MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events

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Activated Ras initiates a cascade of sequential phosphorylation events, including the protein kinases Raf, MEK, and MAP kinase. The Let-60 Ras-mediated signal transduction pathway controls vulval induction in *Caenorhabditis elegans*. Both Lin-45 Raf and Sur-1 MAP kinase have been determined to be essential factors during vulval induction; however, the *C. elegans mek* gene has not been identified. In this paper, we have cloned a *C. elegans mek* gene, *mek-2*, and demonstrated that the MEK-2 protein possesses the biochemical properties of MAP kinase kinases: The *C. elegans* MEK-2 protein can phosphorylate and activate a human MAP kinase (ERK1), and MEK-2 itself can be phosphorylated and activated by immunoprecipitated mammalian Raf. The *mek-2* gene plays a key role in the *let-60 ras*-mediated vulval induction pathway, as loss-of-function mutations in the gene (*ku114* and *h294*) significantly reduce the signal transmitted through Ras. *mek-2(ku114)* completely suppressed the Multivulva (Muv) phenotype of a hyperactive *let-60 ras* mutation, and animals homozygous for *mek-2(ku114)* also displayed a partial larval lethal phenotype. Animals homozygous for *mek-2(h294)* exhibited a highly penetrant sterile and Vulvaless phenotype. Microinjection of a gain-of-function *mek-2* mutation resulted in Muv and other mutant phenotypes, whereas microinjection of a dominant-negative mutation not only suppressed the Muv phenotype of an activated *let-60 ras* mutation but also caused an egg-laying defective phenotype in otherwise wild type animals. Our results demonstrate that *mek-2* acts between *lin-45 raf* and *sur-1/mpk-1* in a signal transduction pathway used in the control of vulval differentiation and other developmental events.

[Key Words: *mek-2*; MAP kinase kinase; vulval induction; *C. elegans*; signal transduction; *ras*]

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A variety of extracellular proliferation and differentiation factors induce protein kinase cascades that result in phosphorylation and regulation of numerous cellular proteins (Crews and Erikson 1993; Davis 1994). Receptors for many of these growth and differentiation factors are receptor tyrosine kinases (RTKs), which transmit signals through the GTP-binding protein Ras (McCormick 1993). Activated Ras initiates a cascade of sequential phosphorylation events in which the serine-threonine kinase Raf phosphorylates and activates MEK (also known as MAP kinase kinase) (Dent et al. 1992; Howe et al. 1992; Kyriakis et al. 1992). MEK, a dual specificity kinase, phosphorylates threonine and tyrosine residues of MAP kinase (also known as ERK), thus leading to the activation of MAP kinase (Boulton et al. 1990; Crews and Erikson 1992). The MAP kinase cascade is highly

conserved in eukaryotic organisms including yeast, *Drosophila*, and mammals (Blumer and Johnson 1994). The activated MAP kinases phosphorylate and regulate many cellular proteins including receptors (Northwood et al. 1991), phospholipase (Lin et al. 1993), other kinases (Sturgill et al. 1988; Stokoe et al. 1992), and nuclear transcription factors (Gille et al. 1992; Hill et al. 1993; Marais et al. 1993). The mammalian MEK displays a high substrate selectivity toward MAP kinase in vitro (Seger et al. 1992a) once it is activated by Raf-mediated phosphorylation of two conserved serine residues, Ser-218 and Ser-222 (Alessi et al. 1994; Zheng and Guan 1994). It has been demonstrated in human MEK1 that substitutions of both Ser-218 and Ser-222 by glutamate or aspartate residues result in a constitutively active form of the protein that is sufficient for NIH-3T3 cell transformation and PC12 cell differentiation (Cowley et al. 1994; Mansour et al. 1994). In contrast, changing these serine residues to alanines results in an interfering mutant

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MEK that could block serum-induced DNA synthesis and revert *ras*- or *src*-transformed cells (Cowley et al. 1994), demonstrating that MEK activation is necessary for cell growth.

Developmental, genetic, and molecular studies in *Caenorhabditis elegans* have shown that multiple intercellular signals are essential for vulval differentiation (Horvitz and Sternberg 1991). During vulval induction, the gonadal anchor cell induces three of the six vulval precursor cells to generate vulval tissue (Fig. 1). The other three cells generate nonspecific epidermis. Several genes necessary for vulval induction encode proteins homologous to factors that act in mammalian RTK/Ras-mediated signal transduction. The *lin-3* gene product, an epidermal growth factor (EGF)-like molecule, is the inductive signal from the anchor cell that induces vulval differentiation (Hill and Sternberg 1992); the *let-23* gene encodes a receptor tyrosine kinase that is likely to be the receptor for Lin-3 (Aroian et al. 1990); the *sem-5* gene, encoding a protein with Src homology domains SH2 and SH3, has been proposed to act between the RTK and Ras (Clark et al. 1992). Activation of Let-60 Ras initiates a kinase cascade including Lin-45, a Raf serine-threonine kinase (Han et al. 1993), and Sur-1, a MAP kinase (Lackner et al. 1994; Wu and Han 1994).

Vulval induction has provided an excellent model system to study the Ras-mediated signaling pathway. Al-

though both Lin-45 Raf and Sur-1 MAP kinase have been determined to be essential factors during *C. elegans* vulval differentiation (Han et al. 1993; Lackner et al. 1994; Wu and Han 1994), the function of the MEK protein has not been identified in *C. elegans*. To further understand the biological function of MAP kinase cascade in a developmental organism, we have cloned and characterized a *C. elegans* *mek* gene, *mek-2*. We show here that the *mek-2* gene encodes a protein that is structurally and biochemically similar to mammalian MEK proteins. Through genetic analysis of loss-of-function mutations, and ectopically expressed dominant mutations, we determined that the *mek-2* gene plays an essential role in the *let-60 ras*-mediated vulval signal transduction pathway. The pleiotropic defects associated with various *mek-2* mutations indicate that this gene is involved in multiple developmental events in *C. elegans*.

Results

The C. elegans mek-2 gene encodes a protein that is homologous to mammalian MAP kinase kinases

To identify *C. elegans* MEK homologs, degenerate oligonucleotides corresponding to the conserved amino acid sequences in the MEK family were used to amplify sequences by polymerase chain reactions (PCR) from a cDNA library (see Material and methods). Sequence analysis of amplified DNA revealed two MEK homologs, *mek-1* and *mek-2*. MEK-2 has high amino acid sequence identity with the mammalian MEK, whereas MEK-1 is more distantly related to mammalian MEK (P. Kayne et al., pers. comm.; K.-L. Guan, unpubl.).

The PCR product corresponding to *mek-2* was used to screen a mixed stage cDNA library (Stratagene). Four positive clones were isolated after screening 300,000 plaques. All four cDNA clones encoded the same protein based on their restriction maps and their 5' sequences. The longest cDNA clone was completely sequenced and had an open reading frame of 385 amino acid residues, which shares a significant sequence identity to mammalian MEK. The 3' end of the cDNA sequences was determined by identifying the poly(A) tails of the cDNA clones. However, the translational start codon ATG was missing in the longest cDNA clone isolated. To identify the 5' end of the cDNA, two primers, one complementary to the vector and the other complementary to the *mek-2* cDNA, were used to amplify sequences by PCR from the cDNA library. A 0.2-kb fragment was isolated and sequenced that contained a putative initiation ATG codon and 11 nucleotides corresponding to the SL1 *trans*-splicing leader sequence that is frequently found at the 5' end of *C. elegans* transcripts (Krause and Hirsh 1987). Thus, we conclude that the full-length *mek-2* cDNA encodes a primary translation product containing 387 amino acids (Fig. 2). As shown in Figure 3, 54% of the amino acids in the *mek-2* gene product are identical to human MEK1 (Seger et al. 1992b; Zheng and Guan 1993), and 55% of them are identical to the *Drosophila* MEK homolog Dsor-1 (Tsuda et al. 1993). All of the con-

Figure 1. A model for vulval cell fate specification in wild-type *C. elegans*. The six multipotent vulval precursor cells (VPCs, P3.p–P8.p, depicted as open ovals) are located just ventral to the gonad. An inhibitory signal from the surrounding epidermis prevents vulval differentiation (Herman and Hedgecock 1990). However, an inductive signal from the anchor cell in the somatic gonad induces the nearby VPCs to adopt vulval cell fates (solid ovals) (Horvitz and Sternberg 1991; Hill and Sternberg 1992). These induced cells divide three rounds, and the progeny generate vulval tissue. The other three precursor cells divide only once and then fuse with the surrounding syncytial epidermis (shaded ovals). The wild-type lineages of each VPC are depicted (Sulston and Horvitz 1977). The precursor P3.p does not divide in 50% of wild-type hermaphrodites.

GAATTCGGCAGCGCAAGTTTGAGATAATGTCGAGCGGAAAACGGCGTAATCCGTTGGG 60
 M S S G K R R N P L G (11)

ACTCAGTCTACCCAGCAGTAAATGAACAAGTGAAGTGGCGAGCGCGGCGGAAGA 120
 L S L P P T V N E Q S E S G E A T A E E (31)

AGCCACCGCAGCTCCCACTAGAAAGACAGCTGAAAAGCTCGGGCTCAGGAGGCCACA 180
 A T A T V P L E E L G H G N G G V V N K C V (51)

AACTCAACGGCTCAGCGAATCTTGCAGGTCAAGGAGGCGATCAAGGAGTTGAGTGAGGA 240
 T Q R L S E F L Q V K E G I K E L S E D (71)

TATGCTACAGACTGAAGGAGAACTTGGCCATGGAATGGAGGAGTTGTTAATAGTTGT 300
 M L Q T E G E L G H G N G G V V N K C V (91)

TCATCGAAAAACCGGTGTAATATGGCTCGGAAGCTTGTACACCTCGAGATCAAGCGGTC 360
 H R K T G V I M A R K L V H L E I K P S (111)

AGTCGACACAGATCGTCAAAGAGCTCGCGCTACTTCAAGTGAATAGTCCATTCAT 420
 V R Q T E I V K E L A V L H K C N S P F I (131)

TGTTGGATTCTACGGGGCTTTTGTGGACAATAACGATATATCAATTTGTATGGAGTATAT 480
 V G F Y G A F V D N N D I S I C M E Y M (151)

GGATGGCTTTTCACTTGATATAGTTTGAAGAGTTGGCCGCTCCCGGAAAGTTTGT 540
 D G L S L D I V L K V G R L P E K F V (171)

CGGACGAATCTCGGTGTCAGTTGTCGGTGAATGACGTACTTGAAGATGAAATTAAGAT 600
 G R I S V A V V R G L T Y L K D E I K I (191)

TTTGCATAGAGATGTTAAACCATGCAATATGCTTCAATAGTAACGAGAAATTAAT 660
 L H R D V K P S N M L V N S N G E I K L (211)

ATGCGATTTTGGAGTCTCGGAATGTTGATTGTCGATGCGCAACTCATTGTCGGAAC 720
 C D F G V S G M L I D S M A N S F V G T (231)

GAGGAGTTATATGGCGCCGCAAGCATCACAGGATCCCACTATACAATTTTCATCGGATAT 780
 R S Y M A P E R L T G S H Y T I S S D I (251)

CTGTCATTGAGTATCACTTGTGCAACTTCTTATGGAGATATCCAGTGCCTGCAAC 840
 W S F G L S L V E L L I G R Y P V P A P (271)

GTCTCAAGCAGAATATGCTACAATGTTTAAATGTGGCTGAAAATGAGATGAACTGGCTGA 900
 S Q A E Y A T M F N V A E N E I E L A D (291)

TAGTCTTGAAGAGCCCACTATCATCCGCGAGCAATCCGGCGTCAATGGCTATTTTGA 960
 S L E E P N Y H P S P S M A I F E (311)

AATGCTTGATTATATTTGTTAATGGACCTCCGCCAACACTCCGAAGAGATTCTTACTGA 1020
 M L D Y I V N G P P P T L P K R F F T D (331)

CGAAGTGATTGGATTGTGTGCAATGCTTCGAAAATTCGAAGTGAACGAGCAACCTT 1080
 E V I G F V S L K R L P S E R A T L (351)

AAAATCTCTCACCAGACGCTCTTTCACACAATATGCAGATCAGGATGATCAGGAGA 1140
 K S L T A D V F F T Q Y A D H D Q G E (371)

ATTCGGGGTTTTGTGAAGGGAACGATTAATCTACCAAGTTGAATCCATAATTAATTTT 1200
 F A V F V K G T I N L P K L N P * (387)

GGATTTTGTATATTTTCATGTCCTTGTCTCTCACTGTCTAGGATGCTCCAGTCCAG 1260

GGGGTGATTCTACGAAATGAGCAATATTTTATTGATATTTTGTGATGGAAAATATTTA 1320

GAAAAATAATCTATATCAGAAAAAAGAAATTC 1356

Figure 2. Nucleotide sequence of *mek-2* cDNA and deduced amino acid sequence. The predicted amino acid sequence (in single-letter code) is shown underneath the nucleotide sequence. Numbers at right indicate the positions of nucleotides or amino acids (in parenthesis). (▼) The positions of introns as determined by genomic DNA sequencing. The putative polyadenylation signal sequences (AAATAA), 11 nucleotides upstream of the poly(A), are underlined. The catalytic essential lysine residue 102 and the activation phosphorylation sites Ser-223 and Ser-227 are indicated by short, thick upward arrows. The longest cDNA isolated from the cDNA library starts at the third codon. DNA sequence of the 5' leader, the first two codons, and intron 1 were obtained from PCR products (see Materials and methods for details).

served structural motifs, including the two phosphorylation target sites, S218 and S222 of human MEK1, are also conserved in *C. elegans* MEK-2 (Figs. 2 and 3).

We used the *mek-2* cDNA as a probe to hybridize a *C. elegans* yeast artificial chromosome (YAC) library (a gift from A. Coulson of Sanger Center, Cambridge, UK) and determined that a YAC clone, Y71F9, contained at least part of the *mek-2* gene. Further Southern analysis showed that another overlapping YAC clone, Y54E10, contained the whole *mek-2* cDNA sequence (data not

shown). These data position *mek-2* on the left arm of chromosome I.

To determine the gene structure of *mek-2*, a genomic DNA library from a yeast strain containing Y54E10 was constructed using λ Ziplox as the vector. This library, which had a complexity of 5×10^6 independent clones, was screened using *mek-2* cDNA as a probe. Genomic clones were isolated and the exon-intron boundaries were sequenced. The *C. elegans* *mek-2* gene contains seven exons and six introns (Fig. 4).

MEK-2 protein functions biochemically between Raf and MAP kinase

Studies from mammalian cells have demonstrated that MEK functions between Raf and MAP kinase (Ahn et al. 1992; Crews and Erikson 1993; Davis 1994; Guan 1994). To determine whether the cloned *C. elegans* MEK-2 behaves biochemically as a MAP kinase kinase, MEK-2 was expressed in *Escherichia coli* as a glutathione S-transferase (GST)-MEK-2 fusion protein. The recombinant fusion protein, GST-MEK-2 was purified by glutathione affinity chromatography to near homogeneity as analyzed by SDS-PAGE. The GST-MEK-2 fusion protein was mostly insoluble under normal inducible conditions (200 μ M IPTG at 37°C). However, when the *E. coli* culture was being induced with a lower concentration of IPTG (20 μ M) at room temperature, most of the expressed fusion proteins were found to be soluble. Approximately 1 mg of GST-MEK-2 fusion protein was obtained from 1 liter of bacterial culture. The purified GST-MEK-2 fusion protein could phosphorylate and activate purified human ERK1 in vitro (Fig. 5A,B). Activation of human ERK1 showed a linear correlation with the concentration of purified GST-MEK-2 fusion protein used in the assay (Fig. 5B). In the control experiments, the GST protein itself could not phosphorylate and activate human ERK1, nor was the GST-MEK-2 fusion protein able to phosphorylate myelin basic protein (MBP) directly. Phosphoamino acid analysis of human ERK1 phosphorylated by GST-MEK-2 fusion protein showed that both tyrosine and threonine residues of ERK1 were phosphorylated (data not shown). These results suggest that the *C. elegans* MEK-2 protein is a dual specific kinase that activates MAP kinase. It is worth noting that the *C. elegans* MEK-2 has a basal kinase activity significantly lower than human MEK1 in the ERK activation assay.

We have also determined that the *C. elegans* MEK-2 can be phosphorylated and activated in vitro by mammalian Raf (Fig. 5C,D). Anti-Raf antibody was used to immunoprecipitate activated Raf from EGF-stimulated Swiss 3T3 cells. Immunoprecipitated Raf was then tested for activation of GST-MEK-2 in vitro. Activation of GST-MEK-2 was determined by a coupled kinase assay in which the GST-MEK-2 activity was measured by the activation of ERK1. In the presence of activated Raf and GST-MEK-2 fusion protein, the kinase activity of human ERK1 increased dramatically as compared with the ERK1 kinase activity in the presence of only the GST-MEK-2 fusion protein or the ERK1 basal activity

Figure 3. Amino acid sequence comparison of the predicted MEK-2 product with members of the MEK family. The deduced amino acid sequence of MEK-2 (residues 72–357) was aligned with human MEK1 (Hmek1, residues 67–358) (Seger et al. 1992b; Zheng and Guan 1993), *Drosophila* Dsor1 (residues 83–358) (Tsuda et al. 1993), *Schizosaccharomyces pombe* Byr1 (residues 65–317) (Nadin-Davis and Nasim 1988), and *Saccharomyces cerevisiae* Ste7 (residues 190–463) (Teague et al. 1986) using the PILEUP program of the Wisconsin Genetic Computation Group. Gaps (denoted by dots) were introduced for maximum alignment. Highly conserved residues (four out of five) are highlighted. Pro-237 and Glu-238, which are mutated in the *mek-2(ku114)* and (*h294*) alleles, respectively, and Arg-348, which should form a salt bridge with Glu-238, are indicated by asterisks (*).

(Fig. 5C,D). In control experiments, neither immunoprecipitated Raf in the absence of the GST–MEK-2 fusion protein nor precipitation with nonspecific serum was able to activate human ERK1. These data suggest that the *C. elegans* MEK-2 can be phosphorylated and activated by Raf kinase. In conclusion, the *C. elegans* MEK-2 functions in vitro as a MAP kinase kinase between Raf and MAP kinase.

ku114 and *h294* are mutant alleles of *mek-2* that affect vulval differentiation

In a genetic screen to identify additional factors acting downstream of Let-60 Ras, we have isolated >60 mutations that suppress the Multivulval (Muv) phenotype of an activated *let-60 ras* mutation, *let-60(n1046 gf)* (Wu

and Han 1994; M. Han, unpubl.). One of the recessive extragenic suppressor mutations, *ku114*, maps to the left arm of chromosome I in a region that corresponds to the position of *mek-2* on the physical map (Material and methods). *ku114* completely suppresses the Muv phenotype of the *let-60(n1046)* *ras* mutation: 0% Muv animals were found in *ku114*; *let-60(n1046)* homozygotes, whereas animals homozygous for the *let-60* mutation alone are ~95% Muv (Table 1). Forty animals homozygous for both *ku114* and *let-60(n1046)* and 50 animals homozygous for *ku114* alone were examined for vulval induction by Normarski microscopy; all of them displayed a wild-type vulval lineage (Table 1; Fig. 6A).

ku114 mapped close to another mutation, *h294*, which was isolated in A. Rose's laboratory (McKim 1990). *h294* homozygous mutant animals were sterile (McKim 1990)

Figure 4. Genomic structure of the *mek-2* gene and molecular lesions of *mek-2(ku114)* and *mek-2(h294)*. The seven exons of *mek-2* are represented by bars. The solid bars indicate the coding region of exons, the open bars denote the 5' and 3' untranslated sequences. Sizes of the six introns are indicated by the numbers above each intron. The *EcoRI* restriction sites (E) are also indicated by a vertical line. Both mutant alleles of *mek-2* contain a nucleotide substitution in exon 6. Sequences at the junction of intron 5 and exon 6 are shown in detail. Nucleotides of intron and exon are shown in lowercase and uppercase, respectively. The position of Thr-241 is indicated by (T²⁴¹). Pro-237 is substituted by a serine because of a nucleotide C → T change in *mek-2(ku114)*. Similarly, Glu-238 is replaced by a lysine because a nucleotide G → A alteration in *mek-2(h294)*.

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Figure 5. (A) Phosphorylation of ERK1 by recombinant MEK-2. Purified recombinant human ERK1 and GST-MEK-2 were phosphorylated in vitro in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. (Lane 1) Autophosphorylation of ERK1 (1 μg); (lane 2) phosphorylation of ERK1 (1 μg) in the presence of GST-MEK-2 (2 μg); (lane 3) autophosphorylation of GST-MEK-2 (2 μg). The positions of molecular weight standards are indicated at left. Arrows denote the positions of ERK1 (Erk) and GST-MEK-2 (Mek). (B) In vitro activation of ERK1 by GST-MEK-2. ERK1 (0.3 μg) was activated by various amounts of GST-MEK-2. Activated ERK1 was then assayed using myelin basic protein (MBP) as a substrate. The activity of ERK was quantitated by scintillation counting of ^{32}P incorporation into MBP. Value of the untreated ERK1 was normalized to 1. Fold of ERK activation (y-axis) is linearly correlated to the amount of GST-MEK-2 (x-axis) used in the experiment. (C) Activation of GST-MEK-2 by Raf as analyzed by SDS-PAGE. Activation of GST-MEK-2 was determined by a coupled kinase activation assay in which the GST-MEK-2 activity was measured by the activation of ERK1. (Lane 1) ERK1 (0.3 μg) alone; (lane 2) basal activity of GST-MEK-2 (0.3 μg ERK1 and 0.3 μg GST-MEK-2); (lane 3) activation of GST-MEK-2 by the immunoprecipitated Raf (0.3 μg ERK1 and 0.3 μg Raf-activated GST-MEK-2). GST-MEK-2 was first activated by immunoprecipitated Raf. The Raf kinase complexed with protein A-agarose was removed by centrifugation, and the activated GST-MEK-2 was then used in the ERK activation assay. Positions of GST-MEK-2 (Mek), ERK1 (Erk), and MBP are indicated. (D) Activation of GST-MEK-2 by Raf. Quantitation of the kinase assay was determined by scintillation counting of ^{32}P incorporation into MBP. Data are from two independent experiments. Conditions for lanes 1, 2 and 3 are identical to those in C.

and defined a gene that is likely to act in vulval signaling pathway (E. Lambie, pers. comm.). We examined 30 *h294* homozygous worms under Normarski microscopy and observed that *h294* has a fully penetrant Vulvaless (Vul) phenotype: All the vulval precursor cells divided once and then fused with the surrounding syncytial hypodermis (Table 1; Fig. 6B). Thus, *h294* defines a gene absolutely required for vulval differentiation.

Because *h294* and *ku114* map to the same chromo-

somal region as the cloned *mek-2* gene, we examined the possibility that the two mutations were alleles of *mek-2*. We first determined that *ku114* and *h294* fail to complement each other for the egg-laying defective (Egl) phenotype (data not shown), suggesting that they are two mutations of the same locus. Second, we tested the ability of *mek-2* to rescue these mutations. Because we were unable to clone the promoter region of the *mek-2* gene, we microinjected the mutant lines with a construct in

Table 1. Effect of *ku114* and *h294* on vulval differentiation

Genotype	Percent vulval induction (n) ^a	Percent Egl (n) ^b	Percent larval lethal (n) ^c
<i>ku114</i>	100 (50)	1 (343)	8 (398)
<i>ku114; let-60(n1046)</i>	100 (40)	28 (398)	0 (400)
<i>h294</i>	0 (30)	N.A.	N.A.

(n) Number of worms examined.

^aPercent of vulval induction as examined by a Normarski DIC microscopy.^bPercent of animals defective in egg-laying.^cPercent of progeny that died as larvae.

(N.A.) Not available.

which the *mek-2* cDNA was fused to a heat shock promoter, *hsp16-41* (Stringham et al. 1992). The HS-*mek-2* plasmid was injected into *ku114; let-60(n1046)* at ~20 µg/ml with a *rol-6(d)* DNA as a dominant marker for transformation (Mello et al. 1991). Because *ku114* completely suppressed the Muv phenotype of *let-60(n1046 gf)*, rescue should cause the reappearance of the Muv phenotype in a *ku114; let-60(n1046 gf)* double mutant strain. Among 22 independent transgenic lines tested, the Muv phenotype ranged from 40% to 100%, with an average of ~73%. A control strain of *ku114; let-60(n1046 gf)* displayed 0% Muv, whereas a strain of *let-60(n1046 gf)* alone displayed ~95% Muv. These data showed that ectopic expression of *mek-2* rescued the mutant phenotype of *ku114*.

To further confirm that *ku114* and *h294* are mutations in the *mek-2* gene, we sequenced *mek-2* in the mutants and identified the molecular lesions. The coding region and intron/exon boundaries of both mutant DNA were amplified by PCR using multiple sets of oligonucleotide primers of *mek-2*, and the PCR products were sequenced directly. *ku114* has a single nucleotide change from C to T in codon 237, which results in the substitution of a serine for proline 237 (Fig. 4). *h294* also has a single nucleotide change from G to A in codon 238, which results in the substitution of a lysine for glutamic acid 238 (Fig. 4). Both the proline and glutamic acid residues are highly conserved in the protein kinase family. Substitution of either amino acid residue is likely to cause a significant change in protein conformation and, therefore, decrease the kinase activity (see Discussion). The molecular lesions of the *mek-2* mutations are also consistent with the genetic analysis that both *ku114* and *h294* are likely to be loss-of-function mutations. Thus, we conclude that *ku114* and *h294* are two mutant alleles of the *mek-2* gene.

Because the mutations of *mek-2* completely suppressed the Muv phenotype of an activated *let-60 ras* mutation, *mek-2* most likely acts downstream of *let-60 ras* in the vulva induction pathway. The MAP kinase cascades are highly conserved among different organisms. In mammalian systems it has been shown that MEK acts between Raf kinase and MAP kinase. Because

MEK-2 resembles MEK, both structurally and functionally, in mammals, we propose that *mek-2* acts downstream of *lin-45* but upstream of *sur-1* in the *C. elegans* vulval signaling pathway (Fig. 7).

MEK-2 functions in multiple developmental processes in C. elegans

Both *ku114* and *h294* display mutant phenotypes in addition to defective vulval differentiation. For example, animals homozygous for *mek-2(h294)* are 100% sterile (this study; McKim 1990). The effects of *mek-2* loss-of-function alleles on germ-line differentiation have been studied in detail by (Church et al. 1995). A similar sterile phenotype is observed in a severe loss-of-function mutation of the *sur-1* MAP kinase gene, *oz140* (Church et al. 1995; T. Schedl, M.R. Lackner and S.K. Kim, both pers. comm.), suggesting that both *mek-2* and *sur-1* play an important role in a signaling pathway that controls *C. elegans* germ-line differentiation. Although animals homozygous for *mek-2(ku114)* displayed no obvious Egl phenotype, *mek-2(ku114); let-60(n1046)* double mutants displayed an Egl phenotype, whereas the mutants showed wild-type vulval differentiation (Table 1). These results are consistent with our previous observations that *sur-1(ku1); let-60(n1046)* mutant animals also displayed an Egl phenotype despite wild-type vulval differentiation (Wu and Han 1994).

Animals homozygous for *mek-2(ku114)* displayed ~8% larval lethality, whereas the *mek-2(ku114); let-60(n1046)* double mutant animals displayed 0% larval lethality (Table 1). The dying *mek-2(ku114)* homozygous animals arrested at different larval stages. The earliest arrest appears at the L1 larval stage. A similar percentage of larval lethality was observed in *sur-1(ku1)* or *sur-1(ku1); let-60(n1046)* homozygous animals (Wu and Han 1994). These data suggest that both *mek-2* and *sur-1* play important roles in early development. The *mek-2(ku114)* mutation appears also to suppress the mutant phenotype in the male tails of the *let-60(n1046 gf)* animals (Chamberlin and Sternberg 1993), as the male mating defect was suppressed in the *mek-2(ku114); let-60(n1046)* double mutants (data not shown). These results suggest that *mek-2* and *sur-1* may act together in multiple aspects of *C. elegans* development.

Microinjection of multiple copies of a mek-2 cDNA construct does not cause a dominant mutant phenotype

To test whether microinjection of multiple copies of *mek-2* was sufficient to generate a Muv phenotype, constructs that contain the wild-type *mek-2* cDNA under the control of the heat shock promoters *hsp16-2* or *hsp16-41* (Stringham et al. 1992) were injected into a wild-type strain (N2) with *rol-6(d)* DNA as a marker for transformation (Mello et al. 1991). We failed to observe Muv animals in 33 independent transgenic lines after heat shock at different developmental stages (Table 2).

Previously, we have shown that injection of high copy numbers of the *sur-1* gene did not cause a dominant phe-

Figure 6. Photomicrographs showing different mutant phenotypes associated with different mutant forms of *mek-2*. (A, B) Animals are at the early L4 larval stage and magnified 590 $\times$; (C–F) animals are at the adult stage and magnified 236 $\times$. (A) The vulva induction of *ku114; let-60(n1046)* homozygous animals is wild type. The arrow indicates the wild-type vulva. (B) Animals homozygous for *h294* display a Vul phenotype. All of the vulva precursor cells only divide once and then fuse with the surrounding epidermis. The bars show that P5.p, P6.p, and P7.p only divide once. (C) Ectopic expression of *mek-2(S223ES227D)* causes a Muv phenotype. The black arrow points to the wild-type vulva; the white arrows point to the two pseudovulvae. (D) Ectopic expression of *mek-2(S223ES227D)* causes hermaphrodite tail abnormality. (E) Activated *let-60* mutation, *let-60(n1046)*, causes a Muv phenotype. The black arrow points to the wild-type vulva, while the white arrows point to the two pseudovulvae. (F) Ectopic expression of *mek-2(S223AS227A)* suppresses the Muv phenotype of the activated *let-60* mutation, *let-60(n1046)*. The white arrow points to a larva hatched inside the hermaphrodite.

nototype (Wu and Han 1994). We have now extended this study by showing that ectopic expression of the *sur-1* cDNA under the control of the heat shock promoters, *hsp16-2* or *hsp16-41*, did not cause a dominant Muv phenotype (Table 2). These *sur-1* fusion constructs were clearly functional, as they were able to rescue the mutant phenotype of *sur-1(ku1)* in 17 independent transgenic lines (data not shown). Because the *mek-2* and *sur-1* genes may act together in a signaling pathway that controls different aspects of worm development, multiple copies of *mek-2* and *sur-1* under the control of the heat shock promoters were also coinjected to test any synergistic effects. None of the 44 transgenic lines tested displayed a Muv phenotype after heat shock at different developmental stages (Table 2).

In conclusion, microinjection of multiple copies of *mek-2*, *sur-1*, or *mek-2* and *sur-1* together in *C. elegans*, unlike microinjection of multiple copy numbers of the *let-60 ras* gene (Han and Sternberg 1990), did not cause a Muv phenotype, suggesting that the quantities of these enzymes are unlikely to be limiting factors in the signaling pathway. Our data agree with previous observations that overexpression of wild-type MEK or MAP kinase in mammalian cell lines caused no transforming phenotype (Seger et al. 1992b; Cowley et al. 1994). These results are consistent with the notion that the regulation of the MEK and MAP kinase activity is at the level of protein activity.

Figure 7. The Let-60 Ras mediated MAP kinase pathway in vulval induction and other development events. Gene products of mammalian homologs are indicated in parentheses. GF and RTK denote for growth factor like molecule and receptor tyrosine kinase, respectively.

Table 2. Phenotypic analysis of microinjection of multiple copies of *mek-2* or *sur-1*

Constructs ^a	Lines with Muv (n) ^b	Percent Muv (n) ^c
HS-mek-2(+)	0/33	0 (>2000)
HS-sur-1(+)	0/40	0 (>2000)
HS-mek-2(+) + HS-sur-1(+)	0/31	0 (>1000)
HS-mek-2(S223ES227D)	55/65	20 (>1500)

(n) Number of transgenic animals examined.

^aConstructs used for microinjection. The concentration of DNA used for microinjection is 20 µg/ml for all plasmids. The host strain for microinjection is N2. For HS-mek-2(+) construct, the wild type *mek-2* cDNA was fused behind heat shock promoter hsp16-2 (pYW89) or hsp16-41 (pYW90). For HS-sur-1(+) construct, the wild type *sur-1* cDNA was fused behind the heat shock promoter hsp16-2 (pYW66) or hsp16-41 (pYW72). For HS-mek-2(+) + HS-sur-1(+) construct, it is pYW89+pYW66 or pYW90+pYW72. For HS-mek-2(S223ES227D) construct, the *mek-2*(S223ES227D) cDNA fused behind the heat shock promoter hsp16-2 (pYW94) or hsp16-41 (pYW95).

^bTransgenic lines displayed Muv phenotype/total transgenic lines examined (n). Among 33 independent transgenic lines examined [HS-mek-2(+)], 18 lines derived from the microinjection of pYW89 and 15 lines from pYW90. Among 40 transgenic lines examined [HS-sur-1(+)], 23 lines derived from the microinjection of pYW66, and 17 lines derived from pYW72. Among 31 transgenic lines examined [HS-mek-2(+) + HS-sur-1(+)], 22 lines derived from the microinjection of pYW89+pYW66, 9 lines derived from pYW90+pYW72. Among 65 transgenic lines examined [HS-mek-2(S223ES227D)], 34 lines derived from the microinjection of pYW94 and 31 lines derived from pYW95. Among 10 non-Muv transgenic lines, 6 of them displayed a high percentage of dead eggs.

^cPercentage of Muv displayed by transgenic animals.

mek-2(S223ES227D) is a gain-of-function mutation

It has been shown recently that a gain-of-function mutation in mammalian MEK can be generated by substituting the two phosphorylation target serines with acidic amino acids, glutamic acid, or aspartic acid (Cowley et al. 1994; Mansour et al. 1994). To test whether such a mutant MEK protein can also be constitutively active in a developmental program such as vulva induction in *C. elegans*, we created corresponding changes in MEK-2 and demonstrated that the *mek-2* gene is not only necessary but also sufficient for vulval differentiation. Mutant constructs that have the *mek-2*(S223ES227D) gene under the control of the heat shock promoters, hsp16-2 or hsp16-41, were microinjected into wild-type (N2) hermaphrodites. The transgenic animals were heat shocked at 37°C for 1 hr at different developmental stages and then scored as adults (Sulston and Horvitz 1977). Most of the transgenic animals displayed pleiotropic defects, including a Muv phenotype and embryonic lethality after heat shock. Among 65 transgenic lines tested, 55 lines displayed a Muv phenotype (Table 2; Fig. 6C). Of the 10 non-Muv transgenic lines, 6 produced a high percentage of dead eggs (data not shown). The phenotypes varied, depending on the stages of the animals being heat shock

treated. When the heat shock was applied during early embryogenesis, severe embryonic lethality occurred at high frequency and very few transgenic animals appeared wild type (data not shown). When the heat shock was applied during early L1, all of the surviving transgenic animals displayed a dumpy (Dpy) phenotype while a few transgenic animals were Muv. When the animals were heat shocked at early L2, transgenic animals displayed mutant phenotypes such as Muv, (Dpy), small (Sma), gonad and hermaphrodite tail abnormality (Fig. 6D), and general sickness. Finally, when the animals were heat shocked at early L3, most of the transgenic animals were very sick with increased Muv percentage, though all of the pleiotropic phenotypes displayed in animals heat-shocked at L2 were still present (data not shown). Non-transgenic control animals subjected to the same heat shock conditions described above were wild type (data not shown).

The Muv phenotype suggests that *mek-2*(S223ES227D) behaves as a gain-of-function mutation, as loss-of-function mutation of *mek-2*(h294) causes a Vul phenotype. The pleiotropic defects caused by *mek-2*(S223ES227D) suggest that *mek-2* plays important roles in many aspects of worm development (Fig. 7). However, we cannot exclude the possibility that some of the dominant phenotypes might be results of the ectopic expression of the mutant *mek-2* gene, as its expression is under the control of heat shock promoters.

mek-2(S223AS227A) causes dominant-negative effects

It has also been shown recently that changing both the phosphorylation target serines (S218 and S222) to alanines in human MEK1 results in a mutant MEK that cannot be phosphorylated and activated by Raf (Alessi et al. 1994; Zheng and Guan 1994). Human MEK1-(S218AS222A) acts as a dominant-negative protein that blocks serum-induced DNA synthesis and reverts *ras*- or *src*-transformed cells (Cowley et al. 1994). To test the effect of an analogous *mek-2* mutation in *C. elegans*, we created the corresponding changes in *mek-2*, *mek-2*(S223AS227A). A plasmid that contains the *mek-2*(S223AS227A) cDNA under the control of a heat shock promoter, hsp16-41, was microinjected into either a wild-type strain (N2) or a strain that contained an activated *let-60* mutation, *let-60*(n1046 gf). In the wild-type background, three independent transgenic lines tested displayed an Egl phenotype after heat shock before L2 or L3: ~30% of the transgenic animals displayed an Egl phenotype, compared with <1% in wild-type animals (Table 3). One possibility for an Egl phenotype is the failure of *mek-2*(S223AS227A) to form a wild-type vulva. To score vulval cell differentiation of the transgenic animals, we microinjected the HS-mek-2(S223AS227A) plasmid into *dpy-20*(e1282) animals along with the *dpy-20* gene (pMH86) as a marker (Han and Sternberg 1991). Among 21 transgenic animals that were Egl, 18 of them displayed a Vul phenotype. In the *let-60*(n1046) background, three of the four transgenic lines tested displayed suppression of the Muv phenotype and an Egl

Table 3. Effects of microinjection of multiple copies of *mek-2*(S223AS227A)

Transgenic line with HS- <i>mek-2</i> (S223AS227A) ^a	Hot strain ^b	Percent Muv (n) ^c	Percent Egl (n) ^d
E2	N2	N.A.	21 (210)
E9	N2	N.A.	45 (286)
D6	<i>let-60</i> (n1046)	23 (95)	N.D.
D8	<i>let-60</i> (n1046)	27 (215)	N.D.

(n) Number of transgenic animals examined.

^aIndependent transgenic lines analyzed. The construct used for microinjection is HS-*mek-2*(S223AS227A): the *mek-2*(S223AS227A) cDNA was fused behind a heat shock promoter hsp16-41 (pYW109). The construct concentration for microinjection is ~50 µg/ml. Heat shock was performed at early L2 for 1 hr at 37°C.^bHost strain for microinjection.^cPercentage of Muv. (N.A.) Not available.^dPercentage of egg-laying defective. (N.D.) Not determined.

phenotype after heat shock before L2 or L3 larval stage (Table 3; Fig. 6F). Two independent transgenic lines displayed ~27% and 28% Muv phenotype after heat shock at 37°C for 1 hr at early L2, compared with ~95% Muv in *let-60*(n1046) homozygous animals (Fig. 6E). The transgenic line that neither suppressed the Muv phenotype of an activated *let-60*(n1046) mutation nor displayed an Egl phenotype might not contain the HS-*mek-2*(S223AS227A) construct. Heat shock itself did not suppress the Muv phenotype of an activated *let-60* *ras* mutation, and the *rol-6*(d) plasmid (pRF4) alone caused neither an Egl phenotype nor the suppression of the Muv phenotype of the activated *let-60*(n1046) (data not shown).

When transgenic animals were heat shocked at an early embryonic developmental stage, a high percentage of dead eggs was observed (data not shown). This result may suggest that *mek-2* plays an important role in *C. elegans* early embryogenesis. However, because the mutant *mek-2* gene was under the control of a heat shock promoter, the embryonic lethal phenotype might be caused by a misexpression of the *mek-2* mutant gene. When transgenic animals were heat shocked at early L1, the dominant-negative phenotypes were much less severe (data not shown). Because vulval induction starts at L3, the MEK-2(S223AS227A) protein expressed in L1 might not be stable enough to cause a dominant-negative effect in L3. These results showed that *mek-2*(S223AS227A) behaved as a dominant-negative mutation as both the suppression of the Muv phenotype of an activated *let-60* *ras* mutation and the Vul phenotype were associated with the *mek-2* loss-of-function mutations. A kinase-deficient mutant, MEK-2(K102M), resulting from the substitution of the catalytic essential lysine residue 102 by a methionine, was also tested as a dominant-negative mutant. Interestingly, *mek-2*(K102M) was not an effective dominant-negative muta-

tion when similar experiments were performed (data not shown).

Discussion

C. elegans MEK-2 functions as a MAP kinase kinase

Because MEK proteins are highly conserved among different organisms, we were able to design degenerate oligonucleotides in the conserved kinase domains and use them to isolate a *C. elegans* MEK homolog. We have shown that the *mek-2* gene product is structurally and functionally similar to the human MEK proteins. By in vitro kinase assays, the MEK-2 protein possesses the biochemical properties of MAP kinase kinase. It can activate the human ERK1 protein by phosphorylation on both threonine and tyrosine, indicating it is a dual specific kinase. Furthermore, MEK-2 itself can be phosphorylated and activated by mammalian Raf, the upstream activator of mammalian MEK. Although the *C. elegans* Lin-45 Raf protein has not been tested for activation of MEK-2 because we are unable to produce active recombinant Raf, it is likely that the *C. elegans* Raf directly activates MEK-2 in vivo. Our genetic analysis of *mek-2* has identified its role in vulval induction and other developmental processes, and the biochemical studies of MEK-2 suggest that it acts between Lin-45 Raf and Sur-1 MAP kinase (Fig. 7).

Loss-of-function mutations in *mek-2* define its roles in multiple developmental events

The role of the *mek-2* gene in specific developmental events is demonstrated by the phenotypes of two *mek-2* loss-of-function mutations and by the dominant phenotypes generated by ectopic expression of different dominant mutant *mek-2* genes. Our genetic analysis of the two *mek-2* loss-of-function mutations, *ku114* and *h294*, is consistent with the genetic analysis of other *mek-2* mutations by Kornfeld et al. (this issue) and Church et al. (1995). *mek-2*(h294) is a severe loss-of-function mutation that displays highly penetrant Vul and sterile phenotypes, suggesting essential roles for the gene in vulval differentiation and germ-line development. Sterility is also associated with mutations in other genes acting in the vulval signal transduction pathway, like *let-23* and *let-60* (Aroian and Sternberg 1991; Han et al. 1993). A mutation in the *sur-1* gene, *oz140*, also caused a highly penetrant sterile phenotype (Church et al. 1995; T. Schedl, M.R. Lackner and S.K. Kim; both pers. comm.).

In contrast to *mek-2*(h294), *mek-2*(ku114) appears to be a relatively weak mutation. Although *ku114* completely suppressed the Muv phenotype of an activated *let-60* *ras* mutation, vulval cell lineages in animals homozygous for *ku114* appear normal. Animals homozygous for both *mek-2*(ku114) and *let-60*(n1046 *gf*) also displayed a partial Egl phenotype that is absent from *mek-2*(ku114) single mutants. This synthetic egg-laying phenotype is likely to be the result of defects in other aspects of the *C. elegans* egg-laying machinery (Thomas

et al. 1990; M. Sundaram and M. Han, in prep.). We have also shown that *mek-2(ku114)* homozygotes display an 8% larval lethality, indicating a role of the gene in early development. Larval lethality is associated with many mutations in most of the key genes acting in the vulval signaling pathway and has been proposed to be the null phenotype for these genes, including *let-60 ras*, *lin-45 raf* and *sur-1* MAP kinase (Han et al. 1990, 1993; Wu and Han 1994). The phenotype of null alleles of *mek-2* is likely to be complete larval lethality.

The molecular lesions of *h294* (a E238K change) and of *ku114* (a P237S change) are consistent with the severity of their phenotype. Based on crystallographic studies of cAMP-dependent protein kinase (PKA) (Knighton et al. 1991), Glu-238 of MEK-2 may form a salt bridge with Arg-348. Substitution of Glu-238 with a lysine residue in *mek-2(h294)* would eliminate the salt bridge with Arg-348, thus severely affecting the protein structure of MEK-2, leading to a reduction of its kinase activity. Pro-237 in MEK-2, adjacent to Glu-238, is also highly conserved in the protein kinase family and corresponds to Pro-207 in PKA. Substitution of Pro-237 with a serine residue may affect the protein structure of MEK-2 in such a way that it weakens the salt bridge between Glu-238 and Arg-348, resulting in a reduction of the protein kinase activity. Consistent with the prediction from three-dimensional structures, we have shown that *mek-2(ku114)* has a relatively weak mutant phenotype: It completely suppressed the Muv phenotype of an activated *let-60* mutation, but animals homozygous for the *ku114* mutation have essentially wild-type vulval differentiation.

Changes in the phosphorylation target sites of MEK-2 lead to dominant mutant phenotypes

In mammalian cells, phosphorylation of the two serine residues at positions 218 and 222 of human MEK1 by Raf is necessary and sufficient for activation (Zheng and Guan 1994; Alessi et al. 1994). Double mutations of these two serine residues to acidic amino acids result in a constitutively active form of MEK, which is necessary and sufficient for NIH-3T3 cell transformation and PC12 cell differentiation (Cowley et al. 1994). We have demonstrated further in this study that a similar mutation of *C. elegans mek-2*, *mek-2(S223ES227D)*, was hyperactive and caused multiple defects in developmental programs. Expression of multiple copies of a *mek-2(S223ES227D)* cDNA construct under the control of a heat shock promoter generated a variety of pleiotropic defects, which included Muv, Dpy, Sma, sickness, gonad and hermaphrodite tail abnormality, and embryonic lethality. Caution should be taken in interpreting the phenotypes because the heat shock promoters were used. These phenotypes are either attributable to hyperactivity of the *mek-2(S223ES227D)* gene in cell signaling pathways in which *mek-2* normally functions or to abnormal expression of the constitutively active mutant gene in cells in which the wild-type *mek-2* does not normally function. We believe that at least some of these dominant pheno-

types are indications of the normal function of *mek-2* in multiple developmental events. For example, the Muv phenotype attributable to ectopic expression of *mek-2(S223ES227D)* is likely to be the result of hyperactivation of *mek-2* in the *let-60 ras*-mediated pathway because the normal function of *mek-2* in the pathway is established by the Vul phenotype of loss-of-function *mek-2* mutations. Other phenotypes, such as larval lethality and sterility, are also seen in loss-of-function mutations in *mek-2*, *sur-1*, *let-60*, and other genes acting in the pathway.

The dominant effects caused by microinjecting *mek-2(S223ES227D)* are a result of the constitutive activation of the MEK-2 kinase. We have shown that ectopic expression of *mek-2(+)*, *sur-1(+)*, or *mek-2(+)* and *sur-1(+)* together results in no obvious phenotypes, although a low percentage of Dpy transgenic animals were observed (data not shown). These studies are consistent with recent results with mammalian MEK that these enzymes are tightly regulated, not at the level of the enzyme quantity, but at the level of protein phosphorylation.

It has been observed with human MEK1 that the substitution of the two serine residues with alanines results in a dominant-negative form of the protein that can block serum-induced DNA synthesis and suppress *ras* or *src* transformation (Cowley et al. 1994). We have demonstrated in this study that a similar change in *mek-2*, *mek-2(S223AS227A)*, also results in a dominant-negative mutant phenotype in *C. elegans*. Interestingly, the kinase-deficient mutant MEK-2(K102M) showed little dominant-negative effects (data not shown). Although the mechanism of such a dominant-negative effect is not clear, MEK-2(S223AS227A) possibly blocks function of the upstream Raf, downstream MAP kinase, or other factors working in the signal transduction pathway. We propose that MEK-2(S223AS227A) is likely to block the function of Raf rather than MAP kinase. First, MAP kinase is expressed in mammalian cells at a level much higher than Raf, and it is easier to sequester a protein that is expressed at a low level such as Raf than a protein expressed at high abundance such as MAP kinase. Second, activation of MEK by Raf involves binding of MEK to Raf, phosphorylating the two serine residues, and releasing the phosphorylated MEK from Raf. Mutant MEK-2(S223AS227A) should be able to bind Raf but not be phosphorylated by Raf. Such a nonproductive complex may result in a stable association of MEK-2(S223AS227A) with Raf, thus titrating Raf away from endogenous MEK protein. In contrast, the kinase-deficient MEK-2(K102M) protein, which shows a much weaker dominant-negative effect than MEK-2(S223AS227A), is expected to bind Raf, be phosphorylated by Raf, and then dissociate from Raf. Therefore, no stable complex between MEK-2(K102M) and Raf should be formed. Our results are consistent with the hypothesis that the dominant-negative effects of MEK-2(S223AS227A) are attributable to the sequestering of Raf. Further biochemical and genetic studies are required to test this hypothesis.

MAP kinase activation has been observed in mammalian cells stimulated with a wide variety of extracellular stimuli, including mitogenic growth factors, differentiation factors, T-cell antigens, cytokines, and tumor promoters (Cobb et al. 1991). The pleiotropic effects of *mek-2* mutation in *C. elegans* further demonstrate the multiple roles of MAP kinase pathways in regulation of cell growth, differentiation, and development. The gain-of-function mutation and dominant-negative mutation of *mek-2* characterized in this report will provide useful tools for understanding the physiological functions of MAP kinase. Future studies to uncover roles of *mek-2* in *C. elegans* developmental events in addition to vulval induction are likely to generate new insights of the MAP kinase pathway and Ras signaling mechanism.

Materials and methods

Cloning of *mek-2*

Two degenerate oligonucleotides were designed on the basis of the conserved amino acid sequences of MEKs. Oligonucleotides CTTGGATCCTA(C,T)AT(A,C,T)GTNTT(C,T)TA (for amino acids YIVGFY corresponding to residues 130–136 of MEK-2; Fig. 2) and

CTTGGATCC(G,T)(C,T)TCNNGN(C,G)(A,T)CAT(A,G)TA (for amino acids YMSPER corresponding to residues 234–239) were synthesized. The underlined sequences denote *Bam*HI restriction sites incorporated in the oligonucleotides. These two primers were used in PCR for 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min using a *C. elegans* cDNA library (Stratagene) as the template.

To isolate *mek-2* cDNA clones, the 0.35-kb PCR clone, which showed strong sequence identity to MEK, was used to screen a mixed stage *C. elegans* cDNA library (Stratagene) under standard conditions (Maniatis et al. 1982). Among 300,000 plaques screened, four positive clones were identified and DNA sequence was determined in both strands using synthetic oligonucleotides.

Because the cDNA clones were incomplete at their 5' ends, the same cDNA library was used as a template for PCR to isolate the 5' region of *mek-2* cDNA. A T7 primer [annealing to the SK(–) vector] and *mek-2*-2N primer (AAGCCCCGTGAATCCA annealing to nucleotides 424–440 in Fig. 2) were used in PCR (94°C for 1 min, 52°C for 1 min, 72°C for 1 min, for 30 cycles). DNA between 0.4 and 0.8 kb was recovered from an agarose gel and used as a template for a second round of PCR in which an SK primer [annealing to the SK(–) vector 3' to the T7 primer] and the *mek-2*-1N primer (TTTCAGCTGTTCTTCTA, annealing to nucleotides 141–158 in Fig. 2) were used for amplification. The PCR product was subcloned into Bluescript SK(–) and then sequenced.

Total chromosomal DNA was isolated from a yeast strain containing the YAC clone Y54E10 and digested with *Eco*RI. The *Eco*RI-digested DNA (0.2 µg) was ligated into *Eco*RI digested λ Ziplox vector (0.5 µg, GIBCO BRL) to construct a yeast genomic library. We obtained a library of 5×10^6 independent clones. This library was directly screened for the *mek-2* gene without amplification. From the *mek-2* cDNA clone, three *Eco*RI fragments (0.15, 1.0, and 0.18 kb) (Fig. 4) were isolated and labeled separately. Screening of the genomic library was performed under the same conditions as for the cDNA library screening. Junctions of exons and introns were determined by DNA sequencing. Intron 1 was determined by direct sequencing of PCR products.

Plasmid construction

To express a GST–MEK-2 fusion protein in *E. coli*, the *mek-2* cDNA clone was amplified by PCR to introduce convenient restriction sites for subcloning into the vector pGEX-KG (Guan and Dixon 1991). The 5'-end and 3'-end primers were GC-TCTAGACTCGAGCGGAAACGGCGT (the underlined sequence denotes an *Xba*I restriction site) and AGCGTCGACAAAGAGACATCGAA (the underlined sequence denotes a *Sal*I restriction site), respectively. The 1.2-kb PCR product was digested with *Xba*I and *Sal*I and subcloned into pGEX-KG to produce pGEX–MEK-2. The cloning junction and the DNA sequence of the *mek-2* portion were confirmed.

To construct a full-length clone of *mek-2* cDNA clone, pYW88, we amplified by PCR a *mek-2* cDNA clone, pCemek-2, which lacks the first two amino acids by using a 5' primer oligonucleotide OYW38: 5'-GCTCTAGATGTCGAGCGGAAACGGCGTAATCC-3'. To construct pYW91, which contains the *mek-2*(S223ES227D) cDNA, we used a modified PCR-based site-directed mutagenesis method. The two internal oligonucleotides are OYW43 (AAAGTCGTTGGCCATCTCATCAATCAACATTCCAG) and OYW44 (GATGAGATGGCCAACGACTTTGTCTGGAACGAGGAG). The constructs were sequenced to ensure that there were no sequence changes attributable to PCR amplifications.

Site-directed mutagenesis was also performed using the Altered Site Mutagenesis System (Promega). The *Xba*I–*Sal*I fragment of *mek-2* was subcloned from pGEX–MEK-2 (described above) into the pALTER vector (Promega). Oligonucleotide TG-GCTCGGAATGCTTGTACA was used to change the lysine at codon 102 to a methionine. Oligonucleotide TTGATTGATCGATGGCCAACGCATTTGTCG was used to change the Ser-223 and Ser-227 to alanines. Nucleotide changes in each mutant were confirmed by DNA sequencing.

pYW94 was constructed as follows: pYW91 [*mek-2*(S223ES227D)] cDNA was cloned into SK(+) *Xba*I and *Eco*RI sites and digested with *Xba*I and *Kpn*I; and the insert band was isolated and then ligated with the band isolated from the vector pPD49.78 (contains hsp16-2), which was digested with *Nhe*I and *Kpn*I. By the same procedure, we constructed pYW95 by using pPD49.83 (contains hsp16-41). Similar methods were used to construct pYW89, pYW90, pYW66, pYW72, and pYW109.

Kinase assay

Plasmid pGEX–MEK-2 was transformed into *E. coli* BL21 to express GST–MEK-2 fusion protein following published procedures (Guan and Dixon 1991). The fusion protein was purified by glutathione agarose affinity chromatography. Recombinant human ERK1 was prepared as described previously (Zheng and Guan 1993).

Purified recombinant human ERK1 (1 µg) was phosphorylated by 2 µg of GST–MEK-2 in 20 µl of kinase assay buffer (18 mM HEPES at pH 7.5, 10 mM magnesium acetate, 50 mM ATP) containing 5 µCi of [γ -³²P]ATP. Phosphoamino acid analysis was performed following published methods (Boyle et al. 1991). Activity of GST–MEK-2 was determined by an in vitro ERK activation assay. Purified recombinant ERK1, which had a very low basal activity, was activated by various amounts of GST–MEK-2 (0.3–1.5 µg) at 30°C for 10 min in 20 µl kinase assay buffer. The activity of ERK was determined using MBP as a substrate (Zheng and Guan 1993).

Swiss 3T3 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum (GIBCO BRL). Cells were grown to confluency and starved for 24 hr in DMEM supplemented with 0.1% serum. The starved cells were stimu-

lated with EGF (100 ng/ml, Sigma) for 2–5 min and harvested in lysis buffer (25 mM HEPES, at pH 7.5, 0.2 mM PMSF, 0.05% 2-mercaptoethanol, 1% Triton X-100, 2 mM sodium vanadate, and 50 mM NaF). Cells were sonicated for 10 sec and centrifuged (10,000g) for 20 min at 4°C. The supernatant was used for immunoprecipitation with anti-Raf antibody. Anti-Raf or control serum (5 μ l) was used in immunoprecipitation of 2 mg protein of Swiss 3T3 cell lysates on ice for 1 hr. Protein A–agarose (20 μ l, Pierce) was added to the immunoprecipitation reaction for 30 min. The immunocomplex was washed three times with cell lysis buffer followed by one washing with the kinase assay buffer. The immunoprecipitated Raf was used to activate 1.5 μ g of GST–MEK-2 in 30 μ l kinase assay buffer for 20 min at 30°C with gentle shaking. The Raf protein complexed with antibody and protein A–agarose was removed by centrifugation. The activated GST–MEK-2 (0.6 μ g) was used to activate 0.6 μ g of ERK following the procedures described above. ERK activity was determined by the myelin basic protein (MBP) kinase assay. Half of the reaction was analyzed by an 18% SDS–PAGE followed by autoradiography. The other half was quantitated by scintillation counting of 32 P incorporation into MBP as described above for the MBP kinase assay. GST–MEK-2 without Raf activation was treated identically as a control.

Isolation and genetic characterization of *mek-2(ku114)* and *mek-2(h294)*

Methods for the culturing, handling, and genetic manipulation were as described previously (Brenner 1974; Wood et al. 1988). All genetic experiments were performed at 20°C. *mek-2(ku114)* was isolated as a recessive suppressor of the Muv phenotype of *let-60(n1046 gf)*. The mutagenesis and screening procedures have been described previously (Wu and Han 1994). *mek-2(h294)* [previously called *let-537(h294)*] was isolated in the laboratory of A. Rose (University of British Columbia, Vancouver, Canada) in a screen for lethal or sterile mutations on the left arm of chromosome I (McKim 1990). To score vulval induction of animals homozygous for *mek-2(h294)*, sterile animals segregated from a strain with genotype *mek-2(h294) +/+ unc-74(e883)* were picked. The methods to score the percentage of vulval induction, egg-laying defective, larval lethal phenotype were performed as described (Wu and Han 1994).

Genetic mapping

Standard two-factor crosses using genetic markers were performed to position *ku114* on the genetic map (Brenner 1974). From mothers of *ku114 +/+ dpy-5(e61) unc-29(e403); let-60(n1046)*, 21 egg-laying defective worms, which were homozygous for *ku114* and *let-60(n1046)*, were picked. Five of them segregated Dpy Unc animals whose genotypes were *ku114 +/+ ku114 dpy-5(e61) unc-29(e403); let-60(n1046)*. From a strain of *ku114 +/+ unc-11(e47) dpy-5(e61); let-60(n1046)*, 21 egg-laying defective worms, homozygous for *ku114* and *let-60(n1046)*, were picked. Four of them segregated Dpy non-Unc animals whose genotypes were *ku114 +/+ ku114 + dpy-5(e61); let-60(n1046)*, whereas none of the 21 Egl[−] animals segregated Unc progenies.

Microinjection experiment

DNA isolation, analysis, and subcloning were performed by standard methods. Microinjection of cloned DNAs into the gonadal syncytia of *C. elegans* hermaphrodites was carried out as described previously (Han and Sterberg 1990; Mello et al. 1991). Each transformation result was scored with multiple indepen-

dent transgenic lines. All plasmids were injected at a concentration of ~20 μ g/ml. Most of the injection experiments stated in this paper were performed by coinjection of a *rol6(d)* plasmid (pRF4) as a dominant marker for transformation (Mello et al. 1991). A strain of *ku114; let-60(n1046)* was used as the host strain to determine whether the *mek-2* gene can rescue the mutant phenotype of *ku114*. A mixed population of transgenic animals (most at embryo, L1, or L2 stages) were heat shock treated at 34°C for 4 hr for 2 consecutive days. The plates were kept in an incubator at 20°C between the heat shocks. The Muv phenotype of roller animals was examined 2 days later.

Experiments analyzing the transgenic lines from the injection of the constructs pYW89, pYW90, pYW89+pYW66, and pYW90+pYW72, were as follows: 1–10 adult transgenic animals were picked to each plate, allowed to lay eggs overnight at 15°C, and removed ~12 hr later. At the time of removal of the adult worms, the plates contained mostly eggs and a few L1 larvae. Heat shock experiments were executed at 34°C for 4 hr at different developmental stages, embryonic, L1, L2, and L3. Adult transgenic animals were scored for their phenotypes. Experiments analyzing the transgenic lines from the injection of the constructs of pYW66, pYW72, pYW71, and pYW73 were as follows: In one set of experiments, adult transgenic animals from each transgenic line were transferred to five different plates, allowed to lay eggs for ~2 hr, and removed. Each of the five plates was heat shocked at 34°C for 1 hr or at 37°C for 2 hr as embryo, L1, L2, L3, or L4 stage accordingly, and the adult roller animals were scored for their phenotypes. In another set of experiments, the adult transgenic animals from different transgenic lines were transferred to new plates and were allowed to lay eggs for 2 hr. These adult worms were then removed. All of the plates were heat shocked at 34°C for ~3 hr at embryo, L1, L2, and L3 stages. The adult transgenic animals were scored for their phenotypes. To determine that the construct pYW66 or pYW72 was able to rescue the mutant phenotype of *sur-1(ku1)*, each construct was microinjected into the host strain, *sur-1(ku1); let-60(n1046)*. The experimental conditions were similar to that used in the rescue experiment of *ku114*.

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Note added in proof

The sequence data for *C. elegans mek-2* have been deposited into the GenBank data library under accession number U21107.

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