A Caenorhabditis elegans JNK signal transduction pathway regulates coordinated movement via type-D GABAergic motor neurons

Masato Kawasaki, Naoki Hisamoto1, Yuichi Iino2, Masayuki Yamamoto2, Jun Ninomiya-Tsuji and Kunihiro Matsumoto

Department of Molecular Biology, Graduate School of Science, Nagoya University, and CREST, Japan Science and Technology Corporation, Chikusa-ku, Nagoya 464-8602, and 2Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, PO Hongo, Tokyo 113-0033, Japan

1Corresponding author

e-mail: i45556a@nucc.cc.nagoya-u.ac.jp

The c-Jun N-terminal kinase (JNK) of the MAP kinase superfamily is activated in response to a variety of cellular stresses and is involved in apoptosis in neurons. However, the roles of the JNK signaling pathway in the nervous system are unknown. The genes for the Caenorhabditis elegans homolog of JNK, JNK-1, and its direct activator, JKK-1, were isolated based on their abilities to function in the Hog1 MAP kinase pathway in yeast. JKK-1 is a member of the MAP kinase kinase superfamily and functions as a specific activator of JNK. Both jnk-1 and jkk-1 are expressed in most neurons. jkk-1 null mutant animals exhibit defects in locomotion that can be rescued by the conditional expression of JKK-1 in mutant adults, suggesting that the defect is not due to a developmental error. Furthermore, ectopic expression of JKK-1 in type-D motor neurons is sufficient to rescue the movement defect. Thus, the C.elegans JNK pathway functions in type-D GABAergic motor neurons and thereby modulates coordinated locomotion.

Keywords: Caenorhabditis elegans/GABAergic motor neurons/JNK MAP kinase pathway/locomotion

Introduction

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases which have been shown to function in a wide variety of biological processes (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). MAPKs are activated by tyrosine and threonine phosphorylation in response to a range of extracellular signals and are regulated via a protein kinase cascade. Both phosphorylation events are catalyzed by a family of dual-specificity MAPK kinases (MAPKKs). MAPKKs are in turn phosphorylated and activated by a family of upstream MAPKK kinases (MAPKKKs). Each of these upstream components plays a role in multiple cell signaling processes.

Three subgroups of the MAPK superfamily have been identified (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998): the extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase (JNK, also known as SAPK) and p38. Distinct amino acid motifs found in the activating phosphorylation site distinguish these three subgroups: TEY for the ERK family, TPY for JNK, and TGY for p38. Furthermore, several subgroups of the MAPKK superfamily have been identified, such as MEK1/MKK1, MEK2/MKK2, MKK3, MKK4/SEK1/JNKK1, MKK6 and MKK7/JNKK2 (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). The ERK group is activated by MEK. While MKK4 can activate both the JNK and p38 subgroups, MKK7 is specific for the JNK subgroup. On the other hand, MKK3 and MKK6 act solely as an activator for the p38 group. These members of the MAPKK superfamily are activated by phosphorylation, catalyzed by members of the MAPKKK superfamily such as Raf, MEKK, TAK1, MLK, Tpl2 and ASK1.

Much has been learned from genetic and biochemical studies of the ERK pathways. In vertebrate cells, Raf MAPKKK triggers the ERK cascade downstream of Ras guanine nucleotide-binding protein, which itself is activated by growth factors that signal through receptor protein tyrosine kinases. Thus, the Raf-MEK-ERK cascade appears to be a component of various growth-promoting pathways (Robinson and Cobb, 1997). In invertebrates, the corresponding MAPK pathway has been elucidated through the genetic analysis of Drosophila and Caenorhabditis elegans, which have proven to be excellent organisms for the genetic analysis of cell signaling. In the Drosophila eye, the MAPK pathway consists of D-Raf (MAPKKK), D-sor1 (MAPKK) and Rolled (MAPK), and this cascade mediates receptor tyrosine kinase signaling which ultimately regulates the differentiation of R7 photoreceptor cells (Zipursky and Rubin, 1994; Wassarman et al., 1995). In C.elegans vulva development, the MAPK pathway mediates the induction of vulval cell fates and includes the factors LIN-45 Raf (MAPKKK), MEK-2/LET-537 (MAPKK) and MPK-1/SUR-1 (MAPK) (Sundaram and Han, 1996).

In contrast to the ERK MAPK pathway, the role of the JNK pathway is less well understood. In vertebrate cell culture systems, the JNK cascade can be activated by a variety of genotoxic or environmental stresses such as alkylating reagents, UV, ionizing radiation and osmotic stress, or by inflammatory cytokines such as tumor necrosis factor α and interleukin 1 (Kyriakis and Avruch, 1996; Ip and Davis, 1998). In most cases, in vitro activation of the JNK cascade primarily inhibits cell growth or induces cell death (Verheij et al., 1996). For example, withdrawal of nerve growth factor from differentiated PC12 cells results in JNK activation and apoptosis (Xia et al., 1995). However, activation of the JNK cascade also has been associated with cell differentiation, cell proliferation and tumorgenesis (Kyriakis and Avruch, 1996; Ip and Davis,
1998). Furthermore, it has been proposed that the JNK cascade may play an important physiological role in neuronal function (Xu et al., 1997). Thus, the biological consequences of JNK activation may depend on cell type and could differ depending on the in vitro and in vivo conditions. Recent genetic studies of Drosophila have demonstrated that the JNK pathway is required for early embryonic development (Noselli, 1998). Two components of the Drosophila JNK (D-JNK) pathway have been identified: D-JNK kinase encoded by hemipterous (hep) (Glise et al., 1995) and D-JNK encoded by basket (bsk) (Riesgo et al., 1996; Sluss et al., 1996). In the absence of Hep or Bsk function, lateral epithelial cells fail to stretch and the embryo develops a hole in the dorsal cuticle. This pathway corresponds to the mammalian MKK7-JNK pathway. The involvement of the JNK pathway in dorsal closure is further emphasized by the observation that mutants for D-jun, a target of D-JNK signaling, fail to complete dorsal closure (Noselli, 1998).

To understand the biological function of the JNK pathway in a genetically amenable multicellular organism, we have undertaken a genetic analysis of the JNK signaling pathway in C.elegans. Here we report the identification of JNK-1, the C.elegans homolog of JNK, and its direct activator, JKK-1, a member of the MAPKK superfamily. jnk-1 and jkk-1 are expressed both in the cell bodies and the axons of most neurons. We found that disruption of the jkk-1 gene caused defects in locomotion, and present evidence that JKK-1 modulates coordinated movement in C.elegans as a result of its role in the function of type-D GABAergic neurons.

Results

Isolation of a C.elegans JNK homolog

The yeast Hog1 MAPK pathway plays a central role in mediating cellular responses to increases in external osmolarity. This signaling cascade consists of the Ssk2, Ssk22, Pbs2 and Hog1 kinases (Figure 1A) (Maeda et al., 1995; Sprague, 1998). Ssk2 and Ssk22 are functionally redundant kinases which are homologous to the mammalian MAPKKK. The downstream target of Ssk2 and Ssk22 is the Pbs2 kinase, which shares high sequence identity with MAPKK. Furthermore, the Hog1 kinase, which has been demonstrated to function downstream of Pbs2, is closely related to MAPK. Thus, high osmolarity triggers a kinase signaling cascade consisting of the Ssk2/Ssk22, Pbs2 and Hog1 kinases, in that order. This leads to the induction of the GPDI gene encoding glycerol-3-phosphate dehydrogenase, and consequent increased synthesis of glycerol, the principal osmolyte (Albertyn et al., 1994). Mammalian JNK has been shown to complement the high osmolarity-sensitive (Osm+) growth phenotype of a hog1Δ mutant (Galcheva et al., 1994). Thus, these components are functionally conserved among species, raising the possibility that yeast defective in the Hog1 pathway may be a useful experimental system with which to identify components involved in the C.elegans JNK pathway.

To identify possible C.elegans JNK homologs, a C.elegans cDNA library was transformed into a yeast hog1Δ mutant and transformants were screened for suppression of the Osm+ phenotype (Figure 1B). Of the $1 \times 10^5$ transformants screened, a total of 33 positives were obtained, and the plasmids recovered from this screen were assigned to two classes based on restriction enzyme analysis. The nucleotide sequence of one class showed that it contained cosmid K11H3.1 encoding glycerol-3-phosphate dehydrogenase. We determined the nucleotide sequence of the second class and found that it encodes a 463 amino acid protein containing the protein kinase subdomains I–XI (Figure 2). Sequence comparisons demonstrated that this C.elegans kinase is most similar to the human JNK3 (70% identity) (Figure 2). Thr276 and Tyr278 residues are found at positions comparable to those found in all MAPKs, where they function as sites of MAPKK phosphorylation and consequent MAPK activation. A distinguishing feature of all MAPKs is the presence of a three-residue sequence found in the activation domain: TPY in the case of JNKs, TEY for ERKs and TGY for p38 kinases (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). By this criterion, this C.elegans kinase appears to belong to the JNK subgroup of the MAPK superfamily, and we therefore termed this protein JNK-1 to indicate that it is a JNK homolog.

Isolation of MAPKK for JNK-1

Although JNK-1 can functionally complement the yeast hog1Δ mutation, it was not clear whether this involves the activation of JNK-1 by Pbs2 located upstream, or the unregulated activation of targets located downstream of JNK-1. To address this issue, we asked whether expression of JNK-1 was able to suppress the osmoregulation defect in yeast associated with loss of Pbs2. A pbs2Δ mutant was transformed with a plasmid that expresses JNK-1, and transformants were tested for their ability to grow in the presence of sorbitol. We found that expression of JNK-1 did not suppress the pbs2Δ defect (Figure 1B), suggesting that Pbs2 is required for the activation of the C.elegans JNK-1. This raised the possibility that the yeast system could be used to identify the C.elegans MAPKKs that activate JNK-1 (Figure 1A).

To identify this upstream kinase, we transformed a pbs2Δ mutant expressing JNK-1 with a C.elegans cDNA library and screened for suppression of the pbs2Δ Osm+ phenotype. We obtained a total of 20 transformants, from among $5 \times 10^5$ screened, capable of growth in the presence of sorbitol. Of these 20 candidates, 11 clones failed to restore sorbitol resistance in the pbs2Δ mutation in the absence of JNK-1 expression, indicating that they suppressed the pbs2Δ mutation in a JNK-1-dependent manner (Figure 1B). These plasmids were of four classes, as determined by restriction enzyme analysis. One class of cDNAs encodes a protein kinase of 435 amino acids that is homologous to members of the MAPKK superfamily and which contains the two characteristic phosphorylation sites required for MAPKK activation (Figure 3). We named it JKK-1 for JNK-1 activator kinase. Suppression of the pbs2Δ phenotype by JKK-1 specifically required JNK-1, as shown by the fact that suppression was not observed when JKK-1 was co-expressed with PMK-1 (corresponding to B0218.3; DDBJ/EMBL/GenBank accession number U58752), a C.elegans p38 homolog that can also complement the Osm+ phenotype of a hog1Δ mutant (Figure 1B). These results suggested that JKK-1 can function specific-
ally in the yeast Hog1 pathway by activating JNK-1, but not PMK-1.

**JNK-1 is a specific activator of JNK**

To determine whether JKK-1 can activate JNK-1, 293 cells were co-transfected with mammalian expression vectors encoding Flag epitope-tagged JKK-1 (Flag-JKK-1) and HA epitope-tagged JNK-1 (HA-JNK-1). HA-JNK-1 was then immunoprecipitated from cell lysates and used in a protein kinase assay with glutathione S-transferase (GST)-c-JUN protein as a substrate. The c-Jun transcription factor is known to be phosphorylated by JNK in mammalian cells (Su *et al*., 1994). As shown in Figure 4A, transfection with JKK-1 resulted in strong activation of JNK-1. Transfection with a kinase-inactive form of JKK-1, in which Lys149 in the ATP binding domain has been mutated to Arg, did not result in JNK-1 activation. This indicates that the kinase activity of JKK-1 is required for activation of JNK-1. Western blot analysis showed that the mutant was expressed at levels comparable to that of the wild-type JKK-1. To examine further the interaction between JNK-1 and JKK-1, we tested the ability of JNK-1 to co-immunoprecipitate with JKK-1 in transfected 293 cells. However, Flag-JKK-1 was not detected in HA-JNK-1
immunoprecipitates (data not shown), suggesting that JKK-1 may not form a stable complex with JNK-1.

We next investigated one aspect of the substrate specificity of JKK-1 by asking if PMK-1 was activated by JKK-1. To do this, we co-expressed Flag-JKK-1 in 293 cells by transient transfection together with HA epitope-tagged PMK-1 (HA-PMK-1). The kinase activity of PMK-1 was determined by immunocomplex kinase assays with GST–ATF2 as a substrate. The co-expression of JKK-1 did not enhance PMK-1 activity (Figure 4A). These results are consistent with the failure of JKK-1 to activate PMK-1 in the yeast Hog1 MAP kinase pathway (Figure 1B).

To investigate further the substrate specificity of JKK-1, we tested the activity of JKK-1 toward mammalian JNK and p38 MAPKs. 293 cells were transiently transfected with Flag-JKK-1 together with HA epitope-tagged JNK or p38. The HA-tagged MAPKs were immunoprecipitated from cell extracts and their kinase activities were measured in vitro using specific substrates (GST–c-JUN and GST–ATF2, respectively). We found that JKK-1 stimulated the kinase activity of JNK but not of p38 (Figure 4B). These results support the idea that JKK-1 can function as a specific activator of JNK.

Expression patterns of jnk-1 and jkk-1 genes

To physically map the positions of jnk-1 and jkk-1 on the chromosome, we used each cDNA as a probe to hybridize a C.elegans yeast artificial chromosome (YAC) library. This analysis localized jnk-1 to the left arm of chromosome I and jkk-1 to the left arm of chromosome X. Sequence information which became available from the C.elegans Genome Consortium during the course of this study showed that jnk-1 and jkk-1 correspond to B0478.1 and F35C8.3, respectively. Comparison of the sequences between the database genomic DNA and the cloned cDNA revealed that the jnk-1 and jkk-1 genes each have 12 exons (Figure 5A).

To determine the expression patterns of JNK-1 and JKK-1, we constructed translational fusions between jnk-1 and jkk-1 and green fluorescent protein (GFP) to generate jnk-1::gfp and jkk-1::gfp (Figure 5A), respectively. Transgenic C.elegans bearing the jnk-1::gfp fusion exhibited fluorescence in most or all of the neurons and their processes, including the nerve ring, the head ganglions, the dorsal and ventral nerve cords, and the tail ganglions (Figure 5B). This fusion gene was expressed in all stages of development. Similar expression patterns were observed in transgenic animals harboring the jkk-1::gfp fusion (Figure 5B). Both the jnk-1::gfp and jkk-1::gfp fusion
Fig. 4. Activation of JNK-1 and JNK by JKK-1. (A) Activation of C.elegans JNK-1 by JKK-1. 293 cells were transfected with Flag-JKK-1, Flag-JKK-1(K149R) (Flag-JKK-1-KN), HA-JNK-1, HA-JNK-1(K148R) (HA-JNK-1-KN) (left panel), and HA-PMK-1 (right panel) as indicated. Immunoprecipitated complexes obtained with anti-HA were used for in vitro kinase reactions with GST–c-JUN for HA-JNK-1 or GST–ATF2 for HA-PMK-1 as a substrate (upper panel). The amounts of immunoprecipitated HA-JNK-1 or HA-PMK-1 were determined with anti-HA (middle panel). Whole cell extracts were also immunoblotted with anti-Flag (bottom panel). (B) Activation of mammalian JNK1 by JKK-1. 293 cells were transfected with Flag-JKK-1, Flag-JKK-1(K149R) (Flag-JKK-1-KN), HA-JNK1 (left panel), and HA-p38 (right panel) as indicated. Immunoprecipitated complexes obtained with anti-HA were used for in vitro kinase reactions with GST–c-JUN for HA-JNK1 or GST–ATF2 for HA-p38 as a substrate (upper panel). The amounts of immunoprecipitated HA-JNK1 or HA-p38 were determined with anti-HA (middle panel). Whole cell extracts were also immunoblotted with anti-Flag (bottom panel).

Isolation of a jkk-1 loss-of-function allele

To investigate the physiological role of JKK-1, we undertook a reverse genetic approach to isolate loss-of-function mutations in jkk-1. Using a transposon-based method (Zwaal et al., 1993), we identified a single deletion allele, jkk-1(km2), and isolated individual worms carrying this mutation. PCR amplification and sequence analysis of the deletion allele using jkk-1-specific primers revealed that the jkk-1(km2) mutation deletes 970 nucleotides of the genomic jkk-1 locus, corresponding to nucleotides 20856–21825 of cosmid F35C8.3 (Figure 5A). This mutation deletes sequences encoding amino acids 139–287 of JKK-1, which includes the kinase domains II–VIII. Thus, km2 is presumably a null allele.

JKK-1 modulates coordinated locomotion

The jkk-1(km2) mutant exhibited defects in body movement. We first tested for defects in the locomotion of the jkk-1 null mutant by placing age-matched wild-type and jkk-1(km2) mutant animals on agar plates coated with Escherichia coli and comparing the tracks left in the bacterial lawn by the movement of the animals. Wild-type N2 moves by propagating waves of alternating dorsal and ventral flexions along its body length, which produces regular sinusoidal tracks on the bacterial lawn. In contrast, the track pattern inscribed by mutant animals on the bacterial lawns was significantly different from those of the wild type. Paths meandered more, seldom running in a straight trajectory for a long distance (Figure 6A). We further compared the behavior of wild-type and jkk-1(km2) mutant animals using a population assay (Figure 6B). In this assay, the mutant animals migrated for a much shorter distance during a given period of time than did wild-type animals. This phenotype was also observed in single animals (see Figure 8). To quantitate the locomotory defects in the jkk-1 null mutant, we photographed tracks made by age-matched jkk-1 and wild-type animals, and then measured the amplitude and the wavelength of the inscribed sinusoidal wave. We found that the amplitude of the body wave was ~2-fold higher in jkk-1(km2) animals compared with wild type (Figure 7). Other behaviors were normal in jkk-1(km2) mutant animals, including pharyngeal pumping, egg laying, foraging and defecation (data not shown). We conclude that the jkk-1(km2) mutant is defective in coordinated locomotion and that jkk-1 is required for maintenance of the wild-type pattern of sinusoidal motion.

To determine whether these locomotion defects are due to abnormal development or abnormal cell function, we generated a plasmid, pMK105, which places the jkk-1 gene under the control of the C.elegans heat-shock promoter hsp16-2. The hsp16-2 promoter directs expression in many tissues including neurons, pMK105 was integrated into jkk-1(km2) mutant animals as a transgenic array.
Fig. 5. Expression of JNK-1 and JKK-1. (A) Structures of the \textit{jnk-1} and \textit{jkk-1} genes. Exons are indicated by boxes. The shaded and open boxes are the translated and untranslated regions, respectively. The black boxes indicate kinase domains. The \textit{trans}-splicing, poly(A) sites and the GFP fusion constructs are also indicated. \textit{jkk-1(km2)} is a 970 bp deletion mutation from which four exons are missing. (B) Expression patterns of the \textit{jnk-1::gfp} and \textit{jkk-1::gfp} constructs. Panels in rows 1 and 3 show Nomarski images of L4 or young adult stage animals of wild-type N2 harboring \textit{jnk-1::gfp} (left panel) or \textit{jkk-1::gfp} (right panel) transgene. Panels in row 2 and 4 show epifluorescence images of the corresponding animals. The panel in row 4 shows intracellular localization patterns of \textit{jnk-1::gfp} and \textit{jkk-1::gfp} in posterior tail ganglia. Some cells expressing \textit{jnk-1::gfp} are different from those expressing \textit{jkk-1::gfp} due to the mosaicism of the extrachromosomal array distribution in each animal.
Fig. 6. Loss-of-function phenotypes of jkk-1. (A) Track patterns inscribed by wild-type and jkk-1(km2) mutant animals. Tracks were carved into a bacterial lawn by wild-type N2 (left panel) and jkk-1(km2) mutant (right panel) animals, each at the L4 stage. (B) Abnormal movement determined in population assay of the jkk-1(km2) mutant animals. Approximately 50 L4 animals were washed three times with M9 buffer and spotted in the center of NGM plates. The worms were killed by chloroform at the indicated times after spotting, and the numbers of worms located outside of the 1.5 cm circle were counted. The fraction of animals (%) at each time point were calculated. Open bars, wild-type N2; black bars, jkk-1(km2) mutant. Each bar represents the mean of three independent assays.

Fig. 7. Abnormal movement in jkk-1 null mutant animals. Tracks were carved into a bacterial lawn by young adult stage animals of wild-type N2, jkk-1(km2) mutant unc-25(e156) mutant, and unc-25(e156); jkk-1(km2) mutant animals. Quantitation of the amplitude and wave length was shown in the right panel. Numbers cited are the average of measurements of individual animals. Scores are reported ± SEM. The number of animals examined is shown in parentheses.

to generate the strain kmIs1. These animals exhibited locomotion defects in the absence of heat treatment. When heat-treated at the young adult stage, movements were still defective up to 12 h after the heat treatment, i.e. even though the wild-type JKK-1 was being produced (data not shown). However, after 24 h the movement defects were rescued (Figure 8), suggesting that complementation by jkk-1 does occur after a certain period of time. Heat treatment per se did not result in the rescue of movement defects in jkk-1(km2) mutant animals, as shown by the control animals kmIs2 carrying the empty vector as an integrated transgenic array (Figure 8). Thus, the movement defects observed in the jkk-1 null mutants are not due to a developmental abnormality, but rather to a defect in neuronal cell function.

**JKK-1 functions in D-type motor neurons**

The locomotion defects in jkk-1(km2) animals were complemented by the introduction of the jkk-1::gfp transgene (data not shown), suggesting that cells expressing the JKK-1::GFP reporter include some or all of those that normally express JKK-1 protein. Since extrachromosomal arrays occasionally fail to segregate to both daughters during cell division, mosaic animals are generated spontan-
Requirement of JKK-1 for normal movement in the adult stage. Transgenic jkk-1(km2) animals bearing pMK105 (P_{hsp16-2};jkk-1; kmls1) or pPD49.78 (P_{hsp16-2}; kmls2) as an integrated array were synchronized at the L1 stage. At late L4 stage, the animals were either left untreated (−) or treated (+) with heat shock for 30 min at 33°C in M9 buffer. Then the animals were cultured at 20°C for 24 h on NGM plates seeded with E.coli. For the assay of movement, single animals were spotted in the center of NGM plates seeded with E.coli and left for 10 min. The tracks on a bacterial lawn were traced by black pen. Ten individual animals were assayed for movement and the numbers of animals showing normal movement (Figure 9A). These results suggest that extrachromosomal array were still defective in movement jkk-1(km2) mutant animals that harbored pMK109 as an

Movement in C.elegans in D-type motor neurons is required for coordinated control of the DD and VD neurons (Jin et al., 1994; Shaham et al., 1996). The plasmid pMK106 containing a P_{unc-30};jkk-1 transgene was introduced as an extrachromosomal array into jkk-1(km2) mutant animals. When we examined locomotion phenotypes in independent lines of ectopic JKK-1-expressing animals, we found that the jkk-1(km2) mutant was constructed and characterized JKK-1, which functions as a specific MAPKK for JNK-1. A jkk-1 null mutant exhibits uncoordinated behavior, suggesting that the C.elegans JNK pathway is required for coordinated movement.

Discussion

We have isolated and characterized a novel JNK, JNK-1, in C.elegans. Sequence analysis reveals high homology to its vertebrate counterparts, especially to JNK3. As is the case for its mammalian homologs, c-Jun was found to be a good substrate for JNK-1 in vitro. We also isolated and characterized JKK-1, which functions as a specific MAPKK for JNK-1. A jkk-1 null mutant exhibits uncoordinated behavior, suggesting that the C.elegans JNK pathway is required for coordinated movement.

Identification of a JNK activator in C.elegans

JNKs are activated by JNK-activating MAPKKs. In vertebrates, two different activators for JNK have been identified, MKK4 and MKK7 (Derijard et al., 1997; Holland et al., 1997; Moriguchi et al., 1997; Tournier et al., 1997). MKK4 is able to activate both JNK and p38 and when overexpressed in COS cells, MKK7 functionally rescues hep mutant flies, suggesting that MKK7 and Hep have some conserved functions (Holland et al., 1997). The Drosophila homolog of JNK, D-JNK, encoded by basket, is also required for dorsal closure (Riesgo et al., 1996; Sluss et al., 1996).
Biochemical analysis of Hep demonstrates that it is a potent activator of D-JNK in vitro. Thus, Hep and D-JNK function in the same signal transduction pathway in Drosophila. Most recently, a Drosophila homolog of MKK4 (D-MKK4) was identified (Han et al., 1998) and shown to be able to activate both D-JNK and a Drosophila homolog of p38, D-p38b, in vitro (unpublished data). In this study, we identified a C.elegans homolog of JNK, JNK-1, and its activator, JKK-1. JKK-1 appears to be a specific activator of JNK-1, since it failed to activate a C.elegans homolog of p38, PMK-1. Furthermore, expression of JKK-1 in mammalian cells specifically stimulates the kinase activity of JNK but not that of p38. Thus, the characteristics of JKK-1 are similar to those of MKK7 and Hep. We have isolated four different cDNAs which can suppress the yeast pbs2Δ mutation in a JNK-1-dependent manner. One of them is JKK-1 and the second, SEK-1, is also homologous to members of the MAPKK superfamily. SEK-1 is able to activate both JNK-1 and PMK-1 in the yeast Hog pathway (unpublished data). Therefore, in vertebrates, Drosophila and C.elegans, there are at least two different JNK activators, MKK4/D-MKK4/SEK-1 and MKK7/Hep/JKK-1. The former can activate both the JNK and p38 subgroups of the MAPK superfamily, whereas the latter is specific for JNK.

Studies from targeted disruptions of the MKK4 gene in mice have demonstrated that activation of JNK in mkk4−/− cells still occurs in response to osmotic shock and UV irradiation, but not in response to anisomycin or heat shock (Nishina et al., 1997; Yang et al., 1997a). Thus, there are MKK4-dependent and-independent intracellular signaling pathways leading to JNK activation, of which the latter may be mediated by MKK7. Loss-of-function mutations in the Drosophila Hep and D-JNK have revealed an essential role for the JNK pathway in dorsal closure (Noselli, 1998). However, D-JNK activity is increased in extracts of hep mutant larvae (Riesgo et al., 1996). In addition to its role in development, D-JNK activity can be stimulated by endotoxic lipopolysaccharide, suggesting that the D-JNK cascade may play a role in the insect immune defense system against bacterial infection (Sluss et al., 1996). D-MKK4 may also be involved in the regulation of insect immunity through its activation of D-JNK. Therefore, in vertebrates and Drosophila, activation of JNK by different environmental stimuli may occur selectively through different JNK activators. Accordingly, it is possible that the JNK-1 signal transduction pathway in C.elegans may also be activated by other MAPKKs in response to specific environmental stimuli.

Caenorhabditis elegans JNK activator is required for coordinated locomotion

The genetic and molecular analyses presented here clearly demonstrated an essential role for JKK-1 in the regulation of coordinated movement. Ectopic expression of JKK-1 in type-D motor neurons was sufficient to rescue the movement defect in jkk-1 mutant animals. JKK-1 expressed from a conditional promoter in adults could also rescue the movement defect, indicating that JKK-1 is involved in the continued functioning of neurons. These results suggest that JKK-1 modulates coordinated movement in C.elegans as a result of its function in type-D motor neurons, presumably through its activation of JNK-1.

How might the JKK-1–JNK-1 cascade act in D-type motor neurons to regulate coordinated movement? The DD and VD motor neurons are inhibitory neurons that utilize the neurotransmitter GABA (McIntire et al., 1993b). Mutations in the unc-25 gene, which encodes the GABA biosynthetic enzyme, cause the simultaneous contraction of the dorsal and ventral muscles such that animals shrink along their body axis (McIntire et al., 1993a,b). In contrast to the phenotypes of unc-25 mutants, jkk-1 null mutants exhibit a subtle modulation of locomotion, suggesting that GABA retains its function in body muscles in the absence of jkk-1 activity. Furthermore, the body tracks left by...
**A neuronal JNK signaling pathway in *C. elegans***

**Materials and methods**

**Screening of *C. elegans* genes in yeast**

Yeast strains TM233 (MATa hog1Δ::URA3 ara3 leu2 his3) and TM334 (MATa phe2::HIS3 ara3 leu2 trp1 his3) carrying pKTINK1 (which expresses JNK-1) were transformed with a *C. elegans* cDNA library constructed in the yeast expression vector pNVLeu, in which expression of the cDNA is under the control of the inducible GAL1 promoter. Osmo-resistant transformants were selected by growing on YPGal plates containing 1 M sorbitol.

**Construction of cDNAs for expression in yeast**

Plasmid pKTJNK1 was constructed by inserting a 1.8 kb *Eco*RI fragment containing the *jnk-1* open reading frame from the original clone pNVJN1 into the *Eco*RI site of pKT10. Plasmid pKTJNK1 expresses the full-length *jnk-1* under control of the *TDH3* promoter. Plasmid pNJVJK1 expresses the full-length *jkk-1* under control of the *GAL1* promoter. Plasmid YCpGPMK1 was constructed by inserting the *Sal/HindIII* fragment of PMK-1 cDNA into the *Sal/HindIII* site of YCPG3 carrying the *GAL1* promoter.

**Construction of cDNAs for kinase assays in 293 cells**

The mammalian expression vector for *Flag* epitope-tagged JKK-1 was constructed by inserting the full-length *jkk-1* cDNA into pFlag-CMV (Kodak). The mammalian expression vector for *HA* epitope-tagged JNK was constructed by inserting a 1.6 kb *Bam*HI fragment containing the full-length *jnk-1* cDNA into the *Bam*HI site of pcDNA3 vector (Invitrogen). The mammalian expression vector for *HA* epitope-tagged PMK-1 was constructed by inserting a 1.3 kb *Not*I fragment containing the full-length *pmk-1* cDNA into the *Not*I site of pcDNA3. Expression plasmids encoding *HA* epitope-tagged JNK1 and p38 were gifts from E.Nishida.

**Assays for kinase activity in 293 mammalian cells**

Human embryonic kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. To assess MAPKs activities, 293 cells (1 × 10⁶) were plated on 10 cm dishes, and transfected with a total 10 μg of DNA containing various expression vectors. After 36 h, cells were collected and washed once with phosphate-buffered saline (PBS), and lysed in 0.3 ml of 0.5% Triton X-100 lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM PMSF and 20 μg aprotinin. Cellular debris was removed by centrifugation at 10,000 g for 5 min. HA-tagged proteins were immunoprecipitated with anti-HA monoclonal antibody (Abcam). Aliquots of immunoprecipitates were incubated with 1 μg of bacterially expressed GST-c-JUN or GST-ATF2 (kindly provided by E.Nishida) in 10 μl of kinase buffer containing 20 mM HEPES (pH 7.4), 1 mM DTT, 5 mM MgCl₂, and 5 μCi of [γ-³²P]ATP (3000 Ci/mmol) at 25°C for 2 min. Samples were analyzed by 10% SDS-PAGE and autoradiography. For immunoblotting, aliquots of immunoprecipitates and whole cell lysates were resolved on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to Hybond-P membranes (Amersham). The membranes were immunoblotted with anti-HA rabbit polyclonal antibody Y-11 (Santa Cruz) or anti-Flag mouse monoclonal antibody M2 (Kodak). The bound antibody was visualized with horseradish peroxidase-conjugated A neuronal JNK signaling pathway in *C. elegans*

**Materials and methods**

**Screening of *C. elegans* genes in yeast**

Yeast strains TM233 (MATa hog1Δ::URA3 ara3 leu2 his3) and TM334 (MATa phe2::HIS3 ara3 leu2 trp1 his3) carrying pKTINK1 (which expresses JNK-1) were transformed with a *C. elegans* cDNA library constructed in the yeast expression vector pNVLeu, in which expression of the cDNA is under the control of the inducible GAL1 promoter. Osmo-resistant transformants were selected by growing on YPGal plates containing 1 M sorbitol.

**Construction of cDNAs for expression in yeast**

Plasmid pKTJNK1 was constructed by inserting a 1.8 kb *Eco*RI fragment containing the *jnk-1* open reading frame from the original clone pNVJN1 into the *Eco*RI site of pKT10. Plasmid pKTJNK1 expresses the full-length *jnk-1* under control of the *TDH3* promoter. Plasmid pNJVJK1 expresses the full-length *jkk-1* under control of the *GAL1* promoter. Plasmid YCpGPMK1 was constructed by inserting the *Sal/HindIII* fragment of PMK-1 cDNA into the *Sal/HindIII* site of YCPG3 carrying the *GAL1* promoter.

**Construction of cDNAs for kinase assays in 293 cells**

The mammalian expression vector for *Flag* epitope-tagged JKK-1 was constructed by inserting the full-length *jkk-1* cDNA into pFlag-CMV (Kodak). The mammalian expression vector for *HA* epitope-tagged JNK was constructed by inserting a 1.6 kb *Bam*HI fragment containing the full-length *jnk-1* cDNA into the *Bam*HI site of pcDNA3 vector (Invitrogen). The mammalian expression vector for *HA* epitope-tagged PMK-1 was constructed by inserting a 1.3 kb *Not*I fragment containing the full-length *pmk-1* cDNA into the *Not*I site of pcDNA3. Expression plasmids encoding *HA* epitope-tagged JNK1 and p38 were gifts from E.Nishida.

**Assays for kinase activity in 293 mammalian cells**

Human embryonic kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. To assess MAPKs activities, 293 cells (1 × 10⁶) were plated on 10 cm dishes, and transfected with a total 10 μg of DNA containing various expression vectors. After 36 h, cells were collected and washed once with phosphate-buffered saline (PBS), and lysed in 0.3 ml of 0.5% Triton X-100 lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM PMSF and 20 μg aprotinin. Cellular debris was removed by centrifugation at 10,000 g for 5 min. HA-tagged proteins were immunoprecipitated with anti-HA monoclonal antibody (Abcam). Aliquots of immunoprecipitates were incubated with 1 μg of bacterially expressed GST-c-JUN or GST-ATF2 (kindly provided by E.Nishida) in 10 μl of kinase buffer containing 20 mM HEPES (pH 7.4), 1 mM DTT, 5 mM MgCl₂, and 5 μCi of [γ-³²P]ATP (3000 Ci/mmol) at 25°C for 2 min. Samples were analyzed by 10% SDS-PAGE and autoradiography. For immunoblotting, aliquots of immunoprecipitates and whole cell lysates were resolved on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to Hybond-P membranes (Amersham). The membranes were immunoblotted with anti-HA rabbit polyclonal antibody Y-11 (Santa Cruz) or anti-Flag mouse monoclonal antibody M2 (Kodak). The bound antibody was visualized with horseradish peroxidase-conjugated...
ated antibody to rabbit or mouse IgG using the enhanced chemiluminescence (ECL) Western blotting system (Amersham).

**References**


