Analysis of the role of the Rac/Cdc42 GTPases during planar cell polarity generation in Drosophila

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ABSTRACT Initial genetic studies in Drosophila suggested that several members of the Rho subfamily (RhoA, Rac1 and Cdc42) are involved in planar cell polarity (PCP) establishment. However, analyses of Rac1, Rac2 and Mtl loss-of-function (LOF) mutants have argued against their role in this process. Here, we investigate in detail the role of the Rho GTPases Mtl, Cdc42, Rac1 and Rac2 in PCP generation. These functional analyses were performed by overexpressing Mtl in eyes and wings, by performing genetic interaction assays and by using a combination of triple and quadruple mutant LOF clones. We found that Mtl overexpression caused PCP phenotypes and that it interacted genetically with other Rho GTPases, such as Rac1 and Cdc42 as well as with several PCP genes, such as stbm, pk and aos. However, Mtl was not found to interact with Rac2, RhoA and other members of the Fz/PCP pathway. Triple mutant clones of Rac1, Rac2 and Mtl were found to exhibit mild PCP defects which were enhanced by reduction of Cdc42 function with a hypomorphic Cdc42 allele. Taken together, these and previous results suggest that Rho GTPases may have partially overlapping functions during PCP generation. Alternatively, it is also possible that the mild PCP phenotypes observed could indicate that they are required at low levels in that process. However, since not all of them function upstream of a JNK cassette, we propose that they may act in at least two parallel pathways.

KEY WORDS: Mtl, Rac1, Rac2, Cdc42, planar cell polarity, redundancy, Drosophila

Introduction

Small GTPases act as signal transducers by switching between inactive GDP-bound and active GTP-bound forms and have been implicated in multiple processes during the development of multicellular organisms (Van Aelst and D’Soucha-Schorey, 1997). In Drosophila several members of the Rho subfamily of small GTPases have been identified: Rac1, Rac2, Cdc42, RhoA, RhoL and Mtl (Luo et al., 1994; Harden et al., 1995; Hariharan et al., 1995; Murphy and Montell, 1996; Sasamura et al., 1997; Strutt et al., 1997, Newsome et al., 2000; Hakeda-Suzuki et al., 2002). Expression of constitutively activated and dominant-negative isoforms of these proteins and analysis of loss-of-function mutants have shed light on their physiological roles. They have been implicated in actin cytoskeleton reorganization (Genova et al., 2000), myogenesis, axonal outgrowth and guidance (Luo et al., 1994; Kaufmann et al., 1998; Hakeda-Suzuki et al., 2002; Ng et al., 2002; Fan et al., 2003), gastrulation (Barrett et al., 1997), oogenesis (Murphy and Montell, 1996), embryonic segmentation (Magie et al., 1999) and cell migration (reviewed by Montell, 1999; Paladi and Tepass, 2004). They also participate in embryonic dorsal closure (Hakeda-Suzuki et al., 2002; Woolner et al., 2005) and epithelial planar cell polarity establishment (Eaton et al., 1995; 1996; Strutt et al., 1997; Fanto et al., 2000; this paper).

In many organs, epithelial cells are polarized not only along the apical-basolateral axis but also within the plane of the epithelium. The acquisition of this planar cell polarity (PCP) is essential for specialized cellular functions (Klein and Mlodzik, 2005). PCP establishment has emerged as a good model to study the role of Rho family small GTPases. In Drosophila, two members of this family, RhoA and Rac1, have been implicated in this process (Eaton et al., 1995; Strutt et al., 1997; Fanto et al., 2000). PCP phenotypes are characterized by the misorientation of cells within the epithelial plane and have been most extensively studied in the context of Drosophila eye and wing development. In the eye, PCP is reflected in the mirror-symmetric arrangement of the ommatidia.

Abbreviations used in this paper: JNK, Jun N-terminal kinase; LOF, loss-of-function; PCP, planar cell polarity.
cell membranes (reviewed in Klein and Mlodzik, 2005). In the eye, Fz/PCP signaling, together with the Notch pathway, is responsible for R3/R4 fate induction and thus for the establishment of ommatidial chirality (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Besides, it has been shown that the ommatidial rotation, depends on the Egfr pathway (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003). Moreover, downstream of Egfr there is a requirement for the Ras/MAPK cascade and Canoe, an adherens-junction-associated protein (Young et al., 1993), that provides a link from Egfr to cytoskeletal elements (Gaengel and Mlodzik, 2003). Recently, it has been also shown that Egfr signaling regulates Cadherin activity in this context (Mirkovic and Mlodzik, 2006).

Several studies have suggested that the small Rho family GTPases are involved in PCP establishment. RhoA loss-of-function mutants display PCP phenotypes in eyes and wings and they dominantly suppress the gain-of-function phenotypes of fz and dsh (Strutt et al., 1997). Conversely, Cdc42 mutants were not found to interact with sev-Fz or sev-Dsh (Boutros et al., 1998), although overexpression of dominant-negative forms of the GT-Pase in the wing affects actin polymerization during wing hair formation resulting in loss or stunting of hairs (Eaton et al., 1995, 1996), but also producing occasional multiple wing hairs (Baron et al., 2000). The role of Rac in PCP establishment was also addressed using dominant-negative and activated isoforms of Rac1, which produce PCP phenotypes in the eye (Fanto et al., 2000). In addition, deficiencies uncovering either Rac1 or Rac2 dominantly suppress sev-E-Dsh (Boutros et al., 1998). However, analyses of loss-of-function mutants in Rac1, Rac2 and Mtl did not reveal clear PCP defects in eyes or wings (Hakeda-Suzuki et al., 2002). Mtl is the Drosophila homolog of the C. elegans MIG-2 GTPase (Zipkin et al., 1997; Newsome et al., 2000). Genetic studies of null and gain-of-function mutations of mig-2 in C. elegans have shown that this GTPase is required for cell migration and axon guidance and that it functions redundantly with other Rho family GTPases in many cells (Zipkin et al., 1997). Similarly, Mlt is functionally related to Rac1 and Rac2 in Drosophila, because these GTPases act redundantly in regulating

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild-type ommatidia (in % ±sd)</th>
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<tbody>
<tr>
<td>sev&gt;Mtl/</td>
<td>75.3 (±1.5)</td>
</tr>
<tr>
<td>Mtl/+</td>
<td>38.1 (±1.1)*</td>
</tr>
<tr>
<td>msp1918/+</td>
<td>75.8 (±3.3)</td>
</tr>
<tr>
<td>hep171/+</td>
<td>72.9 (±3.3)</td>
</tr>
<tr>
<td>bsk super2/+</td>
<td>78.7 (±2.9)</td>
</tr>
<tr>
<td>jun2/</td>
<td>80.2 (±3.3)</td>
</tr>
<tr>
<td>RhoA297/</td>
<td>74.5 (±6.1)</td>
</tr>
<tr>
<td>Cdc42AB/+</td>
<td>92.8 (±3.3)*</td>
</tr>
<tr>
<td>Cdc42AF/+</td>
<td>66.4 (±4.8)*</td>
</tr>
<tr>
<td>Rac2/</td>
<td>78.1 (±4.3)</td>
</tr>
<tr>
<td>Rac1/</td>
<td>63.9 (±4.3)*</td>
</tr>
<tr>
<td>aop2/</td>
<td>47.7 (±0.6)*</td>
</tr>
<tr>
<td>sbt101/</td>
<td>62.7 (±0.1)*</td>
</tr>
<tr>
<td>pkm2W66/+</td>
<td>61.4 (±3.3)*</td>
</tr>
</tbody>
</table>

Percentage of wild-type ommatidia (± standard deviation) of the analyzed eyes of flies heterozygous for the indicated alleles and containing one copy of sev>Mtl. The quantifications of allelic combinations are based on scoring of 3 to 7 independent eyes per genotype. The asterisks indicate significant interactions (p<0.05, t-test).
dorsal closure and axon growth and guidance (Hakeda-Suzuki et al., 2002, Ng et al., 2002).

In order to analyze more precisely the requirement of the Rho GTPases Mtl, Rac1, Rac2 and Cdc42 in PCP establishment, we used several strategies. We show that overexpression of wild-type Mtl in eyes and wings gives rise to PCP defects. Moreover, flies hemizygous for a weak hypomorphic Cdc42 mutant allele also show mild PCP defects in both tissues. In contrast to previous reports (Hakeda-Suzuki et al., 2002), we find that eye clones triple mutant for Rac1, Rac2 and Mtl display mild PCP defects, which are further enhanced when reducing the function of Cdc42 with a hypomorphic mutant allele. Taken together, our data suggest that all four Rho GTPases may have a redundant role during PCP generation. Alternatively, it is also possible that the mild PCP phenotypes observed could indicate that they are required at low levels in that process. However, since we find that Mtl and Cdc42 do not act in the canonical Fz pathway, conversely to Rac1, Rac2 and RhoA, we propose that not all the Rho GTPases act upstream of the JNK module and that there are at least two parallel Rho GTPase family functions.

Results

Overexpression of wild-type Mtl causes PCP phenotypes in eyes and wings

Functional studies of Rac1, Rac2 and Mtl in Drosophila have shown that they have overlapping functions in the control of epithelial morphogenesis, myoblast fusion, and axon growth and guidance (Hakeda-Suzuki et al., 2002; Ng et al., 2002). The same study indicates that animals homozygous for a deletion removing the entire Mtl open reading frame are fully viable, and suggests that these three GTPases are not required for PCP establishment (Hakeda-Suzuki et al., 2002). However, we have established transgenic flies carrying wild-type Mtl under the control of the UAS element (UAS-Mtl lines), and found that overexpression of wild-type Mtl in eyes and wings using several drivers produce PCP defects. sev-GAL4 drives the expression of Mtl in the developing eye disc, in the R3/R4 pair, which is critical for establishment of correct polarity. The eyes of the resulting flies (sev>Mtl), hereafter referred as sev>Mtl are externally rough and reveal typical PCP defects in tangential sections (Fig. 1B). Mtl overexpression resulted in the misorientation of several ommatidia and, at lower frequency, ommatidia with an abnormal complement of photoreceptors. Strikingly, the chirality of the ommatidia was rarely affected and the PCP defects were largely reflected by misrotation (Fig. 1B). As can be seen in Table 1, in sev>Mtl eyes 75.3% of the ommatidia display wild type orientation and the remaining ommatidia show polarity defects (21%), mainly misrotation, as well as defects in photoreceptor differentiation (3.7%). To establish whether the polarity/rotation defects observed with sev>Mtl arise early in development and are thus primary defects, we analyzed polarity generation in sev>Mtl third instar larval eye imaginal discs (when tissue polarity is first apparent and PCP genes are required). sev>Mtl discs were stained with anti-Spalt (as marker for R3/R4 precursors) and anti-Elav (expressed in all photoreceptors). Our results indicate that ommatidial polarity/rotation is affected in sev>Mtl eye discs, since the R3/R4 pairs are often incorrectly oriented with respect to their neighbors (Fig. 1C). The misorientation of the photoreceptor clusters is also evident in an anti-Arm staining of sev>Mtl eye discs (Fig. 1D). We also examined the effect of Mtl overexpression in the wing, using the GAL4 lines C765 and en-GAL4. In wild-type wings, each cell produces a single, distally oriented hair (Fig. 2A). Overexpression of Mtl in the whole wing driven by C765 gave rise exclusively to typical PCP defects and many cells exhibited a multiple wing hair phenotype, producing double or even triple hairs. Moreover, in several areas the wing hairs were not pointing distally, but were misoriented forming waves and whorls (Fig. 2B and data not shown). The same phenotypes were found in the posterior part of adult wings from en-GAL4, UAS-Mtl" files (Fig. 2C). Strikingly, these defects are reminiscent of those of the core PCP genes like ftz, stbm (also known as vang) or pk (Vinson et al., 1989; Taylor et al., 1998; Gubb et al., 1999).

To test whether the PCP eye phenotype observed in sev>Mtl" flies is due to excessive Mtl signaling, we tested the effect of reducing the dosage of Mtl" on such phenotype, using the Mtl" null allele (Hakeda-Suzuki et al., 2002). Surprisingly, we found that the sev>Mtl" phenotype is enhanced in an Mtl" mutant background (Table 1), thus suggesting that this phenotype is not caused by an excessive Mtl signaling but may actually be due to dominant-negative effects. It is possible that the overexpressed Mtl protein may accumulate in an inactive form that could interfere with endogenous Mtl function by directly sequestering it. Alternatively the overexpressed protein could be sequestering or inactivating limiting components or effectors of that GTPase, thus reducing the ability of the cells to signal productively. A similar situation was found when overexpressing the wild type form of Presenilin at very high levels (Ye and Fortini, 1999).

Taken together, our results suggest that Mtl has a role in PCP generation in eyes and wings, but it probably functions redundantly.

Fig. 2. Overexpression of wild-type Mtl causes PCP defects in the wing. All panels show high magnification areas of wings, distal is to the right. (A) Wild-type wing. Note the regular arrangement of hairs, all pointing distally. (B) C765/+; UAS-Mtl/+ wing at 25°C. (C) en-GAL4/+; UAS-Mtl/+ wing at 18°C (this cross is lethal when incubated at 25°C). Overexpression of the wild type form of Mtl in the wing causes typical PCP defects, e.g. duplications and triplications of hairs (black and gray arrows, respectively) and misorientation of hairs. Note many hairs pointing perpendicular to the proximal-distal axis in (B,C).
in this process. Since overexpression of wild-type, activated or dominant-negative isoforms of other Rho GTPases like RhoA and Rac1 (Fanto et al., 2000) also results in PCP defects, they could account for the proposed redundancy of function of Mtl during PCP generation.

**Mtl does not act in the PCP canonical pathway but interacts genetically with Rac1 and Cdc42**

Since our results suggested that Mtl is involved in polarity generation in eyes and wings, we wanted to place it more specifically in the PCP context. To do this we used the sev>Mtl phenotype to test for genetic interactions with mutations in other PCP components and the other GTPases. The results obtained in these experiments are shown in Table 1. Our results indicate that there is no significant genetic interaction between sev>Mtl and the components of the JNK cascade like msn, hep, bsk or jun, suggesting that Mtl does not function upstream of the JNK module. The same result was obtained when reducing the gene dosage of the small GTPase Rho4. Since Mtl is closely related to Rac1 and Rac2, and functionally behaves like both GTPases (Hakeda-Suzuki et al., 2002; Ng et al., 2002), we also tested for genetic interactions with loss-of-function mutations in both genes (Rac1 and Rac2, respectively). We found that Rac1, but not Rac2, interacts genetically with the sev>Mtl phenotype. These results indicate that Mtl might function with Rac1 in the PCP process. Therefore, we wanted to determine whether Mtl and Rac1 act in a hierarchy in the PCP context testing the opposite interaction. We crossed the sev>Rac1V12 transgene (a constitutively active isoform of Rac1), that produces polarity defects and also interferes with photoreceptor differentiation (Fanto et al., 2000), to the Mtl null allele and found that there is a significant suppression of the sev>Rac1V12 phenotype (Table 2), reflected in an increase of the number of correctly oriented wild-type ommatidia. Hence, these results support the idea that Mtl functions cooperatively with Rac1, and that they may have a redundant role during PCP establishment.

In addition we have found that Mtl genetically interacts with Cdc42. Two lethal alleles were tested for genetic interactions with the sev>Mtl phenotype, Cdc42 and Cdc42 (Genova et al., 2000). The Cdc42 allele has been used in previous studies which demonstrated that Cdc42 is not involved in Fz signaling (Boutros et al., 1998), and does not interact with Rac1 (Fanto et al., 2000). We found that both Cdc42 mutant alleles dominantly interact with the sev>Mtl phenotype (Table 1), indicating that Cdc42 might be functionally related to Mtl and suggesting a possible role of Cdc42 in PCP generation (see below). However, while Cdc42 suppressed the sev>Mtl phenotype, the Cdc42 allele enhanced it. Cdc42 is a lethal allele in which the conserved Gly residue at position 114 of the protein is replaced by Asp, probably inactivating the Cdc42 protein (Genova et al., 2000). It is interesting to mention that a mutant allele affecting the same region of the S. cerevisiae Cdc42 protein was reported to be more than simply a null allele, having a dominant negative effect (Ziman et al., 1991). The Cdc42 mutant contains a nucleotide substitution in an splice acceptor site (Genova et al., 2000), probably producing an incomplete protein and thus reducing Cdc42 function. The results obtained with this allele, which is supposed to be a true loss-of-function allele, suggest that Cdc42 and Mtl may act cooperatively during PCP generation (see below).

**Mtl interacts genetically with other genes involved in polarity generation**

We also tested for genetic interactions between Mtl and other genes involved in PCP establishment like strabismus (Stbm, Wolff and Rubin, 1998) and prickle (Pk, Gubb et al., 1999). It has been reported that Stbm and Pk are restricted to the R4 precursor cell to properly modulate Fz signaling (Jenny et al., 2003; Rawls and Wolff, 2003). Pk and Stbm interact physically, leading to the assembly of a Stbm/Pk containing signaling complex that is thought to negatively regulate Fz/Dsh activity and membrane localization (Jenny et al., 2003). Both genes dominantly enhance the sev>Mtl phenotype (Table 1). One of these genes, stbm, was also found to interact genetically with Rac1 (Fanto et al., 2000). Moreover, we also see a dominant enhancement of this phenotype with the rotation-specific allele of the Egfr-inhibitory ligand argos (aos; Gaengel and Mlodzik, 2003). It has been recently reported that Egfr signaling regulates ommatidial rotation through two Ras-effector pathways, the Ras/Raf/MAPK cascade and Ras/Cno signaling (Gaengel and Mlodzik, 2003). Moreover, it regulates Cadherin activity in this context (Mirkovik and Mlodzik, 2006). This interaction and the fact that overexpression of Mtl in the eye mainly causes misrotations, suggests an involvement of Mtl in regulating this aspect of the PCP process.

**TABLE 2**

A NULL MTL MUTANT ALLELE SUPPRESSES THE SEV-RAC1V12 PHENOTYPE

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild-type ommatidia (in % ± sd)</th>
<th>Number of ommatidia scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>sev&gt;Rac1V12; m/+ (Control)</td>
<td>34.1 (14.5)</td>
<td>406</td>
</tr>
<tr>
<td>Mtl+/+</td>
<td>62.3 (13.3)</td>
<td>389</td>
</tr>
</tbody>
</table>

The quantifications of allelic combinations are based on scoring of 3 independent eyes per genotype. The percentage shown in this table is the average number of wild-type ommatidia, with the standard deviation calculated across all eyes of a given genotype scored. The suppression is statistically significant (p<0.01, t-test).
Cdc42\textsuperscript{5} mutant flies exhibit mild PCP defects in eyes and wings

The role of Cdc42 in PCP establishment in the wing has been previously assessed by ectopic expression of different dominant negative isoforms (Eaton \textit{et al.}, 1995; 1996; Baron \textit{et al.}, 2000). These studies suggested that Cdc42 is necessary for the formation of polarized actin structures, since overexpression of Cdc42\textsuperscript{N17} or Cdc42\textsuperscript{Q61L} caused abolishment of both actin polymerization and hair outgrowth, resulting in wings with no hair or with stunted hairs (Eaton \textit{et al.}, 1995; 1996). In addition, expression of Cdc42\textsuperscript{Q61L} producing a multiple wing hair phenotype (Baron \textit{et al.}, 2000). In the eye, it has been demonstrated that flies heterozygous for weak and strong Cdc42 mutant alleles have mild rough eyes, although no defects in ommatidial orientation were reported (Boutros \textit{et al.}, 1998; Genova \textit{et al.}, 2000).

To test more definitively whether Cdc42 plays a role in PCP establishment, we analyzed the effect of reducing Cdc42 function in eyes and wings. As clones of the null allele Cdc42\textsuperscript{5} and Cdc42\textsuperscript{5} do not survive (Genova \textit{et al.}, 2000; data not shown), we analyzed tangential sections of eyes from Cdc42\textsuperscript{5} (a hypomorphic allele) hemizygous males. These show PCP defects, although at very low frequency (2.9%; Table 3), reflected by the presence of misrotated and achiral ommatidia (Fig. 3A and data not shown). Similar results have been found in females in homozygous mitotic eye clones for the Cdc42\textsuperscript{5} allele (Table 3).

Moreover, analyses of wings from Cdc42\textsuperscript{5} individuals also revealed mild PCP defects, with cells exhibiting a multiple wing hair phenotype, producing double or triple hairs (Fig. 3B). These data suggest that Cdc42 has a role in PCP generation in eyes and wings, and could account for the redundancy of function proposed for the Rac and Mtl in this process. In support of this model, a study has demonstrated that Cdc42 acts redundantly with Rac1 and Rac2 during embryonic blood cell migration (Paladi and Tepass, 2004).

Eye clones mutant for different combinations of Mtl, Rac1, Rac2 and Cdc42 alleles display polarity phenotypes

To further investigate the role of Mtl, Rac1, Rac2 and Cdc42 in PCP establishment, we generated eye clones mutant for different allelic combinations of all four genes. We used null alleles for Rac1, Rac2 and Mtl, Rac1\textsuperscript{5}, Rac2\textsuperscript{5} and Mtl\textsuperscript{5}, respectively) and the hypomorphic Cdc42\textsuperscript{5} allele. Phenotypic analyses of eye clones double mutant for either Rac1 and Mtl or Rac1 and Rac2 revealed no PCP defects (data not shown). Besides, clones mutant for either Mtl and Cdc42 or Rac1, Rac2 and Cdc42 showed a low frequency of PCP defects (data not shown), comparable to the results obtained in Cdc42\textsuperscript{5} mutants or mutant clones. Next we generated eye clones triply mutant for Rac1, Rac2 and Mtl. Although previous analyses of such clones suggested that there is no requirement of these GTPases during PCP generation (Hakeda-Suzuki \textit{et al.}, 2002), we detected reproducible ommatidial polarity defects (Fig. 4A). Analyses of such clones revealed that, although many ommatidia display correct polarity, achiral or misrotated triply mutant ommatidia are reproducibly detected.

### Table 3

PERCENTAGE OF ABNORMAL OMMATIDIA IN EYE CLONES MUTANT FOR THE INDICATED ALLELIC COMBINATIONS

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Abnormal ommatidia (in % ± sd)</th>
<th>Number of clones analyzed</th>
<th>Number of ommatidia scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc42\textsuperscript{5}</td>
<td>2.9 (±3.8)</td>
<td>6</td>
<td>422</td>
</tr>
<tr>
<td>Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5}</td>
<td>4.6 (±3.1)</td>
<td>7</td>
<td>486</td>
</tr>
<tr>
<td>Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5}</td>
<td>5.5 (±2.2)</td>
<td>19</td>
<td>1252</td>
</tr>
<tr>
<td>Cdc42\textsuperscript{5}, Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5}</td>
<td>14.2 (±3.5)</td>
<td>12</td>
<td>941</td>
</tr>
</tbody>
</table>

Cdc42\textsuperscript{5} and Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5} quantifications correspond to control clones generated separately. Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5} and Cdc42\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5} quantifications correspond to the results obtained for two populations of different clones obtained from the same cross. The difference in the frequency of abnormal ommatidia between Cdc42\textsuperscript{5}, Rac1\textsuperscript{5}, Rac2\textsuperscript{5} and Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5} and Rac1\textsuperscript{5}, Rac2\textsuperscript{5}, Mtl\textsuperscript{5} #1 or #2 is statistically significant (p < 0.001, Hest). In all cases about 1% of ommatidia show problems in photoreceptor differentiation.
(4.6% of abnormal ommatidia, see Rac1<sup>1/11</sup>, Rac2<sup>1</sup>, Mtl<sup>1</sup> in Table 3). The low penetrance of this phenotype may indicate that the Rac genes play a minor role during PCP generation. However, it can also suggest that their function is largely redundant in this process. Since we have found that Cdc42 also has a role during PCP generation, we asked whether the PCP defects found in eye clones triply mutant for the three GTPases Rac1, Rac2 and Mtl could be modified by reducing the function of Cdc42. We thus generated clones that were quadruple mutant for all four GTPases (see Material and Methods). Strikingly, these quadruple mutant clones display PCP defects at higher frequency. Analysis of tangential sections of such clones revealed typical PCP defects, like symmetrical and misrotated ommatidia (Fig. 4B, C), with a frequency of close to 15% (Table 3, see Cdc42<sup>1</sup>, Rac1<sup>1/11</sup>, Rac2<sup>1</sup>, Mtl<sup>1</sup>). Control clones triply mutant for the three Rac genes (originating from the same cross) showed defects at 5.5% frequency, comparable to the 4.6% obtained in the original triply mutant clones (Table 3, compare Rac1<sup>1/11</sup>, Rac2<sup>1</sup>, Mtl<sup>2</sup> to Rac1<sup>1/11</sup>, Rac2<sup>1</sup>, Mtl<sup>1</sup>). This indicates that the reduction of Cdc42 function is causing the increased PCP defects and that these are not due to genetic background variation in the clones. Armadillo stainings of quadruple mutant eye disc clones confirmed rotation abnormalities and revealed no loss of accessory ommatidial cells that could lead to problems in local cell stacking (Fig. 4D). To support our results in the eye, we have also generated unmarked quadruple mutant clones in adult wings. In these wings we occasionally observed PCP phenotypes, like duplications of wing hairs and groups of hairs that were not pointing distally (data not shown).

Taken together, these results could indicate that the Rac/Cdc42 GTPases may have overlapping functions during PCP establishment in the Drosophila eye. Then, the low penetrance of the mutant phenotype in the quadruple mutant clones, which is comparable to hypomorphic alleles of PCP genes, would be consistent with Cdc42<sup>1</sup> being a hypomorphic allele. However, another explanation for the mild PCP phenotypes observed in the clones could be that these proteins have a minor role during the PCP process. As mentioned above, the closely related RhoA GTPase plays a non-redundant role in PCP establishment (Strutt et al., 1997). One possibility could be that the Rac/Cdc42 GTPases cooperate with RhoA during PCP generation. Then, a reduction of RhoA function could also modify the PCP defects obtained by loss of function of the Rac genes. To test this, we generated clones quadruple mutant for the Rac1<sup>1</sup>, Rac2<sup>1</sup>, Mtl<sup>1</sup> and RhoA genes (using the strong hypomorphic allele RhoA<sup>4117</sup>; Strutt et al., 1997). However, these quadruple mutant clones were not informative as we found mainly photoreceptor loss in such clones, obscuring a potential to score for PCP defects (data not shown). Since the proposed redundancy among the Rho GTPases during PCP generation has not been demonstrated, we can not rule out the possibility that these proteins are required at low levels during this process.

Discussion

In this report we have analyzed in detail the role of Mtl, Rac1, Rac2 and Cdc42, four GTPases of the Rho subfamily in Drosophila. First, we show that overexpression of different Mtl isoforms in eyes and wings produces classical PCP phenotypes, as previously reported for other members of the family, thus suggesting that Mtl also has a role during PCP generation in these tissues. Moreover, genetic interaction assays indicate that Mtl is functionally related to Rac1 and Cdc42, but it does not function in the JNK pathway. Since previous results showed that Rac1 interacts genetically with Rac2 and RhoA (Fanto et al., 2000), we conclude that Rac1, Rac2 and RhoA could act redundantly in Fz/PCP signaling, and Mtl could be acting together with Cdc42, both aspects being connected through Rac1 and Mtl. Regarding this, we also show that flies hemizygous for a hypomorphic Cdc42 allele, as well as mitotic eye clones for the same allele, display typical PCP defects, thus suggesting that Cdc42 may also function in PCP generation. In such a scenario, and in contrast to previously published results (Hakeda-Suzuki et al., 2002), we also demonstrate that mitotic eye clones triply mutant for the Rac GTPases (Mtl, Rac1 and Rac2) show polarity defects, albeit at relatively low frequency. Strikingly, the frequency of the defects is increased in the triple null Rac mutant background by reducing Cdc42 function with a Cdc42 hypomorphic allele. Taken together, all these results suggest that the Rac/Cdc42 GTPases may have a role during PCP generation but probably function redundantly in this process, since only the removal of the four GTPases causes PCP defects. An explanation for the mild PCP phenotypes observed in the quadruple mutant clones, which is comparable to hypomorphic alleles of PCP genes, would be the fact that Cdc42<sup>1</sup> is a hypomorphic allele. In such a scenario, RhoA, another small GTPase of the Rho subfamily, has a well established and non-redundant role in this process (Strutt et al., 1997), and it interacts genetically with Rac1 (Fanto et al., 2000). Since there is a high degree of homology among all these proteins, one possibility could be that the Rac/Cdc42 GTPases cooperate with RhoA during PCP generation. However, since we could not demonstrate the proposed redundancy of function between all these GTPases, an alternative explanation could be that the Rac/Cdc42 GTPases are required at low levels during PCP establishment.
Besides this, the results obtained in the genetic interaction assays indicate that not all the GTPases of the Rho subfamily function upstream the JNK module and that they act in parallel pathways. Our results and previous reports suggest that although Rac1 and Rac2 function downstream of Dsh in the Fz/PCP pathway through JNK/p38 kinases (Boutros et al., 1998; Paricio et al., 1999; Weber et al., 2000), Mtl and Cdc42 might receive a different activating input, as they do not show genetic interactions with gain-of-function Fz and Dsh phenotypes, and Mtl does not interact with JNK components (Boutros et al., 1998; this paper). It is interesting to mention that the rotation-specific phenotype obtained by Mtl overexpression, together with the fact that this GTPase genetically interacts with members of the Egfr pathway and cell adhesion components related to it (data not shown), could indicate that Mtl function in the Egfr pathway regulating ommatidial rotation during the final steps of PCP establishment (this paper; F. Durupt, S.M.-D. and N.P., in preparation). Taken together, all these observations suggest that the requirement of the four GTPases might be subdivided into pairs: Mtl could share a function with Cdc42 (this is supported by data from mammalian tissue culture experiments, where Cdc42 and mammalian Mtl appear to have the same function; A. Hall, personal communication) and Rac1 with Rac2, and both pairs would be connected through the shared functional Rac1-Mtl interaction (Fig. 5). However, whether the GTPase pairs (Mtl-Cdc42, Rac1-Rac2, and Rac1-Mtl) act in parallel or in a hierarchy remains unclear. Although we did not include RhoA in our model, this GTPase functions downstream of Dsh in the Fz/PCP pathway (Strutt et al., 1997) and upstream of the JNK cassette, and interacts genetically with Rac1 (Fanto et al., 2000). Thus, it will function together with the Rac1-Rac2 pair.

In summary, our results are similar to the data obtained from the study of vertebrate gastrulation. Habas et al. (2003) have reported that RhoA and Rac have independent parallel roles during the convergent extension process in vertebrate gastrulation downstream of Fz-Dsh signaling, and that only Rac is able to activate JNK. This is consistent with our data (and previous publications on Drosophila PCP generation; Eaton et al., 1996; Fanto et al., 2000). In addition, our data suggest that Cdc42 could function redundantly with the Rac genes. Supporting this, Cdc42 has also a reported role in convergent extension/vertebrate gastrulation (Choi and Han, 2002). Whether and how this is linked to Fz-PCP signaling remains unclear. Although our results do not provide clear evidence of the redundant function of the GTPases of the Rho subfamily during PCP generation, we can conclude that the situation in vertebrates and Drosophila is similar: not all the GTPases act upstream of a JNK cassette, and there are probably (at least) two parallel Rho GTPase family functions.

Materials and Methods

Generation of flies expressing Mtl transgenes

To generate the UAS-Mtl/wild type construct, the complete Mtl cDNA was cloned into the pUAST Drosophila transformation vector (Brand and Perrimon, 1993). Transgenic flies were generated by standard P-element-mediated transformation (Spradling and Rubin, 1982).

Fly strains and genetic interactions

Flies were grown on standard media at 25°C (unless stated otherwise). GAL4 stocks used were: sev-GAL4 K25 for the third chromosome (gift from Konrad Basler), en-GAL4 and C765-GAL4. Mutant stocks used were: msn102 (Treisman et al., 1997), RhoA+/+RhoAΔV (Strutt et al., 1997), hep575 (Glise et al., 1995), bsk (Riesgo-Escovar et al., 1996), jum2 (Kockel et al., 1997), sbtm (N. Paricio, unpublished), psek-1001 (Gubb et al., 1999), aos (Gaengel and Mlodzik, 2004), Rac1Δ11, Rac2Δ2, MtlΔ (Hakeda-Suzuki et al., 2002), Cdc425, Cdc424 and Cdc423 (Genova et al., 2000).

We also used the sevenless enhancer driven construct sev-RacΔV for interactions with Mtl alleles (Fanto et al., 2000). A sev-Mtl line was generated by recombination of sev-GAL4 and UAS-Mtl chromosomes. Genetic interactions with this line were performed at 25°C. The flies analyzed were heterozygous for sev-Mtl and the mutation of interest. w1118 was used as a negative control.

Generation of mitotic eye clones

Eye clones were generated with the FRT/FLP recombination system (Golic and Linquist, 1989) using ey-FLP lines. To generate clones quadruple mutant for Rac1, Rac2, Mtl and Cdc42 we set up two independent crosses. First, Cdc425/FRT19A females were crossed to Rac1Δ11/Rac2Δ2, MtlΔ and FRT2A/TM6, CyO;2x Ubx sev>Mtl males. Simultaneously, we crossed FRT19A/FM6;ey-FLP females to ey-FLP/Y; spCyO;x2 UbGal4,FRT2A/TM6, Ubx males. From both, we selected the non-balanced progeny and crossed non-balanced males from the first cross to non-balanced females from the second cross. The eye clones are marked by the absence of pigment in adults and by the absence of GFP fluorescence in discs. Adult eye clones were analyzed only in the female offspring, in which 50% will be quadruple mutant for Rac1, Rac2, Mtl and Cdc42 and 50% will be triply mutant for Rac1, Rac2 and Mtl. Among the clones analyzed, we could distinguish two different populations, based on the frequency of the PCP defects they contained. One population exhibited a similar frequency of defects than the control triple mutant clones generated and corresponds to clones mutant only for the Rac genes. The second population showed PCP defects at higher frequency than the controls and corresponds to the quadruple mutant clones. The differences in frequency of defects between both populations are statistically significant.

Histology and immunohistochemistry

Sections of adult eyes were performed as previously described (Tomlinson and Ready, 1987). Wings were dissected from adult flies in SH solution and mounted in Faure medium. Imaginal disc stainings were done in 0.1 M phosphate buffer, 0.2% Triton X-100 and 10% normal goat serum. Primary antibodies used were mouse anti-Elav and mouse anti-β-gal monoclonal from Cappel). Secondary antibodies coupled to fluorochromes were purchased from Calbiochem. Pictures were taken using a Leica TCS-NT confocal laser-scanning microscope.

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