The \textit{Drosophila} STE20-like kinase Misshapen is required downstream of the Frizzled receptor in planar polarity signaling

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The \textit{Drosophila} misshapen (\textit{msn}) gene is a member of the STE20 kinase family. We show that \textit{msn} acts in the Frizzled (\textit{Fz}) mediated epithelial planar polarity (EPP) signaling pathway in eyes and wings. Both \textit{msn} loss- and gain-of-function result in defective ommatidial polarity and wing hair formation. Genetic and biochemical analyses indicate that \textit{msn} acts downstream of \textit{fz} and dishevelled (\textit{dsh}) in the planar polarity pathway, and thus implicates an STE20-like kinase in \textit{Fz}/\textit{Dsh}-mediated signaling. This demonstrates that seven-pass transmembrane receptors can signal via members of the STE20 kinase family in higher eukaryotes. We also show that Msn acts in EPP signaling through the JNK (Jun-N-terminal kinase) module as it does in dorsal closure. Although at the level of \textit{Fz}/\textit{Dsh} there is no apparent redundancy in this pathway, the downstream effector JNK/MAPK (mitogen-activated protein kinase) module is redundant in planar polarity generation. To address the nature of this redundancy, we provide evidence for an involvement of the related MAP kinases of the p38 subfamily in planar polarity signaling downstream of Msn.

Keywords: Dishevelled/ommatidia/p38 kinase/planar polarity/STE20 kinase

Introduction

The responses to extracellular stimuli and intercellular communication are important elements governing the development of higher eukaryotes. The resulting activation of signaling pathways leads to specific responses ranging from cell proliferation to cell shape changes. Although many signaling molecules have been identified through biochemical or genetic experiments, the picture of the respective signal transduction cascades is still incomplete. The genetic analysis of signaling pathways and mechanisms in multicellular model organisms like \textit{Drosophila} or \textit{Caenorhabditis elegans} has helped to identify some of the missing links and address the issues of signaling specificity \textit{in vivo} (Greenwald and Rubin, 1992; Zipursky and Rubin, 1994; Artavanis-Tsakonas \textit{et al}., 1995; Marshall, 1995; Sternberg \textit{et al}., 1995).

Many distinct stimuli and signals lead to the activation of the so-called mitogen-activated protein kinase (MAPK) modules. Several related MAPK cascade modules have been identified in many organisms (including yeast, mammalian cells and \textit{Drosophila}; Marshall, 1994; Herskowitz, 1995; Kyriakis and Avruch, 1996; Treisman, 1996) comprising the ERK (extracellular signal-related kinase)-type modules, the JNK (Jun-N-terminal kinase) or SAPK (stress-activated protein kinase) modules and the kinases of the p38 family. All the MAPK modules consist of a kinase cascade of at least three kinases acting sequentially (Marshall, 1994; Herskowitz, 1995). For any of these kinases there are multiple enzymes with the same (or very similar) specificity and thus it has been postulated that a high degree of redundancy is present within these cascades.

Despite this detailed knowledge about the MAPK modules, their upstream activating mechanisms are only partially known. Only the ERK-type MAPKs have a well defined mechanism of activation from the signal-responsive cell surface receptors; they are effectors of Ras, mediating responses ranging from cell proliferation to cell fate induction (e.g. for \textit{Drosophila}; Zipursky and Rubin, 1994; Freeman, 1997; Karim and Rubin, 1998). In contrast, the molecular mechanisms of activation of the JNK or p38 modules are poorly understood. Although they are potently activated by cellular stress and extracellular signals like LPS and IL-1 (for reviews see Kyriakis and Avruch, 1996; Minden and Karin, 1997; Gutkind, 1998) and factors involved in cell death signaling, such as DAXX and Fas (Coso \textit{et al}., 1995; Minden \textit{et al}., 1995; Yang \textit{et al}., 1997), it is not well established how signals from the cell surface are linked to activation of these MAPKs (Gutkind, 1998).

Genetic experiments in yeast and more recently in \textit{Drosophila}, as well as studies in mammalian cell cultures, have identified the STE20 kinase family as potential upstream activators of the JNK-type MAPK modules (Herskowitz, 1995; Pombo \textit{et al}., 1995; Hu \textit{et al}., 1996; Su \textit{et al}., 1997, 1998), and thus the STE20-like kinases have also been referred to as MKKKKs (MAP kinase kinase kinase kinase). Despite the well documented yeast example where STE20, the founding member of the family, acts in the pheromone signaling pathway and activates the yeast MAPK module (Herskowitz, 1995), it is not known to what membrane receptors STE20-related kinases are linked in higher eukaryotes. Recently, a \textit{Drosophila} STE20-related kinase Misshapen (Msn, the \textit{Drosophila} homolog of mammalian NIK, an SPS1 subfamily STE20-like kinase) was demonstrated to act as an upstream activator of the JNK cascade required for dorsal closure (Noselli, 1998; Su \textit{et al}., 1998). However, the upstream components in the dorsal closure pathway, as well as any link to membrane-associated receptors, are still elusive and thus it is not known how STE20-like kinases are activated in higher eukaryotic organisms.
We analyze the role of the STE20-like Msn kinase in the epithelial planar polarity (EPP) pathway. Our phenotypic and genetic analyses indicate that Msn acts downstream of the Fz receptor, being the first analysis that links an STE20-like kinase to a membrane receptor in higher eukaryotes.

The generation of planar polarity in Drosophila has emerged as a good model system to study signaling pathways mediated by the seven-pass transmembrane Fz receptor (Vinson et al., 1989; Strutt et al., 1997; Axelrod et al., 1998; Boutros et al., 1998). Planar polarity phenotypes are characterized by the misorientation of cells within epithelia, including the primordia of wings, legs and eyes (Adler, 1992; Gubb, 1993; Zheng et al., 1995; Strutt et al., 1997). Besides fz, several other genes have been implicated in planar polarity signaling based on their mutant phenotypes including dishevelled (dsh; Gubb, 1993; Adler et al., 1994; Theisen et al., 1994; Strutt et al., 1997; Wolff and Rubin, 1998). Biochemical and genetic studies have indicated that the planar polarity pathway downstream of Fz includes Dsh and small GTPases of the Rho subfamily (RhoA and Rac) and leads to the activation of a JNK-type MAPK module (Krasnow et al., 1995; Strutt et al., 1997; Boutros et al., 1998). However, it remains unclear how these components are linked, i.e. how Dsh is connected to the JNK cascade.

Here we provide evidence that the STE20-like Msn kinase acts in planar polarity signaling. We show genetically and in biochemical experiments that Msn is required downstream of the Fz receptor and its effector Dsh. This is not only the first analysis that implicates an STE20-like kinase in Fz-mediated signaling, but it is also the first report of higher eukaryotes linking STE20/Msn to a membrane-associated receptor. In addition, we show that in planar polarity, Msn acts through the JNK module as it does in dorsal closure, and also provide evidence for an involvement of the related MAP kinases of the p38 subfamily downstream of Msn.

**Results**

**Identification of misshapen as a component of Dsh-mediated EPP signaling**

In the Drosophila eye, EPP is reflected in the mirror-symmetric arrangement of ommatidial units relative to the dorso-ventral midline (the equator). This pattern is generated posterior to the morphogenetic furrow when ommatidial preclusters rotate 90° towards the equator, adopting opposite chirality depending on their dorsal or ventral positions (Gubb, 1993; Figure 1A). Polarity defects are manifested in the loss of mirror-image symmetry, with the ommatidia misrotating and adopting random chirality or remaining symmetrical (Gubb, 1993; Theisen et al., 1994; Zheng et al., 1995; Strutt et al., 1997; Wolff and Rubin, 1998; see also Figure 1).

The gain-of-function dsh phenotype (sev-Dsh) has been successfully used in previous reports to identify new components of the Fz/Dsh planar polarity pathway (Strutt et al., 1997; Boutros et al., 1998). We have employed the same assay, dominant genetic modification of the sev-Dsh
phenotype (Figure 1B), to screen through a large number of known genes. Among the few mutants that showed a specific interaction, we found two alleles of the previously identified 

misshapen (msn) locus. msn102 and msn72 are X-ray-induced inversions with breakpoints in the msn gene (Treisman et al., 1997). This gene has recently been shown to encode the Drosophila homolog of NIK, a member of the mammalian SPS1 subfamily of the STE20 kinase family (Treisman et al., 1997; Su et al., 1998). Both loss-of-function alleles of msn act as dominant suppressors of sev-Dsh, comparable to other planar polarity-specific Dsh effectors (Figures 1C and 2; Boutros et al., 1998).

In addition, msn was isolated in a gain-of-function screen for genes involved in planar polarity generation (F. Feiguin and S. Eaton, unpublished data). Overexpression of genes required in planar polarity signaling at the relevant time often results in defects that are similar to the loss-of-function mutant phenotypes, e.g. with Fz and Dsh (Krasnow et al., 1995; Strutt et al., 1997; Boutros et al., 1998; see also Figure 1B).

In such a screen, ap-GAL4 flies (ap-GAL4) induces overexpression of the corresponding gene in the notum and the dorsal part of the wing (Calleja et al., 1996) were crossed to the collection of 2200 E/P lines (Rørth et al., 1998) and the progeny were scored for disarranged microchaetae on the notum. One of the lines isolated in this screen, ep(3)0549, showed an abnormal orientation of the microchaetae (Figure 1D) similar to phenotypes obtained with ap driven Fz overexpression (F. Feiguin and S. Eaton, unpublished data). Similarly, ap-GAL4, ep(3)0549 flies showed typical polarity phenotypes on the dorsal surface of the wing where these are manifest in the presence of multiple wing hairs (Figure 1F, see also below).

In situ hybridization experiments to polytene chromosomes (mapping to 62E6-7) and complementation analyses revealed that the EP-element insertion in line ep(3)0549 is in the msn locus and represents a msn allele. Subsequent sequence analyses confirmed that the EP insertion is located 24 bp upstream of the 5′-end of a msn cDNA (data not shown). Taken together, these results suggest that msn is involved in EPP signaling and possibly acts downstream of Dsh.

**A kinase-inactive form of Msn interferes with Dsh-mediated JNK activation**

To gain further confirmation of the role of Msn in Fz/Dsh-mediated polarity signaling, we used an in vitro assay to determine whether Msn acts downstream of Dsh in JNK pathway activation. Previous experiments have shown that expression of Dsh in NIH 3T3 cells activates JNK and Jun phosphorylation, indicating that Dsh is a potent activator of a Jun-kinase pathway (Boutros et al., 1998). Using the same assay, we asked whether co-expression of a dominant-negative (kinase-inactive) Msn protein (DN-Msn) has an effect on Dsh-induced Jun phosphorylation. Significantly, co-expression of DN-Msn reduces this activation and results in lower Jun phosphorylation levels to endogenous background levels. Expression of either wild type or DN-Msn alone caused no significant effects.

**misshapen mutants show planar polarity defects**

Recently, it was shown that msn mutations affect both the morphology of the rhabdomeres in photoreceptors, causing...
malformed, ‘misshapen’ rhabdomeres (Treisman et al., 1997), and also, at lower frequency, the number of photoreceptors. In addition, msn is required for the process of dorsal closure, and embryos mutant for msn display a typical dorsal open phenotype (Su et al., 1998). To analyze its requirements in polarity generation, we looked in detail at msn mutant clones in the eye and the wing. Our phenotypic analysis of eye clones revealed that msn is required for the generation of planar polarity (Figure 4). msn mutant ommatidia containing the normal complement of photoreceptors are often misrotated and display the wrong chiral form or are symmetrical (non-chiral; Figure 4A and B). To confirm that the polarity defects of msn mutant ommatidia are primary defects, and thus implicate msn in polarity generation, we analyzed ommatidial polarity in msn mutant clones at the earliest possible stage in third instar larval imaginal discs (when tissue polarity genes are required). Spalt is expressed in the R3/R4 precursor pair for about two columns at this stage in third instar larval imaginal discs (when tissue polarity genes are required). In msn mutant tissue, ommatidial rotation, and thus polarity, is randomized (e.g. ommatidia rotate in the opposite direction to their wild-type neighbors) showing that these defects result from an early failure in polarity establishment (Figure 4D). Thus in the eye, the msn phenotype (defects in polarity, malformed, misshapen and missing photoreceptors) is very reminiscent of other phenotypes of dorsal closure, and embryos mutant for msn display a typical dorsal open phenotype (Su et al., 1998). To analyze its requirements in polarity generation, we looked in detail at msn mutant clones in the eye and the wing. Our phenotypic analysis of eye clones revealed that msn is required for the generation of planar polarity (Figure 4). msn mutant ommatidia containing the normal complement of photoreceptors are often misrotated and display the wrong chiral form or are symmetrical (non-chiral; Figure 4A and B). To confirm that the polarity defects of msn mutant ommatidia are primary defects, and thus implicate msn in polarity generation, we analyzed ommatidial polarity in msn mutant clones at the earliest possible stage in third instar larval imaginal discs (when tissue polarity genes are required). Spalt is expressed in the R3/R4 precursor pair for about two columns at this stage (Figure 4C and D), and in wild type this reflects the regular arrangement and direction of rotation of the preclusters (Figure 4C). In msn mutant tissue, ommatidial rotation, and thus polarity, is randomized (e.g. ommatidia rotate in the opposite direction to their wild-type neighbors) showing that these defects result from an early failure in polarity establishment (Figure 4D). Thus in the eye, the msn phenotype (defects in polarity, malformed, misshapen and missing photoreceptors) is very reminiscent of other genes involved in both polarity and terminal photoreceptor differentiation (e.g. RhoA; Strutt et al., 1997).

The fz gene has been implicated in the specification of the R3 cell within the R3/R4 pair in the process of chirality generation. The mosaic analysis of both loss-of-function (Zheng et al., 1995) and gain-of-function (Fanto and Mlodzik, 1999) fz alleles has shown that Fz signaling is required in R3 for correct ommatidial chirality generation and also induces R3 fate. The genetic interactions and cell culture experiments have shown that msn acts downstream of Fz/Dsh, and thus we asked whether msn is also involved in the selection of R3 in analogy to the fz requirement. We examined the genotypic composition of mosaic ommatidial clusters within the R3/R4 pair (Figure 5). This analysis revealed that, as is the case for fz, the msn+ cell has a strong preference for adopting the R3 photoreceptor fate. This can often lead to chirality inversions, where the msn+ R4 precursor adopts the R3 position and displaces the original msn+ R3 precursor (examples are shown in Figure 5A and B). In summary, the genetic requirements of msn in single photoreceptors, in particular the R3/R4 pair (Figure 5), are very similar to those of fz.

msn mutant clones in the wing affect the process of hair development and polarity. Phalloidin stainings of msn clones in pupal wings revealed that cells mutant for msn show defects in prehair initiation. These range from a complete failure of actin polymerization in the prehair to approximately wild-type levels of actin (Figure 6). Loss of Misshapen activity specifically affects wing hair actin organization, since adherens junction actin in msn clones appears normal. Although in some mutant cells an actin ‘hair’ is detected in the pupal wing, often at abnormal positions within the cell (Figure 6A), the adult hairs in msn+ tissue are either missing, branched or stunted (Figure 6C; arrows). Some cells that generate stunted hairs initiate them at multiple sites (a typical planar polarity phenotype; arrowhead in Figure 6C). These phenotypes are
Fig. 5. The requirements of msn in the R3/R4 photoreceptor pair. Tangential sections of msn<sup>102</sup> (A) and msn<sup>R17</sup> (B) clones. The high pigment level allows for an analysis with single cell resolution. Examples of ommatidia with mosaic R3/R4 pairs with either wild-type polarity/chirality or inverted chirality (including a symmetrical cluster in (B)) are highlighted with black and white arrows, respectively. The genotype of single photoreceptors can be determined due to the black pigment granules next to the rhabdomeres. Note that the genotype of the R3/R4 pair is critical for establishment of correct polarity. In addition to chirality inversions msn<sup>–</sup> R3 cells can also lead to the formation of symmetrical clusters (an example is highlighted with a black arrow in (B)). In such mosaic clusters the respective pigmented (wild-type) photoreceptors (white arrowheads) or mutant (unpigmented) photoreceptors (black arrowheads) are highlighted. Note that when the msn<sup>+</sup> precursor cell was originally positioned as R4, this cell can adopt the R3 fate and displace the msn<sup>–</sup> cell leading to perfect chirality inversions. (C) Statistical analysis of the R3/R4 requirements of msn. When the R3 precursor is msn<sup>+</sup> in 100% the ommatidium is of wild-type polarity; the opposite scenario, R4 being msn<sup>–</sup>, leads to a high degree of chirality inversions or symmetrical clusters (81%). The number of R3/R4 mosaic clusters analyzed was 46 in 16 clones. The mosaic analysis also showed that for correct 90° rotation there was no bias for any particular photoreceptor (data not shown).

reminiscent of the defects observed either when prehair actin organization is disrupted by dominant-negative Cdc42 (Eaton et al., 1996) or after cytochalasin D treatment of cultured pupal wing discs (Turner and Adler, 1998).

To test whether overexpression of Msn in the eye can cause polarity defects comparable to sev-Fz or sev-Dsh (Strutt et al., 1997; Boutros et al., 1998; see also above), we used the msn E/P-line ep(3)0549 and a UAS-msn strain (Su et al., 1998), and crossed these to sev-GAL4. The eyes of the resulting flies (sev>msn) are externally rough and reveal typical polarity defects in tangential sections (Figure 7A). Taken together with the genetic requirements and the loss-of-function phenotypes, the suppression of the sev-Dsh genotype and the cell culture experiments, these data demonstrate that Msn acts in the Fz/Dsh-mediated polarity signaling downstream of Dsh.

The gain-of-function msn phenotype is modified by JNK pathway components

It has been shown recently that during Drosophila embryogenesis msn acts upstream of the JNK-type MAPK module in dorsal closure signaling (Su et al., 1998). Several lines of evidence support a function of JNK cascade components in the generation of planar polarity (Strutt et al., 1997; Boutros et al., 1998).

To confirm the cell culture experiments and to determine whether components of JNK signaling act downstream of msn in polarity signaling in vivo, we used the gain-
of-function eye polarity phenotype of msn (sev>msn; Figure 7A) to test for dominant interactions with mutations in JNK signaling components. In sev>msn eyes, only 62.6% of the ommatidia are correctly oriented (compared with 100% in wild-type eyes) with the remaining ommatidia showing polarity defects as well as defects in photoreceptor shape and differentiation. We find that reducing the dosage of known components of the JNK cascade causes a strong dominant suppression of sev>msn (Figure 7B and C and Table I). These results are consistent with msn acting upstream of the JNK module in polarity signaling and with the notion that Msn generally acts upstream of JNK-like cascades in higher eukaryotes.

Other components of planar polarity signaling that also participate in the process of dorsal closure upstream of the JNK module are the small GTPases RhoA and Rac (Strutt et al., 1997; Boutros et al., 1998). However, neither RhoA mutants nor deficiencies removing Rac1 show a modification of sev>msn (Table I) suggesting that Msn acts downstream of these small GTPases. This is further supported by the observation that sev-GAL4 driven co-expression of RacN17 and Msn resembles the phenotype of Msn alone (Figure 7A and data not shown).

The Drosophila dreadlocks (dock) gene is highly related to the human proto-oncogene NCK (Garrity et al., 1996). Since Msn is a homolog of NIK which physically interacts with NCK, we tested whether dock alleles can modify sev>msn. None of the dock alleles showed an interaction in this assay (Table I). Moreover, the dock alleles do not interact with sev-Dsh (Figure 2) and do not display a polarity phenotype in homozygous mutant tissue (data not shown; Garrity et al., 1996). Thus, the potential Msn interactor Dock does not play a detectable role in polarity generation.

Table I. Quantification of genetic interactions with the gain-of-function msn phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Correctly rotated ommatidia (% ± SD)</th>
<th>Number of ommatidia scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>sExp(3)0549; +/+ (Control)</td>
<td>62.6 (± 5.1)</td>
<td>752</td>
</tr>
<tr>
<td>hepR35/+</td>
<td>93.5 (± 6.7)</td>
<td>442</td>
</tr>
<tr>
<td>bsk2/+</td>
<td>93.6 (± 2.4)</td>
<td>461</td>
</tr>
<tr>
<td>Df(2L)flp170B (bsk–)</td>
<td>92.4 (± 3.5)</td>
<td>445</td>
</tr>
<tr>
<td>jun2/+</td>
<td>89.7 (± 6.3)</td>
<td>486</td>
</tr>
<tr>
<td>dck3/+</td>
<td>59.6 (± 2.6)</td>
<td>341</td>
</tr>
<tr>
<td>Df(3R)crbR74-4/+ (p38a+)</td>
<td>85.7 (± 6.4)</td>
<td>706</td>
</tr>
<tr>
<td>Df(3R)crbR59-4/+ (p38a+)</td>
<td>84.8 (± 4.4)</td>
<td>564</td>
</tr>
<tr>
<td>Df(2L)bb03e3/+ (p38b+)</td>
<td>85.1 (± 4.2)</td>
<td>437</td>
</tr>
<tr>
<td>Df(2L)bb03e25/+ (p38b+)</td>
<td>86.1 (± 5.8)</td>
<td>475</td>
</tr>
<tr>
<td>Df(3R)rbp13/+ (MKK4–)</td>
<td>88.4 (± 6.1)</td>
<td>542</td>
</tr>
<tr>
<td>Df(3R)cb14/+ (MKK3+, hep+)</td>
<td>96.2 (± 2.5)</td>
<td>550</td>
</tr>
<tr>
<td>Df(3R)H6/+ (MKK3+, hep+)</td>
<td>87.4 (± 2.2)</td>
<td>446</td>
</tr>
<tr>
<td>RhoA2790/+</td>
<td>62.2 (± 9.0)</td>
<td>450</td>
</tr>
<tr>
<td>Df(3L)jnec5/+ (rac1–)</td>
<td>56.0 (± 8.4)</td>
<td>686</td>
</tr>
</tbody>
</table>

Quantification of genetic interaction of the overexpression phenotype of Msn with putative downstream components of EPP signaling. The quantifications of allelic combinations are based on scoring of four to seven independent eyes per genotype. The percentage shown in this table is the average number of correctly oriented ommatidia, with the standard deviation calculated across all eyes of a given genotype scored. In wild-type eyes, all ommatidia are correctly oriented.

The role of JNK-related kinase cascades in polarity signaling

Although there is accumulating evidence that JNK-type MAPK modules are involved in planar polarity signaling (Strutt et al., 1997; Boutros et al., 1998), the analysis of mutant clones of either hep or bsk alleles showed no or
weak phenotypes in imaginal tissues (data not shown). These observations suggest a high degree of redundancy at this level in the polarity signaling pathway.

To address this issue further, we looked for a potential involvement of related kinases that could account for the proposed redundancy. We tested the recently described *Drosophila* kinases, belonging to the JNK/p38 class within the MAPK modules (Han et al., 1998), for genetic interactions with the planar polarity phenotypes of *sev-Dsh* and *sev-msn*. These are obvious candidates to be cooperating with Hep and Bsk in polarity generation. At the level of Hep/JNK, an MKK7 homolog, two other MKKs have been reported (DMKK3 and DMKK4). Similarly, at the level of Bsk/JNK, two p38-like kinases were isolated (Dp38a and Dp38b; Han et al., 1998). Since no mutants have yet been isolated for these genes, we asked whether deficiencies removing these kinases show an interaction with *sev-Dsh*. DMKK3 maps in the vicinity of hep and deficiencies removing DMKK3, Df(3R)p13 and Df(X)G24 and Df(X)H6, also remove hep (S.Noselli; personal communication).

These deficiencies show externally a very strong suppression of *sev-Dsh* (data not shown) with a marked decrease of misrotated ommatidia as observed in tangential sections (Figure 2). Deficiency Df(3R)p13 removes the DMKK4 locus and also dominantly suppresses *sev-Dsh* (Figure 2). Similarly, deficiencies removing either Dp38a, Df(3R)crb77F and Df(3L)crbF954, or Dp38b, Df(2L)b80e3 and Df(2L)b87e25, are suppressors of *sev-Dsh* (Figure 2). We also tested whether the respective deficiencies showed an interaction with *sev>msn*, and found that all of them act as dominant suppressors of this genotype as well (Table 1). It is interesting to mention that the Msn-induced defects in rhabdomere morphology were also suppressed by those deficiencies. These interactions suggest that the p38 kinases are redundant with JNK in the context of planar polarity signaling.

**Discussion**

We have shown that the *Drosophila* STE20-related kinase, Msn, is a component of planar polarity signaling, linking Fz membrane receptors to JNK cascades. This is the first link of an STE20-like kinase to a membrane-associated receptor in metazoans. It was recently shown that Msn acts upstream of a JNK cascade in dorsal closure (Su et al., 1998). Based on genetic interactions with the Dsh and Msn gain-of-function polarity phenotypes, we show that not only JNK but also other related kinase cascades (p38) can act downstream of Fz/Dsh, cooperating with the Hep–Bsk module in planar polarity generation.

**Role of Msn in the generation of polarity**

Our phenotypic analysis of *msn* clones in the eye and wing shows typical planar polarity phenotypes. In the eye, *msn* loss-of-function mutants cause misrotated ommatidia and chirality defects. Similar to *fz* and *dsh*, these polarity phenotypes are already evident in third instar larval discs and display the same genetic requirements in the R3/R4 pair as *fz*. Other defects observed in *msn* mutant clones are malformed rhabdomeres and, at low frequency, defects in photoreceptor differentiation (Treisman et al., 1997; this study). As observed for other members of the polarity pathway (Boutros et al., 1998), overexpression of Msn (sev>*msn*) gives a similar phenotype to loss-of-function alleles. Although mutations in *fz* and *dsh* result in clean planar polarity defects (Theisen et al., 1994; Zheng et al., 1995; Strutt et al., 1997), the additional phenotypes observed in *msn* mutants are comparable to other members of polarity signaling, e.g. *RhoA* alleles also cause loss of photoreceptors or photoreceptors with abnormal morphology and malformed rhabdomeres (Strutt et al., 1997; and data not shown) very similar to *msn*.

The effects of Misshapen overexpression and loss-of-function in the wing suggest that it may represent a branchpoint in the eye and wing polarization pathways. Misshapen overexpression in the wing produces a multiple wing hair phenotype similar to that of the wing-specific polarity genes *inturned* and *fuzzy*. In contrast, *msn* loss-of-function clones have defects similar to those of dominant-negative CDC42 expressing cells; hair actin polymerization is defective and adult hairs are missing or stunted.

One explanation of these data is that Misshapen acts through the JNK pathway, as it does in the eye, but that the targets of transcriptional activation are the components needed for hair formation. Excess production of these components may lead to multiple hairs and their loss to missing hairs. We do not favor this model because Dishevelled, which acts upstream of Misshapen in this pathway, affects only hair polarity and is not required for hair formation. Furthermore, expression in the wing of a kinase-inactive version of JNK, which acts as a dominant negative, has no effect on hair formation or polarity (F.Feiguin, N.Paricio and S.Eaton, unpublished data). Our data are more consistent with a model in which localized Misshapen activity directly promotes polarized cytoskeletal reorganization leading to hair formation; excess Misshapen might then expand the region of the cell competent for hair outgrowth. According to this model, activation of the Fz/Dsh signal transduction pathway in the wing would not necessarily increase the absolute level of Misshapen activity, but rather serve to localize Misshapen activity within the cell. It will be interesting to determine whether molecules such as CDC42, Inturned or Fuzzy represent wing-specific targets of Misshapen.

Taken together with the phenotypes in the eye and its role in dorsal closure (Su et al., 1998), these observations indicate that Msn has a function in both nuclear signaling and cytoskeletal rearrangements.

**Msn links Dsh and JNKs in polarity signaling**

Several studies in yeast and mammalian cells indicate that STE20 kinases function upstream of MKKKs regulating JNK (Herskowitz, 1995; Su et al., 1997). Msn is the *Drosophila* homolog of mammalian NIK, belonging to the SPS1 family of STE20-like kinases (Su et al., 1997, 1998; Treisman et al., 1997). Based on differences in structure and possible regulation, two subfamilies of STE20 kinases have been described: mammalian and *Drosophila* PAKs (p21 Activated Kinases), which are activated by binding GTP-bound Cdc42 and Rac (Manser et al., 1995; Martin et al., 1995; Harden et al., 1996) and contain N-terminal regulatory and a C-terminal kinase domain. Members of the second subfamily, containing the SPS1 kinase in yeast (Friesen et al., 1994), do not interact physically with Cdc42 or Rac and contain an N-terminal
kinase and a C-terminal regulatory domain. Several members of the SPS1 subfamily have been described in mammals, but only a subset of them, such as GCK (Pombo et al., 1995), HPK1 (Hu et al., 1996), GLK (Diener et al., 1997) and NIK (Su et al., 1997) have been shown to activate JNK. Genetic and biochemical studies have recently demonstrated that Msn can activate the JNK module and is required during dorsal closure (Su et al., 1998). Our observations indicate that Msn also acts upstream of the JNK module in polarity signaling in the eye and support this as a general mechanism.

Studies in Saccharomyces cerevisiae have shown that the STE20 kinase acts downstream of the pheromone receptor (Herskowitz, 1995). Our genetic and biochemical studies indicate that Msn acts downstream of Fz/Dsh in the transmission of the polarity signal. This is the first link of this family of kinases to membrane-associated receptors and upstream regulators in higher eukaryotes.

RhoA loss-of-function mutants and a deficiency removing the ral1 gene do not interact with the msn gain-of-function genotype (sev>Msn), suggesting that these genes may act upstream of Msn. Msn does not contain Rac binding sites and does not physically interact with Rac or RhoA (Su et al., 1998). Dock, the Drosophila homolog of Nck (Garrity et al., 1996; Su et al., 1997), is an adapter protein that possibly interacts with Msn through its SH3 domains as has been described for the mammalian homologs NIK and Nck (Su et al., 1997). However, analyses of dock mutants revealed that it is required for photoreceptor axon guidance, but no polarity phenotypes are evident in eye clones (Garrity et al., 1996). Moreover, we have found no interactions between either sev-Dsh or sev>Msn and dock mutants, suggesting that dock is not required in polarity signaling or is redundant in this process. Thus, it remains unclear how Msn is linked to the Rho/Rac GTPases. Our genetic data argue for Msn acting downstream of RhoA/Rac. However, it has also been suggested that Rac and Msn act in parallel pathways (Su et al., 1998).

Redundancy among the JNK/p38 kinases in polarity signaling

Although genetic evidence suggests an involvement of bsk (JNK) and hep (JNKK) in polarity signaling (Strutt et al., 1997; Boutros et al., 1998), phenotypic analyses suggest that the JNK module components are highly redundant in this process. In the search for other kinases involved, we found that deficiencies uncovering the genes encoding the recently described p38 MAP kinases Dp38a and Dp38b, and the MAP kinase kinases DMKK3 and DMKK4 (Han et al., 1998) dominantly suppress the sev>msn and sev-Dsh phenotypes, suggesting that these proteins also function downstream of Dsh and Msn in polarity signaling. It is interesting to note that all phenotypic defects of sev>Msn were dominantly suppressed by mutations in both components of the JNK and the p38 kinase module.

In contrast to these interactions, tissue culture experiments in mammalian cells have shown that NIK overexpression leads to JNK phosphorylation, but no detectable p38 activation was observed (Su et al., 1997). This difference can be explained by cell- and tissue-specific requirements, e.g. in Drosophila during dorsal closure, JNK activation downstream of Msn is not redundant (Su et al., 1998), while redundancy and p38 interactions are observed in polarity signaling. Thus, it is tempting to speculate that both JNK and p38 kinases cooperate in polarity generation.

The reported promiscuity of the kinases at both the MKK and the MAPK levels (Madhani et al., 1997) could account for the redundancy. The Drosophila MKKs and JNK/p38 MAPks also appear to act at (at least partially) on overlapping downstream targets. Whereas DMKK3 appears rather specific for p38 activation (although it activates both p38s), DMKK4 and Hep (the MKK7 cognate) both activate Bsk/JNK (Han et al., 1998). Similarly, Bsk/JNK and both Dp38s can phosphorylate the downstream targets JUn and ATF2 (Han et al., 1998). Thus, a potential downstream target can still be phosphorylated when one of the upstream kinases is removed, and likewise for their upstream activators. An even more complicated picture may emerge when all relevant kinases are identified. Other examples of redundancy are described in yeast MAP kinases. Although KSS1 and FUS3 normally have specific roles in different pathways, it has been shown that they are redundant in the process of mating (Madhani et al., 1997) and in this case KSS1 replaces Fus3 when the latter is not present. The isolation and analysis of all the respective kinases and their mutants will be necessary to understand fully the contribution of each single kinase in planar polarity signaling.

In conclusion, the implication of Msn/STE20 in Fz/Dsh-mediated polarity signaling is an essential first step to unravel the detailed requirements of the relevant downstream kinase cascades.

Materials and methods

Fly strains and genetic interactions

Genetic interactions were performed using a sev-Dsh strain (Boutros et al., 1998); msn122 and msn172 (Treisman et al., 1997) were kindly provided by J.Treisman and are inversions with breakpoints in msn. The ep(3)0549 line is from the pool of E/P lines previously described (Rorth et al., 1998). This line was also used to generate the sev>Msn gain-of-function allele (generated by recombination of sev-GAL4 onto the ep(3)0549 chromosome). UAS-Msn transgenic flies were a gift from J.Treisman. Deficiencies uncovering p38a, p38b and MKK4 genes were obtained from the Bloomington stock center. Deficiencies Di(X;2)G2 and Di(X);H6, which uncover hep and MKK2, were kindly provided by S.Noselli.

Genetic interactions with sev-Dsh (Boutros et al., 1998) and sev>msn were performed at 29°C. The flies analyzed were heterozygous for either sev-Dsh or sev>msn and the mutation of interest. w1118 was used as a negative control.

Mitotic clones

Clones of msn alleles were induced either by the FLP/FRT method (Xu and Rubin, 1993) or with X-rays. Larvae were heat shocked at 38°C for 120 min at 24–48 and 48–72 h after egg laying. Clones were marked by the absence of arm-LacZ staining in imaginal discs and by the loss of the pigment marker in adult eyes. To generate msn clones in pupal wings (marked by the absence of arm-LacZ staining), clones were induced at 48–72 h after egg laying. Such clones were also analyzed in adult wings where they were unmarked. X-ray-induced clones of both msn alleles in adult wings, marked with forked+, were also generated giving rise to the same phenotype.

Histology and cell culture assays

Sections of adult eyes were performed as previously described (Tomlinson and Ready, 1987). Anti-Spalt and anti-beta-gal stainings of eye discs were done in 0.1 M phosphate buffer, 0.2% saponin, 0.3% deoxycholate, 0.2% Triton X-100 and 10% normal goat serum. Spalt was detected with a rat antibody (a gift from R.Barrio) and beta-gal with a rabbit
polyclonal antibody (Cappel). Secondary antibodies were purchased from the Jackson Laboratories. Pupal wing stainings and wing SEM analysis was performed as described (Eaton et al., 1996). The NIH3T3 cell culture assays with DN-Msn and Dsh were performed essentially as described (Boutros et al., 1998). The expression plasmids for the Msn protein were kindly provided by Ed Skolnik (Su et al., 1998).

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