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Transfer of Flexibility between Ankyrin Repeats in IκBα upon Formation of the NF-κB Complex

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The mechanism of inhibition of the transcriptional activator nuclear factor κB (NF-κB) by the inhibitor IκBα is central to the understanding of the control of transcriptional activity via this widely employed pathway. Previous studies suggested that IκBα, a modular protein with an NF-κB binding domain consisting of six ankyrin repeat domains (ANKs), shows differential flexibility, with ANK 1–4 apparently more rigid in solution in the absence of NF-κB than ANK 5 and 6. Here we report NMR studies that confirm the enhanced flexibility of ANK 5 and 6 in free IκBα. Upon binding of NF-κB, ANK 5 and 6 become well structured and rigid, but, somewhat surprisingly, other domains of the IκBα, which were relatively rigid in the free protein, become significantly more flexible. Due to the high molecular masses of the component proteins and the complexes, we employ a hierarchical experimental plan to maximize the available information on local flexibility in the ankyrin repeat domains. Backbone resonances of the 221-residue IκBα protein were assigned firstly in a smaller construct consisting of ankyrin repeats 1–4. These assignments could be readily transferred to the spectra of the construct containing six repeats, both free and complexed with various combinations of the NF-κB p50 and p65 domains. Transverse relaxation optimized spectroscopy-type NMR experiments on differentially labeled proteins enabled information on backbone structure and dynamics to be obtained, even in complexes with molecular masses approaching 100 kDa. Changes in the flexibility and stability of the various ankyrin repeat domains of IκBα complex formation take a variety of forms depending on the position of the domain in the complex, providing a variety of examples of the structural and functional utility of intrinsically unstructured or partly folded protein domains.

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Introduction

The nuclear factor κB (NF-κB) is the prototype of a family of dimeric eukaryotic transcription factors that is primarily responsible for mediating the expression of many genes by binding to upstream κB DNA enhancers.[1,2] In addition, NF-κB responds to many environmental stimuli, such as bacterial toxins, viruses/viral products and apoptotic/necrotic factors. The mammalian NF-κB family contains five members, p50 (p105), p52 (p100), p65 (RelA), RelB and c-Rel.[3,4] The heterodimer of p65 and p50 was the earliest form discovered[5] and represents the most abundant and best-known form of NF-κB.[6]

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Abbreviations used: NF-κB, nuclear factor κB; ANK, ankyrin repeat domain; NLS, nuclear localization signal; PEST, amino acid composition motif rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues; MS, mass spectrometry; HSQC, heteronuclear single quantum coherence; TROSY, transverse relaxation optimized spectroscopy; NOE, nuclear Overhauser enhancement; ps–ns, picosecond to nanosecond; PF, protection factor; EDTA, ethylenediaminetetraacetic acid.

In resting cells, NF-κB is largely sequestered in the cytoplasm in a complex with specific inhibitory proteins of the IκB family, notably IκBα. Inhibition and restriction of NF-κB to the cytoplasm appear to occur through sequestration of nuclear localization signal (NLS) sequences in NF-κB by tight association with IκBα.[6] but IκBα also appears capable of inhibiting NF-κB by binding to it at the DNA binding site.[7]

The p50 and p65 subunits of NF-κB each form two immunoglobulin (Ig)-like domains that together constitute the Rel homology domain of each polypeptide. The N-terminal Ig domain of each subunit makes sequence-specific interactions with the κB DNA sequence, while the C-terminal Ig domains are involved in DNA backbone contacts, dimerization and binding to members of the IκB family.[8] X-ray crystal structures of a number of complexes[9–11] indicate that the NLS peptide of p65 is masked in the IκBα complex, but the NLS of p50 remains accessible.[12] In addition, there are accessible nuclear export signals in IκBα[13] and p65,[14] resulting in a dynamic shuttling of the NF-κB–IκBα complex between nucleus and cytoplasm, with a preponderance in the cytoplasm.[12] When IκBα is degraded following an external signal or cellular stress, the balance between nuclear export and import is disturbed: the p65 NLS is now accessible and the IκBα nuclear export signal has been removed, giving rise to a net increase in the population of NF-κB in the nucleus.[15]

The structure of IκBα in complex with the p50/p65 heterodimer of NF-κB[9,10] includes six ankyrin repeats and a C-terminal PEST sequence. The ANK structural repeat is one of most common structural motifs found in proteins[16] and consists of a β-hairpin followed by two antiparallel α-helices. The PEST is an amino acid composition motif rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues; it has been reported to be related to protein degradation.[17,18] IκBα and NF-κB form an extensive noncontiguous binding surface in which ANK 1–2 contacts the p65 C-terminal NLS of NF-κB and ANK 4–6 is closely associated with the dimerization domains of NF-κB p50 and p65, denoted p50(248–350) and p65 (190–289), respectively.

X-ray crystal structures necessarily give a static picture of the inhibitory process mediated by complex formation among proteins. Although IκBα is well structured in the two crystal structures,[9,10] no crystals have been obtained for free IκBα, suggesting that there may be structural and/or dynamic differences in the free protein. Biophysical studies[19] showed that the ankyrin repeat domains of IκBα are not all well folded in the absence of NF-κB: in the free protein, the third repeat is the most compact, with repeats 1, 5 and 6 the most solvent accessible, according to amide $^{1}H/^{2}H$ exchange experiments.[19] Binding of 1-anilino-8-naphthalene sulfonate and amide exchange indicated that the free protein has considerable molten globule character. An extensive thermodynamic study[20] suggested that parts of the component proteins may fold upon complex formation and this was confirmed by amide $^{1}H/^{2}H$ exchange mass spectrometry (MS) experiments comparing the exchange of free IκBα to NF-κB-bound IκBα.[21] In order to elucidate sites of coupled folding and binding as well as to evaluate the role of IκBα flexibility in its function, we have undertaken an NMR dynamics study of IκBα and several of its complexes with NF-κB domains. Since these complexes are large and in some cases consist of several different polypeptide chains, we have evolved a strategy for labeling and overexpression of the components of each complex that enables site-specific structural and dynamic information to be obtained for IκBα in complexes of increasing size, up to and including the complex with full-length NF-κB.

Protein segmental disorder and flexibility has been recognized as a common occurrence in regulatory systems in the cell, including signaling, cell cycle, transcriptional and translational control.[22–25] A number of binding sites in regulatory molecules show intrinsic disorder and fold upon binding to the partner molecule, which may also have a degree of flexibility or disorder. This paradigm allows for great versatility in the binding interactions of regulatory proteins, bestowing the potential for a high level of selectivity arising from the relatively large contact surface, together with reversibility of the interaction arising from the modest affinity and, frequently, efficient regulation by proteolysis. Here we demonstrate that the significant changes that occur in the backbone flexibility of IκBα when it binds to NF-κB provide a complete program of reciprocal mobility changes that serve to allow the inhibitor to perform each of its functions with exquisite specificity.

Results

Design and differential labeling of IκBα and NF-κB constructs

IκBα consists of a 317-residue polypeptide containing phosphorylation sites in the N- and C-terminal sections (related to the degradation of the inhibitor), and six ankyrin (ANK) repeat domains that function as the binding site for NF-κB. The minimal fragment required for binding and dissociation of DNA-bound NF-κB contains residues K67 to E287 [IκBα (67–287)],[7,26] encompassing the entire six ANK repeats and the first seven residues of C-terminal PEST sequence (Fig. 1).

The p50 subunit used in this study contains the dimerization [p50(248–350)] and N-terminal DNA-binding [p50(39–247)] domains, whereas the p65 construct further contains a NLS sequence at C-terminus in addition to residues 19–321 of p65. A streamlined preparation of heterodimers of p50 (248–350)/p65(190–321) and p50(39–350)/p65 (19–321) exclusive of homodimer contamination is described in the Supplementary Material. Complexes