A *Drosophila* MAPKKK, D-MEKK1, mediates stress responses through activation of p38 MAPK

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In cultured mammalian cells, the p38 mitogen-activated protein kinase (MAPK) pathway is activated in response to a variety of environmental stresses. However, there is little evidence from *in vivo* studies to demonstrate a role for this pathway in the stress response. We identified a *Drosophila* MAPK kinase kinase (MAPKKK), D-MEKK1, which can activate p38 MAPK. D-MEKK1 is structurally similar to the mammalian MEKK4/MTK1 MAPKKK. D-MEKK1 kinase activity was activated in animals under conditions of high osmolarity. *Drosophila* mutants lacking D-MEKK1 were hypersensitive to environmental stresses, including elevated temperature and increased osmolarity. In these D-MEKK1 mutants, activation of *Drosophila* p38 MAPK in response to stress was poor compared with activation in wild-type animals. These results suggest that D-MEKK1 regulation of the p38 MAPK pathway is critical for the response to environmental stresses in *Drosophila*.

Keywords: *Drosophila*/MAPKKK/p38 MAPK/stress responses

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 tyrosine and threonine phosphorylation catalyzed by a family of dual-specificity protein kinase MAPKKs. MAPKK is in turn activated by phosphorylation mediated by MAPKKK.

Three major groups of MAPKs have been identified (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). These include the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs. ERKs often function as downstream effectors of Ras and are central elements

mediating cell proliferation by a variety of growth factors. In contrast, JNKs and p38 MAPKs are activated most potently by cellular stresses and inflammatory cytokines. Furthermore, several subgroups of the MAPKK superfamily have been identified, such as MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). MEK1 and MEK2 activate the ERK group. MKK4 can activate both the JNK and p38 subgroups and MKK7 is specific for the JNK subgroup, while MKK3 and MKK6 act solely as activators of the p38 subgroup. These members of the MAPKK superfamily are activated by phosphorylation catalyzed by members of the MAPKKK superfamily such as Raf, TAK1, Tpl2, ASK1, the MEKK and MLK group of proteins.

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 the insect *Drosophila melanogaster*, the JNK pathway is required for mid-embryonic development (Noselli, 1998). Mutants for two components of the *Drosophila* JNK (D-JNK) pathway, *hemipterous* (*hep*) and *basket* (*bsk*), have been identified and shown to encode *Drosophila* homologs of MKK7 and JNK, respectively (Glise *et al.*, 1995; Riesgo-Escovar *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. Therefore, the D-JNK pathway plays a critical role in the dorsal closure process. D-JNK is also required for some forms of developmental apoptosis in *Drosophila* (Adachi-Yamada *et al.*, 1999a). In contrast, genetic studies of *Caenorhabditis elegans* demonstrate that the JNK pathway regulates coordinated movement via type D GABAergic (GABA: γ -aminobutyric acid) motor neurons, but does not appear to be essential for embryonic morphogenesis (Kawasaki *et al.*, 1999).

Two *Drosophila* p38 MAPKs, D-p38a and D-p38b, may be involved in insect immunity, as well as the response to environmental stresses (S.-J. Han *et al.*, 1998; Z.S. Han *et al.*, 1998). Like mammalian p38, D-p38 MAPKs are activated by stress-inducing and inflammatory stimuli, such as UV irradiation, high osmolarity, heat shock, serum starvation, H₂O₂ and lipopolysaccharide (LPS). However, D-p38 mutants have yet to be isolated. Phosphorylation and activation of D-p38a and b are mediated by the MAPKKs D-MKK3 and D-MKK4 (Z.S. Han *et al.*, 1998). A recent study has shown that D-MKK3 is encoded by the gene *licorne* (*lic*) (Suzanne *et al.*, 1999). Lic plays an essential role in anterior–posterior and dorsal–ventral

patterning during oogenesis by regulating the localization of cell fate determinants (Suzanne *et al.*, 1999). Although *Drosophila* p38 signaling appears to function to control the polarity of the oocyte, full elucidation of the physiological role of the *Drosophila* p38 cascades requires the isolation of mutants in D-p38a and D-p38b.

The role of the JNK and p38 pathways in the response to stress has been established in vertebrate cell culture systems, but not validated by genetic studies in whole animals. Several MAPKKs have been shown to be involved in the stress-induced activation of the p38 and JNK pathways, including members of the MEKK group (MEKK1–MEKK4) (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Gerwins *et al.*, 1997; Takekawa *et al.*, 1997). To understand the biological function of the MEKK group in the whole organism, we isolated and characterized the *D-MEKK1* gene in the genetically amenable *Drosophila* species. The *Drosophila D-MEKK1* gene encodes a MAPKKK that is most similar to mammalian MEKK4/MTK1. *D-MEKK1* is not essential for development, allowing us to evaluate its role in stress responses. We have shown that animals harboring the null allele for *D-MEKK1* are hypersensitive to environmental stresses and have a defect in stress-induced activation of D-p38. Our results suggest that D-MEKK1 mediates stress responses through activation of D-p38.

Results

Isolation of *D-MEKK1* as a *Lic* MAPKK-binding protein

To identify a *Drosophila* MAPKKK, we used the yeast two-hybrid screening system to isolate genes whose protein products associate directly with *Lic* MAPKK (Suzanne *et al.*, 1999). MAPKKs normally activate MAPKKs by phosphorylation within a conserved activation loop between subdomains VII and VIII (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). This phosphorylation and activation motif is conserved within the *Lic* protein as the sequence S²⁰⁰IAKT²⁰⁴. Typically, mutations in the MAPKKK phosphorylation sites of MAPKKs create dominant-negative mutants that interfere with MAPKKK function (Cowley *et al.*, 1994), possibly because these mutants fail to dissociate from MAPKKK. We assumed that similar modifications in *Lic* might enhance its interaction with upstream signaling molecules. Therefore, we generated a dominant-negative mutant of *Lic*-SATA, in which Ser200 and Thr204 are replaced by Ala. Using the yeast two-hybrid system, a 'bait' plasmid containing sequences encoding *Lic*-SATA was used to screen a *Drosophila* cDNA library prepared from imaginal discs. From 1×10^6 transformants, one clone was identified that encoded a novel MAPKKK, which we shall refer to as *D-MEKK1* (see below). The product encoded by the *D-MEKK1* clone interacted with *Lic*-SATA in the yeast two-hybrid system, but not with wild-type *Lic* or a kinase-negative form of *Lic* bearing an amino acid substitution of Lys75 to Arg (Figure 1).

To obtain the full-length *D-MEKK1* cDNA, we screened a *Drosophila* imaginal disc cDNA library using our starting *D-MEKK1* clone as a probe, and obtained a longer cDNA clone. We then carried out 5'-RACE using

LexA -DBD	Gal4-AD	
Lic-SATA	D-MEKK1
Lic-SATA	-	
-	D-MEKK1	
Lic-WT	D-MEKK1	
Lic-KN	D-MEKK1	
		-Leu

Fig. 1. Interaction between D-MEKK1 and *Lic* in the yeast two-hybrid system. Yeast EGY48 cells were co-transformed with expression vectors encoding the indicated LexA DNA-binding domain (DBD) and Gal4 transcription activation domain (AD) fusion proteins. Interactions between the fusion proteins were assayed by growth on leucine-deficient (-Leu) medium.

poly(A)⁺ RNA prepared from pre-pupae as template. Products were screened by sequence analysis and subsequently used to isolate the corresponding cDNA clones from an embryonic *Drosophila* cDNA library. From both the 5'-RACE and library screening, we isolated two types of cDNA, *D-MEKK1a* and *D-MEKK1b*, corresponding to distinct start sites (Figure 2A). The sequences of the *D-MEKK1a* and *D-MEKK1b* cDNAs contain long open reading frames that predict proteins of 1571 and 1497 amino acids, respectively (Figure 2B).

Comparison of the amino acid sequence of the D-MEKK1 protein with the DDBJ/EMBL/GenBank database revealed significant homology to the MAPKKK family (Figure 3). Among members of the MAPKKK family, D-MEKK1 is most similar to mouse MEKK4 (Gerwins *et al.*, 1997) and human MTK1 (Takekawa *et al.*, 1997). D-MEKK1 and MEKK4 share 59% amino acid identity in the kinase domain. In addition, the sequence similarity between these two proteins extends outside the N-terminal non-kinase domain as well. The N-termini of D-MEKK1a and MEKK4 contain a proline-rich region followed by a putative pleckstrin homology (PH) domain (Gibson *et al.*, 1994; Lemmon *et al.*, 1996). However, whereas MEKK4 has a Cdc42/Rac interactive binding (CRIB)-like domain (Burbelo *et al.*, 1995) just upstream of its kinase domain, D-MEKK1 lacks this motif (Figure 3A).

D-MEKK1 is a MAPKKK

To determine whether D-MEKK1 has MAPKKK activity, *D-MEKK1a* was cloned into a mammalian expression vector to generate a Flag epitope-tagged protein (Flag-D-MEKK1). Human embryonic 293 cells were transiently transfected with Flag-D-MEKK1. To avoid the possibility that antibodies against D-MEKK1 might recognize other mammalian protein kinase(s) from 293 cells, we used anti-Flag antibody. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody, followed by kinase assay using recombinant, bacterially expressed MKK6 as a substrate (Figure 4A). D-MEKK1 was found to phosphorylate MKK6 efficiently *in vitro*. To eliminate the possibility that an associated kinase may be co-precipitating with D-MEKK1, we generated a kinase-defective mutant Flag-D-MEKK1(K1311R), in which Lys1311 in the ATP binding domain was mutated to Arg. In contrast to wild-type D-MEKK1, immunoprecipitates of D-MEKK1-(K1311R) did not phosphorylate MKK6, although both

Fig. 2. Primary structure of the *D-MEKK1* gene. (A) Genomic organization of the *D-MEKK1* gene. Exons are indicated by boxes. The shaded and open boxes are the translated and untranslated regions, respectively. The black boxes indicate kinase domains. This locus produces two transcripts, *D-MEKK1a* and *D-MEKK1b*. (B) Amino acid sequences deduced from the nucleotide sequences of the *D-MEKK1* cDNAs. N-terminal 79 amino acids of D-MEKK1a are boxed. N-terminus of D-MEKK1b contains five amino acids (MRKK) in place of the 79 amino acids. The DDBJ/EMBL/GenBank accession Nos for the DMEKK1a and DMEKK1b sequences are AB069961 and AB069962, respectively.

were expressed at comparable levels, as indicated by western blotting probed with anti-Flag antibody. These observations demonstrate that D-MEKK1 acts as a functional MAPKKK.

Having shown that D-MEKK1 phosphorylates MKK6 *in vitro*, we next examined whether D-MEKK1 is able to activate p38 *in vivo* (Figure 4B). 293 cells were transfected with D-MEKK1a together with hemagglutinin (HA) epitope-tagged p38 (HA-p38). HA-p38 was immunoprecipitated from cell lysates, and its kinase activity was measured *in vitro* using glutathione *S*-transferase (GST)-ATF2 protein as a substrate. Cells transfected with D-MEKK1 showed strong activation of p38, while cells transfected with the vector showed little or no activation. Taken together, these results indicate that D-MEKK1 functions as a MAPKKK that can activate the MKK6-p38 MAPK pathway.

Expression of *D-MEKK1* in *Drosophila*

To examine the pattern of *D-MEKK1* expression during embryogenesis, we hybridized whole-mount embryos with an antisense RNA probe synthesized from a *D-MEKK1* cDNA template. We found that the *D-MEKK1* gene is expressed throughout embryonic development (Figure 5A). There is a high level of maternal deposition similar to *D-MKK3/llc* and *D-p38* (Z.S.Han *et al.*, 1998). In the later stages, zygotic expression is present in most tissues. Whole-mount *in situ* hybridization also revealed that

D-MEKK1 mRNAs are homogeneously distributed in imaginal discs (Figure 5B) and the central nervous system (data not shown) of late third-instar larvae. No signal in the embryo or imaginal discs was observed when the control sense probe was used.

Isolation of the *D-MEKK1* mutant

To investigate the *in vivo* function of D-MEKK1, we generated loss-of-function mutations in the *D-MEKK1* locus. First, we performed chromosome *in situ* hybridization to map the cytological location of the *D-MEKK1* gene. *D-MEKK1* was mapped to the 91C region on the right arm of the third chromosome. This localization was further confirmed by hybridization to a polytene chromosome of a heterozygote having the deficiency *Df(3R)Cha7*, which deletes the 91A-91F region. Next, we searched for lines with a P-element insertion in this region from the Szeged stock center and obtained several candidates. By PCR analysis of the genomic DNA of these candidates, we found a line containing *P-lacW* of *l(3)s028102* in the *D-MEKK1* gene. Sequence analysis of the PCR product of this candidate revealed that the P-element was inserted ~280 bp upstream of the kinase domain of D-MEKK1 (Figure 6A). Although this 10 kb insertion reduced the amount of D-MEKK1 protein synthesized within cells, some D-MEKK1 protein could be detected by western blotting analysis (data not shown).

Fig. 3. Comparison of D-MEKK1 and mouse MEKK4. **(A)** Schematic diagrams of the structures of D-MEKK1a, D-MEKK1b and MEKK4. The percentage identity is indicated in each domain. **(B)** Sequence comparison between the N-terminal PH domain of D-MEKK1 and MEKK4. Amino acids that are identical or conserved are indicated by black or gray boxes, respectively. **(C)** Homology between the kinase domain of D-MEKK1 and MEKK4. Roman numerals above the sequences refer to subdomains conserved across the protein kinase family. Amino acids that are identical are indicated by black boxes.

To generate small deletions that partially remove the *D-MEKK1* transcript, a second round of P-element mutagenesis was performed. Excision of a P-element, while sometimes causing mutant reversion by restoring the gene to its original structure, often results in the deletion of sequences flanking the insertion site. Using a PCR-based screen, we examined 89 independent excised lines that had lost the *W*⁺ marker of *P-lacW*, searching for lines that had deleted the kinase domain of the *D-MEKK1* gene. Sequence analysis of one of several deletions, *D-MEKK1*^{Ur36}, revealed that it is lacking 868 nucleotides of the genomic *D-MEKK1* locus (Figure 6A). This mutation deletes sequences encoding amino acids 1194–1483 of D-MEKK1a, which includes the kinase subdomains I–IX (Figure 2B). Thus, *D-MEKK1*^{Ur36} is presumably defective in protein kinase activity.

Polyclonal rabbit antibodies were produced against an N-terminal (1–20 amino acids) and C-terminal peptide (1552–1571 amino acids) from D-MEKK1a (Figure 2B). Anti-D-MEKK1-C antiserum recognized both D-MEKK1a and D-MEKK1b in immunoprecipitation–western blotting analysis of proteins extracted from the third-instar larvae of wild-type animals (Figure 6B). In contrast, D-MEKK1 proteins were not detected in *D-MEKK1^{Ur36}* mutants, confirming that *Ur36* deletion disrupts *D-MEKK1*.

The D-MEKK1 mutant is hypersensitive to environmental stresses

Mutants homozygous or hemizygous for *D-MEKK1*^{Ur36} were viable and showed no obvious morphological aberrations. Since D-p38 is activated in response to heat

shock and osmotic stress in *Drosophila* Schneider cell lines (S.-J.Han *et al.*, 1998), it seemed likely that *D-MEKK1* would function in some aspect of the stress response. To determine whether D-MEKK1 has a role in the response to environmental stress, we examined the effect of increasing temperature on the viability of *D-MEKK1^{Ur36}* mutants (Table I). At the normal

temperature (25°C), *D-MEKK1^{Ur36}* mutants showed normal viability. At a higher temperature (30°C), the viability of homozygous *D-MEKK1^{Ur36}* mutants relative to the heterozygote markedly decreased to ~20%. We also examined the effect of high osmolarity (Table I). Most of the homozygous *D-MEKK1^{Ur36}* mutants died before eclosion when they were bred in the culture medium containing 0.2 M NaCl. Thus, *D-MEKK1* mutants are hypersensitive to these environmental stresses.

To confirm that the phenotypes observed in the *D-MEKK1* mutant animals are indeed due to the *D-MEKK1* mutation, we performed a genetic rescue experiment. We generated *D-MEKK1^{Ur36}* mutant animals harboring a transgene of the wild-type *D-MEKK1a* cDNA under the control of an inducible heat-shock promoter (*hs-D-MEKK1a*). In the absence of heat treatment, basal expression of the *D-MEKK1* transgene was sufficient to weakly rescue the NaCl-sensitive phenotype of *D-MEKK1^{Ur36}* mutants. Heat shock treatment strongly induced expression of D-MEKK1 (see Figure 8A), and effectively rescued the hypersensitivity of this mutant to high NaCl concentration (Table I). This demonstrates that the NaCl-sensitive phenotype of *D-MEKK1^{Ur36}* mutant animals is attributable to loss of *D-MEKK1* function.

Fig. 4. MAPKKK activity of D-MEKK1. (A) Phosphorylation of MKK6 by D-MEKK1. 293 cells were transfected with control vector (-), Flag-D-MEKK1a (WT) or Flag-D-MEKK1a(K1311R) (KN) as indicated. Immunoprecipitated (IP) complexes with anti-Flag were used for *in vitro* kinase reactions with bacterially expressed MKK6 as an exogenous substrate (upper panel). Whole-cell extracts were immunoblotted (IB) with anti-Flag (lower panel). (B) Activation of p38 by D-MEKK1. 293 cells were transfected with control vector (-), D-MEKK1a and HA-p38 as indicated. Immunoprecipitated complexes obtained with anti-HA were used for *in vitro* kinase reactions with GST-ATF2 as a substrate (top panel). The amounts of immunoprecipitated HA-p38 were determined with anti-HA (middle panel). Whole-cell extracts were also immunoblotted with anti-D-MEKK1-N(1–20) (bottom panel).

Environmental stresses activate the *D-MEKK1*–*D-p38* pathway

The increased sensitivity to elevated temperature and increased NaCl in animals lacking D-MEKK1 prompted us to measure the effect of stress on D-MEKK1 protein kinase activity in normal animals. Third-instar larvae were treated with NaCl, and endogenous D-MEKK1 was immunoprecipitated with anti-D-MEKK1 antibody from *Drosophila* extracts. *In vitro* protein kinase assays were

Fig. 5. Expression of the *D-MEKK1* mRNA. Wild-type embryos (A) and late third-instar larval imaginal discs (B) were stained with *D-MEKK1* probes. Sense strand probes were used as a control. (A) Various stages of embryos. (B) Eye-antennal and wing discs.

performed using MKK6 as a substrate. We found that D-MEKK1 activity was indeed upregulated by osmotic stress (Figure 7A).

D-p38 is activated in response to stress stimuli, including osmotic stress and heat shock (S.-J.Han *et al.*, 1998; Z.S.Han *et al.*, 1998; Adachi-Yamada *et al.*, 1999b). To confirm the activation of D-p38 by osmotic stress, we performed western blotting using an anti-phospho p38 antibody that specifically recognizes the phosphorylated, activated form of D-p38 (Adachi-Yamada *et al.*, 1999b). We observed that treatment of wild-type larvae with NaCl stimulated the phosphorylation of D-p38 (Figure 7A). We next addressed whether this activation is mediated by D-MEKK1. Wild-type and *D-MEKK1^{Ur36}* mutant animals were treated with NaCl and examined for D-p38 activation. Significantly, we found that D-p38 activation in response to NaCl was markedly reduced in *D-MEKK1* mutants compared with wild-type larvae (Figure 7B).

Fig. 6. Characterization of the *D-MEKK1* mutation. (A) Genomic organization of the *D-MEKK1^{Ur36}* mutation. Exons are indicated by boxes. The shaded and open boxes are the translated and untranslated regions, respectively. The black boxes indicate kinase domains. In *l(3)s028102*, *P-lacW* is inserted in the *D-MEKK1* gene. *D-MEKK1^{Ur36}* is the deletion mutant derived from *l(3)s028102* by P-element imprecise excision. A dashed line indicates an 868 nucleotide deletion. (B) Western blot of the D-MEKK1 protein. Extracts from third-instar larvae of wild-type (WT) and *D-MEKK1^{Ur36}* mutant were immunoprecipitated with anti-D-MEKK1-N(1–20) (left panel) or anti-D-MEKK1-C(1552–1571) (right panel). The immunoprecipitates were immunoblotted with anti-D-MEKK1-C.

Expression of D-p38 in the mutant larvae was similar to that in wild-type larvae. Activation of D-p38 in response to heat shock was also much lower in *D-MEKK1^{Ur36}* mutants relative to wild-type larvae (Figure 7C). These results indicate that D-MEKK1 is required for the activation of D-p38 in response to stress stimuli.

To confirm that D-p38 is involved in the D-MEKK1-mediated pathway, we tested whether ectopic expression of *D-MEKK1a* leads to activation of D-p38. *D-MEKK1a* was transiently expressed in third-instar larvae using the Gal4-upstream activation sequence (UAS) system (Brand and Perrimon, 1993). We generated a transgenic fly carrying the wild-type *D-MEKK1a* cDNA (*UAS-D-MEKK1a*) and a Gal4 construct driven by the heat-inducible heat shock promoter (*hs-GAL4*). We found that ectopic expression of *D-MEKK1a* induced higher levels of D-MEKK1 protein expression and D-MEKK1 kinase activity compared with control animals (Figure 8A). Western blotting with anti-phospho p38 antibody showed that animals carrying *UAS-D-MEKK1a* had high levels of phosphorylated D-p38 after induction of D-MEKK1 (Figure 8B), whereas the control animals did not. Thus, overexpression of D-MEKK1 can induce activation of D-p38.

Since D-MEKK1 was identified as a specific Lic-binding protein, it was possible that Lic and D-MEKK1 might function in the same signal transduction pathway. To test this possibility, we examined whether Lic protein kinase activity was stimulated by stress stimuli in a manner similar to D-MEKK1 and D-p38 in animals. Wild-type larvae at the third-instar stage were treated with NaCl, and endogenous Lic was immunoprecipitated from *Drosophila* extracts with anti-Lic antibody. *In vitro* protein kinase assays were performed using kinase-negative p38 as a substrate. No Lic activity was detected in these assays, either in the presence or absence of osmotic stress (Figure 9A). In contrast, we observed clear activation of D-p38 by osmotic stress at the same larval stage (Figure 9A). Activation of MAPKKs is dependent on the phosphorylation of specific sites in their catalytic domains (Cowley *et al.*, 1994; Yan and Templeton, 1994). To examine whether *Drosophila* contains a MAPKK that is activated by osmotic stress, we performed western blot analysis using an anti-phospho MKK3 antibody that specifically recognizes the phosphorylated, active form of MAPKKs. Treatment of wild-type animals with NaCl resulted in increased levels of a protein detected with the anti-phospho MKK3 antibody (Figure 9B). However, this protein is unlikely to be Lic, since it migrates at a higher molecular weight. Heat stress also did not induce

Table I. Stress sensitivity in *D-MEKK1^{Ur36}* mutants

Genotype	Stress ^a	HS ^b	Viability (%)	N ^c
<i>D-MEKK1^{Ur36}</i>	25°C	–	95	461.5
<i>D-MEKK1^{Ur36}</i>	30°C	–	20	102
<i>D-MEKK1^{Ur36}</i>	0.2 M NaCl	–	2	558.5
<i>D-MEKK1^{Ur36}</i>	0.2 M NaCl	+	2	387
<i>D-MEKK1^{Ur36}, hs-D-MEKK1a</i>	0.2 M NaCl	–	16	287
<i>D-MEKK1^{Ur36}, hs-D-MEKK1a</i>	0.2 M NaCl	+	68	207.5

^aA final concentration of 0.2 M NaCl was added to the culture medium at 25°C.

^bHeat shock treatment (HS) was carried out at 37°C for 1 h per day during larval period.

^cThe theoretically expected number of each mutant as a result of the crossing was determined from the number of balancer siblings.

Fig. 7. D-MEKK1 mediates environmental stresses to D-p38 in *Drosophila*. (A) Activation of D-MEKK1 by osmotic stress. Third-instar larvae of wild-type animals were injected with 1.2 M NaCl (+) or isotonic solution (-). Extracts prepared from each animal were immunoprecipitated with anti-D-MEKK1-N(1-20). The immunoprecipitates were used for *in vitro* kinase reactions with bacterially expressed MKK6 as a substrate (top panel). The immunoprecipitates were immunoblotted with anti-D-MEKK1-N (second panel). Whole-cell extracts were immunoblotted with anti-phospho p38 (third panel) and anti-D-p38b (bottom panel). (B) Activation of D-p38 by osmotic stress. Third-instar larvae of wild-type (WT) and *D-MEKK1^{Ur36}* mutant animals were injected with 1.2 M NaCl (+) or isotonic solution (-). Extracts prepared from each animal were immunoblotted with anti-phospho p38 (upper panel) and anti-D-p38b (lower panel). (C) Activation of D-p38 by heat shock. Third-instar larvae of wild-type (WT) and *D-MEKK1^{Ur36}* mutant animals were left untreated (-) or subjected to heat shock (HS; 37°C for 1 h). Extracts prepared from each animal were immunoblotted with anti-phospho p38 (upper panel) and anti-D-p38b (lower panel).

phosphorylation of Lic (data not shown). Taken together, these results suggest that Lic is not involved in the D-MEKK1-mediated activation of D-p38 in response to environmental stresses.

Discussion

The p38 MAPK pathway has been implicated in the mediation of stress signals. Proinflammatory cytokines, osmotic shock and bacterial LPS can induce p38 MAPK activity, which leads to an increase in cytokine production (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Ip and Davis, 1998). The p38 MAPKs are also involved in the control of other cellular processes, such as apoptosis and differentiation (Morooka and Nishida, 1998; Cuenda and Cohen, 1999; Zester *et al.*, 1999). *Drosophila* is a good model in which to study the role of MAPK pathways in whole organisms, especially since MAPK signaling

Fig. 8. Effect of D-MEKK1 overexpression on D-p38 activity. (A) Effect of D-MEKK1 overexpression on D-MEKK1 kinase activity. Third-instar larvae carrying both *hs-GAL4* and *UAS-D-MEKK1a* or only *hs-GAL4* were treated with heat shock (37°C for 30 min) and then incubated at 25°C for 120 min. Extracts prepared from these larvae were immunoprecipitated with anti-D-MEKK1-N(1-20). The immunoprecipitates were used for *in vitro* kinase reactions with bacterially expressed MKK6 as a substrate (top panel). Whole-cell extracts were immunoblotted with anti-D-MEKK1-N (middle panel) and anti-D-p38b (bottom panel). (B) Effect of D-MEKK1 overexpression on D-p38 activity. Third-instar larvae carrying both *hs-GAL4* and *UAS-D-MEKK1a* (upper panel) or only *hs-GAL4* (lower panel) were left untreated (-) or subjected to heat shock (37°C for 30 min). After heat shock treatment, larvae were incubated at 25°C for the indicated times (min). Extracts prepared from these larvae were immunoblotted with anti-phospho p38.

cascades homologous to their mammalian counterparts have been shown to exist in *Drosophila* (S.-J.Han *et al.*, 1998; Z.S.Han *et al.*, 1998; Suzanne *et al.*, 1999). In this study, we have isolated and characterized a novel MAPKKK, D-MEKK1, which functions in the p38 MAPK pathway in *Drosophila*. Sequence analysis reveals that this gene has high homology to its vertebrate counterparts, especially to MEKK4/MTK1. D-MEKK1 was found to phosphorylate mammalian MAPKK MKK6, similar to its human homolog. A *D-MEKK1* loss-of-function mutant exhibits hypersensitivity to elevated temperature and increased osmotic stress. These environmental stresses activate D-MEKK1 and D-p38 kinase activities in animals. Stress-induced activation of D-p38 is decreased in *D-MEKK1* mutant animals compared with wild type. Taken together, these results demonstrate that D-MEKK1 is an essential component of the *Drosophila* p38 signal transduction pathway that responds to environmental stresses. Our results provide the first demonstration of the significance of the D-MEKK1-D-p38 signaling pathway in stress responses in whole animals.

One interesting observation is that the homology between D-MEKK1 and MEKK4/MTK1 is found not only in their kinase domains, but also in their N-terminal non-catalytic domains as well. This similarity may indicate

Fig. 9. Activation of MAPKK by osmotic stress in *Drosophila*. (A) Effect of osmotic stress on Lic activity. (Left panels) Third-instar larvae of wild-type animals were injected with 1.2 M NaCl (+) or isotonic solution (-). Extracts prepared from each animal were immunoprecipitated with anti-Lic. The immunoprecipitates were used for *in vitro* kinase reactions with kinase-negative (KN)-p38 as a substrate (top panel). The immunoprecipitates were immunoblotted with anti-Lic (second panel). Whole-cell extracts were immunoblotted with anti-phospho p38 (third panel) and anti-D-p38b (bottom panel). (Right panels) 293 cells were transfected with HA-MKK6. Immunoprecipitated complexes obtained with anti-HA were used for *in vitro* kinase reactions with KN-p38 as a substrate (upper panel). Whole-cell extracts were immunoblotted with anti-HA (lower panel). (B) Phosphorylation of MAPKK by osmotic stress. Third-instar larvae of wild-type animals were injected with 1.2 M NaCl (+) or isotonic solution (-). Extracts prepared from each animal were immunoblotted with anti-phospho MKK3 (left panel) and anti-Lic (right panel).

that these MEKKs utilize a common regulatory mechanism. Known or suggested components upstream of the mammalian MEKKs include a growing family of Ste20-like kinases (Hu *et al.*, 1996; Diener *et al.*, 1997; Su *et al.*, 1997), low molecular weight GTP-binding proteins including Cdc42 and Rac (Minden *et al.*, 1995; Fanger *et al.*, 1997), and trimeric G proteins (Collins *et al.*, 1996; Hooley *et al.*, 1996). The N-terminal regions of D-MEKK1, MEKK4 and MTK1 contain proline-rich sequences that may be involved in SH3 domain or protein-protein interactions. Thus, D-MEKK1 may be regulated by interaction with upstream factors through its N-terminal non-catalytic domain. The identification of other components of the D-MEKK1 pathway in *Drosophila* will undoubtedly provide valuable insights into the signaling pathway regulating the responses to environmental stresses.

Previous studies have shown that D-p38s are involved in the insect immune defense system against bacterial infection and in *decapentaplegic* (*dpp*)-regulated wing morphogenesis (Z.S.Han *et al.*, 1998; Adachi-Yamada *et al.*, 1999b). However, *D-MEKK1* mutants appear normal in both insect immunity and Dpp signaling (data not shown). Possibly other MAPKKK(s) function in a redundant manner to regulate immune responses and the

Dpp pathway by activating D-p38. In fact, several homologs of other distantly related kinases known to function as MAPKKKs have been found in *Drosophila* (Wassarman *et al.*, 1996; Takatsu *et al.*, 2000). Alternatively, it is possible that D-MEKK1 activates only D-p38a or D-p38b, but not both, in response to environmental stress *in vivo*. Supporting this possibility, partial activation of D-p38 was observed in *D-MEKK1* mutant animals subjected to environmental stress. Similar examples of MAPK redundancy have been described in yeast. For example, Kss1 and Fus3 normally have specific roles in different pathways, but have been shown to function redundantly in the mating pathway (Herskowitz, 1995). In this case, Kss1 replaces Fus3 when the latter is deleted. Isolation of D-p38a and D-p38b mutants will be required to understand fully the intricate connection between D-MEKK1 and D-p38 in mediating stress responses.

D-MEKK1 was identified as a protein that specifically interacts with Lic, an MKK that is most similar to the mammalian p38 activators MKK3 and MKK6 (Z.S.Han *et al.*, 1998; Suzanne *et al.*, 1999). Mutations in the *lic* gene result in embryo polarity defects that correlate with changes in two oocyte-localized determinants, Oskar and Gurken, essential for posterior and dorsal specification, respectively (Ray and Schupbach, 1996). Thus, the Lic-D-p38 MAPK signaling pathway plays an important role in the patterning of the *Drosophila* egg. On the other hand, in contrast to *lic* mutants, females homozygous for the *D-MEKK1* loss-of-function mutation lay morphologically normal eggs. These eggs are fertilized and develop to normal adult flies (data not shown). Thus, animals mutant for *D-MEKK1* do not show a *lic*-deficient phenotype. Although a specific interaction can be detected between D-MEKK1 and Lic in the yeast two-hybrid system, D-MEKK1 was unable to phosphorylate Lic proteins purified from *Escherichia coli* *in vitro* (data not shown). These results suggest that D-MEKK1 activates D-p38 in animals via a MAPKK(s) that is distinct from Lic. Consistent with this possibility, D-MEKK1 and D-p38 kinases were activated in late third-instar larvae in response to environmental stress, whereas Lic kinase activity was undetectable. Thus, we currently do not know which MAPKK functions between D-MEKK1 and D-p38 in this signaling pathway. Remarkably, the only additional MAPKK-like kinase in the *Drosophila* DNA database is D-MKK4, which is most similar to mammalian MKK4 (Z.S.Han *et al.*, 1998). D-MKK4 was shown to be able to activate both D-JNK and D-p38b *in vitro* (Z.S.Han *et al.*, 1998; Kawasaki *et al.*, 1999). This raises the possibility that D-MKK4 may be a component of the D-MEKK1-D-p38 pathway required for responses to environmental stresses. Indeed, osmotic stress induces phosphorylation of a protein whose molecular weight (~46 kDa) is similar to that of D-MKK4. Investigations into the functional and genetic associations between D-MEKK1 and other conserved components of this pathway, and the identification of D-p38 substrates, should allow for a more complete understanding of the *Drosophila* D-MEKK1-D-p38 pathway regulating responses to environmental stresses.

Mammalian cells contain four members of the MEKK family (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Gerwins *et al.*, 1997; Takekawa *et al.*, 1997). Recent

results demonstrate that specific MEKKs selectively control specific MAPK pathways. In MEKK1^{-/-} embryonic stem (ES) cells, JNK activation in response to cold shock, oxidative stress and hyperosmolarity is diminished. In contrast, activation of p38 by these stimuli is not affected by the loss of MEKK1 (Yujiri *et al.*, 1999). Thus, targeted disruption of MEKK1 expression results in the selective loss of JNK activation in response to different stimuli. In this study, we show that *Drosophila* D-MEKK1 regulates the D-p38 pathway. Is D-MEKK1 also involved in the D-JNK pathway? Two different developmental processes are known to be controlled by the JNK signaling pathway in *Drosophila*. One is the movement of leading-edge cells during the process of embryonic dorsal closure (Noselli, 1998), and the other is in planar polarity determination of adult tissues (Strutt *et al.*, 1997; Boutros *et al.*, 1998). However, *D-MEKK1* loss-of-function mutants have no effect on either of these processes. Furthermore, in *bsk*^{-/+}; *D-MEKK1*/*D-MEKK1*⁻ embryos, dorsal closure remained unaffected (data not shown). Recent evidence suggests that D-TAK1, a *Drosophila* homolog of the TAK1 MAPKKK, participates in the JNK signaling pathway (Takatsu *et al.*, 2000). Thus, D-MEKK1 and D-TAK1 may specifically regulate the p38 and JNK pathways, respectively. An important concept that emerges from both the mammalian and *Drosophila* studies is that MAPKKK loss-of-function mutations can cause a very specific loss of a cellular function, even though the downstream MAPKs respond to alternative stimuli. Thus, while multiple MAPKKKs may be capable of stimulating a particular downstream target such as JNK or p38, activation of a given pathway in response to a specific stimulus requires a specific MAPKKK member. This may provide greater selectivity and specificity of the responses to different stimuli.

Materials and methods

Yeast two-hybrid screening

Yeast strain EGY48 (*ura3 his3 trp1 LEU2::plexAop6-LEU2*) expressing Lic-SATA fused to the LexA DNA-binding domain was transformed with a *Drosophila* cDNA library (Finley *et al.*, 1996) fused to the Gal4 activation domain.

Molecular cloning of D-MEKK1

Using the *D-MEKK1* DNA fragment obtained in the two-hybrid screening as a probe, an imaginal disc cDNA library was screened and a longer clone was obtained (S7-9) (Brown and Kafatos, 1988). A primer (GT51: 5'-CCCTGGGAGGCCCAACGGGGACGCATTCC-3') was designed from the sequence of S7-9 to perform 5'-RACE, and several clones were obtained. Using one of these clones as a probe (#51-2), a random-primed embryonic cDNA library was screened and several clones were obtained that contain an initiation codon following a stop codon (Umekiya *et al.*, 1997). These cDNA sequences were combined to make the full-length cDNA of *D-MEKK1a* or *D-MEKK1b*.

In situ hybridization

Staining of embryos and imaginal discs was performed as in Ashburner (1989). RNA probes were labeled with DIG labeling kit (Roche Molecular Biochemicals, IN).

Kinase assay

Kinase assay was performed as in Ninomiya-Tsuji *et al.* (1999). Bacterially expressed GST-ATF2, MKK6 or kinase-negative p38 was purified and used as substrate for HA-p38, D-MEKK1 or Lic, respectively.

Genomic DNA PCR

Genomic DNA was prepared from adult flies as described in Ashburner (1989). For screening of a *D-MEKK1* deletion mutant, a pair of primers, GT41 and GT75, was designed to amplify a region of the *D-MEKK1* gene including the P-*lacW* insertion site. GT75: 5'-GACAGCGGTGCTCACACAGTAGAT-3', GT41: 5'-GTCGTGACCCTCTGGGATAG-3'.

Immunoprecipitation

Drosophila larvae were homogenized using a glass homogenizer in extraction buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 12.5 mM β -glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 20 μ M aprotinin, 0.5% Triton X-100). 293 cells were lysed in the same extraction buffer. Larval extracts and 293 cell lysates were clarified by centrifugation at 15 000 g for 3 min. Supernatants were incubated with the appropriate antibody and protein G-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4°C. After immunoprecipitation, immunoprecipitates were washed three times with phosphate-buffered saline (PBS) and then resuspended in 30 μ l of PBS.

Immunoblotting

Drosophila larvae were homogenized in a sample buffer of 65 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol and 5% β -mercaptoethanol. Immunoprecipitates were denatured by addition of sample buffer. Anti-phospho p38 and anti-phospho MKK3 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-D-MEKK1 antibodies (anti-D-MEKK1-N and -C) were raised against synthetic peptides corresponding to the N-terminal 20 amino acids (anti-D-MEKK1-N) and C-terminal 20 amino acids (anti-D-MEKK1-C) of D-MEKK1a, respectively. Anti-Lic antibody was raised against a synthetic peptide corresponding to the N-terminal 20 amino acids of Lic. We used a 1:500 dilution of anti-phospho p38 antibody, 1:250 of anti-phospho MKK3, 1:3000 of anti-D-p38b antibody (Adachi-Yamada *et al.*, 1999b) and 1:1000 of anti-D-MEKK1 and anti-Lic antibodies.

Stresses

For immunoblotting and kinase assays, late third-instar larvae were heat-shocked at 37°C for 1 h in a water bath or were anesthetized with CO₂ and injected with a microcapillary around the posterior pole with an ~150 nl solution of 1.05% NaCl, 5 mM sodium phosphate buffer pH 6.6 and 5 mg/ml xylene cyanol in the presence or absence of 1 M NaCl. In the high temperature experiments, heterozygotes for mutant and balancer chromosomes were crossed and allowed to lay eggs on culture medium in a vial every day. After removing parent flies, the vials were kept at the indicated temperatures. In the high osmolarity experiments, parent flies were allowed to lay eggs every day while feeding on a diet containing the indicated concentrations of NaCl. Viability of the homozygotes was calculated from the number of homozygotes $\times$ 2 and dividing by the number of heterozygotes for both mutant and balancer chromosomes. For the rescue experiment, embryos obtained from crossing *w/Y; D-MEKK1^{Ur36} P[hs-D-MEKK1a, w(+)]/TM3* to *w; D-MEKK1^{Ur36}/TM3* for 24 h were heat shocked at 37°C for 1 h every day during larval period to induce expression of the *D-MEKK1a* transgene.

Transgenic flies

The DNA sequence encoding *D-MEKK1a* was cloned into *pPUAST*, a *GAL4*-responsive vector (Brand and Perrimon, 1993), and *pPCaSpeR-hs*, a heat shock-inducible vector (Thummel and Pirrotta, 1991), to generate *pPUAST-D-MEKK1a* and *pPCaSpeR-hs-D-MEKK1a*, respectively.

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