Exploration of IκB:NFκB binding through Hydrogen Deuterium Mass Spectrometry

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ABSTRACT

NFκB is a pleiotropic family of transcription factors that is often over-expressed in cancerous cells. The study of the binding of NFκB dimers and their inhibitor proteins, IκB, may aid in the understanding the precise role of this over-expression in cancer cells. In the study presented we purified NFκB p50/p65 heterodimer, p65/p65 homodimer, IκBα, and IκBβ to homogeneity and subsequently complexed the IκBs to each of the NFκB dimers. To observe the effects of inhibitor binding to NFκB, the purified proteins were analyzed using Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS), both in their free and bound states in order to investigate the role of IκB binding to the dynamics of NFκB dimers. Preliminary results, presented here, illustrate the role of IκBα binding in altering the dynamics of the NFκB p50/p65 heterodimer. It has been hypothesized that IκB binding to NFκB acts as a chaperone and therefore induces global stabilization of the NFκB proteins. HDX-MS experiments presented here support this postulate by illustrating a decrease in dynamics throughout the entire p50 and p65 proteins when in complex with IκBα.

BACKGROUND

In mammals, the NFκB family consists of five members, RelA (p65), RelB, cRel, p50 and p52, which interact to form distinct homo- and heterodimers. In resting cells, most of the estimated 100,000 NFκB dimers are predominantly cytoplasmic, due to their interaction with the inhibitors of NFκB (IκBs), and therefore remain transcriptionally inactive[1-3]. The IκBs (IκBα, IκBβ, IκBε) effectively sequester the NFκB pool in the cytoplasm by inhibiting both the NFκB nuclear localization sequence and its association with DNA. Activation of most forms of NFκB depends upon phosphorylation-induced ubiquitination of the IκB proteins which are subsequently degraded by the 26S proteasome. Liberated NFκB dimers then translocate to the nucleus where they bind to DNA and participate in transcriptional activation of target genes (Figure 1).

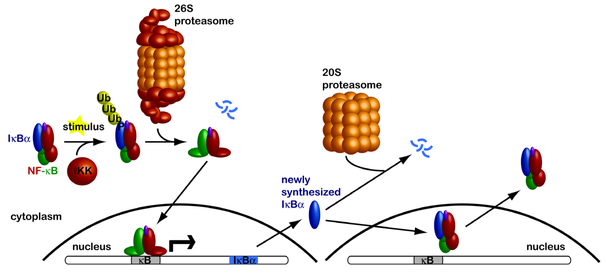


Figure 1. Schematic diagram of the NFκB signaling pathway.

While the NFκB family consists of five members that form distinct homo- and heterodimers, the way in which cells determine the latent pool of NFκB isoforms present in the cytoplasm, and the way in which these distinct isoforms are preferentially activated, has yet to be elucidated. Collaborative research efforts in the Komives, Ghosh and Hoffmann labs has led to the development of the "chaperone hypothesis" which proposes that IκBs serve as chaperones, preferentially stabilizing specific NFκB homo- or heterodimers in the cytoplasm. This effectively determines the latent pool of NFκB isoforms available for activation in resting cells. Upon pathway activation, genes responsive to those specific NFκB isoforms are preferentially activated.

Recent preliminary data collected while investigating the "chaperone hypothesis," suggested that the NFκB RelA/p50 heterodimer had a dissociation constant (K_D) of 500pM while the NFκB RelA/RelA homodimer had a K_D of 40nM. This discrepancy in binding affinity suggests that the RelA subunit of NFκB may play a large role in determining the NFκB:IκB interaction. To investigate this possibility, both homodimeric RelA/RelA NFκB and heterodimeric RelA/p50 NFκB were purified along with IκBα and IκBβ constructs. Purified proteins were then used to perform Hydrogen Deuterium Exchange experiments in an effort to determine the role of the RelA subunit in both NFκB dimer interaction and NFκB:IκB interactions.

PROTEIN PURIFICATION

RelA/p50 NFκB

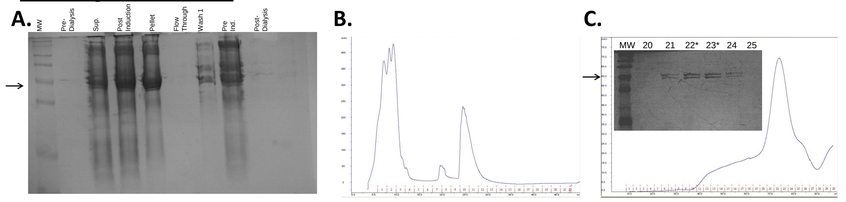


Figure 2. Purification of His-p50/RelA NFκB. A. A full-length NFκB RelA/p50 heterodimer construct (with a hexahistidine tag at the N terminus of the p50 subunit) was expressed in an *E. coli* system. The protein was purified using a Ni-NTA affinity purification protocol. The protein was eluted off of Ni-NTA resin with Imidazole (500mM). **B.** To remove bound DNA from the protein, NFκB RelA/p50 was passed through a MonoS ion-exchange column. **C.** Finally, free NFκB RelA/p50 was purified on a HiPrep 16/60 Sephacryl S200 gel filtration column. Purified protein seen in inset.

RelA/RelA NFκB

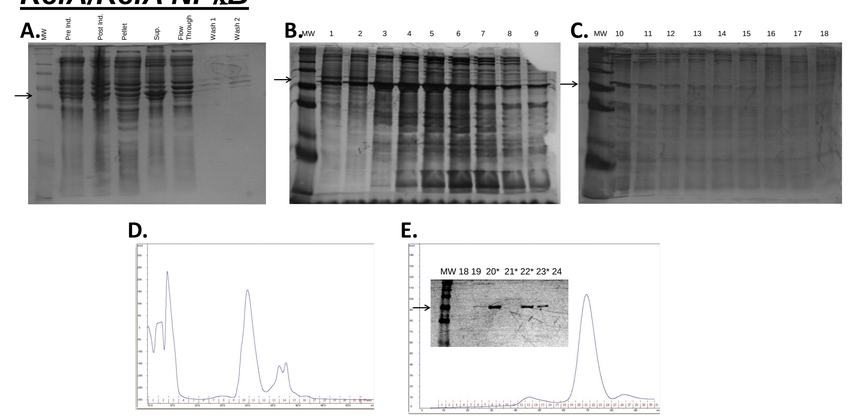


Figure 3. Purification of RelA/RelA NFκB. A. A full-length NFκB RelA/RelA homodimer construct was expressed in an *E. coli* system. **B-C.** To purify homodimeric RelA/RelA NFκB, cell lysate was passed over an SP Sepharose ion exchange chromatography system. The protein was eluted (10mL fractions) off of the SP Sepharose resin using an NaCl gradient (0mM-700mM). **D.** To remove bound DNA from the protein, NFκB RelA/RelA was passed through a MonoS ion-exchange column. **E.** Finally, free NFκB RelA/RelA was purified on a HiPrep 16/60 Sephacryl S200 gel filtration column. Purified protein seen in inset.

IκBα (67-287) and IκBβ (50-359)

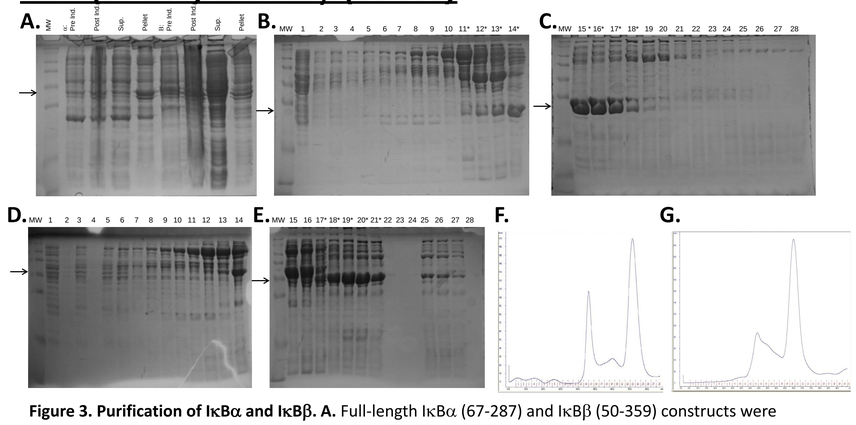
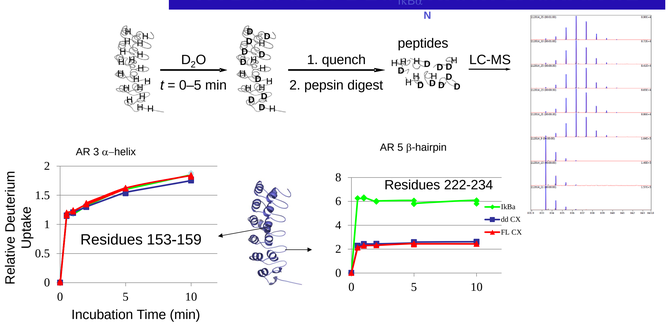
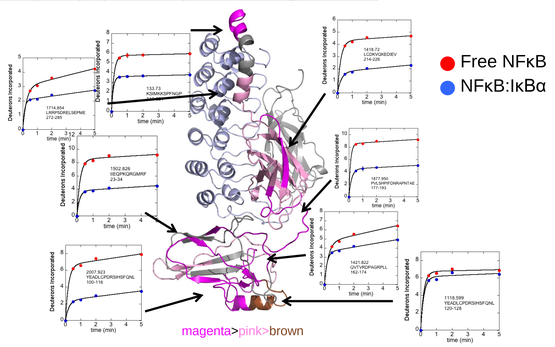


Figure 3. Purification of IκBα and IκBβ. A. Full-length IκBα (67-287) and IκBβ (50-359) constructs were expressed in *E. coli* systems. To purify **B-C.** IκBα (67-287) and **D-E.** IκBβ (50-359) constructs, cell lysates were passed over a HiLoad Q ion exchange chromatography system and eluted using an NaCl gradient (225mM-700mM). Final purification of **F.** IκBα and **G.** IκBβ constructs was done on a HiPrep 16/60 Sephacryl S200 gel filtration column.

HYDROGEN DEUTERIUM EXCHANGE

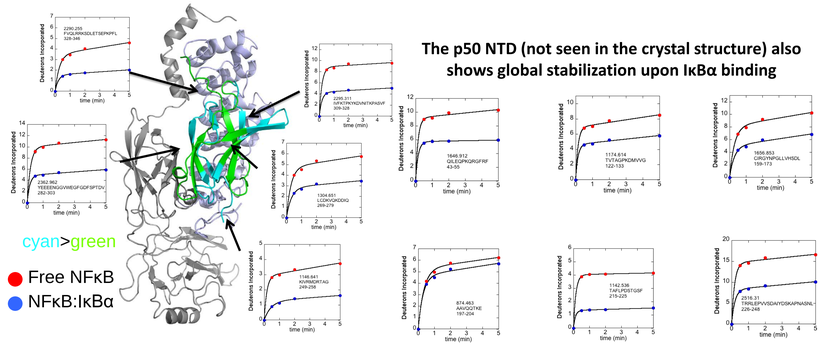


A. Protection of p65 peptides upon binding to IκBα



The overall trend of p65 protection upon IκBα binding shows protection to some extent in all domains of the protein, with the highest levels of protection being localized, for the most part, in the NTD with the exception of the linker region between the dimerization domain (dd) and NTD, a beta strand in the dd on the opposite side of the IκBα binding interface, and the very C-terminus, where the NLS is located.

B. Protection of p50 peptides upon binding to IκBα



In p50, we observed very high levels of protection in peptides throughout the protein indicating that this binding event induces significant stabilization of p50 not only at the dd which is known to directly interact with IκBα but also in the NTD which has, until this point, been postulated to have no role in the binding of IκBα.

FUTURE DIRECTIONS

The main goal of these studies is to investigate the role of RelA (p65) in the different observed affinities of IκBs for each RelA-containing dimer. Currently our lab is performing HDX-MS experiments on free RelA homodimers, RelA homodimers in complex with IκBα, and RelA homodimers in complex with IκBβ.

Further, investigations into IκBβ's role in regulating the NFκB pathway have been largely macroscopic in scale, and there is a paucity of research concerning the biophysics of its interactions with NFκBs. With the protein purified here, we plan to perform the same HDX-MS experiments with the RelA/p50 and RelA/RelA in complex with IκBβ.