Accelerated Publication

IκB Family Members Function by Different Mechanisms*

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**The IκB family of proteins regulates NF-κB-dependent
transcription by inhibiting DNA binding and localizing
these factors to the cell cytoplasm. IκBα does this by
shifting the balance between nuclear import of Rel pro-
teins and their export from the nucleus. Here we show
that, unlike IκBα, IκBβ and IκBε appear to sequester p65
or c-Rel in the cytoplasm by inhibiting nuclear import.
Furthermore, because IκBβ does not undergo nucleocy-
toplasmic shuttling, it cannot remove nuclear proteins
like IκBα does. We conclude that the mechanism of ac-
tion differs among IκB family members.**

The NF-κB[1]/Rel family of transcription factors plays a cen-
tral role in immune and inflammatory responses (1). In most
cell types these proteins are sequestered in the cell cytoplasm
complexed to a family of inhibitory IκB proteins (2, 3). Cellular
activation results in IκB degradation, which leaves the DNA-
binding protein free to translocate to the nucleus and activate
gene expression. Because of the widespread effects of NF-κB
activation, its localization in the cytoplasm must be strictly
maintained. IκBα-deficient mice are a striking example of the
importance of NF-κB sequestration in the cytoplasm; these
mice die of a wasting disease that has been attributed to tumor
necrosis factor-α production (4, 5). Nuclear factor-κB has also
been detected in several diseased tissues, where it has been
proposed to contribute to the pathology in part by inhibiting
apoptosis (6, 7).

The association of Rel with IκBα has been proposed to hide
the NLS of Rel proteins (8, 9), thereby precluding nuclear entry
of the transcription factor. In addition to hiding the NLS,
association with IκBα also inhibits DNA binding by NF-κB.
Thus, association of NF-κB with IκB ensures that NF-κB-de-
pendent gene transcription occurs only when cells are stimu-
lated appropriately.

Recently, IκBα has been shown to shuttle between the nu-
cleus and the cytoplasm. Nuclear entry is mediated by a non-
classical NLS located in the second ankyrin repeat of IκBα
(10–12), and nuclear export is determined by a CRM1-depend-
ent nuclear export sequence located in the N-terminal domain

preceding the first ankyrin domain (13–15). A second nuclear
export sequence has been identified at the C terminus of IκBα,
but its functional significance is unclear at present (16, 17).
The observation that cytoplasmic sequestration of p65·RelA
also required nuclear export was unexpected and led to a reas-
sessment of the existing sequestration model. We and others
(13–15) have proposed that the cytoplasmic location of Rel
proteins by IκBα is a dynamic process that depends on the
active export of Rel·IκBα complexes out of the nucleus.

In our earlier studies we also showed that IκBβ and IκBε,
unlike IκBα, do not shuttle via the CRM1 pathway. Specifi-
cally, the subcellular distribution of GFP-IκBβ or GFP-IκBε in
yeast was not affected by a mutation in the *CRM1* gene, and
leptomycin B (LMB) treatment did not alter the location of
these proteins in transiently transfected mammalian cells. In
addition, we did not detect an association between CRM1 pro-
tein and either IκBβ or IκBε in a yeast two-hybrid assay (15). In
this paper we demonstrate that cytoplasmic retention of Rel
proteins by IκBβ and IκBε involves sequestration rather than
tilting the balance of nuclear import and export as is the case
with IκBα. Furthermore, although newly synthesized IκBβ can
enter the nucleus, it cannot restore nuclear Rel proteins to the
cytoplasm. These observations suggest that IκBβ and IκBε
function differently from IκBα.

MATERIALS AND METHODS

Cell Lines and Strains—D5 h3 T hybridoma cells and A20 mature B
cells were grown in Dulbecco's modified Eagle's medium and RPMI
1640 medium, respectively, with 10% heat-inactivated fetal bovine se-
rum, 50 μM β-mercaptoethanol and antibiotics. COS cells were cultured
in Dulbecco's modified Eagle's medium with 10% newborn calf serum
and antibiotics. Yeast strain W303 and its transformants were gener-
ally grown in synthetic medium with the appropriate amino acid and
nitrogen base supplement.

Plasmids—pGFP-p65 and pCDNA3-HA-IκBα have been described
previously (15). pGFP-cRel contains full-length murine cRel in frame
after GFP. pCDNA3-Myc-IκBβ and pCDNA3-Myc-IκBε were made by
inserting full-length murine IκBβ and IκBε cDNA, respectively, in
frame behind a c-Myc tag (MEQKLISEEDL). Yeast galactose-inducible
plasmid encoding GFP-p65 and copper-inducible HA-IκBα (pCuIκBα)
have been described previously (15). The copper-inducible HA-IκBβ was
made by replacing the IκBα gene with a murine IκBβ full-length gene
in the same vector. All plasmids used in this study were confirmed by
sequencing, and expression of proteins was verified by immunoblotting.

Immunostaining—The procedures for immunostaining adherence
cells were the same as described previously (15). For staining suspen-
sion cells (T and B cells), the procedures were also as described previ-
ously (18).

Protease Digestion—The proteases Asp-N and Lys-C were purchased
from Roche Molecular Biochemicals. Proteases were used according to
the manufacturers' specifications.

Fluorescence Microscopy—The subcellular localization of GFP and
the immunofluorescence signals were observed by fluorescence micros-
copy (Axiophot II, Zeiss) with a GFP generic filter, fluorescein isothio-
cyanate, rhodamine, and DAPI filter.

RESULTS

The nuclear export property of IκBα is essential for cytoplas-
mic location of Rel proteins. However, IκBβ and IκBε, which
are not nucleo-cytoplasmic shuttling proteins, can also effec-
tively localize Rel proteins to the cytoplasm. One possibility
was that cytoplasmic retention by IκBβ/ε may be mediated by
export determinants in the Rel proteins. To test this possibility,
we coexpressed green fluorescent protein (GFP)-tagged Rel
proteins with IκBβ or IκBε in COS cells and assayed the loca-

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[1] The abbreviations used are: NF-κB, nuclear factor-κB; NLS, nuclear
localization signal; GFP, green fluorescent protein; LMB, leptomycin B;
HA, hemagglutinin; DAPI, 4,6-diamidino-2-phenyllindole.

A

B
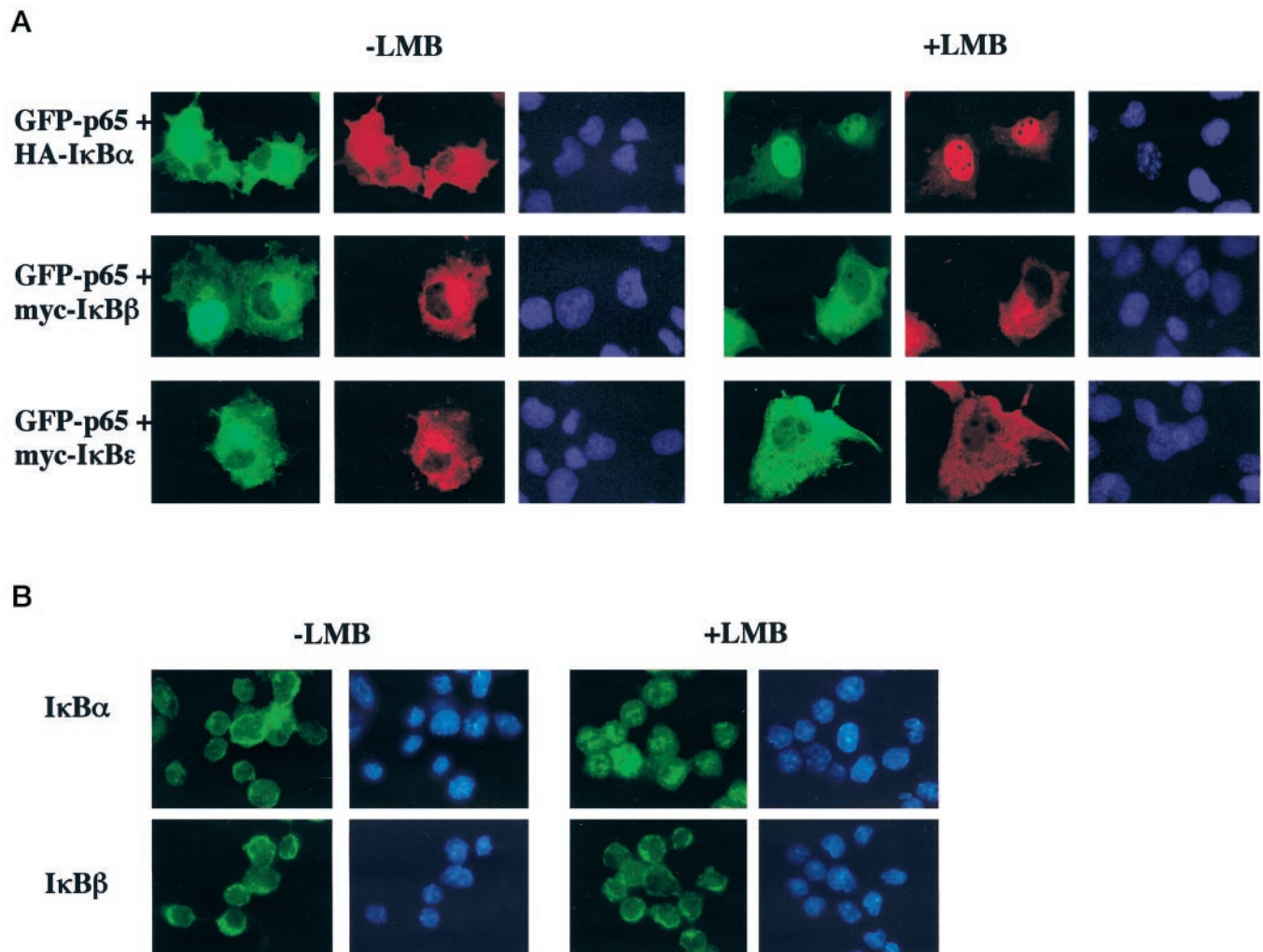
Fig. 1. **Rel·IκBα complexes shuttle continuously, but Rel·IκBβ complexes do not.** *A*, GFP-p65 was transiently transfected with HA-IκBα, Myc-IκBβ, or Myc-IκBε into COS cells. Half the cells were then treated with LMB (10 ng/ml) for 3 h. Untreated (*left panel*) or LMB-treated cells (*right panel*) were fixed for fluorescent visualization. *Green* fluorescence shows a GFP-p65 signal. The *red* fluorescence shows an IκB signal from rhodamine-conjugated antibodies against either the HA tag or the Myc tag. *Blue* fluorescence shows DAPI staining of nuclei. *B*, A20 B cells were settled on specially treated coverslips (Fisher). Half of these were treated with LMB (100 ng/ml) for 45 min. Cells with or without LMB treatment were fixed and permeabilized for immunostaining. *Green* fluorescence shows endogenous IκBα (*first row*) and IκBβ (*second row*) detected by fluorescein isothiocyanate-conjugated antibodies against anti-IκBα and anti-IκBβ respectively. Representative results are shown from one of three independent experiments.

tion of Rel proteins by GFP fluorescence. Both p65 and c-Rel (data not shown) were located in the cytoplasm in the presence of IκBβ (Fig. 1*A, left panel*). However, these complexes did not translocate to the nucleus when the cells were treated with LMB, an inhibitor of CRM1-mediated nuclear export (Fig. 1*A, right panel*). Therefore, CRM1 was not involved in determining the subcellular location of these complexes. Similar results were obtained with IκBε. As expected, IκBα-associated p65, or c-Rel (data not shown), was predominantly nuclear in LMB-treated cells (Fig. 1*A, top row*). Thus, cytoplasmic retention by IκBβ and IκBε may involve true sequestration rather than a balance between import and export as is the case with IκBα.

These observations were confirmed in mammalian cells by investigating the shuttling dynamics of endogenous Rel·IκB complexes. Endogenous proteins in mature B (A20) and mature T (D5 h3) cell lines (data not shown) were visualized by staining fixed, permeabilized cells with anti-IκBα, or anti-IκBβ, antibodies in the presence or absence of LMB to block nuclear export. In untreated cells both IκBs were predominantly cytoplasmic (Fig. 1*B, left panel*). A 1-h LMB treatment induced considerable nuclear translocation of IκBα but not IκBβ (Fig. 1*B, right panel*). Because most of the cellular IκB is associated

with Rel proteins, we concluded that Rel·IκBα complexes shuttled continuously, but Rel·IκBβ complexes did not. Lack of Rel·IκBβ shuttling is consistent with sequestration being the major mechanism of cytoplasmic retention by IκBβ.

We found more direct evidence for differences in interaction between IκBα or IκBβ and p65 through partial proteolysis assays. p65 protein was expressed by transient transfection in BOSC 23 cells in the presence of HA-IκBα or Myc-IκBβ. The p65·IκB complex was immunoprecipitated from whole cell extract with anti-IκBα antibody or anti-IκBβ antibody and digested with different proteases. The p65 fragments were detected using antibodies directed against the N or C terminus of p65 to estimate the cut site from one or the other end of p65. Only two of seven proteases showed significant differences in the pattern of p65 fragments generated in the presence of IκBα or IκBβ.

p65 alone generated one major fragment when treated with Asp-N of ~28 kDa when assayed from the C terminus (Fig. 2, *lanes 5* and *6*); this corresponds to a cut site located 293 amino acids from the N terminus (Fig. 2, *top*). In the p65·IκBα complex, two bands of approximately equal intensity were seen (Fig. 2, *lanes 1* and *2*), whereas in the p65·IκBβ complex the

faster mobility (23 kDa) band was enhanced. Therefore, cutting at residue 293 was reduced in the p65·IκBα complex compared with p65 alone, allowing the detection of the cut site at residue 360 (which was not evident with p65 alone). This is presumably because of the protection of the p65 NLS by IκBα, which lies close to residue 293 between residues 301 and 304. Cutting at 293 was further inhibited in the p65·IκBβ complex as shown by a relative increase in the intensity of the 23-kDa compared with the 28-kDa band. These observation suggest that the region around residue 293, including the NLS, is more protected in the p65·IκBβ complex.

p65·IκBα and p65·IκBβ complexes were also probed using the
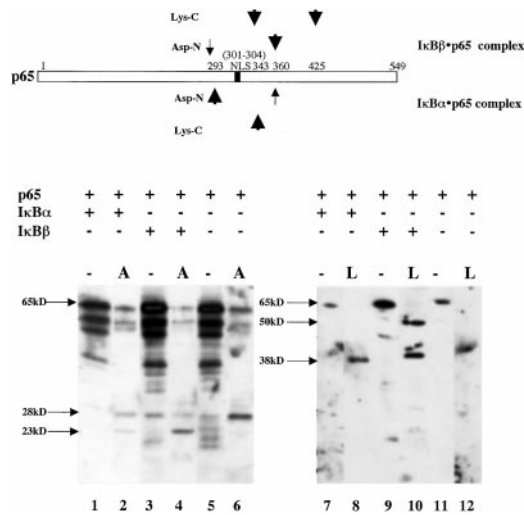
FIG. 2. **Protease digestion of p65·IκBα and p65·IκBβ complexes.** p65 protein was transiently transfected in BOSC 23 cells alone (*lane 1, 2, 7*, and *8*), in the presence of HA-IκBα (*lane 3, 4, 9*, and *10*) or Myc-IκBβ (*lane 5, 6, 11*, and *12*). Anti-IκBα or anti-IκBβ antibodies were used to immunoprecipitate the p65·IκB complex from whole cell extracts. Precipitated materials were digested with Asp-N (*left panel, A*) and Lys-C (*right panel, L*). Undigested samples are indicated by a "−" in the figures. Digested and undigested products were fractionated by SDS-polyacrylamide gel electrophoresis and detected using an antibody against the C terminus of p65 (*left panel*) or antibody against the N terminus of p65 (*right panel*). Relevant protease sites of p65 were predicted by MacVector version 6.0 (*top panel*). NLS represents the nuclear localization signal of p65, with critical residues between residues 301 and 304. *Arrows* (in the *lower panels*) indicate the relative degree of Lys-C or Asp-N cutting in the IκBβ·p65 and IκBα·p65 complexes.

protease Lys-C and p65 antibodies directed against the N terminus. Increased cutting at the residue 425 site was evident in the IκBβ complex compared with the IκBα complex (Fig. 2, *lanes 8* and *11*). These observations also support the interpretation that IκBα and IκBβ interact differently with p65. We suggest that the p65 NLS is better hidden by IκBβ than by IκBα.

The simplest interpretation of the experiments described above was that Rel·IκBβ complexes did not enter the nucleus because the nuclear localization sequences in both proteins were very effectively hidden in the complex. Therefore, the question of nuclear export did not arise. However, the question remained that if any Rel·IκBβ complexes formed in the nucleus, would IκBβ be able to bring the complex out to the cytoplasm? Such a situation may occur at the end of cell stimulation when Rel proteins are already nuclear and new IκBs are synthesized to terminate NF-κB-dependent gene expression. We addressed this question in a yeast model.

We have previously shown that export-dependent cytoplasmic localization of p65 by IκBα can be recapitulated in yeast (15). To test the properties of IκBβ, we coexpressed IκBβ and GFP-p65 from galactose-inducible promoters in wild type or Crm1p-deficient (*crm1-1*) yeast strains. GFP-p65 was located in the cytoplasm under these conditions in both strains (data not shown), correlating closely with the observations in mammalian cells (Fig. 1). In contrast, when GFP-p65 and IκBα were coexpressed in *crm1-1* cells, the complex remained in the nucleus (15). To compare the ability of IκBα and IκBβ to remove nuclear p65, we expressed GFP-p65 using a galactose-inducible promoter, followed by either IκBα, or IκBβ, from a copper-inducible promoter. A 3-h induction with galactose was followed by growth in glucose to suppress GFP-p65 transcription. In cells that did not contain IκB expression vectors, GFP fluorescence was strictly nuclear. Even when cells contained either IκBα or IκBβ expression plasmids, GFP fluorescence was largely restricted to the nucleus, although whole cell expression was observed in ~15% of the cells (Fig. 3, *middle and bottom rows, left panel*). Cytoplasmic expression under these conditions was most likely due to basal IκBα, or IκBβ, expression from the copper-inducible promoter. Induction of IκBα with copper for 2 h resulted in a significant redistribution of GFP-p65 to the cytoplasm, indicating that the newly synthesized IκBα exported nuclear GFP-p65 to the cytoplasm (Fig. 3, *middle row, right panel*). This was mediated by Crm1p because it did not occur in the *crm1-1* strain that contains a mutated

FIG. 3. **Sequential induction of GFP-p65 and IκB.** GFP-p65 was cloned into an expression plasmid with a galactose-inducible promoter. HA-tagged IκBα, or IκBβ, was cloned into an expression plasmid containing a copper-inducible promoter. Yeast strain W303 transformed with both GFP-p65 and HA-IκB expression plasmids was treated with galactose for 3 h to induce GFP-p65 expression (*left panel*). Half of the cells were then treated with glucose to terminate the expression of GFP-p65 followed by 0.75 mM copper sulfate to induce IκB expression (*right panel*). GFP fluorescence was visualized directly with fluorescence microscopy. Whole cell extracts were made from the cells to confirm the induction of GFP-p65, HA-IκBα, and HA-IκBβ proteins by immunoblotting (data not shown). Results shown are from one of three independent experiments.
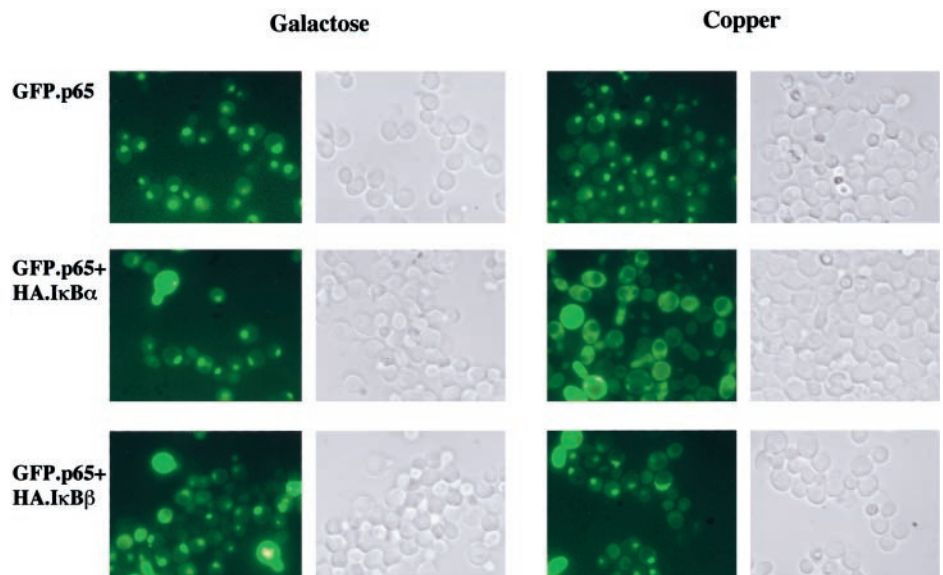
CRM1 gene (data not shown). In contrast, there was little redistribution of GFP-p65 after secondary induction of IκBβ (Fig. 3, *bottom row, right panel*). The small increase in whole cell GFP-p65 expression was probably because of residual GFP-p65 translation during IκBβ induction, which resulted in its cytoplasmic sequestration. These observations indicate that IκBβ cannot remove nuclear Rel proteins to the cytoplasm.

DISCUSSION

We found that p65 or c-Rel associated with IκBβ or IκBε were retained in the cytoplasm, although these IκBs did not shuttle via the CRM1 pathway. We suggest that IκBs, unlike IκBα, sequester rather than shuttle Rel proteins, which implies that there is no available NLS in the Rel·IκBβ (or IκBε) complexes to induce nuclear entry. Conversely, Rel·IκBα complexes must have an available NLS to shuttle. We hypothesize that the Rel and not the IκB component provides the functional NLS of a Rel·IκB complex. Thus, IκBβ or IκBε must hide the Rel NLS more effectively than IκBα. Evidence in favor of this idea was obtained from partial proteolytic studies of p65·IκB complexes.

The sequestration mechanism is based on the lack of an effect of leptomycin B or a mutated CRM1 gene in Rel protein localization by IκBβ. Alternatively, these results could indicate that Rel·IκBβ complexes shuttled by a CRM1-independent pathway. To test this theory, we generated nuclear Rel·IκBβ complexes and determined whether they could reach the cytoplasm by an unidentified pathway. As shown in Fig. 3, IκBβ-mediated GFP-p65 export was inefficient compared with IκBα. We conclude that IκBβ is not an export chaperone like IκBα. Consequently, IκBβ cannot efficiently down-regulate nuclear Rel proteins to restore the resting state of the cell. These results highlight the functional differences between IκBα and IκBβ.

Cheng *et al.* (19) showed that substituting IκBβ for the IκBα gene compensated for the most obvious defects in IκBα$^{-/-}$ mice. They concluded that IκBα and IκBβ were functionally similar and that regulation of expression accounted for most of the phenotype of IκBα-deficient mice. Our contrasting conclu-

sion regarding the mechanism of IκBα and IκBβ function is not at odds with the biological results. Clearly, if sufficient IκBβ is synthesized in a cell, it can retain Rel proteins in the cytoplasm, albeit by a mechanism different from IκBα. The biological results show that retention of Rel proteins by either mechanism is good enough to rescue lethality. That IκBβ is a less efficient nuclear export chaperone than IκBα may be manifest under conditions that were not directly assayed, such as during an immune response or chronic inflammation. We suggest that control of such situations may require the active export-dependent reduction of NF-κB activity.

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