A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*

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The *Drosophila* MAP kinase DJNK is a homolog of the mammalian c-Jun amino-terminal kinase (JNK). Mutations in the DJNK gene correspond to the complementation group *basket*. DJNK is phosphorylated and activated by the *Drosophila* MAP kinase kinase HEP. Substrates of DJNK include the transcription factor DJun. DJNK participates in multiple physiological processes. Exposure to endotoxic lipopolysaccharide initiates an insect immune response and leads to DJNK activation. In addition, embryos lacking DJNK are defective in dorsal closure, a process in which the lateral epithelial cells migrate over the embryo and join at the dorsal midline. These data demonstrate that the DJNK signal transduction pathway mediates an immune response and morphogenesis in vivo.

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The c-Jun amino-terminal kinase (JNK) signal transduction pathway has been implicated in the regulation of gene expression (Dérjard et al. 1994; Kyriakis et al. 1994). Biochemical studies indicate that the effect of JNK to increase gene expression is mediated, in part, by the phosphorylation and activation of the transcription factors c-Jun (Pulverer et al. 1991; Smeal et al. 1991), ATF2 (Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995), and Elk-1 (Cavigelli et al. 1995; Gille et al. 1995; Whitmarsh et al. 1995). Multiple extracellular stimuli, including environmental stress and inflammatory cytokines, activate the JNK protein kinase (Dérjard et al. 1994; Kyriakis et al. 1994; Sluss et al. 1994; Dai et al. 1995; Gupta et al. 1996). Recent studies have indicated that the JNK signaling pathway contributes to the cellular response to environmental stress (Xia et al. 1995; Chen et al. 1996; Verheij et al. 1996). However, the biological role of JNK in the normal development and function of the organism has not been established (Davis 1994).

Exposure of cells to environmental stress causes activation of at least two groups of transcription factors, AP-1 and Rel (Stein et al. 1989). It is established that AP-1 activation is mediated, in part, by the JNK signal transduction pathway (Davis 1994). In contrast, the signaling pathway that activates Rel-related transcription factors during stress has not been established. The Rel

family of transcription factors includes mammalian NF- κ B and the *Drosophila* morphogen Dorsal (Verma et al. 1995). Dorsal is the critical transcription factor that governs the establishment of dorsal/ventral polarity of the embryo (Steward and Govind 1993). The Dorsal-related factor Dif is an important component of the insect immune defense against bacterial infection (Ip et al. 1993) that mediates increased expression of bactericidal peptides, such as Cecropin (Petersen et al. 1995). The Rel family of transcription factors represent conserved molecules that are employed in the immune response of insects and mammals (Ip and Levine 1994). Similarly, AP-1 contributes to the mammalian immune response (Su et al. 1994) and may also participate in insect immunity.

We report the identification of a *Drosophila* homolog of the c-Jun amino-terminal kinase. The DJNK protein kinase is activated by endotoxic lipopolysaccharide (LPS). Therefore, DJNK is a component of the insect immune system that is activated in response to bacterial infection. Molecular genetic analysis of the DJNK locus demonstrated that DJNK is essential for embryonic development. Loss of DJNK function inhibits the movement of the lateral epithelial cells during mid-embryogenesis and blocks dorsal closure. This defect causes a distinctive cuticle phenotype, identified previously as *basket* (*bsk*) (Nüsslein-Volhard et al. 1984). Genetic rescue experiments and sequence analysis provide direct molecular genetic evidence that the *bsk* locus encodes DJNK. This study establishes DJNK as a new member of the MAP kinase group in insects that mediates an im-

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Expression of DJNK during embryonic development

The expression of DJNK was examined using in situ hybridization analysis of embryos at representative developmental stages. A low amount of DJNK staining was detected in early cleavage stage embryos (Fig. 2A). This faint staining appeared in many embryos and may represent expression of maternal mRNA. The zygotic DJNK staining is present in all germ layers during germ-band extension (Fig. 2B). During germ-band retraction, the same intense staining remains in the epidermis and the central nervous system (CNS) (Fig. 2C). When dorsal closure begins (Fig. 2D), DJNK staining was increased in the CNS and was present in the lateral epidermal region extending up to the amnioserosa. A stage 15 embryo, when dorsal closure is complete, shows high levels of DJNK staining in the ventral nerve chord, the brain, and some peripheral neurons (Fig. 2E). A stage 16 embryo, when the ventral nerve chord has been retracted, also shows a high level of DJNK expression in the nervous system (Fig. 2F). In summary, the zygotic pattern of DJNK staining appears to be regulated spatially and temporally. In contrast, the pattern of staining for *Drosophila* Jun

(DJun) in the developing embryo is ubiquitous (Perkins et al. 1990; Zhang et al. 1990). These physical boundaries of DJNK expression, spatial and temporal, may contribute to the regulation of the DJun transcription factor during *Drosophila* development.

The imaginal discs of the third instar larvae were dissected and hybridized with an antisense DJNK riboprobe. All of these discs stained with the DJNK probe (data not shown). The wing disc and eye disc contained high levels of DJNK transcripts (Fig. 2G,I). This observation is consistent with a role for DJNK in the differentiation and function of adult tissues.

Northern blot analysis of mRNA extracted from staged embryos demonstrated that the level of DJNK expression remained relatively constant throughout embryogenesis (Fig. 3). The predominant mRNA species was 1.8 kb. A second, larger mRNA (~1.85 kb) was also detected in the early embryo (Fig. 3). The nature of this larger transcript remains to be determined, but it may represent a maternally transcribed product of the DJNK gene. DJNK was detected in larvae, pupae, and adults (female and male). DJNK expression was also detected in the insect fat body and the *mbn-2* hemocyte cell line

Figure 2. Spatial and temporal distribution of DJNK. (A–F) Various stages of embryos stained with a DJNK antisense riboprobe. The orientation of the embryos is such that anterior is to the left and dorsal is up, unless stated otherwise. (A) An early cleavage stage embryo shows very low levels of DJNK staining. (B) Stage 9 embryo. DJNK staining appears ubiquitous, including the extended germ band and developing anterior and posterior gut. (C) A stage 12 embryo. The focal plane is the lateral surface of the embryo and illustrates the diffuse DJNK staining in the epidermis. The predominant staining is in the developing CNS. (D) A stage 14 embryo. The germ band has retracted and DJNK staining is present in the CNS and in the epidermis extending up to the amnioserosa. (E) Stage 15 embryo. Dorsal closure is complete and the germ band has shortened. DJNK staining is restricted to the ventral nerve chord, brain, and some peripheral neurons. (F) Stage 16 embryo (ventral view). The ventral nerve chord has undergone shortening. This is a ventral view to show the staining of the nerve chord, underlying brain, and peripheral neurons. (G–I) Imaginal discs stained with a DJNK antisense riboprobe. (G) Wing imaginal disc stained with a DJNK antisense riboprobe. (H) Eye imaginal disc stained with a control sense DJNK riboprobe and (I) an antisense DJNK riboprobe. Both imaginal discs contain broad DJNK staining.

Figure 3. DJNK expression during embryonic development and in various tissues. Northern blot analysis of poly(A)⁺ RNA was performed using a random-primed, full-length, DJNK cDNA probe. (Left) Embryo (15 μ g RNA) at 0–4, 4–8, 8–12, 12–14 hr. (Middle) Larvae (10 μ g), adult male (5 μ g), fat body (2 μ g), Toll 10b larvae (15 μ g), pupae (15 μ g), and adult female (15 μ g). (Right) Cultured *mbn-2* hemocytes (30 μ g). The migration of the RNA size markers is indicated on the right (in kb). The blots were reprobed for rp49 mRNA to control for variations in RNA loading (bottom).

(Fig. 3). The fat bodies and hemocytes are the primary immune organs of the insect (Engström 1993; Hoffmann et al. 1993; Hultmark 1993).

DJNK locus in wild-type and deficiency strains

To elucidate the role of DJNK in *Drosophila* developmental and physiological processes, we conducted a molecular genetic analysis of the DJNK genomic locus. Hybridization analysis demonstrated that the DJNK gene lies on the left arm of the second chromosome in the 31B-C interval (Fig. 4A). Several *Jammed*-derived deficiencies in the 31B-C region were analyzed for DJNK expression and genomic lesions. One *Jammed* line, *Df(2L)JR1*, was found by chromosome in situ hybridization analysis to delete the entire DJNK gene (data not shown). Embryos were collected from the *JR1* line, which was carried over the *CyO* balancer chromosome, and RNA in situ hybridization analysis was performed. Approximately one-quarter of the embryos (the expected inheritance) did not stain for DJNK mRNA (Table 1). Two other *Jammed*-derived deletions, *J106* and *J77*, showed normal DJNK staining (Table 1).

The *ME31B*, *flipper* (*flp*), and *Dror* genes have been localized previously to the distal part of 31B-C region (Valoir et al. 1992; Wilson et al. 1993). Sequence analysis of a DJNK genomic clone revealed that it contained the *ME31B* open-reading frame (ORF) in an orientation that was opposite to DJNK (Fig. 4B). Therefore, DJNK is located adjacent to *ME31B*. Embryos were collected from *flp*-derived deficiency lines and stained for DJNK mRNA. One-quarter of the embryos from these lines did

not have DJNK staining, consistent with the expected inheritance (Table 1). Southern blot analysis was performed to confirm the lesions within the DJNK gene. The deficiency *flp170B* probably deletes the entire DJNK gene. Similarly, the deficiency *flp170E* disrupts expres-

Figure 4. Genomic organization of the DJNK locus. (A) In situ hybridization of a DJNK probe with polytene chromosomes from the salivary gland. The DJNK gene maps to the left arm of chromosome 2, region 31 B-C. The line indicates the specific DJNK staining. (B) Schematic illustration of the DJNK locus. The arrows indicate the direction of transcription. The DJNK gene is marked by a hatched box. The DJNK gene is located next to the *ME31B* gene and the P-element insertion *flp*. DJNK is deleted in the strains *flp147E* and *flp170B*, the deletions are represented by lines. The breakpoints of the deficiencies are not precisely defined and are represented by hatched lines. (C) Schematic representation of the intron/exon structure of the DJNK gene. (*) The two *bsk* alleles, corresponding to the mutations in the DJNK gene. *bsk1* has a point mutation in codon 225; GGA (Gly) is replaced with GAA (Glu). *bsk2* has a point mutation at codon 316; AAG (Lys) is replaced by TAG (stop). The sequence of the *bsk1* and *bsk2* genes has been deposited in GenBank under accession nos. U50965 and U50966, respectively. (D) The primary sequence surrounding the *bsk* mutations (kinase subdomains IX–X and subdomain XI) is presented. (#) The stop codon in *bsk2*. These regions are compared with the sequence of other members of the MAP kinase group.

Table 1. Correlation between loss of DJNK function and defects in dorsal closure

	DJNK mRNA	Dorsal closure
<i>Canton S</i>	+	+
<i>Df(2L)JR1</i>	–	–
<i>Df(2L)77</i>	+	+
<i>Df(2L)106</i>	+	+
<i>flp147E</i>	–	–
<i>flp170B</i>	–	–
<i>bsk1</i>	+	–
<i>bsk2</i>	+	–

DJNK mRNA staining was judged by in situ hybridization with a DJNK riboprobe. A total of 500 embryos were scored for DJNK staining. The parent flies are heterozygous and the recessive trait has an expected inheritance of 25%. Strains with >95% embryos staining for DJNK were scored as positive (+). Strains with ~25% embryos without DJNK staining were scored negative (–). The presence (+) or absence (–) of complete dorsal closure was examined in embryo collections (0–12 hr) of each strain. The embryos were aged and the cuticles of unhatched embryos were examined. Fly lines were scored positive (+) if none of the unhatched embryos had defects in dorsal closure and negative (–) if >20% of the embryos exhibited defects in dorsal closure (or 5% for *bsk2*). The *bsk1* and *bsk2* flies contain mutations within the DJNK coding region that do not alter mRNA expression (Fig. 4D).

sion of DJNK mRNA and probably contains a breakpoint within the DJNK gene. A schematic representation of the genomic organization of the DJNK locus within the 31B-C region is shown in Figure 4B.

Loss of DJNK function correlates with defective dorsal closure

The *Jammed* and *flp* deficiencies were examined for abnormalities in cuticle morphology. Embryos were collected from the deficiency strains and aged for 24–36 hr. Cuticles of the unhatched embryos were prepared. For all the deficiencies that deleted DJNK, examination of the cuticles of unhatched embryos revealed a population with an aberrant dorsal cuticle (Fig. 5). These lines produced a population of dead embryos with a range of mutant dorsal cuticle phenotypes (Fig. 5B,C). The severely deformed cuticles had a dramatic reduction in dorsal ridge formation, with the head and anterior segments exposed (Fig. 5B). In other embryos, holes in the dorsal epidermis are observed. The size of the hole varies from a large hole that covers most of the dorsal region (Fig. 5B) to a small hole (Fig. 5C). The deficiency lines where DJNK expression was normal did not contain defects in the dorsal cuticle (Table 1).

Careful examination of the mutant embryos indicated no defects in tissue differentiation (data not shown). Both the amnioserosa and epidermis are present, suggesting that dorsoventral patterning is not defective. Furthermore, the morphogenetic processes of gastrulation, germ-band extension, and germ-band retraction occur

normally. We postulate that the hole in the dorsal cuticle was the result of defective dorsal closure during mid-embryogenesis. During this process, the lateral epithelial cells move as a sheet to cover the dorsal region of the embryo (MacKrell et al. 1988; Ashburner 1989; Ring and Martinez Arias 1993; Young et al. 1993; Fehon et al. 1994). There is no new cell division. Instead, the cells change morphology and migrate. The cells at the leading edge undergo elongation, beginning at germ-band retraction. Subsequently, more cells undergo this morphological change concomitantly with spreading (MacKrell et al. 1988; Ring and Martinez Arias 1993; Young et al. 1993; Fehon et al. 1994). A contiguous layer of cells forms, which results in the closure of the epidermis over the amnioserosa and internal organs.

DJNK rescues the *bsk* phenotype

The correlation between DJNK function and dorsal closure led us to examine the complementation group *bsk* that is defective in dorsal closure and maps to the 31B-F region (Nüsslein-Volhard et al. 1984). We obtained two alleles of *bsk* (*bsk1* and *bsk2*) from the Nüsslein-Volhard laboratory for analysis. Cuticle examination demonstrated that the *bsk* embryos exhibit a defect in dorsal closure that is similar to the *Jammed* and *flp* deficiencies (Table 1; Fig. 5D). Both severely defective cuticles (Fig. 5D) and cuticles with holes (Fig. 5E, F) were observed. The defect in both *bsk* alleles is similar, except that more cuticles with a severe phenotype were observed for *bsk1* than *bsk2*. To identify the genetic location of *bsk*, we crossed the *bsk* alleles with *flp170B* and *flp147E*. Complementation was not observed in the *trans*-heterozygous embryos (data not shown). This finding suggests that *bsk* is located in the same small chromosomal region as DJNK.

To obtain evidence that *bsk* encodes DJNK and participates in the dorsal closure pathway, a genetic rescue experiment was conducted. The DJNK cDNA was placed under the control of a heat shock promoter (hsDJNK) and introduced into the fly genome through P-element-mediated transformation. Nine independent transgenic lines were generated. All of these lines have induced expression of DJNK under heat shock. Control experiments demonstrated that overexpression of the wild-type DJNK protein did not lead to a defect in embryonic development (data not shown). The chromosomal locations of the transgenes were determined and flies with the P-element located on the X or third chromosome were mated with the *bsk1* and *flp170B* flies. Balanced fly stocks were obtained and used to determine if the expression of DJNK could rescue the embryonic lethality or reduce the cuticular lesion.

We found that hsDJNK rescued the dorsal closure phenotype of *bsk1* and *flp170B* (Table 2). Embryos were collected from *flp170B*; *hsDJNK* and *bsk1*; *hsDJNK* and aged until hatching. The cuticle structure of the unhatched embryos was examined. There was a marked reduction in dorsal closure defects. The *flp170B*; *hsDJNK* and

Figure 5. Effect of DJNK on dorsal closure. Comparison of the cuticles of wild-type flies and flies with DJNK defective alleles or *hsDJNK* rescue alleles. The orientation is such that anterior is up. (A) Wild-type cuticle illustrating the regular spacing of the denticle belt on the ventral side (*left*) and complete closure of the epidermis on the dorsal side (*right*). (B) A *flp170B* cuticle which shows a severe defect in dorsal closure. (C) A *flp170B* cuticle (dorsal view) in which dorsal closure began and then terminated leaving a medium size hole. (D) A *bsk1* cuticle illustrating a severe defect in dorsal closure. (E) A *bsk1* cuticle exhibiting a less severe defect in dorsal closure. (F) A *bsk1* cuticle (dorsal view) with a moderate defect that results in a small hole. (G) A *flp170B*; *hsDJNK* cuticle illustrating that the severity of the defect has been reduced. (H) A *bsk1*; *hsDJNK* cuticle that appears to be wild-type. (I) A *bsk1*; *hsDJNK* embryo showing rescue of the dorsal defect, but leaving a tiny hole.

bsk1; *hsDJNK* lines contained very few cuticles with a severe defect in dorsal closure (Table 2). The number of *flp170B*; *hsDJNK* cuticles with defective dorsal closure was greatly reduced (2%) compared with the *flp170B* line (23%). Furthermore, the cuticles presenting defects had a less severe phenotype (Fig. 5G). Wild-type cuticles were observed in the remainder of the unhatched embryos. In the *bsk1*; *hsDJNK* line, most of the unhatched embryos

had wild-type cuticles (Fig. 5H). Some unhatched embryos contained just a pinhole and appeared to develop to a later stage (Fig. 5I). These tiny holes were not apparent in the parent *bsk1* line. Only 1% of the *bsk1*; *hsDJNK* embryos exhibited defective cuticles compared with 22% of the *bsk1* parental line. For both rescued lines (*flp170B*; *hsDJNK* and *bsk1*; *hsDJNK*), there was no significant reduction in the total number of unhatched em-

Table 2. Analysis of DJNK complementation on dorsal closure

	Basket cuticles (%) ^a	Dead embryos (%) ^b
<i>flp170B</i>	23	35
<i>flp170B; hsDJNK</i>	2	25
<i>bsk1</i>	22	28
<i>bsk1; hsDJNK</i>	1	18

Independent *hsDJNK* lines were crossed with *bsk1* and *flp170B* flies. Two lines required heat shock during early to mid-embryogenesis to rescue the cuticle defect of *bsk1* and *flp170B*. Three additional *hsDJNK* lines rescued without heat shock treatment; the data obtained for one *hsDJNK* lines are shown. Five hundred embryos from heterozygous mutant parents were collected and aged until hatching.

^aThe cuticles of the unhatched embryos were examined. The number of basket cuticles (including severe, medium hole, and small hole; Fig. 5B–F) is presented as a percentage of the total embryos collected.

^bThe percentage of unhatched embryos compared with total embryos is presented.

bryos. Therefore, DJNK is probably required for other developmental processes and the *hsDJNK* transgene may not provide correct DJNK expression for complementation of these other functions. We conclude from these genetic rescue experiments, that the dorsal closure phenotype exhibited in the deficiencies and the *bsk* alleles is attributable to the loss of DJNK function.

bsk alleles encode mutant DJNK

RNA in situ hybridization analysis of *bsk* embryos demonstrated that DJNK expression appears to be normal (Table 1). We therefore postulated that the mutations that contribute to the loss-of-function are present within the coding region of DJNK. The wild-type DJNK gene consists of six exons and five introns (Fig. 4C). Sequence analysis demonstrated that the *bsk1* allele encodes a mutated DJNK protein with a substitution of Glu for Gly at codon 225. The G225E mutation is located between subdomains IX and X of the DJNK protein kinase (Fig. 4D). Gly-225 is conserved in the MAP kinase family and is located in a region identified to be required for substrate recognition by JNK protein kinases (Kallunki et al. 1994; Sluss et al. 1994; Dai et al. 1995; Gupta et al. 1996). The *bsk2* allele encodes a truncated DJNK protein kinase that results from the presence of a termination codon within kinase subdomain XI (Fig. 4D). These substitution and truncation mutations in the DJNK gene provide strong evidence that DJNK is encoded by *bsk*. We conclude that dorsal closure requires signaling by the DJNK protein kinase.

Biochemical properties of DJNK

A *Drosophila* MAP kinase kinase homolog, Hemipterous (HEP), was identified recently and demonstrated to be required for dorsal closure (Glise et al. 1995). The HEP

sequence is most similar to the stress-activated mammalian MAP kinase kinases MKK3, MKK4, and MKK6 (Sanchez et al. 1994; Dériard et al. 1995; Lin et al. 1995; Raingeaud et al. 1996). In *hep*[−] embryos, the lateral epidermal cell movement during dorsal closure is suppressed and a defect in dorsal closure is observed. Because the loss of DJNK function also causes defective dorsal closure, it is possible that DJNK and HEP may function in the same signal transduction pathway. To test this hypothesis, we examined whether DJNK is phosphorylated and activated by HEP. Both protein kinases were prepared as recombinant proteins in bacteria and purified (Fig. 6A). HEP and DJNK do not appreciably autophosphorylate (Fig. 6A, lanes 1 and 2). However, when HEP and DJNK are incubated together, there is marked phosphorylation of both proteins (Fig. 6A, lanes 3 and 6). This observation indicates that DJNK is a substrate for HEP and that, like mammalian MKK4 (Dériard et al. 1995), HEP is a substrate for activated DJNK (Fig. 6A). To determine whether the phosphorylation of DJNK by HEP leads to DJNK activation, the protein kinase activity of DJNK was measured. Mammalian JNK phosphorylates the c-Jun transcription factor on the activating sites Ser-63 and Ser-73 (Pulverer et al. 1991; Smeal et al. 1991). A c-Jun homolog in *Drosophila* (DJun) that is expressed throughout development has been identified (Perkins et al. 1988; Perkins et al. 1990; Zhang et al. 1990). The sites of phosphorylation on c-Jun, corresponding to Ser-63 and Ser-73, are conserved in DJun. In vitro protein kinase assays demonstrated that the DJNK protein kinase phosphorylates both c-Jun (data not shown) and DJun (Fig. 6A). Addition of HEP caused a large increase in the phosphorylation of DJun by DJNK (lane 6). These data demonstrate that DJNK is phosphorylated and activated by HEP (Fig. 6A) and provide biochemical evidence that HEP and DJNK are components of a MAP kinase signal transduction pathway.

DJNK is activated during the response to endotoxin

Mammalian JNK has been implicated in a variety of immunity-related signaling pathways, including the response to pro-inflammatory cytokines, the response to endotoxic LPS, and T-cell activation (Dériard et al. 1994; Kyriakis et al. 1994; Sluss et al. 1994; Su et al. 1994; Westwick et al. 1994; Raingeaud et al. 1995; Whitmarsh et al. 1995). Previous studies have demonstrated that insects and mammals employ homologous molecules to mediate immune responses (Engström 1993; Hoffmann et al. 1993; Hultmark 1993; Ip and Levine 1994). We therefore tested whether DJNK contributes to an immune response. Bacterial infection causes insect immune tissues to synthesize a spectrum of antibacterial peptides, including Cecropin and Diptericin, which lyse the invading microorganisms (Hultmark 1993). The response to bacterial infection can be studied by examining the effect of LPS, a component of the bacterial cell wall. Addition of LPS to *Drosophila* cultured cell lines, including *mbn-2* hemocytes and Schneider S2⁺ embryonic cells, causes marked induction of *cecropin* and *dip-*

Figure 6. Regulation of DJNK protein kinase activity. (A) Recombinant HEP was used to phosphorylate and activate DJNK in the presence and absence of the DJNK substrate DJun. (Lanes 1–3) Without the DJNK substrate GST-DJun; (lanes 4–6) with GST-DJun. The samples were examined by PAGE and autoradiography. The numbers on the left represent protein mass markers in kd. (B) Time course of DJNK protein kinase activation by LPS. The *mbn-2* hemocytes were treated with LPS (10 μ g/ml) for the time indicated. The cells were harvested and the endogenous DJNK protein kinase activity was measured in an immune complex kinase assay using a polyclonal antibody to JNK and the substrate GST-DJun. The numbers on the right represent protein mass standards in kd. (C) Treatment with LPS causes increased activity of epitope-tagged DJNK. S2⁺ cells were mock-transfected (lanes 1 and 2) or transfected with the Flag-DJNK expression vector (lanes 3 and 4). LPS (10 μ g/ml) was added to the cells for 15 min. (Top) DJNK protein kinase activity was measured in an immunocomplex kinase assay with the M2 monoclonal antibody (anti-Flag) with the substrate GST-DJun. (Bottom) The expression of Flag-DJNK was examined by protein immunoblot analysis of transfected S2⁺ cell lysates using the M2 antibody. The numbers on the right represent protein mass standards in kilodaltons.

tericin genes (Reichhart et al. 1992; Samakovlis et al. 1992; Engström et al. 1993; Kappler et al. 1993).

The effect of LPS treatment on DJNK protein kinase activity was examined. Hemocytes (*mbn-2* cells) were treated with LPS at a concentration (10 μ g/ml) that is optimal for the induction of the immunity genes *cecropin* and *diptericin* (Engström et al. 1993; Kappler et al. 1993). The cells were harvested after different times of incubation with LPS and the endogenous DJNK was immunopurified with a polyclonal antibody to human JNK. In vitro protein kinase assays were performed with the substrate DJun (Fig. 6B). DJNK was activated within 5

min of LPS addition. However, DJNK activation was transient and returned to basal levels after 1 hr (Fig. 6B). To confirm that the cloned DJNK protein kinase was activated by LPS, we expressed epitope-tagged DJNK (Flag-DJNK) in S2⁺ cells. The expression of Flag-DJNK was confirmed by Western blotting with the M2 monoclonal antibody to the Flag epitope (Fig. 6C). Immune complex kinase assays demonstrated that LPS activated the epitope-tagged DJNK in S2⁺ cells (Fig. 6C). Together, these data indicate that LPS causes rapid activation of DJNK, suggesting that DJNK targets an early event in the response to endotoxin. Mammalian JNK protein kinase activity and AP-1 transcriptional activity are increased during the immune response (Su et al. 1994). By analogy, we propose that the activation of DJun by DJNK in LPS-treated insect cells may lead to increased AP-1 transcriptional activity. Targets of *Drosophila* AP-1 may include the DJun promoter (Zhang et al. 1990; Wang and Goldstein 1994) and other genes related to an immune response (Engström 1993; Hoffmann et al. 1993; Hultmark 1993; Ip and Levine 1994). Genetic analysis of immune responses in *bsk* animals is, however, confounded by the embryonic lethality of the DJNK mutation. As the larval fat body cells grow by endoreplication, a method to achieve mitotic recombination in the fat body precursor cells is required. A goal of future studies will be to develop methods for the genetic analysis of DJNK function during immune responses.

Discussion

Previous studies of *Drosophila* have led to the identification of one MAP kinase, encoded by *rolled* (ERKA), that mediates receptor tyrosine kinase signaling, including the *torso*, *DER*, and *sevenless* pathways (Biggs et al. 1994; Brunner et al. 1994). Here, we report the characterization of a second MAP kinase in *Drosophila* (DJNK) that functions in an independent signal transduction pathway. The MAP kinase DJNK is a homolog of the mammalian c-Jun amino-terminal protein kinase and is encoded by the gene *bsk*. We show that DJNK is activated during the response to endotoxic LPS. In addition, we present biochemical and genetic evidence demonstrating that DJNK participates in dorsal closure, a morphogenetic process during mid-embryogenesis that involves epithelial cell movement. Embryos deficient in DJNK are unable to complete dorsal closure. Consequently, the internal organs are exposed. This defect may account for the embryonic lethality associated with *bsk*.

Role of DJNK in the insect immune response

Insects secrete protective peptides in response to bacterial infection. The genes for two peptides, Cecropin and Diptericin, that are induced in response to infection contain κ B-like motifs (Samakovlis et al. 1990; Reichhart et al. 1992; Engström 1993; Kappler et al. 1993). The NF- κ B-like protein Dif is implicated in insect immunity (Ip et al. 1993). DJNK is activated in response to treatment with LPS (Fig. 6B, C) at concentrations that induce the

protective peptide genes (Reichhart et al. 1992; Samakovlis et al. 1992; Engström et al. 1993; Kappler et al. 1993). Targets of LPS-activated DJNK during the insect immune response may include DJun (Fig. 6B, C) and components of the NF- κ B pathway (Hirano et al. 1996; Meyer et al. 1996). In mammalian cells, the AP-1 transcription factor (Ulevitch and Tobias 1994) and JNK (Rangaud et al. 1995) are activated by LPS. Similarly, mammalian JNK protein kinase activity and AP-1 transcriptional activity are activated during the immune response to antigen stimulation of T cells (Su et al. 1994). By analogy, we propose that the activation of DJun by DJNK in LPS-treated insect cells leads to increased AP-1 transcriptional activity. The activated *Drosophila* AP-1 may induce DJun expression (Zhang et al. 1990; Wang and Goldstein 1994) and other genes related to the insect immune response.

Role of DJNK in epithelial cell movement during embryogenesis

A superficially similar “dorsal open” phenotype is caused by mutations in at least three different groups of genes. These genes may alter dorsoventral patterning, formation of the amnioserosa, or the epidermal cell movement during dorsal closure. The first group of genes includes members of a TGF- β -like signaling pathway, including *decapentaplegic* (*dpp*), *schnurri*, *thick veins*, *saxophone*, and *punt* (Arora et al. 1995; Grieder et al. 1995; Letsou et al. 1995; Ruberte et al. 1995). Mutations in these genes can cause severe ventralization. The second group of genes affect the formation of the amnioserosa, but not dorsoventral patterning (Jurgens et al. 1984; Raiman et al. 1993; Winick et al. 1993). The *pannier* gene, which encodes a GATA-like transcription factor, is representative of this class. The dorsal open phenotype caused by the third group of genes is not associated with defects in the amnioserosa or altered dorsal epidermal cell fate. Instead, these genes affect the cell shape change and movement of the lateral epithelial cells over the dorsal surface of the embryo. The *hep* gene represents an example of this phenotype (Glise et al. 1995).

The *bsk* mutants do not exhibit defects in the dorsal epidermis or amnioserosa, but do cause a severe dorsal open phenotype. The *bsk* phenotype is therefore similar to *hep*. We postulate that *bsk*, like *hep*, is required for cell spreading and movement during dorsal closure. A similar phenotype is observed for recessive zygotic mutations in genes of the *dpp* signaling pathway (Arora et al. 1995; Grieder et al. 1995; Letsou et al. 1995; Ruberte et al. 1995). The *dpp* signaling pathway therefore has both early (dorsoventral patterning) and late (dorsal closure) functions (Affolter et al. 1994). The late functions of the *dpp* signaling pathway may be related to *hep* or *bsk*. However, it has been reported that *bsk* does not enhance the phenotype of a weak *dpp* allele (Arora et al. 1995), indicating that these genes may act independently. Further studies are required to test these hypotheses.

Dorsal closure is regulated by a MAP kinase signal transduction pathway

Evidence obtained from genetic and molecular analyses demonstrate that DJNK is a component of a MAP kinase pathway that regulates dorsal closure. First, the DJNK gene maps to the 31B-C region on the second chromosome. Defective dorsal closure correlates with the loss of DJNK expression caused by deficiencies in the 31B-C region (Table 1). In particular, two small deficiencies, *flp170B* and *flp147E*, link DJNK function and dorsal closure. Second, genetic evidence localizes the *bsk* mutation to this region (Nüsslein-Volhard et al. 1984). The dorsal closure phenotype of *bsk* is rescued by a DJNK transgene (Table 2). This observation represents direct genetic evidence that *bsk* is a mutant allele of DJNK.

Sequence analysis demonstrated the presence of a single amino acid substitution (G225E) in the DJNK gene isolated from *bsk1* flies (Fig. 4D). This point mutation is located in a region of JNK that confers substrate recognition (Kallunki et al. 1994; Sluss et al. 1994; Dai et al. 1995; Gupta et al. 1996). The substitution of a neutral residue (Gly) with a charged residue (Glu) in this region may change substrate recognition by DJNK and therefore the specificity of the kinase. It is also possible that the *bsk1* point mutation changes the interaction of DJNK with other molecules, such as a MAP kinase phosphatase. For example, the *Sevenmaker* gain-of-function mutation of *rolled* inhibits the interaction of MAP kinase phosphatase with ERKA (Bott et al. 1994).

Examination of the DJNK gene isolated from *bsk2* embryos demonstrated the presence of a single point mutation in codon 316 where AAG (Lys) is replaced by TAG (Stop). This mutation causes truncation of DJNK within kinase sub-domain XI (Fig. 4D). The truncated *bsk2* DJNK protein kinase is likely to be functionally defective. The mutant DJNK may fold improperly leading to poor stability or low activity. The presence of weak residual DJNK activity in *bsk2* embryos is consistent with the observation that the number of *bsk2* embryos showing a cuticle phenotype is less than *bsk1*.

The dorsal closure defect caused by loss-of-function mutations in DJNK (Figs. 4 and 5) and HEP (Glise et al. 1995), together with the observation that HEP phosphorylates and activates DJNK (Fig. 6A), provides evidence for a signal transduction pathway that is required for morphogenetic cell movement during embryogenesis. Targets of DJNK in the dorsal closure pathway may include the DJun transcription factor, which is expressed in the lateral epithelial cells of the embryo (Perkins et al. 1990; Zhang et al. 1990). In addition, DJNK may regulate, directly or indirectly, genes required for dorsal closure, including *l(1)myospheroid* (β -integrin), *zipper* (non-muscle myosin heavy chain), *coracle* (membrane skeleton protein), and *puckered* (Nüsslein-Volhard et al. 1984; MacKrell et al. 1988; Ring and Martinez Arias 1993; Young et al. 1993; Fehon et al. 1994). Additional components of the dorsal closure signaling pathway remain to be identified. However, correlative evidence suggests that a Rho-family GTPase and a PAK protein kinase par-

ticipate as upstream elements in this signaling pathway. Mammalian JNK is activated by Rho-family GTPases that regulate the organization of the actin cytoskeleton (Bagrodia et al. 1995; Coso et al. 1995; Minden et al. 1995; Vojtek and Cooper 1995). *Drosophila* Rho-family GTPases (Luo et al. 1994; Harden et al. 1995; Hariharan et al. 1995) could contribute to dorsal closure during elongation of the epithelial cells by causing cytoskeletal rearrangements and/or by activating DJNK. The effect of dominant-negative DRacA to cause defective dorsal closure provides strong evidence in favor of this hypothesis (Harden et al. 1995). The *Drosophila* PAK protein kinase, which is expressed in the lateral epithelial cells during dorsal closure, may mediate the effects of DRacA (Harden et al. 1996). Therefore, the dorsal closure signaling pathway may include a Rho-like GTPase, the protein kinase DPAK and the MAP kinase kinase HEP, leading to the activation of the MAP kinase DJNK.

Materials and methods

Fly strains and genetic analysis

The wild-type strain was *Canton S* (CS). The *Jammed*-derived deficiencies (Valoir et al. 1992; Clegg et al. 1993) were obtained from T. Grigliatti (University of British Columbia, Vancouver, Canada) and K. Beckingham (Rice University, Texas). The *flp147E* and *flp170B* lines were also obtained from K. Beckingham. These deletions were derived by excision of a P-element insertion in *flp*. Two *bsk* strains were obtained from Nüsslein-Volhard's laboratory (Max Planck Institute, Tübingen, Germany). These are stored under the names *bsk[IIJ04]* and *bsk[IIJ71]*, which correspond to *bsk1* and *bsk2*, respectively (Lindsley and Zimm 1992). Another *bsk1* allele was obtained from the Indiana University Stock Center. Sequence analysis demonstrated that the DJNK gene is identical in the two *bsk1* alleles. P-element mediated transformation (Rubin and Spradling 1983) was done using the helper plasmid turbo Δ2–3 (0.1 μg/μl) and the plasmid pCaSpeR-hsFlagDJNK (1 μg/μl) that was constructed by subcloning the Flag-DJNK cDNA into the *Bam*HI and *Not*I sites of pCaSpeR-hs (Thummell and Pirrota 1992). The *yw*⁶⁷ fly used for transformation was made isogenic and does not contain any lethal mutations in the X, second, or third chromosomes. Nine transgenic lines were obtained and balanced with the line *yw; BcElp/CyO; Ki/TM6y*⁺. The chromosomal location of the transgenes was determined and lines with *hsDJNK* located on the X or third chromosome were crossed with flies carrying the *bsk* or *flp*-derived chromosomes. The *hsDJNK* males were crossed with double balancer females and the F₁ +/*CyO*; *hsDJNK/TM6y*⁺ females were collected. The *bsk* and *flp170B* male flies were crossed with double balancer females, and the F₁ *bsk/Elp*; +/*TM6y*⁺ males collected. The F₂ flies from these two crosses were mated. The F₂ generated both female and male flies of the genotype *bsk/CyO*; *hsDJNK/TM6y*⁺. These were collected and mated to establish stocks.

Hybridization analysis and cuticle preparation

Embryos were dechorionated in 100% bleach, fixed, and hybridized at 55°C for 18 hr (Tautz and Pfeifle 1989) to DJNK riboprobes prepared by *in vitro* transcription using digoxigenin-labeled uridine-5' triphosphate (UTP). The probe was detected with an anti-digoxigenin antibody conjugated to alkaline phos-

phatase (Boehringer-Mannheim). Polytene salivary gland chromosomes were hybridized (16 hr) with a digoxigenin-labeled DJNK probe at 37°C. The probe was detected with a peroxidase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim). Cuticle preparations were done by hand rolling the embryos to remove the chorion membrane, treatment with 85% lactic acid, and incubation at 65°C (1 hr). The embryo staining, chromosome staining, and cuticles were examined with a Zeiss Axio-phot Microscope. Photographs were taken using differential interference contrast optics (embryos and cuticles) and phase-contrast optics (chromosomes).

Molecular cloning of DJNK

PCR was performed using the degenerate oligonucleotides TCITC[G/A]TAIGGIGTCATC AT[G/A]AA and CCITT[T/C]CA [A/G]AA[C/T]CA[A/G]ACICA as primers (I = inosine). An embryonic (0–4 hr) cDNA library was used as the template (Brown and Kafatos 1988). The annealing temperature was 42°C and 35 cycles were performed. The PCR product (200 bp) was subcloned into the Bluescript vector (Stratagene, Inc.) and sequenced. Full-length DJNK cDNAs were isolated by screening the 0–4 hr embryonic cDNA library with the PCR fragment as a probe. Five independent clones were obtained, four of which were full length. Genomic DJNK clones were isolated by screening an EMBL4 genomic library (Ip et al. 1993) with the DJNK cDNA. Restriction mapping and Southern analysis demonstrated that a 9-kb *Bam*HI fragment contained all the DJNK-coding region. This *Bam*HI fragment was subcloned in the Bluescript vector (Stratagene, Inc.) and sequenced using oligonucleotide primers and a model 373A machine (Applied Biosystems, Inc.).

Analysis of mutations in the DJNK gene

The embryos obtained by crossing heterozygous *bsk* flies were collected, aged for 24–36 hr, and dechorionated with bleach. Genomic DNA (2–4 μg) from 400 *bsk* embryos was isolated and used to amplify the DJNK genomic locus by PCR. The primer sequences were: BASK1, TACCCTCTTGAAAAGGCCGAT-ATGCTCCTT; BASK2, TTTAGAGTGCGAGTC GGCCTTTTCAACTATA; BASK3, ACGTACAGTATGAATACATCTTTAAAATG; and BASK4, TCTTGTGACTGACTAAGGAGGAAAAGTCG. The BASK1 and BASK4 primers were employed to amplify the DJNK genomic region. The annealing temperature was 60°C and 30 cycles were performed using *Pfu* polymerase (Stratagene, Inc.). The PCR product was re-amplified with the primers BASK1 plus BASK2, or BASK3 plus BASK4. The PCR products were cloned and sequenced with a model 373A machine (Applied Biosystems, Inc.). The sequence of the DJNK gene obtained from four independent PCR reactions was identical.

Expression and purification of recombinant proteins

The DJNK cDNA was sub-cloned into the pGSTag vector (Dressler and Ron 1992) at the *Bam*HI and *Sac*I sites. An expression vector for GST–DJun (residues 1–104) was constructed by sub-cloning a PCR fragment of the DJun gene into the *Bam*HI and *Eco*RI sites of pGEX-3X (Pharmacia-LKB Biotechnology, Inc.). A vector for GST–HEP was constructed by sub-cloning a HEP cDNA (obtained by reverse-transcriptase PCR amplification of poly(A)⁺ RNA obtained from 0- to 12-hr *Drosophila* embryos) into the *Bam*HI and *Eco*RI sites of pGEX-5X-1 (Pharmacia-LKB Biotechnology, Inc.). The plasmids were se-

quenced with an Applied Biosystems model 373A machine. The fusion proteins were purified by affinity chromatography [Smith and Johnson 1988].

In vitro protein kinase assays

Recombinant DJNK (1 μ g), HEP (1 μ g) and DJun (2 μ g) were incubated in 20 μ l of kinase buffer [20 mM HEPES (pH 7.6), 20 mM MgCl₂, 2 mM dithiothreitol, 25 mM β -glycerophosphate, 0.1 mM Na orthovanadate] with 20 μ M [γ -³²P]ATP (10 Ci/mmol). The phosphorylation reaction was terminated after 10 min and examined by polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Characterization of DJNK in insect cells

The *mbn-2* cells were maintained in 1 $\times$ Drosophila Schneider Medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Inc.). LPS (*Escherichia coli* 055:B5, Sigma) was added (10 μ g/ml), the cells incubated for various times at 23°C and harvested in Triton lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM Na pyrophosphate, 25 mM β -glycerophosphate, 1 mM Na orthovanadate, leupeptin (10 μ g/ml), and 1 mM phenylmethylsulfonyl fluoride]. Protein concentration was measured using the BCA assay kit (Pierce Chemical Co.). Endogenous DJNK was purified by immunoprecipitation of cell extracts (500 μ g) with 5 μ l of a rabbit antibody to JNK prebound to protein A-Agarose [Sluss et al. 1994]. The immunoprecipitates were washed four times with HEPES-binding buffer [HB; 20 mM HEPES (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 25 mM MgCl₂, and 0.05% Triton X-100] and once with kinase buffer. The immune complex was resuspended in 25 μ l of kinase buffer with 20 μ M [γ -³²P]ATP (10 Ci/mmol) and 5 μ g of substrate protein (GST-DJun). The reaction was terminated after 10 min and the products were examined by PAGE and autoradiography.

Expression of DJNK in insect cells

Epitope-tagged DJNK was created by PCR using primers containing 5' *Bam*HI and 3' *Sac*I restriction sites. The Flag sequence (Immunex Corp.) was inserted between codons 1 and 2 of DJNK. The PCR product was subcloned in the *Bam*HI and *Sac*I sites of the pAcPA vector, which contains the Actin 5C promoter. Schneider S2* cells were maintained and transfected with the pAcPA expression vector, as described [Han and Manley 1993]. The cells were harvested 24 hr post-transfection in Triton lysis buffer. An aliquot was removed for Western blot analysis using the M2 (anti-Flag) monoclonal antibody (IBI-Kodak). The remainder of the lysate was incubated with the M2 antibody prebound to protein G-Agarose. The immune complexes were washed with HB buffer and kinase buffer. Immune complex kinase assays were done with the substrate GST-DJun.

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Note

The sequence of the DJNK cDNA, DJNK gene, *bsk1* gene, and *bsk2* gene have been deposited in the GenBank data library under the accession nos. U49180, U49249, U50965, and U50966, respectively.

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