Investigation of c-Jun N-terminal Kinase 2 Regulation

Presented by: Jaeeun Go

In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Biochemistry

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Abstract

Mitogen-activated protein kinases (MAPK) allow a cell to respond to external signals to grow, divide or undergo apoptosis. One example is the c-Jun N-terminal kinase 2 (JNK2). This protein is unique in that it can autophosphorylate, or phosphorylate itself. It was found that these proteins tend to form aggregates of four, or tetramers, when inactive and remain as single units, or monomers, when active. In glioblastoma and lung carcinoma cells, JNK2s are reported to be constitutively, or always, active. This study aimed to investigate a selection of JNK2 mutations determine which of these mutations can alter JNK2 activity and oligomerization state.

Results from size-exclusion chromatography and light scattering analysis (SEC-LS) suggested that while the wild type JNK2 exists as a mixture of monomers and tetramers, the activation loop chimera mutant (C177G/N179S) exists only as monomers. Additionally, phosphorylation assay analysis showed increased autophosphorylation activity of the activation loop chimera mutant compared to the wild type. These results suggest that the activation loop may be involved in the mechanism of JNK2 regulation. Elucidation of MAPK regulation mechanisms may allow more effective cancer therapy screening and increase overall understanding of tumor growth mechanisms.

Background:

Kinases are proteins that add phosphate groups to other proteins, a process known as phosphorylation. In general, these proteins serve to transmit signals from the outside of a cell to the inside, a process known as signal transduction. As phosphate groups are transferred, proteins are either activated or inactivated, resulting in some change in cellular activity. A signaling pathway consists of a group of interacting proteins that link a stimulus to the response.
One important signaling pathway in cancer research is the MAPK pathway. This pathway contains a variety of proteins, including a group of kinases called mitogen-activated protein kinases (MAPKs). Normally, this pathway links mitogenic signals (signals to divide) and stress signals to alter transcription of certain genes. Cells then assemble molecular machinery to initiate mitosis or programmed cell death. However, studies have shown that in some cancer cells, certain MAPKs behave abnormally, resulting in uncontrolled cell division.[1]

One important MAPK is the c-Jun N-terminal kinase (JNK). There are three known JNKs: JNK1, JNK2 and JNK3. These kinases are activated by mitogen activated kinase kinase 4 and 7 (MKK4 and MKK7). However, JNK2 is unique in its ability to autophosphorylate, or phosphorylate itself. When JNK2 is doubly phosphorylated by upstream kinases or by autophosphorylation, it becomes active and phosphorylates Activating Transcription Factor 2 (ATF2) and various other proteins.[1] However, in glioblastoma and lung carcinoma cells, JNK2 is found to be constitutively, or always, active. Recent findings have shown that JNK2 can oligomerize (form aggregates) with itself to regulate its activity.[2] Wild-type JNK2 was found as a mixture of monomers, dimers, and tetramers in solution. However, an F170R mutation resulted in inhibition of autophosphorylation activity and stabilization of its tetramer form.[2] Therefore, JNK2 activity may depend on whether the protein is predominately in its monomer or tetramer state. A model of JNK2 regulation is shown in Figure 1. This study aimed to investigate a selection of somatic mutations (mutations that occur in somatic cancer cells) of JNK2. Each mutant was cloned, expressed, purified and tested for activity and oligomerization state. The

results may provide insight on therapeutic approaches of inhibiting JNK2 activity and thereby halting tumor growth.

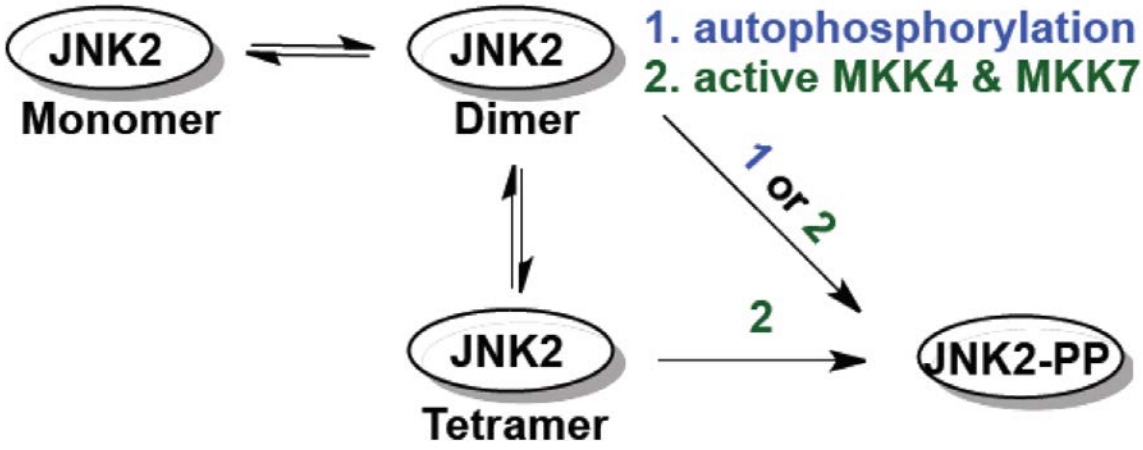

Figure 1: A proposed model of JNK2 regulation.

Materials and Methods:

The protocol for this experiment can be separated into three parts: preparation of phosphate-free JNK2, light scattering analysis, and JNK2 phosphorylation assay analysis.

Preparation of Phosphate-free JNK2

The mutations chosen for investigation were selected from the Catalogue of Somatic Mutations in Cancer (COSMIC) database. Specifically, mutations near the activation loop of JNK2 were chosen. Additionally, an activation loop chimera DNA was prepared according to the protocol in Cui, et. al.[3] The sequences of JNK1 and JNK2 were aligned and differences in the activation loop were mutated onto the JNK2 sequence (C177G/N179S). The full list of mutations chosen are as follows: F170R, A176V, M181I, A193V, E204K, V206L, and activation loop chimera (C177G/N179S). Overlap extension polymerase chain reaction (PCR) was used to produce the mutant *jnk2* constructs. The products of the first round of PCR was run on a 10% agarose gel, excised, and purified. For each mutant, the two products from the first round were then mixed for the second round of PCR, resulting in the complete, amplified product. This product was then ligated into a Qiagen pET28a(+) vector using *NheI* and *HindIII* restriction sites. This vector contained the gene for JNK2 with an N-terminal His-tag cleavable by thrombin. Further details of this construct are described in Madsen et al.[4]

The cloning and purification protocol was followed largely according to Zhan, et. al.[6] *E. coli* strain BL21 (DE3) was electroporated to enhance DNA uptake as described in House et al.[5] Each *jnk2* construct was co-transferred with pCDF-Duet λ-phosphatase into the *E. coli*. Then, a single colony was mixed with 30 mL of Luria-Broth (LB) media with 30 µg/mL Kanamycin and

5 µg/mL Spectinomycin grown overnight at 37 ℃. After overnight growth, the cells were then diluted 100X with LB containing 30 µg/mL Kanamycin and 5 µg/mL Spectinomycin. This diluted culture was grown at 37 ℃ until $O.D_{600}$ reached 0.6. The cells were then induced with IPTG and cultured at 30 ℃ for 3 hours. The cells were then centrifuged at 7000g for 12 minutes, frozen in liquid nitrogen, and stored at -80 ℃.

To purify the expressed protein, the cells were first lysed in buffer A containing 1% Triton X-100 and 0.5 M NaCl (see supplement for buffer details). The lysate was then sonicated with 5 second pulses for 15 minutes at 4 ℃. Temperature was monitored using a thermometer and pulses were regulated to keep the lysate under 10 ℃. Then, the lysate were centrifuged for 30 minutes at 16000 rpm. The supernatant was incubated with Ni-NTA beads for 1 hour at 4 ℃ with a standard 3-D laboratory shaker., and the supernatant was incubated with Ni-NTA beads. The beads were then poured into a 98 mL Econo-Column (by Bio-Rad). The beads were first washed with buffer A containing 10 mM imidazole and eluted with imidazole. Then, the JNK2 was eluted with 25 mL of buffer A containing 200 mM imidazole at pH 8. The elution product was then poured into a Mono Q HR 10/10 anion exchange column (from General Electric). After equilibration in buffer B, the protein was fractionated over 15 column volumes with a gradual increase in NaCl concentration from 0 to 0.5 M. Fractions corresponding to the largest chromatographic peak were combined and dialyzed in buffer C containing 10% glycerol. After dialysis, concentration was measured using standard Bradford assay techniques and a predetermined JNK2 calibration curve. If the concentration was too low, the sample was concentrated by centrifuging it in an Amicon Ultra-15 concentrator for about 10 minutes at 4400 rpm. The dialyzed samples were freezed in liquid nitrogen. Purity of each fraction was assessed by 12% SDS/PAGE gel. A western blot analysis was also carried out on each fraction to assess if any JNK2 remained phosphorylated. Exact buffer concentrations and components are listed in the supplement, which derive from the protocol in Zhan, et. al.[6]

Light Scattering

All buffers and solutions were first filtered through an Anodisc 0.02 µm filter prior to light scattering analysis.

Bovine serum albumin was first injected into a Wyatt DAWN Heleos instrument to normalize the detectors. Between the pump and the QELS detectors, a TSK-GEL G $3000PW_{XL}$ column (by Tosoh Bioscience LLC) allowed separation of any oligomers prior to reaching the detector. The size-exclusion chromatography was performed with buffer F at a flow rate of 0.4 mL/min at 25 ℃. Prior to light scattering analysis, each JNK2 protein was first dialyzed in buffer F. The concentrations of the dialyzed samples were measured using a standard Bradford assay technique with a predetermined calibration curve. Then, each sample was diluted to 25 µM with buffer F, with a final volume of 100 µL. The samples were then centrifuged at 13400 rpm for 30 seconds to precipitate any insoluble material that may produce artifacts during light scattering analysis. 20 µL of each sample was injected into the Heleos instrument. Molar masses were calculated using Astra software (by Wyatt Technology). Specific details of the process are described in Kaoud, et al.[7]

JNK2 Phosphorylation Assay

To estimate the kinase activity of different JNK2 fractions, JNK2 kinase assays were conducted. The assays were performed on a standard 96-well plate with 300 µL capacity per well. Out of all the JNK2 variants purified, 2 were analyzed for the sake of time: wild-type and C177G/N179S. For each variant, 250 nM of the JNK2 was mixed with either 25 nM activated MKK4, 25 nM activated MKK7, 25 nM of both MKK4 and MKK7 or no MKK at all. Each well also contained 11 mM MgCl2, 2 mM DTT, 10 µg/ml BSA, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA in 25 mM HEPES buffer-pH 7.5. The reaction was initiated with addition of 1 mM ATP and incubation at 25 °C for 16 minutes. Then, a 5 µL aliquot from each well was transferred to another set of wells containing 12.5 uM of ATF-2 and buffer F. The reactions were then quenched using buffer D. The quenched samples were then run on a 12% SDS/PAGE gel. Nitrocellulose membranes were prepared by immersing them in methanol, water, then buffer E for 5 minutes each. The proteins were transferred onto the nitrocellulose membranes using a wet blotting system (from Bio-Rad) at 30 V in buffer E for 2 hours.

After transfer, the membranes were briefly washed with methanol, water and then phosphate buffered saline (PBS) for 5 minutes each. The membranes were incubated in LI-COR Odyssey Blocking Buffer for 30 minutes while gently shaking. A primary antibody solution containing 1:5000 anti-phospho-Threonine-Proline (p-Thr-Pro-101) mouse mAb (Cell Signaling Technology) and 1:2000 anti-phospho-Tyrosine (p-Tyr-1000) rabbit mAb (Cell Signaling Technology) in LI-COR Odyssey blocking buffer. After blocking, the 5 mL of the antibody solution was poured over each membrane and incubated for 1 hour. The membranes were then washed with Tris-buffered saline with Tween 20 (TBST). A mixture of secondary antibodies with two different dyes (LI-COR® IRDye 800CW GT anti-rabbit and LI-COR® IRDye 680RD Goat anti-mouse secondary antibodies) was then poured over the membranes and incubated for 1 hour. The membranes were washed with TBST and imaged using the LI-COR imaging system.

Results

Preparation of Phosphate-free JNK2

The use of a lambda phosphatase co-expression protocol enabled the purification of phosphate-free JNK2. Using this form of JNK2, we could monitor its autophosphorylation and relate it to its activity against the downstream substrate, ATF-2. A sample chromatogram produced from the purifications is shown in **Figure 2**. Each pooled fraction was run on a 12% SDS/PAGE gel, which is shown in **Figure 3**. A strong single band at around 50 kDa in each lane indicated presence of relatively pure JNK2 in each fraction. Western blot analysis was performed to assess phosphorylation status of the JNK2 in each pooled fraction. These western blots are shown in **Figure 4**. The absence of phosphorylation signal despite presence of total JNK2 indicated that each fraction contained phosphate-free JNK2.

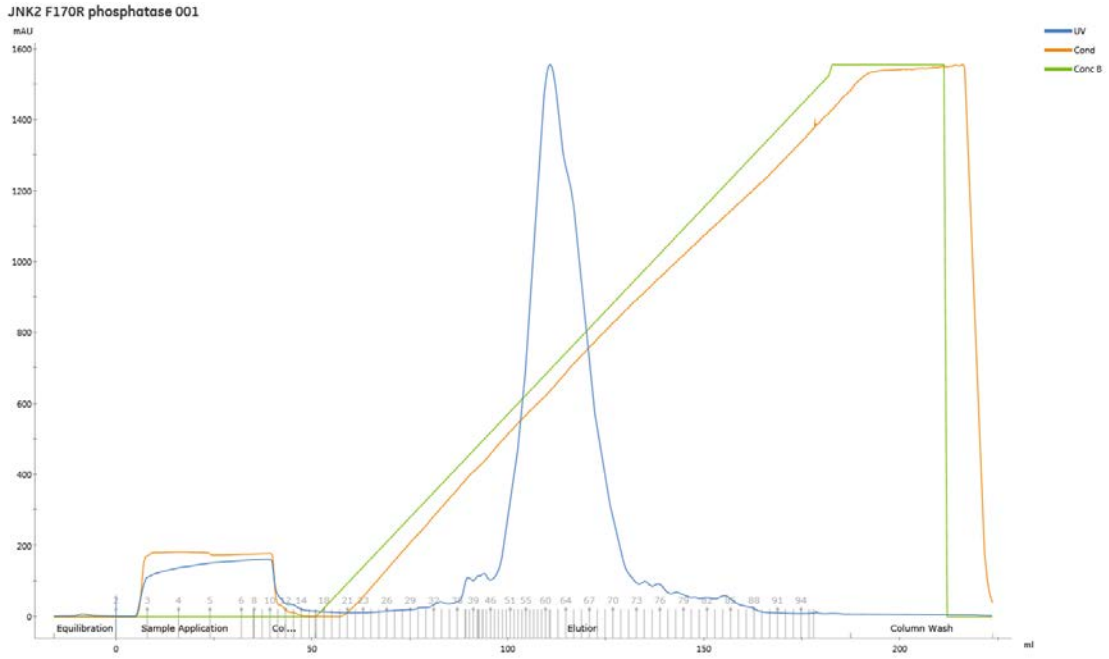

Figure 2: This chromatogram was produced from the purification of JNK2 F170R. Similar chromatograms were produced for the other JNK2 variants purified. The blue line represents UV signal in each fraction and the green line represents increasing salt concentration of buffer B.
from 0 to 0.5 M NaCl.

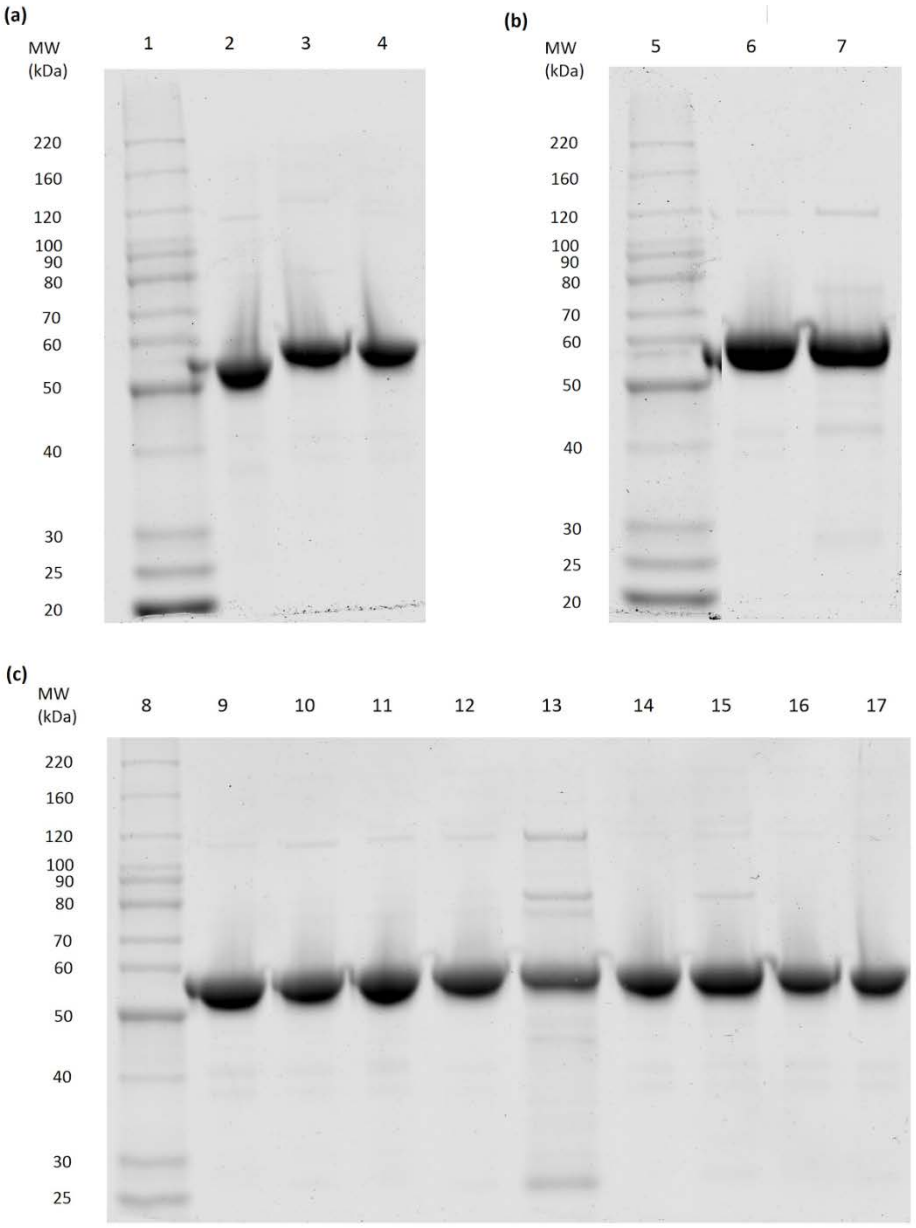

Figure 3: This figure shows the purified JNK2 samples ran on a 12% SDS/PAGE gel. Parts of the gel in **(a)** and **(b)** have been omitted as they contained proteins not relevant to this study. The fractions are labeled as follows: 1 = Ladder = JNK2 F170R, 2 = F170R, 3 = Wild type - fraction 2, 4 = wild type fraction 1, 5 = ladder, 6 = activation chimera loop – fraction 2, 7 = activation loop chimera (C177G/N179S) – fraction 1, 8 = ladder, 9 = A176V – fraction 1, 10 = A176V – fraction 2, 11 = M181I – fraction 1, 12 = M181I – fraction 2, 13 = A193V, 14 = E204K – fraction 1, 15 = E204K – fraction 2, 16 = V206I – fraction 1, 17 = V206I – fraction 2. This image was taken with a LI-COR Odyssey imager.

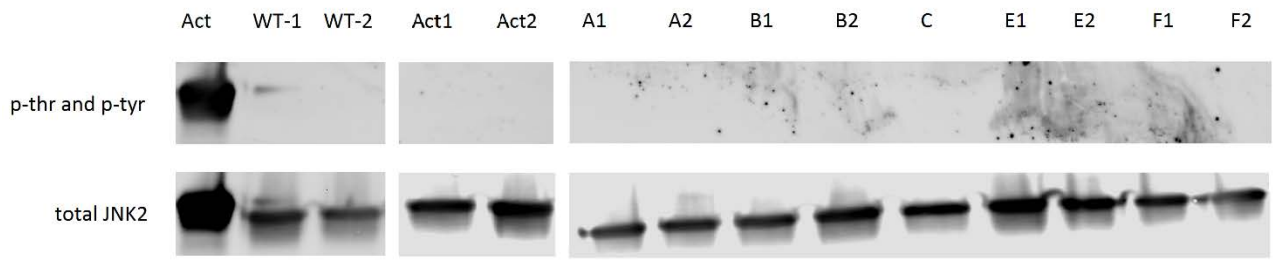

Figure 4: This figure shows the Western Blots of each sample created after the purification step. The proteins are labeled as follows: Act = active JNK2 control, WT-1 = wild type- fraction 1, WT-2 = wild type - fraction 2, Act1 = activation loop chimera (C177G/N179S) – fraction 1, Act2 = activation loop chimera – fraction 2, A1 = A176V – fraction 1, A2 = A176V – fraction 2, B1 = M181I – fraction 1, B2 = M181I – fraction 2, C = A193V, E1 = E204K – fraction 1, E2 = E204K – fraction 2, F1 = V206I – fraction 1, F2 = V206I – fraction 2. This image was taken using a LI-COR Odyssey imager.

Light Scattering

The light scattering analysis (**Figure 6)** showed that the wild type formed a mixture of monomers (50 kDa) and tetramers (200 kDa) in solution. Meanwhile, the activation loop chimera remained as monomers (50 kDa), whether at a moderate concentration of 25 µM or a high concentration of 100 µM.

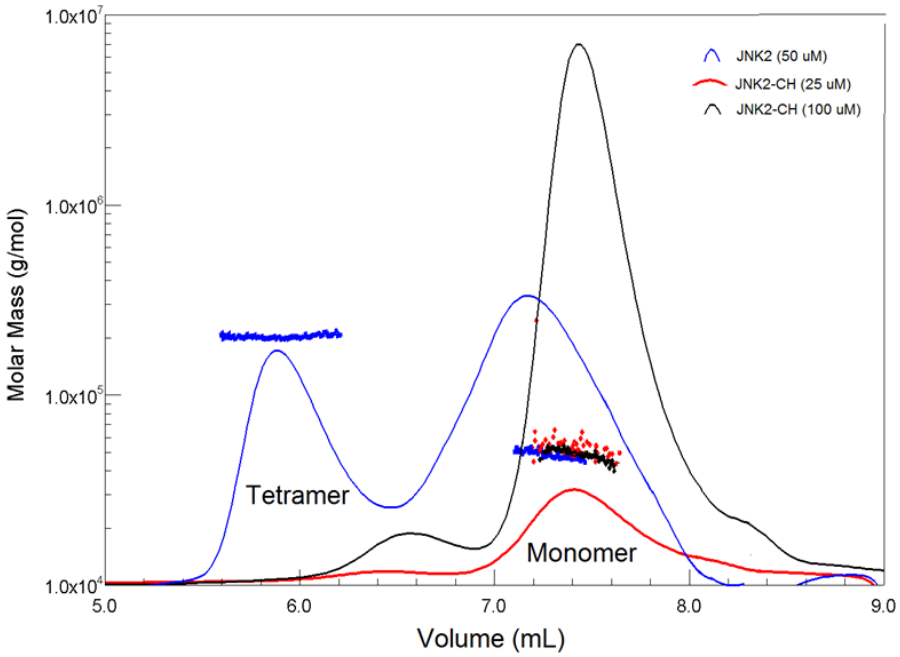

Figure 6: This figure shows the light scattering result for the JNK2 wild type and activation loop chimera (C177G/N179S). The curve lines represent differential refractive index (dRI) signals, while the cluster of dots represent the calculated molar mass within the sample. The wild type enzyme is represented in blue, while the activation loop chimera at 25 µM and 100 µM are represented in red and black, respectively.

JNK2 Phosphorylation Assay

The results of the Western Blot analysis on the JNK2 phosphorylation assay are shown in **Figure 5**. The activation loop chimera (C177G/N179S) mutant (**Figure 5b**) had a greater autophosphorylation activity than the wild type **(Figure 5a)** at both thr-183 and tyr-185.

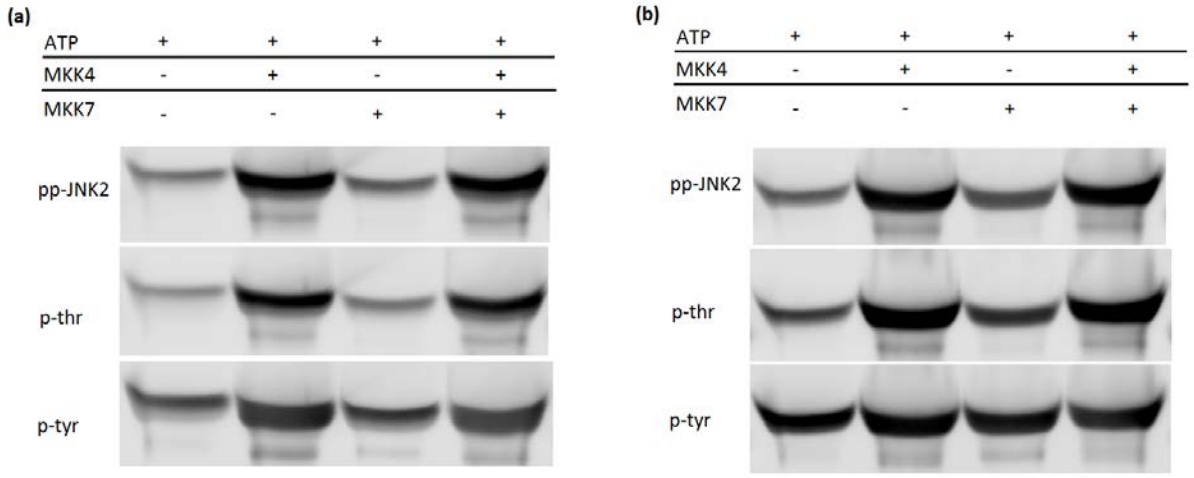

Figure 5: This figure shows the results of the JNK2 phosphorylation assay. The wild type protein is shown in **(a)** while the activation loop chimera (C177G/N179S) is shown in **(b).** The pp-JNK2 row shows the amount of double phosphorylated JNK2, while the p-thr and p-tyr rows show the amount of phosphorylation at thr-183 and tyr-185, respectively. Images were taken using a LI-COR imager.

Discussion

It had been previously demonstrated that JNK2 can potentially regulate its activity through oligomerization.[2] Specifically, it was found that an inactive form, JNK2 F170R, formed exclusively tetramers. Therefore, mutations within the tetramer interface may disrupt the JNK2 tetramer into monomeric units, possibly increasing autophosphorylation and downstream activity. We first investigated several JNK2 mutants to find a mutation that would disrupt the tetramer. Then we assessed autophosphorylation activity of each mutant. However, to accurately measure autophosphorylation activity, preparation of phosphate-free JNK2 was essential. Since JNK2 autophosphorylates in bacterial cells, we co-expressed JNK2 with λ-phosphatase. As demonstrated by the high purity of the protein bands in **Figure 3**, the purification protocol appeared to be an effective method to isolate JNK2 from the co-expressed λ-phosphatase. Additionally, as shown in **Figure 4**, the lack of pp-JNK signal despite presence of JNK2 demonstrates the effectiveness of co-expressing λ-phosphatase to produce phosphate-free JNK2.

Light scattering analysis uses the principles of Brownian motion to determine the size of particles, such as proteins, in solution. When light is shined into a sample and scattered, random particle motion results in temporal fluctuations of the scattered light intensity. Heavier particles tend to move slower, resulting in slower fluctuations. With proper calibration, these fluctuations can be converted into the molar mass of the particles in solution. We used light scattering

analysis to calculate the molar mass of particles for each JNK2 mutant and determine the respective oligomerization states. **Figure 6** showed that the wild type formed a mixture of monomers (50 kDa) and tetramers (200 kDa) in solution. However, the activation loop chimera remained as monomers (50 kDa), whether at a moderate concentration of 25 µM or a high concentration of 100 µM. Therefore, it is possible that the C177G/N179S mutations may lie within the binding interface, resulting in disruption of the tetramer into monomers.

To determine if tetramer disruption and monomer prevalence correlated with autophosphorylation activity, we performed a phosphorylation assay on the two JNK variants. The phosphorylation assay indicated that the activation loop chimera (C177G/N179S) mutant (**Figure 5b**) has a greater autophosphorylation activity than the wild type **(Figure 5a).** Therefore, disruption of the tetramer into monomers resulted in increased activity of JNK2. These results complement a previous study which found that the JNK2 F170R mutant formed exclusively tetramers and exhibited inhibition of its autophosphorylation activity.[2]

If more time was allotted, we would have conducted the light scattering analysis and phosphorylation assay with other JNK2 mutants to further elucidate which somatic mutations can lead to altered JNK2 activity and oligomerization states. Additionally, we would have performed crystallography on the activation loop chimera, JNK2 F170R mutant and wild type to locate the binding interface and corroborate the findings with current data.

Overall, this study has helped elucidate a possible element of JNK2 regulation. Elucidation of MAPK regulation mechanisms may allow more effective cancer therapy screening and increase overall understanding of tumor growth mechanisms.

References

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Supplementary Information

Buffer Legend

Note: Buffers A-C were used according to the protocol in Zhan, *et al.*[6] Buffer F was used according to the protocol in Kaoud, *et al.*[7]

Buffer A (pH 8)
- 20 mM Tris-HCl
- 0.03% Brij-30
- 0.1% (v/v) β-mercaptoethanol
- 5 mM imidazole
- 1 mM benzamidine
- 0.1 mM PMSF
- 0.1 mM TPCK

Buffer B
- 20 mM Tris-HCl
- 0.03% (v/v) Brij-30
- 0.1% (v/v) β-mercaptoethanol

Buffer C
- 25 mM HEPES
- 50 mM KCl
- 0.1 mM EDTA
- 0.1 mM EGTA
- 2 mM DTT

Buffer D (quenching buffer)
- 20 mM HEPES
- 200 mM KCl
- 0.1% BSA
- 50 mM EDTA
- 1 mM EGTA

Buffer E (transfer buffer)
- 25 mM Tris
- 192 mM glycine
- 20% methanol

Buffer F
- 25 mM HEPES (pH 7.5)
- 100 mM KCl
- 0.1 mM EDTA
- 1 mM DTT