Phosphorylation of IκBβ is required to mediate the dissociation of NFκB from DNA

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Nuclear factor κB (NFκB) is responsible for the regulation of genes implicated in tumor suppression, inflammatory response, development, and apoptosis. When NFκB fails to regulate gene expression, diseases such as cancer, chronic inflammation, and Alzheimer's result. NFκB is regulated by its inhibitor, IκB, one of which is IκBβ. Like its more prominent counterpart IκBα, IκBβ prevents gene expression by remaining bound to NFκB in the cytoplasm and masking its nuclear localization sequence (NLS), stopping NFκB from entering the nucleus. Unlike IκBα, however, not all IκBβ immediately masks the NLS when associating with NFκB. Thus, some IκBβ remains bound to NFκB in the nucleus, inhibiting IκBα's ability to bind to NFκB and prolonging gene expression. In this way, IκBβ both inhibits and activates gene expression.[1] Until recently, the mechanism by which IκBβ fulfils both these functions were unclear. *In vivo* work in the Sankar Ghosh lab at Columbia University has shown that phosphorylated IκBβ can regulate NFκB binding to DNA; however, hypophosphorylated IκBβ forms an NFκB:DNA:IκBβ complex insusceptible to regulation by IκBα. Here we present progress towards confirming that phosphorylation of IκBβ is required for IκBβ-mediated dissociation of NFκB from DNA. We have restored a mutated plasmid to express wild type IκBβ and introduced a phosphomimic mutation (S346D) via polymerase chain reaction (PCR). We have also recombinantly expressed and purified *Mus musculus* IκBβ and p65 NFκB and have laid the groundwork for kinetic assays to investigate the significance of the phosphorylation state of IκBβ in NFκB regulation.

IκBs regulate NFκB

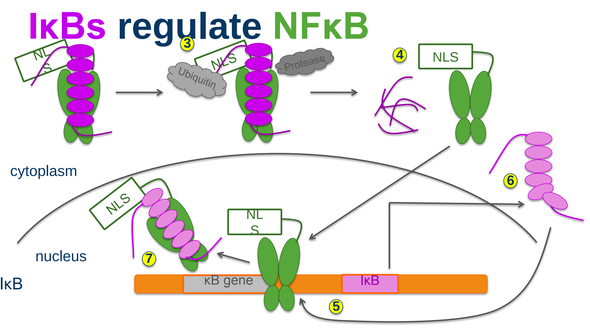
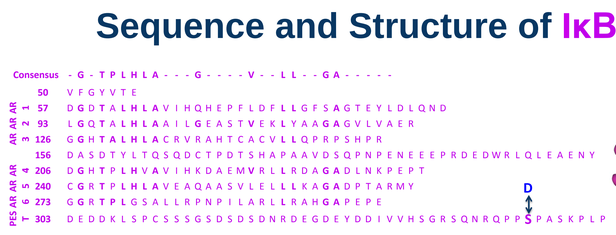


Figure 1. Canonical IκB's regulation of NFκB.

1. Reception of signal (not shown)
2. Transduction (not shown)
3. Response – 1) ubiquitin tags IκB 2) proteases degrade IκB
4. 1) IκB unmasks NFκB's nuclear localization sequence 2) NFκB enters nucleus
5. NFκB activates κB gene and downstream, IκB gene (self-regulation)
6. New IκB created
7. New IκB strips NFκB from DNA

Sequence and Structure of IκBβ



IκBβ is a six ankyrin repeat protein with a C-terminal PEST sequence and an intrinsically disordered linker region between AR3 and AR4. The IκBβ PEST sequence gets phosphorylated *in vivo* by a casein kinase II-like protein, thus increasing the negative charge on the PEST sequence.[2] We hypothesize that the negatively charged PEST sequence is crucial to IκBβ-mediated dissociation of NFκB from DNA by eletrostatically repelling DNA from the IκBβ:NFκB complex. By contrast, hypophosphorylated IκBβ is unable mediate dissociation. We plan to test this by introducing a phosphomimic mutation (S346).

Repairing plasmid

We performed PCR along a gradient of melting temperatures from 71°C to 81°C to repair a mutated version of the pET11a plasmid containing the IκBβ gene. We then ran an agarose gel to determine the temperatures at which the primer annealed most successfully and adjusted future gradients accordingly.

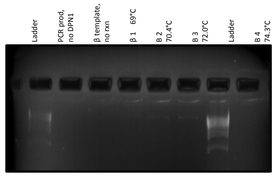


Figure 2. Agarose gel of melting temperature gradient. The subtle bands in the 3rd and 4th lanes indicate that temperatures 70.4°C-72.0°C were most successful.

We then transformed the repaired plasmid into DH5α *E. coli* cells, which produced more of this plasmid.

BEFORE AFTER

Protein expression and purification

Expression

We transformed BL21 *E. coli* with the pET11a plasmid encoding the genes for IκBβ and p65. We grew these cells to OD_{600} = 0.6 and induced protein expression with 0.1 mM IPTG to express IκBβ and NFκB.

Purification

We isolated both proteins from the cells grown by:

1) Centrifuging the cells at 4,000 rpm for 20 min
2) Resuspending the pellets in lysis buffer
3) Sonicating the cellular resuspension
4) Centrifuging the result of the sonication at 12,000 rpm for 45 min
5) Running various columns with the resulting cell lysate

Columns:

IκBβ = negatively charged (pI = 4.71)

HIGH LOAD Q (positively charged)

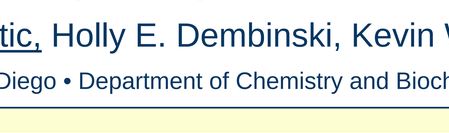
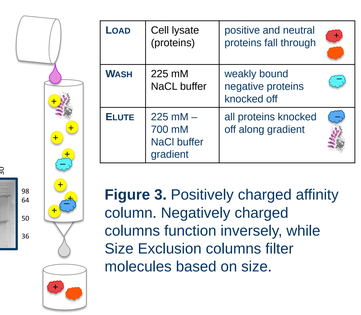


Figure 4. SDS-PAGE of IκBβ HLQ fractions.



LOAD	Cell lysate (proteins)	positive and neutral proteins fall through
WASH	225 mM NaCL buffer	weakly bound negative proteins knocked off
ELUTE	225 mM – 700 mM NaCl buffer gradient	all proteins knocked off along gradient

Figure 3. Positively charged affinity column. Negatively charged columns function inversely, while Size Exclusion columns filter molecules based on size.

S200 (size exclusion)

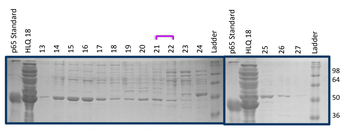


Figure 5. SDS-PAGE of IκBβ S200 fractions.

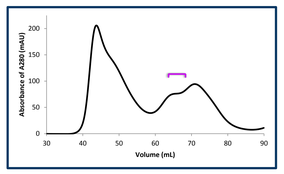


Figure 6. Chromatogram of IκBβ S200 fractions.

p65/p65 = positively charged

SP SEPHAROSE (negatively charged)

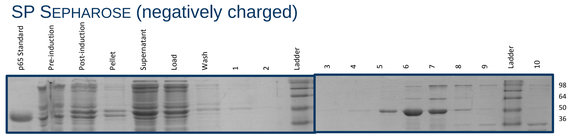


Figure 7. SDS-PAGE of p65/p65 SP Sepharose fractions.

MONOS (negatively charged)

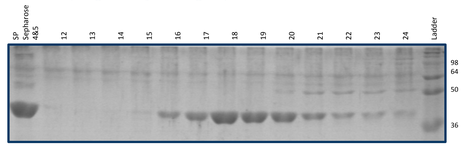


Figure 8. SDS-PAGE of p65/p65 MonoS fractions.

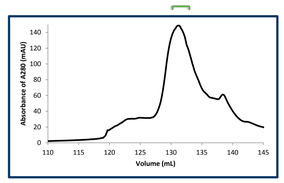


Figure 9. Chromatogram of p65/p65 MonoS fractions.

Protein identification

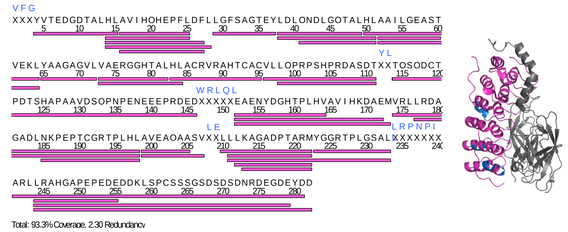
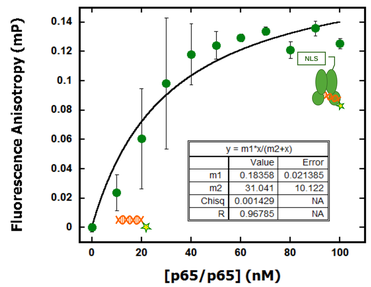


Figure 10. Peptide coverage of IκBβ collected on the Synapt G2S

We used a Synapt mass spectrometer to perform a peptide digestion in order to identify lanes 20 and 21 from the MonoS gel as IκBβ.

Fluorescence Anisotropy

Given a constant concentration of fluorescently labeled DNA (5 nM FITC DNA), as the concentration of p65/p65 increases, the tumbling speed of the molecules in solution decreases. This indicates binding between DNA and p65/p65.



DNA (no p65/p65)

Smaller molecule ⬇ Tumbles more quickly ⬇ Emits less polarized light

p65/p65:DNA

Larger complex ⬇ Tumbles more slowly ⬇ Emits more polarized light

Figure 11. Fluorescence polarization analysis of varying concentrations of p65/p65

Future Directions

Having purified nearly all the necessary proteins (save the S346D mutant IκBβ), we plan to begin kinetic assays using stopped flow fluorescence to measure the relative rates of wild type IκBβ-mediated dissociation and of S346 mutant IκBβ-mediated dissociation of NFκB from DNA. This will allow us to determine whether phosphorylation of IκBβ is crucial to IκBβ's functionality.

Acknowledgements

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References

1. S. Ghosh et al. IκBβ acts to both inhibit and activate gene expression at different stages in the inflammatory response *Nature* (2010).
2. K. Tran, D.T. Distinct Functional Properties of IκBα and IκBβ *Molecular and Cellular Biology* (1997).