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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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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.

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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 Fig. 1) was the first discovered,[6] and is the most abundant form of NF-κB.[1,4]

NF-κB is regulated by association with IκB inhibitors, including IκBα, IκBβ and Iκbε.[3] In unstressed cells, NF-κB associates with IκB to form a stable complex, which is sequestered in the cytoplasm. The classic heterodimer p50/p65 is regulated predominantly by IκBα, [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] lipopolysaccharide,[9] and tumor necrosis factor,[10] the signal response domain at the N-terminus of IκBα is phosphorylated by activated IκB kinase (IKK),[3] which leads to ubiquitination of IκBα and its subsequent degradation by the 26 S proteasome.[4,11,12] NF-κB, released from the IκB complex, is translocated to the nucleus where it can activate gene transcription by binding to the κB DNA site. Since the gene coding for IκBα itself is one of the downstream genes regulated by NF-κB, expression of IκBα

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Abbreviations used: ANK, ankyrin; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TROSY, transverse relaxation optimized spectroscopy; HSQC, heteronuclear single quantum coherence.

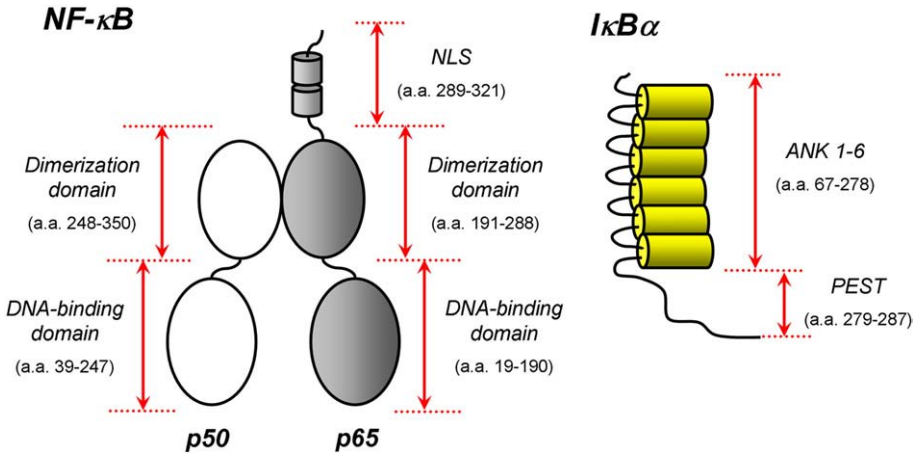

Fig. 1. A schematic representation of the domain boundaries of the NF-κB and IκBα constructs used in this study.

occurs also as a consequence of NF-κB activation,[7] and the newly synthesized inhibitor rebinds to NF-κB, inactivating its transcriptional activity by removing it from the DNA and mediating its export from the nucleus.[7]

Structural studies of the NF-κB/IκBα interaction include X-ray crystal structures of the NF-κB/IκBα complex, determined by two independent groups.[13,14] The IκBα structure in the complex (shown schematically in Fig. 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-κB to DNA.[16,17] The NF-κB protein characterized in the two structures consists of fragments of p65 (including the DNA-binding domain, the dimerization domain and the C-terminal nuclear localization signal peptide NLS) and p50 (dimerization domain only). IκBα and NF-κB 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 top of the p65 DNA-binding domain.[13,14]

More recently, additional information, obtained by NMR spectroscopy and mass spectrometry, about the structure in solution has become available for IκBα both free and bound to NF-κB.[18–20] A streamlined preparation method was adopted to prepare NF-κB/IκBα complexes that are precisely and specifically labeled, with IκBα labeled with ^{13}C, ^{2}H, and ^{15}N and p50 and p65 uniformly deuterated.[20] Combining a fragment-based assignment strategy, complete backbone resonance assignments were made for IκBα (residues 67–287, the minimal fragment required for dissociating NF-κB from DNA[16,17]) in complex with NF-κB (both dimerization and DNA-binding domains of both p65 and p50[20]). The molecular mass of this complex is 94 kDa. Free IκBα 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-κB,[18,20,21] and the center of the molecule, principally ANK3, becomes more flexible in the complex than in the free state.[20]

Although IκBα-mediated down-regulation of the transcriptional activity of NF-κB has been known for many years, the mechanism by which nuclear IκBα competes with the κB DNA sequence for NF-κB binding, thus turning off the transcription of stress-related genes, remains unknown. On the one hand, biochemical and mutagenesis studies implicate binding of IκB to a specific site, the DNA-binding loop on NF-κB.[22] This interaction was found to occur through the PEST motif of IκBα.[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 IκBα and the N-terminal DNA-binding domain of NF-κB p65: the PEST sequence appears to be located too far from the DNA-binding loop on NF-κB 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, its effect might be to lower the affinity of NF-κB for DNA by altering its conformation to an inactive form.[13,16,24] Following our successful characterization of the NF-κB – IκBα system in solution by NMR,[20] we report the elucidation of the interactions of the PEST sequence of IκBα in solution, in complex with NF-kB constructs containing various domains of p50 and p65.

Results

Structural analysis of the IκBα C-terminus

In order to dissect the role of the PEST sequence in the complex between IκBα and NF-κB, we made a detailed examination of the two X-ray structures of this complex, published by two independent groups (PDB 1NFI[14] and 1IKN[13]). 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 IκBα