The N-terminal Nuclear Export Sequence of IκBα Is Required for RanGTP-dependent Binding to CRM1*

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Sang-Hyun Lee and Mark Hannink‡
From the Department of Biochemistry, University of Missouri, Columbia, Missouri 65212

Nuclear export of IκBα is mediated by the CRM1 nuclear export receptor. However, the identity of the nuclear export sequences NESs in IκBα that are responsible for binding of IκBα to CRM1 is controversial. Both a N-terminal NES-like region (amino acids 45–54) and a C-terminal NES-like region (amino acids 265–280) have, in a number of reports from different laboratories, been implicated in CRM1-dependent nuclear export of IκBα. We now demonstrate that the N-terminal NES-like region, but not the C-terminal NES-like region, is required for RanGTP-dependent binding of IκBα to CRM1. IκBα is a relatively weak substrate for CRM1, with an affinity for CRM1 that is 100-fold less than the minute virus of mice NS2 protein, a high affinity cargo protein for CRM1. We also demonstrate that IκBα functions as a physical adaptor between CRM1 and NFκB/Rel proteins. Both free IκBα and Rel-associated IκBα have comparable affinities for CRM1, suggesting that CRM1 does not discriminate between free IκBα and Rel-associated IκBα. Nuclear export of c-Rel by IκBα requires the N-terminal NES-like sequence of IκBα but is not affected by alanine substitutions within the C-terminal NES-like sequence of IκBα. In contrast, nuclear export of the v-Rel oncprotein by IκBα is disrupted by alanine substitutions within either the N-terminal or the C-terminal NES-like sequences. However, alanine substitutions within the C-terminal NES-like sequence significantly reduce the affinity of IκBα for v-Rel, suggesting that loss of export function for this mutant is secondary to reduced association between IκBα and v-Rel. Taken together, our results demonstrate that the N-terminal NES-like sequence in IκBα is required for RanGTP-dependent binding of both free IκBα and NFκB/Rel-associated IκBα proteins to CRM1.

The NFκB/Rel transcription factor family has provided a powerful experimental paradigm for understanding how eukaryotic gene expression is regulated (reviewed in Refs. 1–3). Mammalian cells have five NFκB/Rel proteins, termed p50, p52, RelA(p65), c-Rel, and RelB. The NFκB/Rel proteins are defined by the presence of an 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 NFκB/Rel proteins. NFκB/Rel proteins are regulated, in large part, through association with members of the Inhibitor of kappa B (IκB) family of ankyrin repeat proteins.

A major regulatory mechanism for controlling NFκB/Rel proteins is sequestration in the cytoplasm. Crystalllographic analysis of the ternary p50p65-IκBα complex has revealed that NLSs within the dimeric p50p65 complex are masked by IκBα, suggesting that IκBα inhibits nuclear import of NFκB/Rel proteins by blocking access of the nuclear import machinery to the NLSs of NFκB (4, 5).

Cytoplasmic sequestration of NFκB/Rel proteins arises from a dynamic equilibrium between nuclear import and nuclear export. For example, treatment of unstimulated cells with leptomycin B results in the relocation of both NFκB/Rel and IκBα proteins to the nucleus (6, 7). In addition, leptomycin B treatment results in sustained nuclear localization of NFκB/Rel and IκBα proteins following transient stimulation with activating agents such as tumor necrosis factor-α (6). Because the CRM1 nuclear export receptor is specifically inactivated by leptomycin B (8), CRM1-dependent nuclear export is important for cytoplasmic sequestration of NFκB/Rel and IκBα proteins under conditions of both basal and post-induction repression. CRM1 binds short leucine-rich nuclear export sequences (NESs) in its cargo proteins (8–12). Cooperative binding of RanGTP is required for formation of an export-competent complex between CRM1 and a NES-bearing cargo protein (13). IκBα contains two leucine-rich regions that resemble previously characterized NESs, amino acids 45–54 and 265–280 (Fig. 1). However, there is contradictory evidence in the literature regarding the requirement of these NES-like sequences for nuclear export of IκBα (7, 14–18).

Dargemont and co-workers (14) were the first to show that IκBα could be exported from the nucleus. In these experiments, alanine substitutions for hydrophobic residues within the C-terminal NES-like sequence reduced the rate of IκBα export when injected into nuclei of Xenopus oocytes (14). Subsequently, our laboratory demonstrated that IκBα mediates nuclear export of the v-Rel oncprotein and that this nuclear export function of IκBα is reduced by mutations within the C-terminal NES-like region of IκBα (15).

In contrast, more recent experiments have suggested that the N-terminal NES-like sequence in IκBα is critically required for CRM1-dependent nuclear export of IκBα. For example, Hope and co-workers (7) have shown that alanine substitutions within the N-terminal NES-like sequence abolished nuclear export of IκBα proteins following microinjection into the nuclei of 3T3 cells. Miyamoto and co-workers (16) showed that mutations within the N-terminal NES-like sequence markedly in-
creased nuclear accumulation of a GFP-IκBα protein in transiently transfected COS cells. In agreement with both of these results, our laboratory (17) has recently shown that the entire N-terminal domain of IκBα, including the N-terminal NES-like sequence, is required for nuclear export of GST-IκBα in digitonin-permeabilized HeLa cells. Finally, using both yeast and mammalian cells, Sen and co-workers (18) have showed that the N-terminal NES-like sequence is required for CRM1-dependent nuclear export of IκBα.

There is also contradictory evidence in the literature regarding the requirement of these two NES-like sequences for binding of IκBα to CRM1. In one set of experiments, the C-terminal NES-like sequence of IκBα was shown to be required for binding to in vitro translated CRM1 (12). On the other hand, the N-terminal NES-like sequence has been shown to be required for interaction with CRM1 in the yeast two-hybrid assay (16).

In this study, we now demonstrate that purified IκBα binds to purified CRM1 in a RanGTP-dependent manner. Quantitative analysis of an export-competent ternary complex between IκBα, CRM1, and RanGTP indicates IκBα has a relatively weak affinity for CRM1. The N-terminal NES-like sequence, but not the C-terminal NES-like sequence, is required for the formation of a ternary IκBα-CRM1-RanGTP complex. We also examined the role of the respective NESs of IκBα in IκBα-mediated nuclear export of NFκB/Rel proteins. The N-terminal NES-like sequence of IκBα is required for IκBα-mediated nuclear export of c-Rel, whereas both NES-like sequences of IκBα are required for IκBα-mediated nuclear export of v-Rel. However, alanine substitutions within the C-terminal NES-like sequence markedly reduce the affinity of IκBα for v-Rel, suggesting that loss of IκBα-mediated nuclear export of v-Rel is secondary to reduced binding of IκBα to v-Rel. Taken together, our data indicate that the N-terminal NES-like sequence of IκBα is required for both binding of IκBα to CRM1 and for CRM1-mediated nuclear export of the IκBα-Rel complex.

EXPERIMENTAL PROCEDURES

Construction of Recombinant DNA Molecules—The construction of recombinant DNA molecules was performed by standard techniques (19). An EcoRI fragment containing the avian IκBα cDNA was used as the progenitor for all of the mutants IκBα utilized in this study (20). All point mutations were constructed from phagemid single-strand DNA sequence markedly reduce the affinity of IκBα (1). However, alanine substitutions within the C-terminal NES-like sequences of IκBα, CRM1, and RanGTP indicates IκBα has a relatively weak affinity for CRM1. The N-terminal NES-like sequence, but not the C-terminal NES-like sequence, is required for the formation of a ternary IκBα-CRM1-RanGTP complex. We also examined the role of the respective NESs of IκBα in IκBα-mediated nuclear export of NFκB/Rel proteins. The N-terminal NES-like sequence of IκBα is required for IκBα-mediated nuclear export of c-Rel, whereas both NES-like sequences of IκBα are required for IκBα-mediated nuclear export of v-Rel. However, alanine substitutions within the C-terminal NES-like sequence markedly reduce the affinity of IκBα for v-Rel, suggesting that loss of IκBα-mediated nuclear export of v-Rel is secondary to reduced binding of IκBα to v-Rel. Taken together, our data indicate that the N-terminal NES-like sequence of IκBα is required for both binding of IκBα to CRM1 and for CRM1-mediated nuclear export of the IκBα-Rel complex.

for the CRM1 protein was obtained from Peter Askjaer (University of Aarhus) (13). A baculovirus encoding HA-CRM1 was the generous gift of Alan Diehl (University of Nebraska Medical Center).

Expression and Purification of Recombinant Proteins—The recombinant GST-IκBα proteins were expressed in Escherichia coli strain BL21(DE3)pLysS and purified with glutathione-agarose as previously described (17). The His-tagged IκBα, c-Rel, and v-Rel proteins were expressed in BL21(DE3)pLysS and purified as described in the Novagen pET manual. The Ran proteins were expressed in BLR Rep4 cells and purified by metal-chelate affinity chromatography (Invitrogen) as described (13). The His-CRM1 protein was expressed in BL21(DE3)pLysS cells and purified by metal-chelate affinity chromatography. For His-CRM1 production, a single colony from a freshly transformed plate was inoculated into 50 ml of TB containing 200 μg/ml ampicillin. The cells were grown at 37 °C until A600 reached 0.1, collected by centrifugation, and resuspended in 2 ml of fresh TB. The suspended cells were inoculated in 750 ml of T3 containing 200 μg/ml ampicillin, grown at 37 °C to an A600 of 0.1, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co.) and 1 mM phenylmethylsulfonyl fluoride for 4 h at 20 °C. The cell pellets were lysed with Bugbuster (Novagen) containing 5 mM 2-mercaptoethanol and a protease inhibitor mixture mix (Calbiochem). The His-tagged CRM1 proteins were purified by metal-chelate affinity chromatography.
RanGTP-dependent Association of IκBα with CRM1

(A) GST-ικBα: wt wt CA4 NA4 wt CA4 NA4
HA-CRM1: + + + + + + + +
RanQ69L GTP: - - - - - - - -
Lysates: 1 2 3 4 1 2 3 4

(B) GST-ικBα: - wt CA4 NA4
HA-CRM1: + + + + + + + +
RanQ69L GTP: + + + + + + + +

(C) HA-CRM1 (Arbitrary Unit)
GST-ικBα: wt CA4 NA4

Fig. 3. The N-terminal NES-like region of IκBα is required for binding to CRM1. A, anti-HA-agarose beads bound HA-CRM1 were incubated with 1 μg of either GST-ικBα (lanes 1, 2, and 5) or GST-ικBα-CA4 (lanes 3 and 6) or GST-ικBα-NA4 (lanes 4 and 7) in the presence of 2 μg of RanQ69L GTP except lane 1. For lanes 5, 6, and 7, 200 μg of 293 cell lysates was added during incubation. The beads were washed extensively, and bound proteins were electrophoresed through a SDS-10% polyacrylamide gel, transferred to nitrocellulose, and detected by immunoblot analysis. HA-CRM1, top panel; GST-ικBα, middle panel; RanQ69L, bottom panel. B, glutathione-agarose beads containing 1 μg of GST (lane 1), GST-ικBα (lane 2), GST-ικBα-CA4 (lane 3), or GST-ικBα-NA4 (lane 4) were incubated with 20 μl of insect cell lysates containing HA-CRM1 protein. RanQ69L GTP (1 μg) was added to all samples. The beads were washed extensively, and bound proteins were electrophoresed through a SDS-10% polyacrylamide gel, transferred to nitrocellulose, and HA-CRM1 was detected by immunoblot analysis (top panel). 1/20th of input GST-ικBα proteins were analyzed in parallel (bottom panel, lanes 5–7). C, three independent experiments from panel B were quantified using phosphorimaging analysis, and the relative amounts of bound HA-CRM1 are shown.

(Invitrogen). All purified proteins were dialedyzed against 20 mM HEPES, pH 7.3, 100 mM potassium acetate, 20 mM magnesium acetate, and 1 mM dithiothreitol. HA-CRM1 was purified from Tini insect cell lysates infected at high multiplicity with a baculovirus encoding HA-CRM1. Lysates were collected in 50 mM HEPES, pH 7.5, 300 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol and clarified by centrifugation. A protease inhibitor mix was included during the lysis step. Insect cell lysates were incubated with an anti-HA affinity matrix (Covance) at 4 °C for 2 h. After incubation, the affinity matrix was extensively washed with incubation buffer (10 mM sodium phosphate, pH 7.3, 150 mM NaCl, 0.1% Triton X-100, and 1 mM 2-mercaptoethanol).

Cell Culture and Transfection—COS and 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfections were performed with 1 μg of each pCMV4-based expression plasmid and 15 μl of LipofectAMINE (Life Technologies, Inc.) per 35-mm dish. 293T cells were transfected with 10 μg of a pCMV4-based expression plasmid encoding c-Ret or v-Rel per 100-mm dish using calcium phosphate. 293T lysates were collected in lysis buffer (50 mM HEPES, pH 7.9, 250 mM sodium chloride, 5 mM EDTA, and 1 mM dithiothreitol) containing a protease inhibitor mix. The cellular localization of Rel and IκBα proteins was analyzed 36–48 h after transfection by indirect immunofluorescence as previously described (15). Polyclonal anti-IκBα rabbit antiserum was followed by anti-rabbit FITC-conjugated antibody (Jackson Laboratories) for detection of the ectopically expressed IκBα protein. A monoclonal mouse antiserum directed against Rel (3C1; provided by Henry R. Rose, Jr., University of Texas) was followed by anti-mouse Cy3-conjugated antibody (Jackson Laboratories) for detection of the ectopically expressed Rel protein.

RanGAP Protection Assay—3 μg of Ran was incubated for 30 min on ice with [γ-32P]GTP (10 μCi/ml, 6000 Ci/mmol) in the presence of 20 mM HEPES, pH 7.3, and 100 mM potassium acetate and 5 mM EDTA. Loading was stopped by adding MgCl2 to a final concentration of 20 mM followed by gel filtration on a Bio-Spin 6 column (Bio-Rad) equilibrated with hydrolysis buffer (20 mM HEPES, pH 7.3, 100 mM potassium acetate, 20 mM magnesium acetate, and 1 mM dithiothreitol) containing 0.5 mg/ml bovine serum albumin. Reaction mixtures containing 50 pmol Ran[γ-32P]GTP, 500 nm CRM1, and 0–15 μg GST-IκBα or GST-NS2 proteins in 25 μl 15 °C for 30 min. The samples were placed on ice, 10 mM RanGAP was added, and the samples were immediately placed at 30 °C for 2 min. The reactions were diluted into 1 ml of ice-cold hydrolysis buffer and filtered through nitrocellulose (0.45 Micron, Micron Separations Inc.). Filters were washed twice with 3 ml of hydrolysis buffer, and radioactivity was counted in the presence of 3 ml of scintillation fluid (Ready Safe, Beckman) on a Packard Tri-Carb 2100TR liquid scintillation analyzer.

In Vitro Binding Assays—For HA-CRM1 pull-down assays, 100 ng of immobilized HA-CRM1, 1 μg of GST-IκBα, and 1–2 μg of RanQ69L GTP in incubation buffer was rotated at 4 °C for 2 h. The matrix was extensively washed with incubation buffer prior to addition of SDS-PAGE sample buffer.

For GST-IκBα pull-down of HA-CRM1, the GST-IκBα proteins were loaded onto glutathione-agarose beads in incubation 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 1 μg of RanQ69L GTP at 4 °C for 2 h. The matrix was extensively washed with incubation buffer prior to addition of SDS-PAGE sample buffer. The cells were lysed 48 h after transfection in incubation buffer containing a protease inhibitor mixture.

For GST-IκBα pull-down of v-Rel, 100 μl of 293T cell lysates was incubated with GST-IκBα bound to glutathione-agarose beads in the presence of 0–1 mM His-IκBα protein. The reaction mixtures were incubated at 4 °C for 1 h. After incubation, the beads were extensively washed with incubation buffer prior to 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 by anti-HA mouse monoclonal antibody (Covance), GST-IκBα
proteins were detected by anti-GST mouse monoclonal antibody (Santa Cruz), His-IkBα was detected by a polyclonal rabbit anti-IkBα antiserum, His-v-Rel was detected by a polyclonal mouse antiserum directed against the v-Rel NLS, full-length v-Rel was detected by a polyclonal rabbit anti-Rel antiserum, and Ran was detected by a polyclonal rabbit antiserum (Covance). All primaries were used at dilutions of 1:2000 to 1:4000. The secondary antibodies were either horseradish 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 detect 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 Pharmacia Biotech ECL reagents followed by exposure to XAR-5 film. For quantification, proteins transferred to nitrocellulose membrane were visualized using Super Signal West Dura Extended Duration Substrate (Pierce), and intensity of bands was quantified by phosphorimaging analysis (FXImager, Bio-Rad) using Bio-Rad’s Quantity One software.

RESULTS

**RanGTP-dependent Binding of IkBα to CRM1**—The formation of an export competent complex between CRM1 and a NES-bearing cargo protein requires cooperative binding of RanGTP (13). To determine if IkBα binds to CRM1 in a RanGTP-dependent manner, the ability of purified IkBα to bind purified CRM1 was determined in the absence or presence of RanGTP. HA-tagged CRM1 (HA-CRM1) was immobilized on anti-HA agarose beads, and His-tagged IkBα (His-IkBα) was added in the presence or absence of RanQ69L GTP. The agarose beads were extensively washed, and the presence of bound IkBα was determined by immunoblot analysis. Binding of His-IkBα to HA-CRM1 was markedly increased in the presence of RanQ69L GTP (Fig. 2A, lanes 3 and 4). A 16-fold increase in the amount of His-IkBα bound to CRM1 was observed when RanQ69L GTP was included in the binding reaction (Fig. 2B).

The N-terminal NES-like Sequence, but Not the C-terminal NES-like Sequence, Is Critical for RanGTP-dependent Binding of IkBα to CRM1—To determine which NES-like sequence in IkBα is required for RanGTP-dependent binding to CRM1, the ability of GST-tagged IkBα proteins to associate with HA-CRM1 was determined. Wild-type and mutant GST-tagged IkBα proteins were incubated with immobilized HA-CRM1 in the absence or presence of RanQ69L GTP. After extensive washing, the amount of bound GST-IkBα proteins was determined by immunoblot analysis. As expected, binding of GST-IkBα to HA-CRM1 was markedly increased in the presence of RanGTP (Fig. 3, lanes 1 and 2). Mutant GST-IkBα proteins containing alanine substitutions within either the N-terminal (GST-IkBα-NA4) or the C-terminal (GST-IkBα-CA4) NES-like sequences were still able to associate with CRM1, although a substantial decrease of CRM1 binding was observed with GST-IkBα-NA4 (Fig. 3A, compare lanes 2, 3, and 4). To closely mimic the in vivo situation where CRM1 interacts with multiple cargo proteins, lysates from 293T cells were included during the

![Fig. 4. Quantitative analysis of IkBα-CRM1 interactions.](image)

**Fig. 4.** Quantitative analysis of IkBα-CRM1 interactions. The indicated concentrations of GST-IkBα proteins or GST-NS2 were incubated in the presence of 50 μM Ran-[γ-32P]GTP and 500 nM CRM1 subsequent to addition of 10 nM of RanGAP. GTP bound to Ran was measured by the filter binding assay (see "Experimental Procedures"). Three independent experiments were analyzed for the GST-IkBα proteins, and the error bars represent the standard deviation from the mean. The average of two independent experiments is shown for GST-NS2.

![Fig. 5. IkBα is an adaptor protein that bridges Rel and CRM1.](image)

**Fig. 5.** IkBα is an adaptor protein that bridges Rel and CRM1. A, the indicated concentrations of GST-IkBα or His-c-Rel-(1–320) were incubated in the presence of 50 μM Ran-[γ-32P]GTP and 500 nM CRM1 subsequent to addition of 10 nM of RanGAP. GTP bound to Ran was measured by the filter binding assay (see "Experimental Procedures"). For the IkBα + His-c-Rel sample, the concentration of GST-IkBα was varied in the presence of 1 μM His-c-Rel(1–320). B, the same Ran GAP protection assay using His-v-Rel(1–331) instead of His-c-Rel was performed as described in A. C, anti-HA-agarose beads containing no protein (lanes 1) or 100 ng of HA-CRM1 were incubated with 1 μg of His-tagged v-Rel (lanes 1, 3, and 4), 1 μg of His-tagged v-Rel(1–331) (lanes 1, 2, and 4) and 1 μg of RanQ69L GTP (lanes 1–4). After extensive washing, bound proteins were electrophoresed through an SDS-7.5% polyacrylamide gel and subjected to immunoblot analysis with a monoclonal anti-HA antibody (top panel), a polyclonal anti-Rel-NLS antiserum (middle panel), and anti-Ran (bottom panel) sera.
The ability of the mutant GST-IkBα proteins to inhibit RanGTP-stimulated GTP hydrolysis by Ran was determined. The GST-IkBα-CA4 protein was equally effective as the wild-type GST-IkBα protein for protection against RanGAP, with an apparent affinity of 8 μM. In contrast, 15 μM GST-IkBα-NA4 protein did not provide significant protection against RanGAP-stimulated GTP hydrolysis by Ran.

Taken together, the binding assays and the RanGAP protection assays provide strong evidence that the N-terminal NES-like sequence of IkBα is required for formation of an export-competent complex with CRM1 and RanGTP. In contrast, mutations of hydrophobic residues within the C-terminal NES-like sequence do not affect RanGTP-dependent association of IkBα with CRM1.

**IkBα Is an Adaptor That Bridges CRM1 and Rel Proteins**—There is strong experimental evidence in support of the notion that nuclear export of NFκB/Rel proteins is mediated by CRM1. For example, cytoplasmic sequestration of p65 by IkBα is CRM1-dependent (7, 16). Likewise, our laboratory has previously reported that cytoplasmic retention of the v-Rel oncoprotein by IkBα is abolished by leptomycin B (15). However, it is not clear if IkBα is an adaptor that mediates binding of NFκB/Rel proteins to CRM1 or if NFκB/Rel proteins can bind directly to CRM1. Importantly, a functional NES has been identified in p65 (24).

We utilized the RanGAP protection assay to characterize the adaptor function of IkBα. For these experiments, we used C-terminal truncated c-Rel(1–320) or v-Rel(1–311) proteins that were readily purified as His-tagged proteins from *E. coli*. In this assay, purified His-c-Rel or His-v-Rel protein did not have a measurable affinity for CRM1 in the RanGAP protection assay (Fig. 5, A and B). Significant protection of RanGAP-induced hydrolysis of GTP was observed only in the presence of IkBα. The presence of His-c-Rel or His-v-Rel did not alter the weak affinity of IkBα for CRM1.

To further determine if NFκB/Rel proteins bind directly to CRM1 or require IkBα to act as an adaptor protein, we examined the ability of His-v-Rel to associate with CRM1 in the presence or absence of IkBα. HA-CRMs was immobilized on anti-HA-agarose in the presence of RanQ69L GTP, IkBα, and v-Rel, and the presence of bound v-Rel was determined by immunoblot analysis after extensive washing of the agarose beads. Binding of v-Rel was only observed in the presence of IkBα (Fig. 5C, lane 4), consistent with our RanGAP protection assay results. It is likely that IkBα also functions as a direct physical adaptor between CRM1 and c-Rel, although the specific formation of a CRM1-IkBα-c-Rel complex could not be demonstrated due to high nonspecific binding of the His-c-Rel

### Table: RanGTP-dependent Association of IkBα with CRM1

| Protein Assay | Cotransfected Proteins | Binding | Protection |
|---------------|------------------------|---------|------------|
| IkBα          | c-Rel                  | Positive| Positive   |
| IkBα          | v-Rel                  | Positive| Positive   |
| IkBα + v-Rel  |                        | Negative| Negative   |

### Figure 6: Requirement of each NES-like sequence in IkBα for relocalization of Rel proteins from the nucleus to the cytoplasm.

COS cells were transfected with pcMV4-based mammalian expression vectors encoding wild-type or mutant IkBα proteins alone (A, F, and K) or cotransfected with c-Rel(1–546) (B, C, G, H, I, J, and O). The cellular localization of singly transfected IkBα protein was determined by single-label indirect immunofluorescence with anti-IkBα sera (A, F, and K). The cellular localization of Rel proteins and cotransfected IkBα proteins within the same cell was determined by double-label indirect immunofluorescence with anti-IkBα sera.
protein to anti-HA-agarose beads (data not shown).

N-terminal NES-like Sequence of IκBα Is Required for the Nuclear Export of Rel—To determine which NES-like sequence of IκBα is required for nuclear export of Rel proteins, we examined the ability of wild-type and mutant IκBα proteins to retain c-Rel or v-Rel in the cytoplasm when coexpressed in COS cells. Because the full-length c-Rel protein is predominantly cytoplasmic when singly expressed in a variety of mammalian cell types (3), we utilized a C-terminal truncated c-Rel protein comprising amino acids 1–546. This truncated c-Rel protein lacks a C-terminal transcriptional activation domain and, like v-Rel, efficiently localizes to the nucleus in COS cells (15).

The localization of the wild-type or mutant IκBα proteins when singly overexpressed in COS cells was first determined. The wild-type IκBα protein and both mutant IκBα proteins were predominantly localized to the nucleus (Fig. 6, panels A, F, and K).

The localization of both IκBα and Rel proteins following coexpression in COS cells was next determined. Coexpression of either c-Rel or v-Rel with the wild-type IκBα protein results in the relocalization of both IκBα and the respective Rel protein to the cytoplasm (Fig. 6, panels B–E, Table I). To assess the extent to which cytoplasmic relocalization of the Rel proteins by IκBα requires CRM1-dependent nuclear export, the localization of the coexpressed proteins was determined following a 1-h treatment with leptomycin B. As previously noted (15), cytoplasmic relocalization of c-Rel by IκBα was partially disrupted by leptomycin B treatment, whereas cytoplasmic relocalization of v-Rel by IκBα was completely abolished by leptomycin B (Table I).

The ability of the mutant IκBα proteins to relocalize either c-Rel or v-Rel to the cytoplasm was determined. The IκBα-NA4 protein was not able to completely relocalize c-Rel to the cytoplasm, because both the IκBα-NA4 protein and the c-Rel protein displayed a whole-cell pattern of staining (Fig. 6, panels G and H; Table I). In contrast, coexpression of the IκBα-CA4 protein with c-Rel resulted in the complete relocalization of both the IκBα-CA4 and the c-Rel proteins to the cytoplasm (Fig. 6, panels L and M; Table I). Neither mutant IκBα protein was able to relocalize v-Rel to the cytoplasm (Fig. 6, panels I, J, N, and O).

The failure of mutant IκBα proteins to retain c-Rel or v-Rel in the cytoplasm could arise from a defect in association with CRM1 or from a defect in association with Rel proteins. Our previous analysis of IκBα-CRM1 interactions demonstrated that the IκBα-NA4 protein, but not the IκBα-CA4 protein, is defective in association with CRM1 (Figs. 3 and 4). To determine if the mutant IκBα proteins were able to associate with c-Rel or v-Rel, we determined the ability of GST-tagged IκBα proteins to pull-down c-Rel or v-Rel from lysates of 293T cells transfected with an expression vector for c-Rel or v-Rel. The binding assays were performed in the presence of increasing amounts of His-IκBα as a competitor to obtain an estimate of the relative affinity of the wild-type and mutant GST-IκBα proteins for either c-Rel or v-Rel.

There was no measurable difference between wild-type GST-IκBα and GST-IκBα-NA4 mutant in their ability to associate with c-Rel (Fig. 7, A and B) or with v-Rel (Fig. 7, C and D). Thus, the failure of IκBα-NA4 to efficiently relocalize either c-Rel or v-Rel to the cytoplasm is not due to a defect in association with either c-Rel or v-Rel.

However, the IκBα-CA4 protein was reduced in its ability to associate with either c-Rel (Fig. 7, A and B) or v-Rel (Fig. 7, C and D). At a GST-IκBα concentration of 50 nM, inclusion of 10 nM His-IκBα in the pull-down assay reduced the amount of v-Rel bound to the GST-IκBα-CA4 protein by 30%, whereas the amount of v-Rel bound to the GST-IκBα-CA4 protein was reduced to less than 2%. Because the IκBα-CA4 protein can efficiently relocalize c-Rel, but not v-Rel, to the cytoplasm, these results indicate that a slight reduction in affinity between IκBα and c-Rel does not significantly decrease the ability of IκBα to either inhibit import or mediate export of c-Rel, whereas a significant reduction in affinity between IκBα and v-Rel is sufficient to abolish IκBα-dependent export of v-Rel.

**DISCUSSION**

The distribution of NFκB/Rel proteins between the nucleus and the cytoplasm is a dynamic balance between nuclear import and nuclear export. Although the molecular mechanisms that underlie these competing processes are not fully understood, it is clear that the IκBα protein can exert a profound influence on both nuclear import and nuclear export of NFκB/Rel proteins. To provide further understanding of how nuclear export of both free and NFκB/Rel-associated IκBα is accomplished, we have used two complementary approaches to examine the interaction of IκBα with the nuclear export receptor

![FIGURE](https://example.com/figure.png)
CRM1. In one approach, binding of purified IκBα to purified CRM1 was assessed in the absence or presence of RanQ69L GTP. Because the CRM1 protein in these experiments was extensively used to characterize CRM1 interactions with numerous functional assays that have demonstrated that nuclear export of IκBα is CRM1-dependent (7, 14–18).

We used this binding assay to determine which of two candidate NESs in IκBα are required for binding to CRM1. Despite extensive functional characterization of CRM1-dependent nuclear export of IκBα, the sequences in IκBα that mediate binding to CRM1 have not been conclusively identified (7, 14–18). Using mutant IκBα proteins that contain alanine substitutions for hydrophobic residues within each candidate NES, we found that alanine substitutions within only the N-terminal candidate NES reduced binding of IκBα to CRM1. In contrast, alanine substitutions within the C-terminal candidate NES did not affect association of IκBα with CRM1. It is somewhat surprising that binding of IκBα to CRM1 was not completely abolished by alanine substitutions within the N-terminal NES, because similar mutations have been reported to completely abolish nuclear export of IκBα (7). However, because the wild-type IκBα protein is already a relatively weak substrate for CRM1, a further reduction in the affinity of IκBα for CRM1 would likely reduce the affinity of IκBα for CRM1 such that an IκBα protein containing a mutant NES would no longer effectively compete with other cellular proteins for binding to CRM1. Indeed, the GST-IκBα-NA4 protein was unable to bind to CRM1 when 293T cell lysates were included in the binding reaction.

That the N-terminal NES-like sequence is required for binding of IκBα to CRM1 was substantiated in the RanGAP protection assay. In this assay, protection of RanGAP-induced GTP hydrolysis by Ran requires the formation of a ternary complex between Ran-GTP, CRM1, and a NES-bearing cargo. Alanine substitutions within the N-terminal NES-like sequence markedly reduced protection against RanGAP-induced GTP hydrolysis. In contrast, alanine substitutions within the C-terminal NES-like sequence had no effect on the ability of IκBα to form a ternary complex with RanGTP and CRM1. Taken together, our results provide strong evidence that the N-terminal NES-like sequence of IκBα is an authentic NES that mediates binding of IκBα to CRM1.

Our results indicate that the N-terminal NES-like sequence of IκBα is also required for IκBα-mediated nuclear export of Rel proteins. In the case of c-Rel, mutations with the N-terminal NES-like sequence disrupted the ability of IκBα to relocalize c-Rel from the nucleus to the cytoplasm without significantly altering the ability of IκBα to associate with c-Rel. In contrast, mutations within the C-terminal NES-like sequence had no effect on relocalization of c-Rel by IκBα, despite a slight reduction in the affinity of IκBα for c-Rel. In the case of v-Rel, a mutant form of c-Rel that has lost critical contacts with IκBα that are required for efficient masking of the Rel-derived NLS (15), we find that both NES-like sequences of IκBα are required for relocalization of v-Rel from the nucleus to the cytoplasm. Mutation of the N-terminal NES-like sequence of IκBα did not alter the affinity of IκBα for v-Rel, whereas mutation of the C-terminal NES-like sequence of IκBα resulted in a significant reduction in affinity of IκBα for v-Rel. This result suggests that the inability of the IκBα-CA4 protein to relocalize v-Rel from the nucleus to the cytoplasm is a consequence of impaired association between v-Rel and IκBα rather than a consequence of impaired association with CRM1. Because IκBα is an adaptor protein that bridges Rel proteins and CRM1, loss of association with either the Rel protein or with CRM1 would result in loss of IκBα-mediated export of Rel proteins from the nucleus. We suggest that a reduced affinity of IκBα for Rel proteins, rather than a reduced affinity for CRM1, provides an explanation for why previous reports in the literature, including one
in contrast to the C-terminal NES-like sequence of IkBα was functioning as a bona fide NES.

In the RanGAP assay, the concentration range over which a CRM1-interacting cargo protein provides protection against RanGAP-induced GTP hydrolysis provides an estimate of the affinity of the cargo protein for CRM1 (11). In comparison of IkBα with the NS2 protein, we found that the affinity of IkBα for CRM1 is ~100-fold less than that of NS2. It is likely that the affinity of IkBα for CRM1 is very near the lower limit of exportable substrates, because the GST-IκBα-NA4 protein has only a reduced affinity for CRM1 but is completely defective for CRM1-dependent export.

It is puzzling why IkBα has a relatively weak affinity for CRM1, in light of the notion that IkBα-mediated nuclear export of NFκB/Rel proteins is important for cytoplasmic sequestration of NFκB/Rel proteins. One possibility is that CRM1 might discriminate between free IkBα and NFκB/Rel-associated IkBα proteins by virtue of an increased affinity for NFκB/Rel-associated IkBα proteins as compared with free IkBα. Our results do not support this scenario, because the inclusion of either c-Rel or v-Rel in the RanGAP protection assay did not alter the weak affinity of IkBα for CRM1. An alternative possibility is that the low affinity of IkBα for CRM1 is necessary to allow accumulation of sufficient IkBα to displace NFκB/Rel proteins from DNA prior to binding to CRM1 as a NFκB-Rel-IkBα complex. Under this scenario, a high affinity interaction between IkBα and CRM1 would result in futile shuttling of free IkBα between the nucleus and the cytoplasm, whereas a low affinity interaction between IkBα and CRM1 would ensure that NFκB/Rel-associated IkBα proteins are also available for CRM1-dependent nuclear export. In this regard, it is of interest to note that the Rev protein of HIV is also both an adaptor protein for CRM1-dependent export of human immunodeficiency virus RNA and a low affinity substrate for CRM1 (11). Indeed, it may generally be the case that export adaptor proteins such as IkBα and Rev must be low affinity substrates for CRM1 to enable binding of these adaptor proteins to their targets prior to being removed from the nucleus by CRM1.

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