κB-Ras Binds to the Unique Insert within the Ankyrin Repeat Domain of IκBβ and Regulates Cytoplasmic Retention of IκBβ-NF-κB Complexes

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The IκBα and IκBβ proteins inhibit the transcriptional potential of active NF-κB dimers through stable complex formation. It has been shown that inactive IκBα-NF-κB complexes shuttle in and out of the nucleus, whereas IκBβ-NF-κB complexes are retained exclusively in the cytoplasm of resting cells. The biochemical mechanism underlying this functional difference and its consequences are unknown. Although the two IκB proteins are significantly homologous, IκBβ contains a unique 47-amino acid insertion of unknown function within its ankyrin repeat domain. In this study, we assess the role of the IκBβ insert in regulating cytoplasmic retention of IκBβ-NF-κB complexes. Deletion of the IκBβ insert renders IκBβ-NF-κB complexes capable of shuttling between the nucleus and cytoplasm, similar to IκBα-NF-κB complexes. A small Ras-like G-protein, κB-Ras, participates with the IκBβ insert to effectively mask the NF-κB nuclear localization potential. Similarly, a complex between NF-κB and a mutant IκBβ protein containing four serine to alanine mutations within its C-terminal proline, glutamic acid, serine, and threonine-rich sequence exhibits nucleocytoplasmic shuttling. This suggests a phosphorylation state-dependent role for the C-terminal proline, glutamic acid, serine, and threonine-rich sequence of IκBβ in proper localization of IκBβ-NF-κB complexes. These results are consistent with structural studies, which predicted that binary IκBβ-NF-κB complexes should be capable of nuclear translocation, and with previous observations that hypophosphorylated IκBβ-NF-κB complexes can reside in the nucleus.

Although present in most cell types, NF-κB dimers with transactivation potential are maintained inactive through their stable association with the inhibitor proteins IκBα and IκBβ (4, 5). These IκB proteins share many properties, including domain structure and selectivity toward NF-κB binding partners. However, significant differences exist between them (6–9). Degradation of IκBα leads to rapid but transient activation of NF-κB (10–12). However, many cellular activities, such as lymphoid cell development, endothelial and brain cell function, as well as various pathological conditions and viral infections, require sustained activation of NF-κB (13–25). In each case, IκBβ has been shown to be the critical mediator of persistent NF-κB activity. The mechanism underlying this persistently active NF-κB remains unclear.

In an effort to understand the source of differential NF-κB activation kinetics, several groups have begun investigating the molecular mechanisms of action of the IκBα and IκBβ inhibitor proteins. One clear difference has emerged involving the manner by which these two proteins regulate NF-κB subcellular localization (26). Immunofluorescence studies have revealed that IκBα-NF-κB complexes shuttle between the cytoplasm and nucleus in quiescent cells (27–29). In contrast, inactive IκBβ-NF-κB complexes remain exclusively in the cell cytoplasm (30–32).

A mechanistic explanation for the dynamic shuttling behavior of IκBα-NF-κB complexes has been described recently. X-ray structures of the IκBα-NF-κB p50/p65 heterodimer complex revealed that IκBα masks the nuclear localization signal (NLS) of the NF-κB p65 subunit but fails to mask the p50 subunit NLS (33, 34). Protease protection assays revealed that one NLS in the IκBα-NF-κB p50/p65 heterodimer and IκBα-NF-κB c-Rel homodimer complexes is also exposed to solvent and sensitive to protease cleavage (32). Nuclear import of the IκBα-NF-κB p50/p65 heterodimer complex is imparted by the free p50 NLS, whereas the one free p65 subunit NLS is sufficient to convey similar shuttling properties upon the IκBα-NF-κB p65 homodimer complex (32). Active export of the complex from the nucleus relies on nuclear export signals located in the N-terminal signal response region of IκBα and the transactivation domain of the p65 subunit (27–29, 35).

In an accompanying study, we describe the x-ray crystal structure of an IκBβ-NF-κB p65 homodimer complex (see Ref. 43). The structure reveals that, like IκBα-NF-κB complexes, the NLS of one NF-κB p65 subunit (subunit A) is effectively masked by IκBβ, whereas the second p65 subunit (subunit B)
The NLS of p50 is responsible for shuttling IκBβ/p50/p65 complexes. The left panels show the localization of FLAG-p50ΔNLS/p65 complex. Wild type IκBβ in complex with FLAG-p50ΔNLS/p65 heterodimers is unable to enter the nucleus in both LMB-treated and untreated cells (>80%; middle two panels). The IκBβΔ-(152–192)p50ΔNLS/p65 heterodimer complex is cytoplasmic in both the presence and absence of LMB (>90%).

NLS is largely solvent-exposed. This observation is somewhat unexpected because the second NF-κB subunit NLS is significantly less sensitive to cleavage by proteases when bound to IκBβ (32). Therefore, it remains unclear as to how IκBβ-NF-κB complexes are retained within the cytoplasm of resting cells.

Recently, a small Ras-like protein was identified from a yeast two-hybrid screen as an IκBβ complex with NF-κB activation (36). It was suggested that this protein, named xB-Ras, might function by inhibiting IκBβ degradation. In the present study, we show that xB-Ras is a critical regulator of IκBβ-NF-κB complex subcellular distribution. xB-Ras binds through a unique 47-amino acid insert between ankyrin repeats 3 and 4 of IκBβ and partially masks one p65 subunit NLS in vitro and completely masks it in vivo. The conversion of serines in the IκBβ PEST sequence to non-phosphorylatable residues further alters the subcellular localization properties of IκBβ-NF-κB complexes. These data suggest that additional factors may bind xB-Ras and IκBβ-NF-κB complexes and sequester them to the cytoplasm of quiescent cells. We propose that regulated association and dissociation of xB-Ras determine the subcellular localization of IκBβ-NF-κB complexes.
FIG. 3. The IκBβ insert masks NF-κB subunit B NLS with eB-Ras. A, in vitro immunoprecipitation of wild type and mutant IκBβNF-κB p65 homodimer and IκBαNF-κB p65 homodimer complexes in the presence or absence of eB-Ras (lanes 1–8). Samples were immunoprecipitated with a monoclonal anti-p65 NLS monoclonal antibody and then immunoblotted with an anti-p65 polyclonal antibody. As a negative control, wild type IκBβNF-κB p50/p65 heterodimer complex was also immunoprecipitated with the anti-p65 NLS monoclonal antibody (lanes 9 and 10). B, co-transfection and immunoprecipitation of HA-tagged p65 with IκBα, IκBβ, and insert-deleted IκBβΔ in 293 cells. Complexes were immunoprecipitated with anti-p65 antibody and anti-p65 NLS monoclonal antibody and detected by immunoblot with anti-HA and anti-IκBβ polyclonal antibodies.

EXPERIMENTAL PROCEDURES

Mammalian Cell Transfection—HeLa cell transfection was performed by the LipofectAMINE method (Invitrogen). Leptomycin B (LMB) (5 ng/ml) was added 3 h before harvesting cells. LMB is a generous gift from Prof. M. Yoshida (University of Tokyo, Tokyo, Japan).

Immunofluorescence—Cells (with or without LMB treatment) were fixed in 3% paraformaldehyde for 20 min at room temperature and then permeabilized with phosphate-buffered saline buffer containing 0.5% Nonidet P-40 and 0.01% sodium azide (ISB). Blocking was done using 5 mg/ml bovine serum albumin followed by incubation for 30 min with primary antibodies in ISB. Cells were then washed three times with ISB. Fluorescent-tagged secondary antibody was added in ISB at room temperature.

Immunoprecipitation and Western Analysis—Cells were washed three times in phosphate-buffered saline buffer. Cytoplastmic extracts were made by lysing cells in 1% Triton X-100, 20 mM Tris-HCl (pH 7.6), 200 mM NaCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). Fifty μg of extract was mixed with protein A-agarose and primary antibodies and incubated at 4 °C overnight. The immunoprecipitates were washed three times in lysis buffer and eluted with SDS-PAGE buffer by heating at 100 °C for 5 min. The supernatant was separated by 10% SDS-PAGE. The separated proteins in the gel were transferred to Hybond nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% milk in phosphate-buffered saline with 0.2% Tween and incubated with anti-p65 polyclonal antibody (H-286; Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was washed and incubated with horseradish peroxidase-conjugated anti-rabbit Ig (Santa Cruz Biotechnology). Blots were visualized by use of the ECL reagent kit (Amersham Biosciences).

For in vitro immunoprecipitation experiments, 0.5 μg of NF-κB was mixed with 4 μg of IκB in the presence or absence of 4 μg of eB-Ras in a 15 μl binding reaction. The mixture was incubated on ice for 2 h followed by dilution to 100 μl in lysis buffer. Four μl of this diluted complex was used for the immunoprecipitation reaction using 0.1 μg of the anti-p65 NLS monoclonal antibody (a generous gift from Boche Diagnostics). The immunoprecipitates were then loaded on a Western blot, as described above.

RESULTS

The Unique IκBβ Insert Inhibits Nuclear Import of IκBβNF-κB Complexes—The finding that one p65 subunit (subunit B) NLS is at most weakly bound by IκBβ suggests that this NLS may also be capable of translocating the IκBβNF-κB complex to the nucleus. It has been shown previously that IκBβNF-κB complexes are cytoplastic in quiescent cells (30–32). Therefore, it follows that in quiescent cells the p65 subunit B NLS polypeptide must bind to IκBβ more stably than the binding mode revealed in IκBβNF-κB p65 homodimer complex crystal structure (see Ref. 43).

To test whether the unique 47-amino acid insertion within the ankyrin repeat domain of IκBβ plays any role in determining the subcellular localization of IκBβNF-κB complexes, we deleted this insert from IκBβ (residues 152–192) and observed the co-localization of this mutant (IκBβΔ(152–192)) with NF-κB p65 homodimer in HeLa cells (Fig. 1A). We observe that both complexes are cytoplastic in resting cells. However, in the presence of LMB, an inhibitor of nuclear export receptor CRM1, the mutant IκBβΔ(152–192)NF-κB p65 homodimer complex, but not the wild type complex, is predominantly nuclear (37, 38) (Fig. 1A). Co-immunoprecipitation experiments show that, like the wild type IκBβ, mutant IκBβΔ(152–192) is also associated with p65 (Fig. 1C). Our results thus demonstrate that the insert of IκBβ plays a role in vivo in IκBβNF-κB complex cytoplastic retention.

We have also tested whether the insert of IκBβ is responsible for cytosolic retention of the IκBβNF-κB p50/p65 heterodimer complex (Fig. 1B). As in the case of the IκBβΔ(152–192)NF-κB p65 homodimer complex, we observe that the IκBβΔ(152–192)
protein in complex with NF-κB p50/p65 heterodimer can also enter the nucleus of resting cells. A likely explanation for this phenomenon is that when the insert of IκBβ is removed, the second NLS becomes free (or more loosely bound to IκBβ), enabling the complex to be actively imported into the nucleus. These results suggest that the p50 subunit in the p50/p65 heterodimer acts similarly to p65 subunit B in the NF-κB p65 heterodimer, which possesses an NLS polypeptide that is primarily solvent-exposed.

The p50 Subunit NLS Regulates Nuclear Translocation of IκBβ-NF-κB p50/p65 Heterodimer Complexes—To further test whether nuclear translocation of the IκBβ(152–192)-NF-κB p50/p65 heterodimer complex is mediated by the free p50 NLS, we performed co-transfection experiments with a p50 subunit that lacks its NLS polypeptide (p50ΔNLS), p65, and IκBβΔ(152–192) (Fig. 2). We observe that this complex is cytoplasmic in both the absence and presence of LMB, suggesting that only in the IκBβΔ(152–192)-NF-κB p50/p65 heterodimer complex is the p50 NLS free, whereas it remains masked in the wild type IκBβ-NF-κB p50/p65 heterodimer complex. These results indicate that one NF-κB NLS and the IκBβ insert antagonize one another in directing subcellular localization of all IκBβ-NF-κB complexes.

κB-Ras Regulates the p65 Subunit B NLS of the IκBβ-NF-κB p65 Homodimer Complex in Vitro and in Cells—The above experiments suggest that the IκBβ insert could mask the second (subunit B) NLS of IκBβ-NF-κB complexes in resting cells. Direct contact likely requires modification of one or both of these interacting elements. The small GTPase κB-Ras was recently shown to be involved in regulation of IκBβ degradation (36). To determine whether κB-Ras might be involved in masking the NF-κB subunit B NLS, we have performed immunoprecipitation experiments with a monoclonal antibody directed against the p65 subunit in the p65 NLS used to precipitate free p65, IκBβ-NF-κB p65 homodimer binary complexes, and κB-RasIκBβ-NF-κB p65 homodimer ternary complexes. In parallel experiments, IκBβ-NF-κB p65 homodimer complexes were probed in the presence and absence of κB-Ras. The p65 from the immunoprecipitated complexes was then visualized by anti-p65 polyclonal antibody (Fig. 3A).

We observe that both IκBβ-NF-κB p65 homodimer and IκBβ-NF-κB p65 homodimer complexes can be precipitated by the anti-p65 NLS antibody (Fig. 3A, lanes 2 and 7, respectively). Moreover, we observe that the addition of κB-Ras inhibits the ability of this antibody to bind to IκBβ-NF-κB p65 homodimer complexes but not IκBβ-NF-κB p65 homodimer (Fig. 3A, lanes 3 and 8). To determine whether the insert of IκBβ plays a role in NLS masking by κB-Ras, we performed similar immunoprecipitation experiments with IκBβΔ(152–192)-NF-κB p65 homodimer complexes in the presence and absence of κB-Ras. We observe that the addition of κB-Ras fails to enhance NLS masking of IκBβΔ(152–192)-NF-κB p65 homodimer complexes (Fig. 3A, lanes 4 and 5). To verify specificity of the anti-p65 NLS monoclonal antibody, we tested it against the IκBβ-NF-κB p50/p65 complex. The anti-p65 NLS antibody fails to recognize and precipitate p65 in these complexes. This results from the nearly complete masking of the p65 subunit A NLS polypeptide by IκBβ as observed in the IκBβ-NF-κB p65 complex crystal structure (Fig. 3A, lanes 9 and 10).

To further investigate whether κB-Ras mediates blockade of the p65 NLS in cells, we next co-transfected 293 cells with HA-tagged p65 together with IκBα, IκBβ, or IκBβΔ(152–192). We first confirmed that p65 associates with all three IκB proteins by co-immunoprecipitation using an antibody against the transcriptional activation domain of p65 (Fig. 3B). When the anti-p65 NLS antibody was used for immunoprecipitation, we observed that only IκBα and IκBβΔ(152–192) were pulled down, but not wild type IκBβ. These experiments suggest that in IκBβ-NF-κB p65 homodimer and IκBβΔ(152–192) complexes, at least one p65 NLS is free, whereas both p65 subunit NLS polypeptides are blocked in the IκBβ-NF-κB p65 homodimer complex. We conclude that the IκBβ insert is required for masking of the NF-κB subunit B NLS and cytoplasmic retention of IκBβ-NF-κB complexes.

κB-Ras Directly Interacts with the IκBβ Insert—To identify the role of the IκBβ insert in κB-Ras binding, we have tested
the binding of co-transfected xB-Ras and IxBβ in cells. COS cells were co-transfected with plasmids expressing both wild type IxBβ and xB-Ras. We show that xB-Ras can be precipitated only when wild type IxBβ is present (Fig. 4). However, the association is not observed in cells expressing IxBβΔ(152–192) and xB-Ras. Therefore, the IxBβ insert is required for association with xB-Ras. Taken together, these experiments suggest that the small GTPase xB-Ras may play an in vivo role in blocking the subunit B NLS of dimeric NF-xB by binding directly to the unique insert region of the IxBβ inhibitor protein.

**The IxBβ PEST Functions in Cytoplasmic Retention of IxBβ-NF-xB Complexes—**Because xB-Ras was identified as an IxBβ-interacting protein in a yeast two-hybrid screen with the IxBβ C-terminal PEST used as the bait, it is important to evaluate the role of the IxBβ PEST in NF-xB cytoplasmic sequestration. To test whether phosphorylation of the IxBβ PEST plays any role in cytoplasmic retention of NF-xB, we mutated four phosphorylatable serines (Ser312, Ser313, Ser314, and Ser316) within this region to either alanine (PEST-Ala) or aspartic acid (PEST-Asp). Two serine residues in this region have been shown to be phosphorylated by casein kinase II, and phosphorylation at these sites is important for NF-xB homodimer complex to the cytosol. It is possible that xB-Ras interacts with both the IxBβ insert and phosphorylated PEST. It is also possible that the interaction between the IxBβ PEST and xB-Ras is indirect, mediated by another bridging factor.

**DISCUSSION**

IxBβ-NF-xB complexes are cytoplasmic in quiescent cells (30–32). This suggests that the NF-xB subunit B NLS can exhibit a binding mode, alternative to that observed in the IxBβ-NF-xB p65 homodimer complex crystal structure, in which it is completely masked (see Ref. 43). We have tested this hypothesis derived from our structural analyses of IxBβ-NF-xB complexes by both in vitro and cell-based studies. Transient transfection and immunostaining experiments reveal that the unique and structurally disordered insert between the third and fourth ankyrin repeats of IxBβ functions to regulate nuclear import of the IxBβ-NF-xB p65 homodimer complex in resting cells. These experiments suggest that the insert functions as a nuclear export signal or blocks a nuclear localization sequence. No recognizable export sequence is observed within the IxBβ insert. Interestingly, IxBβΔ(152–192)-NF-xB complexes shuttle between the nucleus and cytoplasm, despite the lack of a nuclear export sequence in IxBβ. We suggest that the partially exposed NLS of NF-xB subunit B and the export potential of the p65 activation domain drive the dynamic shuttling behavior of these complexes (32, 35).

We propose the hypothesis that xB-Ras may act in concert with the IxBβ insert to sequester IxBβ-NF-xB complexes to the cytoplasm. Indeed, we show that the small GTPase xB-Ras is able to reduce access to the NF-xB p65 homodimer subunit B NLS in complexes between p65 and IxBβ. We further present evidence that binding of xB-Ras to IxBβ requires the insert within the ankyrin repeat domain of IxBβ. Because this insert is unique to IxBβ, these results serve to explain the specificity of xB-Ras for the IxBβ-NF-xB complex.

There are several published observations which suggest that phosphorylation of the IxBβ PEST might play an important role in regulation of NF-xB by IxBβ (7, 9, 22). The most important of these is the identification of xB-Ras through the utilization of the IxBβ PEST sequence as bait in a yeast two-hybrid screen (36). Also, it has been shown that PEST phosphorylation...
is important for stable complex formation with NF-κB (39). We show here that PEST phosphorylation of IκBβ functions in cytoplasmic retention of IκBβ-NF-κB complexes. Although we do not observe a clear need for PEST phosphorylation in the interaction between IκBβ and NF-κB or IκB-Ras/IκBβ-NF-κB complex formation, our experiments do suggest that PEST phosphorylation cooperates with IκB-Ras binding and the IκBβ insert in regulating cytosolic retention of IκBβ-NF-κB complexes. We propose that this cooperativity might be mediated through other cellular factors or through further post-translational modification elsewhere in the complex. We favor the first possibility in light of our recent findings that IκBβ is present in the cytoplasm of quiescent cells as large complexes, which include NF-κB and IκB-Ras as well as other as-yet-unknown factors. Working together, the IκBβ insert and the PEST, one NF-κB NLS, and IκB-Ras represent a molecular mechanism for switching between nuclear and cytoplasmic IκBβ-NF-κB complexes (Fig. 6).

Our experiments help to explain the observation that hypophosphorylated IκBβ-NF-κB complexes can localize into the nucleus (7). We suggest that the hypophosphorylated form of IκBβ is functionally equivalent to the IκBβ-PEST-Ala protein construct used in this study.

Finally, our results serve to explain one long-standing puzzle. It has long been thought that IκBα binds NF-κB dimers with a significantly higher affinity than does IκBβ. This conclusion was drawn based on the respective abilities of IκBα and IκBβ to inhibit NF-κB DNA binding. We have shown previously that compared with IκBα, IκBβ binds NF-κB p50/p65 heterodimer and p65 homodimer with only slightly weaker affinity (32, 41, 42). In light of the IκBβ-NF-κB/p65 homodimer complex crystal structure and the biochemical evidence surrounding IκB-Ras binding to IκBβ-NF-κB complexes, we suggest that the in vivo stability of IκBβ-NF-κB complexes is nearly equivalent to that of IκBα-NF-κB complexes. This would explain why both complexes are present in almost all cells in almost equal amounts (5).

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