SEK-1 MAPKK mediates Ca^{2+} signaling to determine neuronal asymmetric development in *Caenorhabditis elegans*

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The mitogen-activated protein kinase (MAPK) pathway is a highly conserved signaling cascade that converts extracellular signals into various outputs. In *Caenorhabditis elegans*, asymmetric expression of the candidate odorant receptor STR-2 in either the left or the right of two bilaterally symmetrical olfactory AWC neurons is regulated by axon contact and Ca^{2+} signaling. We show that the MAPK kinase (MAPKK) SEK-1 is required for asymmetric expression in AWC neurons. Genetic and biochemical analyses reveal that SEK-1 functions in a pathway downstream of UNC-43 and NSY-1, Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and MAPK kinase kinase (MAPKKK), respectively. Thus, the NSY-1–SEK-1–MAPK cascade is activated by Ca^{2+} signaling through CaMKII and establishes asymmetric cell fate decision during neuronal development.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) signal transduction pathways are evolutionarily conserved in eukaryotic cells and transduce signals in response to a variety of extracellular stimuli. Each pathway is composed of three classes of protein kinase: MAPK, MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK) (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). MAPK is activated by phosphorylation of specific tyrosine and threonine residues by a family of dual-specificity protein kinase MAPKKs. MAPKK is in turn activated by phosphorylation of serine and serine/threonine residues by a family of upstream MAPKKKs.

Three subgroups of the MAPK superfamily have been identified (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998): extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Further, several subgroups of the MAPKK superfamily have been identified, such as MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7. The ERK group is activated by MEK1 and MEK2. Whereas MKK4 can activate both the JNK and p38 subgroups, MKK7 is specific to the JNK subgroup. On the other hand, MKK3 and MKK6 act solely as activators of the p38 subgroup. These members of the MAPKK superfamily are activated by members of the MAPKKK superfamily, such as Raf, MEKK, TAK1, MLK, Tpl2 and ASK1.

The JNK and p38 pathways have been implicated in a variety of biological functions in mammalian cells, including apoptosis and the responses to stress. However, the physiological role of these pathways in the normal development and function of the organism has not been fully elucidated. Recent studies using model genetic organisms have revealed some of the physiological roles of the JNK signaling pathway. In *Drosophila*, the JNK pathway is required for the normal morphogenetic process of dorsal closure (Noselli, 1998). In contrast, genetic studies of *Caenorhabditis elegans* demonstrate that the JNK pathway, consisting of JKK-1 (MAPKK) and JNK-1 (MAPK), regulates coordinated movement via type D GABAergic (GABA, γ -aminobutyric acid) motor neurons, but it does not appear to be essential for embryonic morphogenesis (Kawasaki *et al.*, 1999).

Drosophila contains two p38 MAPKs, D-p38a and D-p38b. Phosphorylation and activation of D-p38a/b are mediated by the MAPKKs D-MKK3 and D-MKK4 (Han *et al.*, 1998). A recent

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study has shown that D-MKK3 is encoded by the gene *licorne* (*lic*) (Suzanne *et al.*, 1999), which plays an essential role in anterior–posterior and dorsal–ventral patterning during oogenesis by regulating the localization of cell fate determinants. Thus, the p38 pathway may play an intrinsic role in establishing the initial asymmetry in other organisms. Interestingly, p38 in zebrafish is asymmetrically activated during the cleavage period and plays a role in cleavage control on the prospective dorsal side (Fujii *et al.*, 2000). Although there are two genes, *pmk-1* and *pmk-2*, that encode p38 MAPKs in *C. elegans* (Kawasaki *et al.*, 1999), *pmk-1/2* mutants have yet to be isolated.

The *C. elegans nsy-1* gene encodes a MAPKKK that is most similar to mammalian ASK1, an activator of JNK and p38 MAPKs (Ichijo *et al.*, 1997; Sagasti *et al.*, 2001). The *nsy-1* locus was previously identified as mutations that affect asymmetric olfactory neuron fates (Troemel *et al.*, 1999). Most neurons in *C. elegans* exist as bilaterally symmetric, morphologically similar pairs. However, the two AWC olfactory neurons interact with each other to generate distinct fates (White *et al.*, 1986). The candidate odorant receptor STR-2 is expressed asymmetrically in either the left or right AWC neuron, but never in both. The asymmetric AWC cell fate is regulated by axon contact during development and by Ca²⁺ signaling (Troemel *et al.*, 1999). *nsy-1* mutants express STR-2 in both AWC neurons, disrupting AWC asymmetry. In this study, we show that the MAPKK SEK-1 determines asymmetric cell fates in the AWC neurons by functioning in a pathway downstream of Ca²⁺ signaling and NSY-1. Our results suggest that the NSY-1–SEK-1–MAPK cascade is a central component of the AWC cell fate decision.

RESULTS AND DISCUSSION

SEK-1 functions as a MAPKK

The yeast Hog1 MAPK pathway mediates cellular responses to an increase in external osmolarity (Posas and Saito, 1997). This signaling cascade consists of the Ssk2/22 (MAPKKK), Pbs2 (MAPKK) and Hog1 (MAPK) kinases (Figure 1A). The *C. elegans* JNK homolog, JNK-1, can complement the high osmolarity-sensitive (Osm^s) growth phenotype of a *hog1Δ* mutant (Kawasaki *et al.*, 1999). However, expression of JNK-1 did not suppress the *pbs2Δ* Osm^s phenotype (Kawasaki *et al.*, 1999), suggesting that Pbs2 is required for the activation of the *C. elegans* JNK-1. To identify the *C. elegans* MAPKKs activating JNK-1, we have previously isolated *C. elegans* cDNAs, which can suppress the *pbs2Δ* mutation in a JNK-1-dependent manner (Kawasaki *et al.*, 1999). One of them is JKK-1-homologous to the MAPKK MKK7, an activator specific to JNK. The second, SEK-1, is also related to the MAPKK family (Figure 1B), having similarity to MKK3/6 (50% identity) and MKK4 (43% identity). MKK3 and MKK6 are specific activators of p38, whereas MKK4 can activate both the JNK and p38 subgroups of the MAPK superfamily (Derijard *et al.*, 1995; Moriguchi *et al.*, 1996; Stein *et al.*, 1996). Suppression of the *pbs2Δ* Osm^s phenotype was also observed when SEK-1 was coexpressed with the *C. elegans* p38 MAPK homolog, PMK-1 (Figure 1A). This indicates that SEK-1 can activate both JNK-1 and PMK-1 in the yeast Hog pathway.

Sequence information from the *C. elegans* Genome Consortium showed that *sek-1* corresponds to R03G5.2. Comparison of the sequences between the database genomic DNA and the

cloned cDNA revealed that the *sek-1* gene has seven exons (Figure 2A). To determine the expression pattern of *sek-1*, we constructed a translational fusion between the promoter and coding sequences of *sek-1* and green fluorescent protein (GFP) to generate *sek-1::gfp*. This fusion protein has functional MAPKK activity in *C. elegans* (see below). Transgenic *C. elegans* bearing the *sek-1::gfp* fusion exhibited fluorescence in excretory canal, rectal epithelial cells, uterine-vulval cells and several neurons (data not shown).

To determine whether SEK-1 has MAPKK activity, SEK-1 was cloned into a mammalian expression vector to generate a Flag-tagged protein (Flag-SEK-1). Human 293 cells were transiently transfected with the vector, and cell lysates were subjected to immunoprecipitation with anti-Flag antibody, followed by a kinase assay with kinase-inactive p38 MAPK (p38-KI) as a substrate (Figure 1C). SEK-1 phosphorylated p38-KI *in vitro*, but a catalytically inactive mutant, SEK-1(K79R), in which Lys-79 in the ATP-binding domain was replaced by Arg, did not phosphorylate p38-KI. We next examined whether SEK-1 is able to activate mammalian p38 *in vivo*. Since MAPKs are activated by dual phosphorylation, we performed western-blot analysis using an anti-phospho-p38 antibody that specifically recognizes the dually phosphorylated active form of p38 (Figure 1C). Activation of p38 MAPK *in vivo* was detected in cells expressing Flag-SEK-1 but not Flag-SEK-1(K79R). These results indicate that SEK-1 is a MAPKK that phosphorylates and activates p38 MAPK.

Isolation of a *sek-1* deletion mutant

To examine the biological function of *sek-1*, we generated a *sek-1* deletion mutant, *sek-1(km4)*, by a transposon-based PCR sib-selection method (Zwaal *et al.*, 1993). The *sek-1(km4)* mutant allele is missing an ~2.1-kb region containing three exons, including kinase domains II–XI (Figure 2A). Therefore, *sek-1(km4)* is expected to be a null mutation. *sek-1(km4)* homozygous mutant animals were found to be viable but showed a variety of behavioral defects in egg laying and male turning during mating (data not shown).

SEK-1 regulates asymmetric AWC cell fate

The *C. elegans nsy-1* gene encodes a homolog of the human MAPKKK ASK1 (Ichijo *et al.*, 1997; Sagasti *et al.*, 2001). The *nsy-1* gene is involved in determining asymmetric cell fates in the AWC olfactory neurons (Troemel *et al.*, 1999). AWC neurons exist as bilaterally symmetric pairs in the head (Figure 2B). Asymmetry between the two AWC neurons can be monitored by following expression of the candidate odorant receptor STR-2 (Troemel *et al.*, 1999). A strain carrying a *str-2::gfp* fusion integrated into chromosome I expresses GFP in just one AWC neuron, with about half of the animals in a population expressing GFP in AWC-left and the other half expressing GFP in AWC-right. No animals express GFP in both AWC neurons (Figure 2C, Table I). Similar to *nsy-1*, we found that *sek-1* also affected the asymmetric AWC cell fate. In *sek-1(km4)* mutants, *str-2* was expressed in both AWC neurons (Figure 2C, Table I), indicating that SEK-1 is required for *str-2* asymmetry. With the *str-2::gfp* fusion gene, AWC neuroanatomy was normal in *sek-1(km4)* mutants (data not shown).

Fig. 1. *Caenorhabditis elegans* SEK-1 functions as a MAPKK. (A) Suppression of the *pbs2Δ* mutants by *C. elegans* SEK-1, JNK-1 and PMK-1. *pbs2Δ* mutants were transformed with the indicated *C. elegans* genes. Three independent transformants were streaked onto plates containing 1.2 M sorbitol and incubated at 30°C. The *pbs2Δ* mutants do not grow in the presence of sorbitol. Model for the yeast osmotic stress-activated Hog1 MAPK pathway is shown in the right panel. **(B)** Sequence alignment of SEK-1 with mammalian MAPKKs, MKK3, MKK6 and MKK4. Identical amino acids are shaded. The sites of activating phosphorylation in MAPKKs are indicated by asterisks and the kinase subdomains are marked with roman numbers below the sequences. The DDBJ/EMBL/GenBank accession number for the SEK-1 sequence is AB060731. **(C)** MAPKK activity of SEK-1. HEK 293 cells were transfected with Flag-SEK-1 and Flag-SEK-1(K79R). Anti-Flag antibody immunoprecipitates (IP) were used for *in vitro* kinase reactions with p38-KI as a substrate (top panel). The immunoprecipitates were immunoblotted (IB) with anti-Flag antibody (second panel). Whole-cell extracts were immunoblotted with anti-phospho-p38 MAPK antibody (third panel) and anti-p38 antibody (bottom panel).

To determine whether *sek-1* expression in AWC neurons, or in an another cell type, is required for asymmetrical *str-2* expression,

sek-1 cDNA was expressed under the control of the *odr-3* promoter (Roayaie *et al.*, 1998), which drives expression in the

Fig. 2. Effect of the *sek-1* mutation on *str-2* asymmetry in AWC olfactory neurons. (A) Structure of the *sek-1* gene. Exons are indicated by boxes. The shaded and open boxes are the translated and untranslated regions, respectively. The black boxes indicate kinase domains. *sek-1(km4)* is a 2083-bp deletion mutation in which three exons are missing. (B) The morphology of AWC neurons: a lateral diagram (left panel); a cross-sectional diagram (right panel). (C) Patterns of *str-2::gfp* expression in wild-type N2 animals, *sek-1(km4)* mutants, N2 carrying *odr-3p::nsy-1ΔN* (*Ex[nsy-1ΔN]*) and *sek-1(km4)* carrying *odr-3p::nsy-1ΔN* (*sek-1 Ex[nsy-1ΔN]*). Arrows indicate AWC neurons. Fluorescence posterior to the head is gut auto-fluorescence. The photographs show dorsal views. A, anterior; P, posterior; L, left; R, right.

AWC neurons. For transgenic *sek-1(km4)* mutants harboring the *odr-3p::sek-1* transgene, most animals showed *str-2* expression only in one AWC neuron, but not in both (Table I). Thus, SEK-1 acts within the AWC neurons to determine asymmetric expression of *str-2*.

MAPKKs are activated by phosphorylation within the activation loop between subdomains VII and VIII (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). These phosphorylation sites are conserved in SEK-1 (Ser 204 and Thr208) (Figure 1B). We generated *sek-1(STDD)* mutants, in which both Ser 204 and Thr 208 were replaced with Asp. The *sek-1(STDD)* mutants are predicted to have constitutively active

Fig. 3. SEK-1 MAPKK functions downstream of UNC-43 CaMKII and NSY-1 MAPKKK. (A) Association between SEK-1 and NSY-1. HEK 293 cells were transfected with control vector (-), T7-NSY-1 and Flag-SEK-1(K79R) as indicated. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody. Immunoprecipitates were immunoblotted (IB) with anti-T7 antibody (top panel) and anti-Flag antibody (middle panel). Whole-cell extracts were immunoblotted with anti-T7 antibody (bottom panel). (B) Phosphorylation of SEK-1 by NSY-1. HEK 293 cells were transfected with control vector (-), T7-NSY-1, T7-NSY-1(K703M) and Flag-SEK-1(K79R) as indicated. Immunoprecipitated complexes with anti-Flag antibody were incubated with [γ - 32 P]ATP and analyzed by autoradiography (upper panel). The immunoprecipitates were immunoblotted with anti-Flag antibody (lower panel). (C and D) Effects of *nsy-1* (C) and *unc-43* (D) mutations on SEK-1 activity in *C. elegans*. The *sek-1::gfp* (WT) and *sek-1(K79R)::gfp* (KN) transgenes were injected into wild-type N2 animals, *nsy-1(ky400)* and *unc-43(n1186)* mutants as indicated. Cell lysates from each animal were immunoprecipitated with anti-GFP antibody. The immunoprecipitates were used for *in vitro* kinase reactions with p38-KI (upper panel). The immunoprecipitates were immunoblotted with anti-GFP antibody (lower panel).

kinase activity (Pages *et al.*, 1994). When an *odr-3p::sek-1(STDD)* transgene was introduced into N2 wild-type animals, most animals had no *str-2* expression in either of the AWC neurons, the opposite phenotype of *sek-1(km4)* (Table I). Taken together, the level of SEK-1 kinase activity can determine whether a cell adopts the fate that expresses *str-2* or not.

SEK-1 acts downstream of NSY-1

The above results raised the possibility that NSY-1 and SEK-1 constitute a MAPK cascade that regulates *str-2* asymmetry. To test this possibility, we examined genetic interactions between *nsy-1* and *sek-1*. When a constitutively active form of NSY-1, NSY-1ΔN, lacking the N-terminal 640 amino acids was expressed by the *odr-3* promoter, *str-2* expression was abolished in both AWC neurons (Figure 2C, Table I) (Sagasti *et al.*, 2001). In contrast, when this same *odr-3p::nsy-1ΔN* transgene was expressed in a transgenic *sek-1* null strain, *str-2* expression

Table I. Patterns of *str-2* expression in mutants

	Cells expressing <i>str-2::gfp</i> (%)			<i>n</i>
	2AWC ^{off}	1AWC ^{off} /1AWC ^{on}	2AWC ^{on}	
N2 wild-type	0	100	0	139
<i>nsy-1(ky400)</i>	0	2	98	117
<i>sek-1(km4)</i>	0	2	98	172
<i>sek-1(km4) Ex[odr-3p::sek-1]</i>	5	79	16	56
N2 <i>Ex[odr-3p::nsy-1ΔN]</i>	79	21	0	110
<i>sek-1(km4) Ex[odr-3p::nsy-1ΔN]</i>	0	5	95	61
N2 <i>Ex[odr-3p::sek-1(STDD)]</i>	91	9	0	105
<i>nsy-1(ky400) Ex[odr-3p::sek-1(STDD)]</i>	82	1	17	110
<i>unc-43(n498)</i>	94	6	0	139
<i>unc-43(n498); sek-1(km4)</i>	0	10	90	124

Animals were scored as adults for *str-2::gfp* expression in AWC neurons. The AWC cells can be defined as AWC^{on} and AWC^{off} based on their pattern of *str-2::gfp*.

Fig. 4. Model for *str-2* regulation by the CaMKII–MAPK pathway. See text for details.

appeared in both AWC neurons, similar to the phenotype of *sek-1(km4)* mutant (Figure 2C, Table I). Conversely, a gain-of-function *odr-3p::sek-1(STDD)* transgene increased the numbers of animals with no expression of *str-2* in either of the AWC neurons in loss-of function *nsy-1(ky400)* mutants (Table I). These results indicate that the *sek-1* mutations are epistatic to the *nsy-1* mutations, suggesting that SEK-1 functions in a pathway downstream of NSY-1.

To obtain further support for the possibility that NSY-1 acts as a MAPKKK of SEK-1 MAPKK, we examined the biochemical interaction between NSY-1 and SEK-1. We transiently expressed T7-tagged NSY-1 (T7-NSY-1) together with a kinase-inactive Flag-SEK-1(K79R) in 293 cells. Cell extracts were subjected to immunoprecipitation with anti-Flag antibody, followed by both immunoblotting and *in vitro* kinase assay. NSY-1 was detected

in SEK-1(K79R) immunoprecipitates (Figure 3A), indicating that NSY-1 is in a complex with SEK-1. When the immunoprecipitates were incubated with [γ -³²P]ATP, SEK-1(K79R) became phosphorylated (Figure 3B). We generated a kinase-inactive form of NSY-1, NSY-1(K703M), in which Lys-703 in the ATP-binding domain has been mutated to Met. This mutant did not phosphorylate SEK-1(K79R) (Figure 3B), indicating that the phosphorylation of SEK-1(K79R) is dependent on NSY-1 kinase activity. These results demonstrate that NSY-1 acts as a MAPKKK that phosphorylates SEK-1 MAPKK.

To confirm that NSY-1 activates SEK-1 in *C. elegans*, SEK-1 kinase activity was tested with the *sek-1::gfp* transgene. If NSY-1 functions as MAPKKK for SEK-1 in *C. elegans*, SEK-1::GFP should not show kinase activity in loss-of-function *nsy-1(ky400)* mutant animals. The *sek-1::gfp* transgene was introduced as an extrachromosomal array into both wild-type N2 and *nsy-1(ky400)* mutant animals. SEK-1::GFP was then immunoprecipitated with anti-GFP antibody and tested in a protein kinase assay using p38-KI as a substrate. SEK-1::GFP expressed in wild-type animals was found to have clearly detectable kinase activity, while a catalytically inactive SEK-1(K79R)::GFP negative control protein did not (Figure 3C). When SEK-1::GFP was immunoprecipitated from cell extracts of *nsy-1(ky400)* mutant animals, it had little kinase activity (Figure 3C), indicating that NSY-1 was required for the activation of SEK-1 in *C. elegans*. Taken together, these results indicate that NSY-1 MAPKKK acts in the same pathway upstream of SEK-1 MAPKK and functions to activate SEK-1 kinase activity.

SEK-1 acts downstream of the CaMKII homolog UNC-43

Previous studies have revealed that UNC-43 Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) acts as a switch in determining *str-2* expression in AWC neurons (Reiner et al., 1999; Troemel et al., 1999). While *str-2* is expressed in both AWC neurons in loss-of-function *unc-43(n1186)* mutants, it is

expressed in neither neuron in gain-of-function *unc-43* (*n498*) mutants (Table I). To characterize the role of the *sek-1* gene in AWC asymmetry, we analyzed the epistasis relationship between *sek-1* and *unc-43*. Double mutant analysis with *sek-1(km4)* and *unc-43(n498)* indicated that the *sek-1* mutation was epistatic to the *unc-43* mutation (Table I). This suggests that *sek-1* acts downstream of or in parallel to *unc-43*.

We next tested whether the *unc-43(n1186)* mutation affects SEK-1 kinase activity in *C. elegans*. The *sek-1::gfp* transgene was introduced into *unc-43(n1186)* mutant animals, and SEK-1::GFP immunoprecipitates from animals were assayed for kinase activity. The *unc-43(n1186)* mutation was found to cause a decrease in SEK-1 kinase activity (Figure 3D). These results are consistent with *sek-1* acting downstream of *unc-43* in a linear pathway. Thus, UNC-43 CaMKII functions as a positive mediator of the NSY-1–SEK-1–MAPK cascade regulating AWC asymmetry.

Asymmetric expression of *str-2* creates two independent AWC neurons that each have a distinct olfactory specificity. This organization enables the animal to detect more odors and discriminate between them in complex environments (Wes and Bargmann, 2001). Asymmetrical *str-2* expression in AWC neurons is initiated by regulating Ca²⁺ influx and it is maintained by cGMP signaling (Troemel *et al.*, 1999). Ca²⁺ enters through the UNC-2 and UNC-36 voltage-gated Ca²⁺ channels (Schafer and Kenyon, 1995; Lee *et al.*, 1997) and stimulates UNC-43 CaMKII activity, leading to the inhibition of *str-2* expression in one AWC neuron. Furthermore, we recently showed that NSY-1, a homolog of the human MAPKKK ASK1, is involved in determining AWC asymmetry (Sagasti *et al.*, 2001). In this study we show that SEK-1 MAPKK functions in a pathway downstream of UNC-43 and NSY-1 (Figure 4). Thus, CaMKII is a signal converter, transducing an increase in Ca²⁺ concentration into activation of the NSY-1–SEK-1–MAPK pathway that determines the fate of *str-2* expression in AWC neurons. Mammalian ASK1 activates the JNK and p38 MAPKs (Ichijo *et al.*, 1997). We have not yet identified a *C. elegans* MAPK that functions downstream of the NSY-1–SEK-1 pathway. Since the *C. elegans* JNK homolog *jnk-1* did not affect AWC asymmetry (data not shown), p38 MAPK is a candidate that participates in the AWC cell fate determination. In the absence of lateral signaling between the AWC neurons, both AWC cells do not express *str-2*, and this *str-2*-negative state is probably maintained by the CaMKII–MAPK pathway. During normal development, AWC cells communicate with each other, probably through their axons, which contact each other in the nerve ring (White *et al.*, 1986). This communication inhibits the activity of the CaMKII–MAPK signaling pathway in one of the neurons. Further understanding of this lateral signaling mechanism may be provided by identification of the receptors or ligands that allow the AWC neurons to recognize and signal to each other. The subsequent effects of MAPK on *str-2* expression could be through either transcriptional regulation or the direct phosphorylation of target molecules. Given the power of genetic analysis in *C. elegans*, suppressor screens may lead to the identification of downstream genes encoding targets or downstream effectors of the NSY-1–SEK-1–MAPK pathway that play important roles in the regulation of the asymmetric AWC cell fate decision.

METHODS

Assays of yeast Hog1 MAPK pathway. Yeast strain TM334 (*MATa pbs2Δ::HIS3 ura3 leu2 trp1 his3*) was transformed with yeast expression plasmids encoding PMK-1 and SEK-1. Transformants were scored for osmo-sensitivity on plates containing 1.2 M sorbitol.

Analysis of *str-2* expression in *C. elegans* mutants. A *str-2::gfp* fusion gene and mutants of *unc-43* and *nsy-1* have been described previously (Reiner *et al.*, 1999; Troemel *et al.*, 1999). Germline transformation was carried out as described previously (Mello *et al.*, 1992). Cell identification was based on characteristic morphology and position of GFP-positive cell nuclei as viewed by simultaneous fluorescence and Nomarski differential interference microscopy.

Isolation of the *sek-1* null mutant. A Tc1 insertion mutant *mut-2(r459)* I; *sek-1::Tc1* X was isolated using a sib-selection protocol (Zwaal *et al.*, 1993). This insertion was located in intron VI of *sek-1*. From *mut-2(r459)* I; *sek-1::Tc1* X animals, a 2.1-kb deletion derivative (*km4*) was isolated. The PCR products derived from the deletion were directly sequenced. *sek-1(km4)* was isolated using a sib-selection protocol. The *km4* allele was backcrossed twice to animals of a *lon-2* background and then nine times to animals of wild-type N2 background.

Preparation of GFP immunoprecipitates from *C. elegans*. Sonicated worm lysates were incubated with anti-GFP polyclonal antibody (Clontech) in a buffer containing 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin at 4°C for 3 h. Samples were precipitated with protein A–Sepharose beads (Pharmacia). Immunoprecipitates were subjected to protein kinase analysis and immunoblotting with anti-GFP monoclonal antibody (Clontech).

Immunoblotting. Immunoprecipitation from mammalian cells was carried out as described previously (Kawasaki *et al.*, 1999). The immunoprecipitates and aliquots of total lysates were resolved on SDS–PAGE and transferred to PVDF membranes (Hybond-P, Amersham). The membranes were immunoblotted with antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated antibodies to mouse IgG using the Enhanced Chemiluminescence Western Blotting System (Amersham). Rabbit polyclonal antibodies to p38 and phospho-p38 MAPK (New England BioLabs) and mouse monoclonal antibodies to Flag (Sigma) and T7 (Novagen) were used.

In vitro kinase assays. Aliquots of immunoprecipitates were incubated in 10 μl kinase buffer containing 1 μg of bacterially expressed histidine-tagged p38-K1, 10 mM HEPES pH 7.4, 1 mM DTT, 5 mM MgCl₂ and 5 μCi of [γ-³²P]ATP at 25°C for 2 min. Samples were resolved on SDS–PAGE, and phosphorylated proteins were visualized by autoradiography.

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