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Functional importance of stripping in NFκB signaling revealed by a stripping-impaired IκBα mutant

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Stress-response transcription factors such as NFκB turn on hundreds of genes and must have a mechanism for rapid cessation of transcriptional activation. We recently showed that the inhibitor of NFκB signaling, IκBα, dramatically accelerates the dissociation of NFκB from transcription sites, a process we have called “stripping.” To test the role of the IκBα C-terminal PEST (rich in proline, glutamic acid, serine, and threonine residues) sequence in NFκB stripping, a mutant IκBα was generated in which five acidic PEST residues were mutated to their neutral analogs. This IκBα(5xPEST) mutant was impaired in stripping NFκB from DNA and formed a more stable intermediate ternary complex than that formed from IκBα(WT) because DNA dissociated more slowly. NMR and amide hydrogen-deuterium exchange mass spectrometry showed that the IκBα(5xPEST) appears to be “caught in the act of stripping” because it is not yet completely in the folded and NFκB-bound state. When the mutant was introduced into cells, the rate of postinduction IκBα-mediated export of NFκB from the nucleus decreased markedly.

transcription factor | binding kinetics | intrinsically disordered proteins | nuclear export | hydrogen-deuterium exchange

Stress-response transcription factors turn on hundreds of genes, and their regulation requires robust activation as well as rapid and complete cessation of the ensuing response. A good example is the NFκB family of transcription factors, which responds to a large number of extracellular stress stimuli, including factors controlling inflammation and the immune response (1–3). Aberrant regulation of NFκB results in numerous disease states, including cancer (1, 4). The IκB family of inhibitors keeps NFκB in the cytoplasm (in the “off” state) (5). IκBα is the main temporally regulated IκB. When a stress signal is received, IκBα is degraded rapidly, releasing NFκB, which enters the nucleus, binds to κB DNA sites, and up-regulates gene expression (Fig. L4). In a classic negative feedback loop, the promoter upstream of the IκBα gene is strongly up-regulated by NFκB. We previously showed that in vitro IκBα rapidly accelerates the dissociation of NFκB from many different DNA sequences containing the κB motif in a folding-upon-binding event (6, 7). Thus, removal of NFκB(RelA/p50) from its target sites is kinetically determined, a process we call “molecular stripping” (8). The kinetic control of transcription factor–DNA interactions represents a paradigm shift because these interactions typically are described with equilibrium-binding models (9, 10) and thus would require the formulation of novel models based on stochastic rates.

For IκBα to strip NFκB from DNA, a ternary NFκB–DNA–IκBα complex must form at least transiently. A very transient NFκB–DNA–IκBα complex was indeed observed in stopped-flow fluorescence experiments (11). At high concentrations, signals corresponding to a ternary NFκB–DNA–IκBα complex were also observed by NMR (12, 13). Together the stopped-flow and NMR data showed that IκBα binds to the NFκB–DNA complex so tightly that it effectively does not dissociate, leading to a complex in which the DNA is bound to the N-terminal domains of NFκB and IκBα is bound to the dimerization domains.

IκBα, a protein with six ankyrin repeats (ARs), has a C-terminal sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues (PEST) (Fig. 1B). The similarity of the IκBα PEST sequence to other negatively charged PEST sequences first suggested its involvement in degradation (14); however, more recent studies have not supported these claims and instead revealed degradation signals in the AR domain (15, 16). NMR studies showed that the PEST sequence interacts with positively charged residues in the DNA-binding pocket of NFκB (17), and simulations using a coarse-grained model showed that the IκBα PEST sequence electrostatically repels DNA from NFκB (8). The mutual exclusivity of DNA and PEST sequence binding to NFκB pointed to an involvement of the PEST in the stripping of NFκB from DNA; however, the obvious step of deleting the IκBα PEST sequence weakened NFκB binding (18), making the results of any stripping experiments equivocal. Instead, we neutralized the acidic IκBα PEST residues E282O/E284Q/D285N/E286Q/E287Q to generate the IκBα(5xPEST) mutant, which bound NFκB with nearly WT affinity. This neutralized PEST version of IκBα is stripping impaired, allowing characterization of the NFκB–DNA–IκBα ternary complex. Here we present binding, structural, and single-cell imaging data showing that the IκBα(5xPEST) mutant accelerates the removal of NFκB from DNA at a much lower rate than the WT protein, that the ternary complex of the mutant persists even at low protein concentrations, and that the appearance of the newly formed NFκB–IκBα complex in the cytoplasm of intact cells is slowed for the IκBα(5xPEST) mutant compared with WT IκBα. These data collectively demonstrate the functional importance of IκBα-mediated stripping of NFκB from DNA in the kinetic control of NFκB signaling.

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Significance

Stress-response transcription factors turn on hundreds of genes, and their activity must be turned off completely and quickly. The inhibitor protein IκBα turns off NFκB by forming a transient ternary complex with the NFκB–DNA complex and then promoting DNA dissociation (molecular stripping). Here we report a mutant IκBα that is impaired in its ability to strip NFκB from DNA. The mutant forms a more stable ternary complex, and biophysical characterization shows what IκBα looks like “in the act of stripping.” We also show in single-cell nuclear export assays that the decrease in the rate of DNA dissociation from the mutant ternary complex matches the decrease in the rate of nuclear NFκB export in cells.

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Results

Creation of Stripping-Impaired IκBα. Individual neutralization of each acidic IκBα PEST residue did not affect binding (Fig. S1 A–C) or the ability of each IκBα mutant to strip NFκB from DNA (Fig. S1D) (6). Subsequently, the five acidic IκBα PEST residues were neutralized collectively, generating IκBα(5xPEST). This mutant bound to NFκB dimerization domains with an affinity only fourfold lower than that of WT IκBα (NFκB–IκBα(WT)) \( K_\text{D} = 0.38 \pm 0.02 \) nM; NFκB–IκBα(5xPEST) \( K_\text{D} = 1.5 \pm 0.3 \) nM (Fig. S1C).

The mutant IκBα(5xPEST) appeared not to strip NFκB from DNA completely in stopped-flow fluorescence measurements (Fig. 2 A and B). The fluorescence signal decreased but plateaued at a higher value than for the WT protein. This behavior suggested that the IκBα(5xPEST) mutant was generating a more persistent ternary complex with NFκB and DNA. The leveling of the signal at a higher fluorescence suggested that the equilibrium between the NFκB–IκBα binary complex and the DNA-bound ternary complex had shifted toward the ternary complex compared with the result with WT IκBα. Steady-state fluorescence anisotropy was used to measure the binding affinity of a fluorescein-labeled IFN-κB hairpin DNA oligo (DNA\(^2\)) to NFκB, yielding a \( K_\text{D} \) of 32 ± 13 nM. The addition of IκBα(WT) diminished the DNA binding to a \( K_\text{D} \) of 170 ± 40 nM. DNA binding to the NFκB–IκBα(5xPEST) complex was intermediate, with a \( K_\text{D} \) of 64 ± 10 nM (Fig. 2 C and D).

Size-exclusion chromatography was used to evaluate whether the IκBα(5xPEST) mutant formed a stable ternary NFκB–DNA–IκBα(5xPEST) complex, but the DNA did not remain stably bound. EMSA showed that DNA bound more tightly to the NFκB–IκBα(5xPEST) complex than to the NFκB–IκBα(WT) complex, but neither complex bound DNA as tightly as NFκB alone (Fig. S2). Stopped-flow fluorescence showed that DNA dissociated from NFκB with a \( K_\text{D} \) (dissociation rate constant) of 0.6 ± 0.1 s\(^{-1}\), whereas DNA dissociated from the NFκB–DNA–IκBα(5xPEST) complex only slightly faster with a \( K_\text{D} \) of 1.7 ± 0.1 s\(^{-1}\). DNA dissociation from the NFκB–DNA–IκBα(WT) complex was too fast to measure directly, but using the measured \( K_\text{D} \) of 170 nM for DNA binding to the NFκB–IκBα(WT) complex and the previously measured DNA association rate for the NFκB–IκBα(WT) complex of \( k_\text{a} = 8.6 \times 10^7 \) M\(^{-1}\)s\(^{-1}\) (11), we calculated the \( K_\text{D} \) for DNA dissociation from the ternary NFκB–DNA–IκBα(WT) complex to be 14.0 ± 0.1 s\(^{-1}\). Thus, WT IκBα efficiently mediates molecular stripping of NFκB from DNA by accelerating DNA dissociation from the ternary complex, whereas neutralization of the PEST sequence allows the DNA to remain bound (Fig. 2).

IκBα Accommodates DNA in the NFκB–DNA–IκBα(5xPEST) Ternary Complex with Structural Perturbations Radiating from AR3 and AR6 into AR2. Our group previously showed that AR5 and AR6 are disordered in free IκBα and fold upon binding to NFκB (19, 20). Cross-peaks for most of the amides in AR5 and AR6 were missing from the transverse relaxation-optimized heteronuclear single-quantum coherence (TROSY-HSQC) spectrum of free IκBα (19) but were observed for IκBα bound to NFκB (20). NMR studies in which excess DNA was added to the binary NFκB–IκBα(WT) complex also showed that the addition of DNA did not affect most of the resonances in AR1–AR4 but caused severe broadening of resonances in AR5 and AR6 (12).

We previously showed that DNA binding could be monitored by observing the 1\(^{H}\) spectrum of the distinctive imino resonances of the DNA (13). We used this technique to monitor the formation of the ternary NFκB–DNA–IκBα(5xPEST) complex and found that the complex was fully formed at a slight excess (1.2-fold) of added DNA. Measurement of chemical-shift perturbation upon DNA binding by comparing the 800 MHz TROSY-HSQC spectrum of the binary NFκB–\(^{15}\)N IκBα(5xPEST) complex with the spectrum of the ternary NFκB–DNA–\(^{15}\)N IκBα(5xPEST) complex (Fig. S4) revealed significant N–H chemical-shift perturbations throughout AR5 and AR6 in residues T219 (AR5), R245 (AR5), V246 (AR5), L256 (AR6), T257 (AR6), Q266 (AR6), and E284Q (PEST) (Fig. 3B). Significant chemical-shift perturbations extended up into AR3 to include residue Q165 (AR3). In addition to
chemical-shift perturbations, amide resonances also disappeared upon the formation of the ternary complex; the great majority of the missing peaks were in AR3 (Table S1). Previous work had shown that some amide resonances (i.e., R143, G144, T146, Q154, C156, A158, S159, and T168) corresponding to AR3 are observed in free IkBα but not in the NFkB–IkBα binary complex (20). In addition to these missing peaks, N145, L150, A151, C152, L163, S174, and N180 were missing in the ternary NFκB–DNA–^{2}H^{15}N IkBα(5xPEST) complex.

Although the addition of an excess of DNA caused the peaks corresponding to AR5 and AR6 to disappear in WT IkBα (12), most peaks were observed in the TROSY-HSQC spectrum of our ternary NFκB–DNA–^{2}H^{15}N IkBα(5xPEST) complex except only D226, Y251, S252, S258, G270, L272, and E275 were not observed; the rest of the peaks were observed, and most of the peaks correspond to amide groups in the helical regions of AR5 and AR6 did not undergo significant chemical-shift perturbations.

**Amide Exchange Revealed Changes in the Foldedness of IkBα(5xPEST) in the Ternary Complex.** To explore the dynamic changes in IkBα upon the formation of the ternary complex further, we undertook amide hydrogen–deuterium exchange mass spectrometry (HDXMS) analysis. Consistent with the TROSY-HSQC chemical-shift perturbations, changes in amide exchange were observed in IkBα AR3, AR5, and AR6 (Fig. 4). IkBα residues 158–176 in AR3 showed increased exchange with IkBα(5xPEST) upon formation of the ternary NFκB–DNA–IkBα complex; this region includes Q165, which also showed chemical-shift perturbation (Fig. 4I). IkBα residues 202–236 in AR6 showed increased exchange in the ternary NFκB–DNA–IkBα(5xPEST) complex compared with the binary complex; this region of IkBα also includes the AR5 and AR6 residues that showed significant chemical-shift perturbations (Fig. 4L–P). IkBα residues 202–236 showed larger increases in amide exchange in the ternary complex formed with IkBα(5xPEST) than in the ternary complex formed with IkBα(WT).

Collectively, the chemical-shift perturbation data and amide HDXMS form a consistent structural picture of the stabilized ternary NFκB–DNA–IkBα complex that accommodates the presence of DNA by subtle structural adjustments in AR3, AR5, and AR6 (Fig. 5).

To ascertain whether there were differences in the IkBα(5xPEST) and IkBα(WT) interactions with NFκB, we analyzed the NFκB peptides from the binary NFκB–IkBα and ternary NFκB–DNA–IkBα complexes of both IkBα(WT) and IkBα(5xPEST) (Fig. S3). No discernable differences in amide exchange were observed in the NFκB peptides, providing a strong confirmation that the IkBα(5xPEST) mutant forms a ternary complex that is highly similar to the IkBα(WT) ternary complex.

Previous NMR studies revealed inaccuracies in the IkBα(5xPEST) sequence–NFκB contacts displayed in crystal structures (21, 22) and suggested that the PEST sequence instead contacts R33 and R35 in the RelA DNA-binding domain (17). The HDXMS data showed that the IkBα(WT)–PEST sequence was less dynamic than the PEST sequence of the IkBα(5xPEST) mutant (Fig. 4I). These data support the notion that electrostatic contacts between the IkBα PEST sequence and DNA (25).

**Stripping-Impaired IkBα Is Deficient in Regulating NFκB in Knock-Out Mouse Embryonic Fibroblast Cells.** To ascertain the functional ramifications of the increased ternary complex stabilization, we generated IkBα−/− β−/−/− RelA−/− mouse embryonic fibroblasts (MEFs) that lack all three classical NFκB inhibitors and RelA and reconstituted them with a constitutively expressed fluorescent GFP-RelA and NFκB–responsively expressed IkBα(WT) or IkBα(5xPEST) as described previously (23). EMSA analysis showed the IkBα(5xPEST) normally was localized in resting cells and after TNF stimulation (Fig. S4). As previously shown (23), when WT IkBα was expressed after a pulse of TNFα, single-cell traces showed that GFP–NFκB entered the nucleus and was exported rapidly back into the cytoplasm (Fig. 6). In stark contrast, when IkBα(5xPEST) was expressed following the TNFα pulse, single-cell traces showed that GFP–NFκB remained in the nucleus. In fact, the results from the IkBα(5xPEST) mutant resembled previous results with IkBβ (23), which is known to persist in the nucleus (24). The average rate of GFP–NFκB nuclear export for IkBα(WT) was 2.0 (± 0.2) × 10^{−5}/min, whereas the IkBα(5xPEST) transfectants had a ninefold slower rate of GFP–NFκB nuclear export of 2.2 (± 0.1) × 10^{−7}/min.

**Discussion**

IkBα(WT) efficiently strips NFκB from DNA (6, 11). The mechanism of this process necessitates the formation of a ternary complex, which was extremely transient under stopped-flow conditions but could be observed under the high-concentration conditions of NMR experiments (11–13). Using coarse-grained simulations, we showed that at least part of the driving force for molecular stripping likely comes from electrostatic repulsion between the negatively charged IkBα PEST sequence and DNA (25).
Upon the addition of DNA to form the NFκB ternary complex, we were able to characterize the ternary complex, which is the necessary intermediate in the IκBα-mediated stripping of NFκB from the DNA. Importantly, HDXMS analysis of NFκB in both the IκBα(WT) and IκBα(5xPEST) binary and ternary complexes showed no differences in amide exchange of the NFκB in complex with either IκBα(WT) or IκBα(5xPEST) (Fig. S3). Both kinetic experiments and NMR characterization of the ternary complex in the presence of excess DNA showed that IκBα does not dissociate from NFκB once it is bound (11–13, 26). Here we show that in the stabilized ternary complex formed with the IκBα(5xPEST) mutant, chemical shifts for most IκBα residues in AR1–AR4 were unperturbed in the binary NFκB–IκBα and ternary NFκB–DNA–IκBα complexes. Resonances in AR5 and AR6, particularly in the β-hairpins and loops, had significantly different chemical shifts in the ternary and binary complexes, but the helices remained largely unperturbed (Fig. 5).

The AR domain of IκBα appears to make both structural and dynamic changes to accommodate DNA in the ternary complex. Amide HDXMS experiments revealed that wherever NMR chemical shifts were perturbed, indicating local structural changes, amide exchange increased, corresponding to increased dynamics in the ternary complex (Fig. 5). These results strongly suggest that the structure of the ternary complex is, in fact, an intermediate in the coupled folding and binding of IκBα to NFκB and that IκBα begins to fold during stripping, but folding is not completed until DNA dissociates.

Throughout AR5 and AR6, amide exchange is increased in the ternary complex, indicating that these two repeats, which fold on binding to NFκB (27), are partially unfolded in the ternary complex. These results also help explain the origins of the line broadening previously observed in NMR experiments on the ternary complex (12). The structural accommodation of the DNA by IκBα radiates via long-range allosteric into AR3, which is the most stably folded region of free IκBα (28). Previous NMR experiments showed that resonances in AR3 broaden upon NFκB binding (20),

Here, we undertook a comprehensive experimental characterization of the effects of PEST neutralization on the kinetics of molecular stripping, on the structure of the transient ternary complex, and on the function of IκBα. By neutralizing the PEST sequence, we were able to ascribe a clear function to the IκBα PEST sequence, definitively resolving previously conflicting reports. Our results reveal that the negatively charged PEST residues actually function to promote stripping of NFκB from DNA.

Using stopped-flow fluorescence and equilibrium steady-state anisotropy, we showed that DNA dissociates less readily from the NFκB–DNA–IκBα(5xPEST) ternary complex. On the other hand, a stable ternary complex was not observed. Instead, an equilibrium mixture of the binary NFκB–IκBα and ternary NFκB–IκBα–DNA complexes forms, and the 5xPEST mutations shift the equilibrium toward the ternary complex (Fig. 2D). DNA dissociates eightfold more slowly from the PEST-neutralized ternary complex and also associates more slowly with the WT ternary complex; both are consistent with electrostatic repulsion between the IκBα PEST sequence and the DNA.
and our results suggest DNA binding further weakens the AR3 structure. Previous single-molecule FRET studies suggested that AR3 senses events at the N-terminal domains of NFκB; in these studies, a subtle twisting of the AR domain accounted for the observed decrease in the FRET signal when the N-terminal domains were included in the binary complex and pointed to a slight structural rearrangement upon formation of the binary NFκB–IkBα complex (29). Here we see that when the ternary complex forms, AR3 senses what is bound to the DNA. We knew that IkBα does not dissociate from NFκB once bound, but here we show that DNA association with and dissociation from the ternary complex is very sensitive to electrostatic repulsion by the PEST sequence. When newly synthesized WT IkBα enters the nucleus and binds to DNA-bound NFκB, it causes DNA to dissociate at a rate of ~14/s, which is sufficient to trigger rapid export of all the NFκB from the nucleus. By contrast, the stripping-impaired mutant shows an eightfold slower dissociation rate of DNA from the ternary complex. Intracellular nuclear export assays showed a similar decrease in the rate of export of NFκB from the nucleus when IkBα(5xPEST) replaced IkBα(WT) in intact cells. We conclude that molecular stripping of NFκB plays a very sensitive role in controlling this important, stress-induced transcripational response.

Materials and Methods

Protein Expression and Purification. Human IkBα(29-287) (IkBα) mutants were produced using site-directed mutagenesis, and the proteins were expressed and purified as described (30). 3H-NH2-labeled NFκB(WT) and 3H-NH2-labeled NFκB(5xPEST) were expressed in Escherichia coli BL21(DH5α) grown in M9 minimal medium in D2O (Cambridge Isotope Laboratories) supplemented with 2 g l-1NH4Cl (Cambridge Isotope Laboratories) as described (31). Murine N-terminal hexahistidine-RelA(19-321)/p50(39-350) heterodimer (NFκB) was coexpressed as described previously (20) and was purified by nickel affinity chromatography, cation exchange chromatography (Mono 5, GE Healthcare), and size-exclusion chromatography (Superdex 200; GE Healthcare). Murine dimerization domain RelA(19-321)/p50(39-350) with an N-terminal cysteine and dimerization domain p50(39-350) were expressed, purified, and prepared for surface plasmon resonance (SPR) as described (25, 26, 29, 30). Immediately before the experiments, NFκB was purified by size-exclusion chromatography (Superdex 200) in 25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. Protein concentrations were determined by spectrophotometry at 280 nm (NFκB: ε = 43,760 M/cm; IkBα: ε = 12,950 M/cm).

DNA Labeling and Purification. A hairpin DNA sequence corresponding to the IFN-κB site with a 5’ amino modification, 5’-AmMC6/GGGAAATCTCCCTC- CAGGAAATTTCC-3’ (IDT Technologies), was labeled with pyrene (N-hydroxyl succinimide ester) (DNAγ (Sigma)) or fluorescein (fluorescein isothiocyanate, Sigma) (DNAγ) as described (32).

Stopped-flow Fluorescence. Stopped-flow fluorescence experiments were performed on an Applied Photophysics SX-20 stop-flow apparatus at 25 °C collecting 2,000 points linearly to a final mixing volume of 200 μL. The NFκB-mediated dissociation of DNAγ from NFκB was observed by adding increasing concentrations of IkBα (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, and 2.00 μM) to an NFκB (0.10 μM)–DNAγ (0.12 μM) complex and monitoring the change in fluorescence of the DNAγ. The pyrene label was excited at 434 nm with emission monitored at 476 nm with a 350-nm-cutoff filter. The dissociation of DNA from the ternary complex was observed by adding a 50-fold excess (6 μM unlabeled IFN-κB DNA oligo to the preformed NFκB (0.1 μM)–DNAγ (0.12 μM)–IkBα (0.12 μM) complex. The association of IkBα with NFκB or NFκB-DNA was observed by monitoring emission at 519 nm via the native Trp fluorescence of Trp-258 in IkBα, excited at 280 nm and monitoring emission at 345–355 nm with a 320-nm-cutoff filter. Increasing concentrations of NFκB or NFκB–DNA (0.30, 0.40, 0.50, 0.60, 0.70, and 0.80 μM) were mixed with IkBα (0.10 μM). Data were analyzed with pro Fit 6.1.14 (Quansoft, Inc.) (30).

Fluorescence Anisotropy. DNAγ (5 μM) was incubated with increasing concentrations of NFκB, NFκB–IkBα(WT), or NFκB–IkBα(5xPEST) (0.0, 25.3, 38.0, 57.0, 85.4, 128.1, 192.2, and 288.3 nM) for 4 h at 4 °C before data collection in triplicate at 25 °C on a BioFluor 8500 Microplate Fluorimeter (MDX) or a 1500 Multimode Detector by exciting DNAγ at 495 nm and monitoring emission at 519 nm. Anisotropy values were calculated according to the equation χ = [θ(θH) – Gθ(θH)]/[θ(θH) – 2Gθ(θH)], where χ is anisotropy, θ(θH) is the fluorescence intensity in the parallel direction, Gθ(θH) is the fluorescence intensity in the perpendicular direction, and G of 0.67 (33). The binding curves were fit to the equation: A = (Amax [P]/Kd + [P]) + b, where A is the fluorescence anisotropy value, Amax is the maximum anisotropy, [P] is protein concentration, Kd is the equilibrium dissociation constant, and b is the y-intercept.

SPR Experiments. Sensorgrams were recorded on a GE Biacore 3000 instrument using streptavidin chips (GE Healthcare) by immobilizing 150, 250, and 350 response units of p50(39-350)-biotin-RelA(19-321) on flow cells 2, 3, and 4, respectively, leaving flow cell 1 unmodified for reference subtraction. NFκB-binding experiments were conducted on biotin(WT) and biotin(5xPEST), and the data were analyzed as described (18, 30).

EMSA. Binding reactions contained 30 μM FITC–DNA or 6 μM RelAP50 and 30 μM FITC–DNA (incubated for 20 min in the dark at 25 °C) to which a 1.2-fold excess of biotin(WT), a 1.2-fold excess of biotin(5xPEST), or buffer was added to bring the final reaction volume to 20 μL. The reaction mixtures (10 μL) were added to 10 μL 2x loading buffer (40 mM Tris (pH 8.0), 100 mM NaCl, 2 mM MgCl2, 2 mM DTT, 0.5 mM MgCl2, 10% glycerol, 0.001% (wt/vol) bromophenol blue) and were incubated for 1 h in the dark at 25 °C. The reaction mixtures were run on a 6% polyacrylamide nondenaturing gel containing 25 mM Tris-HCl (pH 8.3), 2.5% glycerol, 19 mM glycerine, and 1 mM DTT at 4 °C for 4 h at 25 °C in the dark. The gel was visualized via UV transillumination and quantified using Image J.

Fig. 6. The IkBα(5xPEST) mutant caused GFP–NFκB to be retained in the nucleus of live IkBα(5xPEST)/p50(WT) MEFs. (A) MEFs expressing IkBα(5xPEST) behind the native κB promoter were visualized for Hoechst-stained nuclei, GFP–RelA, and mCherry-fused IkBα antibody showing colocalization of RelA and IkBα (Composite). (B) After a pulse of TNFα, the same cells showed a markedly diminished rate of export of GFP–NFκB from the nucleus when IkBα(5xPEST) replaced IkBα(WT).
The binary complexes of NFκB with H2O15N15-labeled (WT) or H2O15N15-labeled (5xPEST) were purified by size-exclusion chromatography (Superdex 200). For the ternary complex, NFκB-DNA (5xPEST) was incubated at 1:1.2-fold excess of DNA, and an equimolar concentration of H2O15N15-labeled (WT) was added to the NFκB-DNA complex. Samples were exchanged into 25 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 90% H2O, 10% D2O via PD-10 desalting columns (GE Healthcare) and were concentrated to 0.12 mM using polyethersulfone Vivapure 6 concentrators (Sartorius). TROSY-HSQC spectra were collected at 30 °C on a Bruker Avance 800-MHz spectrometer equipped with a CryoProbe. With these conditions, we observed an intense signal at the TROSY wt-DNA site. The NFκB-H2O15N15-labeled (WT) complex had been assigned previously (20), and these assignments were used to assign the nearly identical TROSY-HSQC spectra of the NFκB-H2O15N15-labeled (5xPEST) complex (76.8% assigned) and the NFκB–DNA–H2O15N15-labeled (5xPEST) complex (74.3% assigned). The four 5xIκBα(5xPEST) mutant residues, E288Q, E289Q, D289N, and E286Q, in the NFκB–IκBα–DNA–H2O15N15-labeled (5xPEST) spectra were also assigned by comparison with the WT spectra. Chemical-shift perturbations were calculated by the equation (ΔδHN) + (ΔδNH2JG)1/2, and those exceeding one SD from the mean were deemed significant.

HDXMS. HDXMS was performed using a Waters G2502 mass spectrometer with HDX technology. The NFκB–DNA–IκBα(WT) and NFκB–DNA–IκBα(5xPEST) samples were prepared by high-intensity deuteration of NFκB in a 10-fold excess of the unmodified IFN-β hairpin DNA followed by the addition of IκBα(WT) or IκBα(5xPEST). The final concentrations were as follows: NFκB (5 μM):DNA (50 μM):IκBα(5xPEST) (5 μM) or NFκB (5 μM):DNA (50 μM):IκBα(WT)(5xPEST) (5 μM). For each deuteration time, 4 μl of the complex was equilibrated to 25 °C for 5 min and then was mixed with 56 μl of D2O buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT) in D2O for 0, 0.5, 1, 2, 5, or 10 min. The resulting mixture was quenched with an equal volume of quench solution (3 M guanidine, 0.1% formic acid, pH 2.66). The quenched sample (50 μl) was injected into an in-line pepsin column (immobilized pepsin; Pierce, Inc.) at 15 °C. The resulting peptides were captured on a BEH C18 VanGuard Pre-column and then were separated on an Agilent UPLC BEH C18 column (1.7 μM, 1.0 × 50 mm; Waters Corporation) using a 75–85% acetonitrile gradient in 0.1% formic acid over 7.5 min and were electrosprayed into the mass spectrometer. Data were collected in the Mobility, ESI+ mode with a mass acquisition range of 200–2,000 (m/z) and a scan time 0.4 s with continuous lock-mass correction. For peptide identification, the mass spectrometer was set to collect data in the MS2, ESI+ mode instead.

The peptides were identified from triplicate MS experiments by searching for nearly identical TROSY-HSQC spectra of the NFκB–IκBα–DNA complexes.

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