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Quantification of GSTpi and JNK protein-protein interactions using backscatter interferometry

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Quantification of GSTpi and JNK protein-protein interactions using backscatter interferometry

by

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ABSTRACT

Protein-protein interactions play a critical role in sustaining life and diseases can often result from mis-regulation of these protein-protein interactions. The interaction between glutathione S-transferase pi (GSTpi) and c-Jun N-terminal kinases (JNKs) is such a system that has been linked to cancer. GSTpi normally functions in the body to detoxify cells by removing foreign compounds, and JNKs participate in the Mitogen Activated Protein (MAP) kinase cascade, whose effects range from cell proliferation to programmed cell death (i.e., apoptosis). GSTpi is known to inhibit the apoptotic behavior of JNK proteins, which in turn can lead to cancer. It is hypothesized that GSTpi does so by binding directly to JNKs, and that this interaction depends upon factors such as the haplotype of GSTpi, or whether the JNKs are phosphorylated. It is then believed that such variables affect the affinity of these proteins for one another. However, these interactions have only been identified qualitatively. Thus, understanding quantitatively the interactions of GSTpi with JNKs in regards to these factors provides crucial insight towards manipulating the pathway for chemotherapies.

This project is aimed at determining the binding affinity constants of GSTpi and JNK proteins with relations to the above variables. To achieve this goal, we will use Backscatter Interferometry (BSI), a very sensitive technique that utilizes very small amount of sample and does not require labeling.

We have successfully expressed and purified a number of the necessary proteins to complete the study. Namely, we have purified GSTpi and the inactive

(unphosphorylated) forms of JNK1α2 and JNK2α2, which are two isoforms that have been shown to bind to GSTpi. We have also obtained the active/phosphorylated form of JNK1α2 and JNK2α2, referred to as pJNK1α2/pJNK2α2 as we plan to study the effect of phosphorylation levels of JNKs on binding to GSTpi. We are currently in the process of purifying pJNKs. We were also able to obtain preliminary BSI data with GSTpi and JNK1α2 alone in solution, demonstrating the efficacy of BSI for use with small quantities of proteins. More testing is underway with GSTpi and the phosphorylated version of JNK1α2 and JNK2α2.

BACKGROUND

The activities of many proteins and enzymes are modulated via protein-protein interactions. A protein can be either inhibited or activated by interacting with other proteins, whether it is through post-translational modifications (such as phosphorylation) or through direct binding of one protein to another. The main focus of this project is on how the phosphorylation of c-Jun N-terminal Kinase (JNK) affects its binding to another enzyme, Glutathione S-Transferase pi (GSTpi).

1. Glutathione S-Transferase pi: GSTpi is a member of the family of cytosolic glutathione S-transferases (GSTs). It is a 25 kDa enzyme that exists as a monomer at lower concentrations and forms a dimer at higher concentrations around 0.1 mg/ml.[1,2] The primary function of GST enzymes is their central role in cellular detoxification. They accomplish this by conjugating glutathione molecules to electrophilic foreign toxins, which prevents them from causing harm to the cell while simultaneously preparing them for excretion.[3] The active site of GSTs consists of a hydrophilic site to bind glutathione (G site), and a hydrophobic site to bind the toxin (H site).[2] The variations in the H sites classify the different GSTs, where GSTpi is more hydrophilic than other classes.[4] GSTpi is the most commonly found GST, and is expressed most in lung, placenta, and esophageal tissues. GSTpi in humans is polymorphic, where residues 105 and 114 vary; wild type is 105I-114A, while "Haplotype C" is 105V-114V. Haplotype C of GSTpi has been associated with various cancers in humans, namely that of the testes and bladder.[5] In addition to detox, GSTs have also been demonstrated to modulate the activities of

enzymes involved in signaling cascades. One such example is the interaction of GSTpi with c-Jun N-Terminal kinases (JNKs).

2. c-Jun N-terminal kinase: c-Jun N-terminal kinases (JNKs) are a class of Mitogen-Activated Protein Kinases (MAPKs), which are proteins that transduce signals sent by cell surface receptors to intracellular targets to control cell proliferation, differentiation, inflammation, and apoptosis.[6,7] They are activated by UV, osmotic shock, and numerous cytokines.[8] JNK enzymes have three major subclasses: JNK1, JNK2, and JNK3; each of these has its own isoforms. JNK1 and JNK2 have four isoforms, while JNK3 has two isoforms.[9] There is 85% sequence identity between JNK families (JNK1 vs. JNK2 vs. JNK3) and more than 90% sequence identity between JNK isoforms (e.g., JNK1α1 vs. JNK1α2).[10] The isoforms differ in N-termini and C-termini length, where JNK1α2 and JNK2α2 are longer. These monomeric proteins both weigh around 47kDa.

JNK proteins are activated within the MAPK protein cascade, involving the following proteins: MAP kinase kinase kinases (MAP3Ks), and MAP kinase kinases (MAP2Ks). These proteins are also called MEKKs and MKKs, respectively. In a linear fashion, activated MEKKs activate MKKs, which then activate JNKs (Figure 1).[11] JNK proteins are activated by MKK4 or MKK7 via a dual phosphorylation of Thr183 and Tyr185, and once phosphorylated, the JNKs phosphorylate specific substrates in the cytosol or the nucleus (e.g., Activating Transcription Factor 2, ATF2), where the signaling pathway continues.[10,12]

Figure 1: Phosphorylation cascade to activate JNK proteins

3. GST-JNK interactions: *In vivo*, in addition to be found in high level in some cancers,[5] GSTpi inhibits JNK apoptotic activity, leading to increased cell proliferation.[13] Aberrant cell proliferation and decreased apoptosis are the hallmarks of cancerous cells. Therefore, studying this interaction in more detail will lead to a better understanding of how GSTpi controls the balance between apoptosis and cell proliferation through modulation of JNK activity, and possibly to a new way to modulate apoptotic pathways in cancer therapy.

Studies have shown that the extended C-terminus of JNK1 and JNK2 is important for GST binding, and thus the longer isoforms (i.e., JNK1α2 and JNK2α2) interact strongest with GSTpi.[14] Interestingly, Haplotype C of GSTpi is the only one that inhibits JNK apoptotic activity *in vivo,* suggesting that this haplotype binds strongest to JNKs.[3] Even though these various active and inactive isoforms of JNK and haplotypes of GSTpi have been shown to have somewhat different affinities for one another, the precise binding affinities for these interactions have not been defined quantitatively. For instance, Thévenin, et al. reported using densitometry analysis of immunoblots that JNK1α2 interacts more strongly with GSTpi than JNK2α2, and that phosphorylation of JNK proteins is essential for interactions with GSTpi.[15] The same authors have also been reported that GSTpi interacts with inactive JNK only if the substrate of JNK, ATF2, is present in solution.[16] However, very recently, De Luca et al. have reported, using changes

in fluorescence spectra, that GSTpi interacts with inactive JNK1α2 even in the absence of ATF2,[17] contradicting the previous findings by Thévenin et al. Thus, there is a serious need for a better characterization of these interactions to clarify the contradicting data. To accomplish this, we take advantage of a relatively novel technique that has shown to provide reliable binding constants between proteins in solution, backscatter interferometry.[18,19]

4. Backscatter interferometry: Backscatter interferometry (BSI) is a novel technique that allows very sensitive analysis of binding interaction between two proteins.[18,19] It relies on the scattering of light to indicate changes in refractive index (RI) within a solution. As illustrated in Figure 2, a laser beam is directed onto a microfluidic channel containing the analytes and is then back-scattered and reflected by a mirror to a charge-coupled device (CCD) detector. An interference fringe pattern is generated from the refraction of light through the glass chip and solvent. The data obtained by the detector is analyzed using a fast Fourier Transform in order to obtain real-time measurements. The generation of the signal is thought to arise from changes in solvation, polarizability, and structural or conformational changes, which changes the RI of the solution.[20] So, by observing the lateral shifts in the fringe patterns, BSI provides a means to indicate changes in solution, namely the binding of molecules. A typical BSI experiment using the glass microfluidic chips is an end-point assay, in which the binding species are premixed and equilibrated prior to injection into the microfluidic chip, with one analyte held at a constant concentration and the second varying in concentration up to excess. With these

experiments, a binding curve can be obtained, and using the rise to max equation for a Michaelis-Menten saturation curve, the dissociation constant (K_D) can be calculated.

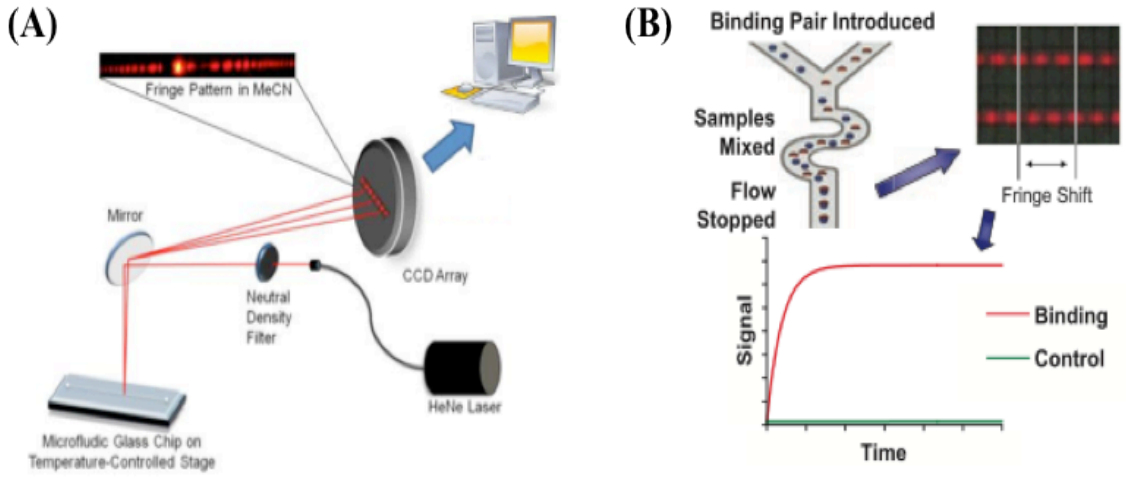

Figure 2: Setup of BSI and typical data output. (A) Experimental BSI setup. (B) Microfluidic chip with serpentine and photo of representative fringe patterns, illustrating the RI-induced position shift of fringes, and a representation of a binding curve obtained.[21]

Unlike other techniques developed to analyze molecular interactions such as surface plasmon resonance, isothermal calorimetry (ITC), and fluorescence-labeled assays, BSI does not require the solutes to be tethered to a surface, labeled or present at high concentration, which can be very time-consuming, expensive and can perturb binding. In addition, due its high sensitivity, BSI only requires very small amount of sample (less than 750 μM in 200 pL).[20] Numerous other studies have already been conducted with BSI, mainly on proteins.[22] For instance, BSI has been used to analyze the binding of the protein calmodulin (CaM) with several small molecule inhibitors, such as calcinuerin (CaN). While the calorimetry results were advantageous in that they yielded a complete

thermodynamic profile, BSI demonstrated the importance of using much lower concentrations for the interaction of CaM with CaN, as these were prone to aggregation at the higher concentrations necessary for ITC.[21]

HYPOTHESIS AND OBJECTIVES

As mentioned, various active and inactive isoforms of JNK and haplotypes of GSTpi have been shown to have different affinities for one another, but the binding affinities for these interactions have only been defined qualitatively. We then propose to use BSI to determine quantitatively the dissociation constants (K_D) for interactions of the GSTpi haplotypes: wild-type and Haplotype C, along with JNK proteins JNK1α2, JNK2α2, and their phosphorylated counterparts. Importantly, since oligomerization can greatly influence protein binding, we will conduct these studies at protein concentrations resulting in monomeric states of the proteins. We believe that BSI is the technique of choice, as only subtle differences are expected and that preparation of large amounts of active kinases can be challenging.

RESEARCH STRATEGY

First, all proteins needed for this study must be expressed, purified, and phosphorylated if necessary. Expression vectors containing the DNA sequences coding for the proteins of interest were a kind gift from Dr. Anastasia Thévenin, and are as follows: JNK1α2/pET15b, JNK2α2/pET15b, His-MKK4/pT75, MEKKC/pBB131, GSTpi-105V-114V/pUC120, and GSTpi-His/pET15b. Prior to over-expression and purification, these plasmids were transformed into BL21(DE3) *E. coli* cells, as these cells showed better protein expression levels compared to other cell types such as pLysS and DH5α (data not shown).

1. Expression and purification of GSTpi, JNK1α2 and JNK2α2: These 3 proteins contain an N-terminal 6x His-tag for purification and are expressed and purified as follows: For the GSTpi construct, 500 mL of lysogeny broth (LB) medium is inoculated with 4.5 ml of BL21(DE3) *E. coli* saturated over-night culture, grown at 37ºC to an OD_{600nm} of 0.6, induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 25°C for approximately 18 hours. JNK1α2 and JNK2α2 production followed a nearly identical process, however the cultures are grown at 18°C after 0.5 mM IPTG induction instead. The cell cultures are then centrifuged and the pellets are resuspended in 5 ml of buffer: 20 mM Tris pH 8, 20 mM imidazole, 150 mM NaCl, 10% glycerol (JNK buffers contain 1mM dithiothreitol) [Lysis Buffer A], and lysed by sonication for 4 min at 4°C. The sonicate is clarified by centrifugation at 10,000 rpm at 4°C for 20 min and applied to a Ni–NTA column that has been pre-equilibrated in the Lysis Buffer A. GSTpi, JNK1α2, and JNK2α2 are then eluted with a linear imidazole gradient using an ÄKTA

purification system (GE Healthcare). The solutions are finally dialyzed to reduce imidazole with: 0.1 M potassium phosphate, 1 mM EDTA, pH 6.6 for GSTpi, and 20 mM Tris, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.6 (Kinase Storage Buffer) for JNKs. Dialysis is accomplished using a 10,000 MWCO dialysis tubing (Thermo Scientific). The purity of the dialyzed proteins is assessed via SDS-PAGE analysis (Figure 3). Measuring the absorbance at 280 nm, the concentrations of the proteins have been determined as: 100 μM for GSTpi, 6.7μM for JNK1α2, and 10.5μM for JNK2α2. Using ImageJ software, purity levels are: >76% for GSTpi, and near 100% for JNKs.

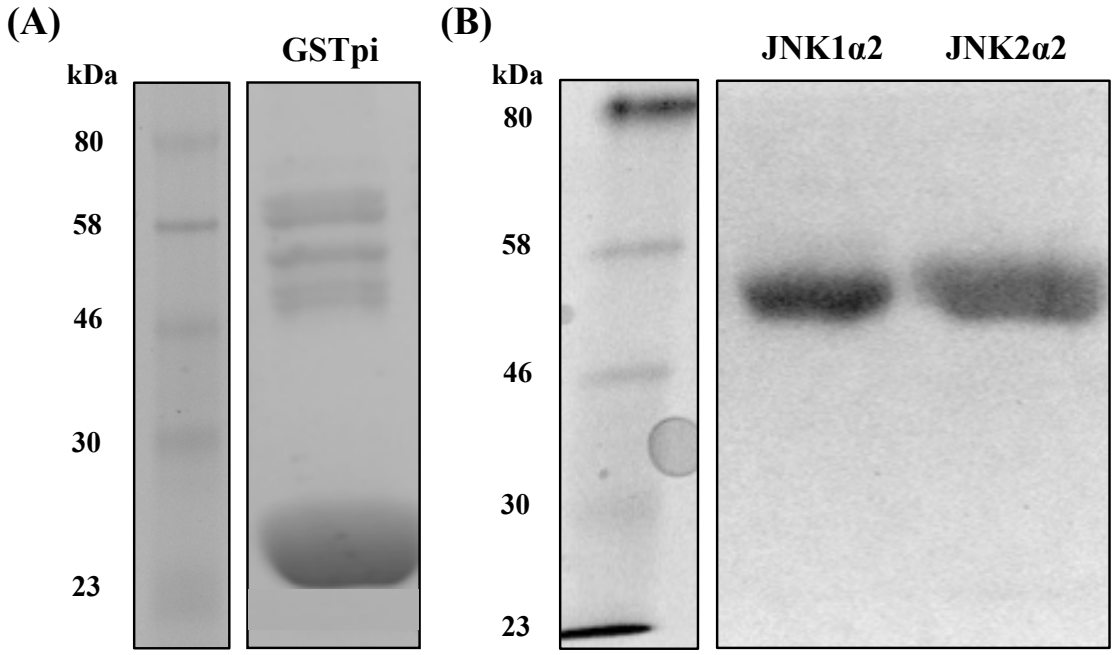

Figure 3: SDS-PAGE stained with Coomassie for GSTpi and JNKs. (A) GSTpi band at 25kDa. (B) Left band is JNK1α2, right band is JNK2α2. Bands are around 47kDa.

2. Expression and purification of MEKKC and MKK4 proteins: As Figure 1 illustrates, JNKs are activated/phosphorylated by the upstream kinase, MKK4, which also must be activated/phosphorylated by MEKK1 to be active. So, to obtain phosphorylated JNKs we

must first co-express MKK4 with its upstream kinase, MEKK1, to produce pMKK4, which is then purified by Ni-affinity chromatography for use in subsequent *in vitro* JNK phosphorylation. Importantly, rather than using native MEKK1, we use MEKKC, which is a constitutively active variant of MEKK1 (i.e., it lacks its regulatory domain). MEKKC and MKK4 are expressed simultaneously in BL21(DE3) cells, following the protocol described above for GSTpi and JNKs. However, upon induction with 0.5 mM IPTG, cells are grown at 37°C for 3 hours, and then pelleted. Cells are then resuspended in 5 mL of: 15 mM imidazole, 1mM DTT, 0.1mM EDTA, Halt protease inhibitor (Thermo Scientific), 150mM NaCl, 10% Glycerol, 1mM Na_3VO_4, 20mM Tris, pH 7.8. Suspended cells are lysed by sonication and purified by nickel column chromatography as previous proteins, however using a linear imidazole gradient in the aforementioned buffer for MKK4 suspension. The protein is finally dialyzed into Kinase Storage Buffer after affinity chromatography. To ensure phosphorylation of MKK4, a western blot is performed using an antibody against phosphorylated MKK4 (pMKK4) at Thr261. Successful purification of pMKK4 is shown in Figure 4.

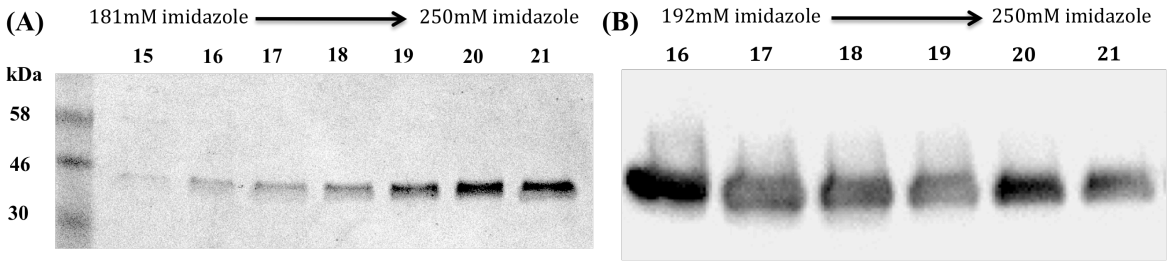

Figure 4: SDS-PAGE and Western Blot images of pMKK4 purification. Numbers above images reflect the fraction collected during nickel affinity chromatography. Corresponding imidazole levels listed above. (A) SDS-PAGE stained with Coomassie. (B) Western Blot using anti-pMKK4 primary antibody of same fractions as (A). Fractions 16 through 21 were pooled together and dialyzed into Kinase Storage Buffer.

3. Activation of JNK proteins: Having obtained pMKK4, activation (phosphorylation) of the purified JNK proteins can be accomplished. First, a small-scale test activation is conducted: 10 μM purified His-JNK is mixed with active 0.3 μM His-pMKK4 in the presence of 10 mM $MgCl_2$, and 6 mM ATP, totaling around 200 μL. The mixture is incubated at 25°C for 3 h. Aliquots (32μL) are removed every 30 min, mixed with 8 μL of 5x SDS sample buffer and boiled for 5 min for subsequent SDS–PAGE and western blot analysis using an anti-phosphorylated JNK antibody. The results shown in Figure 5 indicate a rapid phosphorylation of both JNK1α2 and JNK2α2.

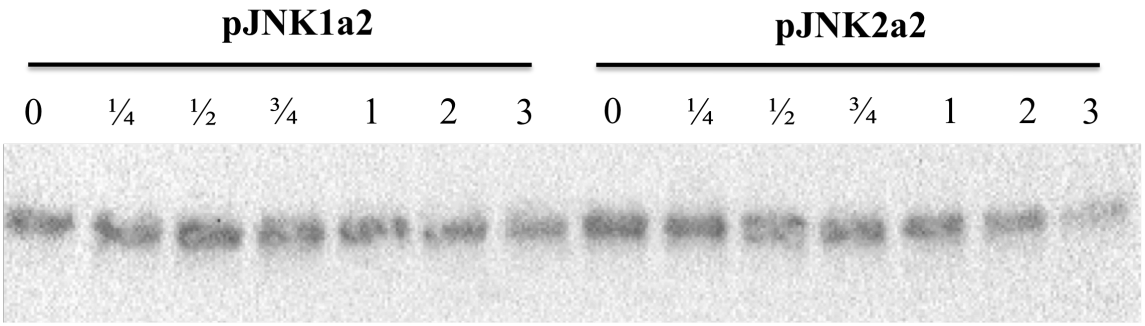

Figure 5: Western Blot (anti-pJNK primary antibody) of JNK activation by pMKK4. For JNK1α2, 2.7μM JNK1α2 and 0.9μM pMKK4 were mixed with 6mM ATP and 10mM $MgCl_2$. For JNK2α2, 4.5μM JNK2α2 and 0.9μM pMKK4 were mixed with 6mM ATP and 10mM $MgCl_2$. Mixture was kept at 25°C. Numbers correspond to time points (hours), at which an aliquot was removed and boiled with SDS dye.

For large-scale activation of JNK, approximately 2 mg of JNK are activated over the course of 2 hours, following the same ratio of reagents as the test-scale activation. After 2 hours, the reaction mixture is dialyzed for 6 hours at 4°C against 15 mM HEPES buffer, pH 7.3, containing 10% glycerol and 1 mM DTT (Cation Exchange Buffer), and then again overnight in fresh Cation Exchange Buffer in order to remove NaCl. Because of differences in the isoelectric points of JNKs (JNK2α2, 5.3; JNK1α2, 5.8) and pMKK4 (~8.3), these proteins can be separated at pH 7.3 using cation exchange chromatography.

Under these conditions, the positively charged pMKK4 binds to the negatively charged CM-52 cation exchange resin, while the negatively charged JNK is eluted in the void volume. The resin is equilibrated first with 150mM HEPES, pH 7.3, then with Cation Exchange Buffer. The mixture is then rocked with the resin at 4°C for 30 min, and the void volume is collected by gravity. The resin is finally washed with three column volumes of Cation Exchange Buffer. Successful separation of pJNK1α2 and pMKK4 is shown in Figure 6. Quantification of pJNK proteins is performed using the Bradford protein assay because ATP present in the mixture absorbs strongly at 260 nm, affecting quantification of protein by UV absorbance at 280 nm. Currently, we have a 1 ml aliquot of 1 μM pJNK1α2.

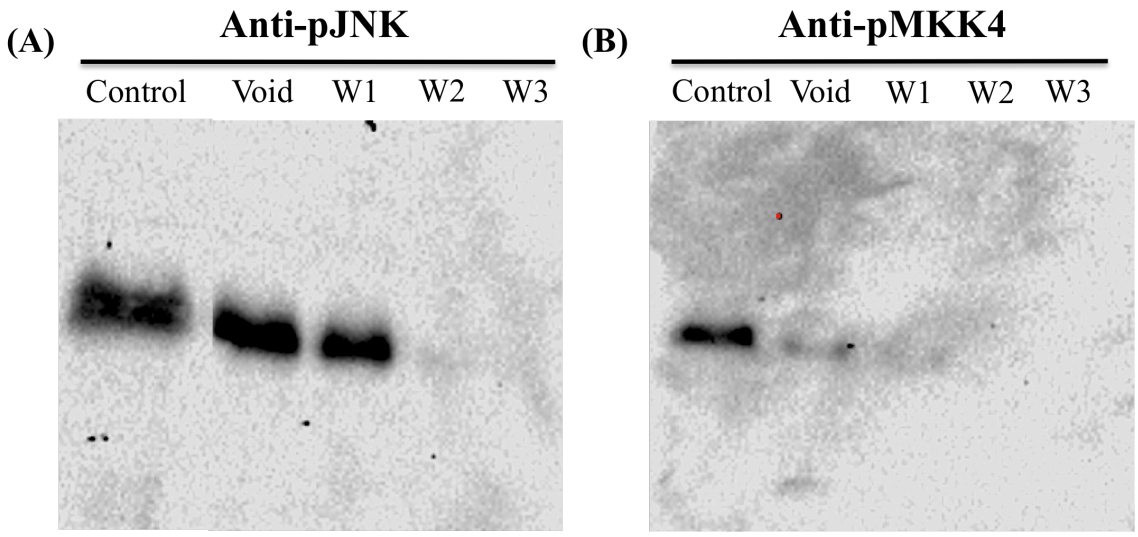

Figure 6: Separation of pJNK1α2 from pMKK4 by cation exchange chromatography. Visualized by immunoblot using (A) anti-pJNK antibody, and (B) anti-pMKK4 antibody. Control is the protein mixture prior to cation exchange. Void corresponds to the void volume. W1, W2, W3 correspond to Wash 1, Wash 2, Wash 3.

So far, we have obtained purified solutions of: GSTpi, JNK1α2, JNK2α2, and pJNK1α2.

4. Preliminary BSI data: For BSI experiments, serial dilutions of the proteins are prepared beginning with the stock solution, and 6 2x dilutions are prepared down from that (i.e., if stock concentration is 10 mM: 10, 5, 2.5, 1.75 mM, etc will be prepared). 2 µL are injected for each data point gathered, and all points are done in repeats of 3, thus 6 µL total of sample is analyzed.

Prior to protein-protein interaction studies, control curves of the proteins will be constructed. These curves are constructed by BSI studies of a protein alone in solution. To date, we have obtained BSI data for GSTpi and JNK1α2 alone in solution (Figure 7 and Figure 8). However, the data is only preliminary as the proteins are in different buffers. Regardless, a linear relationship between concentration and BSI signal is observed for each, which is indicative of a homo-disperse species.

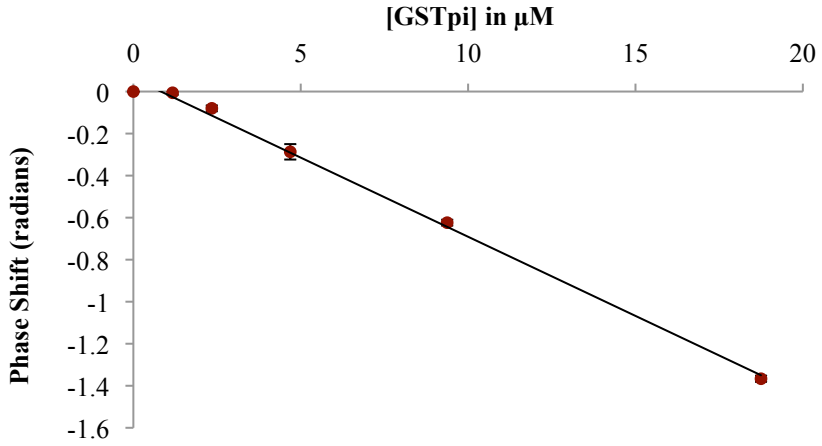

Figure 7: BSI data for GSTpi. GSTpi was diluted in dH_2O. Each data point is the average of 3 measurements, with standard error bars shown. Data points have been minimized to better show error bars.

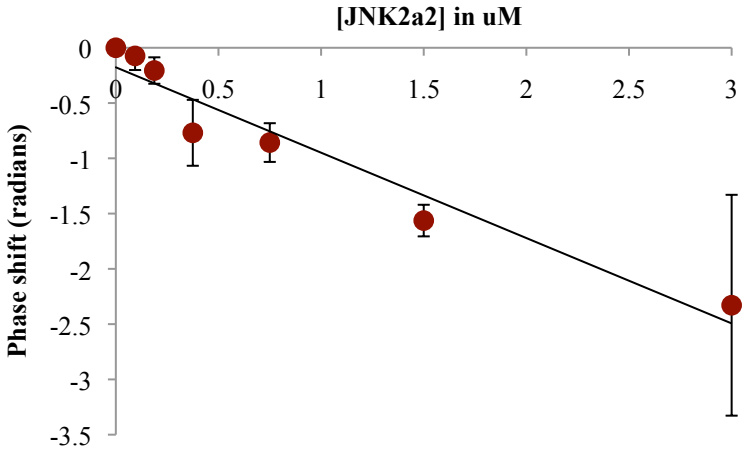

Figure 8: BSI data for JNK2α2. JNK2α2 was diluted in Kinase Storage Buffer (20mM Tris, 150mM NaCl, 10% glycerol, 1mM DTT, pH 7.6). Each data point is the average of 3 measurements, with standard error bars shown.

FUTURE STEPS

1. Using ATF2 to quantify JNK activation: The level of activity of the purified pJNKs will be determined by using ATF2 as a substrate. The experiment will be conducted similarly to the activation of JNKs by pMKK4, by taking aliquots at time points throughout the activation and subsequent analysis via western blotting with an anti-pATF2 antibody. Upon completing this stage, we will have obtained and characterized all necessary proteins to study using BSI.

2. Expression and purification of GSTpi Haplotype C: In the immediate future, we will also express and purify the haplotype of GSTpi, GSTpi-105V-114V using similar methods described above for GSTpi.

3. Determination of binding affinities using BSI: Once all proteins are expressed and purified, we will conduct all BSI combinations shown in Figure 9. In total, 21 runs will be performed to acquire the desired K_D values: Proteins will first be run individually as controls, and then mixed with a constant concentration of another protein to observe the binding curve. For example, using BSI to gather data for GSTpi from 5 μ M to 75 μ M, then running this same concentration range again but each sample also has 10 μ M JNK protein. In order to obtain reliable K_D values, all proteins must be in the same buffer to prevent misleading signals from arising due to different solutes across different buffers. Thus, to keep conditions constant, all proteins will be dialyzed into Kinase Storage Buffer for the study.

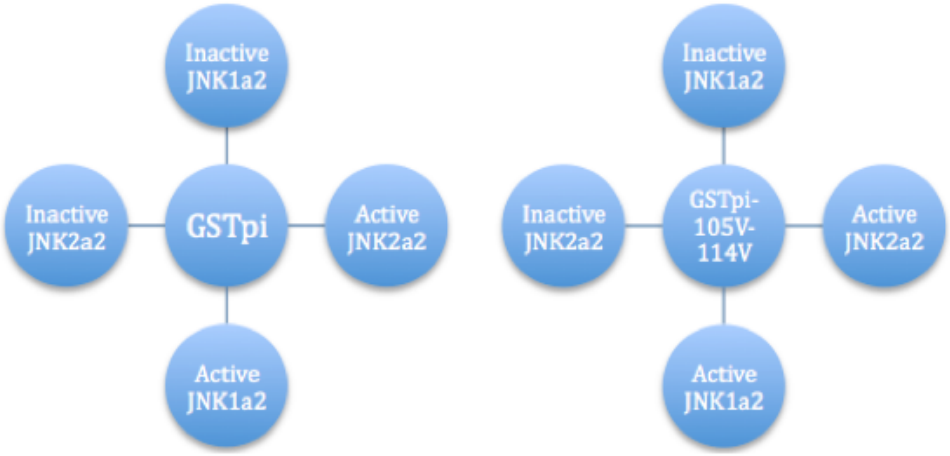

Figure 9: Diagram of the various protein combinations to be studied using BSI. GSTpi and the mutant GSTpi-105V-114V each with 4 combinations of JNK.

The K_D values for each pair will then be determined using the resulting binding curves. The inverse experiment (varying [JNKs] while keeping [GSTpi] constant) will also be performed to compare K_D values. Should issues arise within this portion of the study, the His-tag will be cleaved off GSTpi and see whether it changes the data. Treating the BSI channels with various functional groups (e.g., silano) could also be used to avoid possible protein aggregation on the glass surface. In addition to using the proteins described in this proposal, we can also attempt to express rat His-JNK that is contained in a plasmid with MEKKC/MKK4 already present. Expression of this JNK protein will produce already activated JNK, thus eliminating the bench-top activation step.

4. Using AUC to confirm oligomeric states of proteins: Analytical ultracentrifugation will be used to determine the oligomeric states of proteins at various concentrations. Although the data output from BSI should indicate a change in oligomeric state, as would

seen by a sharp change in the data, analytical ultracentrifugation will be used to show specifically which oligomeric state is present in solution. It also ensures optimal performance for BSI assays, as we want to prevent BSI from picking up self-binding events. By determining which concentrations of the proteins result in monomeric or dimeric states, we can establish a working concentration range where the protein will remain in only one oligomeric form.

5. BSI studies with ATF2 as a JNK substrate: Once the initial BSI study is complete with the aforementioned proteins, studies will be done to examine the effects of ATF2. As reported, ATF2 should promote a greater binding affinity between inactive JNK and GSTpi.[12] Such interactions can be quantified by BSI to determine the accuracy of the report. ATF2 has already been purchased from BPS Bioscience for use with our BSI studies.

OBSTACLES

All research encounters issues along the way, slowing up the collection of data. Some due to human error, while some due to faulty equipment, reagents, etc. Throughout the course of this project, a few obstacles were encountered.

1. Faulty anti-pJNK antibody: The first anti-pJNK antibody (Cell Signaling) we used did not recognize pJNKs. At first, the lack of pJNK bands led to the belief that our sample (testing activation of JNK by pMKK4) simply did not contain pJNK. Upon conducting control experiments with our pMKK4, we confirmed that MKK4 was indeed active. Dr. Lowe-Krentz then provided us with a different anti-pJNK antibody (Santa Cruz), which did reveal pJNK by immunoblot. The subsequent experiments (and the ones presented) were thus conducted the same antibody.

2. CM-52 cation-exchange resin: The cation-exchange resin (kind gift from Dr. Lowe-Krentz) we used to purify pJNK from pMKK4 has also caused issues. The first time using the resin, pJNK1α2 and pMKK4 were successfully separated. However, the next use of the resin to separate pJNK1α2 from pMKK4 resulted in no collection of protein as determined by immunoblot. It led us to believe that the resin was not functioning properly after storage. We are currently repeating these experiments with a new batch of resin.

3. Difficulties with BSI: Finally, it has been difficult to obtain BSI data because the use of the apparatus is limited by the availability of the Flowers' lab, as the apparatus is

under their supervision. Consequently, we must schedule appointments with them to run BSI studies, and the lab members need to have an entire day free to accommodate our samples. As expected, their free time is limited due to priority given to their own experiments.

IMPACT

Obtaining precise binding affinities for these various protein interactions will provide crucial quantitative information regarding the behaviors of the GSTpi-JNK system. Understanding these behaviors can expedite the process to discovering novel ways to manipulate this pathway for therapeutic purposes, given that this pathway involves apoptosis and thus can be linked to cancer. Additionally, success of this goal will help further solidify the validity and usefulness of BSI, a technique that has yet to be popularized despite possessing many benefits.

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VITA

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