IκBβ, but Not IκBa, Functions as a Classical Cytoplasmic Inhibitor of NF-κB Dimers by Masking Both NF-κB Nuclear Localization Sequences in Resting Cells*

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NF-κB dimers, inhibitor IκBα proteins, and NF-κB-IκB complexes exhibit distinct patterns in partitioning between nuclear and cytoplasmic cellular compartments. IκB-dependent modulation of NF-κB subcellular localization represents one of the more poorly understood processes in the NF-κB signaling pathway. In this study, we have combined in vitro biochemical and cell-based methods to elucidate differences in NF-κB regulation exhibited by the inhibitors IκBβ and IκBa. We show that although both IκBα and IκBβ bind to NF-κB with similar global architecture and stability, significant differences exist that contribute to their unique functional roles.

IκBβ derives its high affinity toward NF-κB dimers by binding to both NF-κB subunit nuclear localization signals. In contrast, IκBα contacts only one NF-κB NLS and employs its carboxyl-terminal proline, glutamic acid, serine, and threonine-rich region for high affinity NF-κB binding. We show that the presence of one free NLS in the NF-κB-IκBα complex renders it a dynamic nucleocytoplasmic complex, whereas NF-κB-IκBβ complexes are localized to the cytoplasm of resting cells.

NF-κB represents a paradigm for inducible transcription factors that are regulated, in large part, through their subcellular compartmentalization (1–3). The multiple cellular activities of NF-κB are controlled through interactions with members of the IκB family inhibitor proteins. In resting cells, NF-κB dimers reside in the cytoplasm in complex with IκB proteins, which function by masking the nuclear localization signals (NLSs) of NF-κB. Upon cellular stimulation, the NF-κB-associated IκB proteins are specifically phosphorylated at two amino-terminal serines by the IκB kinase complex (IKK) (4). This leads to the removal of IκBα by a ubiquitin-linked proteosome degradation pathway and to the migration of active NF-κB into the nucleus.

Five homologous polypeptides, p50, p65, c-Rel, RelB, and p52, comprise the mammalian Rel/NF-κB transcription factor family. The subunits associate in a combinatorial fashion to form transcriptionally active homo- and heterodimers. The most prevalent and well characterized species of NF-κB dimer is the p50/p65 heterodimer (Fig. 1a). Rel/NF-κB polypeptides are defined by the presence of the Rel homology region (RHR), a 300-amino acid segment responsible for site-specific DNA binding, subunit dimerization, nuclear translocation, and inhibitor binding (5). The regions responsible for the transcriptional activation lie outside of the RHR (6).

IκBα is a diverse family of transcription factor inhibitors that includes IκBa, IκBβ, IκBe, IκBγ, Bcl-3, p105, and p100 (10). Each of the IκB family proteins contains six or seven imperfect copies of the 33-amino acid structural motif known as ankyrin repeats. Ankyrin repeat-containing domains, present in numerous proteins of diverse functions, are primarily protein-protein interaction modules (11). Principal among IκB family proteins are the inhibitors IκBa and IκBβ (Fig. 1b). Both proteins possess an amino-terminal signal response domain, which contains a pair of conserved serine residues for inducible phosphorylation. IκBa and IκBβ also bear carboxyl-terminal segments rich in the amino acids proline, glutamic acid, serine, and threonine (PEST). This highly acidic PEST region represents a site for constitutive phosphorylation by protein kinase CK2 (casein kinase II) (12–15).

IκBa and IκBβ both bind preferentially to p65- and c-Rel-containing NF-κB homo- and heterodimers (3, 16). Despite extensive primary structural similarities, IκBa and IκBβ exhibit significant functional differences in vivo. The most striking of these differences is that IκBβ activates NF-κB persistently in a cell type- and stimulus-specific manner, whereas regulation of NF-κB by IκBa is rapid but transient (16–18). The distinct functional roles arise due in part to the fact that active NF-κB up-regulates expression of IκBa but not IκBβ. Newly synthesized IκBa can enter the nucleus and dissociate transcriptionally competent NF-κB-DNA complexes (19–22). IκBβ has been shown to be a weaker inhibitor of NF-κB DNA binding as compared with IκBa (18). It is not precisely clear how an inhibitor that exhibits such weak binding affinity is capable of properly regulating NF-κB activity. Finally, several recent studies have shown that both free IκBa and NF-κB-IκBα complexes shuttle between the nucleus and cytoplasm (23–26). Little is known about the subcellular localization status of free IκBα and NF-κB-IκBβ complexes.

In this study, we have investigated the molecular mechanism of NF-κB inhibition by IκBβ and compared it with IκBa. We report that, as with IκBa, IκBβ binds NF-κB dimers with 1:1 stoichiometry. However, the mechanisms by which the two inhibitors interact with NF-κB dimers differ significantly. IκBa

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§ The abbreviations used are: NLS, nuclear localization signal; IKK, IκB kinase complex; BHR, Rel homology region; PEST, proline, glutamic acid, serine, and threonine-rich region; LMB, leptomycin B; GST, glutathione S-transferase; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline.

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and IxBβ vary with regard to their NF-κB DNA-inhibitory binding properties as well as in their abilities to mask the NF-κB nuclear localization sequences. IxBβ gains significant binding energy by contacting both of the NLSs of a NF-κB dimer as opposed to IxBα, which contacts only one NF-κB NLS. We report that NF-κB p50/p65 heterodimer-IxBβ complexes shuttle between the nucleus and cytoplasm by virtue of a free p50 NLS. Inactive NF-κB-IxBβ complexes, on the other hand, reside permanently in the cell cytoplasm as both the NLSs of the NF-κB dimer are masked.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The cloning, expression, and purification of the NF-κB subunits and amino-terminal deletion mutants has been described previously (27, 28). Five serine amino acids within the carboxyl-terminal PEST region of IxBβ (residues 312, 313, 314, 316, and 318) were replaced by glutamic acids to create the E5-IxBβ protein. IxBα-(67–302), IxBβ-(54–309), IxBβ-(54–331), and E5-IxBβ-(54–331) proteins were purified by anion exchange and size exclusion chromatography according to published protocols (29). Full-length GST-IxBβ (residues 1–359) was purified by glutathione-agarose column chromatography following the procedures prescribed by the manufacturer (Amersham Pharmacia Biotech).

**Native Polycarylamide Gel Electrophoresis**—10% native polycarylamide gels were prepared with 0.25× Tris-borate EDTA (TBE) buffer. Proteins and protein complexes were diluted in 10 mM Tris (pH 7.5), 200 mM NaCl, 4% glycerol, and 2 mM β-mercaptoethanol. Reactions were allowed to equilibrate at room temperature for 1 h. Native gel loading buffer (50 mM Tris, pH 7.5, 0.1% bromphenol blue, 10% glycerol, and 1.25 mM β-mercaptoethanol) was then added to each sample. The gels were run in 0.25× TBE for 1.5 h at 3-mA constant current.

**Fluorescence Polarization Competition Assay**—Fluorescence polarization competition assays were performed as described previously (30). Briefly, varying concentrations of IxBβ were mixed with constant amounts of NF-κB pre-equilibrated with fluorescein-labeled DNA. A decrease in polarization was observed with increased IxBβ concentrations. The competition assay binding curves were analyzed for IC₅₀ values, defined as the concentration of IxBβ at 0.5 fractional occupancy. Kᵣ values, the dissociation constant for the NF-κB/IxBβ interaction, were calculated as an average of three experiments. There was less than a 20% error between individual experiments.

**Gel Shift Competition Assay**—A 6% polycarylamide gel was prepared in 0.25× TBE, and IxB was 32P-radiolabeled according to published methods (31). Radiolabeled IxBα (18 nt) in complex with NF-κB p50/p65 heterodimer (35 nt) was incubated with 10–450 unlabeled IxBα and IxBβ and electrophoresed at 120-mA constant current. Gels were analyzed by autoradiography. Similarly, radiolabeled IxBα (100 nt) in complex with NF-κB p65 homodimer (100 nt) was incubated with 10–450 unlabeled IxBα and IxBβ and electrophoretically separated and analyzed.

**Protease Digestion Experiments**—NF-κB p65-(191–321)E5-IxBβ complex at 50 mg/ml was diluted with 20 mM Tris (pH 8.0), 50 mM NaCl, 50% glycerol, and 1 mM dithiothreitol to a final concentration of 8 mg/ml. Thermolysin (Roche Molecular Biochemicals), a zinc metalloproteinase with specificity toward isoleucine, leucine, methionine, and valine, was activated with 2 mM CaCl₂, 0.145 mM ZnSO₄, and 10 mM Tris (pH 8.0). 0.1 μg of thermolysin was added to a 50-μl reaction containing 1.5 mg/ml p65-(191–321)E5-IxBβ complex or p65-(191–321) alone in 10 mM Tris-HCl (pH 8.0), 2 mM CaCl₂, 0.145 mM ZnSO₄, and 1 mM dithiothreitol. The reactions were incubated at 37 °C. The reaction was quenched by the addition of 10 mM EDTA and 1× SDS-polyacrylamide gel electrophoresis loading dye (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) followed by 1 min of boiling and immediate storage at −20 °C. Samples were analyzed on an 18% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie staining. Using a similar experimental design, NF-κB p65-(191–321)IxBα-(67–302) complexes were treated with thermolysin, and their proteolysis patterns were analyzed by SDS-polyacrylamide gel electrophoresis.

**Fluorescence Microscopy**—All cells were grown on eight-well chamber slides (Lab Tek). Cells were transfected with a total of 0.2 μg of plasmid DNA by the LipofectAMINE method (Life Technologies, Inc.). After 24 h, the cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with 0.25% Nonidet P-40 in PBS for 1 min and blocked with 5 mg/ml bovine serum albumin in PBS with 0.1% Tween 20 at room temperature for 30 min. Cells were incubated with rabbit antibody alone or in a mixture as required for the experiment in PBS containing 5 mg/ml bovine serum albumin and 0.2% Nonidet P-40 at room temperature for 2 h. Cells were washed three times with PBS with 0.2% Nonidet P-40 and incubated with secondary antibody at room temperature for 1 h. Finally, cells were washed three times with PBS with 0.1% Tween 20 and covered with a drop of mounting solution (Vector).

**RESULTS**

**Stoichiometry of NF-κB-IxBβ Complexes**—One molecule of IxBα binds to one NF-κB dimer (32). We wished to determine
the stoichiometry of the complex between NF-κB and IκBβ. Size exclusion chromatography on NF-κB-IκBβ and NF-κB-IκBα complexes revealed that they share similar molecular masses (data not shown). This suggests that IκBβ also binds NF-κB dimers with 1:1 stoichiometry. To test this hypothesis, we performed native polyacrylamide gel electrophoretic analysis of NF-κB-IκBβ complexes (Fig. 2). Two different murine IκBβ proteins, IκBβ-(54–331) and GST-IκBβ (residues 1–359), were incubated with the RHR of NF-κB p65 (residues 19–325). The two IκBβ molecules bind NF-κB dimers with identical affinities (see below) but display unique electrophoretic mobilities in complex with p65-(19–325). When equimolar amounts of IκBβ-(54–331), GST-IκBβ, and p65-(19–325) homodimer were mixed and allowed to equilibrate, they ran as two discrete bands on the native polyacrylamide gel, the first representing the IκBβ-(54–331)/p65-(19–325) homodimer complex and the second corresponding to the GST-IκBβ/p65-(19–325) homodimer complex. Higher order binding of IκBβ to NF-κB would have resulted in the appearance of additional, intermediate mobility bands corresponding to combinations of both IκBβ molecules and p65. The absence of any such species confirms that IκBβ binds to NF-κB dimers with 1:1 stoichiometry.

By Comparison to IκBα, IκBβ Is a Weaker Inhibitor of NF-κB DNA Binding—We determined the efficiency by which IκBβ functions as an inhibitor of NF-κB dimer DNA binding. A solution-based competition assay was performed in which fixed amounts of fluorescein-labeled κB DNA bound to the NF-κB p50/p65 heterodimer or p65 homodimer were incubated with increasing concentrations of IκBβ. In this assay, the IκB-dependent removal of NF-κB from DNA and the associated decrease in fluorescence polarization is monitored to determine the DNA binding inhibition constant at equilibrium (30).

First, experiments were performed to confirm that, like IκBα, the amino-terminal signal response domain of IκBβ does not contribute to NF-κB binding affinity (data not shown). IκBβ-(54–331) inhibits the wild type DNA binding of p50-(39–376)/p65-(19–325) heterodimer with an apparent inhibition constant (Kᵢ) of 150 nM (Fig. 3a and Table I). This value is ~10-fold weaker than that exhibited by IκBα. We next mutated all five serines within the PEST sequence of IκBβ to phosphomimetic glutamic acids (E5-IκBβ) (33). This resulted in only a slight enhancement of Kᵢ for E5-IκBβ-(54–331). Using the same fluorescence polarization competition assay, we determined the inhibition constants of these IκBβ proteins for the NF-κB p65 homodimer in complex with κB DNA (see Fig. 3b). IκBβ-(54–331) exhibited a 2.5-fold lower affinity for p65-(19–325) homodimer as compared with the p50/p65 heterodimer (Table I). E5-IκBβ-(54–331) disrupts the p65 homodimer-DNA complex only marginally better.

IκBβ Relies More on the NF-κB NLS Polypeptide(s) for Stable Complex Formation—X-ray crystal structures of the NF-κB-IκBα complex reveal how the segment encompassing the NLS of the NF-κB p65 subunit (murine residues 291–319) is important for the stability of this complex (34, 35). Specifically, IκBα mediates numerous ionic interactions with the NF-κB p65 subunit NLS residues and makes van der Waals contacts with several hydrophobic side chains carboxyl-terminal to it. This region of the NF-κB RHR does not exhibit an ordered structure in the DNA-bound NF-κB crystal structures (36–38) and will be referred to as the NLS polypeptide (see Fig. 1b). To determine whether the p65 NLS polypeptide is as important for IκBβ binding, we generated a shorter version of the p65 subunit RHR that ends at residue Arg304. Fluorescence polarization competition assays were performed to determine the binding affinity of IκBβ for both the wild type p65-(19–325) RHR homodimer and the truncated p65-(19–304) (Fig. 3b).

As seen in Table II and Fig. 3b, the binding affinity of IκBβ for p65-(19–325) is 24-fold tighter than for p65-(19–304). In contrast, IκBα binds to the corresponding p65 homodimers with similar affinities. In a separate study, in which direct binding of recombinant NF-κB and IκB proteins was assayed, we observed that IκBα binds to p65-(19–325) homodimer with 5–6-fold higher affinity than p65-(19–304) (31). We currently have no explanation for the discrepancy in the values obtained from the two methods. Regardless, both assays indicate that this NLS polypeptide segment of NF-κB plays a lesser role in binding to IκBα as compared with IκBβ.

We also determined the binding affinities of IκBβ-(54–331) for both the wild type NF-κB p50-(39–376)/p65-(19–325) heterodimer and the truncated heterodimer p50-(39–363)/p65-(19–304). As summarized in Table II, the binding affinity of the truncated heterodimer is nearly 10-fold weaker as compared with the wild type p50/p65 heterodimer. In contrast, IκBα binds both the long and short NF-κB heterodimers with nearly equal affinities (Table II). Again, analysis of IκBα/NF-κB complex formation by direct binding assay showed a 4-fold higher...
Fig. 3. IxBβ is a weak inhibitor of NF-κB DNA binding. a, curves represent fluorescence polarization competition binding assays, where 60 nM NF-κB p50-(39–376)/p65-(19–325) and 6 nM IxB DNA were incubated with increasing concentrations of IxBβ-(54–331) (blue), E5-IxBβ-(54–331) (magenta), and IxBβ-(54–309). b, 1 μM NF-κB p50-(19–325) homodimer (magenta) and 1 μM NF-κB p65-(19–325) homodimer (blue) were incubated with 10 nM IxB DNA and increasing concentrations of IxBβ-(54–331). These curves identify the importance of the p65 NLS polypeptide for interaction with IxBβ.

| TABLE I | Relative inhibition constant (K_i) values of IxBβ for NF-κB dimer-DNA complexes as calculated from fluorescence polarization competition experiments |
|-----------------|-----------------|-----------------|-----------------|
| IxBβ            | NF-κB           | K_i (nM)        | Relative K_i    |
| IxBβ-(54–331)   | p50-(39–376)/p65-(19–325) | 150 ± 29        | 1               |
| E5-IxBβ-(54–331)| p50-(39–376)/p65-(19–325) | 144 ± 14        | 1               |
| IxBβ-(54–309)   | p50-(39–376)/p65-(19–325) | 1533 ± 230      | 10              |
| IxBβ-(54–331)   | p65-(19–325)/p65-(19–325) | 414 ± 149       | 2.8             |
| E5-IxBβ-(54–331)| p65-(19–325)/p65-(19–325) | 375 ± 92        | 2.5             |
| IxBβ-(54–309)   | p65-(19–325)/p65-(19–325) | 6700 ± 480      | 45              |

a K_i = [IxBβ]/[NF-κB]/[IxBβ]/[NF-κB].

The affinity for the longer p50/p65 heterodimer as compared with p50-(39–363)/p65-(19–304) (31). In these experiments indicate that the NLS polypeptide segment of NF-κB p65 homodimers and p50/p65 heterodimers makes greater contributions to overall binding affinity in the case of IxBβ but not IxBα. In addition to fluorescence polarization experiments, the stability of these complexes was analyzed by qualitative analytical size exclusion chromatography. A 5-fold molar excess of IxBβ was incubated with either the truncated p65-(19–304) or p65-(19–325) homodimer, and the complexes were separated on a...
size exclusion column (data not shown). The NF-κB p65-(19–304)/IκBα complex exhibits one broad peak, suggestive of an unstable complex. The p65-(19–325) homodimer/IκBα complex, on the other hand, displays one sharp peak corresponding to the NF-κB p65/IκBα complex followed by a second peak that contains free IκBα. These experiments lend support to the hypothesis that the NLS polypeptides of NF-κB subunits play a greater role in binding to IκBα than they do with IκBβ.

**IκBβ Interacts Strongly with NF-κB**—The fact that IκBβ inhibits the DNA binding of NF-κB more weakly than does IκBα may not necessarily indicate that interactions between IκBβ and NF-κB dimers are of low affinity. To test the affinity of direct physical association between IκBβ and NF-κB dimers, we employed an electrophoretic gel mobility shift competition assay (31). In this assay, radiolabeled IκBα in complex with NF-κB p50/p65 heterodimer was competed out by increasing amounts of unlabeled IκBβ (Fig. 4a). We performed similar experiments using IκBα as the unlabeled competitor. Each experiment was performed at least four times, and the concentrations of unlabeled IκBβ and IκBα required to dissociate half of the radiolabeled IκBα were calculated.

It is clear from these experiments that IκBα is a stronger competitor than IκBβ, suggesting that IκBα binds more tightly to the NF-κB p50/p65 heterodimer. However, the difference in affinity of IκBα and IκBβ toward NF-κB is only on the order of 2–3-fold compared with a much larger disparity observed in the abilities of these inhibitors to disrupt NF-κB DNA binding. Furthermore, the pattern of competitive replacement of radiolabeled IκBα differs between the unlabeled IκBα and IκBβ. IκBβ begins to interfere with NF-κB-IκBα binding at a lower concentration and exhibits a more gradual competitive binding.
mation (Fig. 5, lanes 2–5). A molecular weight marker was run as a reference (lane 1). The incubation times for the proteolysis reactions are listed in minutes above the gel. The asterisks denote the stable proteolytic fragment of p65. b, thermolysin-treated NF-κB p65-(191–321) homodimer alone (lanes 6–8) and in complex with IκBβ-(54–331) (lanes 2–5). A molecular weight marker was run as a reference (lane 1).

IκBβ Interacts with Both NF-κB NLSs—A model that accounts for both the increased dependence of IκBβ on the NF-κB NLS polypeptides and its weaker ability to inhibit NF-κB DNA binding is that, in contrast to IκBa, IκBβ forms high affinity complexes with NF-κB by contacting both NLSs. To test this hypothesis, we performed controlled proteolysis experiments and observed the protection patterns of NF-κB subunit NLSs. E5-IκBβ-(54–331) was complexed with a p65 subunit construct containing only the protease-resistant dimerization domain with the p65 NLS polypeptide removed, suggesting that both p65 NLS polypeptides are required to form stable complexes. We have analyzed the proteolytic digestion of the complex by mass spectrometry and observed that the lower band of the doublet corresponds to p65 amino acids 191–311. By comparison, both NLS polypeptides of free p65-(191–321) homodimer are proteolyzed to the stable dimerization domain fragment within minutes of thermolysin treatment (Fig. 5a, lanes 6–8). Interestingly, when the p65-(19–321)IκBa complex is treated with thermolysin, approximately half of the p65 is proteolyzed to the dimerization domain fragment within minutes (Fig. 5b, lanes 2–8). The resulting doublet remains stable indefinitely. This suggests that, unlike IκBβ, IκBa is capable of protecting only one p65 NLS polypeptide from proteolytic cleavage.

IκBβ Is a Cytoplasmic Inhibitor of NF-κB, whereas IκBa Is Nucleocytoplasmic—Having established that IκBβ masks both NF-κB subunit NLSs, we investigated the in vivo consequences of differential NF-κB interactions by IκBa and IκBβ. First, we determined the subcellular distribution of endogenous IκBβ in p65−/− MEF cells (Fig. 6a). We observe that IκBβ is a predominantly cytoplasmic protein, while IκBa is present both in the cytoplasm and nucleus, although a larger fraction is present in the cytoplasm. While the vast majority of IκB proteins exist as complexes with NF-κB, it is possible that at least a fraction of IκBa and IκBβ are present as free proteins in p65−/− MEF cells. Compartmental distribution of IκBa and IκBβ was also determined in cells treated with the nuclear export inhibitor leptomycin B (LMB) (39–42). The subcellular distribution of IκBβ does not change significantly upon the addition of LMB. This result indicates that endogenous IκBβ is cytoplasmic irrespective of its status as a free protein or in complex with NF-κB dimers such as the p50/c-Rel heterodimer or c-Rel homodimer in p65−/− MEF cells. On the other hand, the addition of LMB results in nuclear staining of IκBa. This result suggests that the entire population of IκBa, both free and in complex with NF-κB, participates in dynamic shuttling between the nuclear and cytoplasmic cellular compartments.
Because it is unclear in these studies whether the IkB proteins are free or in complex with NF-kB dimers, we further tested for IkB compartmentalization in the presence of excess NF-kB binding partner. MEF p65−/− cells were transfected with p65 cDNA to test whether nuclear localization of IkBβ and IkBα is influenced by the presence of excess p65 homodimer (Fig. 6b). Immunofluorescence staining of p65-transfected cells with anti-IkBα antibody indicated that IkBα localizes exclusively to the cytoplasm. It appears that overexpressed p65 localizes mostly to the nucleus; we do, however, observe some cytoplasmic staining of p65. Treatment with LMB, however, resulted in nuclear IkBα staining. Immunofluorescence staining of p65 revealed that p65 co-localizes with IkBα. This result is consistent with reports published previously (23–25). In contrast, IkBβ exhibits a unique pattern of subcellular localization in p65-transfected cells (Fig. 6c). We observe that, in p65-transfected p65−/− MEF cells, IkBβ remains primarily cytoplasmic in both the LMB-treated and -untreated cells. This result is even clearer when both IkBβ and p65 are co-transfected in HeLa cells (Fig. 6d). The NF-κB p65 homodimer-IkBβ complex is, therefore, a resident cytoplasmic complex.

The p50 Subunit NLS Is Responsible for Nuclear Translocation of the NF-κB p50/p65 Heterodimer—IkBα Complex—In an effort to determine whether NF-κB p50/p65 heterodimer-IkBα complexes behave similarly to p65 homodimer-IkB complexes, p65−/− MEF cells were co-transfected with both p65 and p50 cDNAs. Immunofluorescence staining revealed that the NF-κB p50/p65-IkBα complex is cytoplasmic (Fig. 7a, left panels). We observe staining of overexpressed p50 in both the cytoplasm and nucleus. Nuclear p50 is likely to be due to excess p50 homodimer, which is not retained in the cytoplasm by IkB proteins (23). However, similar to complexes between p65 homodimer and IkBα, LMB treatment resulted in nuclear complex staining. Similar experiments performed with the NF-κB p50/p65 heterodimer-IkBβ complex revealed that it is primarily cytoplasmic and that LMB treatment of cells does not alter this localization profile (Fig. 7b, left panels).

We next tested whether nuclear translocation of the NF-κB p50/p65 heterodimer-IkBα complex is dependent on the free p50 NLS as suggested by the x-ray crystal structures (34, 35) and protease sensitivity studies of the NF-κB p65 homodimer-IkBα complex presented in this study. p65−/− MEF cells were transfected with p65 and an excess of NLS-deficient p50 (residues 1–352). We observe that the NF-κB p50-(1–352)/p65 heterodimer-IkBα complex localizes to the cytoplasm in both LMB-treated and -untreated cells (Fig. 7a, right panels). As expected, the analogous NF-κB p50-(1–352)/p65-IkBα complex displays the same cytoplasmic staining pattern as the wild type NF-κB-IkBβ complex (Fig. 7b, right panels). These results confirm that the free p50 subunit NLS of the NF-κB p50/p65 heterodimer-IkBα complex is a necessary component for dynamic nucleocytoplasmic shuttling of the complex. Further-

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**Fig. 6.** IkBβ is a cytoplasmic inhibitor of NF-κB, whereas IkBα is nucleocytoplasmic. a, the subcellular distribution of IkBα (left panels) and IkBβ (right panels) in resting p65−/− MEF cells in the presence (above) and absence (below) of nuclear export receptor inhibitor LMB. Note that IkBα localizes to the nucleus only upon LMB treatment. b, localization of IkBα in p65−/− MEF cells transfected with HA-tagged p65. Immunofluorescence staining is directed toward IkBα proteins (left panels, green) and HA (right panels, red). c, IkBβ localization (left panels, red) in p65−/− MEF cells transfected with HA-p65 (right panels, green). Note that IkBβ remains primarily cytoplasmic in both the presence and absence of LMB. d, co-transfection and immunofluorescence detection of IkBβ (left panels, red) and p65 (right panels, green) in HeLa cells.
more, our studies suggest that in contrast to IκBα, IκBβ retains NF-κB dimers in the cytoplasm by masking both the nuclear localization signals of NF-κB.

DISCUSSION

IκBβ Binds Strongly to NF-κB Dimers but Inhibits NF-κB DNA Complexes Weakly—It is generally believed that IκBα is a stronger inhibitor of NF-κB dimers than IκBβ. This conclusion is based principally on their relative abilities to inhibit NF-κB-DNA complexes (18). Inhibition of NF-κB-DNA complexes may not, however, directly correlate with the ability of these inhibitors to associate with NF-κB dimers. We have shown previously by direct binding assay that IκBα exhibits high binding affinity toward NF-κB dimers with dissociation constants in the low nanomolar range (31). It is clear, however, that in most cell types both IκBα and IκBβ are present and capable of forming stable complexes with NF-κB p50/p65 in roughly similar ratios (16). This suggests that IκBβ must also be capable of high affinity NF-κB binding.

In this study, we determined that, as with IκBα, IκBβ binds NF-κB dimers at a 1:1 molar ratio. We determined the magnitude of NF-κB DNA binding inhibition by IκBβ and observed that IκBβ inhibits DNA binding by both the NF-κB p50/p65 heterodimer and p65 homodimer with ∼30-fold lower efficiency than does IκBα. In contrast, both IκBα and IκBβ compete away radiolabeled IκBα from NF-κB with only marginal differences. The estimated variation in NF-κB-IκBβ and NF-κB-IκBα complex binding affinity is 2–4-fold. These results suggest that, although IκBα and IκBβ differ in their abilities to inhibit NF-κB DNA binding, both inhibitors are capable of forming high affinity complexes with NF-κB dimers.

Fig. 7. Nuclear localization of NF-κB/IκBα relies on the p50 subunit NLS. a, subcellular distribution of IκBα in p65+/− MEF cells co-transfected with HA-tagged p65 and either FLAG-tagged p50 (left panels) or FLAG-tagged p50 with the NLS polypeptide removed (p50ΔNLS, right panels). b, subcellular distribution of IκBβ in p65+/− MEF cells co-transfected with HA-tagged p65 and a FLAG-tagged p50 or p50ΔNLS.
The PEST Sequences of IκBα and IκBβ Play Different Roles—Three-dimensional x-ray structures of the NF-κB-IκBα complex revealed a mechanism by which IκBα blocks the DNA binding of NF-κB (34, 35). The PEST sequence and the sixth ankyrin repeat of IκBα interact with the regions of NF-κB that are involved in DNA recognition and binding. The removal of this carboxy-terminal PEST region renders IκBα incapable of inhibiting NF-κB-DNA complexes (30, 32). We reported previously that the binding constants of NF-κB-IκBα complexes measured using direct binding assay and DNA binding competition assays produce roughly similar results (30, 31). We concluded from these experiments that both the ankyrin repeat domain and the PEST sequence of IκBα are critical in forming stable complexes with NF-κB p50/p65 heterodimer and the p65 homodimer.

In this report, we show that the two activities of binding to NF-κB and inhibition of NF-κB DNA binding are not directly linked in the context of recombinant IκBβ. It has been shown previously that phosphorylation at serine 313 and serine 315 of human IκBα is essential for efficient inhibition of DNA binding by NF-κB family homodimers p65 and c-Rel (12, 18, 33). We have converted five carboxy-terminal serine residues, including those corresponding to human positions 313 and 315, to glutamic acid, however, and this E5-IκBβ behaves similarly to murine IκBβ with regard to NF-κB binding and inhibition of DNA binding. It is, therefore, possible that glutamic acid substitution does not adequately mimic phosphorylation in this case and that the phosphorylated IκBβ functions differently than does the E5-IκBβ.

An explanation for the apparent discrepancy between the ability of IκBβ to bind tightly to NF-κB dimers and its relative inability to disrupt NF-κB DNA binding comes from previous observations that wild type and PEST-deleted IκBβ are capable of forming ternary complexes with NF-κB and DNA (17, 18). These experiments lend support to the idea that the PEST sequence of IκBα is not a critical component for stable complex formation with NF-κB dimers. They also indicate that in the presence of both NF-κB and DNA, IκBα participates in two somewhat independent binding equilibria; one is for formation of the NF-κB-IκBα complex, and the other involves the ternary complex of NF-κB-DNA-IκBα. Depending on the reaction conditions, reactant concentrations, and, possibly, the post-translational modification status of the factors involved, the active fraction of IκBβ can shift from one equilibrium to the other. As a result, the presence and status of target DNA fail to provide an accurate measure of IκBβ binding affinity toward NF-κB dimers.

The NLSs of NF-κB Dimers Interact Differently with IκB Proteins—Within the context of the p65 homodimer, we have demonstrated that the NF-κB NLS polypeptide contributes a 24-fold enhancement in binding affinity for IκBβ. By contrast, this same NLS polypeptide segment contributes only 5–6-fold enhancement toward IκBα binding affinity. This greater dependence by IκBβ on the p65 NLS polypeptides results from the involvement of both NLSs in NF-κB binding to IκBβ. We have shown that both p65 homodimer NLSs are protected from proteolytic digestion in the NF-κB p65 homodimer-IκBβ complex. In contrast, IκBα protects only one p65 homodimer NLS. As was first revealed by the NF-κB p50/p65 heterodimer-IκBα crystal structure, IκBα directly contacts only the p65 subunit NLS (34, 35). Interestingly, our results also indicate that IκBβ does not identically protect both p65 NLSs. One NLS is clearly better protected. This is not surprising, since the symmetric p65 homodimer and pseudosymmetric p50/p65 heterodimer are contacted asymmetrically by one IκB molecule. It is likely that, in complex with IκBβ, the fully protected NLS of the p65 homodimer and p50/p65 heterodimer makes similar contacts to those observed between IκBα and the p65 subunit NLS polypeptide (residues 291–319) in the x-ray crystal structures. We speculate that a second p65 NLS or the p50 NLS of the p50/p65 heterodimer partially contacts IκBβ in a manner that might expose some flanking sequences to proteases (Fig. 8). There is little doubt, however, that IκBα does not contact the second NLS in either of the two complexes.

Analogously to the NF-κB p50/p65 heterodimer-IκBα complex x-ray crystal structure places the second p65 homodimer NLS polypeptide within the vicinity of the first and second ankyrin repeats of IκBα. This is a region of relatively high sequence homology between IκBα and IκBβ. IκBβ, however, contains a unique 47-amino acid insertion between its third and fourth ankyrin repeats. IκBα-based modeling of the IκBβ ankyrin repeats places the IκBβ insertion on the same side of IκBβ as the second NF-κB p65 subunit NLS polypeptide.2 Therefore, we suggest that the IκBβ insert region could be involved in masking the second NF-κB NLS polypeptide.

Compartmentalization of Free and NF-κB-complexed IκB in Resting Cells—IκBα has long been characterized as a strictly cytoplasmic inhibitor of transcription factor NF-κB. It functions as such by masking the NLSs of the NF-κB dimers (43). After crystallographic analysis of the NF-κB p50/p65 heterodimer-IκBα complex revealed that only the NF-κB p65 subunit NLS is masked via direct contacts with the first two ankyrin repeats of IκBα, several groups reported that NF-κB-IκBα complexes are dynamic in nature and distribute between the nucleus and cytoplasm (23–26). Here, we have shown that the free p50 subunit NLS is responsible for transport of NF-κB-IκBα complexes to the nucleus. Interestingly, nuclear detection of NF-κB-IκBα occurs only when the nuclear export receptor CRM1 is blocked by treatment with LMB. These observations suggest that the nuclear export of the complex is a more efficient process than entry (Fig. 8). This is perhaps due to the combined effect of multiple nuclear export signals identified in IκBα and the activation domain of p65, which cancel the opposing effect of the one free NLS (44).

Here we report that free IκBβ is a strictly cytoplasmic protein in p65−/− cells. It is not clear why IκBβ, in the absence of any nuclear export sequence, remains cytoplasmic, while IκBα, by contrast, can enter the nucleus freely. We further report that complexes between NF-κB and IκBβ remain primarily within the cell cytoplasm. We propose that the combined effects of three different factors, the masking of both NF-κB NLSs by IκBβ, the absence of any inherent IκBβ nuclear localization potential, and the putative p65 activation domain nuclear export signal, result in the strict cytoplasmic retention of NF-κB-IκBβ complexes. This cytoplasmic NF-κB in complex with IκBβ represents a unique pool of transcriptional activators that could be regulated by a subset of signaling pathways and other similarly localized effector molecules.

Unique Functional Roles for IκBα and IκBβ—Substitution of the IκBα gene with the gene encoding IκBβ restores the wild type phenotype to IκBα knockout mice (45, 46). This finding has contributed to the belief that IκBβ plays a functionally redundant role in regulating NF-κB activity. However, altered tissue distribution patterns and expression levels of IκBβ under the IκBα promoter could account for the observed rescue phenotype. It remains impossible to assign a precise function to IκBβ in the absence of IκBα−/− mice.

Based on our study, we propose that although both IκBα and IκBβ are capable of inhibiting NF-κB activity, they do so by differing mechanisms. The most striking of these differences

2 T. Huxford, unpublished data.
involves the ability of IκBα/IκBβ to mask both NLS polypeptide sequences of the NF-κB p65 homodimer as opposed to IκBα/IκBβ, which masks one of the two. As a result of this difference, NF-κB/IκBα/IκBβ complexes shuttle between the nucleus and cytoplasm, whereas NF-κB/IκBα/IκBβ complexes remain principally in the cell cytoplasm. On a final note, during the preparation of this manuscript, Tam and Sen (47) reported that unlike IκBα/IκBβ, IκBα/IκBβ and IκBβ inhibit basal nucleocytoplasmic shuttling of NF-κB p65 and c-Rel dimers.

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IκBβ Is a Resident Cytoplasmic NF-κB Inhibitor

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IκBβ, but Not IκBα, Functions as a Classical Cytoplasmic Inhibitor of NF-κB Dimers by Masking Both NF-κB Nuclear Localization Sequences in Resting Cells
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