Investigation of the role of L66 in the internal dynamics of TrkB

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Question of Interest

The activation of neuroreceptor TrkB is a key regulator of many neuronal health processes. Our goal is to understand the path of signal transmission through the protein by studying the intermolecular dynamics, as well as identify the residues that determine binding specificity. We focus specifically on domain 5 (d5), the extracellular domain containing the neurotrophin binding interface.

Background and Biological Significance

- Human neuroreceptor TrkB (tyrosine related kinase) interacts with specific neurotrophins to activate signaling pathways that maintain neuronal health and survival, as well as pathways promoting long term potentiation and memory formation [1].
- TrkB is a transmembrane neuronal protein. Extracellular neurotrophin binding elicits an intracellular autophosphorylation response and kinase cascade [2].
- Disturbances to these interactions have been implicated in neuronal diseases such as Parkinson's and Huntington's.
- A better understanding of the binding selectivity between TrkB and its binding partners is hoped to lead to improved drug therapies targeting this neuroreceptor.

Objectives

To understand the binding selectivity this protein displays, we have been using nuclear magnetic resonance (NMR) spectroscopy to perform dynamics experiments examining possible allosteric changes. Previous work has identified several residues of interest on which this project is focused (Fig. 1). Finding appropriate mutations of these residues is key in determining their importance.

Hypothesis

By mutating dynamically significant residues one at a time, we will better understand the importance of that residue to both the structure and internal movement of TrkB.

Approach

- Nuclear Magnetic Resonance spectroscopy is an ideal method to use in this study, as it is sensitive to the dynamics of individual residues.
- Our current goal was to use chemical shift perturbation experiments to evaluate the structural properties and stability of mutants compared to unmutated (wild type) TrkB-d5.
- Our long term project goal is to run R_2/R_1 experiments on the mutants, to observe changes in flexibility compared to wild type.

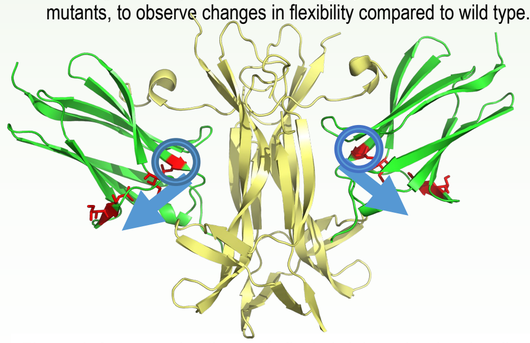


Figure 1. A proposed pathway of allosteric intramolecular signaling, plotted on the crystal structure of the neurotrophin NT4 dimer (yellow) bound on each side to domain five of human TrkB (hTrkB-d5, green). The residues of interest to this study are shown in red. Circled in blue are the L66 residues in the binding interface that were mutated in this project. Figure adapted from reference [3]

Materials and Methods

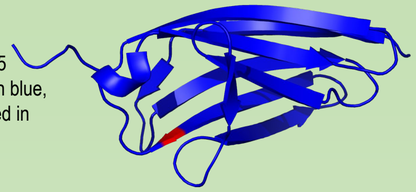


Figure 2. hTrkB-d5 monomer shown in blue, with L66 highlighted in red.
(PDB ID 1HCF)

All methods were per the protocol described in reference [3]. In brief:

Gene preparation

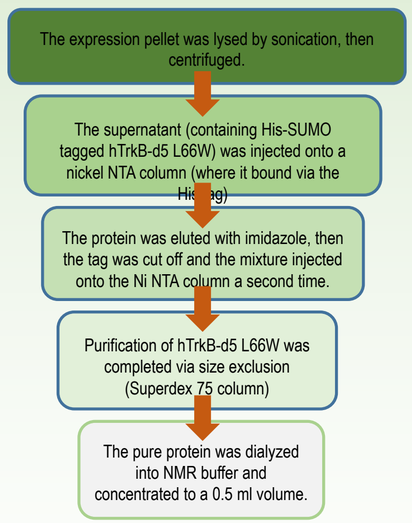
- Using site directed mutagenesis, the leucine at position 66 was exchanged for an alanine in the case of mutant L66A, or for a tryptophan in the case of mutant L66W. The gene for each mutant was inserted into the pET SUMO plasmid carrying a lac promoter, His-tag, and a kanamycin (KAN) resistance region.

Protein expression:

BL21 *E. coli* cells were transformed with the plasmid using electroporation and plated on LB/KAN plates. From these plates, a colony was picked to inoculate an overnight 50 mL LB/KAN growth. The cells were then harvested via centrifugation and resuspended in 1L of minimal media to control carbon and nitrogen sources.

- Once the culture reached the right density, the culture was induced with IPTG, promoting expression of TrkB. Growth continued for three hours, and the resulting mixture was pelleted.

Purification:



NMR:

- The samples were prepared using ^{15}N ammonium chloride as nutritive source to make the nitrogen nuclei of the proteins NMR visible.
- All experiments were run on an Agilent 600 MHz NMR spectrometer.
- ^{15}N-HSQC experiment:
  - correlates the chemical shift of a backbone amide hydrogen with its directly bound nitrogen.
  - results in a 2D spectrum (^1H and ^{15}N on the two axes).
  - Can provide detailed and high resolution structural information, with a probe at each amino acid.

Data and Results

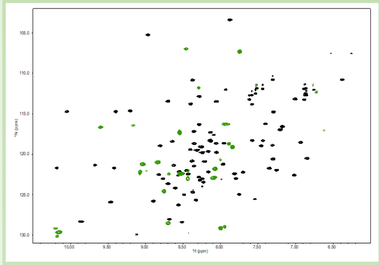


Figure 3. The ^{15}N-HSQC of wild type TrkB-d5 (black) is overlaid with that of the TrkB-L66A mutant (green). There are widespread differences in chemical shift between wild type and mutant, which is unusual for a single point mutation.

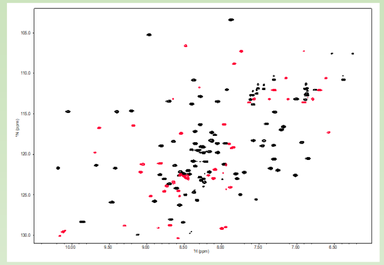


Figure 4. The ^{15}N-HSQC of wild type TrkB-d5 (in black) is overlaid with that of the TrkB-L66W mutant (red).

Chemical shift perturbation calculations provide a comparison of wild type spectra to those of the mutants. These effects caused by the mutations denote changes in the the chemical environment (and therefore structure) of each affected amide group. (**Figs. 5 and 6**).

Chemical shift perturbation was calculated by the following formula:

$$\mathrm{CSP} = \Delta\delta = \sqrt{\frac{(\Delta H)^2 + ((H/N)(\Delta N))^2}{2}}$$

where ΔH and ΔN correspond to the difference in amide proton and nitrogen chemical shifts, respectively. H/N is a scaling factor used to ensure the chemical shift change of each nucleus is weighted equally

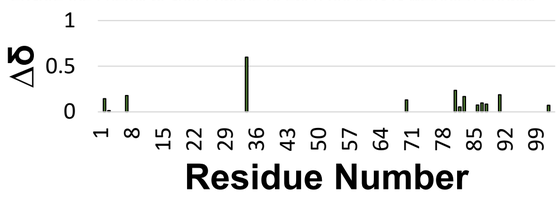


Figure 5. Chemical shift perturbation (Δδ) between hTrkB-d5 L66A and wild type as a function of residue number. The loss of a bulky residue at this position appears to be extremely disruptive, as many peaks in the NMR spectrum of the mutant have disappeared (leading to an inability to measure Δδ in those cases) and the peaks that are visible have shifted significantly, suggesting large structural changes and widespread increases in flexibility

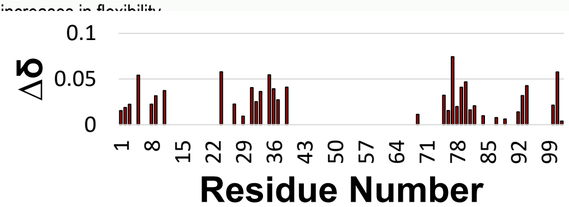


Figure 6. Chemical shift perturbation (Δδ) between hTrkB-d5 L66W and wild type as a function of residue number. It is clear that increasing the size of the side chain at this position is much less disruptive (than L66A): fewer peaks are missing from the spectrum, and the CSP is significantly smaller.

Conclusions and Future Work

- The loss of so many peaks in the HSQC spectra may imply that both the L66A and L66W mutations were overly disruptive to the protein. While the more conservative tryptophan proved to be much less troublesome than the alanine in place of the leucine, it is yet unclear the structural importance of position L66.
- The other residues of interest noted from previous work (shown in red in **Fig. 1**) are I21, I48, and L79. Future work will include similar investigations into their influence on the dynamics of the protein.
- Another aspect of the protein implicated in the binding specificity of TrkB is the unstructured linking portion that joins TrkB-d5 to the transmembrane domain. Future studies will focus on similar dynamics work using the elongated version of the protein, starting with a reconfirmation of dynamic importance of the residues noted above.

References

1. Minichiello, L. (2009) "TrkB Signalling Pathways in LTP and Learning." *Nature Reviews Neuroscience,* **10***:* 850–860.
2. "TrkB Receptor." TrkB Receptor - an Overview | ScienceDirect Topics, www.sciencedirect.com/topics/neuroscience/trkb-receptor.
3. Surinarintr, A. (2017) "Investigation of Internal Motions in TrkB-d5 Provides Insight into Molecular Recognition and Allostery", MS Thesis, California State University Northridge.

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