Characterization of the Nuclear Import and Export Functions of IκBe*

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Control over the nuclear localization of nuclear factor κB/Rel proteins is accomplished in large part through association with members of the inhibitor of κB (IκB) protein family. For example, the well studied IκBα protein actively shuttles between the nucleus and the cytoplasm and both inhibits nuclear import and mediates nuclear export of NF-κB/Rel proteins. In contrast, the IκBβ protein can inhibit nuclear import of NF-κB/Rel proteins but does not remove NF-κB/Rel proteins from the nucleus. To further understand how the IκB proteins control the nuclear-cytoplasmic distribution of NF-κB/Rel proteins, we have characterized the nuclear import and nuclear export functions of IκBe. Our results indicate that the IκBe protein, like the IκBα protein, actively shuttles between the nucleus and the cytoplasm. Similar to IκBα, nuclear import of IκBe is mediated by its ankyrin repeat domain and is not blocked by the dominant-negative RanQ69L protein. However, the nuclear import function of the IκBα ankyrin repeat domain is markedly less efficient than that of IκBα, with the result that nuclear shuttling of IκBe between the nucleus and the cytoplasm is significantly slower than IκBα. Nuclear export of IκBe is mediated by a short leucine-rich nuclear export sequence (NES)-like sequence (343VLLPFDDLKI352), located between amino acids 343 and 352. This NES-like sequence is required for RanGTP-dependent binding of IκB to CRM1. Nuclear accumulation of IκBe is increased by either leptomycin B treatment or alanine substitutions within the IκBe-derived NES. A functional NES is required for both efficient cytoplasmic retention and post-induction control of c-Rel by IκBe, consistent with the notion that IκBe-mediated nuclear export contributes to control over the nucleocytoplasmic distribution of NF-κB/Rel proteins.

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The NF-κB/Rel transcription factor family has provided a powerful experimental paradigm for understanding how eukaryotic gene expression is regulated (reviewed in Refs. 1–5). Mammalian cells have five NF-κB/Rel proteins, termed p50, p52, RelA (p65), c-Rel, and RelB. The NF-κB/Rel proteins are defined by the presence of a N-terminal, highly conserved region of 300 amino acids known as the Rel homology domain. The Rel homology domain is responsible for dimerization, nuclear localization, and DNA binding by NF-κB/Rel proteins. Nuclear localization and DNA binding of NF-κB/Rel proteins are regulated in large part through association with members of the inhibitor of κB (IκB) family. In mammalian cells, the major regulatory IκB proteins are IκBα, IκBβ, and IκBe, which share a common domain structure. The N terminus of these IκB proteins constitutes a signal response domain, which is targeted for phosphorylation and ubiquitination by a variety of extracellular stimuli (6–8). The central portion of IκB proteins contains an ankyrin repeat domain comprised of six ankyrin repeats that functions as a protein-protein interaction domain (9–13). The C-terminal regions of IκB proteins are generally acidic, are important for inhibition of DNA binding by NF-κB/Rel proteins, and may regulate the basal degradation rate of IκB proteins (14–17).

Although the IκB proteins were first identified as cytoplasmic inhibitors of NF-κB/Rel proteins, it is now clear that IκB proteins also act in the nucleus. Thus, for example, an important nuclear function for IκBe is post-induction repression of NF-κB activation (18–32). Increased nuclear localization of IκBe has been demonstrated after cytokine-induced degradation of the cytoplasmic pool of IκBe proteins (22, 25). Nuclear import of IκBe is mediated by sequences within the ankyrin repeat domain and does not require the importin-β nuclear import complex utilized by conventional nuclear localization sequences (NLSs) (26). In the nucleus, IκBe is able to displace NF-κB/Rel proteins from their cognate DNA elements and direct the export of NF-κB from the nucleus to the cytoplasm (18–29). IκBe binds to the nuclear export receptor CRM1 via a conventional nuclear export sequence (NES) within its N-terminal domain and functions as an adaptor protein to bridge CRM1 and NF-κB/Rel proteins (20, 28). Although the importance of IκBe-mediated nuclear export of NF-κB/Rel proteins in controlling NF-κB/Rel-dependent transcription is not fully understood, one consequence of IκBe-mediated nuclear export of NF-κB/Rel proteins is to regenerate the cytoplasmic pool of NF-κB in preparation for subsequent reactivation (30).

The IκBβ protein has also been detected in the nucleus (10, 27, 31). In contrast to IκBα, IκBβ forms a ternary complex with NF-κB and DNA and is thought to protect NF-κB from inactivation by IκBα in the nucleus (10, 27). A further contrast between IκBα and IκBβ is the absence of a functional NES in IκBβ (20). IκBα-mediated cytoplasmic retention of NF-κB/Rel proteins is disrupted by the CRM1-specific inhibitor, leptomycin B, whereas IκBβ-mediated cytoplasmic retention of NF-κB/Rel proteins is independent of CRM1 (16, 32). Taken together,

1 The abbreviations used are: NLS, nuclear localization sequence; NF-κB, nuclear factor κB; IκBα, inhibitor of κBα; IκBβ, inhibitor of κBβ; IκBe, inhibitor of κBe; CRM1, coat-lined membrane receptor of post endoplasmic reticulum S-transferase; RanBP1, Ran-binding protein 1; RanGAP, RanGTPase-activating protein; CMV, cytomegalovirus; HA, hemagglutinin; TNF, tumor necrosis factor; wt, wild type; ARD, ankyrin repeat domain.
these data suggest that IxBα and IxBβ have distinct and non-overlapping nuclear functions.

The third major IxB protein, IxBε, is generally regarded as a cytoplasmic protein. However, the possibility that IxBε can shuttle between the cytoplasm and the nucleus has not been examined. We now report that the IxBε protein is able to shuttle between the cytoplasm and the nucleus. Nuclear import of IxBε is mediated by its ankyrin repeat domain, although the IxBε-derived ankyrin repeat domain is less efficient at promoting nuclear localization of a heterologous reporter protein than the ankyrin repeat domain of IxBα. Similar to IxBε, nuclear import of IxBε is not blocked by a dominant-negative Ran protein that otherwise disrupts classical NLS-dependent nuclear import. The IxBε protein interacts with CRM1 in a RanGTP-dependent manner and contains a canonical “leucine-rich” NES-like motif (amino acids 343–352) that is required for Ran-GTP-dependent binding to CRM1. Mutation of this NES-like motif in IxBε results in increased nuclear localization of IxBε. Importantly, the C-terminal NES-like motif of IxBε is required for both efficient cytoplasmic retention and post-induction repression of c-Rel by IxBε. Taken together, our data indicate that IxBε is not restricted to the cytoplasm as previously suggested but actively shuttles between the nucleus and the cytoplasm. Furthermore, our results suggest that one of the functions of IxBε in the nucleus is to direct the export of NF-κB/Rel proteins back to the cytoplasm.

EXPERIMENTAL PROCEDURES

Construction of Recombinant DNA Molecules—The construction of DNA molecules was performed by standard techniques (33). The cytomegalovirus (CMV)-derived vector encoding human IxBα was obtained from Nancy Rice (11). Point mutations of IxBα were expressed as His-tagged proteins in BL21(DE3)pLysS and purified with glutathione-agarose (26).

The PCR-derived DNAs were cloned into pGEX-5X-2 vector as XhoI fragments. All GST expression vectors for IxBα were constructed using PCR with appropriate oligonucleotide primers, and the PCR-derived DNAs were cloned into the pETToh vector as NdeI-XhoI fragments.

The Myc-IxBα protein expression vectors were constructed using PCR, and PCR-derived DNAs were cloned into a CMV-derived 5’GFP-3’ vector as NdeI-XhoI fragments. The Myc-IxBε protein expression vectors were constructed using PCR, and PCR-derived DNAs were cloned into a CMV-derived 5’Myc epitope-tagged vector as BamHI and XhoI. The Myc-NPC-ARD fusion protein containing the IxBα ARD (amino acids 59–243) has been described previously (29). The Myc-NPC-ARD fusion protein containing the IxBβ (amino acids 47–309) and IxBε ARD (amino acids 109–339) was constructed using PCR with appropriate primers, and the PCR-derived DNA was cloned into a CMV-derived mycNPF expression vector (29).

Expression vectors for GST-NLS, RanQ69L, RanBP1, and HA-CRM1 have been described previously (26, 28). Bacterial expression vectors for importin-α and importin-β were obtained from Dirk Gorlich (University of Heidelberg).

Expression and Purification of Recombinant Proteins—The recombinant GST-IxBα were expressed in Escherichia coli strain BL21(DE3)pLyS3 and purified with glutathione-agarose (26).

The RanQ69L, RanBP1, importin-β, importin-α, and CRM1 proteins were expressed as His-tagged proteins in E. coli strain BL21(DE3)pLyS3 and purified with metal-chelate affinity chromatography. HA-CRM1 was purified from insect cells infected with a baculovirus vector (28). The labeling of proteins with fluorescein was carried out as described previously (25).

Cell Culture and Transfection—Either COS1 cells or 3T3 cells derived from IxBα nullizygous mice were grown in Dulbecco’s modified eagle medium containing 10% fetal bovine serum. Transfections were performed with 1 μg of the indicated plasmids and 15 μl of LipofectAMINE (Invitrogen)/35-mm dish. The cellular localization of c-Rel, IxBα, Myc-tagged IxBα, and the Myc-NPC-ARD fusion proteins were analyzed 40 h after transfection by indirect immunofluorescence as previously described (28). The cells were fixed with ice-cold methanol and washed with phosphate-buffered saline before antibody incubations. For TNF-α treatment, the transfected 3T3 cells derived from IxBα nullizygous mice were reseeded with complete medium (Dulbecco’s modified eugle medium containing 10% fetal bovine serum) containing cycloheximide (100 μg/ml; Sigma) and TNF-α (10 ng/ml; Chemicon International) for 2 h. The transfected cells were washed three times with Dulbecco’s modified Eagle’s medium and incubated with complete medium either lacking or containing 20 μg leptomycin B for 3 h before fixation and analysis by double-label indirect immunofluorescence. For detection of the IxBε proteins, a rabbit polyclonal anti-IxBε antibody (Santa Cruz Biotechnology) was followed with a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Jackson Laboratories). For detection of either c-Rel or the Myc-NPC-ARD fusion proteins, mouse monoclonal antibodies directed against either c-Rel (Santa Cruz) or the Myc epitope (Santa Cruz) were followed by a Cy3-conjugated anti-mouse antibody (Jackson Laboratories). For detection of c-Rel and Myc-tagged IxBε in 3T3 cells derived from IxBα nullizygous mice, a rabbit polyclonal antibody directed against c-Rel (Santa Cruz) and a mouse monoclonal antibody directed against the Myc epitope (Santa Cruz) were followed by a fluorescein isothiocyanate-conjugated anti-rabbit and a Cy3-conjugated anti-mouse antibody, respectively.

In Vitro Binding Assay—To measure binding of GST-IxBα to immobilized CRM1, anti-HA-agarose beads containing 100 ng of HA-CRM1 was incubated with 2 μg of GST-IxBα and 2 μg of RanQ69L GTP in binding buffer (10 mM sodium phosphate, pH 7.3, 150 mM NaCl, 0.1% Triton X-100, and 1 mM 2-mercaptoethanol) and rotated at 4 °C for 2 h. The matrix was extensively washed with binding buffer before the addition of SDS-PAGE sample buffer. To measure binding of HA-CRM1 to immobilized IxBα proteins, 2 μg of each GST-IxBα protein was loaded onto glutathione-agarose beads in binding buffer at 4 °C for 30 min. The beads were extensively washed and incubated with 20 μl of insect cell lysates containing HA-CRM1 in the presence of 2 μg of RanQ69L-GTP at 4 °C for 2 h. The matrix was extensively washed with binding buffer before the addition of SDS-PAGE sample buffer. In all binding assays, immunoblot analysis was used to measure proteins that remained bound to the respective solid supports after extensive washing. The samples were electrophoresed through SDS-polyacrylamide gels and transferred to nitrocellulose. HA-CRM1 was detected with a mouse monoclonal antibody against the HA epitope (Covance), GST-IxBα proteins were detected with a mouse monoclonal antibody against GST (Santa Cruz), and the RanQ69L protein was detected with a rabbit polyclonal antibody against Ran (Covance). All primary antibodies were used at dilutions of 1:2000–1:4000. For all immunoblot analyses, the secondary antibodies were either horseshadish peroxidase-coupled anti-mouse IgG (New England Biolabs) used at a dilution of 1:20,000 or horseradish peroxidase-coupled anti-rabbit IgG (Sigma) used at a dilution of 1:10,000. To allow detection of multiple proteins on the same nitrocellulose filter, some filters were stripped with IgG elution buffer (Pierce) before subsequent antibody incubations. The proteins were visualized using Amersham Biosciences ECL reagents followed by exposure to XAR-5 film.

FIG. 1. Schematic illustration of the IxBε protein. The amino acid sequence of the C-terminal NES-like motif is shown. The conserved consensus motif of essential hydrophobic amino acids in C-terminal NES-like motif of IxBε is compared with other well characterized NES motifs (bold and underlined), including the N2 protein of the Minute virus of mice (MVM NS2) (39), MAPKAP kinase 2 (MK2) (40), and the protein kinase inhibitor (PKI) (41).
on coverslips were treated with 10 nM leptomycin B for 30 min before the coverslips were fixed with 4% paraformaldehyde and mounted panels D analysis. Immunoprecipitation of Myc-tagged I alent amounts of protein lysates were used for the immunoprecipitation rabbit polyclonal antibody directed against c-Rel and or a mouse mono- noblot analysis. the presence of c-Rel in the immunoprecipitates was determined by immu- to a final concentration of 100

FIG. 2. Nuclear accumulation of IxB in digitonin-permeabilized cells is leptomycin B-sensitive and independent of GTP hydrolysis by Ran. Digitonin-permeabilized HeLa cells were incubated with 5 µg of fluorescein-labeled His-IxB or fluorescein-labeled GST-NLS for 20 min at room temperature in the presence of reticulocyte lysate and an energy regenerating system. For panels B, C, F, and G, 3 µg of CRM1 and RanBP1 were added. For panels D, H, and J, 5 µg of RanQ69L preloaded with GTP was added. For nuclear import of fluorescein-labeled GST-NLS (panels I and J), 3 µg of importin-α and importin-β were added. For panels C and G, HeLa cells were treated with 10 nM leptomycin B (LMB) for 30 min before permeabilization with digitonin, and leptomycin B (10 nM) was included in the import reactions.

**RanGAP Protection Assay**—RanGAP protection assay was performed as described previously except when using His-tagged IxB proteins purified from E. coli (28). In brief, 0–15 µM His-tagged IxB proteins were incubated in the presence of 100 nM Ran-[γ-32P]GTP and 1 µM CRM1 at 15°C for 30 min. The samples were placed on ice, 10 nM RanGAP was added, and the samples were immediately placed at 30°C for 2 min. The amount of [γ-32P] GTP bound to Ran was measured by the filter binding assay (28).

**Immunoprecipitation Analysis of IxB and c-Rel Proteins**—Cell lysates for the immunoprecipitation experiments were prepared in ELB (50 mM Tris-HCl, pH 7.9, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, and 1 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Calbiochem). To inhibit phosphatase activity, 0.4 mM sodium orthovanadate and 1 mM sodium fluoride were included in the lysis buffer. Expression of c-Rel and of the Myc-tagged IxB proteins was confirmed by immunoblot analysis using a rabbit polyclonal antibody directed against c-Rel and or a mouse monoclonal antibody directed against the Myc epitope (Santa Cruz). Equivalent amounts of protein lysates were used for the immunoprecipitation analysis. Immunoprecipitation of Myc-tagged IxB proteins was performed with 20 µl of anti-Myc-agarose beads/sample (Santa Cruz). The presence of c-Rel in the immunoprecipitates was determined by immunoblot analysis.

**In Vitro Nuclear Import Assay**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Approximately 16 h before the transport assays, 6 × 10⁵ HeLa cells were placed onto 35-mm diameter plates containing glass coverslips. Cells on coverslips were permeabilized with 50 µg of digitonin/ml (Calbiochem) in transport buffer for 5 min on ice. The transport reactions were conducted for 20 min at room temperature. A standard 50-µl transport reaction contained an energy-regenerating system (1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, 20 units of creatine phosphokinase/ml), protease inhibitor mix, 2 mM dithiothreitol, and 15 µl of rabbit reticulocyte lysate. The fluorescein-labeled import substrates were added to a final concentration of 100 µg/ml. After nuclear transport reactions, the coverslips were fixed with 4% paraformaldehyde and mounted onto glass slides with Mowiol containing 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma). When indicated, HeLa cells growing on coverslips were treated with 10 nM leptomycin B for 30 min before digitonin permeabilization, and 10 nM leptomycin B was included throughout the transport reactions. The CRM1 and RanBP1 dependence experiments were performed in the presence of 2 µg each of CRM1 and RanBP1. The RanQ69L experiments were performed in the presence of 5 µg of RanQ69L preloaded with GTP. The GST-NLS nuclear import reactions were performed in the presence of 3 µg each of importin-α and importin-β. The panels shown in Fig. 2 were taken with a 60× oil immersion lens on a Nikon Optiphot-2 equipped with a Diagnostic Imaging Spot II camera. Images were collected using software from Diagnostic Imaging and transferred to Adobe Photoshop. No manipu- lation of the images was performed except for converting the original color images to black and white. Equivalent exposure time periods were used for all panels in Fig. 2.

**RESULTS**

**Nuclear Import of IxB in Digitonin-permeabilized HeLa Cells**—The IxB protein is located in the cytoplasm, yet shares along with IxαB and IxBβ a central ankyrin repeat domain that is responsible for nuclear import of IxB (Fig. 1) (29). To determine whether IxB is capable of entering the nucleus, in vitro nuclear import assays were performed using fluorescein-labeled IxB and digitonin-permeabilized HeLa cells. In the presence of reticulocyte lysate and an energy regenerating system, nuclear accumulation of IxB was readily observed (Fig. 2A). Nuclear accumulation of IxB was energy-dependent and was enhanced in the presence of reticulocyte lysate (data not shown).

**Nuclear Import of IxB Is Independent of GTP Hydrolysis by Ran**—The directionality of nuclear export and nuclear import pathways is determined by the asymmetric distribution of the GTP-bound and GDP-bound forms of Ran between the nucleus and the cytoplasm. To determine whether nuclear import of IxB is sensitive to perturbation of the asymmetric Ran-nucleotide gradient, nuclear accumulation of IxB was determined in the presence of the dominant-negative RanQ69L protein bound to GTP. RanQ69L GTP efficiently blocked nuclear import of a protein containing a classical NLS (GST-NLS; Fig. 2J). In contrast, RanQ69L GTP did not block nuclear import of IxB (Figs. 2, D and H). Thus, like IxBα and IxBβ and in contrast to nuclear import of proteins that contain a classical NLS, nuclear import of IxB is not disrupted by perturbation of the asymmetric Ran-GTP gradient.

Because nuclear export reactions can also be monitored using the digitonin-permeabilized cell assay, the possibility that
IκB may also undergo nuclear export was examined. Nuclear accumulation of IκB was abolished when the nuclear import reactions were carried out in the presence of CRM1 and RanBP1 (Fig. 2B). In contrast, the inclusion of leptomycin B in the import reaction enhanced nuclear accumulation of IκB, even in the presence of exogenous CRM1 (Fig. 2C).

The ability of exogenous CRM1 to block nuclear accumulation of IκB in digitonin-permeabilized HeLa cells suggested that IκB can be exported from the nucleus via the CRM1-dependent nuclear export pathway. To define the region(s) of IκB required for CRM1-dependent nuclear export, several mutant IκB proteins were characterized for their ability to accumulate in the nucleus of digitonin-permeabilized cells in a CRM1-independent manner (data not shown). Removal of 21 amino acids from the C terminus of IκB was sufficient to allow

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**Fig. 3. The C-terminal NES-like region interacts with CRM1 in RanGTP-dependent manner.** A, anti-HA-agarose beads containing equivalent amounts of HA-CRM1 were incubated with 2 μg of either GST-IκB (lanes 1 and 2), GST-IκB(L350A/I352A) (lane 3), or GST-IκB(L340–352) (lane 4) in the absence (lane 1) or presence of 2 μg of RanQ69L-GTP (lanes 2–4). The beads were washed extensively, and bound proteins were electrophoresed through a SDS, 10% polyacrylamide gel, transferred to nitrocellulose, and detected by immunoblot analysis using GST-IκB, RanBP1 (Fig. 2B), L350A/I352A, or L340–352 of IκB was sufficient to allow CRM1-independent nuclear accumulation of IκB (His-IκB1–340; Figs. 2, E and F).

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**IκB binds to CRM1 in a RanGTP-dependent manner.** The formation of an export-competent complex between CRM1 and a NES-bearing cargo protein requires cooperative binding of RanGTP (34). To determine whether IκB binds to CRM1 in a RanGTP-dependent manner, the ability of purified IκB protein to bind CRM1 was determined in the absence or presence of RanGTP. For this purpose, GST-tagged IκB (GST-IκB-wt) was prepared. HA-tagged CRM1 (HA-CRM1) was immobilized on anti-HA-agarose beads, and GST-IκB was added in the presence or absence of RanQ69L-GTP. To closely mimic the in vivo situation where CRM1 interacts with multiple cargo proteins, lysates from 293 cells were included during binding reaction. After incubation, the beads were extensively washed, and bound proteins were determined with immunoblot analysis. 1/50 of input insect cell lysates containing HA-CRM1 was analyzed by immunoblot analysis in parallel (lane 1).
His-IkBε was prepared, a C-terminal deletion mutant (GST-IkBεL350A/I352A) and a point mutant containing alanine substitutions for leucine 350 and isoleucine 352 (GST-IkBεL350A/I352A) of IkBε. Binding of these mutant IkBε proteins to CRM1 in the presence of RanGTP was determined. Binding of both mutant GST-IkBε proteins to CRM1 in the presence of RanQ69L GTP was significantly reduced compared with wild-type GST-IkBε (Fig. 3A, compare lanes 2–4). To confirm this observation, the respective GST-IkBε proteins were immobilized on glutathione-agarose and incubated with insect cell lysates containing HA-CRM1. An equivalent amount of RanQ69L GTP was added to each sample. The agarose beads were extensively washed, and an equivalent amount of RanQ69L GTP was added to each sample. The agarose beads were extensively washed, and bound HA-CRM1 was determined with immunoblot analysis. Binding of HA-CRM1 to the immobilized GST-IkBεL350A/I352A and GST-IkBεL340 proteins was markedly reduced compared with wild-type GST-IkBε (Fig. 3B, compare lane 3–5).

Quantitative Analysis of IkB-CRM1 Interactions—A RanGAP protection assay was used to provide a quantitative measure of the affinity of IkB for CRM1. The slow intrinsic GTPase activity of Ran is markedly stimulated by the GTPase-activating protein, RanGAP. However, upon formation of an export-competent ternary complex between RanGTP, CRM1, and a NES-bearing cargo protein, RanGAP is no longer able to stimulate the GTPase activity of Ran. In this assay, the ability of a NES-bearing cargo protein to inhibit RanGAP-stimulated GTP hydrolysis in a concentration-dependent manner provides an estimate of the affinity of the cargo protein for CRM1.

His-tagged IkBα, IkBβ, and IkBε proteins were assayed for the inhibition of RanGAP-stimulated GTP hydrolysis by Ran. 8 μM His-IkBβ protein provided 50% protection against RanGAP-stimulated GTP hydrolysis. His-IkBε protein was equally effective as His-IkBβ protein for protection against RanGAP. In contrast, 15 μM His-IkBβ protein did not provide significant protection against RanGAP-stimulated GTP hydrolysis by Ran (Fig. 4A).

The ability of the mutant His-IkBε proteins to inhibit RanGAP-stimulated GTP hydrolysis by Ran was determined. 15 μM His-IkBεL340 protein did not provide significant protection against RanGAP-stimulated GTP hydrolysis by Ran, although 8 μM His-IkBεL350A/I352A protein provided 20% protection, suggesting that His-IkBεL350A/I352A protein has an ability of partial interaction to CRM1 (Fig. 4B).

Taken together, the binding assays and the RanGAP protection assays provide strong evidence that the C-terminal NES-like sequence of IkBε is required for formation of an export-competent complex with CRM1 and RanGTP. Furthermore, IkBα and IkBε proteins have comparable affinities to CRM1. In contrast, under this experimental condition, IkBβ does not have a measurable affinity to CRM1.

The C-terminal NES-like Motif Is Required for Cytoplasmic Localization of IkBε—To determine whether the NES motif in IkBε contributes to the cytoplasmic localization of IkBε, expression vectors encoding either wild-type or mutant IkBε proteins were transiently transfected into COS1 cells. Localization of the ectopically expressed IkBε proteins was determined by indirect immunofluorescence. The wild-type IkBε protein was predominantly localized to the cytoplasm (Fig. 5A; Table I). In contrast, the mutant IkBε proteins displayed a whole-cell pattern of staining (Figs. 5, C and D). Furthermore, the staining pattern of the wild-type IkBε protein shifted from a cytoplasmic pattern to a whole-cell pattern after a 3-h treatment with leptomycin B (Fig. 5B). The staining pattern of the IkBε mutant proteins was not altered by leptomycin B treatment (Table I). Taken together, our results indicate that the C-terminal NES-like motif of IkBε contributes to cytoplasmic localization of IkBε.

Inefficient Nuclear Import of IkBε—The relatively slow nuclear accumulation of IkBε in leptomycin B-treated cells is in contrast to the rapid nuclear accumulation of IkBα after leptomycin B treatment (21, 25). To determine whether this difference between IkBα and IkBε is a consequence of impaired nuclear import of IkBε relative to IkBα or reflects the ability of IkBε to access a CRM1-independent nuclear export pathway,
we compared the nuclear import function of the respective ARDs from IxBa and IxBe along with the ARD from IxBβ. The ankyrin repeat domains of each of the IxB proteins were fused to the core domain of nucleoplasmin containing an N-terminal Myc epitope tag (Myc-NPc). Expression vectors encoding the respective Myc-NPc-ARD fusion proteins were transfected into COS1 cells, and the ability of the respective fusion proteins to localize to the nucleus was determined. As previously reported, the IxB ARD efficiently relocalized Myc-NPc from the cytoplasm to the nucleus (Figs. 6, A and B; Table II) (29). The IxB ARD also redistributed Myc-NPc from the cytoplasm to the nucleus, although with less efficiency compared with the ARD of IxBa (Fig. 6C; Table II). In contrast, the ARD of IxBβ did not significantly relocalize Myc-NPc from the cytoplasm to the nucleus (Fig. 6D; Table II). Thus, the relatively slow nuclear accumulation of IxB in leptomycin B-treated cells is likely due to the relatively weak nuclear import function of the IxB ARD.

The C-terminal NES Motif of IxBe Is Required for Cytoplasmic Localization of NF-κB/Rel Proteins—To determine the effect of IxB on localization of NF-κB/Rel proteins, a CMV-derived expression vector encoding c-Rel was cotransfected with expression vectors encoding wild-type or mutant IxB proteins into COS1 cells. For this purpose, a C-terminal truncated c-Rel comprising amino acids 1–362 was utilized. The c-Rel1362-truncated c-Rel proteins lack a C-terminal transcriptional activation domain. Importantly, the c-Rel1362 protein still associates with both wild-type and mutant IxB proteins (Fig. 7).

This C-terminal truncated c-Rel protein was strongly localized to the nucleus when ectopically expressed in COS1 cells (Table I). As expected, coexpression of c-Rel1362 with the wild-type IxBe proteins resulted in the colocalization of c-Rel and IxBe in the cytoplasm (Figs. 8, A and B). In 15% of cells treated with 20 nM leptomycin B, both c-Rel1362 and IxBe remained in the cytoplasm (Figs. 8, C and D, Table I). To determine the extent to which the IxBe NES contributes to cytoplasmic retention of c-Rel by IxBe, expression vectors for either IxBa362 or IxBβ350A/352 were cotransfected along with an expression vector for c-Rel362 into COS1 cells, and the localization of the c-Rel1362 protein was monitored by indirect immunofluorescence. Nuclear staining of the c-Rel1362 protein was readily detected despite coexpression of the mutant IxB proteins (Figs. 8, E, F, I, and J; Table I). The IxB proteins colocalized with the c-Rel1362 protein. The nucleocytoplasmic distribution of the c-Rel1362 protein and of the mutant IxB proteins was insensitive to leptomycin B (Figs. 8, G, H, K, and L, Table I). IxB is one of the major determinants of nucleocytoplasmic localization of NF-κB/Rel proteins. Therefore, the endogenous IxB protein might affect the nucleocytoplasmic localization of ectopically expressed c-Rel1362 in COS1 cells considering a marginal nuclear translocation of c-Rel1362 and IxB proteins was observed in leptomycin B-treated COS1 cells (Table I).

To avoid this possibility, 3T3 cells derived from IxB nullizygous mice were used for cotransfection assay. Coexpression of c-Rel1362 with the Myc-tagged wild-type IxBe proteins resulted in the colocalization of c-Rel1362 and Myc-IxBe in the cytoplasm (Figs. 9, A and B). In contrast, nuclear staining of the c-Rel1362 protein was readily detected despite coexpression of the Myc-tagged mutant IxB proteins (Figs. 9, C–F, Table III).

To examine the possibility that IxBex nuclear export might contribute to post-induction repression of c-Rel, 3T3 cells de-
induction period resulted in a marked shift of both c-Rel1
contrast, the inclusion of leptomycin B during the 3-h post-
calized in the cytoplasm (Figs. 10, A
were cotransfected with expression vectors encoding the indicated Myc-
TNF-/H9260
were analyzed 3 h after removal of TNF-/H9260
sion vectors for I
1362 were treated with TNF-/H9260
of I
362 protein and the I
362 protein and the I
nullizygous mice cotransfected with expres-
nullizygous mice derived from I
nullizygous mice. After 2 h, 

FIG. 9. The C-terminal NES-like motif of IκBe is required to retain either IκBe or IκBe and c-Rel complexes in the cytoplasm in 3T3 IκBe+/− cells. 3T3 cells derived from IκBe nullizygous mice were cotransfected with expression vectors encoding the indicated Myc-tagged wild type or mutant IκBe proteins and an expression vector encoding c-Rel1–362. Forty hours after transfection, the cellular localization of the c-Rel1–362 protein and the IκBe proteins within the same cell were determined by double-label indirect immunofluorescence. The panels C, D, G, H, K, and L, cells were treated with leptomycin B for 3 h at a final concentration of 20 nM before analysis by double-label indirect immunofluorescence. LMB, leptomycin B.

Myc-IκBe + c-Rel

α-Myc

α-c-Rel

IκBe-wt

A

B

IκBe

C

D

IκBeL350A/352A

E

F

IκBe1–340

H

I

J

K

L

FIG. 8. The C-terminal NES-like motif of IκBe is required to retain either IκBe or IκBe and c-Rel complexes in the cytoplasm. COS1 cells were cotransfected with expression vectors encoding the indicated wild-type or mutant IκBe proteins and an expression vector encoding c-Rel1–362. Forty hours after transfection, the cellular localization of the c-Rel1–362 protein and the IκBe proteins within the same cell were determined by double-label indirect immunofluorescence. For the panels C, D, H, K, and L, cells were treated with leptomycin B for 3 h at a final concentration of 20 nM before analysis by double-label indirect immunofluorescence. LMB, leptomycin B.

Under the same condition, the nucleocytoplasmic distribution of the c-Rel1–362 protein and of the Myc-tagged mutant IκBe proteins was insensitive to leptomycin B (Figs. 10, E–L, Table III).

DISCUSSION

The IκBe protein, first identified in a two-hybrid screen using the RelA subunit of NF-κB as bait, is emerging as an important regulator of NF-κB/Rel proteins (11, 35, 36). Most notably, loss of IκBe results in specific defects in T-cell development, Ig isotype switching, and cytokine gene expression (37). IκBe has been suggested to act exclusively in the cytoplasm to inhibit nuclear localization of NF-κB/Rel proteins (36), although nucleocytoplasmic shuttling of IκBe has not been examined in detail. In this report, we provide evidence that IκBe contains nuclear import and export functions that enable shuttling of IκBe between the nucleus and the cytoplasm. Furthermore, IκBe is able to mediate CRM1-dependent nuclear export of NF-κB/Rel proteins.

Our results provide several lines of evidence for a nuclear import function in IκBe. First, although IκBe displays a predominant cytoplasmic pattern of localization, disruption of nuclear export with the CRM1-specific inhibitor, leptomycin B, results in a significant relocation of IκBe to the nucleus. Second, fusion of the ankyrin repeat domain of IκBe is able to target a heterologous protein to the nucleus. Finally, IκBe is able to accumulate in the nucleus of digitonin-permeabilized cells in standard in vitro nuclear import assays. Taken together, our results indicate that the ankyrin repeat domain of IκBe, like the ankyrin repeat domain of IκBα, is a functional nuclear import domain. It is likely that IκBe and IκBα utilize a similar mechanism for transit through the nuclear pore complex, as nuclear import of IκBe, like that of IκBα, is not disrupted by the dominant-negative RanQ69L-GTP protein (26). Despite the overall similarities in nuclear import between IκBe and IκBα, one important difference is the relative effectiveness of the ankyrin repeat domains of IκBα and IκBe at promoting nuclear import. Fusion of the ankyrin repeat domain of IκBα to the core domain of nucleoplasmin results in a marked relocalization of nucleoplasmin from the cytoplasm to the nucleus. The ankyrin repeat domain of IκBe is also effective at relocalization of the nucleoplasmin core from the cytoplasm to the nucleus, although it is less efficient

derived from IκBe nullizygous mice cotransfected with expression vectors for IκBe and c-Rel1–362 were treated with TNF-α and cycloheximide to induce degradation of IκBe. After 2 h, TNF-α and cycloheximide were removed to allow re-synthesis of IκBe, and the cellular distributions of IκBe and c-Rel1–362 were analyzed 3 h after removal of TNF-α and cycloheximide. In the absence of leptomycin B, c-Rel1–362 and Myc-IκBe colocalized in the cytoplasm (Figs. 10, A and B, Table III). In contrast, the inclusion of leptomycin B during the 3-h post-induction period resulted in a marked shift of both c-Rel1–362 and Myc-IκBe to the nucleus (Figs. 10, C and D, Table III).
Nuclear Import and Export Functions of IκBε

Table III
Sub-cellular distribution of cRel1–362 proteins in 3T3 IκBa−/− cells

| c-Rel1–362 protein | IκBε proteins | %N | %N-C | %C |
|-------------------|---------------|----|------|----|
| +                 | +             | 100| 0    | 0  |
| +                 | IκBε-wt       | 0  | 5    | 95 |
| +                 | IκBεL350A/L352A| 26 | 74   | 0  |
| +                 | IκBε1–340     | 37 | 63   | 0  |

Percentages of cells that displayed predominantly nuclear (N), whole cell (N-C), and cytoplasmic (C) staining of the c-Rel1–362 protein are presented. A total of 30 cells positive for expression of the c-Rel1–362 protein in the presence of the indicated IκBε protein were examined. The relative weakness of the IκBε functions only in the cytoplasm (20, 36). Our results agree with the notion that nuclear import of IκBε is less efficient than IκBa. Nevertheless, the ability of IκBε to shuttle between the cytoplasm and the nucleus suggests that IκBε may function in both compartments to regulate NF-κB/Rel proteins.

Fig. 10. The NES of IκBε is required for the efficient post-induction repression of c-Rel. 3T3 cells derived from IκBa nullizygous mice were cotransfected with expression vectors encoding the indicated Myc-tagged wild type or mutant IκBε proteins and an expression vector encoding c-Rel1–362. Forty hours after transfection, the transfected cells were refed with complete medium containing TNF-α (10 ng/ml) and cycloheximide (100 μg/ml) for 2 h. The TNF-α and cycloheximide were subsequently removed, and the transfected cells were chased in complete medium for 3 h in the absence (panels A, B, E, F, I, J) or presence of leptomycin B (LMB) at a concentration of 20 nM before fixation of 3T3 cells derived from IκBa nullizygous mice for indirect immunofluorescence.

than the ARD of IκBa. In contrast, the ARD of IκBβ is ineffective at relocalization of nucleoplasmin core to the nucleus. This finding suggests that although the ankyrin repeat domains of IκB proteins share overall structural similarities, the ankyrin repeat domains of each IκB proteins are markedly different in their abilities of mediating nuclear import.

The relative weakness of the IκBε ankyrin repeat as a functional nuclear import signal is also reflected in the behavior of the full-length IκBε protein in leptomycin B-treated cells. In the case of IκBa, 30 min of leptomycin B treatment is sufficient to relocalize the bulk of IκBa to the nucleus (21, 25), whereas only a partial relocalization of IκBε is achieved even after several hours of leptomycin B. Several other reports also demonstrate that nuclear localization of IκBε is markedly diminished relative to IκBa, leading to the prevailing suggestion that IκBε functions only in the cytoplasm (20, 36). Our results agree with the notion that nuclear import of IκBε is less efficient than IκBa. Nevertheless, the ability of IκBε to shuttle between the cytoplasm and the nucleus suggests that IκBε may function in both compartments to regulate NF-κB/Rel proteins.

One potential nuclear function of IκBε might be to mediate nuclear export of NF-κB/Rel proteins. In agreement with the results of Tam and Sen (38), we find that inhibition of CRM1-dependent nuclear export only marginally alters the steady-state cytoplasmic localization of the c-Rel/IκBε complex. However, we find that CRM1-dependent nuclear export is required for efficient IκBε-mediated cytoplasmic retention of c-Rel after TNF-α/cycloheximide-induced degradation of IκBε. We have
identified a canonical NES in IkBα that is required for RanGTP-dependent binding of IkBα to CRM1. Furthermore, IkBα and IkBα proteins have comparable affinities to CRM1. In contrast, IkBβ does not have measurable affinity to CRM1. The NES of IkBα is also required for cytoplasmic localization of both IkBα and for IkBα-dependent cytoplasmic retention of c-Rel. These results suggest that removal of NF-κB/Rel proteins from the nucleus is an important nuclear function of IkBα.

Post-induction repression of activated gene expression provides an effective mechanism for temporal regulation of gene expression. In the NF-κB/Rel system, it is clear that a major function of the IkBα protein is to participate in an autoregulatory circuit that attenuates NF-κB-dependent transcription. Thus, for example, the IkBα gene is rapidly induced by NF-κB/Rel proteins, and IkBα is able to efficiently enter the nucleus, displace NF-κB/Rel proteins from DNA, and mediate their removal from the nucleus (18–29). Our present results together with the previous demonstration that expression of the IkBα gene is induced by NF-κB/Rel proteins (11) support the notion that IkBα also participates in post-induction repression of NF-κB/Rel proteins. However, as suggested by Whiteside et al. (11), it is likely that IkBα and IkBβ have distinct roles in the post-induction repression of NF-κB/Rel proteins. IkBα expression is induced with markedly slower kinetics than IkBβ, and nuclear import of IkBα is less efficient than IkBβ (11, 35, 36).

IkBα and IkBβ both share the ability to inhibit DNA binding of NF-κB/Rel proteins and to direct their removal from the nucleus, although these IkB proteins have distinct specificities for different NF-κB/Rel proteins (11). A plausible scenario is that IkBα controls expression of NF-κB-dependent genes that must be activated and subsequently repressed in a relatively short time frame, whereas IkBβ is specialized for control over NF-κB-dependent genes that are activated and subsequently repressed over a longer time period.

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