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

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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 *GPD1* 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^s) growth phenotype of a *hog1*Δ 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 *hog1*Δ mutant and transformants were screened for suppression of the Osm^s phenotype (Figure 1B). Of the 1×10^5 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 criteria, 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 *hog1*Δ 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 *pbs2*Δ 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 *pbs2*Δ 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 *pbs2*Δ mutant expressing JNK-1 with a *C.elegans* cDNA library and screened for suppression of the *pbs2*Δ Osm^s phenotype. We obtained a total of 20 transformants, from among 5×10^5 screened, capable of growth in the presence of sorbitol. Of these 20 candidates, 11 clones failed to restore sorbitol resistance in the *pbs2*Δ mutation in the absence of JNK-1 expression, indicating that they suppressed the *pbs2*Δ 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 *pbs2*Δ 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^s phenotype of a *hog1*Δ mutant (Figure 1B). These results suggested that JKK-1 can function specific-

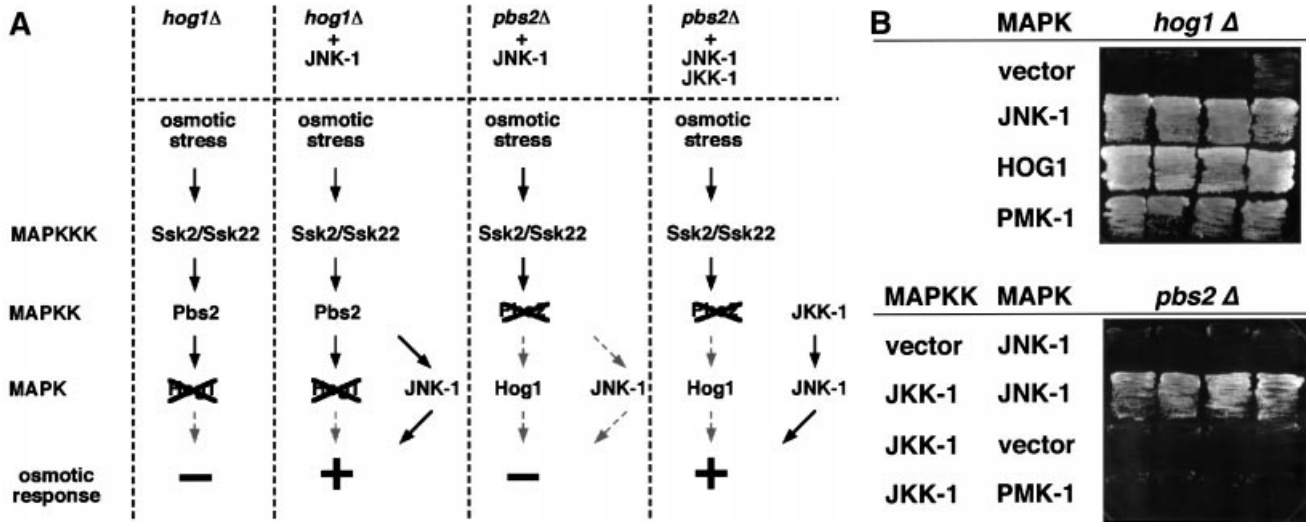

Fig. 1. Screening for *C.elegans* JNK homolog and its activator MAPKK in yeast. (**A**) Model for the yeast osmotic stress-activated MAPK pathway. *hog1*Δ or *pbs2*Δ mutants are sensitive to high osmolarity (Osm^s). Expression of a *C.elegans* JNK homolog, JNK-1, complements the Osm^s growth phenotype of the *hog1*Δ mutant. Expression of JNK-1 is unable to suppress the Osm^s phenotype of the *pbs2*Δ mutant. Co-expression of JNK-1 with its activator JKK-1 can suppress the *pbs2*Δ Osm^s phenotype. (**B**) Suppression of the *hog1*Δ and *pbs2*Δ mutants by *C.elegans* genes. Strains TM233 (*hog1*Δ, upper panel) and TM334 (*pbs2*Δ, lower panel) were transformed with various plasmids. Transformants were streaked onto YPGal plates containing 1 M sorbitol and incubated at 30°C. Each patch represents an independent transformant. Plasmids were as follows. (Upper panel) row 1, pKT10 (vector); row 2, pKTJNK1 (JNK-1); row 3, pJB30 (HOG1); and row 4, YCpGPMK1 (PMK-1). (Lower panel) row 1, pNVLeu (vector) and pKTJNK1 (JNK-1); row 2, pNVJKK1 (JKK-1) and pKTJNK1 (JNK-1); row 3, pNVJKK1 (JKK-1) and pKT10 (vector); and row 4, pNVJKK1 (JKK-1) and YCpGPMK1 (PMK-1).

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JNK-1   1:MEERLSTTSSYPSHPGRSVEEDHWTLLASSSISSIIRGTRGHLNNFIESVGNWLVPSSSGRDDDAVSLDSCQSVYSPVRHHINSGTGGGILMEPSSIHVP 100
JNK1    1:----------------------------------------------------------------------------------------M.RSKRD  7
JNK2    1:----------------------------------------------------------------------------------------M.DSKCD  7
JNK3    1:-------------------------------------------------M.LHFLYYCSEPTLDVKIAFCQGFDKQVDVSYIAKHYNM.KSK.D  45

                                  I                  II              III          IV
JNK-1 101:ENYYSVTIGEAQMVVLKRYQNLRLIGSGAQGIVCSAFDTVRNEQVAIKKLSRPFQNVTHAKRAYRELKLMSLVNHKNIIGILNCFTPQKKLDEFNDLYIV 200
JNK1    8:N.F...E..DSTFT........KP..........A.Y.AILERN............Q..........V..KC........L..V.....S.E..Q.V... 107
JNK2    8:SQF...QVADSTFT......Q.KP..........A.....LGIS..V.........Q..........V.LKC.......SL..V.....T.E..Q.V.L. 107
JNK3   46:NQF...EV.DSTFT........KP..........A.Y.A.LDRN............Q..........V..KC.......SL..V.....T.E..Q.V.L. 145

            V                    VIA               VIB             VII            * *  VIII
JNK-1 201:MELMDANLCQVIQMDLDHERLSYLLYQMLCGIRHLHSAGIIHRDLKPSNIVVRSDCTLKILDFGLARTAIEAFMMTPYVVTRYYRAPEVILGMGYKENVD 300
JNK1  108:.............E.....M..........K.....................K.............GTS.......................... 207
JNK2  108:............H.E.....M..........K.....................K.............CTN.......................... 207
JNK3  146:.............E.....M..........K.....................K.............GTS.......................... 245

          IX                          X                                                          XI
JNK-1 301:VWSIGCIFGELIRGRVLFPGGDHIDQWTRIIEQLGTPDRSFLERLQPTVRNYVENRPRYQATPFEVLFSDNMFPMTADSSRLTGAQARDLLSRMLVIDPE 400
JNK1  208:L..V...M..MVCHKI....R.Y....NKV.......CPE.MKK......T......K.AGYS..K..P.VL..ADSEHNK.KAS.......K.....AS 307
JNK2  208:I..V...M...VK.C.I.Q.T......NKV.......SAE.MKK.............K.PGIK..E..P.WI..SESERDKIKTS.......K......D 307
JNK3  246:I..V...M..MV.HKI....R.Y....NKV.......CPE.MKK.............K.AGLT.PK..P.SL..ADSEHNK.KAS.......K......A 345


JNK-1 401:RRISVDDALRHPYVNVWFDEIEVYAPPPLPYDHNMDVEQNVDS-WREHIFRELTDYARTHDIYS                                  463
JNK1  308:K.....E..Q...I...Y.PS.AE....KIP.KQL.EREHTIEE.K.L.YK.VM.LEERTKNGVIRGQPSPLGAAVINGSQHPSSSSSVNDVSSMSTDPT 407
JNK2  308:K.....E......IT..Y.PA.AE....QI..AQLEEREHAIEE.K.L.YK.VM.WEERSKNGVVKDQPSDAAVSSNATPSQSSSINDISSMSTEQTLAS 407
JNK3  346:K........Q...I...Y.PA..E....QI..KQL.EREHTIEE.K.L.YK.VMNSEEKTKNGVVKGQPSPSAQVQQ                        422


JNK1  408:LASDTDSSLEAAAGPLGCCR                                                                               427
JNK2  408:DTDSSLDASTGPLEGCR                                                                                 424
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Fig. 2. Sequence alignment of JNK-1 with mammalian JNKs. Identical residues are indicated with a period. Gaps were introduced into sequences to optimize alignments. The conserved TPY motif is marked with asterisks and the kinase subdomains are marked with roman numbers above the sequences. The DDBJ/EMBL/GenBank accession number for the JNK-1 sequence is AB024085.

ally in the yeast Hog1 pathway by activating JNK-1, but not PMK-1.

JKK-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 JKK-1 and JNK-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

A

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                                                                    I              II           III
JKK-1   1:------------------------MENVCFQQRLRDLETRVRKWKFLKLGLTEVRLRPRDRRSTSVDQKHKECSSTSSSPQHQRPNNIGYLTSPMERK-FT  76
MKK4    1:-------------------------------------------MAAPSPSGGGGSGGGSG.GTPGPVGSPAPGHPAVSSM.GKRKALK.NFANPPFKS.  56
MKK7    1:MAASSLEQKLSRLEAKLKQENREARRRIDLNLDISPQRPRPIIVITLSPAPAPSQ.AALQLPLAN.GGSRSP..E..-...PT.PTRPRHMLGLPSTL..  99

                                                                    I              II           III
JKK-1  77:PLSMKPSPSRRDTEKDALEYEFLEGYKKSGTLEIDGEKQVVDPNEIHIISLLGSGSCGVVESATVR--SKLMAVKTMYKNDNKENLKRILRDVRI-MSMC 173
MKK4   57:ARFTLNPNPTGVQNPHIERLRTHSIESSGKLKISPEQHWDFTAEDLKDLGEI.R.AY.S.NKMVHKPSGQI....RIRSTVDEKEQ.QL.M.LDVV.RSS 156
MKK7  100:.----------.SM.SIEIDQKLQ.IM.QT.Y.T.G.QRYQAEI.DLENLGEM...T..Q.WKMRF.KTGHII...Q.RRSG...EN....M.LDVVLKSH 190

          IV                V                                                VI                  VII
JKK-1 174:NSPFIVTSYGYFMFDSSVKICMQIMSACCEKLLRRIYHSKLDFFPEFVAGHIVYSAISALDYLKEKHSIIHRDIKPSNILFDDSGNVKLCDFGISGFMTD 273
MKK4  157:DC.Y..QF..ALFREGDCW...EL..TSFD.FYKYV.SVLDDVI..EIL.K.TLATVK..NH...NLK.............L.R...I.........QLV. 276
MKK7  191:DC.Y..QCF.T.ITNTD.F.A.EL.GT.A...KK.----MQGPI..RIL.KMTVAIVK..Y......GV....V......L.ER.QI.........RLV. 286

        *   *     VIII                IX                          X                          XI
JKK-1 274:SMAHSKSAGCPPYMAPERLTIETNSK--YDVRSDVWSLGITVYQLVTGLYPFPLNDMEFTTLTIIANLNLPSPSLREETKRSFSPLFIEFLDLCLRKDVR 371
MKK4  277:.I.KTRD...R.......IDPSASRQ-G.............L.E.A..RF.Y.KWNSV.DQ..QVVKGDP.QL.NS..-.E...S..N.VN...T..ES 353
MKK7  287:.K.KTR....AA......IDPPDPT.PD..I.A.......SLVE.A..QF.YKNCKTD.EV..KVLQEEP.LLPGHMG-----..GD.QS.VKD..T..H. 382

JKK-1 372:ERPEYRQLMKHDFYLDYDPASGSAYKFKAINGKCNQVADWFVDVIRLSKTEDELKSIPNTPCVN                                      435
MKK4  354:K..K.KE.L..P.I.M.EERAVEVACYVCKILDQMPATPSSPMYVD                                                      399
MKK7  383:K..K.NK.LE.S.IKH.EILE----------VD..S..K..MAKTESPRTSGVLSQHHLPFFSGSLEESPTSPPSPKSFPLSPAIPQAQAEWVSGR  468
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B

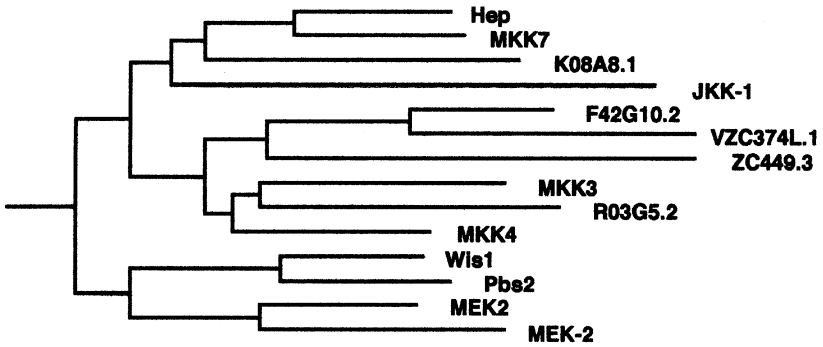


Fig. 3. JKK-1 sequence analysis. (**A**) Sequence alignment of JKK-1 with mammalian JNK-activating MAPKKs. The sites of activating phosphorylation in MAPKKs are indicated by asterisks and the kinase subdomains are marked with roman numbers above the sequences. The DDBJ/EMBL/GenBank accession number for the JKK-1 sequence is AB024086. (**B**) Alignment dendrogram generated using the CLUSTAL algorithm.

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

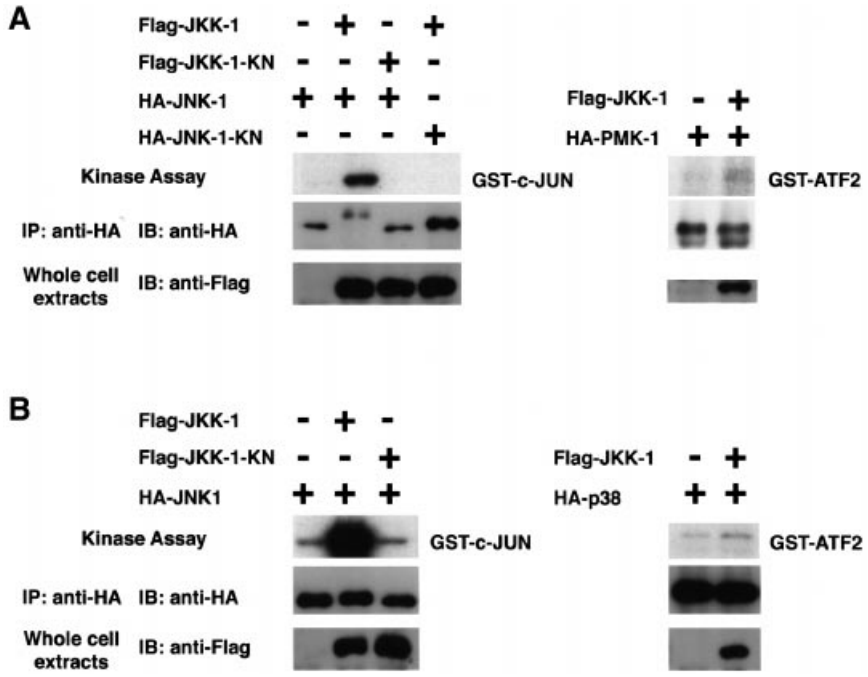


Fig. 4. Activation of JNK-1 and JNK by JKK-1. (**A**) Activation of *C.elegans* JNK-1 by JKK-1. 293 cells were transfected with Flag-JKK-1, Flag-JKK-1(K149R) (Flag-JKK-1-KN), HA-JNK-1, HA-JNK-1(K148R) (HA-JNK-1-KN) (left panel), and HA-PMK-1 (right panel) as indicated. Immunoprecipitated complexes obtained 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).

genes were also expressed in cell bodies and axons. Cells expressing JNK-1::GFP exhibited cytoplasmic as well as nuclear staining, whereas the JKK-1::GFP fusion protein was excluded from the nucleus (Figure 5B). Thus, JNK-1 and JKK-1 appear to be co-expressed in most neurons, consistent with the possibility that they constitute a functional unit. This suggested that the *C.elegans* JNK pathway is likely to be involved in some aspect of neuronal function.

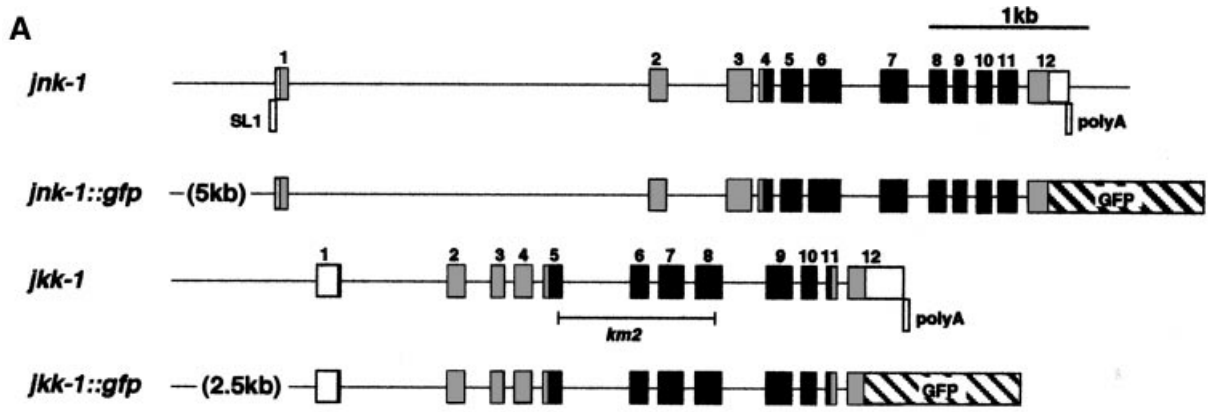
Isolation of a jkk-1 loss-of-function allele

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



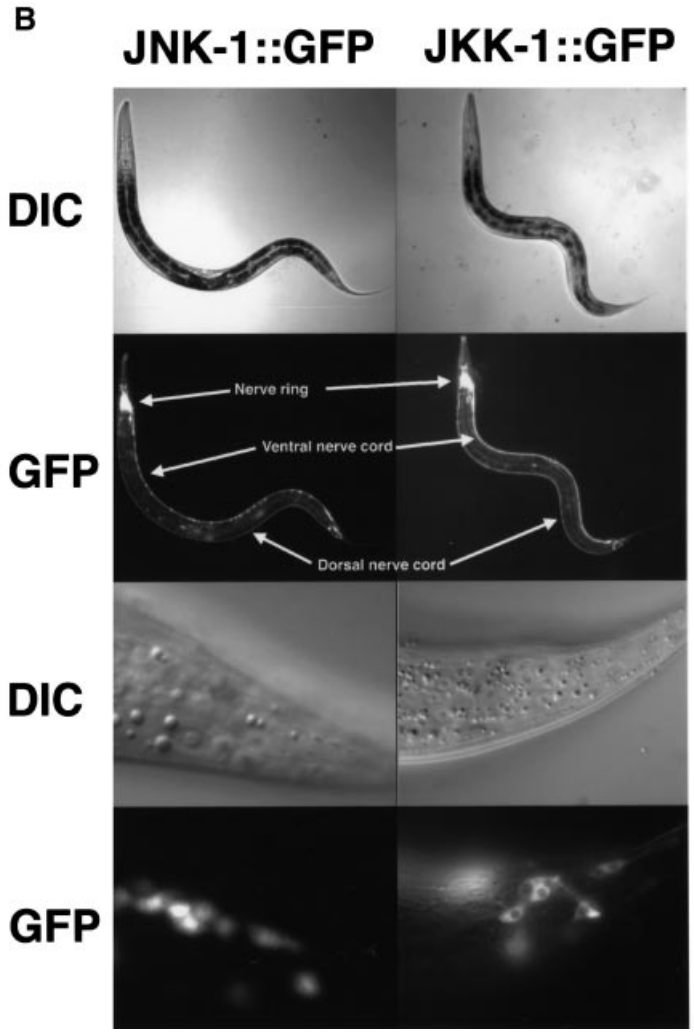


Fig. 5. Expression of JNK-1 and JKK-1. (**A**) Structures of the *jnk-1* and *jkk-1* genes. Exons are indicated by boxes. The shaded and open boxes are the translated and untranslated regions, respectively. The black boxes indicate kinase domains. The *trans*-splicing, poly(A) sites and the GFP fusion constructs are also indicated. *jkk-1(km2)* is a 970 bp deletion mutation from which four exons are missing. (**B**) Expression patterns of the *jnk-1::gfp* and *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 *jnk-1::gfp* (left panel) or *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 *jnk-1::gfp* and *jkk-1::gfp* in posterior tail ganglions. Some cells expressing *jnk-1::gfp* are different from those expressing *jkk-1::gfp* due to the mosaicism of the extrachromosomal array distribution in each animal.

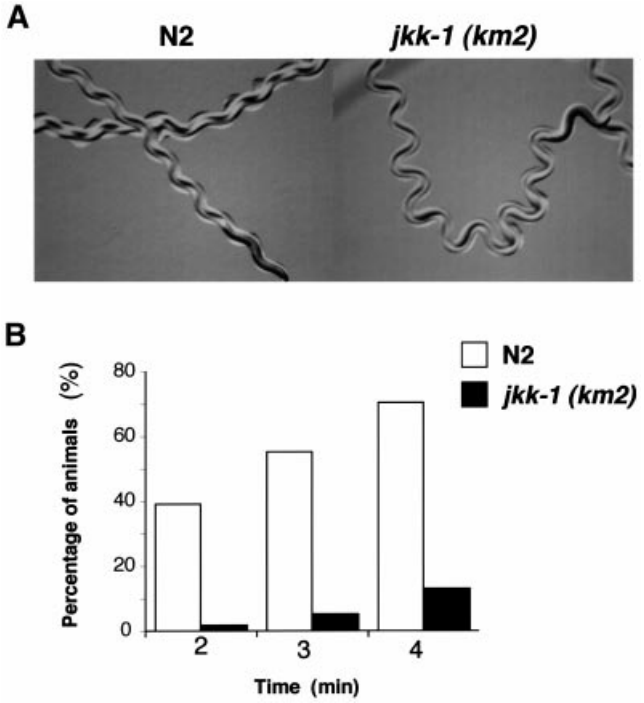


Fig. 6. Loss-of-function phenotypes of *jkk-1*. (**A**) Track patterns inscribed by wild-type and *jkk-1(km2)* mutant animals. Tracks were carved into a bacterial lawn by wild-type N2 (left panel) and *jkk-1(km2)* mutant (right panel) animals, each at the L4 stage. (**B**) Abnormal movement determined 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.

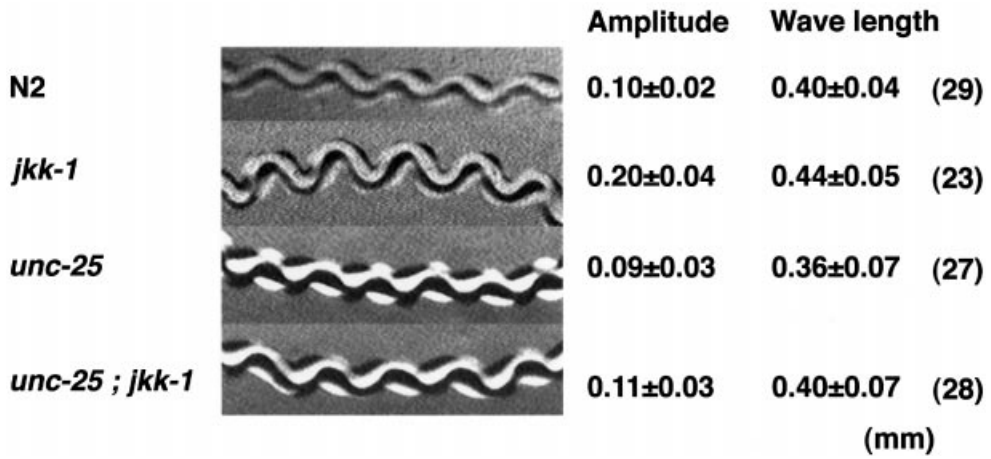


Fig. 7. Abnormal movement in *jkk-1* null mutant animals. Tracks were carved into a bacterial lawn by young adult stage animals of wild-type N2, *jkk-1(km2)* mutant *unc-25(e156)* mutant, and *unc-25(e156); jkk-1(km2)* mutant animals. Quantitation of the amplitude and wave length was shown in the right 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-

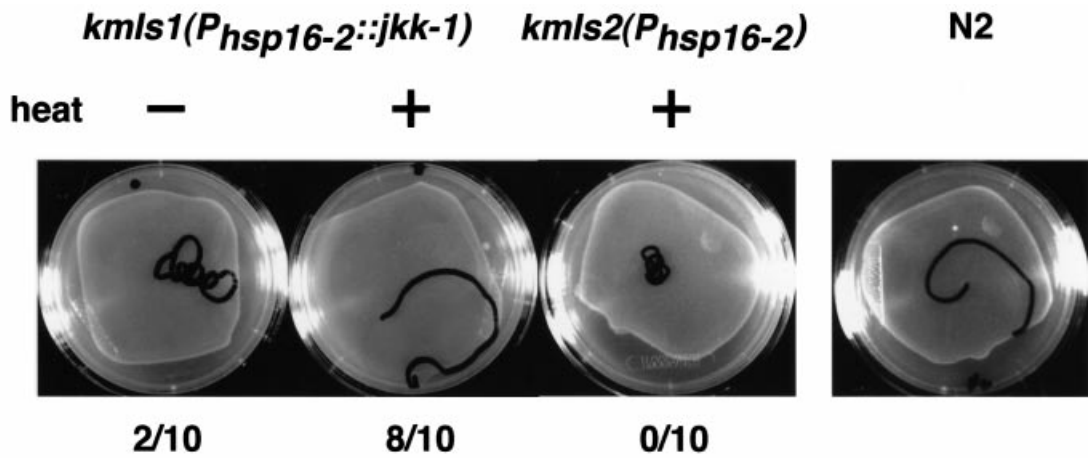


Fig. 8. Requirement of JKK-1 for normal movement in the adult stage. Transgenic *jkk-1(km2)* animals bearing pMK105 ($P_{hsp16\text{-}2}$*::jkk-1; kmIs1*) or pPD49.78 ($P_{hsp16\text{-}2}$*; kmIs2*) as an integrated array were synchronized at the L1 stage. At late L4 stage, the animals were either left 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 are shown in the lower panel. As a control, the track of wild-type N2 on bacterial lawn is shown in the right panel.

eously within a population, and these can be used to analyze which of the many neurons that express *jkk-1* are required for coordinated locomotion. We allowed the *jkk-1(km2)* animals to propagate for several generations and then selected mosaic animals that still expressed the rescue phenotype using a population assay described in Figure 6B. Upon examination of individual stained neuronal cells, we found that staining of motor neurons in the ventral nerve cord appeared to correlate with coordinated locomotion (data not shown). This is consistent with the fact that ventral nerve cord motor neurons are important for coordinated movement in the animal (McIntire *et al*., 1993b).

D-type GABAergic (GABA, γ-aminobutyric acid) motor neurons have been implicated in the regulation of wave amplitude (Mclntire *et al*., 1993a,b). To investigate whether expression of *jkk-1* in D-type motor neurons is important for its effect on locomotion, we expressed the *jkk-1* cDNA under the control of the promoter for the *unc-30* gene, whose expression is restricted primarily to the DD and VD neurons (Jin *et al*., 1994; Shaham and Horvitz, 1996). The plasmid pMK106 containing a $P_{unc\text{-}30}$*::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 $P_{unc\text{-}30}$*::jkk-1* transgene could rescue the *jkk-1* movement defects, including the altered body wave amplitude (Figure 9). To confirm that JKK-1 is required specifically in the D-type motor neurons for normal locomotion, we expressed *jkk-1* cDNA in the VA and VB motor neurons using the promoter from the *del-1* gene (Tavernarakis *et al*., 1997). For this purpose, we constructed the plasmid pMK109 in which expression of *jkk-1* was placed under the control of the *del-1* promoter ($P_{del\text{-}1}$::jkk-1). Transgenic *jkk-1(km2)* mutant animals that harbored pMK109 as an extrachromosomal array were still defective in movement (Figure 9A). These results suggest that *jkk-1* expression in D-type motor neurons is required for coordinated movement in *C.elegans*.

The *unc-25* gene, which encodes the GABA biosynthetic enzyme glutamic acid decarboxylase (Jin *et al*., 1999), also affects locomotion via its effect on D-type motor neurons (McIntire *et al*., 1993b). To investigate the functional relationship between *unc-25* and *jkk-1*, a double *unc-25(e156); jkk-1(km2)* mutant was constructed and characterized. *unc-25(e156); jkk-1(km2)* double mutants traversed the lawn in a manner similar to that of *unc-25(e156)* mutants; the amplitude of the path was consistently reduced (Figure 7). Thus, the increased amplitude phenotype in *jkk-1* mutants requires *unc-25* activity, raising the possibility that the *jkk-1* phenotype is caused by excessive release of GABA.

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*., 1995; Holland *et al*., 1997; Moriguchi *et al*., 1997; Tournier *et al*., 1997). MKK4 is able to activate both JNK and p38 MAPKs *in vitro* and when overexpressed in COS cells, whereas MKK7 is a specific activator for JNK. MKK7 is most similar to the *Drosophila* Hep, which is required in the embryo for dorsal closure, a process involving coordinate shape changes of ectodermal cells (Noselli, 1998). Furthermore, 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).

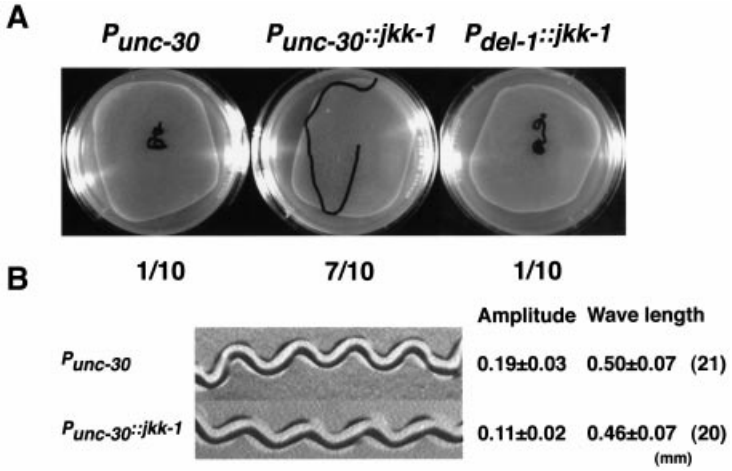


Fig. 9. Suppression of the movement defect in *jkk-1* null mutants by ectopic expression of *jkk-1* with the *unc-30* promoter. (**A**) The tracks on a bacterial lawn. Transgenic *jkk-1(km2)* animals bearing pSC157 (P_{unc-30}; left panel), pMK106 (P_{unc-30}*::jkk-1*; middle panel) or pMK109 (P_{del-1}*::jkk-1*; right panel) as an extrachromosomal array were cultured at 20°C 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 are shown in the lower panel. (**B**) Quantitation of the amplitude. Tracks were carved into a bacterial lawn by L4 stage *jkk-1(km2)* animals bearing pSC157 (P_{unc-30}) or pMK106 (P_{unc-30}*::jkk-1*) as an extrachromosomal array. Quantitation of the amplitude and wave length was shown in the right panel. Numbers cited are the average of measurements of individual young adult animals. Scores are reported ± SEM standard deviation. The number of animals examined is shown in parentheses.

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 *pbs2*Δ 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

jkk-1 mutant animals have an increased amplitude, whereas double mutants of *jkk-1; unc-25* move in a manner similar to *unc-25* mutants. Therefore, it is unlikely that the *jkk-1* mutation causes any decrease in GABA levels. Instead, the *jkk-1* phenotype may be caused by the excessive release of GABA by the nervous system. This latter possibility is consistent with the double mutant analysis, which suggests that JKK-1 acts as an upstream negative regulator of UNC-25. Alternatively, it is possible that the JKK-1–JNK-1 cascade may affect the timing of the release of GABA from D-type motor neurons to the muscles by regulating the motor neuron structure.

The processes of DD and VD neurons terminate abruptly at close proximity to the end of a neighboring process of the same class, and they are usually jointed by gap junctions (White *et al*., 1986). In mammalian cells, ERK MAP kinase phosphorylates the connexin gap junction protein, resulting in the down-regulation of gap junctional communication (Warn–Cramer *et al*., 1998). These findings raise the possibility that the JKK-1–JNK-1 cascade may regulate coordinated locomotion by modulating phosphorylation of connexin-like proteins, which in turn alters gap junction-mediated communication between adjacent motor neurons. In fact, at least 16 members of the connexin-like OPUS family are present in *C.elegans* genomic sequences. It will be interesting to examine whether JNK-1 can phosphorylate the connexin-like protein expressed in D-type motor neurons and whether such phosphorylation would affect these neurons' activity. Further studies will be needed to determine the precise role of the neuronal JKK-1–JNK-1 pathway in the regulation of locomotion by D-type motor neurons.

In mammalian cells, JNK activity is substantially higher in the central nervous system, and the JNK cascade can be activated throughout the brain by non-invasive stimuli (Xu *et al.*, 1997). Furthermore, the JNK pathway has been implicated in the stress-induced apoptosis of neurons (Yang *et al*., 1997b). These observations raise the possibility that the JNK cascade functions as an important physiological signal transduction pathway in the nervous system. In mammalian cells, 10 JNK isoforms have been identified, each resulting from alternative splicing of three genes, *JNK1*, *JNK2* and *JNK3* (Gupta *et al*., 1996). Although JNK1 and JNK2 are expressed ubiquitously in a variety of human tissues, JNK3 is selectively expressed within neurons in the brain (Gupta *et al*., 1996). This restricted expression suggests that the JNK3 cascade may play an important physiological role in neuronal activities. Experimental disruption of the *JNK3* gene in mice has revealed that the JNK3-mediated signaling pathway is involved in kainate-induced hippocampal neuron apoptosis (Yang *et al*., 1997b). However, a comparable number of motor neurons were found in the facial nuclei of wild-type and *jnk3*$^{-/-}$ mice, indicating that *jnk3*$^{-/-}$ mice have no apparent development abnormalities, including cell death. Similarly, the *jkk-1* null mutation has no effect on normal development or programmed cell death in neurons. It is therefore possible that, like the *C.elegans* JKK-1–JNK-1 cascade, the JNK3 signaling pathway is required for neuronal function. Most recently, DENN was identified as a substrate for JNK3 (Zhang *et al*., 1998). The *C.elegans* homolog of DENN, AEX-3, has been isolated as the GDP/GTP exchange factor specific for Rab3 subfamily members to regulate exocytosis of neurotransmitters (Iwasaki *et al*., 1997). *aex-3* mutants exhibit an abnormal defecation motor program and poor male mating efficiency. On the other hand, *jkk-1* mutants do not show such defects, suggesting that AEX-3 does not function in the JKK-1 cascade. It is therefore possible that one of the targets of the JNK-1 pathway may modulate neurotransmitter release. Screens of extragenic suppressors or enhancers of the *jkk-1* phenotype in *jkk-1* null mutants might be used to identify other components that participate in regulating the JNK-1 signaling pathway. Further characterization of these components should provide additional insight into the mechanism by which the JNK cascade regulates coordinated behavior in *C.elegans*.

Materials and methods

Screening of C.elegans genes in yeast

Yeast strains TM233 (*MAT***a** *hog1*Δ*::URA3 ura3 leu2 his3*) and TM334 (*MAT***a** *pbs2*Δ*::HIS3 ura3 leu2 trp1 his3*) carrying pKTJNK1 (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 pNVJNK1 into the *Eco*RI site of pKT10. Plasmid pKTJNK1 expresses the full-length *jnk-1* under control of the *TDH3* promoter. Plasmid pNVJKK1 expresses the full-length *jkk-1* under control of the *GAL1* promoter. Plasmid YCpGPMK1 was constructed by inserting the *Sal*I–*Hin*dIII fragment of PMK-1 cDNA into the *Sal*I–*Hin*dIII site of YCpG33 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-1 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 (1×10^6) 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 $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM PMSF and 20 μM aprotinin. Cellular debris was removed by centrifugation at 10 000 *g* for 5 min. HA epitope-tagged proteins were immunoprecipitated with anti-HA monoclonal antibody HA.11 (Babco). 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 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 5 μCi of [γ-^{32}P]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-conjug-

ated antibody to rabbit or mouse IgG using the enhanced chemiluminescence (ECL) Western blotting system (Amersham).

Recombinant DNAs

A 13 kb *Sph*I–*Bam*HI fragment from the cosmid B0478 was subcloned into the *Sph*I–*Bam*HI sites of the GFP reporter vector pPD95.75 (kindly provided by A.Fire), generating plasmid pMK101. This construct contained the *jnk-1* coding region and all introns of the gene flanked by 5 kb of DNA at the 5′ end and 2 kb of DNA at the 3′ end. To fuse GFP to the 3′ end of the *jnk-1* coding region, two synthetic oligonucleotides (5′-CACAGATTACGCAAGAACCCATGACATTTATTC-AGATCTG and 5′-GATCCAGATCTGAATAAATGTCATGGGTTCT-TGCGTAATCTGTGAGCT), which contained the DNA sequences encoding the C-terminal 11 amino acids of JNK-1 and the *Sac*I and *Bam*HI sites, were annealed and substituted for the *Sac*I–*Bam*HI fragment of pMK101. The resulting plasmid pMK102 contains the *jnk-1::gfp* fusion. To construct the *jkk-1::gfp* fusion (pMK104), a 3.6 kb *Hin*dIII–*Xba*I fragment from the cosmid F35C8 was subcloned into the *Hin*dIII–*Xba*I sites of pPD95.75, generating plasmid pMK103. This construct contained the 2.5 kb of *jkk-1* upstream sequence, the first 99 amino acids of the *jkk-1* open reading frame, as well as the first three introns. The *Xba*I–*Sma*I fragment, which contained the remaining 334 amino acids of JKK-1 protein, was obtained from *jkk-1* cDNA and then subcloned into the *Xba*I–*Sma*I sites of pMK103. The resulting plasmid pMK104 lacked the C-terminal two amino acids of JKK-1. This *jkk-1::gfp* construct was shown to be functional because pMK104 could rescue the phenotypes of the *jkk-1* null mutation. To construct another *jkk-1::gfp* fusion (pMK110) which contained all introns of *jkk-1* genomic sequence, a partially digested 2 kb *Xba*I–*Sal*I fragment from F35C8 was subcloned into the *Xba*I–*Sal*I site of pMK104. The plasmid pMK105 was constructed by inserting the full-length *jkk-1* cDNA downstream of the *hsp16-2* promoter in the vector pPD49.78 (kindly provided by A.Fire). The plasmid pMK106 was constructed by inserting the full-length *jkk-1* cDNA downstream of the *unc-30* promoter in the vector pSC157 (kindly provided by Y.Jin). These constructs were co-injected with the pMK107 plasmid, which contains the EF1α promoter (kindly provided by M.Koga) fused to GFP. The plasmid pMK108 was constructed by inserting a 1.6 kb *Hin*dIII fragment from $P_{del\text{-}1}$*::lacZ* (kindly provided by M.Driscoll) to the vector pPD49.26 (kindly provided by A.Fire). The plasmid pMK109 was constructed by inserting the full-length *jkk-1* cDNA into the downstream of the *del-1* promoter in pMK108.

Isolation of jkk-1 null mutant

A Tc1 insertion mutant *jkk-1::Tc1 (km1)* X; *mut-2(r459)* I was isolated using a sib-selection protocol (Zwaal *et al*., 1993). This insertion was located between amino acid positions 286 and 287 in exon 8 of *jkk-1*. With the outer set of primers, 5′-GTGTCAATCAGCGCCAAACACAC and 5′-GCACCAGTGATGATATCAGACACG, and the nested set primers, 5′-TCATTTCCTCCGTGTTGAGTCTG and 5′-TTGACAGA-AGGCGTGTTTGGG, spanning a genomic region of 2.6 kb, 1.0 and 1.2 kb deletion derivatives (*km2* and *km3*, respectively) of *km1* were detected. The PCR products derived from the deletions were directly sequenced. *jkk-1(km2)* was isolated using a sib-selection protocol. The *km2* allele was backcrossed nine times to animals of a *lon-2* background and then twice to animals of an N2 background. We named this strain KU2.

Strain construction

Transgenic *jkk-1(km2)* lines bearing pMK105 ($P_{hsp16\text{-}2}$*::jkk-1*) or pPD49.78 ($P_{hsp16\text{-}2}$) as an integrated array (*kmIs1* and *kmIs2*, respectively) were generated by treatment of animals with 40 Gy (1 Gy = 100 rads) from an X-ray source. Each transgenic strain was backcrossed six times to animals of a *jkk-1(km2)* background. To generate the *unc-25(e156); jkk-1(km2)* double mutant KU3, the KU2 males were mated to the CB156 hermaphrodites [genotype: unc-*25(e156)*]. Then, the *unc-25(e156)*/*unc-25(e156); jkk-1(km2)*/+ Unc F2 hermaphrodites were selected by PCR and allowed to self-fertilize to generate the *unc-25(e156); jkk-1(km2)* double mutant.

Locomotion assays

Individual animals were transferred to fresh NGM plates seeded with *E.coli* and allowed to cut tracks for 10–20 min before paths were measured. Tracks were measured from photographs or video recordings. The amplitude of the path and the distance between successive peaks in the path (the wave length) were measured.

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