Development of an Mst2 construct for future studies of conformational regulation

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Abstract:

The serine/threonine protein kinases Mst2 and Mst1 are key members of the Hippo pathway, a signaling pathway that regulates apoptosis by inhibiting the transcriptional cofactor yes-associated protein (YAP).[1] Mst2 is activated by phosphorylation at threonine-180, allowing it to phosphorylate large tumor suppressor kinases 1/2 (LATS1/2) which can then inhibit YAP.[1,2] The conformation of Mst2, particularly at the activation loop, is predicted to change when it is activated by phosphorylation. We believe that the stability and relatively small size of Mst2 may allow us to farther study its conformation using fluorescence-based techniques. Proteins can be labeled for these techniques using cysteine-maleimide crosslinking, where a "Cys-lite" background is prepared, and cysteine residues are inserted at specific positions that can be labeled under typical lab conditions. Using a Quikchange Lightning site-directed mutagenesis kit, we have made constructs of the His-tagged Mst2 kinase domain with a cysteine labeling position in a Cys-lite background to direct a fluorescent donor or acceptor tag. We have validated a high-yield purification protocol and checked the folding of our construct using circular dichroism (CD), and the nucleotide binding of our construct using fluorescence-based thermal denaturation. We have also prepared this construct with and without co-expression with lambda phosphatase.

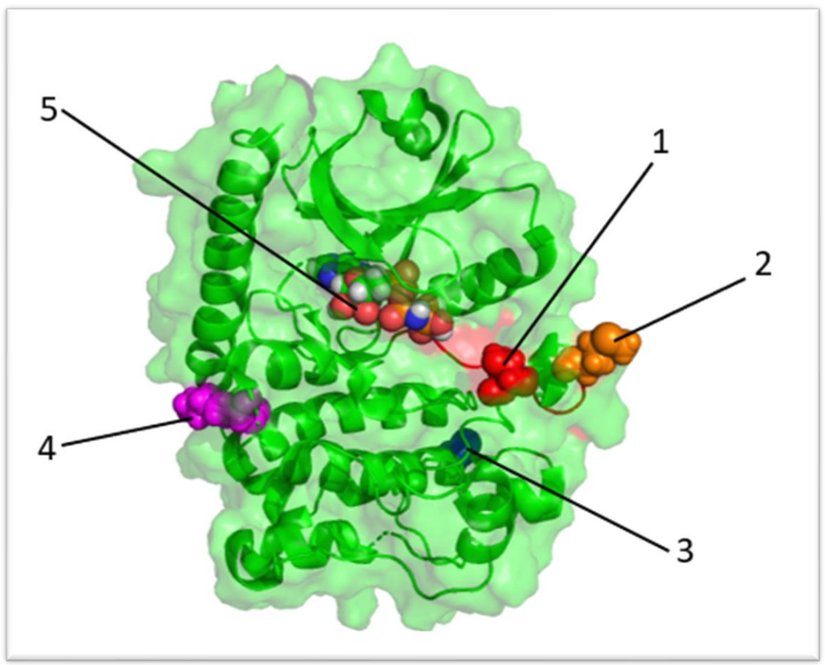


Figure 1: Model of Cys-lite Mst2 C200A M175C K116C unphos. background to be made. 1: T180, 2: M175C, 3: C200A, 4: K116C not yet mutated on our model. 5: AMP-PNP

Methods and Results:

Week 1-4 •Mutagenesis to direct C200A and M175C into wild type Mst2 •Transform into XL10 Gold ultracompetent cells and purify Mst2 C200A plasmid	Week 3-4 •Repeat mutagenesis procedure to direct M175C into Mst2 C200A and purify Mst2 C200A M175C plasmid
Week 5 •Transform Mst2 C200A M175C plasmid into BL21 DE3 RIL E. coli cells and purify protein	Week 6-7 •Bradford assay and CD tests
Week 8-11 •Repeat mutagenesis procedure to direct λ phosphatase into Mst2 •Repeat protein purification procedure to purify Mst2 C200A M175C unphosphorylated protein	Week 12-13 •Bradford assay, SDS PAGE, CD, and thermal denaturation tests. Sent plasmids for sequencing

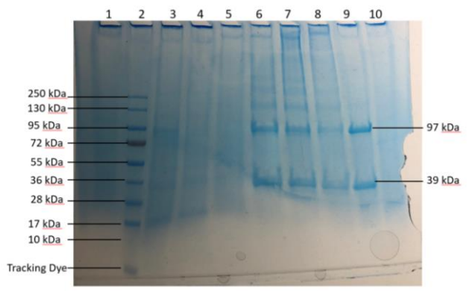
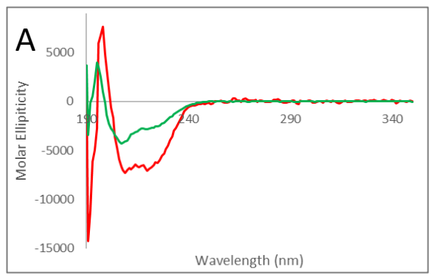


Figure 2: SDS-PAGE gel. Lane 1: buffer, 2: PageRuler Plus ladder, 3: lysate, 4: flowthrough, 5: wash, 6-8: elution fractions, 9: dialyzed Mst2 C200A M175C unphosphorylated, 10: Mst2 C200A M175C phosphorylated.



Mst2 C200A M175C	Helix	Sheet	Turn/Other
Phosphorylated	41.4%	11.7%	46.9%
Unphosphorylated	16.9%	28.9%	54.2%

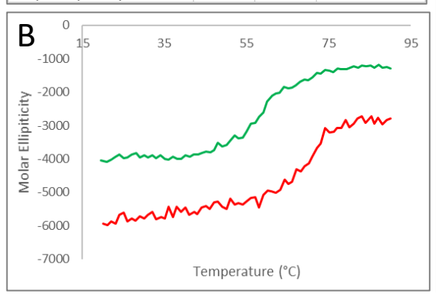
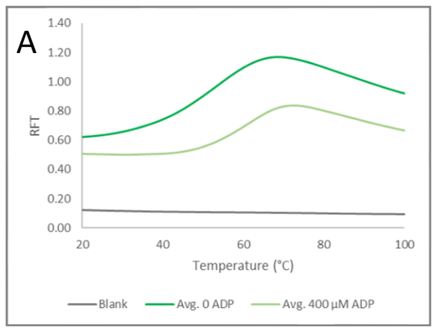


Figure 3: Wavelength CD and secondary structure percentages (A) and thermal denaturation (B) spectra of Mst2 C200A M175C phos. (red) and Mst2 C200A M175C unphos. (green)



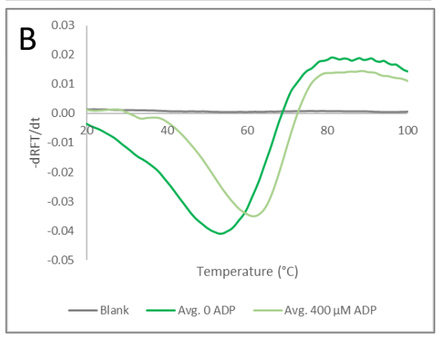


Figure 4: (A) Thermal denaturation RFU vs Temperature (°C) spectra of Mst2 C200A M175C unphos. (B) Derivative –dRFU/dt vs Temperature (°C) spectra of and Mst2 C200A M175C unphos.

Conclusions and Future Directions:

SDS-PAGE:

- Mst2 C200A M175C unphos. had a molecular weight of 39 kDa which is relatively consistent with literature value of our 321-residue long sequence (36.7 kDa)[3]
- Second band indicating 97 kDa possibly due to disulfide linkages between monomers
- Mst2 C200A M175C phos. Band was too faint to be quantified

Circular Dichroism (CD):

- Unphosphorylated construct (Tm = 58 °C) appears to be less stable than phosphorylated counterpart (Tm = 68 °C)
- Phosphorylated construct is 41.4% α-helices, 11.7% β-sheets, and 46.9% other. Similar to literature values (based on PyMol) of 52.3% α-helices, 15.4% β-sheets, and 32.2% other

Thermal Denaturation Assay:

- Mst2 C200A M175C unphos. binds tightly to ADP.
  - T_m with no ADP bound = 54 °C
  - T_m with ADP bound = 62 °C

The significant change in Tm for the unphosphorylated construct does suggest a conformational change. Our phosphorylated and unphosphorylated constructs overall appear to be well folded and stable enough to continue constructing a Cys-lite background with cysteine labeling positions for future FRET tests to study the conformation of Mst2. We intend to mutate K116 into a cysteine to complete the His-tagged Mst2 kinase domain Cys-lite construct. We also intend to test the validity of the final construct with more SDS-PAGE and CD tests.

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Citations:

(1) Galan, J. A.; Avruch, J. MST1/MST2 Protein Kinases: Regulation and Physiologic Roles. *Biochemistry* **2016**, *55* (39), 5507–5519. DOI: 10.1021/acs.biochem.6b00763.

(2) Ni, L.; Li, S.; Yu, J.; Min, J.; Brautigam, C. A.; Tomchick, D. R.; Pan, D.; Luo, X. Structural Basis for Autoactivation of Human Mst2 Kinase and Its Regulation by RASSF5. *Structure* **2013**, *21* (10), 1757–1768. DOI: 10.1016/j.str.2013.07.008.

(3) Albanese, S. K.; Parton, D. L.; Işık, M.; Rodríguez-Laureano, L.; Hanson, S. M.; Behr, J. M.; Gradia, S.; Jeans, C.; Levinson, N. M.; Seeliger, M. A.; Chodera, J. D. An Open Library of Human Kinase Domain Constructs for Automated Bacterial Expression. *Biochemistry* **2018**, *57* (31), 4675–4689. https://doi.org/10.1021/acs.biochem.7b01081.