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Interaction of the IκBα C-terminal PEST sequence with NF-κB: Insights into the Inhibition of NF-κB DNA binding by IκBα

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Abstract

The transcription factor NF-κB (p50/p65) binds either a κB DNA element or its inhibitor protein, IκBα, but these two binding events are mutually exclusive. The reason for this exclusivity is not obvious from the available crystal structure data. The C-terminal PEST-like sequence of IκBα appears to be involved in the process, but it is located in both of the published X-ray structures of the IκBα/NF-κB complex at a significant distance away from the DNA contact loop in the NF-κB DNA-binding domain. We have used nuclear magnetic resonance spectroscopy and differential isotopic labeling to probe the interactions between the p50/p65 NF-κB heterodimer and IκBα in solution. Our measurements are able to resolve a local structural discrepancy between the two crystal structures, and we confirm that the primary interaction of the IκBα PEST domain is with the DNA-binding domain of the p65 subunit. Mutagenesis of key arginine residues in the DNA contact sequence results in the loss of specific interaction of the PEST sequence with the p65 subdomain. We conclude that the local structure of the IκBα/NF-κB complex in the region of the PEST sequence is consistent with a direct interaction of this acidic sequence with the basic DNA contact sequence in p65, thus reducing the affinity of NF-κB for DNA by a competitive mechanism that is still to be elucidated fully.

Keywords

transcriptional activation; NMR

INTRODUCTION

NF-κBs constitute a family of transcriptional activators with five members in mammalian systems, p50 (p105 precursor), p52 (p100 precursor), p65 (RelA), c-Rel and RelB.^{1–3} Each member of the NF-κB family contains a highly conserved ~300 residue segment at the N-terminus. This segment, termed the Rel homology region (RHR) consists of two immunoglobulin-like domains, an N-terminal DNA-binding domain and a C-terminal dimerization domain, which are critical for nearly all NF-κB functions.^{4,5} Different combinations of components derived from different members of the NF-κB family promote the formation of a variety of homo- and heterodimers.^{1,2} The p50/p65 heterodimer (shown schematically in Figure 1) was the first discovered⁶ and is the most abundant form of NF-κB.^{1,4}

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NF- κ B is regulated by association with I κ B inhibitors, including I κ B α , I κ B β and I κ B ϵ .³ In unstressed cells, NF- κ B associates with I κ B to form a stable complex, which is sequestered in the cytoplasm. The classic heterodimer p50/p65 is predominantly regulated by I κ B α ,^{3,7} and this cytoplasmic complex mediates rapid response to activating signals.³ Within minutes after the application of extracellular stimuli such as pro-inflammatory cytokines,⁸ lipopolysaccharide⁹ and tumor necrosis factor,¹⁰ the signal response domain at the N-terminus of I κ B α is phosphorylated by activated I κ B kinase (IKK),³ which leads to ubiquitination of I κ B α and its subsequent degradation by the 26S proteasome.^{4,11,12} NF- κ B, released from the I κ B complex, is translocated to the nucleus where it can activate gene transcription by binding to the κ B DNA site. Since the gene coding for I κ B α itself is one of the downstream genes regulated by NF- κ B, expression of I κ B α also occurs as a consequence of NF- κ B activation,⁷ and the newly synthesized inhibitor rebinds to NF- κ B, inactivating its transcriptional activity by removing it from the DNA and mediating its export from the nucleus.⁷

Structural studies of the NF- κ B/I κ B α interaction include X-ray crystal structures of the NF- κ B/I κ B α complex, determined by two independent groups.^{13,14} The I κ B α structure in the complex (shown schematically in Figure 1) includes six stacked ankyrin (ANK) repeats and a C-terminal PEST sequence.^{13,14} PEST sequences are rich in proline, glutamic acid, serine and threonine residues and are thought to be related to protein degradation^{12,15} and to inhibit binding of NF- κ B to DNA.^{16,17} The NF- κ B protein characterized in the two structures consists of fragments of p65 (including the DNA-binding domain, dimerization domain and C-terminal nuclear localization signal peptide, NLS) and p50 (dimerization domain only). I κ B α and NF- κ B form an extensive noncontiguous binding surface, with ANK 1–2 contacting the p65 NLS and ANK 4–6 closely associated with the p50 and p65 dimerization domains; the C-terminal PEST sequence was found to pack on the top of p65 DNA-binding domain.^{13,14}

More recently, additional structural information has become available in solution for I κ B α both free and bound to NF- κ B, using NMR spectroscopy and mass spectrometry.^{18–20} A streamlined preparation method was adopted to prepare NF- κ B/I κ B α complexes that are precisely and specifically labeled, with I κ B α labeled with ²H, ¹³C and ¹⁵N and p50 and p65 uniformly deuterated.²⁰ Combining a fragment-based assignment strategy, complete backbone resonance assignments were made for I κ B α (residues 67–287, the minimal fragment required for dissociating NF- κ B from DNA^{16,17}) in complex with NF- κ B (both dimerization and DNA-binding domains of both p65 and p50²⁰). The molecular weight of this complex is 94 kDa. Free I κ B α is apparently fluxional or molten globular in the region of ANK5–6;^{20,21} this region undergoes a structural and dynamic transition as it folds upon binding to NF- κ B,^{18,20,21} and the center of the molecule, principally ANK3, becomes more flexible in the complex than in the free state.²⁰

Although I κ B α -mediated down-regulation of the transcriptional activity of NF- κ B has been known for many years, the mechanism by which nuclear I κ B α competes with the κ B DNA sequence for NF- κ B binding, thus turning off the transcription of stress-related genes, remains unknown. On the one hand, biochemical and mutagenesis studies implicate binding of I κ B to a specific site, the DNA-binding loop on NF- κ B.²² This interaction was found to occur through the PEST motif of I κ B α .²³ On the other hand, a completely different interaction was observed in the X-ray crystal structures^{13,14} between the C-terminal PEST sequence of I κ B α and the N-terminal DNA-binding domain of NF- κ B p65: the PEST sequence appears to be located too far from the DNA-binding loop on NF- κ B to be responsible for the specific effects noted in the biochemical experiments.^{22,23} If the PEST sequence is indeed located far from the DNA-binding loop, it was suggested that its effect might be to lower the affinity of NF- κ B for DNA by altering its conformation to an inactive form.^{13,16,24} Following our successful characterization of the NF- κ B – I κ B α system in solution by NMR,²⁰ we report the elucidation

of the interactions of the PEST sequence of I κ B α in solution, in complex with NF- κ B constructs containing various domains of p50 and p65.

RESULTS

Structural Analysis of the I κ B α C-terminus

In order to dissect the role of the PEST sequence in the complex between I κ B α and NF- κ B, we made a detailed examination of the two X-ray structures of this complex, published by two independent groups (PDB 1NFI¹⁴ and 1IKN¹³). The protein components of the two structures differ only slightly in length, and the two structures are highly similar.²⁵ In this study, we focus on the structural features of the I κ B α C-terminal region and their relationship to the function of I κ B α in the p50/p65 complex. A careful comparison of the C-terminal structure of the complexed I κ B α in the two sets of X-ray coordinates shows two discrepancies (Figure 2). First, there is a difference in the orientation of the side chain of W258 (Figure 2B). In structure 1NFI, a hydrogen bond is shown between W258(ϵ H) and the backbone carbonyl of L277, but this hydrogen bond does not appear in structure 1IKN because the W258 side chain is in an orientation incompatible with its formation. Second, there is a 1-residue frame shift in the backbone position between 1IKN and 1NFI at residue 270 (Figure 2C). The dotted circle indicates the position of the side chain of Q271 in 1IKN; nothing is fit to the corresponding position in 1NFI. Instead, the Q271 side chain in 1NFI was fitted to the same position as the side chain of L272 in 1IKN, and L272 (1NFI) is fitted in the position of T273 (1IKN), and subsequent residues are frame shifted. This is illustrated by a comparison of the backbone dihedral angles (colored entries in Table 1). Regardless of the discrepancies in side chain position, the overall orientation of bond sin backbone and side chain are very similar between the two structures (backbone RMSD < 1 Å) (Figure 2C), indicating the similarity of electron densities in the local regions. It is unlikely that these differences indicate the presence of two different conformations in the two structures, but rather reflects the difficulty of fitting residues into the marginal electron density of a region that is highly mobile in solution²⁰ and likely disordered in the crystal.

Solution structure of the I κ B α C-terminus

Local structure in the I κ B α C-terminus was determined by two sets of NMR parameters: NOE connectivities identified from NOESY spectra and dihedral angles predicted from chemical shifts using the program TALOS.²⁶ [²H, ¹⁵N]-labeled I κ B α (67–287) in complex with [²H]-labeled p50(248–350)/p65(190–321) was used for acquiring the NOEs. NOE strips from a ¹⁵N-edited TROSY-NOESY-HSQC spectrum at the backbone ¹⁵N chemical shifts of residues L277 to L280 and the N ϵ side chain of W258 are shown in Figure 3A. The dotted lines indicate the NOE cross peaks that denote short distances between backbone amide protons (NH) and the W258 indole proton (ϵ H). The relative intensities of the cross peaks can be correlated with internuclear distances via the r^{-6} distance dependence of the NOE. A relatively intense cross peak (distance estimate ~ 4.5 Å) is observed for the W258(ϵ H)-to-L280 NH connectivity, and there are weak NOEs (distance estimate ~ 5–6 Å) for the W258(ϵ H)-to-Q278 NH and W258(ϵ H)-to-M279 NH connectivities. These distances are consistent with the presence of a hydrogen bond between the N ϵ H of W258 and the backbone CO of Q278, illustrated in Figure 3B.

Neither of the X-ray coordinates matches the NOE constraints in this region (Figure 3B). In 1IKN, there is no hydrogen bond to the N ϵ H of W258: the W258 side chain is rotated around the β - γ bond and the indole proton points away from the backbone of the PEST sequence (Figure 2B, 3B); the distance is thus too great for a detectable NOE to be expected (Table 2). In 1NFI, the N ϵ H of W258 makes a hydrogen bond, but it is with the CO of L277 rather than that of Q278. This close approach should yield significant NOEs, but the strongest NOE is

expected to be between W258(ϵ H) and M279 NH instead of the observed connectivity to L280 NH (Figure 3B). Both structures match the NOE data imperfectly, but can be reconciled with it if elements of the two structures are incorporated into a hybrid structure. The hydrogen bonding of the W258 N ϵ to a backbone CO is incorporated from 1NFI, but the register of the backbone is incorporated from 1IKN: instead of the hydrogen bond being to the CO of L277 (as in 1NFI), it is to the CO of Q278. The two structures are merged to generate a hybrid coordinate set: the coordinates of residues from the N-terminus to L269 (including W258) are taken from 1NFI, and from G270 to the C-terminus is taken from 1IKN. The distances derived from 1IKN/1NFI hybrid structure match the observed NOEs (Table 2): the distance between W258(ϵ H) and L280 NH is 4.4 Å, corresponding to the relatively intense long-range NOE shown in Figure 3A, and the two weak NOEs of proton pairs of W258(ϵ H)/Q278 NH and W258(ϵ H)/M279 NH correspond to the distances of 6.0 and 5.0 Å, respectively in the hybrid structure.

Further information on the backbone structure in the C-terminal region of I κ B α is provided by the dihedral angle information predicted by TALOS from the measured NMR chemical shifts and the measured (ϕ , ψ) angles of 1IKN and 1NFI (Table 1). The angles derived from 1IKN are generally similar to those from the TALOS prediction, while the angles derived from 1NFI correspond to the predicted ones only if the backbone is frame-shifted by one residue, consistent with the NOE results.

Dynamic properties of the C-terminal PEST sequence

The local dynamics at the C-terminus of I κ B α in the NF- κ B complex (exploded view from the previously-published study²⁰) is shown in Figure 4. After residue Q278, the ^{15}N -R₂ and [^1H]- ^{15}N NOE show a gradual decrease from the values typical of a folded protein, while ^{15}N -R₁ values are increased. This result demonstrates that the backbone motions are gradually increased after Q278, consistent with our hypothesis that residue Q278 is immobilized by hydrogen bonding to the side chain of W258. We conclude that the region ending at Q278 can be treated as part of the fold of ANK 6 and the residues after Q278 should be considered random-coil-like, with high flexibility in solution.

Influence of C-terminal PEST on ANK 6

In order to function in the inhibition of DNA binding by NF- κ B, a minimal length of I κ B α , residues 67–287, is required:¹⁶ a shorter fragment of I κ B α where the C-terminal PEST sequence had been deleted (I κ B α 67–277) fails to dissociate DNA from NF- κ B.¹⁶ Two possible roles for the PEST sequence are suggested by these results: (a) the PEST sequence competes with DNA for binding to NF- κ B, so that removing the PEST sequence abolishes the interaction, or (b) the absence of the PEST sequence destabilizes the structure of the C-terminal ankyrin repeats in the complex, promoting the incompletely-folded and fluxional state of this region that is present in free I κ B α .^{18,20}

In order to determine the structural basis for the effects of I κ B α C-terminal deletion, a truncated fragment, I κ B α (67–275) was constructed and the [^2H , ^{13}C , ^{15}N]-labeled I κ B α (67–275)/p50 (248–350)/p65(190–321) complex was prepared. Backbone assignments for this complex were made with reference to those of the longer fragment described above, and confirmed using a TROSY-type triple-resonance HNCA experiment. The resonances of ANK 6 were mostly assigned from the HNCA spectrum; the backbone NH resonance of T263 is missing.

The TROSY-HSQC of p50(248–350)/p65(190–321) complexed I κ B α (67–287) and I κ B α (67–275) are superimposed in Figure 5A; residues with significant perturbations are indicated. The composite amide chemical shift difference, $\Delta\delta_{(\text{N}+\text{NH})} = [(\delta_{\text{N}}^2/25 + \delta_{\text{NH}}^2)/2]^{1/2}$, between the two complexes is shown in Figure 5B. It is noticeable that perturbations are present only

in ANK 6, with local maxima at residues T247, S252 and E275. Negligible chemical shift differences are observed for the other ankyrin repeats, even for ANK 5, which shows $\Delta\delta_{(N+NH)}$ generally less than 0.1 ppm, indicating a lack of significant structural variation between the two complexes. Since ANK 5 is immediately adjacent to ANK 6, with significant non-covalent contact between the two repeat domains, any significant conformational change in ANK 6 should induce detectable chemical shift perturbation in ANK 5. The results shown in Figure 5B therefore imply that there is no major structural change in ANK 6 when the C-terminal tail is removed. This conclusion is supported by a comparison of the $^{13}\text{C}_\alpha$ chemical shift differences $\Delta\delta_{(C\alpha)}$, which confirm the secondary structural similarity between the two complexes (Figure 5C). The residues perturbed by truncation of the PEST sequence (Figure 5D) are present mainly at the C-terminus of ANK 6, in the immediate neighborhood of residue E275, or in the region (including S252 and W258) that contacts the PEST sequence in the hybrid structure derived above. Deletion of residues 276–287 has perturbed the local environment and caused variations in the chemical shift, but the fold of ANK 6 remains the same. This is consistent with the reported K_d value of 12 nM (25°C) for the complex between I κ B α (67–275) and p50(248–350)/p65(190–321)²⁷, which, although significantly weaker than that of I κ B α (67–287) (0.32 nM at 37°C), remains strong enough to induce slow-exchange NMR behavior for the complex as a whole. We conclude that the presence of the PEST sequence does not contribute significantly to the structure of ANK 6, since ANK 6 adopts similar secondary and tertiary structures in the presence and absence of the C-terminal PEST. Thus, inhibition of NF- κ B DNA binding by I κ B α most likely involves direct contact between the PEST sequence and NF- κ B.

The reason for the 250-fold decrease of binding affinity upon removal of the PEST sequence²⁷ is unclear. Based on the similarity of structures, the binding difference might relate to local structural stability in the C-terminal ANKs of I κ B α . Perhaps the presence of residues 276–287 stabilizes these ankyrin repeats through a capping mechanism in the complex²⁸. If so, we would expect that the affinity decrease should be due primarily to lowered ANK6 affinity. Nevertheless, the pattern of chemical shift changes indicates that ANK6 remains fully stable (rather than becoming imperfectly folded as it would be in the free state). This observation may indicate that both ANK5 and ANK6 have to be dissociated in order for the instability of the free form to be realized. In addition, the free form of the truncated I κ B α aggregates more readily, reflecting its lower stability.

The behavior of the W258(ϵ H) in the NMR spectrum of the truncated fragment (Figure 5A) provides further evidence for the presence of the hydrogen bond between W258(ϵ H) and the Q278 backbone carbonyl, described above. The ^1H resonance of the W258(ϵ H) shows a dramatic upfield shift in Figure 5A, indicative of the loss of the hydrogen bond when residues 276–287 are missing.

Dynamics of the PEST sequence in complex with NF- κ B

It is clear from the relaxation data acquired for the complex I κ B α (67–287)/p50(248–350)/p65 (190–321) (Figure 4) that the backbone of the I κ B α PEST sequence increases in flexibility following Q278, the site of the hydrogen bond to the W258 side chain. This complex contains only the dimerization domains of p50 and p65. The two X-ray structures^{13,14} contain the DNA-binding domain of p65 in addition to the two dimerization domains, and the PEST sequence shows a similar backbone structure (with variations described above) in each of these structures. Its position has been used to form hypotheses as to the function of the PEST sequence in the removal of NF- κ B from DNA by I κ B α . However, the full complex of I κ B α and NF- κ B contains both the DNA-binding and dimerization domains of both p65 and p50. We asked whether the presence of the p50 DNA-binding domain would cause changes in the behavior

of the PEST sequence, and addressed this question by comparing the NMR spectra of the I κ B α PEST domain in four complexes containing different domains of p50 and p65.

The four complexes are: I κ B α (67–287)/p50(248–350)/p65(190–321), I κ B α (67–287)/p50(248–350)/p65(19–321), I κ B α (67–287)/p50(39–350)/p65(190–321) and I κ B α (67–287)/p50(39–350)/p65(19–321). These complexes are shown schematically in Figure 6, together with regions of TROSY-HSQC spectra containing the PEST assignments. The influence of the addition of the p50 DNA-binding domain is shown by the difference between the spectra in Figures 6A [I κ B α (67–287)/p50(248–350)/p65(190–321)] and 6B [I κ B α (67–287)/p50(39–350)/p65(190–321)], while the effect of addition of the p65 DNA-binding domain is shown by the difference between Figures 6A and 6C [I κ B α (67–87)/p50(248–350)/p65(19–321)]. The effect of addition of both DNA-binding domains is shown in Figure 6D. A histogram illustrating the relative size of the perturbations in the resonances of the PEST sequence is shown in Figure 7A.

The comparison leads to four major observations. Firstly, the p50 N-terminal domain (red bars in Figure 7A) makes a slight but significant perturbation in the chemical shifts of residues S283, D285 and E286, suggesting the presence of a weak interaction between the p50 N-terminal domain and this region of the I κ B α PEST sequence. Secondly, the p65 N-terminal domain (yellow and orange bars) produces similar but more significant perturbations in the resonances of the PEST sequence. The cross peak of residue S283 is shifted in the same way and to the same extent as for the complex containing the p50 N-terminal domain (Figure 6B), but D285 and E286 show multiple cross peaks (yellow and orange bars in Figure 7A) and a shift to higher field in both ^1H and ^{15}N dimensions. Other residues in the PEST sequence show slight chemical shift perturbations. Thirdly, the presence of both p65 and p50 N-terminal domains (Figure 6D, green bars in Figure 7A) induces chemical shift perturbations similar to those caused by addition of the p65 N-terminal domain alone. The broadened cross peaks for residues D285 and E286 of Figure 6D are in the same position as the split resonances in Figure 6C. The similarity between Figures 6C and D implies that the PEST sequence binds preferentially to the p65 N-terminal domain. The only major difference between the two spectra is that residue S283 shows an additional cross peak (blue bar in Figure 7A), additional line broadening and weaker intensity in Figure 6D, which may suggest that this residue makes additional interactions in the full complex. Finally, there is negligible perturbation of the resonances of remaining portions of I κ B α , including all of the ANK repeats, indicating that, consistent with the X-ray structures, there is no contact in the complex between the NF- κ B N-terminal domains and the ANK repeats.

The complexes in Figure 6A–C behave similarly in solution, reflected in the consistent and narrow linewidths of the resonances. However in the largest complex (Figure 6D), which incorporates both N-terminal domains, the resonances are dramatically broadened, even though a lower protein concentration (0.15 mM) was used to minimize intermolecular contact between the complexes. It is unlikely that this broadening is due only to an increase in molecular weight of ~ 20 kDa. This observation may instead be an indication of differential dynamics within the larger complex. The I κ B α PEST sequence recognizes preferentially the p65 N-terminal domain, which will serve to anchor this domain to some extent to the main bulk of the complex. However, the p50 N-terminal domain is likely to be relatively mobile in this complex (presumably less so in the complex shown in Figure 6B since it interacts weakly with the PEST sequence in that complex). The presence of a flexibly-tethered domain will increase the effective radius of the entire complex, resulting in a reduction of the overall tumbling rate. This effect would be consistent with the difficulty of crystallizing the I κ B α /NF- κ B complex containing both N-terminal domains, such that the available X-ray structures contain only the p65 N-terminal domain.

Contact site for IκBα PEST on NF-κB

The involvement of the acidic PEST sequence in the inhibition of NF-κB DNA binding is well established. Our results confirm that the PEST sequence makes direct contact preferentially with the p65 N-terminal domain. However, there is some inconsistency in the literature between reports that the RXXRXXRXC DNA-binding motif is the site of interaction of the IκBα PEST sequence^{22,23} and the two X-ray structures,^{13,14} which show that the PEST is closest to (but not within ~ 10 Å of) the first arginine at the N-terminal end of the DNA-binding motif on the p65 DNA-binding domain. In these structures, all of the other arginines are greater than 25 Å from the PEST domain.

In order to investigate the interaction site for the IκBα PEST sequence on the p65 DNA-binding domain, and in particular to ascertain whether the DNA-binding loop is involved, we constructed three NF-κB mutants where arginine residues in the conserved DNA-binding motif were substituted with alanine: p65(19–321, R30A/R33A/R35A), p65(19–321, R33A/R35A) and p65(19–321, R30A). The [²H, ¹⁵N]-labeled IκBα(67–287) in complex with the individual p50(248–350)/p65(19–321) mutants were prepared and the corresponding TROSY-HSQC spectra are plotted in Figure 8. The complexes with the double and triple mutants R33A/R35A and R30A/R33A/R35A (Figure 8B,C) show differences in the resonances of the PEST sequence, compared to the wild-type heterodimer complex (Figure 8A). The single mutation R30A (Figure 8D) does not affect the resonances of the PEST sequences. The spectra of the double and triple mutants (Figures 8B and C) are identical to each other, and are almost identical to those observed for the complex where the NF-κB N-terminal domain is absent (Figure 6A). This is illustrated in Figure 7B, where the yellow and orange bars represent the same data as in Figure 7A for the difference between the (wild-type) p50(248–350)/p65(19–321)-IκB complex and the p50(248–350)/p65(190–321)-IκB complex. The triple (blue bars) and double (purple bars) mutants are almost identical to the complex in the absence of the p65 N-terminal domain, while the single mutant (pink and brown bars) closely resembles the wild-type complex (yellow and orange bars). We conclude that R33 and R35 are critical for the binding of the acidic PEST sequence of IκBα, whereas R30, which appears to make no additional contribution in the triple mutant compared to the double mutant, is likely not involved. These same two arginine side chains are inserted into the major groove of the DNA in the NF-κB/DNA complex, mediating direct sequence-specific contact with the DNA bases GUA14 and GUA15 of the κB recognition element,²⁹ while the R30 side chain points away from the DNA. Our results indicate that the κB recognition element and the PEST sequence compete for the same binding site on NF-κB, which includes residues R33 and R35, but likely not R30.

DISCUSSION

Evidence for interaction between the p65 DNA-binding loop and the IκBα PEST

The conserved NF-κB DNA-binding loop (corresponding to residues 30 to 45 in p65) appears to interact directly with the PEST sequence of IκBα, based on the following evidence. Firstly, the structure of the NF-κB/DNA complex²⁹ showed four residues, R33, R35, Y36 and E39 in p65, mediating direct DNA base-specific contacts. A peptide with sequence derived from the DNA-binding loop of v-Rel (EQPRQRGTRFRY containing the critical residues underlined) has been used successfully to recover DNA binding following IκBα-mediated inhibition.²² These experiments led to the identification of the conserved sequence motif RXXRXXRXC (equivalent to residues 30 to 38 in p65) that appears to be required for IκBα recognition.²² Secondly, an antibody raised against the conserved sequence motif prevented association between c-Rel and IκBα,²³ and mutants where residues 280–290 of IκBα were deleted were unable to inhibit DNA binding by p65.²³ Thirdly, replacement of the 15-amino acid DNA-binding loop of p65 by a sequence containing three glycines resulted in a 57-fold reduction in the binding affinity between IκBα and p65.¹⁶ These results appeared to offer strong evidence

for direct interaction between the conserved DNA-binding motif of the Rel proteins and p65 and the PEST sequence of I κ B α .

Position of the PEST sequence in the 3D structure

The published X-ray structures of the p65/p50/ I κ B α complex^{13,14} provide an important resource for the understanding of the interactions of NF- κ B and I κ B α , but have limitations in areas of the complex with backbone flexibility. The two X-ray structures were obtained with very similar protein constructs, but the 1NFI structure¹⁴ shows the N-terminal nuclear localization signal portion of p65 in a two-helix structure, while the C-terminus of I κ B α is not defined in this structure after residue 282 (283 of the hybrid structure); the last 5 residues of the 69–288 construct are undefined. The 1IKN structure¹³ does not show the nuclear localization sequence so clearly, but defines coordinates for a longer portion of the C-terminus of I κ B α (residues 284–292, sequence E₂₈₄DEESYDTE₂₉₂), where the last 10 residues of the 67–302 construct are undefined. Our results show that the C-terminal PEST sequence of I κ B α is never really well-structured in solution.²⁰ Even in the 94 kDa complex of I κ B α with p50(39–350) and p65(19–321), the resonances of the PEST sequence remain strong and their chemical shifts are located in the central “random coil” region of the ¹H-¹⁵N TROSY-HSQC spectrum (Figure 6D). The multiple cross peaks observed for D285 and E286 (Figure 6C) and for S283 in the largest complex (Figure 6D) may be an indication of the presence of several different local structures that are exchanging on a slow-to-intermediate time scale. The presence of both the p50 and p65 N-terminal domains restricts the mobility of the PEST sequence, without, however, inducing a single structured conformer. In addition, the observation of significant chemical shift perturbations in D285 and E286 is consistent with the mutation study²³ that showed that changing E284, D285 and E286 caused a significant loss of activity; these authors concluded that three consecutive acidic residues are critical for inhibitory activity.

According to both of the X-ray structures, the PEST sequence is located in the vicinity of the p65 N-terminal domain, but does not specifically interact with the DNA contact loop. The structures show that the I κ B α molecule uses the bottom of ANK 6 and the C-terminal PEST region to interact with the p65 N-terminal domain. The main interactions are between the highly acidic patch of the PEST sequence: residues E282, E284, D285, E286 and E287, and a basic region of p65 N-terminal domain: residues K28, R30, K79, R158 and H181. Other residues in I κ B α , W258, Q278, M279 and P281 also stabilize the interface through polar and van der Waals contacts. It was proposed¹³ that these interactions promoted allosteric regulation of DNA binding by NF- κ B: binding of I κ B α changes the orientation of the p65 N-terminal domain, locking the N-terminal domain into a closed conformation that interferes with DNA binding by NF- κ B, which is in an “open” configuration in the DNA complex.²⁹ Our NMR studies show chemical shift perturbations, induced mainly by the p65 N-terminal domain, in the same residues of I κ B α that would be expected from its position in the X-ray structures. However we observe evidence for an additional interaction not compatible with the X-ray structures: since the binding of the I κ B α C-terminus is specifically influenced by mutations in the DNA-binding loop of p65, we infer that this region also recognizes the R33 and R35 residues, whereas R30 is not critical. In the X-ray structure 1IKN,¹³ these two arginines are located at a distance of 25 to 30 Å from the acidic PEST residues, E284, D285 and E286 (Figure 9). A similar distance is observed in the structure 1NFI¹⁴ despite a slightly different orientation of the p65 N-terminal domain. The X-ray studies employed slightly different I κ B α constructs (67–302 in 1IKN and 69–288 in 1NFI), but these differences are unlikely to be the cause of the altered domain orientations since I κ B α (67–317) and (67–287) have been reported to be able to recognize NF- κ B with equal affinities and kinetics.^{17,30} the residues after E287 probably play a negligible role in NF- κ B binding. We believe that there is significant contact between R33/R35 and the C-terminal PEST sequence of I κ B α in solution, since alanine substitution of R33 and R35 is

sufficient to reverse the changes in the NMR spectrum of the PEST region that are identified with recognition of p65, and that the orientations in the X-ray structures, possibly manipulated by crystal packing forces, do not truly reflect the structure of the complex in solution.

Interactions between the IκBα PEST and p65 are specific but weak

The chemical shift perturbations caused by the addition of the p50 and p65 N-terminal domains to the complex of IκBα with the C-terminal dimerization domains are slight (Figure 6), indicating that the interaction between PEST sequence and NF-κB N-terminal domains is a weak one. Comparison of Figures 6B and 6C indicates that the greater contribution to the interaction is made by p65. This result is consistent with the relative binding affinities reported for binding of IκBα(67–287) to p50(248–376)/p65(19–325) (0.039 nM) versus p50(248–350)/p65(190–321) (0.32 nM).³⁰ The dynamic nature of the interaction is quite consistent with previously-reported binding¹⁷ and cross-linking¹⁶ results.

The binding preference for p65 might be explained by the spatial position and orientation of the PEST tail in the complex. Although the NMR relaxation measurements show that the C-terminus of IκBα is not well-structured even in the largest complex, the hydrogen bond between the side chain of W258 and the backbone carbonyl of Q278 imparts a significant rigidity to the polypeptide in this region, and the backbone of residue Q278 (identified as L277 in 1NFI) is relatively well defined in the X-ray structures. This rigidity limits the conformational space sampled by the C-terminal tail of IκBα to the vicinity of the p65 N-terminal domain. Without breaking the hydrogen bond, the region including the critical acidic side chains E284, D285 and E286 is not free to extend towards the p50 N-terminal domain. It should be noted that there is no direct information on the location of the p50 N-terminal domain, since it is absent from both X-ray structures. Our inference from the increase in NMR linewidth in Figure 6D is that the p50 N-terminal domain is not well docked onto the remainder of the complex, so the preference of the IκBα PEST sequence for the DNA binding sequence of the p65 N-terminal domain might simply be that the distance to the analogous region of p50 is too great.

We recognize that, although IκBα is a general inhibitor for NF-κBs, the IκBα inhibitory effect is probably more complex than the interpretation of results derived only for the p65/p50 heterodimer. All members of the NF-κB family contain a DNA-binding loop with a similar sequence, for instance, Rel B (RGMRFRYEC) and p52 (RGFFRYGC), but the RelB-p52 heterodimer is not effectively inhibited by the IκBα but rather by the atypical IκB domain present as the C-terminal domain of p100 (the precursor of p52).³¹

CONCLUSIONS

The presence of different orientations for the N-terminal domain of p65 in the two X-ray structures, together with our observations in solution, which confirm the flexibility of the C-terminus of IκBα after the hydrogen bonded residue Q278, argues against a strong and specific interaction between the PEST sequence and the p65 N-terminal domain. Neither do these observations support the idea that the interaction would be strong enough to promote an allosteric domain rearrangement of the type suggested by Huxford et al.¹³ to explain the location of the PEST sequence in the X-ray structures. Following the solution results obtained here, we suggest that the role of the PEST sequence in DNA dissociation from NF-κB by IκBα may involve direct electrostatic interactions with the basic DNA-binding loop, leading the dissociation of the p65 N-terminal domain from DNA.

MATERIALS AND METHODS

Expression and Purification of Proteins

Complexes of differentially labeled I κ B α with deuterated p50/p65 heterodimers were prepared by a streamlined method to obtain specific triple-component complexes.²⁰ Briefly, the p50 and p65 NF- κ B subunits were coexpressed in *E. coli*; the subunit with the lower expression yield incorporates a hexahistidine tag at the N-terminus. [²H]-labeled p50 and p65 components and [²H, ¹³C, ¹⁵N] or [²H, ¹⁵N]-labeled I κ B α are prepared in separate cultures. Cell lysates derived from the two cultures are mixed before purification. The high affinity ($K_d \sim 10^{-9}$) between I κ B α and the NF- κ B heterodimer promotes formation of a stable triple-component complex in the mixed lysate. Purification of the correct complex is achieved by passing the mixed cell lysate through a nickel affinity chromatography column followed by gel filtration purification. This procedure allows efficient and precise separation of excellent yields of intact, differentially labeled complexes.

Four vectors of coexpressing p50(248–350)/His-p65(190–321), p50(248–350)/His-p65(19–321), His-p50(39–350)/p65(190–321) and His-p50(39–350)/p65(19–321), were generated for the NF- κ B heterodimer. The p50(248–350) fragment includes the dimerization domain whereas p50(39–350) further includes the N-terminal DNA-binding domain. The p65(190–321) fragment includes the dimerization domain and C-terminal NLS peptide (residues 289–321) and p65(19–321) additionally contains the DNA-binding domain. The coexpression vector for His-p65(19–321)/p50(39–350) gives the largest complex in the previous study;²⁰ this dimer starts to aggregate at protein concentrations > 0.1 mM, probably due to the instability of the p50(39–350) subunit. The alternative construct incorporating the N-terminal hexahistidine tag on the N-terminus of p50(39–350) stabilized the p50 subunit and the p50/p65 heterodimer, and was therefore employed for preparation and characterization of the largest complex.

Proteins were expressed in *E. coli* strain BL21(DE3). Coexpression of uniformly-deuterated NF- κ B heterodimer was carried out in D₂O-adapted *E. coli* cells harboring one of the constructed coexpression vectors in M9 minimal medium in D₂O with 0.1 mg/ml carbenicillin at 37°C. NF- κ B showed dramatically reduced expression yield in D₂O medium, especially for the construct of His-p50(39–350)/p65(19–321), the largest complex. The medium was adjusted to increase yield by doubling the ingredients, including NH₄Cl, glucose and other salts, to allow the cell culture to grow to high density. Cultures were routinely grown to OD₆₀₀ ~ 1.2 and induced by 0.5 mM isopropyl-1-thio- β -D-thiogalactoside (IPTG). After an additional 24 hours of induction at 15°C, the cells grew to OD₆₀₀ of ~ 2.5 and were harvested by centrifugation (6,000 g). The yield was as high as two-fold compared to the culture in regular D₂O M9 medium. Expression of [²H, ¹³C, ¹⁵N]-labeled I κ B α (67–287) was carried out in regular M9 minimal medium in D₂O supplemented with ¹⁵NH₄Cl (0.5 g/L), ¹⁵NH₄SO₄ (1 g/L) and ²H, ¹³C-labeled glucose (2 g/L). The culture was induced at OD₆₀₀ of 0.6 by 0.2 mM IPTG and with additional 24 hours incubation at 15°C.

Protein complexes purified to homogeneity by the streamlined method outlined above were exchanged into NMR buffer (25 mM Tris pH 7.5/50 mM NaCl/1 mM EDTA/ 1 mM dithiothreitol (DTT) in 90% H₂O/10% D₂O). The purities of the final protein products were judged to be higher than 95 % by SDS-PAGE. The sample ²H enrichment is estimated to higher than 90% in I κ B α and 70 % in NF- κ B, adjudged by a 1D NMR spectrum. Sample concentrations of 0.3 ~ 0.5 mM were used throughout the study, except for the largest complex, I κ B α /His-p50(39–350)/p65(19–321), where 0.15 mM was used.

Mutants to probe sites of I κ B α binding included p50(248–350)/His-p65(19–321, R30A/R33A/R35A), p50(248–350)/His-p65(19–321, R33A/R35A) and p50(248–350)/His-p65(19–321,

R30A), where arginine residues in the DNA-binding interface of the p65 N-terminal domain were substituted with alanines. The mutants were introduced by using the Quickchange site-directed mutagenesis methodology with the properly designed mutagenic primers and the coexpression plasmid, p50(248–350)/His-p65(19–321). The solution behavior of the mutants is similar to the wild type protein; the same procedures were therefore adopted for preparing the complexes containing mutant proteins.

NMR experiments and structural characterization

Resonances of bound IκBα(67–275) were assigned using [²H, ¹³C, ¹⁵N]-IκBα(67–275) complexed with [²H]-p50(248–350)/[²H]-p65(190–321). NMR spectra were acquired at 25° C on a Bruker Avance800 equipped with cryoprobe. A TROSY-HNCA spectrum³² was acquired for the assignment, with data size = 2048 (t3) × 64 (t2) × 84 (t1) complex points, number of scans = 40 and 1.5s delay time between each scan.

To elucidate the structure of IκBα C-terminal PEST region, we performed 3D ¹⁵N-edited NOESY-TROSY spectra^{33,34} for [²H, ¹⁵N]-IκBα(67–287) in complex with [²H]-p50(248–350)/p65(190–321); the sample contained 0.5 mM protein in NMR buffer. The spectra were acquired with 32 scans per FID, 2048 (t3) × 64 (t2) × 144 (t1) complex points, 1.5 s delay time between each scan, and mixing times of 100 ms and 200 ms on the Bruker DRX600 and Avance900 spectrometers, respectively. Data were processed using NMRpipe³⁵ and analyzed using NMRView.³⁶ Backbone NH-NH NOE cross-peaks were assigned manually based on the backbone ¹⁵N, NH chemical shifts reported previously.²⁰

Backbone dihedral angles of IκBα residues G270 to E282 were predicted by the program TALOS²⁶ by analysis of ¹³C_α, ¹³C_β, ¹³CO and ¹⁵N chemical shifts, derived from the backbone TROSY triple-resonance experiments, including HNCA, HN(CO)CA, HNCACB, HN(CO)CACB and HNCO.³² The ¹H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm and the ¹⁵N and ¹³C chemical shifts were referenced using consensus ratios of 0.101329118 for ¹⁵N/¹H and 0.251449530 for ¹³C/¹H.³⁷ The ¹³C chemical shifts were further corrected for isotope effects.^{38,39} Only the TALOS predictions that are identified as “good” were included in the comparison.

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Figure 1.
Schematic representation of the domain boundaries of the NF- κ B and I κ B α constructs used in this study.

Figure 2.

Comparison of IκBα structures in the p50-p65 complex. A. Overlay of the two structures of IκBα (colored ribbons) from the two published X-ray structures (PDB 1NFI¹⁴ and 1IKN¹³). The NF-κB dimerization domain, p50 fragment (residues 249–355) and p65 fragment (residues 190–320), derived from structure 1NFI is shown by the grey surface, and the backbone of the IκBα fragment (residues 70–282) from the same structure is represented by the magenta ribbon. The green ribbon shows the backbone of the IκBα (residues 73–292) from 1IKN superimposed on the equivalent region of the 1NFI structure. Structural differences near the C-terminal PEST sequence are indicated by two dotted boxes, which are enlarged in B and C. B. Structural difference in the side chain orientation of W258. A hydrogen bond (dashed line) is present in structure 1NFI between the side chain indole proton of W258 and the backbone carbonyl of L277. The hydrogen bond is absent in 1IKN due to flipping of the side chain orientation by 180°. C. One-residue frame shift of the backbone after G270 in 1NFI. The dotted circle indicates the position of the Q271 side chain in 1IKN; no residue is fitted in the corresponding position in 1NFI.

Figure 3.

NMR identification of C-terminal structure of bound IkBa. A. Strip plots in the HN-HN region of the ^{15}N -NOESY-TROSY-HSQC spectrum (100ms, 600 MHz, 25°C) of $[^2\text{H}, ^{15}\text{N}]$ -IkBa(67–287) in complex with $[^2\text{H}]$ -p50(248–350)/p65(190–321). The strips are taken at the ^{15}N frequencies of W258(εH) and NHs of residues 277–280. Long-distance NOE cross peaks between W258(εH) and other NHs are indicated by dashed lines and triangles. Diagonal peaks are denoted by *. B. Diagram showing the spatial relationship between the protons expected for the region represented by the NOEs in part A. Distances shown for the structures 1NFI and 1IKN are derived from the published crystal coordinates^{13,14} and for the hybrid structure derived from a combination of these two coordinate sets. Only the hybrid structure yields distances consistent with the NOE intensities shown in part A.

Figure 4. Backbone amide ^{15}N relaxation parameters for C-terminal PEST sequence of bound $\text{I}\kappa\text{B}\alpha$ at 30°C , measured at 600 MHz (expanded from the data of Sue et al.²⁰). A. R_1 , B. R_2 and C. $\{^1\text{H}\}^{15}\text{N}$ -NOE for residues 270–287. The triangles indicate residue Q278.

Figure 5.

Influence of C-terminal PEST sequence on ANK 6. A. Superposition of the ^1H - ^{15}N TROSY-HSQC of $[\text{}^2\text{H}, \text{}^{15}\text{N}, \text{}^{13}\text{C}]\text{-I}\kappa\text{B}\alpha(67\text{--}287)$ (blue) and $[\text{}^2\text{H}, \text{}^{15}\text{N}, \text{}^{13}\text{C}]\text{-I}\kappa\text{B}\alpha(67\text{--}275)$ (red) complexed with $[\text{}^2\text{H}]\text{-p50(248--350)/p65(190--321)}$. Backbone amide resonances with significant chemical shift differences are indicated by arrows and labels. The spectra were taken at 25°C and 800 MHz. B. Variation of the composite backbone NH and ^{15}N chemical shift differences obtained from the spectra in part A, between bound $\text{I}\kappa\text{B}\alpha(67\text{--}275)$ and bound $\text{I}\kappa\text{B}\alpha(67\text{--}287)$, $\Delta\delta_{(\text{N}+\text{NH})} = [\Delta\text{N}^2/25 + \delta_{\text{NH}}^2/2]^{1/2}$, where δ_{N} and δ_{NH} represent the chemical shift differences for ^{15}N and NH. The residues with the largest local differences are labeled. C. Evaluation of secondary structure in ANK 6 and the C-terminal PEST sequence in bound $\text{I}\kappa\text{B}\alpha(67\text{--}275)$ (upper, red bars) and bound $\text{I}\kappa\text{B}\alpha(67\text{--}287)$ (lower, blue bars). The parameter $\Delta\delta_{(\text{C}\alpha)}$ was calculated from the difference between the experimental $^{13}\text{C}_{\alpha}$ chemical shift and the corresponding random coil values^{40,41} are plotted against residue number for residues 242–287. The secondary structure seen in the two X-ray structures^{13,14} is indicated at the top for comparison. The missing value of $\Delta\delta$ of T263 in bound $\text{I}\kappa\text{B}\alpha(67\text{--}275)$ is marked by asterisk. D. Spatial distribution of residues with significant $\Delta\delta_{(\text{N}+\text{NH})}$ values, indicated by colored balls: red ($\Delta\delta > 0.3$ ppm), orange ($0.3 > \Delta\delta > 0.2$ ppm) and yellow ($0.2 > \Delta\delta > 0.1$ ppm). Structure of ANK 5, ANK 6 and PEST sequence of the $\text{I}\kappa\text{B}\alpha/\text{I}\kappa\text{B}\beta$ hybrid structure are plotted. The triangle indicates the position of residue 275, the C-terminus of the truncated construct.

Figure 6. 900-MHz TROSY-HSQC spectra of $[^2\text{H}, ^{15}\text{N}]$ -IkB α (67–287) in complex with NF- κ B fragments. A. $[^2\text{H}]$ -p50(248–350)/p65(190–321), B. $[^2\text{H}]$ -p50(39–350)/p65(190–321), C. $[^2\text{H}]$ -p50(248–350)/p65(19–321), D. $[^2\text{H}]$ -p50(39–350)/p65(19–321). Schematic diagrams representing the complexes are shown at the top of the spectra. The red lines in parts B, C and D represent the superimposed outline of the cross peaks in part A for comparison.

Figure 7.

A. Variation of the composite backbone NH and ^{15}N chemical shift differences for the PEST sequence of I κ B α , obtained from the spectra in Figure 6. The effect of addition of the N-terminal domains of p50 (red bars, corresponding to differences between Figures 6B and 6A), p65 (yellow bars; Figures 6C and 6A) and both p50 and p65 (green bars; Figures 6D and 6A) is shown by the chemical shift differences $\Delta\delta_{\text{N+NH}} = [(\Delta\text{N}^2/25 + \delta_{\text{NH}}^2)/2]^{1/2}$, where δ_{N} and δ_{NH} represent the chemical shift differences for ^{15}N and NH. Orange bars for residues 285 and 286, and the blue bar for residue 283 show variant values obtained for some of the alternate cross peaks in Figure 6C and D. B. Variation of the composite backbone NH and ^{15}N chemical shift differences for the PEST sequence of I κ B α , obtained from the spectra in Figure 8, illustrating the effect of mutations in the DNA-binding loop of p65: wild-type (yellow and orange bars, identical to the corresponding data in part A), triple mutant (blue bars), double mutant (purple bars) and single mutant (pink bars). The brown bars for residues 285 and 286 show variant values obtained for some of the alternate cross peaks in Figure 8D.

Figure 8.

900-MHz TROSY-HSQC spectra for $[^2\text{H}, ^{15}\text{N}]$ -IkB α (67–287) in complex with NF- κ B mutants, substituting Arg residues to Ala in the p65 N-terminal DNA-binding domain. A. with wild-type $[^2\text{H}]$ -p50(248–350)/p65(19–321), B. with $[^2\text{H}]$ -p50(248–350)/p65(19–321, R30A/R33A/R35A), (C) $[^2\text{H}]$ -p50(248–350)/p65(19–321, R33A/R35A), (D) $[^2\text{H}]$ -p50(248–350)/p65(19–321, R30A). Schematic diagrams representing the complexes are shown at the top of the spectra. The red lines in parts B, C and D represent the superimposed outline of the cross peaks in part A for comparison.

Figure 9.

Ribbon diagram showing the backbone structure of the IκBα/NF-κB complex (PDB 1IKN¹³). Domains are colored green (IκBα, ANK 3 to residue 287), yellow (p50 C-terminal dimerization domain), orange (p65 C-terminal dimerization domain) and blue (p65 N-terminal DNA binding domain). Side chains of E284, D285, E286 in the PEST sequence and of R30, R33, R35 in the N-terminal DNA binding domain of p65 are represented by sticks colored green or blue according to the domain from which they are derived, with N and O colored blue and red respectively. The estimated distance between the two residue groups is labeled.

Table 1
Comparison of dihedral angles for the PEST sequence of IkBa

Residue	Dihedral Angles from 1IKN ¹		Dihedral Angles from 1NFI ²		Predicted Dihedral		Angles ³
	ϕ	ψ	ϕ	ψ	ϕ	ψ	
G270	-41.5	-57.0	-57.3	42.5	-	-	resembles
Q271	-46.9	-55.4	-133.9	38.2	-72 ± 12	-35 ± 13	11KN
L272	-66.8	-38.4	-126.8	153.5	-93 ± 22	-22 ± 30	11KN
T273	-66.9	139.2	-34.5	128.4	-	-	
L274	-53.0	139.8	-53.2	-30.8	-	-	
E275	-47.7	-39.9	-75.5	-5.6	-57 ± 8	-47 ± 9	11KN
N276	-101.4	27.6	-117.1	26.4	-62 ± 7	-36 ± 12	neither
L277	-132.3	20.5	-103.1	-52.2	-78 ± 16	-35 ± 12	1NFI
Q278	-105.8	-38.9	-92.5	162.0	-61 ± 9	-27 ± 7	11KN
M279	-92.6	163.3	-76.0	-158.7	-	-	
L280	-98.5	149.0	-131.2	167.7	-83 ± 20	139 ± 24	both
E282	-116.7	89.0	119±55	28±48	-	-	

¹ reference¹³

² reference¹⁴

³ from chemical shifts using TALOS²⁶

Table 2

Expected distances from the indole proton of W258 to various backbone NH

Distance (Å) to W258(εH)	1IKN	1NFI	Hybrid
L277(NH)	12.8	6.0	8.2
Q278(NH)	10.9	4.6	6.0
M279(NH)	9.3	3.8	5.0
L280(NH)	6.9	7.8	4.4