Investigation of activity and nucleotide binding of vaccinia-related kinase 3 (VRK3)

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Abstract

Kinases play important roles in the regulation of mitosis and cell signaling. Overexpression and misregulation of many kinases can lead to disease, and kinases have made highly successful drug targets for the treatment of cancer. The vaccinia-related kinase (VRK) family are serine/threonine kinases which have higher levels of expression in cells that are actively dividing. Two members of this family, VRK1 and VRK2, are functional kinases which play roles in the cell cycle, transcription regulation, and cell signaling. However, the third member of this family, VRK3, is classified as a pseudokinase, meaning it does not behave like most conserved kinases in the human kinome. It only weakly binds ATP due to substitutions in its sequence compared to VRK1 and VRK2. In this study, point mutations were made to the structure of the VRK3 kinase domain in an attempt to increase its activity. These point mutations were of the large hydrophobic residues thought to block VRK3's binding site to residues conserved in VRK1 and VRK2. Mutant VRK3 kinase domains were then purified and stability was tested *in vitro* using circular dichroism. Nucleotide binding was tested using circular dichroism, and enzyme activity was quantified using a coupled kinase assay.

Methods

Protein Purification:

Plasmids encoding the sequences of the 10His-tagged VRK1 and VRK3 kinase domains were obtained from Addgene. Plasmids were introduced to BL21 DE3 RIL *E. coli* cells on LB + ampicillin plates. Cells were then grown up in TB + ampicillin, spun down, and resuspended in lysis buffer (50 mM Tris HCl pH 8.0, 500 mM NaCl, 10% glycerol, 20mM imidazole). Purification consisted of breaking up the cells using sonication, spinning down the lysate and running a NiNTA column. The protein was His-tagged so a nickel column was chosen to purify (elution buffer: 50 mM Tris pH 7.5, 200 mM NaCl, 10% glycerol, 500 mM imidazole). Fractions were dialyzed into a storage buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10% glycerol) using SnakeSkin Dialysis and then flash frozen in aliquots using liquid nitrogen.

Mutagenesis:

Using the Quickhange Lightning kit from Agilent, point mutations were introduced to VRK3. VRK3 plasmids were transformed into XL10 Gold ultracompetent cells using the kit and the appropriate primers for the mutation ordered from IDT. Mutant plasmids were then prepped for VRK3 L180I, VRK3 L262F, and VRK3 F313L using the GeneJET Plasmid Miniprep kit from ThermoFisher Scientific. These proteins were prepped and purified using the above procedure as well. The plasmids were sent in for sequencing to confirm the success of the mutations.

Activity Assay:

Using the ADP Quest Kit from DiscoverX, a coupled kinase activity assay was run on VRK1, VRK3, L180I, and F313L in the presence of 1 mg/mL myelin basic protein (MBP) and 100 μM ATP. The assay was run with an emission wavelength of 590 nm by exciting at 530 nm. The activity of each protein was determined in the presence and absence of protein substrate. In addition, an assay was run on VRK3 at varying concentrations of ATP in the absence of MBP in order to quantify ATP hydrolysis activity.

Circular Dichroism:

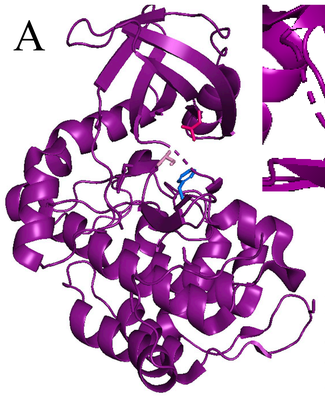
The proteins' secondary structure and melting temperature were determined using circular dichroism. The samples were diluted to 0.3 mg/mL using phosphate buffer and then analyzed for helical content. In addition, a thermal melt was done on each sample and the melting temperatures were calculated and compared to each other. Thermal melts were also done in the presence and absence of ADP for VRK3, F313L, and L180I. The melting points were used to determine whether ADP binds and stabilizes the protein.

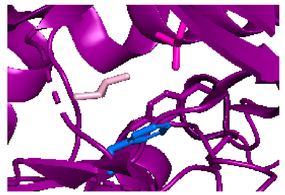
Acknowledgements

Thank you to Winona State University and the Winona Foundation for a Student Research & Creative Project Grant for the funding of this project. Thank you, also, to them for a Student Travel Support Grant awarded to present at the American Chemical Society National Meeting 2020.

Results

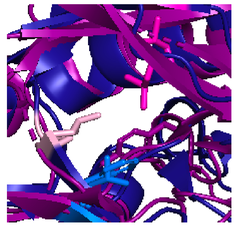
A



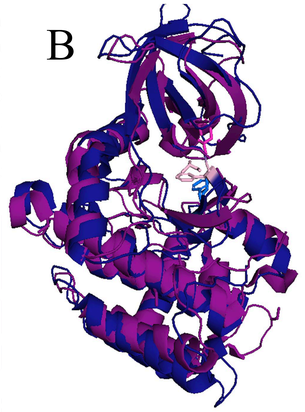


A: Structure of VRK3 (2JII) in purple, L180 in dark pink, L262 in light pink, and F313 in blue and a highlight of these residues in the active site. Images were prepared in PyMOL.

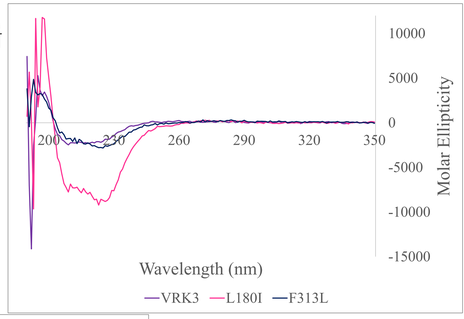
B: Structure of VRK3 in purple aligned with VRK1 (2KUL) in dark blue. Highlight of the active site below. L180I in dark pink, F313L in blue, and L262F in light pink.



B

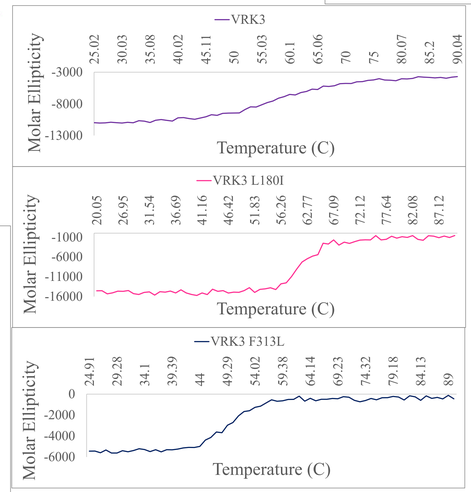


G



G: Circular dichroism of VRK3, VRK3 L180I, and VRK3 F313L.

H

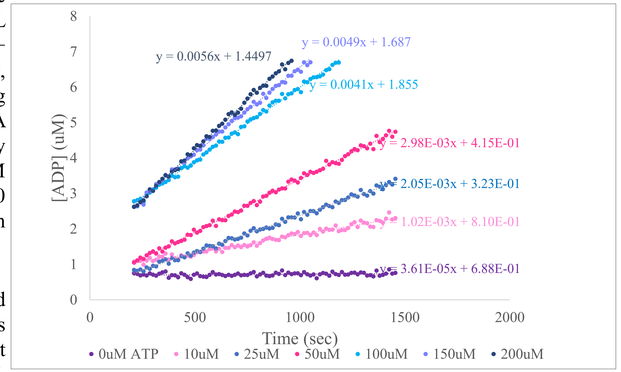


H: Thermal melts of VRK3, L180I, and F313L. Melting points of the proteins were determined for these melts and recorded in table 1.

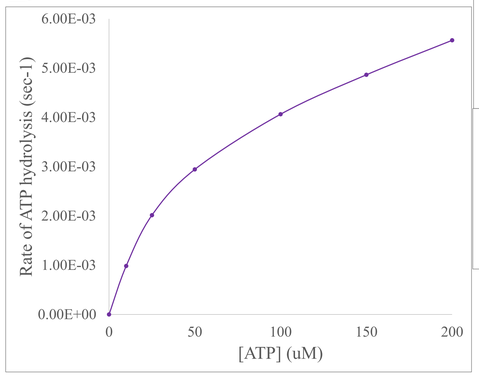
Table 1. Melting temperatures of VRK3 wt and mutants determined using circular dichroism (G).

Protein	T_m (°C)
VRK3	57.9
L180I	60.8
F313L	49.4

C

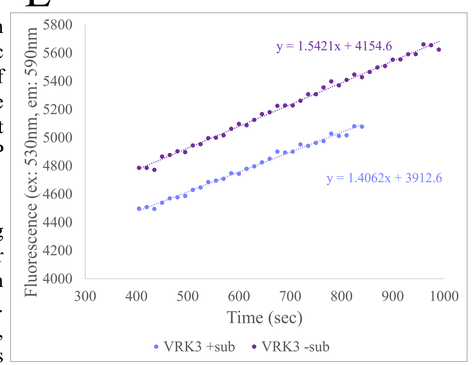


D

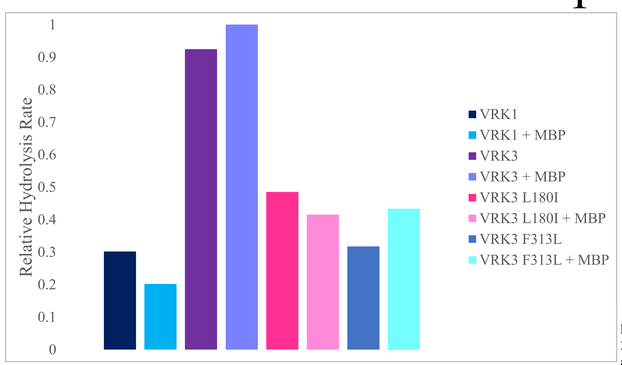


C: Activity of VRK3 in the presence of varying concentrations of ADP ranging from 0 uM to 200 uM. The steeper the slope, the faster VRK3 hydrolyzes ATP. This experiment suggests VRK3 binds and hydrolyzes ATP.
D: Graph of [ATP] and relative rate of hydrolysis for VRK3. K_m is 71.4 ± 8.6.

E



F



E: Activity Assay of VRK3 with and without ADP. The slope of the line of the activity of VRK3 with no substrate was 1.5421 while the slope of the line for VRK3 with substrate was 1.4062. This is not a significant difference. MBP may not be the appropriate phosphorylation substrate to use, and this will be tested in the future.
F: Relative ATP hydrolysis activity for VRK1, VRK3, VRK3 L180I, and VRK3 F313L with and without MBP substrate.

Conclusions

- After receiving the sequence of each mutant, it was determined that the mutagenesis was effective for F313L and L180I.
- The activity assay of VRK3 with varying concentrations of ADP (figures C and D) suggests that VRK3 does appear to bind and hydrolyze ATP. The K_m for this binding was found to be 71.4 ± 8.6. However, VRK3 doesn't appear to attach the hydrolyzed phosphate to a substrate.
- The activity assay was successful, however, MBP may not have been the appropriate substrate. Figures E and F suggest that once the mutations, F313L and L180I, are introduced to the protein, the effective hydrolysis activity of VRK3 appears to decrease and the protein then behaves more like VRK1. This experiment was inconclusive and more testing is required.
- The steady-state CD traces of VRK3 and the mutants suggest that the mutated proteins have a different fold. This is because the CD trace of both F313L and L180I differ from that of VRK3 significantly.
- According to the thermal melt, VRK3 and L180I appear to have similar stability due to their similar melting temps. However, F313L significantly destabilizes the protein. This likely supports Scheef's claim that this residue is an important part of the hydrophobic spine of the kinase.

References

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