encoding CAT (hatched box) and Rev responsive element (RRE) are contained within a single intron defined by the splice donor (SD) and the splice acceptor (SA). (**B**) Domain organization of IκBα and IκBα derivatives tested by RevΔNES complementation and microinjection for export and a summary of results. (**C**) Identification of IκBα sequences that restore export to RevΔNES. Results shown are the mean (± SEM) of four independent experiments. (**D**) Western analysis of transiently transfected 293T cells. Two hundred and fifty microliters from normalized lysates were resolved on a 14% SDS–PAGE gel and probed with a cocktail of anti-Rev rabbit serum (Kim *et al*., 1996). Arrow indicates fusion proteins. RevDNES-IκBα 186–316 can not be seen in this Western blot. It migrates with the background band, but was visualized with a different antibody cocktail (data not shown). (**E**) IκBα aa 45–55 contains a 'leucine-rich' NES with the conserved consensus motif of four essential hydrophobic amino acids, the core tetramer (in bold) typically leucines, and essential hydrophobic amino acids immediately upstream (shaded boxes). The IκBα N-terminal NES mutant (I52A,L54A) is also shown. (**F**) The N-terminal NES of IκBα, but not the C-terminal NES is conserved in chicken pp40.

localized in the cytoplasm. Surprisingly, 73–316, which includes the putative C-terminal NES, did not export (Figure 2D). 73–316 was still competent for import, accumulating in the nuclei after it was injected into cytoplasm (Figure 2E), corroborating previously published data that identified NLS function within the ARD of IκBα (Sachdev *et al*., 1998; Turpin *et al*., 1999). These results show that aa 1–72 of IκBα are necessary and sufficient

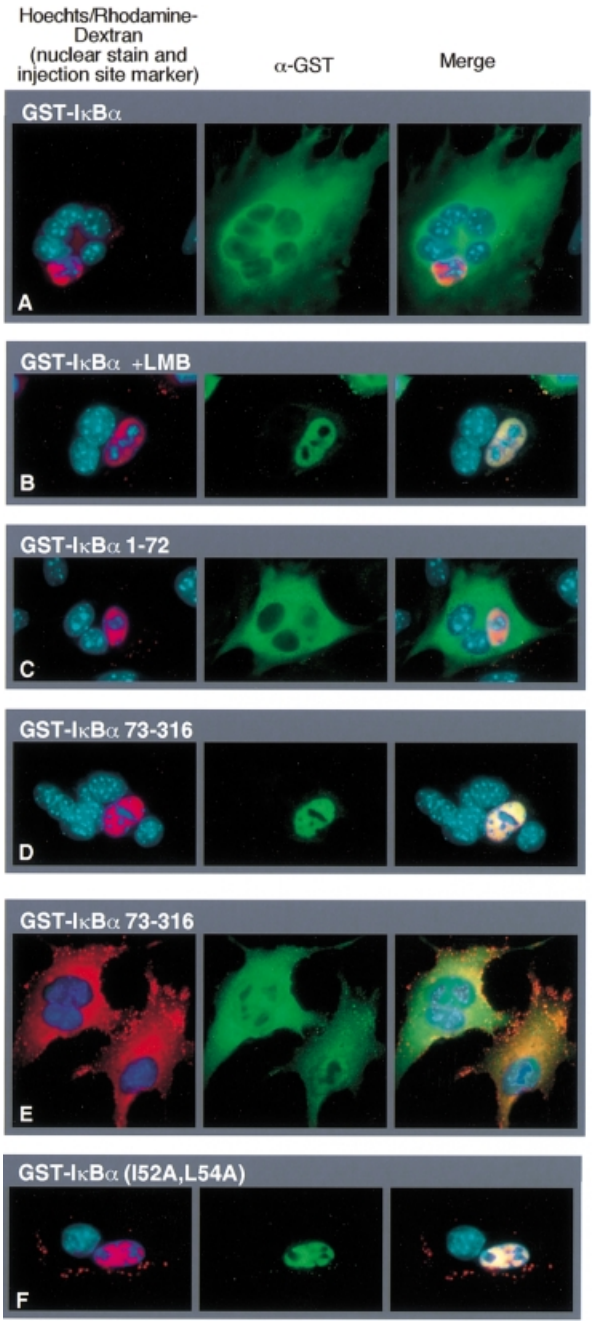


Fig. 2. The N-terminal NES is necessary for the nuclear export of GST–IκBα fusion proteins. (**A**) Wt IκBα fused to GST, injected into a nucleus relocalizes to the cytoplasm. (**B**) LMB blocked nuclear export of GST–IκBα. (**C**) The N-terminus of IκBα is sufficient to mediate export of a GST–IκBα fusion protein. GST–IκBα 1–72 injected into a nucleus relocalizes to the cytoplasm. (**D**) GST–IκBα 73–316 injected into nuclei does not export. (**E**) A deletion of the N-terminal NES, GST–IκBα 73–316, injected into the cytoplasm imports into nuclei and accumulates there. (**F**) A two point mutation within the N-terminal NES (I52A,L54A) abrogates export activity of GST–IκBα.

for nuclear export and that the NLS activity of IκBα is within aa 73–316. To determine whether nuclear export was dependent on the NES at aa 45–55 or an unidentified N-terminal sequence, we tested full-length IκBα with point mutations of core hydrophobic residues in the N-terminal NES (I52A,L54A). This mutation abolished

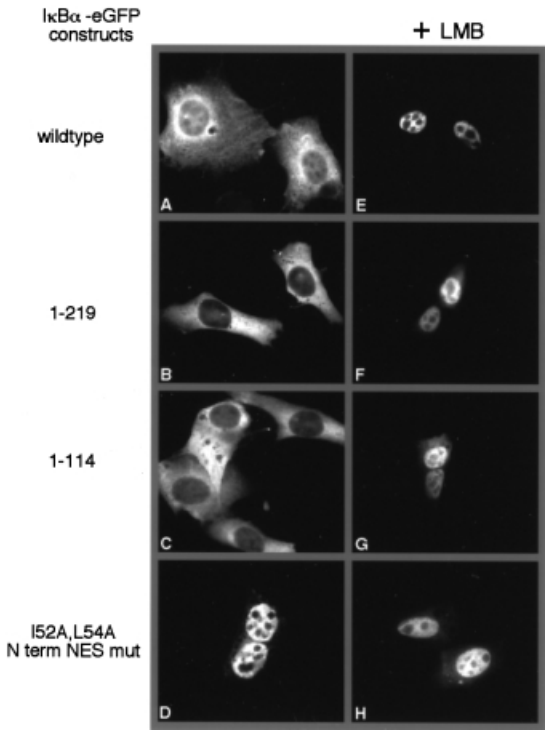


Fig. 3. IκBα shuttles. The N-terminal NES and ARD repeats 1 and 2 through to amino acid 114 enable IκBα–eGFP to import and export. IκBα derivatives that include the N-terminal NES have a cytoplasmic steady-state localization (**A**–**D**) and continuously shuttle, accumulating in the nucleus within 2 h of treatment with 5 nM LMB (**E**–**G**). (**H**) LMB does not affect the nuclear localization of the NES mutant.

export, revealing that the N-terminal NES at aa 45–55 is required for export of IκBα (Figure 2F).

The N-terminal NES is required for the cytoplasmic localization of IκBα

To investigate nucleocytoplasmic transport of IκBα expressed in living cells, we determined subcellular localization of different IκBα–eGFP fusion proteins (Figure 3) in 3T3 cells by fluorescent microscopy. Full-length wt IκBα 1-316, a C-terminal deletion 1–219, and the N-terminus through the first ARD repeat 1–114 were cytoplasmic in transient transfections (Figure 3A–C) and stable lines (data not shown). To determine whether the fusion proteins were capable of shuttling, we treated cells with LMB and cycloheximide (CHX). LMB would trap actively shuttling proteins in the nucleus and CHX would ensure that we were observing the movement of extant IκBα. Within 2 h of administering LMB and CHX, the fusion proteins were nuclear (Figure 3E–G). Deleting the C-terminal and all but the first 114 amino acids did not affect import or export function, suggesting that aa 1–114 are sufficient for export and import (Figure 3C and G). In contrast, the N-terminal NES mutant (I52A,L54A) was nuclear (Figure 3D). This data reiterates the pre-eminence of the N-terminal NES, suggesting that all IκBα passes through the nucleus. To ensure eGFP was not responsible for the localization of these proteins, we examined the localization of non-fusion IκBα wt and mutant proteins. We found that the non-fusion proteins showed the same subcellular localization as the eGFP constructs (data not shown).

We also considered that by fusing eGFP to IκBα or by introducing mutations we might alter its localization by ablating its function as an inhibitor of NF-κB. Utilizing the NF-κB responsive elements (κ-B sites) within the 5′ LTR of HIV-1, we asked whether the IκBα NES mutant and IκBα–eGFP fusion proteins could inhibit NF-κB. We co-transfected 293T cells with different IκBα constructs, p65 and HIV-CAT, where CAT transcription is initiated by p65 binding to the κ-B sites (Nabel and Baltimore, 1987). Forty-eight hours after transfection, cell lysates were assayed for CAT activity to determine the degree of NF-κB inhibition. As a negative control, we used a mutant HIV-CAT (mut κB) lacking the p65 binding sites. The results in Figure 4A show that the N-terminal NES mutant and fusion proteins (wt and mutant) inhibited p65 transcriptional activation of HIV-CAT to the same degree and in the same concentration-dependent manner as wt IκBα. Next, we examined the responsiveness of the IκBα N-terminal NES mutant to signal-induced degradation. The oncogenic Tax protein from HTLV-1 is a strong activator of NF-κB/Rel transcription factors. The presence of Tax increases both the signal-induced degradation and constitutive turnover of IκBα (Maggirwar *et al.*, 1995; Geleziunas *et al.*, 1998; Petropoulos and Hiscott, 1998). Co-transfection of Tax and HIV-CAT in 3T3 cells doubles CAT activity (Figure 4B). Tax enhancement was inhibited by co-expressing either IκBα wt or NES mutant (I52A,L54A) (Figure 4B). This inhibition of Tax-mediated activation was overcome in a dose-dependent manner by increasing the concentration of the Tax expression vector. At 1.0 μg, Tax activated HIV-CAT by 2-fold, overcoming the inhibition of both wt and NES mutant IκBα proteins (Figure 4C), and indicating that the NES mutant is as responsive to Tax-induced degradation as wild type.

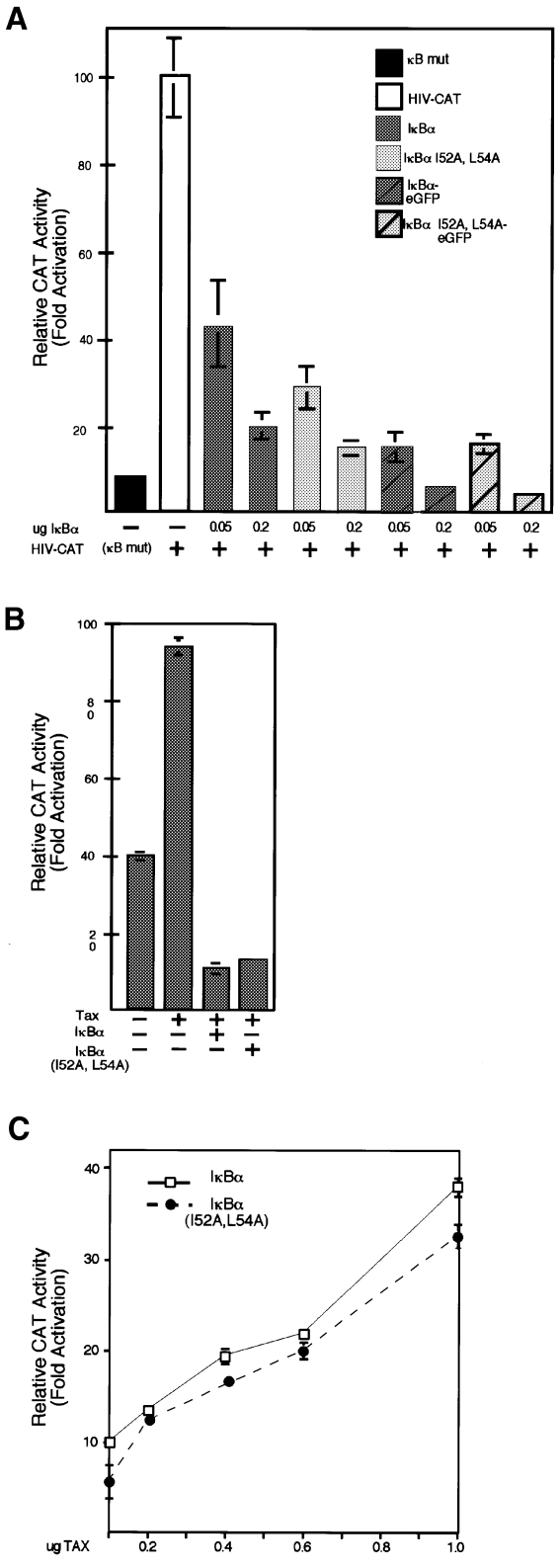


Fig. 4. NF-κB inhibition by IκBα NES mutant. (**A**) The IκBα–eGFP fusion proteins and the NES mutant, IκBα (I52A,L54A), inhibit transcription of HIV-CAT with the same efficiency and in the same concentration-dependent manner as wild type. HIV-CAT mut κB with deletions of the κ binding sites was used as a negative control. 293T cells were transfected with HIV-CAT, β-gal, p65, poly(A) for promoter balance, and the specified quantity of the different IκBα derivatives. Results shown are the mean (± SEM) of three experiments. (**B**) Exogenous expression of IκBα (I52A,L54A) inhibits TAX-mediated activation of NF-κB transcription with the same efficiency as wild type. CV1 cells were transfected with HIV-CAT, β-gal, poly(A), 4 μg of TAX and 1 μg of either IκBα wt or IκBα (I52A,L54A). Normalized CAT values shown are the mean (± SEM) of three experiments. (**C**) TAX can overcome inhibition by both wild-type IκBα and the NES mutant. 3T3 cells were transfected with HIV-CAT, β-gal, poly(A), p65, 0.05 μg of either IκBα wt or IκBα (I52A,L54A), and the indicated quantity of TAX expression vector. Normalized CAT values shown are the mean (± SEM) of three experiments.

Import of IκBα is temperature dependent and efficient

To ascertain whether nuclear accumulation of IκBα was due to passive diffusion or active transport, we treated 3T3 cells transfected with wt IκBα–eGFP or 1–114 with 5 nM LMB and incubated the cells at either 4 or 37°C. At 37°C, both fusion proteins accumulated in the nucleus (Figure 5A) within 60 min. But at 4°C, the fusion proteins remained cytoplasmic. The nuclear import of IκBα–eGFP proteins could be restored simply by returning the cultures containing LMB to 37°C (data not shown). In conclusion, IκBα is actively imported in a temperature-dependent manner.

To determine the rate of IκBα shuttling, we observed stable cell lines expressing the eGFP fusion proteins, wt IκBα–eGFP and 1–114, in the presence of CHX and LMB. Figure 5B shows that relocalization is detected within 10 min for both wt (1–316) and 1–114, and is almost complete in 30 min. Similar results were seen in cells transiently expressing fusion proteins (data not

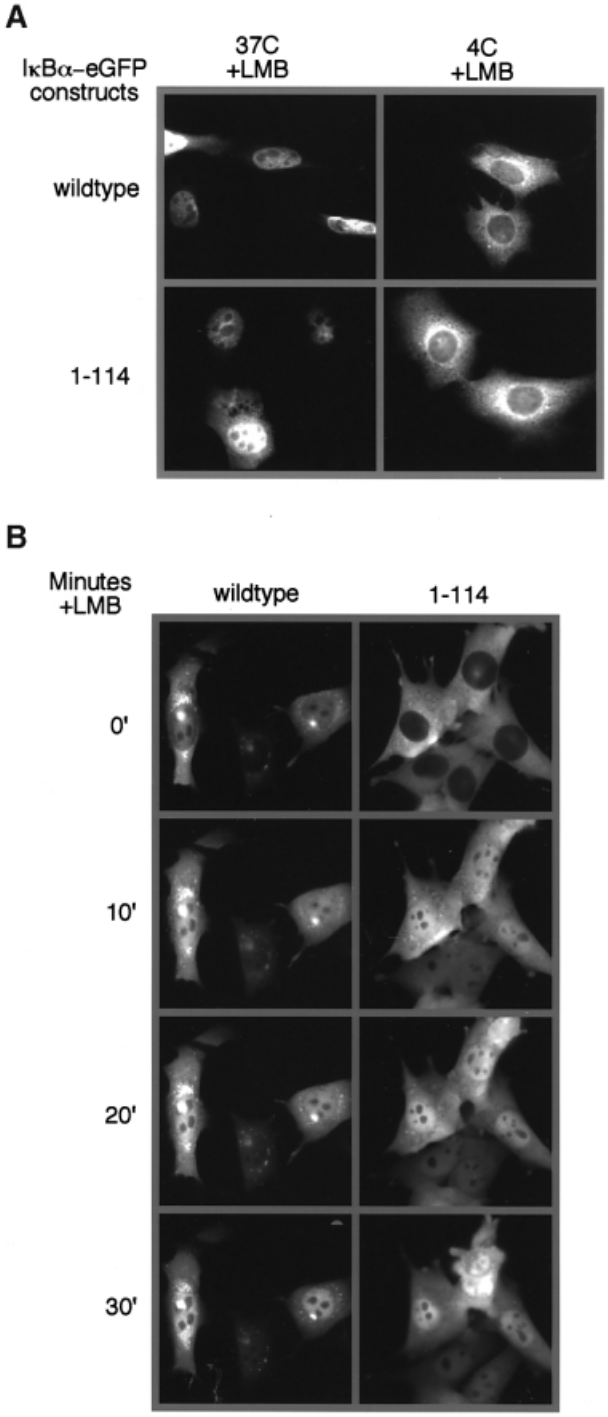


Fig. 5. The shuttling of IκBα is quick and energy dependent. (**A**) IκBα–eGFP does not import at 4°C. 3T3 cells transfected with the IκBα–eGFP construct indicated were treated with 5 nM LMB at 37 or 4°C for 1 h. (**B**) 3T3 stable cell lines expressing the IκBα–eGFP derivatives were cultured on large cover slips for Bioptics FSC2 live cell chamber and maintained at 37°C. At time 0, 5 nM LMB and 100 μg/ml CHX were added. A picture was taken every 10 min using the Delta Vision system.

shown). This demonstrates that IκBα is imported efficiently, amassing in the nucleus after nuclear export is blocked by LMB, and indicates that an NLS sequence is contained within aa 1–114.

IκBα–p65 complexes shuttle

According to current tenets of IκBα–NF-κB regulation, formation of the IκBα–NF-κB complex determines the subcellular localization of NF-κB and its subsequent transcription activities. In the absence of IκBα, p65 is nuclear, whereas at steady-state the complex is cytoplasmic, purportedly due to cytoplasmic retention signals within IκBα (Baeuerle and Baltimore, 1988; Beg *et al*., 1992). In addition, several properties of IκBα, e.g. stability and expression levels, are affected by NF-κB binding. In view of the conjoined biological activities of IκBα and NF-κB, we considered the possibility that NF-κB influences the steady-state subcellular localization of IκBα. To address this, we co-transfected excess p65 with several IκBα–eGFP constructs in 3T3 cells. For all co-transfection experiments, the p65 expression vector was present in 4:1 excess to IκBα derivatives.

Wt IκBα–eGFP co-transfected with p65 is cytoplasmic (Figure 6G), but it continues to shuttle, accumulating in the nucleus in response to LMB (Figure 6M). This argues against a 'cytoplasmic retention' model of regulation for IκBα–NF-κB and suggests that localization may not be a 'static position' but a dynamic steady-state, the net result of competing export and re-import rates. Unlike wt IκBα, the localization of the N-terminal NES mutant was altered dramatically from nuclear to cytoplasmic by the co-transfection of p65 (Figure 6H). Alone, this result would support the 'cytoplasmic retention model', where newly synthesized mutant IκBα might bind exogenous p65, forming a cytoplasmic anchored complex before it has the opportunity to import. However, this was disproved when we found that the NES mutant shuttles in the presence of excess p65, relocalizing to the nucleus after the addition of LMB (Figure 6N). This suggested that there was a second NES, one dependent on p65, which could maintain shuttling function and restore cytoplasmic steady-state localization to the IκBα NES mutant. The previously reported C-terminal NES was a likely candidate. We generated a C-terminal NES mutant substituting alanines for leucines in the putative core tetramer (L272A,L274A) and a double NES mutant (I52A,L54A, L272A,L274A). The C-terminal NES mutant was cytoplasmic with or without exogenous p65 (Figure 6C and I) and the protein was import competent (Figure 6O). The double NES mutant was nuclear (Figure 6D), but likewise relocalized to the cytoplasm when co-transfected with p65 (Figure 6J) and retained import function (Figure 6P). Thus, the C-terminal NES was not responsible for restoring the cytoplasmic steady-state localization to the N-terminal NES mutant. Was p65 the source of the second NES?

To address the question of p65-mediated export, we generated two additional IκBα N-terminal NES mutant eGFP fusion proteins: a deletion of the C-terminal NES that retains the majority of sequences involved in interaction with p65, 1–219 (I52A,L54A) (Jaffray *et al*., 1995; Malek *et al*., 1998); and a larger deletion, 1–114 (I52A,L54A), which includes the N-terminus through to the beginning of the second ARD repeat. Transfected alone, both proteins were nuclear (Figure 6E and F). Co-transfected with p65, NES mutant 1–219 relocalized to the cytoplasm (Figure 6K). In contrast, p65 did not alter the nuclear localization of NES mutant 1–114 (Figure 6L). Transfections in 293T cells produced the same results

(data not shown). These data suggest that: (i) IκBα and NF-κB can shuttle as a complex; and (ii) it is interaction with p65 and not another protein expressed or activated as a result of the excess p65 that causes the cytoplasmic localization of the IκBα NES mutants. Moreover, the rate of shuttling for the IκBα NES mutant +p65 (Figure 6S) was similar to the relocalization of the wild-type constructs (Figure 5B).

Because our data suggests that p65 contains some level of intrinsic export function independent of the IκBα NES, we analyzed p65 for export function in the absence of IκBα. For this experiment, we used $p65^{-/-}$ murine embryo fibroblasts (MEFs) and HeLa cells in a heterokaryon assay. Prior to fusion, we degraded IκBα (Figure 7D) and concentrated p65 within the HeLa nuclei. This analysis would allow us to determine whether p65 would relocalize from the HeLa nuclei to the $p65^{-/-}$ MEF nuclei. Figure 7B shows that within a heterokaryon, p65 levels are the same in MEF and HeLa nuclei. Nuclear HeLa p65 exported and subsequently re-imported equally into both MEF and HeLa nuclei. In contrast, LMB treatment of the heterokaryons prevented the nuclear export of HeLa p65 and subsequent import and accumulation in the MEF nuclei (Figure 7C). Together, these results reveal that: (i) p65 shuttles, exporting and re-importing in the absence of IκBα or new protein synthesis; and (ii) p65 export is LMB sensitive. Our conclusions are concordant with a recent report in which a putative CRM1-dependent NES was identified in p65 (Harhaj and Sun, 1999).

IκBα and p65 shuttle as a complex and can be activated in the nucleus by TNFα-induced degradation

Our studies show that IκBα–p65 complexes resulting from exogenous expression shuttle continuously between the nucleus and cytoplasm. Next, we sought to determine whether the same was true for the endogenous complex. As previous studies have indicated, IκBα and p65 co-localize in the cytoplasm in unactivated cells. But after 2 h of LMB treatment, IκBα and p65 co-localize to the nucleus (Figure 8). To determine if p65 accumulating in the nucleus in response to LMB was transcriptionally active, we transfected 3T3 cells with the HIV-CAT and p65, then treated the cultures with LMB, TNFα or both. CAT activity was the same in the presence or absence of LMB, indicating that p65 localized in the nucleus by these conditions is transcriptionally inhibited (Figure 9A). Treatment of transfected cultures with LMB, however, did not prevent activation of NF-κB by TNFα. Pre-treating cells with LMB decreased TNFα-mediated activation by 50% (Figure 9A). These results suggest that: (i) blocking CRM1 export results in the nuclear accumulation of inhibited p65, presumably complexed with IκBα; and (ii) inhibited nuclear IκBα–p65 complexes can be activated.

Next, we used an electrophoretic mobility shift assay (EMSA) to test our hypothesis that IκBα–p65 complexes

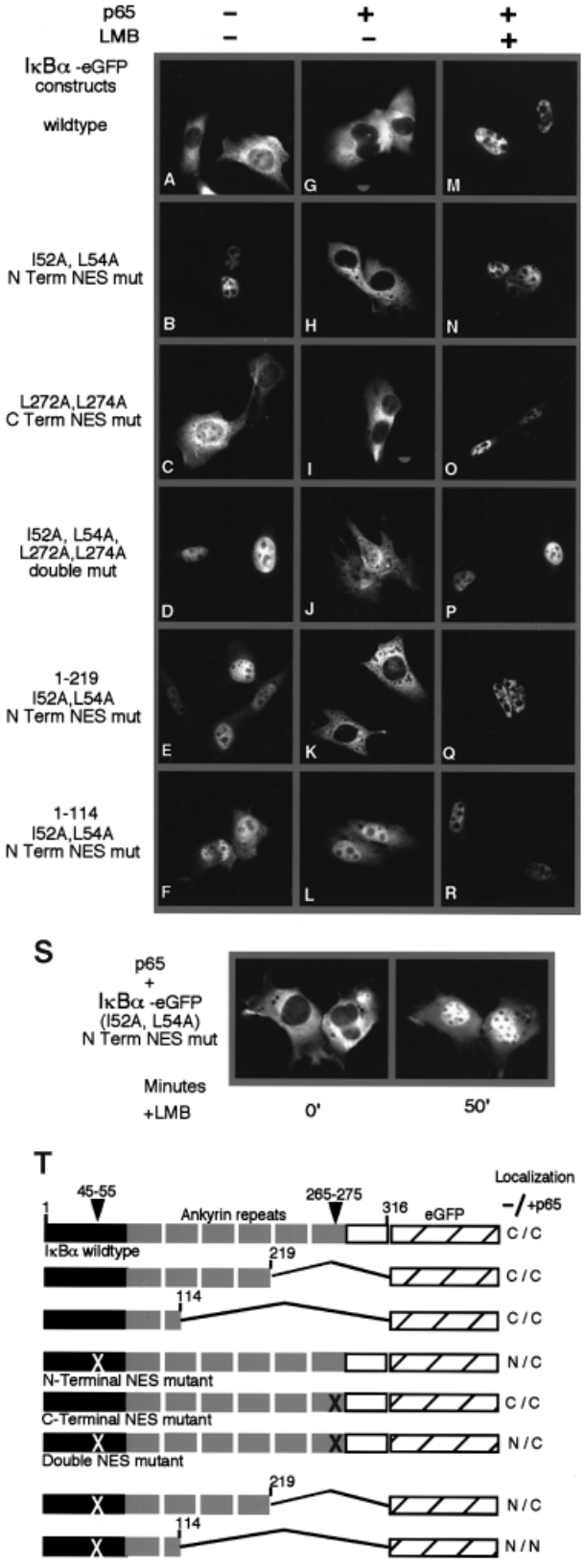


Fig. 6. p65 can alter the subcellular localization of IκBα NES mutants. (**A**–**F**) N-terminal NES mutants are nuclear, but not the C-terminal NES mutant. (**G**–**K**) Co-expressing p65 results in the cytoplasmic localization of all NES mutants except for the C-terminus/ARD deletion 1–114 (**L**). (**M**–**Q**) The relocalized NES mutants continue to shuttle, accumulating in the nuclei within 2 h of 5 nM LMB treatment. 3T3 cells were transfected with 0.4 μg of the indicated IκBα–eGFP vector ± 01.6 μg of p65. (**R**) LMB does not affect the localization of the nuclear mutant 1–114. (**S**) NES mutant shuttles at rates similar to that of wt IκBα in 3T3 cells when transiently co-expressed with excess p65. At time 0, 5 nM LMB and 100 μg/ml CHX were added to the media. A video of this time-lapse can be seen at http://www.salk.edu/LABS/idl/hope.html. (**T**) Summary of IκBα–eGFP derivatives tested for export.

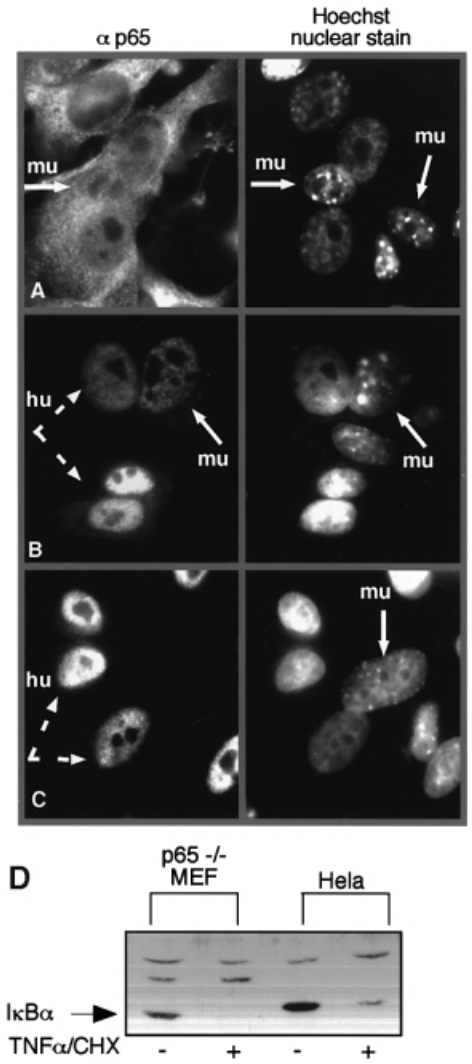


Fig. 7. Heterokaryon assay. p65 can shuttle in the absence of IκBα and new protein synthesis, and its shuttling is LMB sensitive. HeLa cells (hu) were co-cultured with $p65^{-/-}$ MEFs (mu) prior to PEG fusion. (**A**) p65 is found in the cytoplasm, and both human and murine nuclei in untreated heterokaryons. Un-fused murine nuclei are at the bottom right of the panel. (**B**) p65 shuttles from the HeLa nuclei into the $p65^{-/-}$ nuclei after IκBα has been degraded. Prior to fusion, co-cultures were treated with 10 ng/ml TNFα and 100 μg/ml CHX for 30 min. LMB (5 nM) was added and cells were incubated for an additional 90 min. After fusion, cells were rinsed with >1 l of PBS, incubated for 2 h in media containing 100 μg/ml CHX then analyzed. HeLa polykaryon is shown at the bottom of the panel. (**C**) p65 does not shuttle to murine nuclei in the presence of LMB. Prior to fusion, cells were treated as in (B). After fusion, heterokaryons were incubated for 2 h in media containing 100 μg/ml CHX and 5 nM LMB. (**D**) Western blot showing that IκBα is degraded in both HeLa and MEF cells after 30 min of TNFα and CHX treatment. Total protein (75 μg) for each sample was resolved on an 11% SDS–PAGE gel.

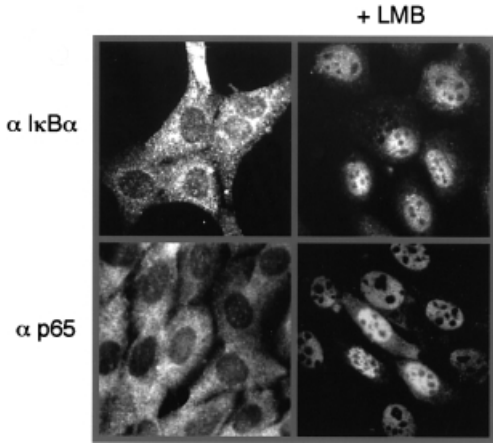


Fig. 8. Endogenous IκBα and p65 shuttle, and their shuttling is sensitive to LMB. 3T3 cells were treated with 5 nM LMB at 37°C for 2 h.

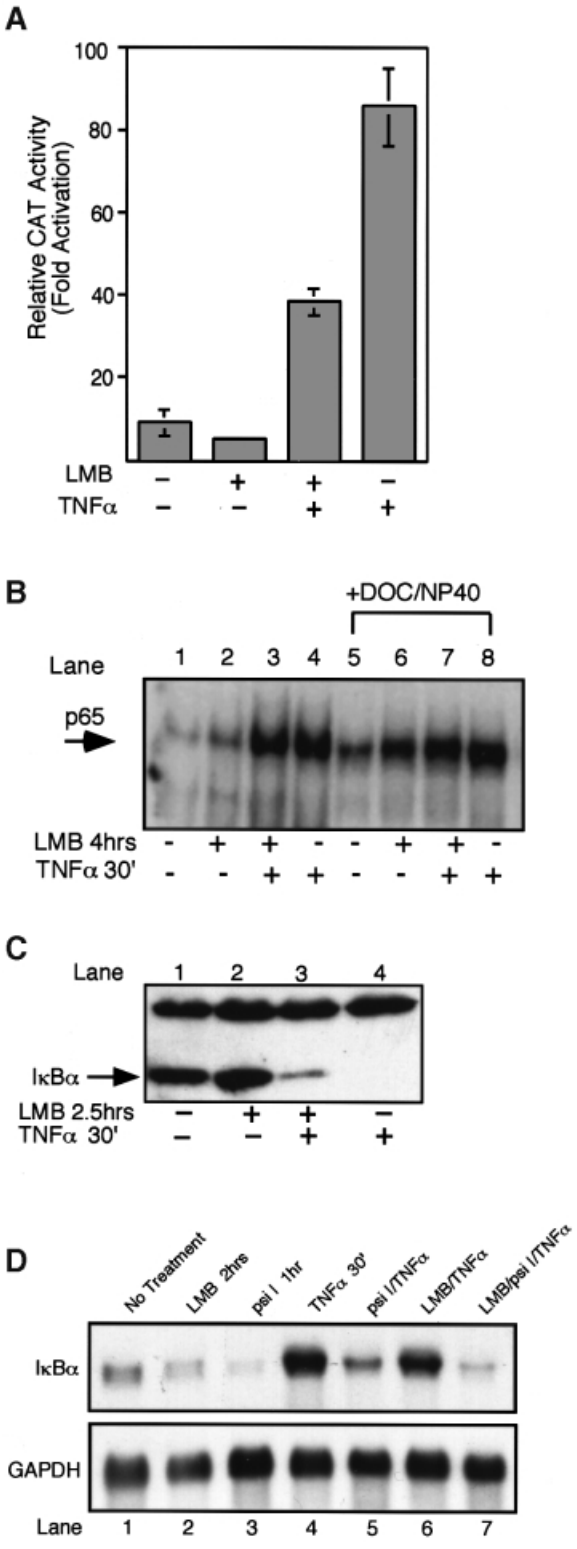
shuttle and can be activated in the nucleus. We assayed nuclear extracts derived from 3T3 cells treated with LMB, TNFα or both for NF-κB binding of DNA. Figure 9B (lane 2) shows that LMB did not activate NF-κB in 3T3 cells since no difference is observed relative to untreated cells (lane 1). In contrast, specific NF-κB–DNA binding activity is induced by 30 min of TNFα treatment in the presence or absence of LMB pre-treatment (Figure 9B, lanes 3 and 4). Additional aliquots of nuclear extracts were treated with deoxycholine/Nonidet P-40 (DOC/NP-40), disrupting the IκBα–NF-κB complexes, thereby freeing previously inhibited nuclear NF-κB for DNA binding. Administration of DOC/NP-40 to LMB-treated extracts increased NF-κB–DNA binding (Figure 9B, compare lanes 2 and 6), demonstrating that LMB treatment leads to the nuclear accumulation of IκBα–NF-κB complexes. Furthermore, DOC/NP-40 treatment of nuclear extracts shows that a majority of the IκBα–NF-κB complex pool becomes trapped in the nucleus upon LMB addition. When nuclear IκBα–NF-κB complexes are disrupted by DOC/NP-40, there is little difference in DNA binding between cases of LMB treatment alone, LMB followed by TNFα, or TNFα alone (Figure 9B, lanes 6–8). These data support our hypothesis that IκBα and NF-κB shuttle as an inhibited complex and indicate that it is possible to activate the nuclear pool of IκBα–p65 with TNFα. The same results were obtained with 293T cells (data not shown).

To investigate the mechanism of activation of the nuclear NF-κB pool by TNFα, we analyzed the levels of IκBα in the presence or absence of LMB by Western blot analysis. To ensure that LMB resulted in nuclear localization of IκBα and p65, cover slips were included in plates. Cells on cover slips were fixed (and subsequently stained for IκBα and p65) immediately preceding harvest (data not shown). 3T3 cells were treated with LMB for 2 h followed by 30 min exposure to TNFα. Western blot analysis (Figure 9C) showed that LMB treatment alone had no effect on IκBα levels (lane 2). In contrast, TNFα treatment reduced IκBα to undetectable levels (Figure 9C, lane 4). Finally, most of the IκBα was degraded as a consequence of TNFα treatment after LMB induced nuclear localization (Figure 9C, lane 3). These results reveal that TNFα can induce the degradation of nuclear IκBα.

Activation of nuclear NF-κB is dependent on proteasome function

To test whether TNFα activated nuclear IκBα–p65 complexes by the same mechanism as cytoplasmic complexes, we tested the ability of proteasome inhibitor (Proteasome Inhibitor 1, Calbiochem) to inhibit activation in the presence of LMB. For this study, we used Northern blot analysis to ascertain NF-κB activation by measuring transcription of NF-κB target genes. We probed for IκBα,

an early target for NF-κB-activated transcription (Sun *et al*., 1993; Cheng *et al*., 1994; Chiao *et al*., 1994). We also probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to show equal loading. Northern blot results supported our previous findings. Figure 9D shows that LMB did not activate transcription (lane 2), but nuclear IκBα–p65 could be activated by TNFα (lane 6). Northern data also showed that proteasome inhibitor blocked TNFα activation of cytoplasmic IκBα–p65 (Figure 9D, lane 5) and nuclear complexes (lane 7). A cover slip was included in each plate of cells harvested for a Northern blot. The cells on these cover slips were fixed and stained for IκBα and p65 as a control to ensure that both TNFα and LMB were functional (data not shown).



Discussion

Currently, it is believed that IκBα and NF-κB associate in the cytoplasm forming a complex that is incompetent for nuclear localization. After signal-induced degradation of IκBα, the NLS of Rel dimers are exposed, facilitating their relocalization to the nucleus. The results presented here indicate that the localization of IκBα is very dynamic. IκBα contains both nuclear localization and nuclear export function and shuttles continuously between the nucleus and cytoplasm. Our studies suggest that the N-terminal NES, aa 45–55, is the sequence responsible for nuclear export and subsequent cytoplasmic localization of IκBα. Identification of an N-terminal NES also corroborates previously published observations that sequences within the N-terminus, but not the C-terminus, inhibited the function of HIV-1 Rev in an NF-κB-independent manner (Wu *et al*., 1997). The presence of an LMB-sensitive NES in the N-terminus suggests that Rev inhibition was a consequence of competition for the CRM1 export pathway. We find no evidence of NES function in the previously reported C-terminal NES. It can not complement the function of an NES mutant of HIV Rev (Figure 1C) or mediate export of GST fusion proteins in microinjection analysis (Figure 2). In addition, point mutations within the putative C-terminal NES or its deletion have no effect on the steady-state cytoplasmic localization of IκBα (Figure 6C). In contrast, point mutations disrupting the N-terminal NES cause IκBα to be nuclear (Figures 2F and 3D).

Previous reports have shown the IκBα N-terminus mediated cytoplasmic localization of p50 and c-Rel homodimers (Latimer *et al*., 1998; Luque and Gelinas, 1998) ostensibly resulting from the N-terminus masking one or both of the NLSs in the dimer. But recent reports on the crystal structure of the IκBα–NF-κB complex show that

Fig. 9. Endogenous IκBα and p65 shuttle as a complex and can be activated in the nucleus. (**A**) TNFα can induce transcription of HIV-1-LTR-CAT reporter even when IκBα–p65 complexes are localized in the nucleus. 293T cells were transfected with HIV-CAT and β-gal. Forty-eight hours after transfection, 5 nM LMB was added. Three and a half hours later, 10 ng/ml TNFα was added. Cells were harvested 14.5 h after TNFα addition. Normalized CAT values shown are the mean (± SEM) of three experiments. (**B**) Gel shift assay shows that IκBα–p65 shuttles as inhibited complexes (lane 2) and that nuclear complexes can be activated by TNFα (lane 3). 3T3 cells were pre-treated with 5 nM LMB for 3.5 h then treated with 10 ng/ml TNFα and incubated for an additional 30 min. Nuclear fractions were incubated with HIV-LTR κB oligo probe and were resolved on a 4% native polyacrylamide gel. (**C**) Western analysis shows that TNFα induces degradation of nuclear IκBα. 3T3 cells were pre-treated with 5 nM LMB for 2 h and CHX for 30 min. Then 10 ng/ml TNFα was added and cells were incubated for an additional 30 min. Total protein (75 μg) was resolved on 11% SDS–PAGE. (**D**) Northern analysis shows that proteasome inhibitor blocks activation of nuclear IκBα–p65 complexes (lane 7). 3T3 cells were pre-treated with 5 nM LMB for 2 h and 50 μM proteasome Inhibitor 1 (PSI 1) for 1 h. Then 10 ng/ml TNFα was added and cells were incubated for an additional 30 min. Total RNA (15 μg) was resolved on a 1.2% agarose/formaldehyde gel.

sequences within the first and second ARD repeat, but not the N-terminus, mask the NLSs of the dimer (Huxford *et al*., 1998; Jacobs and Harrison, 1998). Our identification of the N-terminal NES in IκBα overlapping exactly the same region necessary for cytoplasmic retention of the Rel homodimers suggests a re-interpretation of the results (Latimer *et al*., 1998). We propose that it is the export function of the IκBα N-terminal NES that mediates the steady-state cytoplasmic localization of Rel dimers rather than NLS masking.

Our analysis reveals that nuclear localization of IκBα is mediated by sequences located within the ARD. Hannink and co-workers showed that ARDs of diverse proteins, including IκBα, could function as NLSs when fused to a cytoplasmic protein. They found that three point mutations within a hydrophobic cluster (aa 114–124) of the second repeat disrupted nuclear accumulation of exogenous IκBα (Sachdev *et al*., 1998). However, our data indicate that the hydrophobic cluster of ARD repeat 2 is not necessary for import and that ARD repeat 1 through to aa 114 is sufficient for temperature-dependent nuclear import of IκBα (Figure 5A). It is likely that the mutations within repeat 2 alter the kinetics of shuttling. By decreasing the rate of import, steady-state localization of the exogenous IκBα will favor the cytoplasm. Consistent with this model, we find that IκBα derivatives containing ⩽4 ARD repeats are more cytoplasmic than constructs with the full complement of six repeats (Figure 5B). Regardless of steady-state localization, all derivatives that include aa 1–114 continue to shuttle, illustrated by relocalization to the nucleus after treatment with LMB (Figure 3E–H).

Our results support the paradigm that interaction with NF-κB influences the kinetics of IκBα shuttling. Overexpression of p65 will dramatically alter the steady-state localization of IκBα NES mutants (Figure 6H–K). Simultaneous overexpression of p65 results in cytoplasmic localization of the usually nuclear NES mutants with the exception of 1–114 (I52A,L54A) (Figure 6F), and this mutant derivative does not include the ARD repeats where specific IκBα residues contact p65 in a complex (Huxford *et al*., 1998).

Our current analysis reveals NES and NLS activity in both IκBα and p65, supporting a shuttling model for IκBα–NF-κB (p65/p50) complexes. The rapid nuclear accumulation of IκBα NES mutants and p65 in response to LMB treatment (Figure 6N–Q) demonstrates that the complex is shuttling. Likewise, endogenous IκBα–NF-κB complexes relocalize from the cytoplasm to the nucleus after LMB treatment (Figure 8). Moreover, we find that endogenous p65 shuttles continuously between nucleus and cytoplasm in the absence of IκBα (Figure 7B and C). LMB-induced nuclear accumulation of IκBα–NF-κB does not activate NF-κB transcription as detected by a number of assays including the expression of transfected CAT reporters, Northern analysis of induced genes and EMSA analysis of NF-κB specific DNA binding (Figure 9). Suprisingly, we find that nuclear complexes of IκBα and NF-κB are susceptible to signal-induced degradation of IκBα (Figures 4C, 9C and D) and, consequently, can be activated through a proteasome-dependent mechanism. Although activation of nuclear complexes was unanticipated, it is not altogether implausible since it is known that proteasomes are present within the nucleus (Reits *et al*., 1997).

It was reported recently that LMB inhibited the activation of NF-κB as detected by a transfected reporter (Rodriguez *et al*., 1999). However, we believe that the reduced activation observed in the presence of LMB may be due to non-specific inhibition resulting from long exposure to the CRM1 inhibitor. In short-term experiments such as the induction of the IκBα gene by TNFα, LMB had little effect (Figure 9D, lane 6). Furthermore, proteasome inhibitor blocked TNFα activation of LMB-pre-treated cultures equally as well as untreated cultures (Figure 9D, lanes 5 and 7). Together with Western data showing that IκBα from cultures pre-treated for 3 h with LMB is degraded after TNFα stimulation (Figure 9C, lane 3), these data support our conclusion that nuclear IκBα, complexed with NF-κB, can be specifically degraded by proteasomes in the nucleus following stimulation with TNFα.

We propose a dynamic model where the IκBα–NF-κB complex shuttles continuously between the nucleus and cytoplasm; the observed cytoplasmic localization is the result of net nucleocytoplasmic shuttling kinetics in which the rate of export exceeds the rate of import. Ultimately, the shuttling complexes are biologically active, capable of responding to activating stimuli whether the complex is nuclear or cytoplasmic. Ultimately, the ability to activate nuclear complexes may provide clues to why IκBα–Rel complexes shuttle. Shuttling of individual proteins may facilitate the generation of IκBα–NF-κB complexes by increasing the rate at which the factors associate. From this perspective, shuttling IκBα may act as a buffer against 'leaky' NF-κB transcription, preventing any NF-κB that evades binding a cytoplasmic inhibitor from inappropriately activating transcription. The presence of nuclear IκBα would not inhibit a rapid transcription response to stimuli because, as we have demonstrated, nuclear IκBα is not protected from signal-induced proteolysis.

One consequence of IκBα–NF-κB complex shuttling would be the presence of small pools of IκBα–NF-κB in the nucleus at all times. The degradation of IκBα in these complexes would allow rapid binding of NF-κB with its target DNA without any delay associated with the time it takes for nuclear translocation (Tenjinbaru *et al*., 1999). For proper regulation, some genes might require such rapid induction after specific stimulation that translocation of cytoplasmic pools of NF-κB may be a bottleneck (Scheinman *et al*., 1995). Therefore, the nuclear pools may act as initiators of the genes most sensitive to NF-κB activation.

The ubiquitous IκB–Rel transcription system is undoubtedly regulated by numerous interacting parameters to direct precisely such a diversity of cellular processes in a tissue-specific and signal-dependent manner. The variable kinetics of protein and complex shuttling demonstrated here present a new level of complexity in the subcellular localization and concentration and offer potentially novel modes of IκBα–NF-κB regulation yet to be investigated.

Materials and methods

Plasmid constructs

GST and RevΔNES IκBα derivatives were generated by PCR of human IκBα with oligonucleotides creating flanking 5′ *Bgl*II and 3′ *Xba*I sites.

IκBα derivatives were cloned into RevΔNES vector as described previously (Kim *et al*., 1996). GST–IκBα fusion proteins were based on pGEX-2T expression plasmid (Pharmacia). For eGFP fusion proteins, a *Bgl*II site was introduced at the desired 3′ end of IκBα in pCMX-murine IκBα (Chiao *et al*., 1994). eGFP cDNA from pEGFP-N1 (Clontech) was cloned in-frame to the C-terminus of IκBα. Plasmids, HIV-CAT and HIV-CAT mut κB, were gifts from Dr D.Trono (University of Geneva, Switzerland). All mutations of IκBα were constructed by site-directed mutagenesis with PCR.

Cell culture and transfections

3T3, 293T, CV1 and HeLa cells were grown in Dulbecco's modified Eagle's medium, and MEF cells were grown in RPMI-1640 (Bio-Whittaker). Both media were supplemented with 10% fetal calf serum and streptomycin. 293T cells were transfected by the calcium phosphate method. 3T3 and CV1 cells were transected with Superfect (Qiagen). Stable cell lines were generated as described previously (Izumi *et al*., 1991) using 3 μg/ml blastocidin (Invitrogen) in NIH 3T3 cells.

CAT assays

For RevΔNES complementation, 293T cells in six-well plates were transfected with 0.2 μg of CAT reporter, pDM128, 0.2 μg of pCMV β-gal, 1 μg of the indicated RevΔNES-IκBα construct and sufficient pUC19 for 2 μg of total DNA. For the NF-κB inhibition CAT assay (Figure 4A), 293T cells in six-well plates were transfected with 0.2 μg of HIV-CAT reporter, 0.2 μg of pCMV β-gal, 0.1 μg of pCMXp65 (Chiao *et al*., 1994), pCMVpoly(A) for promoter balance and the specified quantity of IκBα derivative. In Figure 4B, CV1 cells in 10 cm plates were transfected with 5 μg of HIV-CAT, 2 μg of CMV β-gal, pCMVpoly(A), 4 μg of TAX and 1 μg of either IκBα wt or IκBα (I52A,L54A). In Figure 4C, 3T3 cells in six-well plates were transfected with 0.2 μg of HIV-CAT, 0.2 μg of CMV β-gal, 0.02 μg of pCMXp65, 0.05 μg of either IκBα wt or IκBα (I52A,L54A), pCMVpoly(A) and the indicated quantity of TAX expression vector. β-gal activity was used to normalize for transfection efficiency. Samples were resolved by thin-layer chromatography and CAT activity was quantified by PhosophorImager (Molecular Dynamics) and Imagequant software. All results shown were confirmed in three or more separate transfections.

Western blots

Cell pellets from 10 cm plates were resuspended in 250 μl of lysis buffer [10 mM NaCl, 10 mM Tris pH 7.5, 0.5% NP-40, 1% SDS and 1 protease inhibitor cocktail tablet/l (Boehringer Mannheim)]. Lysates were normalized for total protein using BCA Protein Assay (Pierce). For RevΔNES derivatives, lysates were normalized using co-transfected β-gal activity. Protein samples were separated on SDS–PAGE gels then transferred to PVDF-Plus membranes (MSI). Blocked membranes (7% non fat milk/Tris-buffered saline with Tween-20) were incubated with the appropriate primary antibody, washed, then incubated with anti-rabbit Ig horseradish peroxidase (Amersham Life Science). Proteins were visualized by Super Signal WestPico (Pierce).

Fluorescence microscopy

All cells were grown on glass cover slips or two-well chamber slides (Lab Tek). Cells were fixed with 4% paraformaldehyde/PBS, rinsed, and blocked with blocking solution (BLK) [PBS, 15% normal donkey serum (Jackson ImmunoResearch), 0.2% Triton X-100]. Cells were incubated with primary antibody (1′ ab) diluted 1:100 in BLK. Cells were rinsed and incubated with 2′ ab at 1:250 and 100 μg/ml Hoechst 33258 (Sigma) in BLK. Samples were mounted with Gel/Mount (Biomeda). Unless specified, pictures were taken with a Sensicam camera (Cooke Corp.) and Slidebook software (III Denver) with a 40× or 60× objective of a Nikon Diaphot 300. 1′ abs used include: mu monoclonal α-GST, goat α-p65, and a cocktail of rabbit α-IκBα (Santa Cruz Biotechnology).

Microinjection analysis

Concentrated GST fusion proteins ~1 mg/ml (Amicon) with 2.5 mg/ml Rhodamine-Dextran (Sigma) were injected into 3T3 cells plated on fibronectin (0.4 mg/ml)-treated coverslips. For fusion, coverslips were inverted onto 50 μL of polyethylene glycol (PEG) 3200/PBS for 2 min. The coverslips were rinsed in >1 l PBS, then incubated in fresh media for 2 h prior to injection. Microinjection was performed by a semi-automated microinjection system (Eppendorf). After injection, cells were incubated for 60 min and then analyzed by indirect immunofluorescence.

Heterokaryon analysis

HeLa cells were co-cultured with $p65^{-/-}$ MEFs on fibronectin-treated coverslips. Heterokaryons were generated by PEG fusion as above. To degrade IκBα in both cell types and translocate p65 into HeLa nuclei, co-cultures of HeLa and MEF cells on cover slips were treated with 10 ng/ml TNFα and 100 μg/ml CHX for 30 min prior to fusion. Previously, we noted that 60 min of 5 nM LMB treatment resulted in a more complete nuclear localization of p65 than TNFα/CHX alone (data not shown). Hence, LMB was added to the TNFα/CHX media and cells were incubated for an additional 90 min. The cover slips were then rinsed extensively with large volumes of PBS, and cells were fused with PEG and incubated for 120 min in CHX. For LMB treatment, we repeated the above conditions except that after PEG fusion we re-added LMB for the post-fusion incubation period. Two independent heterokaryon assays were performed for each experimental condition. The results of 20 heterokaryons were recorded per experiment.

Northern blots

Fifteen-centimeter plates of 3T3 cells at 70% confluence were harvested. Cell pellets were immediately frozen in a dry ice/EtOH bath. Pellets were resuspended in 250 μl of PBS and 1 ml of RNA STAT-50LS (TEL-TEST, Inc.). Total RNA was resolved on 1.2% agarose/formaldehyde gel and transferred to Duralon UV (Stratagene). DNA probes were prepared with Prime it II kit (Stratagene) using [α-^{32}P]dCTP (Amersham) and IκBα or GAPDH cDNA. DNA probes were hybridized separately to Northern blot using UTRAhyb (Ambion).

Gel shift assay

EMSA was performed as described (Van Antwerp *et al*., 1996). Briefly, nuclear extracts were prepared by the micropreparation technique (Andrews and Faller, 1991). For the pre-binding reaction, 5 μg of nuclear extract were mixed with 0.5 μg of poly dI-dC (Pharmacia) and DNA binding buffer in a total volume of 10 μl and incubated on ice for 20 min. Double-strand oligonucleotide encoding the HIV-LTR κB site was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Dupont NEN). The oligonucleotide probe was then added to the pre-binding reaction at 6000 c.p.m. per sample. Binding was brought to equilibrium by further incubation at room temperature for 30 min. The samples were loaded onto a 4% native polyacrylamide gel and run for 2 h at 150 V in 0.25× TBE. Dried gels were visualized by autoradiography. For DOC/NP-40 treatment of nuclear extracts, 0.8% DOC was added for the first 10 min of the pre-binding reaction, followed by the addition of 1.2% NP-40 for the final 10 min. The remaining procedure was identical to untreated extracts.

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