Identification of c-Jun NH$_2$-terminal Protein Kinase (JNK)-activating Kinase 2 as an Activator of JNK but Not p38*

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Xianghuai Lu, Shino Nemoto, and Anning Lin‡
From the Department of Pathology, Division of Molecular and Cellular Pathology, the University of Alabama at Birmingham, Birmingham, Alabama 35294

c-Jun NH$_2$-terminal protein kinase (JNK), a distant member of the mitogen-activated protein (MAP) kinase family, regulates gene expression in response to various extracellular stimuli. JNK is activated by JNK-activating kinase 1 (JNKK1), a dual specificity protein kinase that phosphorylates JNK on threonine 183 and tyrosine 185 residues. Here we show that JNKK2, a novel member of the MAP kinase kinase family, was phosphorylated and activated by MEKK1, a MAP kinase kinase kinase in the JNK signaling cascade. JNKK2 activity was also stimulated by constitutively active forms of Rac and Cdc42Hs, members of the Rho small GTP-binding protein family. Unlike JNKK1 that activates both JNK and p38 MAP kinases, JNKK2 stimulated only JNK. Transient transfection assays demonstrated that JNKK2 potentiated the stimulation of c-Jun transcriptional activity by MEKK1. The existence of multiple JNK-activating kinases may contribute to the specificity of the JNK signaling cascade.

The mitogen-activated protein (MAP) kinase family is an essential part of the signal transduction machinery and occupies a central position in cell growth, differentiation, and transformation (1–3). To date, several mammalian MAP kinases have been identified, including extracellular signal-regulated kinase (ERK) (4, 5), c-Jun NH$_2$-terminal protein kinase (JNK), also known as stress-activated protein kinase (SAPK) (6–8), and p38 (also known as Mkp2/CBSP) (9–11). Each MAP kinase belongs to a distinct MAP kinase module which consists of a MAP kinase kinase kinase, a MAP kinase kinase (MKK), and a MAP kinase. The MAP kinase kinases in the ERK signaling cascade are MAPK/ERK kinase (MEK) 1 and 2 (12–14), and the MAP kinase kinase kinases include Raf-1, Mos, and MEK kinase (MEKK) 1 (15–19). In the JNK signaling cascade, the MAP kinase kinase is JNK-activating kinase (JNKK) 1 (also known as SAPK/ERK kinase, SEK, and MKK4) (20–22), and the MAP kinase kinases are the MEKKs (23–25). In the p38 signaling cascade, the MAP kinase kinases include JNKK1, MKK3, and MKK6 (20, 22, 26, 27). The MAP kinase kinase kinases for p38 may include MEKK1, TAK1, and Ask (28–30). These individual MAP kinase modules may provide a structural basis for different signaling cascades to relay extracellular stimuli to specific effectors.

A challenge in understanding the mammalian MAP kinase cascade is how signaling specificity is achieved. Despite the MAP kinase modules, cross-talk still exists. The cross-talk may allow the cells to coordinate the activity of different signaling cascades to produce a specific physiological response. However, it also makes the signaling cascades prone to lack of specificity. Other mechanisms are needed to ensure the specificity of each MAP kinase cascade, including subcellular localization, specific associated proteins, high enzymatic specificity, and selective responsiveness to extracellular stimuli.

The JNK cascade is activated by various stimuli such as growth factors, cytokines, tumor promoters, protein synthesis inhibitors, ultraviolet (UV) light irradiation, and oncogenes (1). JNK in turn stimulates the activity of several transcription factors including c-Jun, ATF-2, Elk, and Sap-1 (6–8, 31–33). It is not completely understood how the specificity of the JNK cascade is maintained. One plausible mechanism is through the existence of multiple JNKKs that have high substrate specificity and respond to distinct upstream signals. To test this hypothesis, we have isolated a novel JNK-activating kinase, JNKK2, which is a close homologue of JNKK1. In contrast to JNKK1, which is selectively expressed in skeletal muscle and brain, JNKK2 is expressed in many tissues examined. Unlike JNKK1 which activates both JNK and p38, JNKK2 stimulated only JNK. Furthermore, JNKK2 did not respond to several extracellular stimuli that activate JNKK1. These data suggest that JNKK2 is a specific JNK activator and may contribute to the specificity of the JNK cascade.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, 2 mm glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin.

cDNA Cloning—A human JNKK1 cDNA (20) was used as a probe to isolate JNKK1 homologues by screening a ZAPII HeLa cDNA library (Stratagene) at low stringency. Thirty positive clones were obtained after screening 2 × 10$^8$ phage. These clones were grouped and sequenced on both strands by the dyeoxy chain termination method using Sequenase Version 2.0 (U. S. Biochemical Corp.). Nucleotide sequence comparison of clones 4 and 23 with the GenBank database reveals five sets of nucleotide sequences (GenBank accession numbers AA194047, AA019720, AA194193, AA258025, and AA252650) that are nearly identical. These clones were partially sequenced by the Washington University-Merck EST project. The clones AA194047 and AA019720 were requested and completely sequenced.

cDNA Constructs—The JNKK2 expression vector was constructed by inserting a polymerase chain reaction-generated NcoI-BglII fragment encoding JNKK2 into NcoI and BglII sites of pSR3-hemagglutinin (HA) vector (20). To construct pGEX-KG-JNKK2, the BglII fragment of the NcoI-BglII fragment of JNKK2 was blunted, and the fragment was inserted between the NcoI site and blunted HindIII site of pGEX-KG vector. A Chameleon mutagenesis kit (Stratagene) was used to re-
Cloning of JNKK2

Fig. 2. Activation of JNKK2 by activated MEKK1, Rac, and Cdc42Hs but not UV and anisomycin. A, HeLa cells were transfected with pSR3-HA-MEKK2 (1 μg) without expression vectors of MEKK1 or the indicated small GTPases (0.5 μg each). HA-JNKK2 was immunoprecipitated, and its activity was measured by kinase assays with GST-JNK1 as a substrate. Fold stimulation is indicated. An aliquot of each sample was analyzed for expression of HA-JNKK2 by Western blot analysis using an anti-HA antibody (Santa Cruz). B, HeLa cells were transfected with pSR3-HA-MEKK1 or empty vector (0.5 μg each). HA-MEKK1 was immunoprecipitated, and its activity was measured by kinase assays with GST-JNKK2 or GST-JNKK2 (S272A/T276A) as a substrate. C, HeLa cells were transfected with expression vectors of HA-JNKK1 (top panel) or HA-JNKK2 (middle panel) (1 μg each). After 48 h, the cells were treated with UV (80 J/m², 20 s) or anisomycin (Aniso.) (50 ng/ml, 15 min) or left untreated (C, control). The activity of HA-JNKK1 or HA-JNKK2 was determined as described in A. Endogenous JNK (bottom panel) was isolated from JNKK2-transfected HeLa cells by immunoprecipitation with an anti-JNK1 antibody (PharMingen), and its activity was measured by kinase assays with GST-c-Jun as a substrate.

RESULTS

Isolation of JNKK2 cDNA—Sequence analysis revealed that the JNKK2 clone encodes a complete open reading frame which appears to be a novel MAP kinase kinase (Fig. 1). The new MAP kinase kinase is closest to hep, a Drosophila JNK-activating kinase (36) (56.2% identity), followed by human JNKK1 (20) (42.4% identity), based on the comparison of all known MAP kinase kinases (PILE-UP program, Wisconsin Genetics Computer Group). Northern blot analysis revealed that unlike JNK1, which is expressed mainly in skeletal muscle and brain, JNKK-2 is widely expressed in many tissues, with the highest level of expression in skeletal muscle (data not shown), suggesting that the two JNK activators may have different roles in different cells.

JNKK2 Can Be Activated by MEKK1, Rac, and Cdc42Hs—We and others have shown that MEKK1 acts as a MAP kinase kinase kinase which directly phosphorylates and activates JNK1, p38, MKK6, MEK1, ERK2, c-Jun, and ATF2 have been described (AA). The expression vectors of MEKK1, Rac1, Rac2, Cdc42Hs, RhoA, place Ser-272 and Thr-276 with alanines, to create pGEX-KG-JNKK2 JNKK2 is AF006689. The protein sequence is presented in single-letter code. The GenBank™ accession number of JNKK2 is AF006689.

Fig. 1. Primary structure of JNKK2. Shown is the cDNA sequence and deduced amino acid sequence of JNKK2. The protein sequence is presented in single-letter code. The GenBank™ accession number of JNKK2 is AF006689.
The specificity of the JNK cascade may also be achieved by limiting the responsiveness of JNKKs to a certain subset of upstream stimuli. It has been shown that various extracellular stimuli and proto-oncogenes stimulate JNK activity, some of which do so through activating JNKK1. JNKK2 can be significantly activated by MEKK1, Rac, and Cde42/Hs, which are activators of JNKK1 (Fig. 2A). However, JNKK2 was only weakly activated by UV and anisomycin (Fig. 2C), both of which strongly activate JNKK1 (Fig. 2C). Osmotic stress stimulates JNKK1, but only weakly stimulated JNKK2 activity (data not shown). The differential response of JNKK1 and JNKK2 to extracellular stimuli suggests that signaling leading to JNK activation may diverge upstream of JNK at the level of JNKKs. It is possible that JNKK2 may mediate the activation of JNK by extracellular stimuli that stimulate only JNK but not p38. Identification of extracellular stimuli that specifically activate JNKK2 should provide insights into the specificity of the JNK signaling pathway.

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FIG. 3. JNKK2 selectively phosphorylates and activates JNK but not p38 or ERK2. A. HeLa cells were transfected with pSRα3-HA-JNKK2 (1 μg) together with pSRα3-MEKK1 (0.2 μg) or with pCMV5-HA-MEK1 (EE) or pcDNA3-HA-MKK6 (1 μg each) expression vectors. After 48 h, the cells transfected with MKK6 were irradiated with UV (80 J/m², 20 s). The transfected kinases were immunoprecipitated, and their activities were determined by kinase assays with GST-JNK1, GST-p38, and histidine-ERK2 as substrates, as indicated. B. MEKK1-activated HA-JNKK2 was isolated as described in A, and its activity was measured by kinase assays with GST-JNK1 wild-type (WT), GST-JNK1 (APY), and GST-JNK1 (APP) mutants as substrates. C. MEKK1-activated HA-JNKK2 was isolated as described in A, and its activity was measured in a coupled kinase assay with GST-c-Jun as a substrate. D. HeLa cells were transfected with expression vectors encoding M2-JNK1 and M2-p38 (1 μg each), HA-JNKK2 (2 and 3 μg), and MEKK (20 ng), as indicated. M2-JNK1 or M2-p38 were immunoprecipitated with anti-M2-Flag antibody, and their activities were measured by kinase assays with GST-c-Jun or GST-ATF2 as substrates, respectively. Phosphorylated GST-c-Jun shows a slower migration in the SDS gel, as indicated.

JNKK activation, as measured in a coupled kinase assay (Fig. 3C).

The effect of JNKK2 on JNK in vivo was examined in transient transfection assays. In HeLa cells, cotransfection of JNKK2 stimulated M2 Flag-tagged JNK1 (M2-JNK1) activity (Fig. 3D). The stimulation by JNKK2 was further potentiated by a suboptimal amount of cotransfected MEKK1 (Fig. 3D). Under the same conditions, JNKK2 or JNKK2 plus MEKK1 did not stimulate M2-p38 activity (Fig. 3D). These data demonstrate that JNKK2 is a specific activator of JNK in vivo.

JNKK2 Potentiates the Stimulation of c-Jun Transcriptional Activity by MEKK1—To determine the effect of JNKK2 on c-Jun transcriptional activity, HeLa cells were cotransfected with the active form of MEKK1 and GAL4-c-Jun fusion protein (20). MEKK1 stimulated GAL4-c-Jun activity 18-fold, as measured by a luciferase reporter gene driven by a GAL4 responsive promoter (Fig. 4). Coexpression of wild-type JNKK2 potentiated the stimulatory effect of MEKK1 on GAL4-c-Jun, resulting in 28-fold activation (Fig. 4). Neither MEKK1 alone nor MEKK1 plus JNKK2 stimulated the activity of GAL4-c-Jun Ala-63/73, in which both Ser-63 and Ser-73 have been replaced with alamines (Fig. 4). These results demonstrate that JNKK2 participates in stimulation of e-Jun transcription activity.

FIG. 4. JNKK2 potentiates c-Jun transcriptional activity. HeLa cells were cotransfected with a 5 × GAL4-Luc reporter plasmid (1 μg) and expression vectors of GAL4-c-Jun(1–223), GAL4-c-Jun(1–223) (Ala-63/73), MEKK1, JNKK1, and JNKK2 (10 ng each). Luciferase activity was determined as described (20). The averages of three experiments are shown. Luciferase activity expressed by cells transfected with pSRα was given an arbitrary value of 1.

DISCUSSION
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