Phosphorylation of the PEST Domain of IκBβ Regulates the Function of NF-κB/IκBβ Complexes*

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Timothy A. McKinsey, Zhi-Liang Chu, and Dean W. Ballard‡
From the Howard Hughes Medical Institute, Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Activation of transcription factor NF-κB involves the signal-dependent degradation of basally phosphorylated inhibitors such as IκBa and IκBβ. The gene encoding IκBa is under NF-κB control, which provides a negative feedback loop to terminate the induced NF-κB response. However, recent studies have identified a hypo-phosphorylated pool of IκBβ that shields nuclear NF-κB from inhibition by newly synthesized IκBa. In the present work, we provide three lines of evidence indicating that this protection mechanism is regulated by the C-terminal PEST domain of IκBβ. First, disruption of two basal phosphoacceptors present in the IκBβ PEST domain (Ser-313 and Ser-315) yields a mutant that forms ternary complexes with NF-κB and its target DNA-binding site. Second, based on in vitro mixing experiments, these ternary complexes are resistant to the inhibitory action of IκBa. Third, mutants of IκBβ that are defective for phosphorylation at Ser-313 and Ser-315 fail to efficiently block NF-κB-directed transcription in vivo, whereas replacement of these two IκBβ residues with a phosphoserine mimic generates a fully functional repressor. Taken together, our findings suggest that the functional fate of NF-κB when bound to IκBβ is critically dependent on the phosphorylation status of the IκBβ PEST domain.

Inducible members of the NF-κB/Rel family of transcription factors mediate rapid cellular responses to stress-related, mitogenic, and pro-apoptotic signals (reviewed in Ref. 1). The most well characterized form of NF-κB isotypes of IκB, including IκBa and IκBβ (1). Although both of these inhibitors are basally phosphorylated and subject to signal-dependent degradation, recent studies indicate that IκBa and IκBβ are regulated by distinct mechanisms that affect the duration of nuclear NF-κB activity (1–3). Specifically, activation cues that selectively trigger IκBα degradation are typically associated with a transient pattern of nuclear NF-κB expression, whereas the concomitant loss of IκBβ correlates with a persistent NF-κB response (2, 3). In further contrast to IκBα, the gene encoding IκBa is rapidly induced by NF-κB (1). This relationship establishes a negative feedback pathway in which newly synthesized IκBa can facilitate a transient mode of NF-κB action (4). However, under stimulatory conditions that lead to prolonged NF-κB activation, cells may express a hypophosphorylated form of IκBβ that binds NF-κB and shields it from feedback inhibition by IκBa (5, 6). The precise molecular mechanism by which NF-κB/IκBβ complexes escape from the inhibitory action of IκBa during a persistent NF-κB response remains unclear.

In this report, we provide evidence for the presence of regulatory serine residues within the C-terminal PEST domain of IκBβ that affect its capacity to either terminate or preserve the functional expression of nuclear NF-κB. Specifically, when overexpressed in mammalian cells, mutants of IκBβ containing alanine substitutions for Ser-313 and Ser-315 fail to efficiently block NF-κB-directed transcription relative to the wild type inhibitor. These PEST mutants of IκBβ are hypophosphorylated in vivo and form ternary complexes with NF-κB and DNA that are resistant to dissociation by IκBa in vitro. In contrast, replacement of Ser-313 and Ser-315 with aspartic acid, which can act as a phosphoserine mimic (7, 8), rescues the NF-κB inhibitory function of IκBβ. These findings indicate that the PEST domain of IκBβ plays dual roles in the control of NF-κB activity, depending on the phosphorylation status of Ser-313 and Ser-315.

Experimental Procedures

Plasmids—cDNAs encoding RelA (9), p50 (10), IκBβ (11), and IκBa (12) were cloned into pCMV4 (13). IκBα cDNAs were fused in-frame with oligonucleotides encoding the FLAG epitope as described (14, 15). Colon substitutions were introduced into IκBβ by site-directed mutagenesis (Muta-Gen kit, Bio-Rad) and confirmed by DNA sequencing. The chloramphenicol acetyltransferase (CAT) reporter plasmid containing two β enhancer elements (HIV-β-CAT) has been described (16).

Metabolic Radiolabeling Studies—Human A2093T cells (17) were transfected using calcium phosphate precipitation (18) and metabolically radiolabeled with 32P, 1 μCi/ml (ICN). Whole cell extracts were prepared and subjected to immunoprecipitation with anti-FLAG antibodies (M2; IBI-Kodak) as described (14). Immunoprecipitates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by sequential autoradiography and immunoblotting (14). For phosphoamino acid analysis, membrane-bound IκBa was

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† To whom correspondence should be addressed: Howard Hughes Medical Inst., Vanderbilt University School of Medicine, 802 Light Hall, Nashville, TN 37232-0295. Tel.: 615-343-1548; Fax: 615-343-5743; E-mail: dean.ballard@mcmail.vanderbilt.edu.

‡ The abbreviations used are: CAT, chloramphenicol acetyltransferase; CKII, casein kinase II; HIV-1, type 1 human immunodeficiency virus; PAGE, polyacrylamide gel electrophoresis.
hydrolyzed in 6 M hydrochloric acid (1 h, 110 °C) and subjected to two-dimensional electrophoresis on thin-layer cellulose plates (7).

Functional and Biochemical Assays—CAT assays were performed as described (14) using a liquid scintillation counting method (19). To measure NF-κB DNA binding activity, nuclear extracts were prepared (20) and incubated with a 32P-labeled b-pd probe using published reaction conditions (21). Nucleoprotein complexes were resolved on native 5% polyacrylamide gels and visualized by autoradiography (20). Protein-DNA cross-linking experiments (14) were conducted with a photoactive derivative of the b-pd probe containing 5-bromo-2′-deoxyuridine 5′-triphosphate (22). DNA-protein adducts were fractionated by immunoprecipitation with either RelA-specific antisemir or monoclonal anti-FLAG antibodies and resolved by SDS-PAGE.

RESULTS

The PEST Domain of IκBβ Is Basally Phosphorylated at Ser-313 and Ser-315—Primary structural (2, 11) and functional (3, 14, 23) analyses have established that IκBβ is a tripartite inhibitor containing (i) an N-terminal response domain required for signal-dependent breakdown, (ii) a central NF-κB-interactive domain composed of six ankyrin repeat motifs, and (iii) a C-terminal PEST domain that harbors two consensus phosphorylation sites for casein kinase II (CKII). We recently demonstrated that the PEST domain of IκBβ is basally phosphorylated at serine residues (14). As shown in Fig. 1A, five serines in this domain are clustered within or near the two consensus CKII motifs (Ser/Thr-Xaa-Xaa-(Glu/Asp); Ref. 24).

To initially explore which of these PEST residues are phosphoacceptors, a series of specific amino acid substitutions were introduced into the PEST domain of wild type IκBβ (WT) and C-terminal PEST domain (PEST, amino acids 306–356) are shown. Consensus CKII motifs are underlined, and potential phosphoacceptors within these motifs (Ser-313 and Ser-315) are indicated with asterisks. Amino acid substitutions introduced into the PEST domain of wild type IκBβ are indicated with underlined (designated S313T and S315T) mutants. A293T cells (1 × 10^6) were transfected with expression vectors (7.5 μg) for either wild type IκBβ (WT) or the IκBβ mutant S313T/S315T. After 36 h of culture, transfecants were metabolically labeled with [32P] (4 h), and ectopic IκBβ proteins were immunopurified using anti-FLAG antibodies. Immunoprecipitates were subjected to phosphoamino acid analysis in parallel with standards. The positions of phosphorylated (P-) amino acids and the sample origin (Ori) are indicated.

In contrast, replacement of Ser-313 and Ser-315 with the phosphoserine mimetic aspartic acid (7, 8) partially restored the phosphorylation defect of Mu-1 (75% of wild type; Fig. 1B, lane 3). However, these aspartic acid mutations failed to rescue phosphorylation when introduced into the serine-depleted PEST background (Mu-4; Fig. 1B, lane 5). All of these PEST mutants were expressed at comparable levels in vivo (data not shown). These data suggest that the IκBβ PEST domain is phosphorylated on multiple serines by a cooperative mechanism that is dependent on phosphoryl group transfer to Ser-313 and/or Ser-315.

To directly test whether IκBβ is phosphorylated at Ser-313 and Ser-315, site-directed mutants containing threonine rather than serine at both positions were prepared and overexpressed in A293T cells. After biosynthetic radiolabeling with [32P]P, ectopic IκBβ proteins were immunopurified and subjected to two-dimensional phosphoamino acid analysis. Consistent with our prior findings (14), control experiments performed with wild type IκBβ revealed phosphorylation exclusively at serine residues (Fig. 1C). In contrast, analysis of the threonine mutant (designated S313T/S315T) revealed the presence of both phosphoserine and phosphothreonine. Similar qualitative results were obtained with IκBβ mutants harboring single threonine substitutions at either position (data not shown). These findings indicate that Ser-313 and Ser-315 both serve as phosphoacceptors in vivo.

Phosphorylation-defective Mutants of IκBβ Form Stable Complexes with NF-κB and DNA—Suyang et al. (Ref. 5 and reviewed in Ref. 6) have identified a hypophosphorylated species of endogenous IκBβ that forms stable complexes with NF-κB. Unlike wild type IκBβ, this species fails to block NF-κB DNA binding activity in vitro. Having defined phosphorylation-defective variants of IκBβ (Fig. 1), we next explored their potential to recapitulate the unusual functional phenotype of the hypophosphorylated form of IκBβ described by Suyang et al. (5).

For these studies, A293T cells were cotransfected with expression vectors for the p50 and RelA subunits of NF-κB along with plasmids encoding FLAG epitope-tagged IκBβ proteins. Nuclear extracts were prepared and cross-linked to [32P]P-labeled κB oligonucleotides. To specifically detect NF-κB/IκBβ complexes bound to target DNA, the resultant adducts were immunoprecipitated with anti-FLAG antibodies and analyzed by SDS-PAGE for the presence of the NF-κB/IκBβ complex (Fig. 2A). PEST mutants containing alanine substitutions for Ser-313 and Ser-315 formed stable ternary complexes with NF-κB and DNA, as evidenced by co-immunoprecipitation with RelA (Mu-1; Fig. 2A, lane 2). Similar results were obtained with the serine-depleted PEST mutant of IκBβ (Mu-3; Fig. 2A, lane 5). In contrast, PEST mutants containing phosphoserine mimetics at positions 313 and 315 associated with DNA-bound NF-κB at much lower
were prepared from A293T cells transfected with expression vectors for kPEST-directed phosphorylation events, lysates were prepared whether this protection mechanism is regulated specifically by standards are given in kilodaltons. by SDS-PAGE, and visualized by autoradiography. Molecular mass adducts were immunoprecipitated with anti-FLAG antibodies, resolved linked by exposure to UV light (300 nm for 30 min). Photoreactive ktures containing a photoreactive 32P-labeled nuclear extracts were prepared from recipient cells, added to reaction mixtures containing a photoreactive 32P-labeled kPEST mutants of IκB that formed ternary complexes with NF-κB and DNA (Mu-3; Figs. 1A and 2A). Extracts containing NF-κB were incubated with a photoreactive kB-pd probe in the presence of Mu-3, and the resultant nucleoprotein complexes were cross-linked and immunoprecipitated with RelA-specific antiserum. As shown in Fig. 2B, radiolabeled RelA-DNA adducts were readily detected in IκB-deficient reactions (Fig. 2B, lane 1, top panel). The addition of IκB to these reaction mixtures failed to disrupt the DNA binding function of NF-κB when associated with Mu-3 (Fig. 2B, lanes 2–5, top panel). In contrast, IκBb potently inhibited NF-κB DNA binding activity in control reaction mixtures lacking Mu-3 (Fig. 2B, lanes 2–5, bottom panel), which confirmed the integrity of IκBb preparations used in these studies. As such, ternary complexes containing this phosphorylation-defective PEST mutant of IκBb are resistant to dissociation by IκBb, a property consistent with that proposed for hypophosphorylated IκBb in vivo (5, 6).

Regulation of NF-κB-directed Transcription by IκBb PEST Mutants—In addition to its capacity to bind DNA, the RelA subunit of NF-κB contains a C-terminal transactivation domain (1). Although the data shown in Fig. 2A demonstrate that complexes containing NF-κB and phosphorylation-defective forms of IκBb bind target DNA in vitro, the in vivo activity of NF-κB in the presence of these PEST mutants remained untested. To address this critical issue, A293T cells were cotransfected with a CAT reporter plasmid driven by two kB enhancer elements along with expression vectors for p50, RelA, and IκBb. Under our overexpression conditions, NF-κB stimulated reporter gene activity ~35-fold over basal levels in control transfectants lacking ectopic IκB. As shown in Fig. 3A, wild type IκBb completely blocked the transactivation function of NF-κB. Similar results were obtained with a PEST mutant harboring phosphoserine mimetics in place of Ser-313/Ser-315 (Mu-2). In contrast, phosphorylation-defective mutants of IκBb that formed ternary complexes with NF-κB and DNA in vitro failed to efficiently block NF-κB-dependent transcription in vivo. All of these functional results correlated strongly with the pattern of NF-κB DNA binding activity observed in gel shift experiments that were conducted with nuclear extracts from the same transfectants (Fig. 3B). Taken together, these in vivo data suggest that the functional fate of NF-κB when bound to

**FIG. 2.** Phosphorylation-defective PEST mutants of IκBb form ternary complexes with NF-κB and DNA that are resistant to dissociation by IκBb. A, A293T cells (10^5) were cotransfected with pCMV4 expression vectors for RelA (2 μg), p50 (2 μg), and the indicated FLAG-tagged derivatives of IκBb (1 μg). After 36 h of growth, nuclear extracts were prepared from recipient cells, added to reaction mixtures containing a photoreactive 32P-labeled kB probe, and cross-linked by exposure to UV light (300 nm for 30 min). Photoreactive adducts were immunoprecipitated with anti-FLAG antibodies, resolved by SDS-PAGE, and visualized by autoradiography. Molecular mass standards are given in kilodaltons. WT, wild type. B, whole cell extracts were prepared from A293T cells transfected with expression vectors for RelA and p50 (3.75 μg each), IκBb (7.5 μg), or IκBb mutant Mu-3 (7.5 μg). Extracts enriched for NF-κB (2.5 μg) were added to reaction mixtures containing a photoreactive 32P-labeled kB probe in the presence (upper panel) or the absence (lower panel) of IκBb mutant Mu-3 (5 μg). Following a 10-min incubation, the indicated DNA-binding mixtures were supplemented with increasing doses (0.25, 0.5, 1.0, and 2.5 μg) of whole cell extracts derived from IκBb-expressing transfectants (lanes 2–5). Products were cross-linked with UV light (300 nm, 30 min) and subjected to immunoprecipitation with RelA-specific antiserum. DNA-protein adducts were resolved by SDS-PAGE and visualized by autoradiography.

**FIG. 3.** Effects of IκBb PEST mutations on NF-κB-directed transcription in vivo. A, A293T cells (1 × 10^5) were cotransfected with HIV-κB-CAT (0.5 μg) along with expression vectors for RelA (2 μg), p50 (2 μg), and the indicated forms of IκBb (1 μg). After 36 h of culture, whole cell extracts were prepared from half of each culture and assayed for CAT activity. Induction of reporter gene expression over basal levels (fold induction) is reported as a percentage of the activity induced by NF-κB in ectopic IκB-deficient cells (mean fold induction = 36 ± 3.6). The error bars depict standard errors of the mean percentage value derived from three independent transfections. B, nuclear extracts were prepared from the remaining half of each culture and added to reaction mixtures containing a 32P-labeled kB probe. Resultant DNA-protein complexes were resolved by electrophoresis on a native 5% polyacrylamide gel and visualized by autoradiography.
IκBβ is critically dependent on the phosphorylation status of the IκBβ PEST domain.

**DISCUSSION**

Recent studies have established that the PEST domain of IκBβ is basally phosphorylated on serines in vivo (14). However, the precise role of this domain in the regulation of NF-κB activity remains unclear. One clue to its function emerged from experiments showing that bacterially expressed forms of IκBβ lacking these basal modifications associate with NF-κB but fail to prevent DNA binding (5). Consistent with this, phoshatase treatment of purified IκBβ from mammalian cells results in the loss of NF-κB inhibitory activity (25). Furthermore, specific serine mutations within the PEST domain of IκBβ have been identified that disrupt its ability to associate efficiently with c-Rel, another member of the NF-κB family of transcription factors (14).

The present report provides strong evidence that the PEST domain of IκBβ controls whether NF-κB/IκBβ complexes are either latent or competent for transcription. We have found that serines clustered within the IκBβ PEST domain are phosphorylated via a cooperative mechanism involving requisite phosphorylation at Ser-313 and Ser-315. In keeping with the regulatory conditions. In support of their proposal, in vitro mixing experiments with bacterially expressed proteins showed that recombinant IκBβ associates efficiently with NF-κB and shields it from neutralization by IκBα (5). However, these prior studies did not define the sequences in endogenous IκBβ that mediate this protective function.

In the present work, we identified phosphorylation-defective PEST mutants with properties that closely mimic this unusual functional phenotype. As determined with IκBα proteins from mammalian cells, mutants defective for phosphorylation at Ser-313 and Ser-315 form stable ternary complexes with NF-κB and target DNA that are resistant to dissociation by IκBα in vitro. These results highlight a potential regulatory role for the IκBβ PEST domain in the repression of NF-κB-directed transcription by IκBα. Further resolution of this issue awaits insights into the phosphorylation status of the PEST domain of IκBβ during a transient versus persistent NF-κB response.

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