

Pre-folding I κ B α Alters Control of NF- κ B Signaling

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Transcription complex components frequently show coupled folding and binding but the functional significance of this mode of molecular recognition is unclear. I κ B α binds to and inhibits the transcriptional activity of NF- κ B via its ankyrin repeat (AR) domain. The β -hairpins in ARs 5–6 in I κ B α are weakly-folded in the free protein, and their folding is coupled to NF- κ B binding. Here, we show that introduction of two stabilizing mutations in I κ B α AR 6 causes ARs 5–6 to fold cooperatively to a conformation similar to that in NF- κ B-bound I κ B α . Free I κ B α is degraded by a proteasome-dependent but ubiquitin-independent mechanism, and this process is slower for the pre-folded mutants both *in vitro* and in cells. Interestingly, the pre-folded mutants bind NF- κ B more weakly, as shown by both surface plasmon resonance and isothermal titration calorimetry *in vitro* and immunoprecipitation experiments from cells. One consequence of the weaker binding is that resting cells containing these mutants show incomplete inhibition of NF- κ B activation; they have significant amounts of nuclear NF- κ B. Additionally, the weaker binding combined with the slower rate of degradation of the free protein results in reduced levels of nuclear NF- κ B upon stimulation. These data demonstrate clearly that the coupled folding and binding of I κ B α is critical for its precise control of NF- κ B transcriptional activity.

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Introduction

The nuclear factor κ B (NF- κ B) family of transcription factors have key roles in normal growth and development, in inflammatory and immune responses, and in numerous human diseases.^{1,2} While the most abundant NF- κ B is the p50/p65 heterodimer, the NF- κ B family is composed of homo- and heterodimers formed from the combinatorial assembly of the p65 (RelA), RelB, c-Rel, p50,

and p52 subunits.¹ The inhibitor proteins I κ B α , I κ B β , and I κ B ϵ tightly regulate the transcriptional activity of p65 and c-Rel containing NF- κ B dimers.³ In resting cells, I κ B α binds extremely tightly to NF- κ B, preventing its nuclear accumulation and association with DNA.^{4–6} Upon stimulation, NF- κ B-bound I κ B α is specifically phosphorylated (by the I κ B kinase, IKK), ubiquitinated, and degraded by the proteasome.^{7–11} NF- κ B then enters the nucleus, binds DNA, and regulates transcription of its numerous target genes.¹² NF- κ B activates transcription of its own inhibitor, I κ B α , resulting in a negative-feedback loop.^{13–16} The newly synthesized I κ B α enters the nucleus and is responsible for rapid post-induction repression of NF- κ B transcriptional activity.¹⁷ I κ B regulation of NF- κ B transcriptional activity is so critical that misregulation results in many different diseases.² In fact, constitutive activation of NF- κ B is observed in many types of cancer, and improper I κ B α function is observed in B-cell and Hodgkin's lymphomas.¹⁸

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Abbreviations used: AR, ankyrin repeat; WT, wild-type; YL/TA, Y254L/T257A; CP/AP, C186P/A220P; YL/TA/CP/AP, Y254L/T257A/C186P/A220P; SASA, solvent-accessible surface area; HSQC, heteronuclear single quantum coherence; MEF, mouse embryonic fibroblast; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; EMSA, electrophoretic mobility-shift assay.

Free I κ B α has marginal thermodynamic stability, and it is degraded rapidly by the proteasome in a process that does not require phosphorylation or ubiquitination.^{19,20} The *in vivo* half-life of free I κ B α is less than 10 min.^{20–22} However, NF- κ B-bound I κ B α is stable for hours, and its degradation requires phosphorylation and ubiquitination.^{20,21} These distinct degradation pathways for free and NF- κ B-bound I κ B α appear to be critical for signal-responsive NF- κ B activation. Decreases in NF- κ B-bound I κ B α phosphorylation reduce NF- κ B activation upon stimulation in a mathematical model of NF- κ B signaling.²¹ Furthermore, I κ B α mutants with slower basal degradation rates result in slower activation of NF- κ B upon stimulation with TNF- α .²⁰ A recent study shows that rapid synthesis and degradation of I κ B α provides a mechanism for resistance to metabolic stresses.²³ Additionally, this study showed that both degradation pathways are critical for proper control of NF- κ B activation in response to UV.

I κ B α is composed of an N-terminal signal response region where phosphorylation and ubiquitination occur, an ankyrin repeat (AR) domain that binds to NF- κ B (Fig. 1a), and a C-terminal PEST sequence.^{24,25}

The PEST sequence is important for basal degradation of free I κ B α .²⁰ The AR, a structural motif of ~30–40 amino acids composed of a β -hairpin followed by two antiparallel α -helices and a variable loop, is found in more than 3000 different proteins with highly varied functions.²⁶ AR domains function by mediating specific protein–protein interactions.²⁷ AR consensus sequences based on statistical analyses were developed.^{28,29} Many consensus designed AR proteins have been made, and they are generally more stable than naturally occurring AR proteins.^{28–31} The GXTPLHLA motif (Fig. 1b) is the most prevalent signature in the consensus sequence, and mutation of I κ B α ARs 4 and 5 to these residues resulted in a stability increase of ~1.5 kcal/mol.³²

The I κ B α •NF- κ B interface buries more than 4000 Å² and all six I κ B α ARs contact NF- κ B (Fig. 1a).^{24,25} In free I κ B α , only ARs 1–4 of I κ B α are folded compactly, whereas, ARs 5 and 6 are folded weakly and are highly flexible.^{32–34} ARs 1–4 fold cooperatively,³² and show protection from amide H/²H exchange, which is consistent with a compact structure.³³ In fact, the extent of exchange in ARs 1–4 correlates with the solvent-accessible surface area (SASA) calculated for I κ B α from the

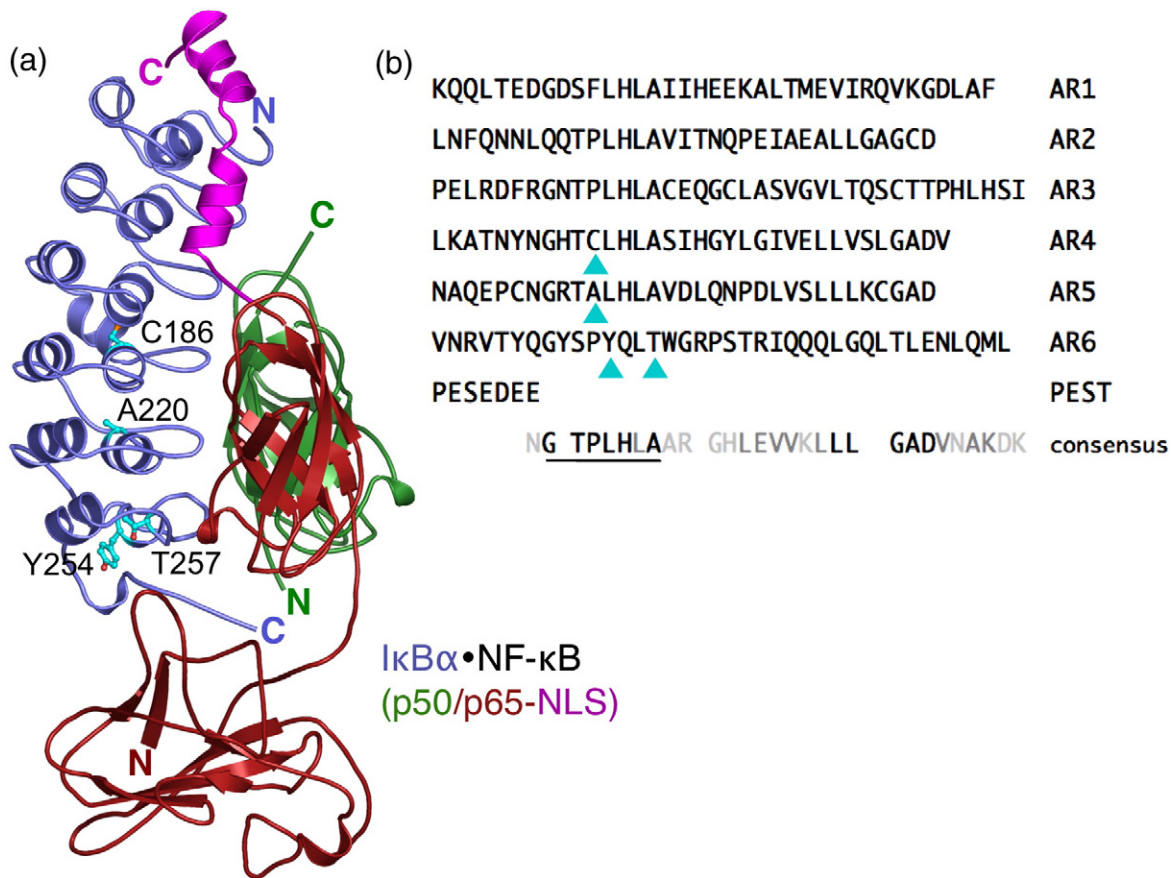


Fig. 1. (a) The crystal structure of I κ B α (blue) bound to NF- κ B (p50, green; p65, red; p65 nuclear localization sequence (NLS), magenta).²⁴ Residues mutated in this study, Y254, T257, C186, and A220, do not contact NF- κ B; they are depicted with ball-and-stick representation and colored cyan. The figure was prepared using PyMOL (<http://pymol.sourceforge.net/>). (b) The sequences of the I κ B α ankyrin repeats (ARs) are aligned with the consensus sequence for a stable AR.²⁹ Cyan triangles indicate residues mutated in this study. In the consensus sequence, black letters indicate highly conserved residues and gray letters indicate weaker conservation.

I κ B α •NF- κ B crystal structure with NF- κ B removed, suggesting that ARs 1–4 adopt the same conformation in free and NF- κ B-bound I κ B α .³³ In contrast, ARs 5–6 do not fold cooperatively.³² The β -hairpins in ARs 5–6 exchange nearly all of their amide protons and they exchange more than predicted by

their SASA, suggesting that they are flexible.³³ However, when I κ B α is bound to NF- κ B, the β -hairpins in ARs 5–6 show large decreases in amide H/²H exchange. The extent of exchange in NF- κ B-bound I κ B α correlates with the SASA calculated from the I κ B α •NF- κ B crystal structure, suggesting

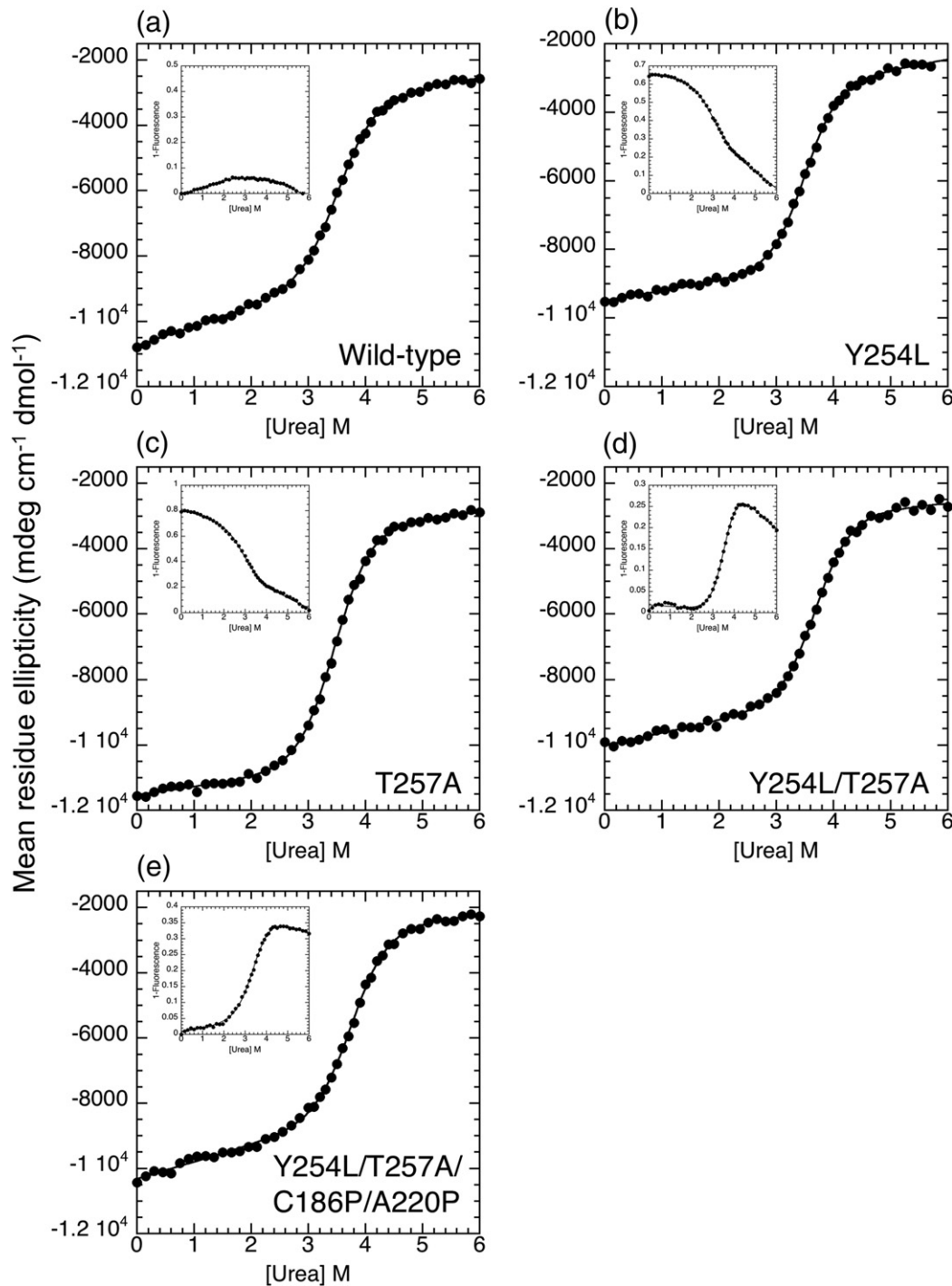


Fig. 2. Equilibrium unfolding of WT and mutant I κ B α using urea as a denaturant. The CD signal and the fluorescence of the single tryptophan in I κ B α , W258, located in AR 6 (insets) were recorded simultaneously for urea titrations of various I κ B α proteins. The cooperative CD unfolding transition shows that the Y254L (b), Y254L/T257A (d), and Y254L/T257A/C186P/A220P (e) mutants are slightly more stable than WT (a) I κ B α , but the T257A (c) mutant has the same thermodynamic stability. Only mutants containing both Y254L and T257A (d and e) show cooperative unfolding transitions in the fluorescence of W258, which is located in AR 6 (insets).

that ARs 5–6 are folded compactly when bound to NF- κ B. Thus, free I κ B α is partially folded, ARs 1–4 are folded compactly and ARs 5–6 are folded weakly. ARs 5–6 adopt a fully folded conformation only when I κ B α binds to NF- κ B.³³

Dynamic structures that fold upon binding to their targets are observed in many transcription factors,^{35–38} and cell-cycle regulators.^{39,40} Many eukaryotic transcription factors are predicted to have extended regions of intrinsic disorder.⁴¹ Coupled folding and binding also appears to be important for the recruitment of co-activators for transcriptional activation. Recent NMR studies elucidated the mechanism of coupled folding and binding of CREB with CBP,⁴² and p160 co-activators and CBP/p300 show mutual synergistic folding.⁴³

Despite the wealth of biophysical characterization of coupled folding and binding, the biological consequences of this process remain unclear. Folding-on-binding of the cyclin-dependent kinase (Cdk) inhibitor p27 was shown to confer binding specificity for only Cdks that regulate cell division.⁴⁴ Additional possibilities, such as facilitating rapid degradation, ability to bind multiple targets, and rapid binding kinetics, have been proposed, but functional characterizations remain elusive. We proposed that the coupled folding and binding of ARs 5–6 in I κ B α might modulate the binding affinity between I κ B α and NF- κ B,⁶ and might be the switch between the basal and stimulated degradation mechanisms.³³

In experiments presented here, we took advantage of the stable consensus sequence to rationally design I κ B α mutants with pre-folded ARs 5–6. We demonstrate that mutation of as few as two amino acids in AR 6 causes pre-folding of the two C-terminal ARs of I κ B α . Evolution apparently selected for weakly folded sequences in ARs 5–6 in I κ B α , and this region confers at least two functions that are critical for proper control of NF- κ B signaling: high-affinity binding to NF- κ B and rapid degradation of free I κ B α .

Results

Rational design of stable, folded I κ B α mutants

To make AR 6 of I κ B α conform more closely to the consensus sequence for a stable AR,^{28,29} we introduced the Y254L and T257A substitutions (Fig. 1),

both individually and in combination, into the AR domain of I κ B α (residues 67–287). These two amino acids in AR 6 do not contact NF- κ B in the I κ B α •NF- κ B crystal structure.^{24,25} The Y254L/T257A (YL/TA) substitutions were also combined with two other mutations located in ARs 4 and 5, C186P and A220P, which were previously shown to increase the overall stability by ~ 1.5 kcal/mol.³² The far-UV circular dichroism (CD) spectra of WT I κ B α and the mutants showed no significant differences (data not shown), indicating that the secondary structure of the mutants is unchanged. To confirm that the mutations did not change the binding interface appreciably, we calculated the spectral similarity factor from the NMR chemical shifts of ¹⁵N-NF- κ B resonances bound to wild-type (WT) or YL/TA I κ B α .⁴⁵ Spectral similarity factors of less than 10 Hz are considered to be insignificant, and the spectral similarity factor (monitoring NF- κ B) that we obtained was 2.3 Hz. Thus, no significant difference in the binding interface was introduced by the mutations.

CD shows WT I κ B α folding by a cooperative transition involving ARs 1–4, and a non-cooperative transition involving ARs 5–6.³² The overall stability of the proteins (ΔG_{CD}) obtained from the CD measurements shows that the Y254L single mutation is sufficient to stabilize the I κ B α AR domain, and the T257A mutation does not change the overall level of stability (Fig. 2 and Table 1). Although the C186P/A220P (CP/AP) mutant is more stable than WT I κ B α , combination of the CP/AP mutations with YL/TA does not result in additional stability (Table 1). Folding studies of other AR domains show that increasing the number of repeats in the cooperatively folded unit does not necessarily add to the overall level of stability.^{46,47}

While CD monitors the entire AR domain, a single tryptophan, W258, in AR 6 monitors the folding transition of only the C-terminal part of the I κ B α AR domain. Therefore, measuring the equilibrium folding of I κ B α using CD and fluorescence signals simultaneously enables us to distinguish between the cooperative folding transition and the non-cooperative folding of ARs 5–6 (Fig. 2). In WT I κ B α , the CD and the W258 fluorescence both show a non-cooperative transition that can be assigned to ARs 5–6.³² Although the Y254L mutation was sufficient to increase the overall level of stability as

Table 1. I κ B α equilibrium folding by urea

Protein	ΔG_{CD} (kcal/mol)	m_{CD} (kcal/mol•M)	m_{pre}^a (mdeg/cm•dmol•M)	ΔG_{FL} (kcal/mol)	m_{FL} (kcal/mol•M)
Wild-type	6.5 $\pm$ 0.2	1.8 $\pm$ 0.1	590 $\pm$ 20	N/A ^b	N/A
Y254L	7.1 $\pm$ 0.2	2.0 $\pm$ 0.1	280 $\pm$ 20	N/A	N/A
T257A	6.3 $\pm$ 0.2	1.8 $\pm$ 0.1	260 $\pm$ 30	N/A	N/A
Y254L/T257A	7.2 $\pm$ 0.3	2.0 $\pm$ 0.1	380 $\pm$ 30	6.7 $\pm$ 0.2	1.9 $\pm$ 0.1
Y254L/T257A/C186P/A220P	7.0 $\pm$ 0.3	1.9 $\pm$ 0.1	520 $\pm$ 30	5.0 $\pm$ 0.2	1.4 $\pm$ 0.1
C186P/A220P ^c	8.3 $\pm$ 0.8	2.2 $\pm$ 0.2	400 $\pm$ 30	N/A	N/A

^a m_{pre} is the slope of the pre-transition baseline.

^b ΔG_{FL} and m_{FL} could not be calculated for non-cooperative folding transitions.

^c Taken from Ferreiro *et al.*³²

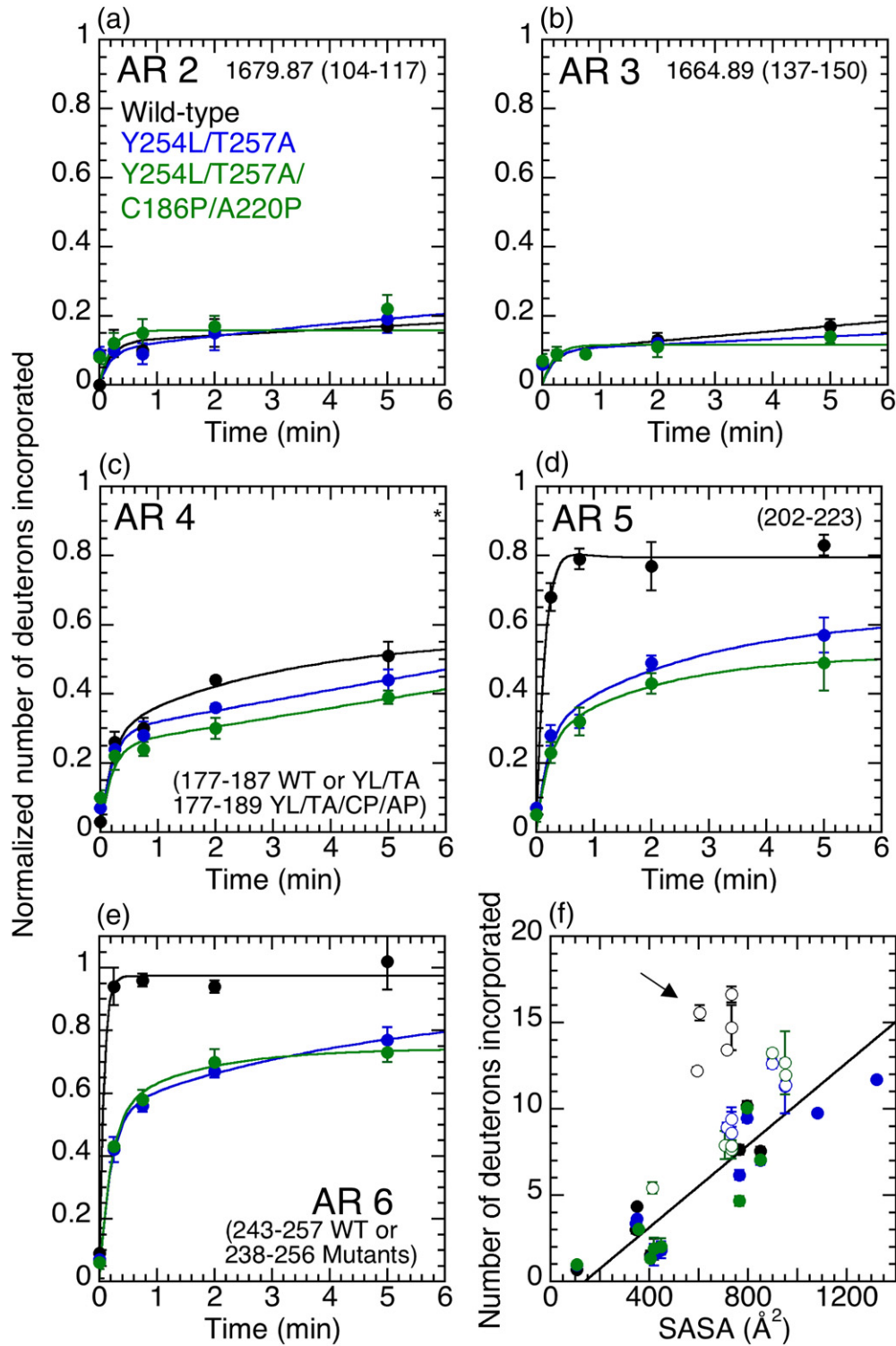


Fig. 3. Amide H/ 2 H exchange in wild-type (black), Y254L/T257A (blue), and Y254L/T257A/C186P/A220P (green) I κ B α . Deuterium incorporation into the β -hairpins in ARs 2 (a), 3 (b), and 4 (c) is similar in all three proteins; however, the β -hairpins in ARs 5 (d) and 6 (e) incorporate much less deuterium in the pre-folded mutants than in WT I κ B α . The deuterium incorporation is normalized according to the number of backbone amides in the peptide. (f) The number of deuterons incorporated in each peptide in ARs 1–4 (filled circles) correlates extremely well with the calculated solvent-accessible surface area (SASA) of the corresponding region of I κ B α . The β -hairpins in ARs 5 and 6 (open circles) in free WT I κ B α exchange to a much greater extent than predicted by their SASA (see cluster indicated by arrow), whereas the extent of exchange in these regions in the mutants are well correlated with their SASA. The average of three independent exchange experiments is reported, and the error bars represent the standard deviation of these experiments.

measured by CD, it was not sufficient to pre-fold AR 6 as measured by a cooperative folding transition of W258. In contrast, both of the YL/TA-containing mutants did show a cooperative folding transition of W258, indicating that ARs 5–6 now fold cooperatively (Fig. 2). The W258 fluorescence revealed that the C-terminal ARs in the YL/TA mutants are more stable (ΔG_{FLS}) than those in the YL/TA/CP/AP mutant (Table 1).

I κ B α mutants have compactly-folded ARs 5–6

To further probe the "foldedness" of the YL/TA and YL/TA/CP/AP mutants, we measured their conformational flexibility using amide H/ 2 H exchange, which probes the solvent accessibility of the amide protons in the protein. In non-globular proteins, regions that are compact exchange fewer amide protons, whereas regions that are weakly-folded exchange more amide protons.⁴⁸ The β -hairpins in free WT I κ B α are compactly-folded in ARs 1–4, and exchange only a few amide protons in these regions, but they are weakly-folded in ARs 5–6, and exchange nearly all of their amide protons in these regions.³³ All three I κ B α proteins show similar exchange behavior in ARs 1–4. Remarkably, the YL/TA and YL/TA/CP/AP mutants both show much less exchange in the β -hairpins in ARs 5–6 compared to WT I κ B α (Fig. 3; Supplementary Data Table 1).

Calculations of SASA of regions in the protein can be used to account for the structural determinants of their exchange.⁴⁸ If the extent of exchange correlates with the calculated SASA, then the structure of the region is the primary determinant of the exchange. However, exchange that is much greater than predicted by the SASA indicates conformational flexibility. SASA calculations using a model for the free I κ B α structure from the I κ B α •NF- κ B crystal structure with NF- κ B removed showed that the β -hairpins in WT I κ B α ARs 5–6 exchange much more than predicted by their SASA, indicating that they are flexible in free I κ B α .³³ Similar analyses show that the exchange in all regions of the mutant I κ B α proteins is well correlated with their SASA (Fig. 3f), suggesting that the mutants adopt a folded structure similar to that of NF- κ B-bound I κ B α .

NMR 1 H, 15 N heteronuclear single quantum coherence (HSQC) spectra of WT I κ B α showed only 169 of the 208 expected cross-peaks, nearly all of which have been assigned to ARs 1–4 (Supplementary Data Fig. 1). In contrast, the HSQC spectrum of the YL/TA mutant shows all of the expected cross-peaks (Supplementary Data Fig. 1). The cross-peaks assigned to ARs 1–4 in WT I κ B α

show significant overlap with those in the YL/TA mutant spectrum. The spectral similarity factor calculated for ARs 1–4 comparing WT and YL/TA I κ B α of 4.1 Hz is less than 10 Hz, suggesting that the structures are similar in these regions.⁴⁵ This high degree of similarity is especially striking since the presence of the folded ARs 5–6 is expected to perturb the chemical shifts of ARs 1–4 slightly. The presence of a large number of new cross-peaks that likely correspond to ARs 5–6 provide additional evidence that these ARs are compactly folded in the YL/TA mutant.

Pre-folded I κ B α mutants are degraded more slowly *in vitro* and *in vivo*

Robust NF- κ B activation in response to extracellular signals depends on the basal degradation rate of free I κ B α .^{20,21} Degradation of free I κ B α is independent of phosphorylation and ubiquitination and instead appears to be mediated by its C-terminal PEST sequence.²⁰ Free I κ B α degradation is about five times slower when the PEST sequence is deleted.²⁰ I κ B α is readily degraded *in vitro* by the 20S proteasome.^{20,49} A common feature of ubiquitin-independent substrates of the 20S proteasome is that they require an unfolded region to initiate degradation.⁵⁰ Since the C-terminal ARs are more compact in the YL/TA and YL/TA/CP/AP pre-folded mutants than in WT I κ B α , we tested to see if the degradation of the free proteins was altered. *In vitro* degradation experiments utilized proteins that were purified by size-exclusion chromatography, since the presence of aggregates slows degradation (data not shown). Although WT I κ B α was degraded almost completely within 30 min, the mutants persist for longer than 60 min (Fig. 4a).

To measure the *in vivo* half-lives of full-length WT, YL/TA, and YL/TA/CP/AP I κ B α we introduced these transgenes into stable mouse embryonic fibroblast (MEF) cell lines deficient in the NF- κ B proteins known to associate with I κ B α (nfkb3KO: *nfkb1*^{-/-}*rela*^{-/-}*crel*^{-/-}),³ since NF- κ B binding slows the degradation of I κ B α .^{13,20,49} Transgenic free WT I κ B α is degraded at the same rate as endogenous free I κ B α .²⁰ After treatment with cycloheximide to stop translation, the amount of I κ B α remaining was measured by Western blot. WT I κ B α was degraded with a half-life of ~7 min, whereas the YL/TA and YL/TA/CP/AP mutants were degraded more slowly, with half-lives of ~23 min and ~11 min, respectively (Fig. 4b). Importantly, the *in vivo* degradation rate is inversely correlated with the stability of AR 6 (ΔG_{FL}) in each protein (Fig. 4b and Table 1).

Fig. 4. Y254L/T257A (blue) and Y254L/T257A/C186P/A220P (green) are degraded more slowly than WT I κ B α (black) *in vitro* and *in vivo*. (a) Purified 20S proteasome was incubated with WT and mutant I κ B α and the amount of protein remaining was detected by Western blot (top) and quantified by densitometry measurements (bottom). (b) Stable cell-lines containing I κ B α transgenes were treated with cycloheximide to stop translation and the amount of protein remaining over time was detected by Western blot (top). Densitometry quantification of two independent experiments is shown (bottom) with a combined fit of the data. (c) The C186P/A220P mutant (orange) is degraded faster than WT I κ B α (black) in cells. An α - β -actin Western blot, shown in b and c, shows the equivalent loading of all samples.

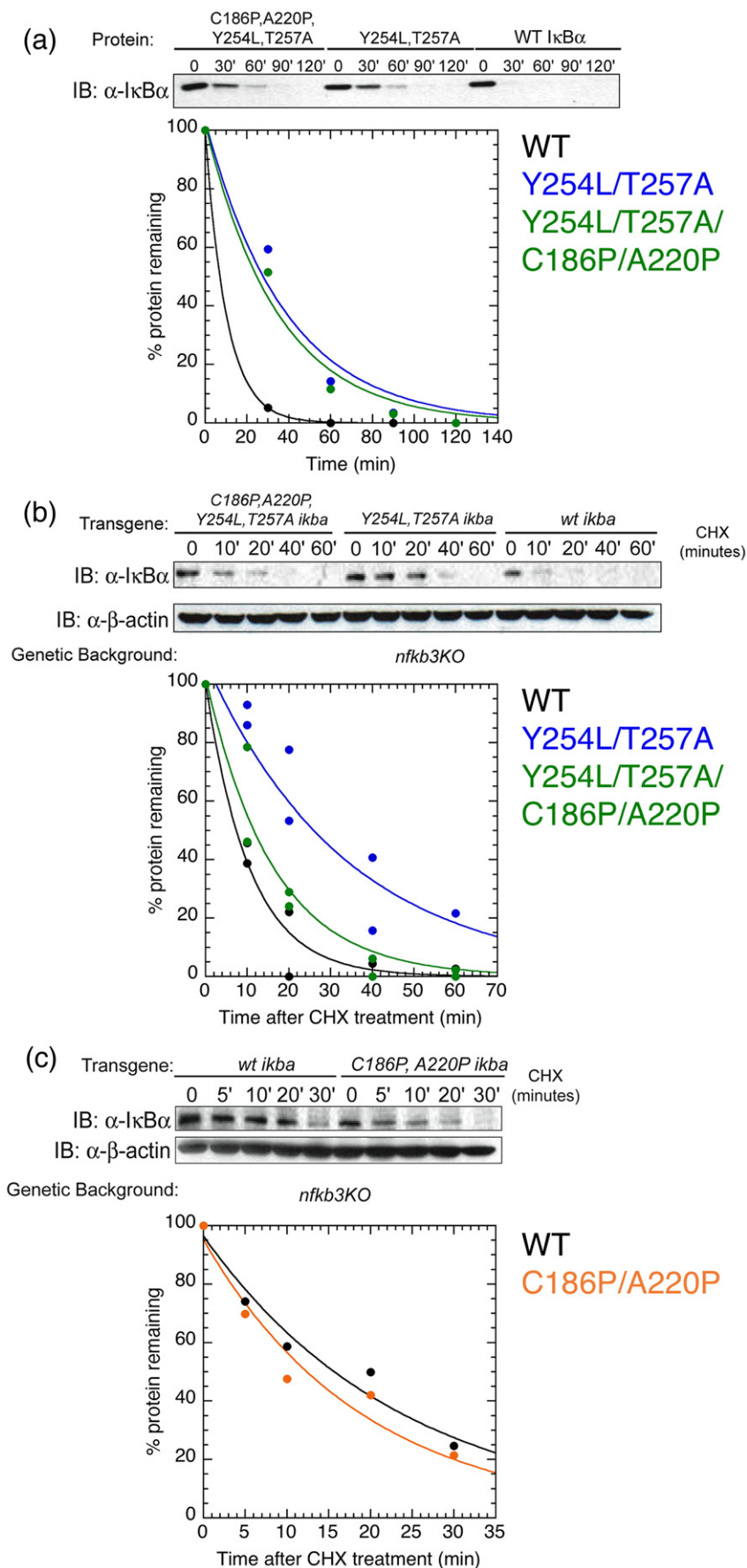


Fig. 4 (legend on previous page)

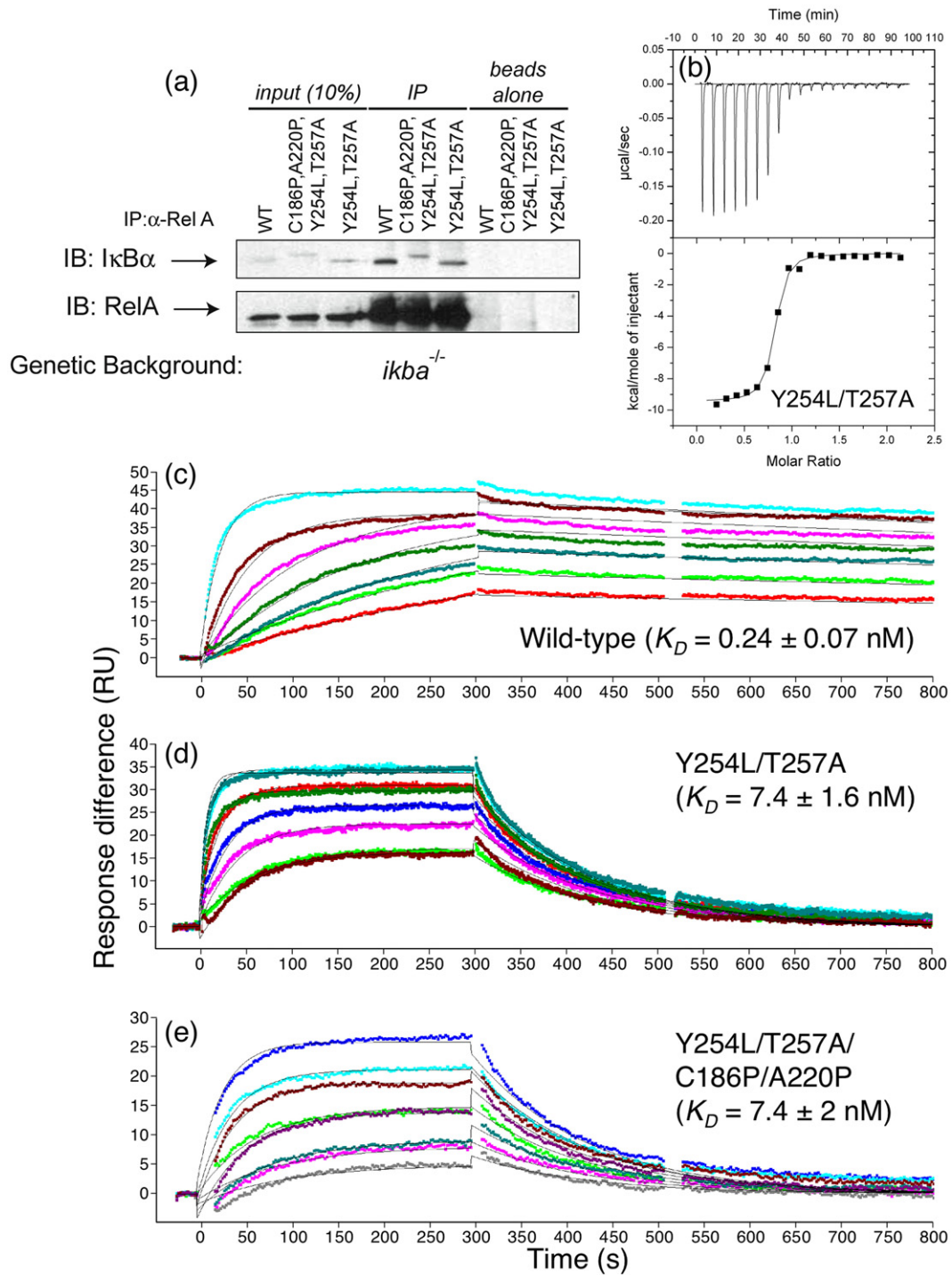


Fig. 5. Y254L/T257A and Y254L/T257A/C186P/A220P bind more weakly than WT I κ B α to NF- κ B (p50_{248–350}/p65_{190–321}) *in vitro* and *in vivo*. (a) NF- κ B (p50/p65 and p65/p65) was immunoprecipitated from lysates of stable cell-lines containing I κ B α transgenes. Total I κ B α (10% input samples) and NF- κ B-bound I κ B α (IP samples) levels were detected by Western blot (top). The starting levels of I κ B α are higher in the Y254L/T257A and Y254L/T257A/C186P/A220P mutants compared to WT I κ B α , but much lower levels of NF- κ B-bound I κ B α are observed for both mutants compared to WT I κ B α . The starting and immunoprecipitated levels of NF- κ B (p65) are similar in all three cell-lines (bottom). There is no non-specific binding of I κ B α or NF- κ B to the protein G beads (beads alone samples). (b) ITC binding isotherm of NF- κ B titrated into Y254L/T257A I κ B α at 25 °C. Data were analyzed using a model for a single set of identical binding sites, and the observed K_D is 23 nM. c–e, Surface plasmon resonance (Biacore) was used to determine the binding kinetics of NF- κ B (immobilized via an N-terminal biotin tag on the p65 subunit) with (c) wild type I κ B α (at concentrations of 1.55–59.7 nM), (d) Y254L/T257A I κ B α (at concentrations of 6.89–118 nM) and (e) Y254L/T257A/C186P/A220P I κ B α (at concentrations of 1.40–106 nM). The pre-folded mutants both dissociate much faster than WT I κ B α . Data were analyzed using a 1:1 Langmuir binding model.

Comparison of the results from the stabilized mutants allows us to show whether the proteasome degradation rate depends on overall I κ B α stability or on the local stability of AR 6. The YL/TA and YL/TA/CP/AP mutants show increases in both their overall stability (ΔG_{CD}) and the local stability of AR 6 (ΔG_{FL}) (Fig. 2 and Table 1). In contrast, the previously characterized CP/AP mutant has increased overall stability, but does not show cooperative folding of AR 6 by fluorescence.³² This mutant is degraded at approximately the same rate as WT I κ B α , both by the 20S proteasome *in vitro* (data not shown) and in stable nfkb3KO cell lines treated with cycloheximide *in vivo* (Fig. 4c). This result clearly points to local stability in AR 6 as a key determinant of the susceptibility of I κ B α to proteasome degradation.

Pre-folded I κ B α mutants bind NF- κ B with reduced affinity

The transcriptional activity of NF- κ B is highly regulated,⁵¹ in part, through the extremely tight binding of I κ B α to NF- κ B.⁴⁻⁶ To determine whether pre-folding of I κ B α alters its NF- κ B binding affinity, we first introduced WT, YL/TA, and YL/TA/CP/AP I κ B α into stable MEF cells lines deficient in endogenous I κ B α (*ikba*^{-/-}). We then immunoprecipitated RelA (p65) and measured the amount of NF- κ B-bound I κ B α by Western blot. Consistent with their slower rates of degradation (Fig. 4a and b), the steady-state levels of I κ B α are slightly higher for the mutants compared to WT I κ B α (input samples, Fig. 5a). In contrast, the levels of NF- κ B-bound I κ B α in the immunoprecipitated samples are much lower for the mutants than for WT I κ B α (Fig. 5a), indicating a significantly weaker binding affinity. Densitometric quantification of the amounts of total (input samples, Fig. 5a, multiplied by 10) and NF- κ B-bound I κ B α (IP samples, Fig. 5a) shows that the amount of free I κ B α is twice as high for the pre-folded mutants.

We measured the binding kinetics by surface plasmon resonance (SPR) using biotinylated NF- κ B (p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁) immobilized on a streptavidin chip. All of the I κ B α proteins associated with

NF- κ B at exactly the same rapid rate of 1.1×10^6 M⁻¹s⁻¹; however, the mutants dissociated from NF- κ B much more rapidly than WT (Fig. 5c to e). The YL/TA and YL/TA/CP/AP mutants dissociate 28 times faster than WT I κ B α (Table 2A). The much faster dissociation of the pre-folded mutants results in reversible NF- κ B binding, unlike the nearly irreversible binding seen for WT I κ B α .

The dissociation constants (K_D) determined by isothermal titration calorimetry (ITC) for the YL/TA and YL/TA/CP/AP mutants were 23 nM and 21 nM, respectively (Fig. 5b and Table 2B). As in previous studies,⁶ the affinities determined by ITC are about three times weaker than those determined in SPR experiments, which is within the expected range (Table 2). For both the YL/TA and YL/TA/CP/AP mutants, binding to NF- κ B is driven mainly by favorable enthalpy at 298 K, but the entropy is also favorable (Table 2B). WT I κ B α binding to NF- κ B has a much larger favorable enthalpy; however, unlike the mutants, the entropy of binding is slightly unfavorable at 298 K (Table 2B).

NF- κ B transcriptional activation

In resting cells, the extremely tight binding of I κ B α to NF- κ B retains the transcription factor in the cytosol.⁴⁻⁶ We measured the levels of nuclear NF- κ B in resting cells containing WT I κ B α or the pre-folded mutants to determine the effects the weaker binding affinity and the slower basal degradation rates of the free pre-folded mutants have on basal NF- κ B activation. NF- κ B is inhibited almost completely in resting cells containing WT I κ B α , as shown by the extremely small amount of nuclear NF- κ B measured by electrophoretic mobility-shift assays (EMSAs). In contrast, resting cells containing the pre-folded I κ B α mutants have a significant amount of nuclear NF- κ B, similar to that seen for cells completely lacking I κ B α (pBABE vector) (Fig. 6a). All of these cells still contain I κ B β and ϵ , which can compensate, but not completely, for I κ B α deficiency.^{21,52}

NF- κ B is activated when, in response to extracellular stimuli, the I κ B that is bound to it is phosphorylated, ubiquitinated, and degraded by

Table 2. I κ B α and NF- κ B (p50₁₉₁₋₃₂₁/p65₂₄₈₋₃₅₀) binding kinetics and thermodynamics

A. SPR binding kinetics and affinities				
Protein	k_a (10 ⁶ M ⁻¹ s ⁻¹)	k_d (10 ⁻³ s ⁻¹)	K_D (nM)	χ^2
Wild-type	1.1 $\pm$ 0.2	0.28 $\pm$ 0.05	0.24 $\pm$ 0.07	1.5
Y254L/T257A	1.1 $\pm$ 0.2	7.9 $\pm$ 0.1	7.4 $\pm$ 1.6	0.44
Y254L/T257A/C186P/A220P	1.1 $\pm$ 0.2	8.0 $\pm$ 0.3	7.4 $\pm$ 2	0.27
B. ITC binding thermodynamics				
Protein	$K_{D,ITC}$ (nM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol) (K_D from ITC)	$-T\Delta S$ (kcal/mol) (K_D from SPR)
Wild-type	N/A ^a	-15	N/A ^a	1.9
Y254L/T257A	23	-9.4	-1.0	-1.7
Y254L/T257A/C186P/A220P	21	-9.8	-0.7	-1.5

^a The $K_{D,ITC}$ and corresponding $-T\Delta S$ for WT I κ B α binding to NF- κ B could not be determined due to the high c value for the interaction, where c is defined by Wiseman *et al.*⁷³

the proteasome (Fig. 6b). We tested NF- κ B activation in cells containing the pre-folded mutants, which have altered NF- κ B binding affinities and basal degradation rates (for the free protein), to understand the effects of these parameters. We first sought to verify that the signal-dependent degradation rate was unaffected by the mutations. Since phosphorylation of I κ B α initiates signal-dependent degradation, followed by rapid ubiquitination and proteasomal degradation of I κ B α ,^{8,11,53} we measured the phosphorylation rate of the different

I κ B α proteins in cells stimulated with 0.1 ng/mL of TNF- α . Both of the pre-folded mutants are phosphorylated at the same rate as WT I κ B α (Fig. 6c). Therefore, any changes in the observed activation of NF- κ B in cells containing the pre-folded mutants should reflect the alterations in their stability and binding affinities.

We measured the amount of nuclear NF- κ B in cells containing different I κ B α proteins after stimulation with 0.1 ng/mL of TNF- α . Cells containing WT I κ B α show a robust increase in nuclear NF- κ B levels upon

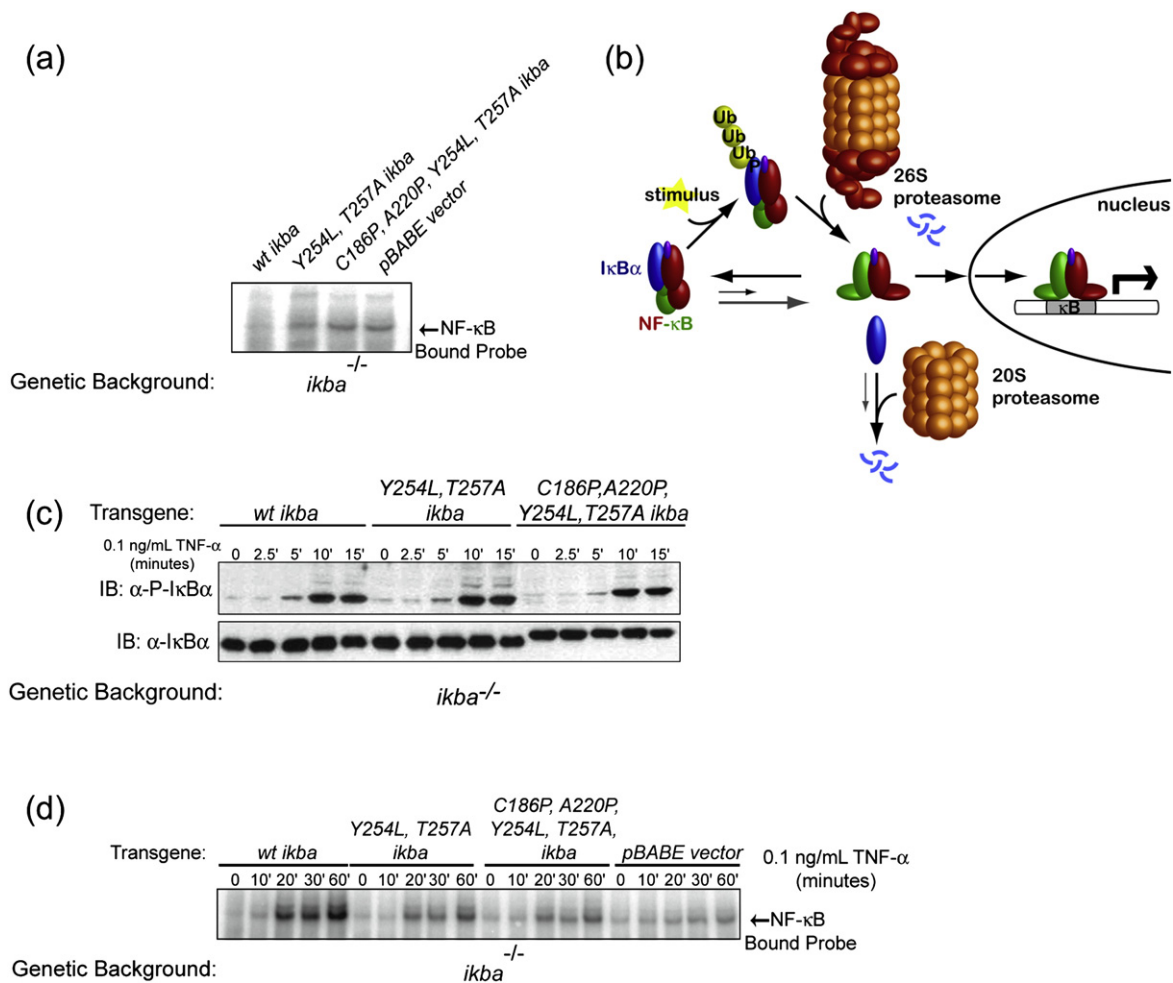


Fig. 6. Cells containing pre-folded mutants show altered amounts of nuclear NF- κ B compared to WT I κ B α . a, Nuclear NF- κ B levels in resting cells, measured by EMSA, show an extremely small amount of nuclear NF- κ B in cells containing WT I κ B α , whereas a significant amount of nuclear NF- κ B is seen in cells containing the pre-folded mutants, which is equivalent to the amount seen in cells deficient in I κ B α (pBABE vector). b, Schematic outlining stimulus-induced activation of NF- κ B. I κ B α binds to NF- κ B and, in resting cells, this prevents its nuclear localization. However, the faster dissociation rates for the pre-folded mutants (gray arrow) result in a significant amount of free I κ B α and unbound NF- κ B, which can translocate into the nucleus. Furthermore, free I κ B α basal degradation is slower in cells containing the pre-folded mutants (gray arrow), resulting in a further increase in free I κ B α levels. Upon stimulation, NF- κ B-bound I κ B α is phosphorylated, which initiates rapid ubiquitination and degradation by the 26S proteasome. This releases NF- κ B, which can then translocate into the nucleus, bind DNA, and activate transcription. c, Measurement of the amount of phosphorylated I κ B α after stimulation with TNF- α shows that the pre-folded mutants are phosphorylated at the same rate as WT I κ B α . Since phosphorylation initiates signal-dependent degradation of NF- κ B-bound I κ B α , we expect that the pre-folded mutants will be degraded at the same rate as WT I κ B α in response to stimulus, in contrast to the slower basal degradation rates of the free pre-folded mutants. d, Upon stimulation with TNF- α , cells containing WT I κ B α show a robust increase in nuclear NF- κ B, as measured by EMSA. Cells containing the pre-folded mutants also show an increase in nuclear NF- κ B upon stimulation; however, the response is reduced compared to cells containing WT I κ B α , but higher than that observed in cells deficient in I κ B α (pBABE vector).

stimulation, due to the signal-dependent degradation of I κ B α that releases NF- κ B, which translocates into the nucleus. Cells containing the pre-folded mutants also show an increase in nuclear NF- κ B levels upon stimulation; however, these levels are reduced compared to those in cells containing WT I κ B α (Fig. 6d). This may be due to the fact that there are lower levels of NF- κ B-bound I κ B α in cells containing the pre-folded mutants. The nuclear NF- κ B levels were higher upon stimulation in cells containing the pre-folded mutants than in cells lacking I κ B α , suggesting that the I κ B α mutants continue to play a role in NF- κ B activation (Fig. 6d).

Discussion

Like many other transcriptional activators and cell-cycle regulators that fold upon binding to their targets,⁵⁴ the folding of I κ B α is coupled to its binding to NF- κ B.³³ In most cases, the functional significance of coupled folding and binding remains a mystery. The simplicity of the ankyrin-repeat architecture of I κ B α presents a unique opportunity to rationally perturb the "foldedness" of free I κ B α , since many determinants of folding and stability in AR proteins are understood. In addition, the repeat architecture allows engineering of local changes in stability.^{55,56} The wealth of prior characterization of the NF- κ B signaling module provides the biological framework within which we should be able to interpret the functional consequences of our rational perturbations of I κ B α .

Only two mutations are required to fold ARs 5–6 in I κ B α

The GXTPLHLA motif is the strongest signature in the AR consensus sequence.^{28,29} Mutations to this consensus stabilize AR domains, and mutations away from it destabilize them.^{32,57,58,59,60} I κ B α deviates from this consensus signature in ARs 1, 2, 4, 5, and 6 (Fig. 1b). Interestingly, these deviations are generally conserved among species. While some of these deviations are amino acids that contact NF- κ B (F77, Q111, and Q255),^{24,25} many do not contact NF- κ B and can be substituted without affecting NF- κ B binding.³² We show here that mutation of only two residues, Y254 and T257, to their consensus counterparts causes a dramatic increase in the foldedness of ARs 5–6 (Figs. 2 and 3), which are weakly-folded in free WT I κ B α , but are compactly-folded in NF- κ B-bound I κ B α .³³ In similar experiments, the tetratricopeptide repeat (TPR) domain of protein phosphatase 5, which contains three repeats, required only one mutation to fold before binding.⁶¹ These results emphasize the utility of the non-globular architecture of repeat proteins to address questions of coupled folding and binding.

CD experiments show that the helical secondary structure is fully formed in free WT I κ B α .³⁴ Therefore, it seems that ARs 5–6 in free WT I κ B α are poised to fold, but lack a few stabilizing interactions.

While WT I κ B α gains this additional foldedness upon interaction with NF- κ B,³³ two substitutions (Y254L/T257A) provide sufficient local stability for the pre-folded mutants to attain essentially the same folded structure as the NF- κ B-bound form, but in the absence of NF- κ B. Y254L and T257A do not contact NF- κ B (Fig. 1a), and the similarity of ¹⁵N-NF- κ B chemical shifts when bound to WT or YL/TA I κ B α suggests that the binding interface is unchanged.

While many consensus-designed AR proteins have been made,^{28–31} it is unclear exactly how these sequences stabilize the protein. Our data show that both the Y254L and T257A substitutions are required to pre-fold I κ B α (Fig. 2). The packing of residues in these conserved positions in I κ B α ARs suggests that the Y254L substitution may be important for intra-repeat stabilization and the T257A substitution may be important for inter-repeat stabilization.

I κ B α foldedness controls its intracellular half-life

Free WT I κ B α is rapidly degraded in cells, with an *in vivo* half-life of ~7 min (Fig. 4b). In fact, it is degraded so quickly that phosphorylation and ubiquitination of free I κ B α is unnecessary.²⁰ Binding to NF- κ B slows the degradation of I κ B α dramatically, making phosphorylation and ubiquitination required for the degradation of NF- κ B-bound I κ B α . We found that pre-folding the C-terminal repeats of I κ B α caused it to be degraded more slowly than WT I κ B α (Fig. 4a and b). The disordered PEST sequence C-terminal to the AR domain appears to mediate the basal degradation of free I κ B α .²⁰ Pre-folding the C-terminal ARs in the mutants may cause a conformational change in the PEST sequence or may have a direct role in influencing the degradation rate.

Comparison of the CP/AP mutant with the pre-folded mutants, which all have similar increases in overall protein stability, shows that the determining factor in susceptibility to degradation is not overall stability, but instead the local stability at the C-terminus. Local stability of specific regions in many proteins controls their degradation rates,⁶² although in some cases the overall thermodynamic stability of the protein is also influential in the degradation process.⁶³ The 20S proteasome core, without any regulatory subunits, degrades some proteins, including I κ B α , in an ubiquitin-independent "default" degradation mechanism.⁶⁴ A unifying characteristic of substrates of the 20S proteasome is the presence of unstructured regions.⁵⁰ It is likely that local rather than global stability will be the predominant determinant of protein degradation for substrates of ubiquitin-independent proteasomal degradation.

I κ B α foldedness controls NF- κ B binding affinity

Wild-type I κ B α binds to NF- κ B extremely tightly, preventing its nuclear localization.^{4–6} Interestingly, *in vitro* binding kinetics and thermodynamics show

that pre-folding I κ B α substantially reduces the overall binding energy, which is consistent with the weaker binding that is observed for the pre-folded mutants *in vivo* (Fig. 5). Remarkably, the overall affinities of the pre-folded mutants for NF- κ B (7.4 nM) are weaker than the affinity of NF- κ B for DNA (4.7 nM).⁶⁵ This result suggests that coupled folding and binding is necessary to achieve the high-affinity binding that is required for effective inhibition of NF- κ B transcriptional activity.

Since folding events are generally accompanied by a large entropic penalty, our observation that the pre-folded mutants bind NF- κ B with weaker affinity compared to WT I κ B α may be somewhat unexpected. Indeed, a similar study of coupled folding and binding in a TPR protein found that increases in the favorable folding enthalpy, due to the coupled folding reaction, were not realized in the binding affinity due to nearly equivalent entropic penalties.⁶¹ In our study, we find that NF- κ B binding is accompanied by a much larger favorable change in enthalpy in WT I κ B α compared to the pre-folded mutants (Table 2B). This additional enthalpy most likely arises from interactions within WT I κ B α that are realized in the folding of ARs 5–6 that is coupled to NF- κ B binding. As expected, WT I κ B α binding to NF- κ B is accompanied by an unfavorable change in entropy; however, the magnitude of this entropic penalty is quite small (Table 2B). This may be due to the fact that free I κ B α is partially folded. Only the folding of ARs 5–6 is coupled to NF- κ B binding, and ARs 5–6 in free I κ B α are folded weakly, but not unfolded completely.^{32–34} Intriguingly, a human growth hormone (hGH) variant with a helix that is highly flexible in the unbound state, but is folded compactly in the unbound wild-type hGH, actually binds to the cognate receptor ~400 times more tightly.⁶⁶ Similar to I κ B α binding to NF- κ B, hGH binding is enthalpically driven and the high-affinity variant shows a much larger favorable change in enthalpy for binding that is not fully compensated by its unfavorable change in entropy, resulting in the higher overall binding energy for the variant hGH. These data suggest that there may be a more complex thermodynamic balance in the binding of partially folded proteins that, in some cases, allows for an increase in binding affinity due to coupled folding and binding.

All three I κ B α proteins bind to NF- κ B with the same association rate, but the pre-folded mutants dissociate from NF- κ B 28-times faster compared to WT I κ B α (Fig. 5c to e and Table 2A). Since dissociation would then require unfolding and disruption of the favorable intra-I κ B α interactions, it is possible to understand how a favorable enthalpy for folding WT I κ B α can result in a marked slowing of its dissociation from NF- κ B. A previous investigation of the thermodynamics of two protein–protein interactions with different binding kinetics found that the dissociation of the complex was slow in the enthalpically driven interaction.⁶⁷ Coupled folding and binding in I κ B α appears to be optimized to slow dissociation through increased favorable enthalpy, which requires mini-

mization of the associated entropic penalty to result in an increase in binding affinity.

I κ B α foldedness controls NF- κ B transcription activation

In resting cells, extremely tight binding to I κ B α retains NF- κ B in the cytosol, effectively eliminating NF- κ B transcriptional activity.^{4–6} However, the weaker binding of the pre-folded I κ B α mutants results in incomplete inhibition and a significant amount of nuclear NF- κ B is present (Fig. 6a). In fact, there is nearly as much nuclear NF- κ B in cells containing the pre-folded mutants as there is in cells deficient in I κ B α . This is a situation similar to that seen in Hodgkin's disease, where altered forms of I κ B α are unable to bind NF- κ B, resulting in sustained NF- κ B transcriptional activity.¹⁸

Upon stimulation, subsequent phosphorylation, ubiquitination, and proteasomal degradation of the NF- κ B-bound I κ B releases NF- κ B, which translocates into the nucleus (Fig. 6b).^{7–11} Accordingly, cells containing WT I κ B α show a robust increase in nuclear NF- κ B in response to stimulation (Fig. 6d). Importantly, we found no difference in the stimulus-induced phosphorylation, which initiates signal-dependent degradation of I κ B α , among any of the I κ B α proteins (Fig. 6c). Thus, any difference observed in the activation of NF- κ B reflects their different *in vivo* stabilities and NF- κ B binding properties. Cells containing the pre-folded mutants show an increase in nuclear NF- κ B when stimulated; however, the response is reduced compared to that in cells containing WT I κ B α (Fig. 6d). This reduction is likely due to a number of factors. The amount of NF- κ B-bound I κ B α is lower in cells containing the pre-folded mutants (Fig. 5a), due to their weaker NF- κ B binding. While this leads to an increase in nuclear NF- κ B levels in resting cells, I κ B β and ϵ probably bind some of the excess free NF- κ B, since they can compensate partially for the lack of I κ B α in *ikba*^{−/−} cells.^{21,52} Stimulus-induced degradation of I κ B β and ϵ also lead to NF- κ B activation; however, the response is delayed compared to I κ B α .^{21,52} Since stimulation increases the amount of nuclear NF- κ B in cells containing the pre-folded mutants compared to the empty vector control, the pre-folded I κ B α mutants appear to still have a role in NF- κ B activation in response to stimulation (Fig. 6d). I κ B α mutants with truncations of their C-terminal PEST sequence show slower basal degradation rates (for the free protein), but they show no change in NF- κ B binding.²⁰ Stimulation of cells containing these degradation mutants also results in less nuclear NF- κ B compared to cells containing WT I κ B α , due to the higher levels of free I κ B α resulting from their slower basal degradation rates.²⁰ A similar phenomenon may be contributing to the observed weaker response to stimulation observed for the pre-folded mutants. Clearly, cells containing the pre-folded mutants show misregulation of NF- κ B.

We have shown that the weakly folded C-terminal repeats of I κ B α and their coupled folding and binding

are required for full repression of NF- κ B in resting cells and robust activation of NF- κ B upon stimulation. Coupled folding and binding of WT I κ B α to NF- κ B results in extremely high-affinity binding. The weakly folded C-terminal repeats of I κ B α are also determinants of the rapid basal degradation rate. Both of these properties effectively eliminate free I κ B α in the cell and facilitate a robust activation response upon stimulation. These results also demonstrate the diverse functional consequences of coupled folding and binding. This phenomenon allows a single protein to develop extremely different functional properties in its free and bound states, and provides a rapid mechanism to switch between these distinct functional states. Undoubtedly, coupled folding and binding will play a critical role in other highly regulated cellular signaling systems.

Materials and Methods

Protein expression and purification

Human I κ B α _{67–287} was expressed and purified essentially as described, except cultures were induced at 18 °C.^{6,34} I κ B α mutations were introduced using Quik-Change mutagenesis.⁶⁸ NF- κ B (p65_{190–321}, with an added N-terminal Cys and p50_{248–350}), were expressed, purified, and quantified as described.⁶ I κ B α protein concentrations were determined by spectrophotometry, using molar absorptivities of 12,950 M^{–1} cm^{–1} for WT and T257A I κ B α and 11,460 M^{–1} cm^{–1} for Y254L, Y254L/T257A, and Y254L/T257A/C186P/A220P I κ B α .

Cell line preparation

Full-length human I κ B α (WT, YL/TA, and YL/TA/CP/AP) was introduced into immortalized 3T3 mouse embryonic fibroblasts using the pBabe-puro retroviral transgenic system.⁶⁹ 293 T cells (80% confluent) in Dulbecco's modified Eagle's medium (DMEM) were transiently transfected with 20 μ L of Lipofectamine 2000 (Invitrogen). Retroviral vector (8 μ g) was co-transfected with 3 μ g of pCI-Eco (Imgenex). After 3 h of growth, the medium was changed and these cells were allowed to grow for 40–48 h in DMEM supplemented with penicillin/streptomycin/gentamycin (Invitrogen) and 10% (v/v) fetal bovine serum. The supernatant was then filtered and placed onto the target 40–50% confluent 3T3 mouse embryonic fibroblasts (*ikba*^{–/–} or *nfkB3KO*: *nfkB1*^{–/–} *rela*^{–/–} *crel*^{–/–}) along with 8 μ g/mL of polybrene (Sigma) in DMEM supplemented with penicillin/streptomycin/gentamycin (Invitrogen) and 10% (v/v) bovine calf serum. These cells grew for another 48 h before selection with 10 μ g/mL of puromycin (Calbiochem). Cells containing the transgenes were grown in DMEM supplemented with penicillin/streptomycin/gentamycin and 10% bovine calf serum. Protein concentrations of cellular extracts and nuclear extracts were determined by Bradford assay.

Equilibrium folding

Equilibrium folding curves were measured using CD and fluorescence simultaneously as described,³² except

2 μ M I κ B α was used in all cases. Additionally, 50 mL of 8 M urea was deionized by stirring for 1 h with 2.5 g of AG501X8 mixed-bed resin (BioRad). After removal of the resin, buffer was added to a final concentration of 25 mM Tris, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT (pH 7.5). The concentration of urea in the denatured samples (7.21–7.39 M) was determined by refractometry.⁷⁰ Folding curves were fit to a two-state folding model, where the pre- and post-transition baselines were treated with a linear dependence on the concentration of denaturant, as described.³²

Amide H/²H exchange

Exchange reactions were performed essentially as described, except the reactions proceeded for 0 min, 0.25 min, 0.75 min, 2 min, or 5 min.³² For WT I κ B α , 14 peptides that cover 60% of the sequence were analyzed. For YL/TA I κ B α , 17 peptides yielded 70% coverage. For YL/TA/CP/AP I κ B α , 14 peptides yielded 63% coverage. The β -hairpin in AR 4 contains C186, which is mutated to proline in the YL/TA/CP/AP mutant, resulting in a peptide that covers two extra residues (188–189) of the protein. (All peptides are given in Supplementary Data Table 1.) The SASA of each peptide was calculated as described,⁴⁸ using the structure from the I κ B α •NF- κ B crystal structure with NF- κ B removed as a model.²⁴ Similar correlations are observed whether or not mutated peptides were included, even though SASA was calculated from the WT protein model.

NMR spectroscopy

Free ¹⁵N-I κ B α _{67–287} (0.1 mM) or 0.08 mM ²H-¹⁵N p50_{248–350}/p65_{190–321} bound to 0.1 mM I κ B α _{67–287} were prepared in 25 mM Tris, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 2 mM NaN₃ at pH 7.5 in 90% H₂O/10% ²H₂O. ¹H,¹⁵N transverse relaxation optimized spectroscopy (TROSY)-HSQC NMR spectra were acquired at 20 °C on Bruker AVANCE 750 and Bruker AVANCE 800 spectrometers. Spectra were processed with NMRPipe,⁷¹ and analyzed with NMRView.⁷²

Proteasome degradation assay

In vitro experiments were performed with human 20S proteasome (a gift from Drs Rechsteiner and Pratt, University of Utah). I κ B α _{67–287} (1 μ M), purified by size-exclusion chromatography within 30 h, was incubated with 20S proteasome (56 nM) for 0 min, 30 min, 60 min, 90 min, or 120 min at 25 °C in 20 mM Tris, 200 mM NaCl, 10 mM MgCl₂, 1 mM DTT (pH 7.0). Degradation reactions were quenched by boiling with SDS-PAGE sample buffer. Intact I κ B α was separated by SDS-PAGE (12.5% polyacrylamide gel) and visualized using Western blots probed with sc-847 (Santa Cruz Biotechnologies) followed by anti-rabbit HRP conjugate. Reactions without proteasome did not degrade (data not shown). Densitometry measurements were performed using ImageQuant TL (GE Healthcare).

Full-length I κ B α transgenes were introduced into mouse embryonic fibroblasts deficient in the NF- κ B proteins known to associate with it (*nfkB3KO*: *nfkB1*^{–/–} *rela*^{–/–} *crel*^{–/–}), since NF- κ B binding slows the degradation of I κ B α .^{13,20,49} Cells were grown to 70% confluency and treated with 10 μ g/mL of cycloheximide resuspended in 50% (v/v) ethanol. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in 100 μ L of 20 mM Tris (pH 7.5),

200 mM NaCl, 1% (v/v) Triton X-100, 2 mM DTT, 5 mM *p*-nitrophenylphosphate, 2 mM sodium phosphate, 1 mM phenylmethanesulfonylfluoride, and Protease Inhibitor Cocktail. Cell extract (50 μ g) was separated using SDS-PAGE (12.5% polyacrylamide gel) and visualized using Western blots probed with sc-371 (Santa Cruz Biotechnologies) followed by anti-rabbit horseradish peroxidase (HRP) conjugate. Densitometry measurements were performed using ImageQuant TL (GE Healthcare), and I κ B α measurements were normalized for loading using an α - β -actin control.

Immunoprecipitation

NF- κ B was immunoprecipitated from mouse embryonic fibroblasts deficient in endogenous I κ B α (*ikba*^{-/-}) containing full-length I κ B α transgenes grown to 95% confluency. Cell lysates were prepared as described above, and 500 μ g of total cellular protein was treated with sc-372-G (Santa Cruz Biotechnologies) overnight. Immunoprecipitates were captured with protein G agarose (Upstate), washed three times with 10 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and analyzed by SDS-PAGE. I κ B α and NF- κ B were visualized by Western blot, probed with sc-371 and sc-372 (Santa Cruz Biotechnologies) followed by anti-rabbit HRP conjugate. Densitometry measurements were performed using ImageQuant TL (GE Healthcare).

Surface plasmon resonance

Sensorgrams were recorded on a Biacore 3000 instrument using streptavidin chips as described.⁶ NF- κ B was biotinylated and immobilized as described;⁶ 150 RU, 250 RU, and 350 RU of NF- κ B (p50_{248–350}/p65_{190–321}) were immobilized. For NF- κ B (p50/p65) binding, WT I κ B α _{67–287} (1.55–59.7 nM) was injected for 5 min and dissociation was measured for 20 min at 25 °C at 50 μ L/min. Regeneration was achieved by a 1 min pulse of 3 M urea in 0.5 $\times$ running buffer, as determined by repeat injections. YL/TA I κ B α _{67–287} (6.89–118 nM) and YL/TA/CP/AP I κ B α _{67–287} (1.40–106 nM) were injected for 5 min, dissociation was measured for 15 min at 25 °C and 50 μ L/min, and no regeneration was required.

Isothermal titration calorimetry

ITC experiments for I κ B α _{67–287} binding to NF- κ B (p50_{248–350}/p65_{190–321}) were performed as described.⁶ The $K_{D,obs}$ for WT I κ B α binding to NF- κ B could not be determined due to the high *c* value for the interaction, where *c* is defined by Wiseman *et al.*;⁷³ therefore, the value of $-T\Delta S$ was calculated from the affinity obtained by SPR.

I κ B α phosphorylation assay

Mouse embryonic fibroblasts deficient in endogenous I κ B α (*ikba*^{-/-}) containing full-length human I κ B α transgenes were grown to 95% confluency. Cells were stimulated with 0.1 ng/mL of TNF- α , and lysed as described above. Cell extract (50 μ g) was separated using SDS-PAGE (12.5% polyacrylamide gel) and visualized using Western blots probed with 5a5 (antibody for S32/36 phosphorylated I κ B α from Cell signaling) and sc-371 (antibody for total I κ B α from Santa Cruz

Biotechnologies) followed by anti-mouse and anti-rabbit HRP conjugate, respectively. Densitometry measurements were performed using ImageQuant TL (GE Healthcare), and phosphorylated I κ B α measurements were normalized according to the total I κ B α level in each sample.

Electrophoretic mobility-shift assay

EMSAs were performed essentially as described,¹⁷ except cells were grown to confluency, stimulated with 0.1 ng/mL of TNF- α , and 6 μ g of nuclear protein was used.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2008.02.053](https://doi.org/10.1016/j.jmb.2008.02.053)

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