The *Drosophila* Ste20-related kinase misshapen is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway

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Dorsal closure in the *Drosophila* embryo occurs during the later stages of embryogenesis and involves changes in cell shape leading to the juxtaposition and subsequent adherence of the lateral epidermal primordia over the amnioserosa. Dorsal closure requires the activation of a conserved c-jun amino-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) module, as it is blocked by null mutations in JNK kinase ([hemipterous (hep)] and JNK [basket (bsk)]. *Drosophila* JNK (DJNK) functions by phosphorylating and activating DJun, which in turn induces the transcription of decapentaplegic (dpp). We provide biochemical and genetic evidence that a Ste20-related kinase, misshapen (msn), functions upstream of hep and bsk to stimulate dorsal closure in the *Drosophila* embryo. Mammalian (NCK-interacting kinase [NIK]) and Caenorhabditis elegans (mig-15) homologs of msn have been identified; mig-15 is necessary for several developmental processes in *C. elegans*. These data suggest that msn, mig-15, and NIK are components of a signaling pathway that is conserved among flies, worms, and mammals to control developmentally regulated pathways.

[Key Words: *Drosophila*; Ste20 kinase; NIK; misshapen; dorsal closure; JNK]

Received December 23, 1997; revised version accepted June 2, 1998.

Multiple mitogen-activated protein kinase (MAPK) modules have been identified as components of signal transmission pathways, including at least six in yeast and three in mammalian cells (Herskowitz 1995; Kyriakis and Avruch 1996). All MAPK modules are composed of three sequentially acting protein kinases: a MAP kinase, an enzyme that activates MAP kinase [known as MAP kinase kinase (MKK), MAP extracellular signal-related kinase (ERK) kinase (MEK), or Ste7-related kinase], and an enzyme that activates MKK [known as a MAP kinase kinase (M KK), MAP ERK kinase kinase (MEKK), or Ste11-related kinase] (Davis 1994; Cobb and Goldsmith 1995; Herskowitz 1995). The defining characteristic of these MAP kinase modules is the MAP kinase itself, and in mammalian cells the MAP kinases described thus far include the p42 and p44 MAP kinases (also known as ERK1 and ERK2), c-jun amino-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and p38. In contrast to p42 and p44 MAP kinases, which are frequently downstream effectors of Ras and are central elements mediating cell proliferation by a variety of growth factors, JNK and p38 are activated most potently by cellular stresses and inflammatory cytokines (Kyriakis and Avruch 1996).

Genetic epistasis analysis in yeast as well as studies in mammalian cells have indicated that Ste20-related kinases function upstream of MKKs to regulate the JNK MAPK module, and Ste20 kinases have been considered to be MAP kinase kinase kinase kinases (M KK K) (Herskowitz 1995; Hu et al. 1996; Pombo et al. 1995; Su et al. 1997). Two families of protein kinases, which are closely related to Ste20 in their kinase domain, have been identified based on their structure and regulation. The first family includes the yeast Ste20 protein kinase and the mammalian and *Drosophila* p21-activated protein kinases (PAKs) (Man ser et al. 1994; Martin et al. 1995; Harden et al. 1996). Kinases in this group contain a conserved p21Rac and Cdc42-binding domain in their amino terminus (Burbelo et al. 1995; Martin et al. 1995) and are activated by binding GTP-bound Cdc42 and Rac (Man ser et al. 1994; Bagrodia et al. 1995; Herskowitz 1995; Martin et al. 1995). The second family lacks...
Because of the difficulty in studying Ste20 kinases in the understanding of the Ras–Raf–MAP kinase signaling pathway. Organisms proved invaluable in furthering our understanding, especially helpful in unraveling complex signaling pathways (Dickson and Hafen 1994). For example, studies of these kinases expressed at very high levels and therefore, studies may not be conclusive because JNK activation might be regulated by the photoreceptor transcription factor Glass. Its name derives from the finding that functions of these kinases. Recently, we have identified a Drosophila homolog of NIK (a member of the mammalian SPS1 family of Ste20 kinases), called misshapen (msn). Msn was identified in a screen for genes that are essential for NIK to fully activate JNK in mammalian cells (Su et al. 1997). To determine whether msn activates JNK, 293 cells were transfected with msn together with an epitope-tagged JNK, and kinase activity assays were performed on JNK precipitates. Overexpression of either msn or NIK led to about a four- to fivefold increase in JNK kinase activity as assessed by in vitro kinase reaction (Fig. 1B). In agreement with previous studies using NIK, a mutation abolishing msn's ability to activate JNK. The ability of msn to activate JNK was confirmed by examining its effect on an activated transcription factor 2 (ATF2)-stimulated luciferase reporter gene; JNK has been shown to phosphorylate and activate ATF2 (Gupta et al. 1995). Overexpression of msn in 293 cells led to about a 10-fold increase in the transcriptional activity of ATF2 (Fig. 1C). These findings indicate that msn and NIK are both structurally and functionally similar and suggests that msn may function to activate JNK in Drosophila.

Results

msn is the Drosophila homolog of NIK

The Drosophila msn and C. elegans mig-15 proteins are highly homologous to mammalian NIK, an activator of the JNK module (Fig. 1A). These three proteins share the same overall structure, containing an amino-terminal kinase domain and a carboxy-terminal putative regulatory domain. Moreover, these three proteins are highly conserved within both the kinase domain and the carboxy-terminal regulatory domain. The high conservation within these two domains suggested that msn and mig-15 would activate the JNK MAPK module, because previously we have shown that both of these domains are essential for NIK to fully activate JNK in mammalian cells (Su et al. 1997). To determine whether msn activates JNK, 293 cells were transfected with msn together with an epitope-tagged JNK, and kinase activity assays were performed on JNK precipitates. Overexpression of either msn or NIK led to about a four- to fivefold increase in JNK kinase activity as assessed by in vitro kinase reaction (Fig. 1B). In agreement with previous studies using NIK, a mutation abolishing msn's ability to activate JNK. The ability of msn to activate JNK was confirmed by examining its effect on an activated transcription factor 2 (ATF2)-stimulated luciferase reporter gene; JNK has been shown to phosphorylate and activate ATF2 (Gupta et al. 1995). Overexpression of msn in 293 cells led to about a 10-fold increase in the transcriptional activity of ATF2 (Fig. 1C). These findings indicate that msn and NIK are both structurally and functionally similar and suggests that msn may function to activate JNK in Drosophila.

Mutations in msn impair dorsal closure in the Drosophila embryo

Drosophila embryos mutant for JNK (bsk), JNK kinase
(hep), or Djin display a dorsal open phenotype, indicating that activation of this pathway is essential for normal embryonic development (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996; Hou et al. 1997; Kockel et al. 1997). To test whether msn activates the JNK MAPK module in Drosophila, we determined whether embryos zygotically mutant for msn also display a dorsal open phenotype. Two inversion alleles of msn, msn\textsuperscript{102} and msn\textsuperscript{172}, were used in this analysis (Treisman et al. 1997). Embryos homozygous for either msn allele or transheterozygous for the two alleles display a defect in dorsal closure, resembling embryos zygotically mutant for bsk (Fig. 2). The observed defect in dorsal closure was observed at a frequency of ∼15%, which is similar to that found for bsk\textsuperscript{1} (data not shown). Expression of a msn cDNA in the epidermis rescues the dorsal closure defect in msn mutant embryos, allowing survival of all homozygotes to the pupal stage; this demonstrates that the phenotype is attributable to loss of msn function. GAL4 driven by the ectoderm-specific
promoter at 69B (Brand and Perrimon 1993) was used to direct the expression of UAS-msn in msn mutant embryos. msn mutant pupae that are rescued because of ectopic expression of msn are longer as the result of the lack of the dominant Tubby marker on the balancer. We found that 47 of 126 pupae obtained from a cross between UAS-msn; msn102/+ and msn102/TM6B and 69B–GAL4, msn102/TM6B were longer; the percentage of long pupae (36%) is very close to the 33% that is predicted if ectopic expression of msn rescued msn homozygous embryos.

msn regulates dpp expression in the leading edge cells surrounding the amnioserosa

The role of DJNK in dorsal closure is to phosphorylate and activate Djun, resulting in transcriptional activation of the dpp gene at the leading edge of the dorsal epidermis (Glise and Noselli 1997; Hou et al. 1997; Riesgo-Escovar and Hafen 1997a). Binding of dpp to its receptors thick veins (tkv) and punk (put) on the ventrally adjacent epithelial cells in turn induces reorganization of the cytoskeleton, leading to epithelial cell elongation and subsequent closure over the amnioserosa (Glise and Noselli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997a). This is supported by the findings that dpp expression is decreased in the dorsal-most epithelial cells in embryos lacking bsk and hep, and that expression of activated forms of Djun or tkv rescues embryos zygotically mutant for bsk or Djun (Glise and Noselli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997a). Therefore, if msn functions to initiate dorsal closure by activating DJNK, dpp expression in the leading edge epithelial cells should be decreased in msn mutant embryos. In agreement with the idea that msn functions upstream of bsk, we observed that dpp expression in leading edge cells surrounding the amnioserosa was decreased in embryos lacking msn to a comparable degree to bsk1 embryos (Fig. 3; data not shown). We found that ∼20% of embryos derived from the msn102/+; msn102/+cross displayed a decrease in dpp expression in the dorsal leading edge. The decrease in dpp staining in msn mutant embryos is limited to the dorsal rim cells; dpp expression in the visceral mesoderm and lateral ectoderm is normal in these embryos and serves as a control for dpp staining (Fig. 3).

Figure 3. dpp expression in embryos lacking msn. Embryos (5 to 11 hr old) from a cross between msn102/+ and msn102/+ or between msn102/+ and +/+ flies were hybridized with a dpp antisense probe. About 20% of embryos derived from the msn102/+; msn102/+cross displayed a decrease in dpp expression in cells at the dorsal leading edge (arrow).

Genetic evidence that msn functions upstream of the JNK MAP kinase pathway in Drosophila

The above findings suggest that msn and bsk act in the same pathway and that msn functions upstream of DJNK activation. Further evidence that msn and bsk function in the same pathway comes from the observation that some embryos doubly heterozygous for msn and bsk displayed a dorsal open phenotype. We found that ∼10% of embryos derived from a cross between msn/+ and bsk/+; flies exhibited a dorsal open phenotype (Fig. 4; Table 1). The defect in dorsal closure in embryos doubly heterozygous for msn and bsk is not a dominant effect of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of either gene; a defect in dorsal closure was very rarely observed when msn/+ or bsk/+; flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway.
number of embryos with a dorsal open phenotype from ~50% (36 of 76) to <10% (6 of 65). In addition, we found that expression of an activated form of tkv, tkvQ253D (Nellen et al. 1996), also rescued the dorsal open phenotype in msn mutant embryos. GAL4 driven by the ectoderm-specific promoter at 69B (Brand and Perrimon 1993) was used to direct the expression of UAS–tkvQ253D in msn mutant embryos. This expression of activated tkv rescued partially the dorsal open phenotype caused by msn; it also had a dorsalizing effect on the ventral ectoderm of the embryos related to the earlier function of dpp in establishing the dorsoventral axis, which served to mark embryos expressing activated tkv (Fig. 5). Thus, these findings provide genetic evidence that msn functions upstream of the JNK MAP kinase module in leading edge cells.

GTP–Rac is required for msn and NIK to fully activate JNK

Rac activation is thought to be important for stimulating dorsal closure because expression of dominant negative forms of Rac (DN Rac) or Cdc42 inhibit dorsal closure in the Drosophila embryo (Harden et al. 1995; Riesgo-Escobar et al. 1996). The finding that activated Djun rescues the defect in dorsal closure induced by expression of DN Rac indicates that Rac probably functions upstream of JNK activation to stimulate dorsal closure (Hou et al. 1997). To begin to address the mechanism whereby Rac and msn cooperate to activate JNK, 293 cells were transfected with msn or NIK together with DN Rac and an epitope-tagged JNK, and kinase activity assays were performed on JNK precipitates. Although overexpression of NIK or msn led to a four- to fivefold increase in JNK activation, coexpression of DN Rac decreased markedly JNK activation (Fig. 6).

The PAK family of Ste20 kinases contain a conserved p21Rac and Cdc42-binding domain and are activated by

### Table 1. Embryos with defects in dorsal closure

| Cross          | No./total no. (%) |
|----------------|-------------------|
| +/+ × msn102/+ | 1/143 (0.7)       |
| +/+ × bsk1/+   | 1/108 (0.9)       |
| bsk1/+ × msn102/+ | 15/137 (10.9)   |
| bsk2/+ × msn102/+ | 20/196 (10.1)   |
| flp147/+ × msn102/+ | 39/388 (10.1) |
| hep1/+ × msn102/+ | 31/88 (35.2)    |
| hep75/+ × msn103/+ | 22/61 (36.0)    |

Embryos were collected and aged for 24 hr. Cuticles were prepared from unhatched embryos, and the number of embryos with an aberrant dorsal cuticle was determined. The total number of hatched and unhatched embryos was determined by resuspending all of the larvae and embryos in Hoyer’s solution and counting under a microscope.
binding GTP-bound Cdc42 and Rac (Burbelo et al. 1995; Martin et al. 1995). To determine whether Rac may regulate directly msn or NIK in a similar manner to the way it activates PAK, we assessed whether msn or NIK bound activated Rac in vitro. Although msn and NIK do not contain a Rac-binding domain, other proteins without a Rac-binding motif have been found recently to bind Rac (Fanger et al. 1997). We were unable to detect binding of activated Rac to either msn or NIK using an overlay assay with GTP-bound Rac or by using the yeast two hybrid system, whereas binding to a positive control, mammalian PAK1, or Drosophila p21-activated protein kinase (DAPK), was easily detected (data not shown). These findings suggest that although Rac cooperates with both NIK and msn to activate JNK, Rac is unlikely to activate directly msn or NIK.

**Discussion**

At least two distinct MAPK modules have now been identified in Drosophila. Genetic evidence has established that these pathways function independently and are essential for the regulation of distinct developmentally regulated processes (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). The first MAPK module to be described is activated by several receptor tyrosine kinases including the sevenless, Drosophila epidermal growth factor (DEGF), and torso receptors (for review, see Dickson and Hafen 1994; Perrimon 1994). These receptors signal through DRas to activate DRaf (MKKK), which in turn phosphorylates and activates D Sor1 (MKK), which phosphorylates and activates the Drosophila MAP kinase rolled (DERK). Activation of this MAPK module is essential for the sevenless receptor to specify the fate of R7 photoreceptors, the DEGF receptor to specify embryonic dorsoventral polarity or the development of other photoreceptors, and torso to specify embryonic termini. The second MAPK module to be described in Drosophila is the JNK pathway (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). Activation of this pathway stimulates changes in the cytoskeleton that enable lateral ectodermal cells to spread over the amnioserosa and join in the dorsal midline. Failure to activate the JNK MAP kinase module leads to a dorsal open phenotype and embryonic lethality. The JNK module is also activated during the Drosophila immune response to lipopolysaccharide (LPS) (Sluss et al. 1996). Unlike the Ras–Raf–MAPK module for which the upstream signals are well known, very little is known about the receptors or signaling molecules that mediate activation of the JNK MAP module in either Drosophila or mammalian cells under normal biological conditions. Our findings indicate that the Ste20-related kinase msn functions upstream of the JNK MAP module and is essential for initiating changes in cell shape that regulate dorsal closure of the Drosophila embryo. We found that msn mutant embryos exhibit a defect in dorsal closure that is similar to the defect in embryos lacking components of the JNK pathway. Furthermore, the finding that flies doubly heterozygous for msn and either bsk or hep display a defect in dorsal closure, coupled with genetic epistasis analysis showing that activated D Jun and tkv at least partially rescue the defect in dorsal closure in msn mutant embryos, indicates that msn and bsk function in the same pathway and that msn functions upstream of bsk (JNK) activation.

Although several members of the SPS family of Ste20-related kinases have been shown to be specific and potent activators of the JNK pathway when overexpressed transiently in mammalian cells (Pombo et al. 1995; Hu et al. 1996; Su et al. 1997), little is known about the function or regulation of these kinases under normal physiological circumstances. Our finding that msn functions upstream of the JNK MAP kinase module in a genetically defined system indicates that kinases in this family are important activators of JNK under physiologically relevant conditions. The data put to rest the idea that activation of the JNK MAPK module observed in transient transfection assays by SPS1 Ste20 family members is an experimental artifact related to misexpressing these proteins at high levels.

As a result of several recent studies, a number of signaling molecules that are critical for stimulating dorsal closure can now be ordered on a signaling pathway. Genetic epistasis analysis in yeast, as well as studies in mammalian cells, lead us to predict that msn functions as a MKKK in Drosophila and activates the JNK pathway by activating a yet to be defined Drosophila MKKK (Fig. 7). Therefore, msn becomes the most proximal molecule identified so far on this signaling pathway. Studies over the past several years have clarified which signaling molecules acting downstream of this putative Drosophila MKKK are important in stimulating dorsal closure. A Drosophila MKKK would be likely to phosphorylate and activate the JNK kinase hep, which in turn phosphorylates and activates JNK (bsk) (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). D JNK mediates its changes in the cytoskeleton primarily by regulating the expression of target genes; DJNK phosphorylates and activates DJun, which in turn cooperates with D Fos to stimulate transcription of dpp, a member of the transforming growth factor-β (TGF-β) family (Glise and
Figure 7. Schematic diagram showing the function of msn, NIK, and mig-15 (see text for details). mig-15 has not yet been shown to activate C. elegans JNK (SAK1) or JNK kinase (SEK). In addition, neither SAK nor SEK have yet been shown to function upstream of Q-neuroblast migration or muscle arm targeting in C. elegans.

Noselli 1997; Hou et al. 1997; Riesgo-Escovar and Hafen 1997a,b; Noselli 1998). Signaling of dpp through its receptors tkv and put then stimulates changes in cell shape, thereby enabling the lateral epidermal cells to stretch over and subsequently close over the amnioserosa dorsally (Affolter et al. 1994; Brummel et al. 1994; Terracol and Lengyel 1994; Letsou et al. 1995; Ruperte et al. 1995).

Currently very little is known about the downstream MKKKs/MEKKs that are activated by Ste20 kinases or the mechanisms whereby these MKKKs are activated. The identification of a large number of kinases in mammalian cells that are capable of functioning as MKKKs, coupled with the finding that many of these kinases are constitutively active when overexpressed in cells, has hindered the identification of the specific downstream targets of Ste20 kinases or the mechanisms by which they activate MKKKs (Kyriakis and Avruch 1996). Recent evidence has suggested that direct interaction of Ste20 kinases with specific downstream MKKKs may be a critical component of their regulation (Hu et al. 1996; Su et al. 1997). We have found that the carboxy-terminal domain of NIK, which is highly conserved between NIK, msn, and mig-15, is critical for NIK to activate the JNK pathway and to associate with MEKK1 in cells (Su et al. 1997).

Our findings also indicate that Rac cooperates with SPS1 family Ste20 kinases to mediate activation of JNK. Previous studies have suggested that Rac functions upstream of the DJNK MAPK module in Drosophila; expression of DN Rac or Cdc42 results in a dorsal open phenotype (Harden et al. 1995; Riesgo-Escovar et al. 1996) and the defect in dorsal closure induced by expression of DN Rac can be reversed by expressing an activated form of Djun (Hou et al. 1997). Because PAK family Ste20 kinases are activated by GTP-bound Cdc42 and Rac, it had been assumed that this family of Ste20 kinases rather than an SPS1 Ste20 kinase family member would cooperate with Rac to activate JNK (Noselli 1998). Thus, these findings have led us to consider new paradigms for how Rac functions to activate JNK. We do not think that Rac activates msn directly. Unlike PAK family members, msn does not contain a consensus Rac-binding motif and we did not detect binding of msn to activated Rac in vitro (data not shown). Rather, we favor the hypothesis that Rac cooperates with msn to activate a downstream MKKK. MKKKs of the mixed lineage kinase family as well as MEKK1 and MEKK4 have been shown to bind GTP-bound Cdc42 or Rac (Teramoto et al. 1997; Fanger et al. 1997). Thus, Rac may cooperate with msn to regulate a downstream MKKK in a manner similar to the way Ras cooperates with a yet to be defined kinase to activate RAF. In this model, binding of a MKKK to activated Rac would facilitate interaction of this MKKK with msn, thereby enabling its activation by msn (Fig. 7). However, we cannot exclude the possibility that Rac and msn activate parallel pathways converging on JNK activation.

It is intriguing that the C. elegans homolog of msn, mig-15, is also an essential gene in development and, like msn, functions to regulate processes that undoubtedly require changes in the cytoskeleton and cell shape in developing worms (E. Hedgecock, pers. comm.). mig-15 mutants have a variety of developmental defects including defects in Q-neuroblast migration and muscle arm targeting. Although it is not yet clear whether any or all of the phenotypes apparent in worms lacking mig-15 are attributable to defective activation of the C. elegans JNK, these findings suggest a common theme in which JNK activation plays a central role in a variety of developmental processes by coordinating changes in cell shape and the cytoskeleton (Fig. 7). It is likely, however, that some of the phenotypes observed in embryos lacking these Ste20 kinases are independent of their effect on JNK activation. In addition to defects in dorsal closure, some embryos mutant for msn displayed a ventral defect (data not shown). Moreover, although msn, like bsk and Djun, is not required for specifying the fate of photoreceptor cells, clones of msn mutant photoreceptor cells display an abnormal shape (Riesgo-Escovar et al. 1996; Hou et al. 1997; Treisman et al. 1997). These defects are never observed in embryos mutant for bsks and therefore indicate that msn has other essential functions that are independent of JNK activation (Riesgo-Escovar et al. 1996). We were unable to evaluate the maternal contribution of msn to dorsal closure because of its requirement for oogenesis (Treisman et al. 1997).

Our findings also support the idea that the regulation of Ste20 kinases in mammalian cells is likely to be more complex than previously recognized. Several mammalian Ste20 kinases related to msn and NIK, which specifically activate the JNK pathway, such as GC kinase and HPK1, have been identified (Pombo et al. 1995; Hu et al. 1996; Su et al. 1997). It has not been clear whether the
function served by these kinases is redundant or whether each may function only under specific circumstances. Although the full repertoire of Ste20 kinases in Drosophila is not known, our results support the idea that members of this family are subject to different modes of regulation and, for at least some functions, are not redundant with other family members. Studying msn and mig-15 in defined genetic systems will be a critical tool in the effort to unravel these complex pathways in mammalian cells.

The placement of msn, mig-15, and NIK on a MAP kinase pathway that is likely to be conserved between worms, flies, and mammals is reminiscent of the better studied Ras–Raf–MAPK module. Genetic analysis of this MAP kinase pathway in both Drosophila and C. elegans was critical in eventually elucidating key components of this pathway. Despite the large number of Ste20 kinases that have now been identified in mammalian cells, virtually nothing is known about either the upstream regulation of this family of kinases or their downstream targets. Thus, the identification of other components of this pathway in Drosophila and C. elegans will undoubtedly provide valuable insights into the signals that activate Ste20 kinases and their subsequent effects on cells.

Materials and methods

Constructs, mutagenesis, and JNK assays

All constructs for tissue culture were myc-epitope tagged in their carboxyl terminus and subcloned into the vector pRK5 (Marcusohn et al. 1995; Su et al. 1997). To assess JNK activation, 2 µg of NIK, msn, or vector control were transfected into 293 cells together with 2 µg of GST-tagged JNK. GST-JNK was precipitated from 500 µg of cell lysates using glutathione agarose and subjected to an in vitro kinase assay using GST-ΔJun (Su et al. 1997). Reaction products were separated by SDS-PAGE and visualized by autoradiography. To make a kinase inactive form of msn, the msn cDNA was subcloned into the vector pALTER (Promega) and site directed mutagenesis was performed according to the manufacturer’s specifications. The kinase inactive msn contained a substitution of N for D at position 160 (msn D160N). Transactivation of ATF2 was assessed by cotransfecting a fusion protein consisting of ATF2 (amino acids 1-250) and the GAL4-DNA-binding domain together with a vector control or msn and a reporter plasmid containing 5x GAL4-DNA-binding domains. All transfections were standardized by cotransfecting a control plasmid expressing β-galactosidase (Promega). Dominant negative N17 Rac was expressed using the vector RKS as described (Marcusohn et al. 1995).

Fly strains and genetic analysis

msn102 and msn172 are x-ray-induced inversions with breakpoints within the msn gene (Treisman et al. 1997). Both inversions break within the coding region, although msn102 causes a larger disruption of the msn gene. The Df(2L)flp 147E line, a deletion of part of the bsk-coding region, was obtained from K. Beckingham (Rice University, Houston, TX). The bsk1 and bsk2 lines were obtained from the Nusslein-Volhard laboratory by way of T. Ip (University of Massachusetts Medical School, Worcester, MA). The hep1 and hep2 lines were obtained from S. Noselli [Centre de Biologie du Development, Centre National de la Recherche Scientifique (CNRS), Toulouse, France]. The activated Djun line has been described previously (Tréier et al. 1995).

Genetic interactions

To test for genetic interaction between msn and bsk, or between msn and hep, embryos were collected from a cross between msn/+ flies with either hsr, bsk1, or hep1/+ flies. Embryos were collected for 6 hr and aged for an additional 16 hr and the total number of embryos with a dorsal open phenotype was determined.

To test whether activated Jun (hs-SEJunAsp) rescues msn mutant embryos, activated Djun was introduced into the msn102 mutant background using the compound balancer SM6.TM6B to generate msn102; hs-SEJunAsp/SM6.TM6B. To induce expression of hs-SEJunAsp, 5- to 7-hr-old embryos were heat-shocked for 45 min. Cuticle preparations were then performed 16 hr after heat shock.

To target expression of activated tkv, the activated type 1 dpp receptor, to the dorsal ectoderm we used the Gal4/UAS system (Brand and Perrimon 1993). A UAS–tkvQ253D transgene on the third chromosome was recombined with msn102 to generate msn102; UAS–tkvQ253D/TM6B (Hoodless et al. 1996). To induce ectodermal expression of tkvQ253D, msn102; UAS–tkvQ253D/TM6B flies were crossed to 69B–GAL4, msn102/TM6B.

Ectopic expression of msn

The Gal4/UAS system was also used to test whether ectopic expression of msn rescues the dorsal defect in msn mutant embryos. The msn cDNA was cloned into the vector pUAST (Brand and Perrimon 1993). Germ-line transformations were then performed using standard methods following injection of pUAS–msn with a helper plasmid turbo ß2-3 (Spradling and Rubin 1982). Transgenic lines containing UAS–msn on the second chromosome were crossed to msn102 to generate UAS–msn; msn102/SM6.TM6B. To induce ectodermal expression of msn, UAS–msn; msn102/SM6.TM6B were crossed to 69B–GAL4, msn102/TM6B.

Cuticle preparations

Embryos were collected on yeasted agar plates, dechorionated in 100% bleach, rinsed with water, and then fixed for 10 min at 65°C in a solution containing acetic acid and glycerol at a 3:1 ratio. Embryos were then mounted in Hoyer’s medium and incubated for 24 hr at 65°C.

In situ hybridization

In situ hybridization to embryos was performed using digoxigenin-labeled dpp RNA probe according to the method of Tautz and Pfeifle (1989) as modified by Ronchi et al. (1993).

Acknowledgments

We thank Tony Ip of The University of Massachusetts Medical School for helpful discussions and for kindly providing the dpp cDNA and the bsk1 and bsk2 stocks. We thank S. Noselli for hep fly stocks and Gary Struhl for UAS–tkvQ253D. We thank X. Steven Hou and Norbert Perrimon of Harvard Medical School for helpful discussions and hsSEJunAsp fly stocks, and Kate Beckingham of Rice University for providing the Df(2L)flp 147E
stocks. We thank Hongzhi Liu for performing the yeast two-hybrid analysis of activated Drac and msn. The manuscript was improved by the critical comments of Corinne Zaffran. This work was supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and American Diabetes Association to E.Y.S.

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*Genes Dev.* 1998, 12:
Access the most recent version at doi:10.1101/gad.12.15.2371