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Measuring the Constitutive Activation of c-Jun N-terminal Kinase Isoforms

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Abstract

The c-Jun N-terminal kinases (JNK) are important regulators of cell growth, proliferation, and apoptosis. JNKs are typically activated by a sequence of events that include phosphorylation of its T-P-Y motif by an upstream kinase, followed by homodimerization and translocation to the nucleus. Constitutive activation of JNK has been found in a variety of cancers including non-small cell lung carcinomas, gliomas, and mantle cell lymphoma. *In vitro* studies show that constitutive activation of JNK induces a transformed phenotype in fibroblasts and enhances tumorigenicity in a variety of cell lines. Interestingly, a subset of JNK isoforms was recently found to autoactivate rendering the proteins constitutively active. These constitutively active JNK proteins were found to play a pivotal role in activating transcription factors that increase cellular growth and tumor formation in mice. In this chapter, we describe techniques and methods that have been successfully used to study the three components of JNK activation. Use of these techniques may lead to a better understanding of the components of JNK pathways and how JNK is activated in cancer cells.

1. Introduction

The c-Jun N-terminal kinases (JNK) are a subgroup of the mitogen-activated protein kinases (MAPK) that translate extracellular stimuli into nuclear responses through the phosphorylation of transcription factors. The MAPK signaling modules have been conserved throughout evolution and adapted for various purposes from sporulation in yeast to regulation of the cell cycle in mammalian cells (Chen *et al.*, 2001b). The JNK pathway is characterized by a modular signaling cascade that comprises three phosphorylation events (Fig. 26.1A; Cuenda, 2000; Davis, 1999; Minden *et al.*, 1995; Olson *et al.*, 1995). Upon activation from growth factors or environmental stresses, the serine/threonine MAP kinase kinase kinases (MAPKKK) will phosphorylate and activate a dual specificity MAPKK, so named because they are capable of phosphorylating both threonine and tyrosine. Subsequently, these MAPKK will phosphorylate the threonine and tyrosine of the T-P-Y motif on JNK. After phosphorylation, a portion of the cytoplasmic JNK pool homodimerizes and translocates to the nucleus where it influences gene expression by regulating the

activities of specific transcription factors such as c-JUN, ATF family members, c-MYC, p53, and NFAT4 (Chow *et al.*, 1997; Gupta *et al.*, 1995; Kallunki *et al.*, 1996; Khokhlatchev *et al.*, 1998). JNK signaling can initiate events of cell cycle progression or apoptosis, depending on the stimuli and cell type.

There are three distinct genes encoding JNK, *JNK1*, *JNK2*, and *JNK3*, and 10 different isoforms (Gupta *et al.*, 1996; Fig. 26.1B). The JNK1 and JNK2 proteins are expressed in all tissue, while JNK3 expression is restricted to the brain, heart, and testis (Bode and Dong, 2007; Cuevas *et al.*, 2007). The 10 isoforms are produced by alternative splicing of the *JNK* genes. The α and β forms differ by an alternatively spliced exon in the middle of the transcript, while the -1 versus -2 forms differ by differential splicing at the 3' end. There is now accumulating information that each of the *JNK* genes and isoforms mediates different functions. For example, JNK1 activity is associated with apoptosis and tumor suppression, while JNK2 activity can stimulate cell proliferation and tumor formation (Chen *et al.*, 2001a; Yang *et al.*, 2003). Consequently, each *JNK* gene and individual isoform most likely mediates different functions and there is compelling evidence indicating that JNK activity plays an important role in tumorigenesis (Bogoyevitch, 2006).

Recent research suggests that it is the constitutive activation of specific *JNK* genes that can enhance tumorigenesis within a variety of cancers. For instance, a constitutively active JNK mutant in which an upstream activator kinase was fused to JNK was shown to transform NIH-3T3 fibroblasts (Rennefahrt *et al.*, 2002). In addition, cellular transformation by viral or parasite infection was found to be dependent on constitutive activation of JNK (Wang *et al.*, 2009; Xu *et al.*, 1996). Immunohistochemical studies also demonstrated that JNK is constitutively active in the majority of gliomas (Li *et al.*, 2008), non-small cell lung (Khatlani *et al.*, 2007), mantle cell lymphoma (Wang *et al.*, 2009), and squamous cell carcinomas (Ke *et al.*, 2010). Together these findings indicate that constitutively active JNK proteins can induce tumorigenesis in a variety of cancers.

Additional studies analyzing the role of constitutive JNK activation in cancer revealed that the JNK2 isoforms have the unique ability to auto-phosphorylate and autoactivate (Tsuiki *et al.*, 2003). Several *in vitro* kinase studies have shown that JNK2 can phosphorylate itself on the T-P-Y motif in the absence of upstream kinases and phosphorylate downstream substrates such as c-JUN (Cui *et al.*, 2005; Nitta *et al.*, 2008; Pimienta *et al.*, 2007; Tsuiki *et al.*, 2003). A closer analysis of the mechanism of constitutive activation revealed that JNK2 isoforms initially dimerize, then the bound monomers phosphorylate each other in a *trans*-manner, and finally the phosphorylated homodimer translocates into the nucleus thereby regulating gene expression (Fig. 26.1A; Nitta *et al.*, 2008). Interestingly, these constitutively active JNK2 isoforms are highly expressed in a variety of cancers including brain and non-small cell lung carcinomas suggesting that these constitutively active genes directly lead to altered cell proliferation and tumor formation (Cui *et al.*, 2006; Khatlani *et al.*, 2007).

This chapter will focus on providing protocols and pointers for studying the constitutive activation of JNK isoforms. In order for JNK to be constitutively active, three main events must occur: (1) phosphorylation of the T-P-Y motif, (2) formation of a homodimer, and (3) translocation into the nucleus. Upon activation, JNK then regulates gene expression by

phosphorylating transcription factors. To measure the constitutive activity of JNK, we describe several techniques that can be used to monitor the occurrence of each of these three events *in vitro* and *in vivo*.

2. Important Reagents for Studying JNK Activity

In this section, we describe the reagents that are necessary to accurately measure the constitutive activity of JNK.

2.1. Immunoreagents

The following materials have been especially useful for studying activation of the JNK pathway. Antibodies specific for phosphorylation on the T-P-Y motif of JNK (#9255), phosphorylated c-JUN (#9261), and JNK2 (#4672) are available from Cell Signaling Technology, Beverly, MA. Anti-JNK1 (#sc-1648), -JNK2 (#sc-827), and -JNK3 (#sc-130075) antibodies are available from Santa Cruz Biotechnologies, Santa Cruz, CA.

2.2. Important buffers

- Qiagen His Tag Lysis Buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 100 µg/mL PMSF, 50 mM β-glycerolphosphate, pH 8.0
- Qiagen 6× His wash buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0
- Qiagen elution buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0
- Kinase buffer: 25 mM HEPES (pH 7.4), 25 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM NaVO₄, and 25 mM β-glycerophosphate

2.3. Kinase inactive JNK constructs

To accurately measure the constitutive activity of JNK, a well-known kinase inactive mutant should be used as a negative control. Two common mutations that inactivate JNK are the mutation of the T-P-Y motif to A-P-F and the K55R mutation. The mutation of the threonine to alanine and tyrosine to phenylalanine prevents the phosphorylation of the activation loop and subsequently inhibits JNK kinase activity (Pimienta *et al.*, 2007). The second mutation, K55R, mutates a lysine that is critical for phosphotransfer in the ATP-binding motif thereby inhibiting the kinase activity of JNK. Both mutations are commonly used as inactive JNK mutants and have also been used as dominant negatives for *in vitro* and *in vivo* assays, although exactly which mutant to be used depends on the context of the experiment. When overexpressed, these forms will compete with the corresponding wild type endogenous proteins for access to substrates and activating partners.

3. Protein Expression and Purification of JNK Proteins and c-JUN

In this part, we provide the optimal conditions to express and purify recombinant JNK proteins and a well-known JNK substrate, c-JUN, from *Escherichia coli* using 6× His or GST tags. The protocols are adapted from the Qiagen 6× His tag expression handbook and

GST fusion system from Amersham Biosciences (for additional details please consult the Qiagen and Amersham company webpages). Once the recombinant proteins are purified, they can be used for *in vitro* kinase assays, gel-filtration analysis, and binding assays.

3.1. Expression and purification of bacterially expressed 6× His-tagged JNK protein

Based on our experience, we recommend using the pET28 (Novagen) construct to express 6× His-tagged JNK isoform proteins in BL21(DE3) pLysS *E. coli* strain (Novagen). The following protocol will yield 4–10 mg of purified JNK (Fig. 26.2A).

1. A single colony of transformed *E. coli* is shaken overnight at 37 °C in 5 mL of LB medium containing suitable selection antibiotics.
2. The 5 mL starting culture is added to 200 mL of LB medium containing antibiotics and shaken for another 2–3 h until the OD600 reaches approximately 0.6–0.9. Collect a baseline sample (see below for additional information).¹
3. The culture is chilled on ice for 10 min and isopropyl-β-D-thiogalactopyranoside (IPTG) is added to a final concentration of 0.5 mM and shaken for an additional 3–5 h at 30 °C. Collect an induced sample.¹
4. The bacteria are pelleted, the supernatant removed, and then resuspended in 10 mL of Qiagen His Tag Lysis Buffer. The bacteria are then sonicated on ice for 20 s at the highest power with additional 20 s sonication intervals until the lysate clarifies. The lysate is then centrifuged at 15,000 × g for 20 min. The induced protein should be in the supernatant. Collect an input sample from the supernatant.¹
5. Add 1 mL of the 50% Ni-NTA agarose bead slurry (Qiagen) to the 10 mL of cleared lysate and mix gently by shaking in an end-over-end manner at 4 °C for 1 h to overnight.
6. Load the lysate–Ni-NTA mixture onto a column (Bio-Rad, econo-Pac disposable chromatography column) and allow the lysate to flow through the column. Collect a flow-through sample as a control.¹
7. Wash the column twice with 4 mL of Qiagen 6× His wash buffer.
8. Elute the protein from the column three times with 1.0 mL Qiagen elution buffer and collect into separate tubes.
9. Analyze all column samples by SDS-PAGE followed by staining with Coomassie blue.
10. Combine all the relevant eluates and concentrate the protein using Microcon Ultracel YM-10 (Millipore) and dilute with glycerol to obtain a final concentration of 20% glycerol at 1 mg/mL. Store recombinant protein at –80 °C.

¹To verify that protein expression and purification conditions are optimized, numerous samples should be obtained throughout the procedure. For the uninduced and induced samples, 0.5 mL of the bacteria solution is centrifuged for 30 s at 10 K rpm, after which the supernatant is removed. The remaining pellet is resuspended in protein sample buffer and boiled for 5 min. For the remaining samples, collect 200 μL, add sample buffer, and boil for 5 min.

3.2. Expression and purification of bacterially expressed GST-tagged c-JUN protein

The pGEX (Clontech) construct was used to efficiently express a GST-tagged domain of c-JUN in BL21(DE3)pLysS *E. coli* strain (Novagen). The following protocol will yield 5–10 mg of purified GST-c-JUN (Fig. 26.2B).

Steps 1 and 2 are the same as previously described.

- 3 The culture was chilled on ice for 10 min and isopropyl- β -D-thiogalactopyranoside (IPTG) is added to a final concentration of 0.5 mM and shaken for additional 3–5 h at 37 °C. Collect 500 μ L for an induced sample.¹
- 4 The bacteria are centrifuged and resuspended in cold 10 mL of PBS containing 2 μ g/mL aprotinin, 2 μ g/mL leupeptin, 100 μ g/mL PMSF, and 50 mM β -glycerolphosphate. Bacteria are sonicated on ice for 20 s at highest power with additional 20 s sonication intervals until the lysate clarifies. The lysate is then centrifuged at 15,000 $\times g$ for 20 min. The induced protein should be in the supernatant. Collect an input sample.¹
- 5 Add 1 mL of 50% Glutathione Sepharose 4B slurry (GE Scientific) to the chromatography column (Bio-Rad, econo-Pac disposable chromatography column) and wash with 5 mL of cold PBS to remove residual ethanol.
- 6 Add the sonicated lysate to the column containing the washed Glutathione Sepharose and incubate for 1 h at 4 °C using an end-over-end rotation.
- 7 Allow the lysate to flow through the column. Collect a 500 μ L flow-through sample.¹
- 8 Wash the column by adding 10 mL of 1 $\times$ PBS. Repeat twice more for a total of three washes.
- 9 Elute the fusion protein by adding 0.5 mL of elution buffer. Incubate the column at room temperature for 5 min and then collect the eluate. Repeat three to four times and collect samples in separate tubes.
- 10 Analyze all the samples by SDS-PAGE followed by staining with Coomassie blue.
- 11 Combine all the relevant eluates and concentrate the protein using a Microcon Ultracel YM-10 (Millipore) to a concentration of 1 mg/mL. Add glycerol to obtain a final concentration of 20% glycerol. Store recombinant protein at –80 °C for 6 months.

4. Measuring the Autophosphorylation Ability of the JNK Isoforms

One element of constitutive JNK activity is the ability of JNK proteins to autophosphorylate in the absence of upstream kinases. We have used two different *in vitro* kinase assays to analyze JNK autophosphorylation: a Western immunoblot assay and a radioactive assay. The Western analysis determines whether a JNK protein is being phosphorylated at the T-P-Y motif by using a specific phospho-JNK antibody. This technique is useful since phosphorylation of the T-P-Y motif has been widely shown to regulate the kinase activity of

JNK. However, the use of ^{32}P ATP in the *in vitro* kinase assay is more sensitive than using Western immunoblotting; however, it cannot be used to identify which JNK residues are being phosphorylated. An example of the radioactive *in vitro* kinase is depicted in Fig. 26.2B.

4.1. Western immunoblot *in vitro* kinase assay

1. One microgram of the 6 $\times$ His-JNK fusion protein was incubated in 25 μL of the kinase buffer containing 30 μM ATP at 30 $^{\circ}\text{C}$ for 30 min.
2. The reactions were terminated by adding protein loading buffer and boiling for 5 min.
3. The kinase solution was loaded onto a SDS-PAGE and subsequently transferred onto a nitrocellulose membrane.
4. After protein transfer, the membrane is incubated in Ponceau S solution for 1–2 min to visualize protein levels. Two washes with PBST are done to remove the Ponceau S stain from the membrane.
5. The membrane is then incubated in Blocking buffer (5% milk in PBST (PBS and 0.2% Tween 20)) for 1 h.
6. After washing the membrane with PBST, primary antibodies are diluted in 1% BSA and 0.01% thimerasol in PBST, and incubated at room temperature for 1–2 h or 4 $^{\circ}\text{C}$ for overnight incubations.
7. The membrane is washed three times with PBST, incubated with appropriate secondary antibody in blocking buffer for 30 min at room temperature followed by three additional washes.
8. The membranes are incubated with a chemiluminescence reagent and then developed using Kodak film.

4.2. Radioactive *in vitro* kinase assay

1. One microgram of the 6 $\times$ His-JNK fusion protein is incubated in 25 μL of the kinase buffer containing 30 μM ATP and 5 μCi of γ - ^{32}P ATP (3000 Ci/mmol) at 30 $^{\circ}\text{C}$ for 30 min.
2. The reaction is terminated by adding protein loading buffer and boiling for 5 min.
3. The kinase solution is then loaded onto a SDS-PAGE and subsequently transferred onto a nitrocellulose membrane.
4. After protein transfer, the membrane is incubated in Ponceau S solution for 1–2 min to visualize protein levels.
5. The nitrocellulose membrane is exposed to Kodak X-ray film and developed.

5. Determining the Kinase Activity of the JNK Isoforms

To verify that a JNK protein is constitutively active, we optimized the *in vitro* kinase assay to measure phosphorylation of a well-known JNK substrate c-Jun. This protocol can also be

used to test other potential JNK substrates for their ability to interact with and be phosphorylated by a constitutively active JNK isoform. Previous research has shown that constitutively active JNK2 isoforms can phosphorylate oncoproteins such as Akt (Cui *et al.*, 2006), β -catenin (Wu *et al.*, 2008), Sirt1 (Ford *et al.*, 2008), and Ras (Nielsen *et al.*, 2007) and tumor suppressors such as p53 (Maeda and Karin, 2003; Oleinik *et al.*, 2007). Additional research is ongoing to identify additional JNK substrates that may be altered in cancer.

To measure the kinase activity of the purified 6 $\times$ His-JNK fusion protein toward a new substrate, a similar *in vitro* kinase assay is conducted where 1 μ g of the recombinant protein substrate (such as GST-c-Jun) is added with 1 μ g of the 6 $\times$ His-JNK isoform. As a control to verify that the phosphorylation is specific to the JNK isoform, a kinase dead JNK mutant (K55R) should also be tested. This radioactive kinase assay will quickly demonstrate the ability of the JNK protein to phosphorylate the potential downstream substrate, but in order to specifically identify the residue being phosphorylated, more elaborate methods, such as phosphoamino acid analysis or HPLC/mass spectrometric analysis of peptide fragments, would be required.

6. Monitoring the Formation of JNK Homodimers

Previous research has shown that MAPK homodimerization is an important element of MAPK activity. Extensive studies in *Xenopus* and human cell lines have shown that MAPK exists both as a dimer and a monomer, but upon phosphorylation the MAPK will dissociate from the MAPK kinase, form homodimers, and then translocate into the nucleus (Adachi *et al.*, 1999; Khokhlatchev *et al.*, 1998). We have previously shown that dimerization is necessary for JNK autophosphorylation and nuclear translocation (Nitta *et al.*, 2008). The following protocol will enable the identification of the JNK homodimers.

6.1. Generating a standard curve

Initially a standard curve is established to determine which protein fractions correspond to specific-sized proteins. The following protocol was adapted from the Sigma molecular weight gel-filtration markers protocol.

1. A prepacked gel-filtration column containing 90 mL of Sephacryl S-400 (GE Healthcare) is initially equilibrated by running PBS through the column at a flow rate of 0.2 mL/min at 4 °C. *Note:* The packing of a column is very critical. For additional instructions on packing your own column, refer to the protocol described by GE Healthcare.
2. 1 mL of 2 mg/mL of Blue Dextran (reconstituted in PBS and 5% glycerol) is loaded gently onto the column, and 1 mL fractions are collected.
3. The protein concentration of each fraction is determined by measuring the OD at 280 nm.
4. The fraction that has the highest concentration of the Blue Dextran is known as the void volume (V_o). For example, if the 40th fraction contains the highest concentration of Blue Dextran, then the V_o is 40. We collected 1 mL for each

fraction; consequently the 40th fraction would mean the V_o is 40 mL. The V_o is used to normalize the molecular weight standards.

5. Load 1 mL of 2 mg/mL of a specific molecular weight standard and begin collecting 1 mL fractions.
6. Determine which fraction contains the molecular weight standard by measuring the OD at 280 nm. The fraction that contains the protein is known as the elution volume (V_e).
7. Repeat steps 6 and 7 for each molecular weight standard.
8. Plot the molecular weight versus V_e/V_o for each respective protein standard on semilog paper to derive the standard curve (Fig. 26.3A).

To determine if the 6 $\times$ His-JNK fusion proteins exist as a homodimer or monomer, 1 mg of the 6 $\times$ His-JNK fusion protein (at 1 mg/mL) is applied to the column and 1 mL fractions are collected as previously described. The protein concentrations are determined by measuring the OD₂₈₀ for each fraction to determine the elution volume (V_e). To determine if the 6 $\times$ His-JNK fusion protein is present in the fractions, a fixed amount of each fraction (usually 50 μ l) is run on SDS-PAGE and then stained with Coomassie blue. The size of the protein is determined by plotting the V_e/V_o points on the standard curve. As seen in Fig. 26.3B, 6 $\times$ His-JNK2 α 2 exists in two different states, a dimer and monomer. The 59-mL fraction contained proteins approximately 110–120 kDa in size, consistent with a JNK2 α 2 dimer, while the 67-mL fraction contained proteins 50–60 kDa in size, consistent with a JNK2 α 2 monomer.

7. Measuring Nuclear Translocation of JNK Protein

One hallmark of constitutive JNK activity is the ability to translocate to the nucleus. JNK does not contain an obvious nuclear localization sequence, but studies using the fission yeast *Schizosaccharomyces pombe*, indicate that the yeast homologue of JNK, Spc1, is actively transported to the nucleus by Pim1, a homologue of the guanine nucleotide exchange factor RCC1 (Gaits and Russell, 1999). To determine if the constitutively JNK protein can readily translocate to the nucleus, we have transfected a GFP-tagged JNK protein into a variety of cancer cell lines. The following protocol will efficiently transfect the exogenous protein in cells and enable easy identification of nuclear localization.

7.1. Transfection of cells for studying constitutive activity of JNK Pathway

Certain biochemical studies involving the JNK pathway require high-efficiency transfections. To this end, we were able to obtain high-efficiency transfections using the Mirus TransIT[®]-LT1 Reagent for a variety of cell lines (HEK293, HCC-827, NCI-H2009, and several others).

1. Approximately 24 h prior to transfection, plate cells to obtain a cell density of 50–70% confluence the following day on a 100 mm plate with 10 mL of the appropriate media.

2. In a sterile plastic tube, add 1 mL of serum free media followed by 24 μ L of TransIT[®]-LT1 Reagent. After mixing by gentle pipetting, the solution is incubated at room temperature for 5–10 min.
3. Add 4 μ g of plasmid DNA to the diluted TransIT[®]-LT1 Reagent and mix by gentle pipetting and incubate at room temperature for 10–20 min.
4. Add the TransIT[®]-LT1 Reagent/DNA complex mixture, drop wise to the cells in complete growth medium. Incubate for 24–48 h.

7.2. Immunofluorescence staining of cultured cells

To monitor the nuclear localization of JNK, the following protocol is used. Constitutively active JNK isoforms have a strong nuclear localization compared to the kinase dead JNK mutant (Fig. 26.4).

1. Cells are cultured on coverslips (12 mm diameter grade) and washed with 1 $\times$ PBS to remove residual media.
2. The cells are fixed with 4% paraformaldehyde for 10 min at room temperature, washed with PBS, and then permeabilized with 0.5% Triton X-100 in 1 $\times$ PBS for 10 min and blocked with blocking buffer (1 $\times$ PBS, 0.2% Tween 20, and 10% goat serum) for 30 min at 37 °C. If your cells are tagged with a fluorescent protein, then skip to step 5.
3. 200 μ L of blocking buffer containing the primary antibody is incubated at 37 °C for 1 h, followed by 2–3 washes with the blocking buffer.
4. Fluorochrome-conjugated secondary antibody is applied in 200 μ L of blocking buffer for 1 h at 37 °C.
5. Cells are then washed once with PBS before counter-staining the nuclei with 1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI) dye in PBS, washed twice with PBS, and covered with mounting medium (Gel/Mount, Biomedica Corp.) and a cover glass.

8. Future Directions

8.1. Therapeutic potential of JNK inhibition in human disease

The MAPKs are involved in a variety of diseases and are critical in cell-cycle regulation (Dhillon *et al.*, 2007; McCubrey *et al.*, 2006; Torii *et al.*, 2006). Recently, constitutive activation of JNK has been found in a variety of cancers including non-small cell lung carcinomas, gliomas, mantle cell lymphoma, and squamous cell carcinomas (Ke *et al.*, 2010; Khatlani *et al.*, 2007; Li *et al.*, 2008; Wang *et al.*, 2009). Interestingly, a group of JNK isoforms possess the ability to autophosphorylate rendering them constitutively active. Overexpression of one constitutively active isoform, JNK2 α 2, was found to enhance cell proliferation, anchorage independent growth, and tumor formation in mice (Cui *et al.*, 2006). While the *JNK* genes have been linked to tumorigenesis, they have been shown to influence other diseases as well. For example, JNK has been shown to be involved in Parkinson's, Alzheimer's, diabetes, and renal disease (Kim and Choi, 2010; Yang and Trevillyan, 2008).

These findings suggest that the JNK family is an important target for therapeutic intervention.

8.2. Methods to inhibit JNK

Using a small molecule screen to inhibit JNK activity has been a popular method for elucidating potential JNK targets. Current JNK inhibitors, like SP600125, target the highly conserved ATP-binding sites of JNK (Ishii *et al.*, 2004). Perhaps as a result of targeting this conserved binding site, SP600125 has been shown to inhibit 13 of 30 tested protein kinases, indicating a potential problem with specificity issues (Bain *et al.*, 2003). Consequently, since recent reports demonstrate that each JNK gene and isoform can mediate different functions, the promiscuous nature of SP600125 decreases its potential as a successful therapeutic reagent to study JNK inhibition.

To enhance the specificity of JNK inhibition, non ATP-binding site inhibitors such as BI-78D3, a small molecule mimic of JNK-interacting protein 1 (JIP1), which is a scaffolding protein that binds JNK, have also been used (Stebbins *et al.*, 2008). By targeting a specific binding site located preferentially within *JNK* genes, the specificity of inhibition is increased. However, by targeting these binding partners, only a small subset of the various pathways affected by JNK during disease progression will be successfully inhibited. Since JNK is found to regulate a wide variety of transcription factors in a cell type- or tissue-specific manner, it would be difficult to identify the specific JNK interaction that is the direct cause of tumorigenesis and disease.

Therefore, in lieu of using small molecule and binding partner inhibition, another exciting potential target for JNK inhibition is to block JNK homodimerization. As previously discussed, JNK homodimerization is critical for JNK activity and inhibition of dimer formation was shown to decrease JNK activity, and ameliorates JNK-induced tumorigenesis in glioma cell lines (Nitta *et al.*, 2008; Wilsbacher *et al.*, 2006). By targeting homodimerization of JNK, the need to inhibit effectors or binding partners of JNK to inhibit activity is reduced. Currently, there exists no defined method for blocking homodimerization, but a potential method is the utilization of peptide inhibitors.

Peptide inhibition of JNK is a more specific method to blocking JNK activity. By constructing short peptides against binding sites between JNK binding partners, such as a cell-permeable peptide against the sigma domain for c-JUN, JNK activity is preferentially inhibited (Holzberg *et al.*, 2003). Since the crystal structure of JNK2 and JNK3 suggests that the JNK protein have different binding domains, peptide inhibition could be used to specifically target JNK isoforms thereby reducing cross-reactivity (Shaw *et al.*, 2008; Xie *et al.*, 1998).

The methods described above can be applied to the different JNK genes and isoforms, thanks in part to the similarity across the JNK family of genes. The variety of methods to inhibit JNK action enhances its widespread appeal as a therapeutic target, and its importance in the cellular milieu serves as a desirable target for scientists and clinicians.

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Figure 26.1.

(A) Above: Schematic of the signaling cascade for JNK activation. Below: Model for constitutive JNK activation. (B) Diagram illustrating the 10 isoforms of JNK derived from *JNK1*, *JNK2*, and *JNK3* genes.

Figure 26.2.

(A) Purification of recombinant JNK2 α 2 from *E. coli*. 6 $\times$ His-tagged JNK2 α 2 was purified from cleared bacterial lysate and the samples were run on a SDS-PAGE followed by a Coomassie Blue stain. (B) Radioactive *in vitro* kinase assay using purified 6 $\times$ His-JNK2 α 2 protein.

Figure 26.3.

Measuring JNK homodimerization using size exclusion chromatography. (A) Standard curve using Sigma molecular weight standards. (B) Elution profile of 6x His-JNK2 α 2. The two peaks correspond to the monomeric and dimeric forms of JNK.

Figure 26.4.

Direct immunofluorescence of GFP-tagged JNK constructs in U87-MG cells measuring nuclear translocation. Top: Nuclear localization of the constitutively active GFP-tagged wild type JNK2α2 construct. Bottom: Nuclear localization of the kinase dead GFP-tagged K55R JNK2α2 mutant. Arrows indicate cells with strong nuclear localization (top) or cytoplasmic stain (bottom).