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Role of disorder in IκB-NFκB interaction

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Abstract

The paradigmatic transcription factors of the NFκB family provide an increasingly complex view of the mechanism of signal-mediated transcriptional activation. Although the primary event, phosphorylation and subsequent ubiquitin-dependent degradation of IκBα, the inhibitor of the canonical NFκB (p50/p65), is reasonably well understood, the means whereby the activation is turned off by post-induction repression is less well understood. Recent work highlighted in this review suggests that the inhibitor IκBα participates in the “stripping” of NFκB from the DNA, and that this process relies heavily on the disordered and weakly ordered segments of IκBα. Kinetic and equilibrium measurements in vitro as well as genetic screens in vivo convincingly demonstrate not only that IκBα greatly increases the dissociation rate of NFκB from DNA, but that further control of the process is mediated by the extremely short half-life of free IκBα, doubtless a result of the overall weakly folded nature of the free protein. These studies illustrate the versatility of protein systems that use not only well-structured proteins and protein complexes, but employ also the full range of available weakly structured and disordered states to maximize functional efficiency and metabolic control.

Introduction

The transcription factor NFκB, originally described as a nuclear protein in B lymphocytes that bound to κB target DNA sites (1), controls transcription of a large number of cellular activation events critical for development, proper control of cell growth and proliferation, the immune response, control of apoptosis and survival, and stress responses to a variety of noxious stimuli (2, 3). The NFκB network is critical for health, and aberrant NFκB activity plays a role in a variety of human cancers (e.g. Hodgkin’s and non-Hodgkin’s lymphoma) (4) and chronic inflammatory diseases (e.g. arthritis and Crohn’s disease) (5, 6). An understanding of the underlying molecular mechanisms of the control of signaling by NFκB may well lead to the design of modulators of chronic inflammation and better chemotherapeutic agents (5, 7).

In mammals, the NFκB family contains five homologous members: Rel (also known as c-Rel), RelA (also known as p65), RelB, p50 (encoded by the *nfκb1* gene) and p52 (encoded by the *nfκb2* gene). These proteins interact combinatorially to form a family of homo- and heterodimers that constitute the transcriptionally-active proteins. All five proteins have a Rel homology domain (RHD), which functions in dimerization and DNA binding (8). An amino acid sequence alignment of the RHDs of the NFκB family members is shown in Figure 1. The RHD has a nuclear-localization sequence (NLS) at its C-terminus. Transcriptional activation functions are mediated by C-terminal regions of the NFκB proteins outside the RHD (9).

Specific NF κ B inhibitor proteins, known as I κ Bs, control NF κ B signaling. I κ Bs have an ankyrin repeat domain (ARD) composed of six to seven ankyrin repeats (ARs), which binds to the RHD of p65 and masks the NLS. Defects in I κ B α have been implicated in a number of diseases, the most recent example being the demonstration that it is deleted in 25% of glioblastoma cancers (10). The ARD is a common protein-protein interaction motif that adopts an elongated fold in which the ankyrin repeats stack against each other in a linear fashion by folding into two antiparallel α -helices connected by a short loop, followed by a β -hairpin that protrudes away from the helical stack (Figure 2).

The homeostasis of NF κ B and I κ B in the cell allows for a rapid and specific response to external signals. A schematic view of the canonical pathway of signaling is shown in Figure 3. The resting cell contains NF κ B (commonly the heterodimer of p50 and p65) in complex with the inhibitor I κ B α in the cytoplasm. The activation of NF κ B has two aspects: signal-dependent phosphorylation and subsequent ubiquitin-dependent degradation of I κ B α by the proteasome, and targeting of NF κ B to the nucleus following release of the p65 NLS following degradation of I κ B α . Many aspects of these processes, as well as the I κ B α -mediated stripping of NF κ B from its cognate DNA site to turn off transcription after response to the signal, rely on changes in the folded states of all of these molecules. This conformational plasticity and its application in streamlining the vital functions of the NF κ B system is the subject of this review. Other recent comprehensive reviews target other aspects of this system (11, 12).

X-Ray Crystal Structures

The first insight into the structure of NF κ B was obtained by crystallography in 1995 (13), in a complex of the p50 homodimer with a DNA κ B site. This structure showed the canonical two immunoglobulin-like domains per p50 monomer, and identified the C-terminal dimerization domain and N-terminal DNA-binding domain. Structures of DNA-bound p50/p65 heterodimer (14) and p65 homodimer (15) showed that the pattern of DNA binding was similar no matter which members of the NF κ B family were present in the complex. The structure of the p65/p50 heterodimer DNA complex is shown in Figure 4A. The dimerization domains (dd) of p50 and p65 interact with each other, while the DNA-binding domains (DBD) make intimate interactions with the major groove of the DNA, mediated by the side chains of a number of conserved arginine residues.

Two independent structures of the complex between I κ B α and the p50/p65 heterodimer form of NF κ B were published in 1998 (16, 17). These two structures were remarkably similar in the protein constructs used and in the coordinates eventually obtained for most, but not all, of the protein components. Both groups were able to crystallize only a complex in which the DNA-binding domain of p50 had been truncated. Perhaps as a result of the absence of this domain, a significant difference was observed for NF κ B in the structures of the inhibitor complex compared to the DNA complexes: the DNA-binding domain of p65 was rotated to a more central position (Figure 4B, C). Differences between the two I κ B α structures included the definition of the NLS at the C-terminus of the p65 construct and the position of the PEST-containing sequence at the C-terminus of I κ B α . These structural variations are likely a consequence of disorder in the crystal and/or flexibility in the polypeptide chain, and are consistent with evidence, described in the following sections, for functional disorder and molecular motion in these complexes. Subsequent theoretical studies predicted that I κ Bs would fold upon binding to NF κ B (18).

Disorder in the p65 NLS Mediates Nuclear Localization

The nuclear localization sequence of p65 targets it to the nucleus. In the I κ B α -NF κ B complex, the p65 NLS is sequestered by formation of a two-helix structure bound to AR1-2

of I κ B α (17, 19). Upon stimulation, I κ B α is phosphorylated and ubiquitinated, and degraded, likely while still bound to the NF κ B (20), by the 26S proteasome (Figure 3). The DNA complex of NF κ B shows no density for the NLS in the X-ray crystal structure (14), which suggests that this region of p65 is disordered in the absence of I κ B α . Disorder in the NLS would allow it to take up alternative conformations upon binding to alternative partners such as importin α , which appears to require binding in an extended structure (21). A recent study by NMR and ITC showed that the p65 NLS is indeed disordered in solution and that it folds into a 2-helix structure upon binding to I κ B α (19).

Free I κ B α is Disordered – or is it?

An interesting feature of the crystallographic studies of NF κ B and I κ B α is how difficult these molecules are to crystallize, particularly in the free state, and even in complex with each other, since the p50 DBD must be removed before the complex can be crystallized. In order to obtain insights into the nature of free I κ B α , Croy et al. (22) performed a series of biophysical experiments on free I κ B α , including circular dichroism (CD) spectroscopy, hydrogen-deuterium exchange and fluorescence studies of the binding of 8-anilino-1-naphthalenesulfonic acid (ANS) to the protein. These results indicated that many of the ankyrin repeat (AR) modules in free I κ B α were incompletely folded, and that the protein showed the characteristics of a molten state: near-native secondary structure but significant increase in the fluorescence of ANS.

An extensive and systematic amide H/D exchange study (23) showed that ARs 2-4 are compact and sequestered from solvent, but ARs 1, 5 and 6 are solvent exposed, likely due to conformational flexibility. The amide exchange rates in ARs 5 and 6 in the complex with NF κ B indicated that these regions fold into compact domains upon binding to NF κ B. The results are illustrated in Figure 5.

The results shown in Figure 5 were obtained by analyzing the mass spectra of peptides obtained from the exchanged protein samples by enzymatic digestion. As can be seen from the prevalence of gray areas representing sequences where no data were available by this method, the results are incomplete. To obtain a more comprehensive site-specific picture of the nature of the free I κ B α structure, NMR spectroscopy was employed (24). The ^1H - ^{15}N HSQC spectrum of free I κ B α (Figure 6A) shows cross peaks with variable sizes, some that are well-resolved and quite sharp and others that are weak and broadened. This behavior is consistent with partial folding of the molecule, with part incompletely folded, likely containing a molten globule-like state. By contrast, the HSQC spectrum of I κ B α when it is bound to NF κ B (Figure 6B) shows uniform, sharp cross peaks characteristic of a protein that is well folded throughout. Resonance assignments for the free protein could readily be made for the AR1-4, but the broadened signals for ARs 5 and 6 made the task extremely difficult.

The reason for the lower proportion of stable folded structure in ARs 5 and 6 is not immediately apparent from a comparison of the amino acid sequences of the I κ B α ankyrin repeats (Figure 7), although these two repeats deviate the most from the consensus sequence shown in Figure 7. In order to test the hypothesis that the amino acid sequences were responsible for the lowered stability of ARs 5 and 6, Ferreiro et al. (25) designed a system to report on the stability towards chemical denaturation of free I κ B α and mutant forms engineered to more closely resemble the consensus sequence in ARs 4 and 5. Mutation of C186 and A220 to P resulted in a protein that was considerably more stable to denaturation; it also showed increased protection in H/D exchange experiments and a somewhat less broadened NMR spectrum, indicating that the molten globule nature of ARs 5 and 6 had been stabilized by the mutations to better approximate a well-folded structure in the free state. Subsequently, Truhlar et al. focused on stabilizing β -hairpins of AR5 and 6, which

were observed to be among the least stable parts of free I κ B α (26). Mutations were therefore designed to stabilize these regions. It was found that the addition of two stabilizing mutations in the β -hairpin of AR6 enhances the cooperative folding of free I κ B α , resulting in marked amide protection of AR5 and 6 to a degree comparable to that seen in the NF κ B complex (26).

The C-terminus of I κ B α is disordered; indeed, its amino acid sequence resembles the cellular interaction motif called PEST after its content of Pro, Glu, Ser and Thr residues. The tight binding between I κ B α and NF κ B (p50/p65) is at least partly mediated by the presence of the PEST domain in I κ B α : truncation of even a few residues from this region resulted in significantly weaker binding to NF κ B (27). Similarly, the C-terminal nuclear localization sequence (NLS) of NF κ B p65 is disordered in the free dimeric protein, but becomes ordered upon interaction with I κ B α , forming a pair of helices that interact primarily with AR1 (17). The presence at either end of the interface of the two regions where binding-induced folding occurs in the I κ B α /NF κ B interaction was interpreted as a “squeeze” mechanism that increased the affinity of the complex by stabilizing the structure of the ankyrin repeats (27). In describing what is clearly a very similar observation, this time obtained from NMR experiments, changes in flexibility in the ARs of I κ B α were inferred from the presence or absence of cross peaks in ^1H - ^{15}N HSQC spectra. Many cross peaks corresponding to AR5 and 6 were absent in the spectra of free I κ B α , but reappeared in the spectrum upon complexation with NF κ B (Figure 6). Further analysis of the spectrum revealed that cross peaks corresponding to AR3, which were present in the spectrum of free I κ B α , were missing from the spectrum of the complex, suggesting that the area of flexibility had migrated from AR5 and 6 to AR3 upon complex formation (24). It was suggested that such a mechanism might provide compensation for entropy loss as the disordered areas in the two molecules were ordered upon complex formation. It is also perhaps intuitively obvious that the center of the I κ B α molecule should be the most flexible in the complex, since the two ends of the interface, the sites of the interaction “hot spots” (27), are the sites of the most intimate interactions. Recent single-molecule FRET experiments (28) showed that free I κ B α populates a disordered state that contains a higher proportion of extended structure than that observed in the X-ray crystal structures of the NF κ B complex (16, 17). Fluctuations between collapsed and extended states occur on a millisecond time scale according to extended real-time SM-FRET observations (28). This time scale is consistent with the observation of relatively broad resonances in the NMR experiments on free I κ B α (24).

Disorder in I κ B α is Necessary for Function

It is clear from all of the solution studies on I κ B α (and by implication, the crystal studies as well) that areas of the molecule are disordered. This disorder takes the form of a complete lack of folded structure only in the PEST sequence. Disorder in AR5 and 6 is most likely due to the presence of molten globule-like forms that exist in a state where multiple conformers with different structures interchange on a timescale of milliseconds. For NMR spectroscopy, such a time scale is intermediate between “fast exchange” where peaks are broadened and appear at chemical shifts intermediate between those of the component forms, and “slow exchange” where duplicate peaks are observed for a resonance corresponding to two different chemical environments. A state of intermediate exchange is a severe disadvantage for NMR, since the resonances broaden and disappear, although information on the locations of such exchange processes can be inferred from the absence of particular peaks (24).

The mutations that stabilized the β -hairpins of AR5 and 6 resulted in marked stabilization of AR5 and AR6 in free I κ B α (26). These consensus mutations, Y254L, T257A in the β -

hairpin of AR5 and 6 enhanced the cooperative folding of free I κ B α , resulting in the stabilization of AR5 and 6 to a degree comparable to that seen in the NF κ B complex (26). The Y254L, T257A stabilized mutant showed an increased half-life in cells, providing strong evidence that the weakly-folded nature of AR5 and 6 is directly related to the rapid ubiquitin-independent degradation of I κ B α (8, 11).

I κ B α Functions in Removing NF κ B from DNA

One of the most distinctive features of the NF κ B signaling pathway is the rapidly deployed negative feedback loop that results in post-induction repression. This phenomenon results from the strong activation of the I κ B α gene by NF κ B. Newly synthesized I κ B α protein enters the nucleus and successfully competes with DNA for the NF κ B. The newly-formed I κ B α -NF κ B complex is transported to the cytoplasm, where it resumes its status in the resting cell ready for future activation. Early work suggested that I κ B α may be responsible for active dissociation of NF κ B from DNA (29), but equilibrium measurements alone (2) could not distinguish an active dissociation mechanism from mere competition resulting from the greater affinity of the I κ B α for NF κ B (30). Recent studies have shown that the I κ B α indeed plays an active dissociating NF κ B from DNA by a kinetic mechanism, enhancing the dissociation rate (31). Interestingly, the rate enhancement depended both on the presence of the disordered PEST domain and weak structure in the C-terminal ankyrin repeats (AR5 and 6) (31).

Observation of ternary complex

If the “stripping” function of I κ B α is primarily a result of differences in the equilibrium dissociation/association constants between the I κ B α -NF κ B and DNA-NF κ B complexes, we would expect that even the stronger I κ B α -NF κ B complex could be competed out by the addition of excess DNA. A test of the kinetic mechanism of stripping therefore involved an *in vitro* attempt to observe this competition. A sample of the I κ B α -NF κ B complex with the I κ B α portion differentially labeled for NMR spectroscopy was titrated with excess κ B DNA (32). If the DNA in excess was capable of removing the NF κ B from the I κ B α , the distinctive NMR resonances of the free I κ B α should be observed in the spectrum. Instead, several different observations were made. Addition of small amounts of DNA resulted in the observation of signals arising from the free PEST domain of I κ B α . That is, the DNA successfully competed for the region of NF κ B associated with the PEST. However, the remainder of the NMR signals remained typical of the NF κ B-bound form of I κ B α , becoming weaker and broader as more DNA was added. The distinctive NMR signals of the DNA were shifted and broadened, indicating that it was associated with a higher-molecular weight complex. These results suggested that the I κ B α remained bound to the NF κ B even in the presence of DNA, forming a loose ternary complex with the I κ B α PEST sequence dissociated from the complex. Since the signals of AR5 and 6 were among those that disappeared first, it is possible that this region of the I κ B α is also dissociated, forming the molten-globular structure seen in the free protein. Importantly, there is no evidence that AR1-4 dissociate from NF κ B in the presence of excess DNA. Stopped flow fluorescence measurements also show the presence of a ternary complex under these conditions (32).

The experimental conditions where the ternary complex was observed are never likely to be reproduced *in vivo*. Nevertheless, these observations can be used to envisage a molecular mechanism for the stripping process that takes into account the clear requirement for disorder and weak structure in the C-terminal region of I κ B α . In a reversal of the above process, the addition of I κ B α to the NF κ B-DNA complex results first in the association of AR1-4, possibly by capture of the disordered C-terminal NLS sequence of p53, resulting in the formation of a ternary complex. Now that the disordered AR5-6 and PEST are in

proximity to the NF κ B DNA-binding site, they are successful in peeling off the DNA and forming the tight NF κ B-I κ B α complex for export to the cytoplasm. These ideas are illustrated in Figure 8.

Perspectives and Conclusions

The studies described in this brief review illustrate that the machinery of NF κ B transcriptional activation is complex and requires the interaction of a number of components in unexpected ways, including the participation of disordered and weakly-structured regions that appear to play a critical role in the kinetic control of the post-induction repression process for NF κ B transcriptional activation. The lower structural stability of certain regions of I κ B α doubtless also plays a role in the regulation of the process by reducing the half-life of free I κ B α in cells by some 2000-fold compared to its stability in the NF κ B complex (33).

The role of disorder in the NF κ B cycle is becoming increasingly visible. The order-disorder transition of the p65 NLS (helical in the I κ B α complex, disordered when the I κ B is phosphorylated and degraded, allowing the NLS to interact with the importin proteins in the required extended conformation) precipitates the transport of NF κ B to the nucleus upon activation. It now appears that this region of the protein may also function in a “fly-casting” mechanism to engage newly-synthesized I κ B α and initiate the post-induction stripping of NF κ B from the DNA by I κ B α .

These observations illustrate the versatility of proteins, which utilize many different states for their functions: fully folded, partly folded and unfolded forms of the same protein all have important roles to play.

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p65 18 GPYVEIIEQPKQGRMRFRYKCEGRSAGSIPGERSTDTTKHTPIKINGYTGPCTVRIS--IVTKDPPHRPHELVGKIC-PDCTYAEICPPRCI-HSFONIGICVKKRDEQAISOR
 c-Rel 7 NPYIIEIIEQPKQGRMRFRYKCEGRSAGSIPGERSTNNRTYPSQIMNYYGCKVRIT--IVTKNDPYKPHPHLVGKIC-RDGYEAEERGQERRP-LFQNGIRCVKKKEVKELIIR
 p50 41 GPYIQIIEQPKQGRMRFRYKCEGRSAGSIPGERSTNNRTYPSQIMNYYGCKVRIT--IVTNGNIIHHAHSLVGKIC-PDGTCTVTAGPKDMV-VGEANGLHVTKKKVFETEAR
 p52 39 GPYIVIVEQPKQGRMRFRYKCEGRSAGSIPGERSTNNRTYPSQIMNYYGCKVRIT--IVTHSDPPRAHSLVGKICSEIGICAVSVGPKDMT-AQFNNGLHVTKKKMMGMTMIOR
 RelB 124 QPHIVITEQPKQGRMRFRYKCEGRSAGSIPGERSTNNRTYPSQIMNYYGCKVRIT--IVTKDPPHRVPHSLVGKIC-TDGICRVRPHVSPRHSFNNIGICVKKKEIEAAIERK

 p65 134 IQT-----NNNPFQVPIEEQ--RCIYDINAVRCEQVTVRPSG-RPIRPPVISHPIFDNRAPNTAEIKICRVNRNSGSGGDEIFLLCKVQ
 c-Rel 123 IKA-----GINPFNVPEKQINDIECDINVVRCEQVLPDEHGNLTALPPVVSNIYDNRAPNTAEIKICRVNKNCGSVRGDEIFLLCKVQ
 p50 157 MTEACIRGYNPGIVHPDIAVQAEGGGRCIGREKEIRQAAQOTKEMDLSVVRMFAFLPSTGSEFTRREPVVSDAIYDSKAPNASNKKIVRMRTAGCVTGGEIYLLCKVQ
 p52 154 IQRQRER-----SRPQGTAEQRETEQEAKEKKVMDLSIVRREFSAFIRASGSEFSLPKPVISQPIHDSKSPGASNKKISRMRTAGSVRGDEIYLLCKVQ
 RelB 243 IQG-----IDPYNAG--SKNHQEVDMNVVRICQASYSRQQG-QMRRMPPVISEPVYDKKSTNTSEIRICRINKESGPGTGGEIYLLCKVQ

 p65 221 KEDIEVYETCP---GWEARGSESQADVHRQVAIVERTPPYADESQAQPVVRVSMQRRPSDRESEPMEEFOYIPDIDRRHRIEKKRRTYETFKSIMKKSPFSGPTD
 c-Rel 213 KDDIEVRVFIN---DWEAKGIFSQADVHRQVAIVERTPPYCK-AITEPVTVMQRRPSDQEVSESMDFRYIPDEKDTYGNRAKQKTTLLFQKLCQHHVETGERH
 p50 277 KDDIQIRFYEEENGWVGEGDFSPDVHRQVAIVERTPPYKYNITKPAVSVFQRRKSDSETSEPKPFLYYPEIKDKEEVQRKQKMPNFSSEGGSGAGAGG
 p52 255 KDDIEVRFYEDDENG--WQARGDFSPDVHRQVAIVERTPPYHKMKIERPVTVFQRRKRGQVSDSKQFTYYPIVEDKKEEVQRKRRKALPTFSQPFSGGSHMGGGS
 RelB 330 KEDISVVSRSAS---WEGRADFSQADVHRQVAIVERTPPYEDIEIVEPVTNVVLRQRTIGVCSEPIPETYIPRHHHSYGVDRKKRRGMPPVGEINSSPHGLIES

Figure 1.

Amino acid sequence alignment of the Rel homology domains of the 5 monomeric forms that combine as homo- or heterodimers of NF κ B.

Figure 2.
Ribbon structure of the backbone of the Notch ankyrin repeat domain (34) showing the secondary structure elements for a single repeat (red).

Figure 3.

Schematic diagram showing the cycle of signal-activated NFκB transcriptional activation. At rest in the cytoplasm, NFκB (shown as its common isoform, the heterodimer of p50 and p65) remains in a tight complex with the inhibitor (in this case, IκBα), which contains 6 ankyrin repeats. Upon receipt of an external signal, the bound IκB is phosphorylated and subsequently ubiquitinated and targeted for degradation by the 26S proteasome. The freed NFκB is translocated to the nucleus where it binds to the κB DNA site and initiates transcription of downstream genes, which include that for IκBα. Newly synthesized IκBα, localized to the nucleus, strips NFκB from the DNA and returns it to the cytoplasm and the resting state.

Figure 4.

X-ray crystal structures of NFκB in complex with DNA and with IκBα. A. Complex of the heterodimer of p50 (green) and p65 (red) with double-stranded DNA (pink, yellow) (structure 1VKX (14)). B. Complex of the heterodimer of p50 (green) and p65 (red) with IκBα, with ankyrin repeats 1–6 colored as indicated on the right (structure 1NFI (17)). C. Complex of the heterodimer of p50 (green) and p65 (red) with IκBα (gray) (structure 1IKN (16)). Figures were generated using MolMol (35).

Figure 5.

Ankyrin repeat domains of $I\kappa B\alpha$ colored according to the rate of exchange of backbone amide protons (23), according to the scale at the bottom of the figure. Gray indicates areas where no data were available. Both of the structures shown were derived from the X-ray crystal structure of the NF κ B complex (1NFI (17)).

Figure 6. ^1H - ^{15}N HSQC spectra of I κ B α AR1-6 (residues 67–287) A. free. B. in complex with fully-deuterated heterodimer of p50/p65 (24).

Figure 7.

Amino acid sequence alignment of the six ankyrin repeats of IκBα showing the consensus sequence and the sequence variation of AR6.

Figure 8.

A. Schematic representation of the model for the formation of the ternary complex between NFκB, IκBα and DNA (32). B. Schematic representation of the mechanism for stripping of NFκB from DNA by IκBα.