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Activation by phosphorylation and purification of human c-Jun N-terminal Kinase (JNK) isoforms in milligram amounts

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Abstract

c-Jun N-terminal kinases (JNKs) are part of the mitogen activated protein kinase (MAPK) signaling cascade. They are activated through dual phosphorylation of two residues in the activation loop, a threonine and a tyrosine, by MAP2 kinases (MKK4 and 7) in response to various extracellular stresses such as UV or osmotic shock, as well as by cytokines and growth factors. Only small amounts of phosphorylated, active JNKs have previously been produced because of difficulties in expressing these phosphorylated kinases in *E. coli*, which lack the appropriate upstream kinases. We have now established a novel activation and purification method that allows for reproducible production of milligram amounts of active, phosphorylated JNKs suitable for a variety of enzymatic, biophysical and structural characterizations. We utilize N-terminally His-tagged MKK4 that is coexpressed in *E. coli* with a constitutively active form of MEKK1. This phosphorylated, active His-MKK4 is purified by Ni-NTA chromatography and used to phosphorylate milligram amounts of three different isoforms of human JNKs (JNK1 α 1, JNK1 α 2 and JNK2 α 2) that had separately been expressed and purified from *E. coli* in their inactive forms. These *in vitro* activated JNKs are phosphorylated on both residues (T183, Y185) in their activation loops and are active towards their substrate, ATF2.

Keywords

Phosphorylation; MAP Kinase; c-Jun N-terminal Kinases; Signaling Cascade

Mitogen-activated protein kinases (MAPKs)¹ are eukaryotic enzymes that transduce signals sent by cell surface receptors to important intracellular targets, controlling cell proliferation, differentiation, inflammation and apoptosis [1,2]. In mammals, at least three types of MAPKs are known: extracellular signal-regulating kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases [3]. Even though the components of each MAPK cascade vary depending on the MAPK, the overall organization is highly conserved. MAPKs occupy the third tier in the MAPK cascade, below MAP2K (MKKs) and MAP3K (MEKKs, ASK1, MLK), where MAP3Ks activate MAP2Ks, which in turn activate the MAPKs [4,5,6].

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¹Abbreviations used: MAPK, mitogen-activated protein kinase; JNK, c-jun N-terminal kinase; MEKK, MAPK-ERK kinase kinase; MKK4, MAP kinase kinase 4; IPTG, Isopropyl β -D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol; PTP1, protein tyrosine phosphatase 1; ATF2, activating transcription factor 2; GST, glutathione S-transferase; TBS-T, Tris-buffered saline with 0.1% Tween-20.

C-Jun N-terminal kinases (JNKs), one class of MAPK, are activated by various extracellular stress stimuli, such as UV or osmotic shock, as well as by cytokines and growth factors [7,8]. Three JNK genes exist: JNK1, JNK2 and JNK3. Both JNK1 and JNK2 are expressed ubiquitously, while JNK3 is predominantly located the brain, testis and heart tissue [7]. Differential splicing of the JNK genes gives rise to ten isoforms that vary in the length of their N and C termini, as well as in the substrate binding sites and specificities. There are 4 isoforms for JNK1, 4 for JNK2 and 2 for JNK3 [9,10].

JNKs are activated by their upstream MAP2Ks, MKK4 and MKK7, through dual phosphorylation of threonine and tyrosine in the TPY motif found in the activation loop [4,11]. MKK4 and MKK7 are phosphorylated by several different MEKKs (MEKK1-4, ASK1, ASK2, MLK2, MLK3 and many others) [6]. Upon phosphorylation and activation by MKK4 and MKK7, JNKs in turn phosphorylate their cytosolic substrates, or enter the nucleus, where they activate c-Jun, ATF2, Elk1, c-Myc, and p53 transcription factors, leading to increased gene expression and regulation of various cellular processes [6,7,12,13].

For full kinase activity, both T183 and Y185 of the activation loop of JNK must be phosphorylated [14,15]. Experiments both *in vivo* and *in vitro* indicate that MKK4 and MKK7 activate JNKs synergistically, where the tyrosine residue is preferentially phosphorylated by MKK4, while the threonine residue is phosphorylated by MKK7 [15,16,17]. However, JNK phosphorylation experiments *in vitro* showed that higher concentrations of active MKK4 effectively phosphorylated both the Tyr and Thr of JNK. In this case, addition of MKK7 did not produce any additional increase in JNK activity [7,18].

Several approaches have been employed to produce active, phosphorylated JNKs. Since *E. coli* lacks the necessary upstream activators, JNKs have been transiently expressed and purified from human 293T cells that have been appropriately stressed to induce activation of the MAPK cascade and JNK phosphorylation. The main drawback to this method, which relies on mammalian cell culture expression, is the low yield of active MAPKs (usually in µg levels) [19]. Inactive JNK2B2 has been expressed and purified from Sf9 insect cells by utilizing a baculovirus infection method and was later shown to be suitable for a small scale phosphorylation using commercially obtained active MKK4 and MKK7 *in vitro* [20]. Rat JNK2 has also been coexpressed in *E. coli* with the appropriate upstream kinases (MEKKs and MKKs), which allowed for purification of active, His-tagged JNK2 [5]; however, this method was limited to production of only one specific isoform of JNK. Activation of any other JNK isoforms in this expression system would require the replacement of the rat JNK2 gene with a gene of interest, as was done by Niu *et al.*, 2008 to produce active, human JNK2α2 [2].

Here, we describe a modified expression, activation and purification procedure that allows for production of active, phosphorylated human JNK isoforms. Instead of coexpressing the JNK isoforms of interest with upstream kinases in *E. coli*, as described by Khokhlatchev *et al.*, 1997 [5], we coexpressed His-tagged MKK4 with its upstream kinase, MEKK1 and then used this active, purified MKK4 in subsequent *in vitro* JNK phosphorylation experiments. We describe here the activation of three different human JNK isoforms using this method: JNK2α2, JNK1α2 and JNK1α1, and their catalytic activity is compared to rat JNK2 expressed and purified following the expression method of Khokhlatchev *et al.*, 1997 [5]. Because of its ease of use, flexibility and reproducibility, we consider that our method will generally be useful for the production of milligram amounts of any activated JNK isoforms for a variety of enzymatic and structural characterizations.

Materials and Methods

Expression of active rat JNK2 α 2 from *E. coli*

Active rat JNK2 α 2, to be used as a positive control, was expressed in *E. coli* with its upstream kinases MEKK1 and MKK4 [5]. Plasmids containing rat JNK2 α 2, human MKK4 and a constitutively active form of MEKK1 were a kind gift from Dr. Melanie Cobb of University of Texas, Southwestern Medical Center. Briefly, the plasmid (NpT7-5), carrying an ampicillin resistance gene, contains N-terminally His-tagged rat JNK2 α 2 and non-tagged human MKK4, under the control of the T7 promoter. A low copy number pBB-131 plasmid, which has a kanamycin resistance gene, contains the MEKK-C gene (constitutively-active C-terminal kinase domain fragment of rat MEKK-1) [21]. Coexpression of these three proteins in *E. coli* allows for activation of rat JNK2 α 2 *in vivo* using the provided upstream kinases: constitutively-active MEKK-1 phosphorylates MKK4, which in turn phosphorylates JNK2 α 2. Plasmids were transformed into BL21(DE3) cells in 1:1 molar ratio and grown on ampicillin/kanamycin plates containing 100 μ g/ml of each antibiotic. Various conditions (including the ones used by Khokhlatchev *et al.* [5] and by Niu *et al.* [2]), of growth temperature and induction time were screened to obtain optimal yields of active rat JNK2 α 2. The best yields were obtained when 2 L cultures were grown at 37 °C to an OD_{600nm} of 0.6, induced with 0.25 mM IPTG and grown at 37 °C for 4 hours. The cell culture was centrifuged at 8,000 rpm and resuspended in 12 ml of 50 mM NaH₂PO₄ buffer, pH 7.8, containing 300 mM NaCl, 15 mM Imidazole, 10% glycerol, 1 mM Na₃VO₄, 1mM DTT (termed “Lysis Buffer A”), and lysed by sonication. The sonicate was clarified by centrifugation at 10,000 rpm at 4 °C for 20 minutes and applied to a 10 ml Ni-NTA (Qiagen) column that had been pre-equilibrated in the Lysis Buffer A at 4 °C. The column was washed with 800 ml of Lysis buffer A, and rat His-JNK2 α 2 was then eluted using a linear imidazole gradient consisting of 50 ml of Lysis buffer A and 50 ml of the same buffer containing 250 mM imidazole. Eluted rat His-JNK2 α 2 was dialyzed at 4 °C into 20 mM Tris chloride buffer, pH 7.6, containing 150 mM NaCl, 10% glycerol, 1 mM DTT (termed “Kinase Storage Buffer”), and stored in 1 ml aliquots at –80 °C.

Plasmid modification, expression and purification of active MKK4

In the construct described above, the rat JNK2 α 2 gene is flanked by *Nco*-I restriction sites on both 3' and 5' ends. To remove the rat JNK2 α 2 gene, double restriction enzyme digestion was carried out using *Nco*I (Invitrogen). Re-ligation of the digested plasmid (excluding the JNK2 α 2 gene) places the Histidine tag that was upstream of the JNK2 α 2 sequence, in frame with MKK4, allowing for expression of N-terminal Histidine-tagged MKK4. Plasmids were re-ligated using T4 DNA ligase (Invitrogen) and the DNA from resulting colonies was sequenced at the University of Delaware DNA Sequencing and Genotyping Center to ensure proper in-frame translation of His-MKK4. BL21(DE3) *E. coli* cells were transformed with His-MKK4-NpT7-5 and MEKK-C-pBB-131 as described above. A few colonies (3-4) were picked and 2 liters of culture were grown at 37 °C for each colony to OD_{600nm} of 0.6 at 37 °C, then induced with 0.5 mM IPTG and grown at 37 °C for 3 h. Aliquots (50 ml) of each culture were centrifuged separately. The cell pellets from the residual 1,950 ml of cell culture were stored at –80 °C, while the 50 ml pellets were resuspended in 2 ml 20 mM Tris chloride buffer, pH 7.8, containing 150 mM NaCl, 10% glycerol, 1 mM DTT, 0.1 mM EDTA and Halt protease inhibitor cocktail (Pierce). Cell densities were compared by measuring the OD_{600nm}, and additional buffer was added as necessary to ensure equal cell densities among the various samples. Cells were lysed by sonication and sonicates were clarified by centrifugation at 10,000 rpm at 4 °C for 20 min. SDS-PAGE and Western blotting analysis of soluble fractions from different MEKK-C/MKK4 coexpression colonies were carried out in order to select the colonies with the highest level of MKK4 phosphorylation using phospho-threonine-261 MKK4 antibody (Cell Signaling

Technology). Pellets (from 2 L cultures) with the highest levels of MKK4 phosphorylation were then used for subsequent purification of active MKK4. Each pellet was resuspended in 15 ml of 20 mM Tris chloride buffer, pH 7.8, containing 150 mM NaCl, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 1 mM Na₃VO₄ and 15 mM Imidazole ("termed Lysis Buffer B") with Halt protease inhibitor cocktail (Pierce) and was lysed by sonication. Sonicates were clarified by centrifugation at 10,000 rpm at 4 °C for 20 min and the supernatant was placed on a pre-equilibrated 10 ml Ni-NTA column. The column was washed with Lysis Buffer B and then with additional Lysis Buffer B containing 25 mM Imidazole. Active, N-terminally histidine-tagged MKK4 was eluted with Elution Buffer (Lysis Buffer B containing 250 mM imidazole) and was dialyzed into Kinase Storage Buffer. Proteins were stored at -80 °C in 1 ml aliquots.

Cloning of JNK1α1 and JNK1α2 into pET15b vector

Flag-JNK1-pcDNA3 was a generous gift from Dr. Roger Davis (UMass Medical School). DNA sequencing revealed that it encoded the shorter isoform of JNK1: JNK1α1. The cDNA of JNK1α1 is distinguished by three features, as compared to the full-length gene of JNK1α2: a five base pair insertion, a base pair deletion and a base pair substitution. These changes cause a frame shift and introduce a stop codon, leading to translation of a shorter isoform of the protein (JNK1α1 with 379 amino acids, 45 kDa in molecular weight, instead of JNK1α2 with 427 amino acids, 48 kDa) (Source - NCBI database:

<http://www.ncbi.nlm.nih.gov/>). To convert the cDNA of JNK1α1 to that of the longer splicing isoform (JNK1α2), a 3 step PCR overlap extension method was utilized [22]. The following primers were used in this procedure: 1-Forward (1F), 1-Reverse (1R), 2-Forward (2F) and 2-Reverse (2R). In reaction 1, primer 1F was used together with primer 1R. Primer 1F contained a complementary 5' JNK1 sequence along with a *XhoI* recognition sequence, while Primer 1R removed the 5 base pair insertion and introduced an overlap region. In the second PCR reaction, primer 1F and primer 2R were used. Primer 2F corrected for the base pair deletion and substitution, and introduced an overlap region, while Primer 2R was complementary to the 3' end of JNK1 cDNA, and introduced a *BamHI* recognition sequence along with a stop codon. Primer sequences (from 5' to 3') are given below: 1F: AAAAAACCGCTCGAGCGGAGCAGAAGCAAGCGTGACAACAAT; 1R: CTGAGAGCCATTGATCACTGCTGCACCTAAAGGAGAGGGCTGCCCCCG; 2F:GCAGTGATCAATGGCTCTCAGCATCCATCATCATCGTCGTCTGTCAATGATGT GT CTTCA; 2R: AAAAACGGGATCCCGTCATCATCTACAGCAGCCCAGAGGCCAGC.

The PCR reactions were conducted according to the following conditions: 1 minute for denaturation at 94 °C, 2 minutes for annealing at 55 °C and 8 minutes for extension at 50 °C. A total of 25 cycles was used for the PCR reactions. The products of both PCR reactions were run on a 1% agarose DNA gel along with TrackIt 1kb Plus DNA standard (Invitrogen) to check for the size of the products (expected size of PCR1: 1143bp and PCR2: 138bp). The products of PCR1 and PCR2 were subsequently mixed in a 1:1 ratio for the PCR3 reaction. The overlap regions found in both PCR products allowed for annealing of the PCR1 and PCR2 products to one another, and Primers 1F and 2R were used for amplification to yield the full length JNK1α2. The same PCR conditions that were used for PCR1 and PCR2, were also used in PCR3. In all the PCR reactions, JNK1α1-pcDNA3 served as a template and Platinum Taq polymerase (Invitrogen) was used together with MgCl₂ (final concentration: 1.5 mM) and dNTPs (final concentration: 0.2 mM) (Invitrogen). The final product of this reaction was also purified on a 1% agarose DNA gel and compared with a positive control: PCR product obtained by amplifying JNK1α1-pcDNA3 using Primer 1F and Primer 2R. The PCR product of the newly engineered JNK1α2 gene, along with the JNK1α1 cDNA control, were digested with *BamHI* and *XhoI* and inserted into a

pET15b vector (Novagen) that was digested with the same restriction enzymes. T4 DNA ligase (Invitrogen) was used and ligation products were transformed into DH5 α electro-competent cells (Invitrogen), colonies were grown, and DNA was isolated. All samples were sequenced in both directions at the University of Delaware DNA Sequencing & Genotyping Center to identify samples with the correct JNK1 α 1 or JNK1 α 2 sequences.

Expression and purification of inactive JNK2 α 2, JNK1 α 2 and JNK1 α 1

JNK2 α 2-pET15b was a generous gift from Dr. Ze'ev Ronai (Sanford-Burnham Institute for Medical Research, La Jolla, CA). All three constructs: JNK1 α 1, JNK1 α 2 and JNK2 α 2-pET15b were separately expressed and purified following the same protocol. JNK-pET15b constructs were transformed into *E. coli* BL21(DE3) cells (Invitrogen) and 1-liter cultures were grown at 37°C until OD_{600nm} reached 0.6. Protein expression was induced with 0.5 mM IPTG and the cells were grown overnight at 18 °C. Cells were centrifuged and resuspended in Lysis Buffer C: 20 mM Tris chloride, pH 8.0, containing 10% glycerol, 150 mM NaCl, 1 mM DTT and 20 mM imidazole. The cell suspensions were sonicated, clarified by centrifugation and supernatants were loaded on a 10 ml Ni-NTA column pre-equilibrated with the Lysis Buffer C. Na₃VO₄ (a phosphatase inhibitor) was not used since these JNKs were in their unphosphorylated forms. The column was washed with 1 liter of Lysis buffer C and the His-tagged JNKs were eluted using a linear gradient consisting of 50 ml Lysis Buffer C and 50 ml of same buffer containing 250 mM imidazole. Fractions with JNK proteins (as determined by SDS-PAGE analysis) were pooled and dialyzed into Kinase Storage Buffer, and stored at -80 °C in 1 ml aliquots. For the long isoforms of JNK (JNK1 α 2 and JNK2 α 2) it was important to keep protein concentrations below 1 mg/ml, as these isoforms are more prone to aggregation, compared to JNK1 α 1.

In-vitro JNK phosphorylation and isolation of active JNK

The activation of the JNKs was accomplished by incubating 10 μ M His-JNK, 0.3 μ M active His-MKK4, 10 mM MgCl₂, and 6 mM ATP in Kinase Storage Buffer, pH 7.6, in a total volume of 12 ml. The mixture was incubated at 25 °C for 3 hours. Aliquots (30 μ l) were removed every 30 minutes, mixed with 30 μ l of 2x SDS sample buffer and boiled for 5 min for subsequent SDS-PAGE and Western Blot analysis. After 3 hours, the reaction mixture was dialyzed overnight at 4°C against 15 mM HEPES buffer, pH 7.3, containing 10% glycerol and 1 mM DTT (Cation Exchange Buffer) in order to remove NaCl. Because of differences in the isoelectric points of JNKs (JNK2 α 2, 5.3; JNK1 α 2, 5.8; JNK1 α 1, 7.4) and MKK4 (~8.3), these proteins can be separated at pH 7.2 using cation exchange chromatography. Since the predicted isoelectric point for JNK1 α 1 is higher (7.4) than other JNKs, removal of MKK4 by cation exchange was carried out at pH 7.9 for this particular JNK isoform. Under these conditions, the positively charged MKK4 binds to the negatively charged resin, while the negatively charged JNK is eluted in the void volume.

Pre-swollen carboxymethyl cellulose cation exchange resin (20 ml, Millipore) was equilibrated with Cation Exchange Buffer. The dialyzed kinase activation mixture was added to the resin and His-MKK4 proteins were allowed to bind for 30 min at 4 °C by gentle rocking. The resin was then centrifuged at 4,000 rpm and the void volume was collected. The resin was washed a total of 4-5 times with the Cation Exchange Buffer. Samples from the void volume, as well as from the subsequent washes were analyzed by SDS-PAGE for the presence of His-tagged active JNKs. Phosphorylated JNKs were dialyzed into the Kinase Storage Buffer and stored in 1 ml aliquots at -80 °C.

Protein Tyrosine Phosphatase treatment of active JNK1 α 2

Active and pure His-JNK1 α 2 (0.5 μ M) was incubated with 2 μ M protein tyrosine phosphatase 1 (PTP1) (BPS Biosciences) in 50 mM HEPES buffer, pH 7.4, containing 100

mM NaCl, 2 mM EDTA, and 3 mM DTT, for 30 min at 30 °C. Aliquots (30 µl) were removed at various time points, mixed with 30 µl of 2x SDS sample buffer and boiled for 5 min for subsequent SDS-PAGE and Western blot analysis.

Preparation of non-tagged ATF2

N-terminally GST-tagged ATF2 with a PreScission Protease cleavage site between the GST tag and ATF2 was purchased from BPS Biosciences and dialyzed into 50 mM Tris-Cl, pH 7, containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT (Cleavage Buffer). PreScission Protease (GE Healthcare) with a GST tag (but with no cleavage site) was used to cleave GST-ATF2 according to the manufacturer's protocol. The cleavage reaction was carried out for 3 hours at 5 °C. Glutathione-Sepharose resin (GE Healthcare) was equilibrated in Cleavage Buffer and the digestion mixture was added to the resin (1 mg of digested GST-ATF2 fusion for 1 ml of settled resin). The GSTs (both GST-PreScission Protease and that cleaved from GST-ATF2 fusion protein) bind to the glutathione sepharose, which was accomplished by incubation of the digestion mixture with the resin for 30 min at room temperature with gentle rocking. The resin was then centrifuged at 4,000 rpm and the supernatant was collected. Two to three additional washes with Cleavage Buffer (2 ml each) were carried out to collect all unbound, cleaved ATF2 (residues 19-96).

Determination of protein concentration

Protein concentration of untagged ATF2 and active His-JNKs was determined using the Bio-Rad dye reagent diluted according to the manufacturer's protocol, with inactive, His-JNK1α2 as a standard. Protein concentrations of inactive His-JNKs, GST-ATF2 and active His-MKK4 were determined by measuring the absorbance at 280 nm.

SDS PAGE and Western blot analysis

Samples were resolved by SDS-PAGE at room temperature and 140 V on a 12% Tris-Glycine gel, pH 8.8 and transferred to Hybond ECL nitrocellulose membrane (Amersham) using Transfer Buffer (25 mM Tris, 192 mM glycine, pH 8.3, containing 20% (v/v) methanol) in an XCell-II blotting module (Invitrogen) at 60 mA overnight at 4 °C. Membranes were then blocked in 5% dry milk (Fisher Sci.) in Tris-buffered saline, pH 7.6, containing 0.1% Tween-20 (TBS-T buffer) for 1 hour. Membranes were subsequently washed for 10 min in TBS-T buffer 3 times. To test for JNK dual phosphorylation, antibodies specific to JNK phosphorylated at both Thr and Tyr residues (Sigma-Aldrich) (pThr183/pTyr185 for short isoforms of JNK correspond to pThr221 and pTyr223 for long isoforms) were used. To show that Thr 183 was indeed phosphorylated, antibodies specific to Thr183 of JNK (Signalway Antibody) and to phosphorylated threonine followed by a proline (pThr-Pro) (Cell Signaling Technology) were also used. For screening MKK4 phosphorylation levels, antibodies specific for pThr261 of MKK4 (Cell Signaling Technology) were used. To test for phosphorylation of ATF2, antibodies specific for ATF2 phosphorylated at both Thr69 and Thr71 (Sigma-Aldrich) were utilized. In the case of each primary antibody, dilutions of 1:1,000 were incubated with the membrane for 1 hour at room temperature. The membrane was then washed in TBS-T buffer 3 additional times and treated with the appropriate anti-IGg secondary antibody (1:2,000 dilution) tagged with horseradish peroxidase (Cell Signaling Technology) for 30 min at room temperature. After extensive washing (6 times, 10 min each), the membrane was treated for 5 min with ECL Western Blotting substrate (Pierce) and imaged for a 5 min using a luminescence filter on an ImmunoChem™8800 Imager (AlphaInnotech).

JNK enzymatic assays and determination of specific activity

To generate a calibration curve using known concentrations of phosphorylated ATF2, 6 μ M ATF2 was phosphorylated by incubation at 25 °C for 1 hour with 200 nM activated human JNK1 α 2 in 20 mM Tris-Cl buffer, pH 7.6, containing 2 mM ATP, 10 mM MgCl₂, 150 mM NaCl, 10% glycerol and 0.5 mM DTT. Aliquots (30 μ l) were withdrawn at various time points, mixed with 30 μ l of 2x SDS sample buffer and boiled for 5 min. Samples were then separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed using dually phosphorylated ATF2 antibodies. Phosphorylation by JNK1 α 2 for 1 hour allowed for complete phosphorylation of ATF2. Serial dilutions of phosphorylated ATF2 were then used to prepare SDS-PAGE samples of known concentrations, which were separated by SDS-PAGE. Samples were then transferred to a nitrocellulose membrane and probed with antibodies to dually phosphorylated ATF2. Bands were analyzed by ImageJ 1.41o software and data in the linear range was fit to a linear function. The slope of this calibration curve (in luminescence units/ μ M of phosphorylated ATF2) was then used to calculate JNK specific activities (in μ mol of phosphorylated ATF2/min/mg of kinase). Calibration curves were obtained from two independent experiments and averaged to calculate the final slope value. Kinase assay reactions were as follows: ATF2 (2 μ M final concentration) was added to 200 μ L reaction mixture of 20 mM Tris-Cl buffer, pH 7.6, containing 150 mM NaCl, 10% glycerol, 2 mM ATP, 10 mM MgCl₂, 0.5 mM DTT and an appropriate amount of kinase to ensure linearity of ATF2 phosphorylation over a 10 min time course. Concentrations of both rat and human JNK2 α 2 used in the assays were 100 nM, while 25 nM was used for both human JNK1 isoforms. Aliquots (30 μ l) were withdrawn at various time points (from 2-10 min), mixed with 30 μ l of SDS-PAGE sample buffer and boiled for 5 min. Samples were then separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed using antibodies to dually phosphorylated ATF2. Densitometric analysis of samples was carried out and the data was fit to a linear function. The slope of the line (in luminescence units/min) was then used to calculate JNK specific activity. Kinase assays were replicates of at least two independent experiments.

Results and Discussion

To develop an expression and activation method that can be applicable to production of any JNK isoforms, we sought to first obtain active MKK4 and to use it in *in vitro* phosphorylation experiments with JNKs purified separately in their inactive forms (Fig. 1). Development of this *in vitro* activation protocol allowed us to have full control of the phosphorylation reaction and provided good yields of three active JNK isoforms (JNK1 α 1, JNK1 α 2 and JNK2 α 2). Importantly, each isoform obtained was shown to have high specific activities towards the JNK substrate, ATF2.

Expression and purification of inactive human JNK isoforms from *E. coli*

To express human JNK1 isoforms in *E. coli*, the JNK1 α 1 gene from a mammalian expression vector was used. To engineer the long isoform of JNK1 (JNK1 α 2), the JNK1 α 1 gene was modified using the “3 Step PCR” method, as described in Materials and Methods. Introduction of three modifications (a five base-pair insertion, a base-pair deletion and a base-pair substitution) resulted in the gene encoding JNK1 α 2. The JNK1 α 1 and JNK1 α 2 genes were then separately cloned into a pET15b vector in order to produce N-terminally Histidine-tagged proteins in *E. coli*. All human JNKs were separately expressed using a pET15b vector in BL21(DE3) *E. coli* cells and purified by Ni-NTA chromatography in high yields (18-50 mg/liter of culture) (as recorded in Fig. 2C) and >99% purity, as shown by SDS-PAGE (Fig. 2B). Large amounts of soluble JNK protein were observed after cell lysis and only small amounts were not able to bind to the Ni-NTA resin and were lost in the flow-through (Fig. 2A). The 5 kDa molecular weight difference between the short and long

isoforms of human JNK can be seen in Fig. 2B; His-JNK1 α 1 is 45 kDa, while both His-JNK1 α 2 and His-JNK2 α 2 are 50 kDa. The long isoform of JNK1 (JNK1 α 2) was not as stable as the short isoform (JNK1 α 1), and it tended to precipitate if concentrations above 1 mg/ml were used. Its aggregation-prone nature can be seen in Fig. 2B, as it was the only purified JNK that exhibited a weak dimer band at around 100 kDa on a reducing SDS-PAGE.

Active, rat JNK2 α 2 was expressed in *E. coli* with its upstream kinase activators as was described by Khokhlatchev *et al.* [5] but following an expression and purification protocol developed by Niu *et al* [2]. In addition, colonies were screened using antibodies to dually phosphorylated JNK, and rat JNK2 α 2 was purified by Ni-NTA chromatography from a colony with the highest amount of active protein (data not shown). Purified rat JNK2 α 2 was of high purity (Fig. 2B) and was obtained with a yield of 5-6 mg of active protein/liter of *E. coli* culture (Fig. 2C). It is difficult to compare the yields of active rat JNK2 α 2 produced here, with those of previous workers, since others did not report specific yields of JNK2 α 2 [2,5].

Vector modification, expression and purification of active MKK4

To phosphorylate large amounts of human JNKs *in vitro* and to have full control of the phosphorylation reaction, we sought to produce and isolate phosphorylated, active MKK4. Therefore, the ratJNK2 α 2 gene was excised from NpT7-5 vector, and the vector was religated.

The Histidine tag, originally in frame with ratJNK2 α 2, was now in frame with the human MKK4 gene, creating a Histidine-tagged MKK4-NpT7-5 vector. This modification of the original construct enabled coexpression of His-MKK4 with its constitutively active upstream activator, MEKK-C. Activated His-MKK4 could then be purified by Ni-NTA chromatography (Fig.1). As the expression levels of MKK4 were initially low, several growth temperatures were tested to produce the highest amount of soluble, active MKK4. As shown in Fig. 3A, shorter growth times (3 hours) at 37 °C following IPTG induction proved to give the highest levels of soluble, phosphorylated MKK4, while all overnight cultures gave lower yields of soluble MKK4 and also exhibited proteolysis of MKK4 (Fig. 3A). In the initial experiments, it was noted that levels of active MKK4 varied from colony to colony, leading to subsequent variation in the effectiveness of JNK phosphorylation. Therefore, it was imperative to screen for total phosphorylated MKK4 in the cultures generated from each colony to obtain the highest levels of phosphorylated MKK4 (Fig. 3B). In addition, use of protease inhibitors during MKK4 purification was important, as MKK4 purified in the absence of inhibitors exhibited high levels of proteolysis (Fig. 3B) and very low activity towards JNK (data not shown). These screening steps allowed us to obtain MKK4 with reproducible phosphorylation efficiencies to be used in subsequent JNK activation reactions.

Activation of Human JNKs by active MKK4

Activation of human JNK isoforms was achieved by incubating purified, inactive Histidine-tagged JNKs with low μ M concentrations of active MKK4. Prior to larger scale experiments, small volume pilot activation experiments were carried out for each JNK. These were then scaled up to 12 ml reaction mixtures, as described in Materials and Methods. As can be seen from Fig. 4 (panels A-C), all three human JNK isoforms reached full phosphorylation by 3 hours. Activation was monitored by Western blotting using two different sets of antibodies: 1) specific for JNKs phosphorylated at both Thr-183 and Tyr-185 in the activation loop; and 2) antibodies specific for JNKs phosphorylated at Thr-183. Since others have noted that MKK4 has preference for the tyrosine residue, while

MKK7 has a preference for the threonine residue [16], we felt that additional Western blot analysis with antibodies against phospho-threonine was necessary to demonstrate that threonine was indeed phosphorylated in our activation reactions with MKK4 alone. Full phosphorylation of all three JNKs after 3 hours was observed using these antibodies (Fig. 4A-C). Moreover, we have monitored our activation reactions using antibodies specific to phosphorylated threonine followed by a proline (pThr-Pro). Since the activation loop of JNK contains a Thr-Pro-Tyr motif, these antibodies were suitable for monitoring phosphorylation of JNK by MKK4 at Thr-183, as exemplified by the results for JNK1 α 2 and JNK1 α 1 in Fig. 4D (Fig. 4D). In addition, to show that tyrosine was phosphorylated in the activated JNK products, JNK1 α 2 was incubated with Protein Tyrosine Phosphatase 1 (PTP-1) and subsequent Western blotting analysis was carried out using antibodies against doubly-phosphorylated JNK to monitor dephosphorylation of Tyr 185. A drastic decrease in dual JNK phosphorylation levels was observed after treatment with PTP-1, demonstrating that Tyr 185 had been phosphorylated by MKK4 (Fig. 4E). To remove the active His-tagged MKK4 from our target protein product, active His-JNKs, we took advantage of the difference in the isoelectric points of MKK4 and JNK and used cation exchange chromatography. As evaluated by SDS-PAGE stained with Coomassie Blue (Fig. 5), the samples of active human JNK isoforms obtained from CM52 resin were of high purity. Final yields of 3-5 mg of active human JNK isoforms were obtained from each activation reaction. However, the amount of JNKs activated by MKK4 could be scaled up in future experiments, if needed.

Determination of JNK specific activity

To measure specific activity of JNKs, ATF2 was used as a substrate. ATF2 (residues 19-96) is commercially available as an N-terminal fusion with GST. Commercially obtained GST-tagged ATF2 is expressed and purified as a full-length fusion, as well as a slightly shorter molecular weight fusion truncated at the ATF2 C-terminus. GST is a relatively large protein with a subunit molecular weight of about 24,000 Da and has been shown to occur in a monomer-dimer equilibrium [23]. We were concerned that the presence of the GST-tag may affect the measured activity, and chose to use untagged ATF2 by cleaving the N-terminal GST fusion with PreScission protease. Removal of both the GST tag and the PreScission protease was straightforward, since the protease itself had a GST tag, allowing for simple separation of cleaved ATF2 with Glutathione-Sepharose resin. Cleavage and removal of the GST-tag leads to production of untagged ATF2. Like the original commercial GST-ATF2 fusion, the untagged ATF2 consists of two molecular weight species (one containing residues 19-96 of ATF2, and the second several amino acids shorter at the C-terminus). However, both species were able to become phosphorylated on Thr-69 and Thr-71 (Fig. 6A). Therefore, both ATF2 bands were included in all densitometry data analyses.

To calculate ATF2 phosphorylation by JNK as specific activity (in μmol of ATF2/min/mg of kinase), we generated a calibration curve of relative luminescence intensity of known concentrations of phosphorylated ATF2 using antibodies specific to dually phosphorylated ATF2 (Fig. 6A and 6B). Samples representing the linear range of phosphorylated ATF2 (from 0.03 μM to 0.4 μM) were fit to a linear function to determine the slope ($80,350 \pm 6,000$ luminescence units/ μM of phosphorylated ATF2, $r^2 = 0.96$) to be used in specific activity calculations for activated JNK isoforms (Fig 6B). The specific activity of human JNK1 α 2 was the highest of all the isoforms tested: 13.4 ± 0.3 $\mu\text{mol}/\text{min}/\text{mg}$ (Fig. 6 C-D and Table 1). Furthermore, the specific activities of two separate preparations of active JNK1 α 2 were very similar (13.2 ± 2.0 and 13.8 ± 1.6 $\mu\text{mol}/\text{min}/\text{mg}$), showing that activation of JNK using this method is reproducible. Until now, the specific activity and the k_{cat} of active JNK1 α 2 have never been reported.

The specific activity of the short isoform of JNK1 (JNK1 α 1) was 11.4 ± 0.2 $\mu\text{mol}/\text{min}/\text{mg}$ with a k_{cat} value of 8.6 s^{-1} (Table 1). This isoform has been previously characterized by others with a k value of $0.13 \text{ s}^{-1} \text{ cat}$ [9]. This previous result is more than 60-fold lower when compared to the k_{cat} reported here. The preparation of JNK1 α 1 used by Ember and LoGrasso, was expressed in Sf21 insect cells and was obtained commercially by the authors [9]. It is likely that the *in vitro* activation of JNK1 α 1 in the present study is more efficient and provides more highly phosphorylated kinase as compared to that achieved in an insect cell expression system.

The specific activity of human JNK2 α 2 was comparable to rat JNK2 α 2 (3.8 ± 0.2 and 4.5 ± 1.0 , respectively) (Table 1). Notably, the activities of both rat JNK2 α 2 and human JNK2 α 2 reported here are higher than those reported by others [2,5]. Khokhlatchev *et al.* reported the specific activity for rat JNK2 α 2 coexpressed with MKK4 and MEKK in *E. coli* to be $1.5 \mu\text{mol ATP}/\text{min}/\text{mg}$ of kinase [5], while Niu *et al.* reported the human JNK2 α 2 specific activity to be $7.5 \text{ pmol of phosphate}/\text{min}/\text{pmol of kinase}$ ($1.2 \mu\text{mol ATP}/\text{min}/\text{mg}$ of kinase) with the k_{cat} value of 0.51 s^{-1} [2]. The former used GST-tagged c-Jun, while the latter used GST-tagged ATF2. Since these specific activities were reported in terms of ATP and we report our values in terms of ATF2, previously reported value of 1.2 - $1.5 \mu\text{mol ATP}/\text{min}/\text{mg}$ of kinase would equal 0.6 - $0.75 \mu\text{mol of ATF2 or c-Jun}/\text{min}/\text{mg}$ of kinase. Therefore, our specific activities and k_{cat} values are about 6-fold higher than those reported previously. One possible reason our JNK2 proteins are more active could be the fact that we used an untagged substrate, rather than a GST-tagged substrate, which may exist in dimeric form [23]. In addition, rat JNK2 α 2 coexpressed with MKK4 and MEKK-C in *E. coli* is more active, potentially because we carried out a series of colony screens by Western blot analysis for those producing the highest amount of phosphorylated JNK2. We then selected the colony with an optimized expression of phosphorylated MKK4 to be used in subsequent purification by Ni-NTA.

While crystal structures of low activity JNK1, JNK2 and JNK3 have been solved, no crystal structure of JNK in its active, phosphorylated form exists [8,13,24]. Lack of crystal structures of active JNKs is partly because there has been no activation method that is generally applicable to activation of JNK isoforms. Here, we describe an activation method that will be generally suitable for activation of any JNK isoform. We were able to successfully produce three highly active and pure full-length isoforms of JNK: JNK1 α 1, JNK1 α 2 and JNK2 α 2. Our activation method was equally effective for all three JNK isoforms tested.

Since the active JNK isoforms described here were full length, they were only stable below concentrations of $1 \text{ mg}/\text{ml}$. We were able to obtain sufficiently high amounts of active JNKs suitable for a variety of biochemical and biophysical studies. However, these full-length kinases may not be appropriate for crystallographic studies, where high mg concentrations (1 - $10 \text{ mg}/\text{ml}$) are required. To produce stable and homogeneous JNK proteins that are well-behaved at high protein concentrations, others have already tested various truncations and mutations of human JNKs during crystallization trials [8,25]. Therefore, we believe that our activation method, when used on inactive JNKs that have been pre-screened to yield successful crystals, will lead to the generation of crystal structures of active JNK isoforms in the future.

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Fig. 1.

Scheme for expression, purification and activation of human JNKs to yield active, pure enzymes. Plasmids containing His-tagged JNK isoforms were separately transformed into *E. coli*, expression was induced with IPTG and inactive His-tagged JNK was purified using Ni-NTA chromatography. In parallel experiments, plasmids containing constitutively active MEKK1 (MEKK-C) and His-tagged MKK4 were transformed into *E. coli*, expression was induced with IPTG, and active, His-tagged MKK4 was purified. Purified, inactive, His-tagged JNK was then incubated with purified, active His-MKK4 along with $MgCl_2$ and ATP at 25 °C. Mixtures of activated, His-tagged JNK and His-tagged MKK4 were then placed on CM52 cation exchange resin to remove His-MKK4 and to obtain pure and active His-JNK.

Fig. 2.

A. Coomassie Blue stained SDS-PAGE gel of inactive, His-JNK1α2 to demonstrate JNK protein levels in various lysate and Ni-NTA flow-through fractions. Lane 1, positive control – purified human inactive His-JNK2α2; lane 2, total cell lysate; lane 3, insoluble fraction; lane 4, soluble fraction; lane 5, soluble fraction diluted 5-fold; lane 6, early flow-through from the Ni-NTA column; lane 7, late flow-through from the Ni-NTA column. B. SDS-PAGE gel of c-Jun N-Terminal kinases (JNKs) purified using Ni-NTA chromatography. The gel was stained with Coomassie Blue. Lanes 1-3 show inactive, human, His-tagged JNKs (Lane 1, JNK1α1; lane 2, JNK1α2; lane 3, JNK2α2). Lane 4 shows His-tagged, active rat JNK2α2 coexpressed in *E. coli* with MKK4 and MEKK-C, after purification by Ni-NTA chromatography. C. Purification yields (in mg/liter of culture) of JNK proteins shown in panel B.

Fig. 3.

Western blot analysis for active, phosphorylated MKK4. His-tagged MKK4 was coexpressed with MEKK-C to produce active His-MKK4. Antibodies against MKK4 phosphorylated at Thr-261 were used to monitor expression levels of active MKK4. A. Expression screen at various temperatures (after IPTG induction) to obtain the highest levels of active and soluble MKK4. B. Screening of three *E. coli* cultures expressing both MEKK-C and MKK4. After IPTG induction, *E. coli* was grown at 37 °C for 3 h and levels of phosphorylated MKK4 were assessed in the soluble fraction of the culture from each colony. Comparison of active MKK4 purified in the presence and absence of protease inhibitors during cell lysis and Ni-NTA chromatography, is also shown.

Fig. 4. Phosphorylation of human JNK isoforms by active MKK4. A-C, Western blotting analysis of activation reactions using antibodies against dually phosphorylated JNK (Thr-183 and Tyr-185) or against JNK phosphorylated at Thr-185. Activation reactions (10 μ M inactive JNK and 0.3 μ M active MKK4) were incubated at 25 °C for 3 h. A, Activation of JNK2α2; B, Activation of JNK1α2; C, Activation of JNK1α1. D, Western blotting analysis of unphosphorylated, inactive (I), and phosphorylated, active (A) JNK1 isoforms (His-JNK1α2 and His-JNK1α1) using antibodies against phosphorylated Thr followed by Pro (pThr-Pro). E, Protein Tyrosine Phosphatase 1 (PTP1) treatment of active, His-JNK1α2 to demonstrate the presence of phosphorylation on Tyr-185 of JNK. Active JNK1α2 was incubated with PTP1 for 30 min at 30 °C and the dephosphorylation of Tyr-185 was monitored by Western blot with antibodies against dually-phosphorylated JNK (Thr-183 and Tyr-185). Coomassie Blue staining is also shown to demonstrate total protein amounts of His-JNK1α2 and of PTP1 in the dephosphorylation reaction.

Fig. 5. SDS-PAGE of human c-Jun N-terminal kinases (JNKs) activated *in vitro* by incubation with active, His-MKK4, and after removal of His-MKK4 using CM52 cation exchange resin. The gel was stained with Coomassie Blue. Lane 1, His-JNK1α1; lane 2, His-JNK1α2; lane 3, His-JNK2α2.

Fig. 6. Phosphorylation of ATF2 by active JNKs and determination of JNK specific activity. **A.** Representative Western blot of fully phosphorylated ATF2 samples (in serial dilutions) probed with antibodies specific to dually-phosphorylated ATF2 (Thr-69 and Thr-71). To produce fully phosphorylated ATF2, untagged ATF2 (6 μM) was incubated with 200 nM active JNK1α2 for 1 h until full saturation. **B.** Phosphorylated ATF2 calibration curve using densitometry analysis results from **A**. Densitometry data was fit to a linear function and the slope (in Luminescence units/μM of phosphorylated ATF2) was used in subsequent specific activity calculations. The data is the average of two independent kinase reactions and Western blots. **C.** Representative activity assays of activated and purified human His-JNK1α2 and His-JNK1α1. 25 nM active JNK was incubated with 2 μM untagged ATF2 in assay buffer containing 6 mM ATP and 10 mM MgCl for 10 min at 25 °C. Phosphorylation levels of ATF2 over time were monitored by Western blot with antibodies specific to dually-phosphorylated ATF2. **D.** Plots of ATF2 phosphorylation rates by JNK1α2 and JNK1α1 as determined by densitometry analysis of Western blots shown in **C**. Data was fit to a linear function and the slopes (in Luminescence units/min) were used to calculate JNK specific activity using the slope value determined in **B**. For each JNK, the data is an average of two independent kinase assays and Western blots.

Table 1 Specific activity and k_{cat} values for active JNK isoforms.

MAP Kinase	Specific Activity ^a ($\mu\text{mol ATF2/min/mg}$)	k_{cat} ^b (s^{-1})
Human JNK1α2	13.4 $\pm$ 0.4	11.2 $\pm$ 0.3
Human JNK1α1	11.4 $\pm$ 0.2	8.6 $\pm$ 0.2
Human JNK2α2	3.8 $\pm$ 0.2	3.2 $\pm$ 0.7
Rat JNK2α2	4.5 $\pm$ 1.0	3.6 $\pm$ 0.8

^a Specific activities were calculated from at least two independent experiments. The errors shown are standard errors.

^b The k_{cat} values were calculated by converting specific activity values from μmol of ATF2/min/mg of kinase to μmol of ATF2/min/ μmol of kinase and dividing by 60 seconds.