Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early Drosophila development

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SUMMARY

Rho GTPases play an important role in diverse biological processes such as actin cytoskeleton organization, gene transcription, cell cycle progression and adhesion. They are required during early Drosophila development for proper execution of morphogenetic movements of individual cells and groups of cells important for the formation of the embryonic body plan. We isolated loss-of-function mutations in the Drosophila Rho1 (Rho1) gene during a genetic screen for maternal-effect mutations, allowing us to investigate the specific roles Rho1 plays in the context of the developing organism. Here we report that Rho1 is required for many early events: loss of Rho1 function results in both maternal and embryonic phenotypes. Embryos homozygous for the Rho1 mutation exhibit a characteristic zygotic phenotype, which includes severe defects in head involution and imperfect dorsal closure. Two phenotypes are associated with reduction of maternal Rho1 activity: the actin cytoskeleton is disrupted in egg chambers, especially in the ring canals and embryos display patterning defects as a result of improper maintenance of segmentation gene expression. Despite showing imperfect dorsal closure, Rho1 does not activate downstream genes or interact genetically with members of the JNK signaling pathway, used by its relatives dRac and dCdc42 for proper dorsal closure. Consistent with its roles in regulating actin cytoskeletal organization, we find that Rho1 interacts genetically and physically with the Drosophila formin homologue, cappuccino. We also show that Rho1 interacts both genetically and physically with concertina, a Gα protein involved in cell shape changes during gastrulation.

Key words: Rho GTPase, Drosophila, Morphogenesis, Cytoskeleton, Dorsal closure, Head involution, concertina, cappuccino

INTRODUCTION

During embryogenesis in Drosophila, morphogenetic movements of individual cells and groups of cells are important for the proper formation of the three germ layers and for the structures that are subsequently derived from them. These activities are driven in part by differentiation of specific cells followed by cell shape changes and coordinated cell movements, many of which have been shown to be a direct result of signaling pathways whose targets are capable of effecting the reorganization and relocalization of various cytoskeletal components.

One family of signaling proteins shown to be important in mediating cytoskeletal changes in response to extracellular cues is Rho and its relatives, Rac and Cdc42. Rho, Rac and Cdc42 are small GTPases in the Ras superfamily initially shown through work on fibroblasts to affect the organization of the actin cytoskeleton (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). Specifically, microinjection of constitutively active Rho causes the rapid formation of stress fibers, actin-myosin filaments anchored to integrin complexes in the cell membrane (Ridley and Hall, 1992), while activated Rac induces lamellipodial (membrane ruffle) formation (Ridley et al., 1992) and activated Cdc42 results in filopodial extension (Kozma et al., 1995; Nobes and Hall, 1995). Moreover, Cdc42 can induce Rac, which in turn induces Rho, suggesting that, although each family member mediates particular aspects of cytoskeletal function, they are linked hierarchically (Nobes and Hall, 1995). In addition to its role in mediating actin-myosin interactions, Rho GTPases have been implicated in many seemingly disparate cellular processes such as cell cycle progression, cadherin assembly, MAP kinase signaling and phospholipid turnover through activation of PI-3 kinase (reviewed in: Narumiya, 1996; Van Aelst and D’Souza-Schorey, 1997; Hall, 1998; Mackay and Hall, 1998). The diversity of functions exhibited by Rho and its relatives suggests that they may operate within a number of different pathways at particular times and places during development. The ability of Rho family members to regulate the cytoskeleton, in particular, makes them excellent candidates for genes involved in morphogenesis.

To date five Rho family members have been identified in Drosophila: Rho1, RhoL (Rho-like), dRacA, dRacB and dCdc42 (Harhiran et al., 1995; Murphy and Montell, 1996). Studies using constitutively activated (ca) and dominant-negative (dn)
forms of these proteins indicate roles for these GTPases in a variety of processes linked to cell shape changes and actin cytoskeleton regulation during Drosophila development. During oogenesis, expression of dCdc42(dn) and RhO(dn) causes nurse cell collapse and subcortical actin breakdown, while expression of RhO(ca) disrupts nurse-cell–follicle-cell contacts (Murphy and Montell, 1996). dRac(dn) was also shown to affect the migration of border cells, a specialized subset of follicle cells in the oocytes (Murphy and Montell, 1996). In the embryo, ectopic expression of dRac(dn) and dCdc42(dn) reveals roles for these proteins in dorsal closure, by affecting the JNK signaling pathway (Glise et al., 1995; Harden et al., 1995, 1996; Riesgo-Escovar et al., 1996; Noselli, 1998). Disruptions are also seen during neurogenesis and myogenesis where dRac(dn) affects axonal outgrowth of the peripheral neurons and fusion of the myoblasts, respectively (Luo et al., 1994). Other family members play roles later in development such as during the formation of the compound eye where RhO(dn) was shown to cause a collapse of the actin network important for proper ommatidial morphogenesis (Hariharan et al., 1995). Because there is a high level of conservation among Rho family members, the specificity of each member for the different developmental processes is not yet clear.

In Drosophila, a single RhoA homologue (Rho1) has been identified molecularly (Hariharan et al., 1995; Sasamura et al., 1997). Consistent with what is known in mammalian cells, studies of Rho1(dn) and Rho1(ca) suggest that Rho1 is involved in regulation of actin cytoskeleton, transcriptional regulation and cell cycle progression/cell proliferation. We have identified P-element insertions within the Drosophila Rho1 gene during a genetic screen for maternal-effect mutations, allowing us to investigate the specific roles Rho plays in the context of the developing organism. These same P-element-induced mutations were also identified in a genetic screen for rough eye mutants where they were shown to affect ommatidial polarity during eye development and were placed downstream of a Frizzled receptor (Strutt et al., 1997). Here we report that loss of Rho1 function also results in both maternal and embryonic phenotypes. Embryos homozygous for mutant Rho1 exhibit a characteristic zygotic phenotype, which includes imperfect dorsal closure and severe defects in head involution. The Rho1 maternal effect phenotypes consist of disorganized actin cytoskeletal structures in ovaries, especially the outer ring canals, and defective segmentation gene expression. Together, these phenotypes suggest that a Rho-mediated signaling cascade is required for the proper execution of many morphogenetic events during Drosophila oogenesis and embryogenesis.

MATERIALS AND METHODS

Fly stocks

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract medium at 25°C. The alleles used in this study are: Rho1(W3), Rho1(k07236) (from I. Kiss, T. Laverty and G. Rubin); ctcAC10, ctc(O835) (S. Parks); capu8R12, capu27L (L. Manseau); DRhOGEF2 (K. Barrett); hep1, hep75S (S. Noselli); arm199S, armD105S (M. Peifer); pucAC16 (A. Martinez-Arias); and bsk1, aopP, Egfl1, Egfl2, wgp2 (Bloomington Stock Center). Revertants of the Rho alleles were generated as described in Török et al. (1993). One of these, RhoD220, was determined by Southern analysis to remove a portion of the coding region thereby affecting all Rho1 transcripts.

Embryo/ovary phenotypic analysis

Embryos were prepared and immunohistochemical detection of proteins was performed as described previously (Parkhurst et al., 1990) using alkaline-phosphatase-coupled secondary antibodies (Jackson Labs) visualized with Substrate Kit II reagents (Vector Labs, Inc.), or Alexa 488- or 594-conjugated fluorescent secondary antibodies (Molecular Probes). Antiserum used were as follows: anti-Hb from D. Tautz; anti-Kr from S. Carroll; anti-Eve, anti-En and anti-FasIII from N. Patel and C. Goodman; anti-Fz from H. Krause; anti-Wg from R. Nusse; anti-β-tubulin (N357) from Amersham; and anti-phosphotyrosine (clone 4G10) from Upstate Biotechnology Inc. Embryos were prepared and stained with acridine orange as described by Abrams et al. (1993).

Embryos to be stained with phallolidin were fixed for 3 minutes in a 37% formaldehyde/heptane bilayer. Embryos were then incubated in 400 μl of DAPI (1 μg/ml) + 1 unit rhodamine- or Alexa 594-conjugated phallolidin (Molecular Probes) for 20 minutes, washed 3× in PBS + 0.025% Tween and mounted in 80% glycerol with 20 mM sodium carbonate buffer (pH 9.5) + 4% n-propyl gallate to inhibit photobleaching. Ovaries were dissected by hand, then fixed in 4% formaldehyde in PBS + 0.05% Tween for 20 minutes. Following fixation, ovaries were washed 3× in PBS-Tween, permeabilized in PBS-Tween + 1% Triton X-100 for 1 hour; then stained with 3 units rhodamine- or Alexa 594-conjugated phallolidin in 400 μl DAPI (1 μg/ml) for 1.5 hours.

Immunohistochemical whole-mount in situ hybridization was performed according to the protocol of Tautz and Pfeifle (1989). Digoxigenin-substituted probes were obtained by PCR amplification of the dpp cDNA.

Embryos for scanning electron microscopy analysis were fixed in a 25% glutaraldehyde (Polysciences)/heptane bilayer for 15 minutes. The vitelline membrane was removed by replacing the glutaraldehyde layer with methanol. Embryos were hydrated in 1× PBS then incubated in 2% osmium (Polysciences) in 1× PBS for 30 minutes. Embryos were dehydrated in an ethanol series then placed in hexamethyldisilazane (HMDS; Ted Pella). The HMDS was allowed to evaporate, then the embryos were mounted on carbon black tape and sputter-coated using gold-palladium.

Larval cuticle preparations were prepared and analyzed as described by Wieschaus and Nüsslein-Volhard (1986).

Genomic characterization

Isolation of the genomic DNA flanking the P-element insertion site was carried out as described by Cooley et al. (1988). These flanking sequences were used to screen an EMBL3 Drosophila melanogaster genomic library (from R. Blackman). Restriction enzyme and Southern analyses were used to characterize and compare the DNA flanking the P elements with that of the overlapping genomic phage clones. The DNA flanking the k07236 P element was also used to screen an ovary genomic characterization by Abrams et al. (1993).

Genomic and cDNA fragments were subcloned into Bluescript and overlapping subclones were generated using ExoIII nuclease (Henikoff, 1984). Sequencing was carried out manually using Sequenase (United States Biochemical) or with Taq DyeDeoxy terminator AutoSequencing (Applied Biosystems). Rho1 genomic and three cDNA sequences (Rho1 m3, Rho1 m7, Rho1m8) are available from the GenBank database under accession numbers AF177871, AF177872, AF177873 and AF177874, respectively.

Northern analysis

Developmental staged RNAs were prepared as previously described (Mozer et al., 1985). Northern production and hybridization was as described (Mozer et al., 1985). Using 5 μg of poly(A)+-selected RNA probe and Maganagraph membrane (Micro Separations Inc.), DRho1 is expressed ubiquitously during development and was used as a loading control (Mozer et al., 1985).
In vitro interactions of GST fusion proteins
The full-length cta ORF (58A, Parks and Wieschaus, 1991) was obtained by PCR then subcloned into the BamHI-EcoRI site of pCite 4C* (Novagen). capu was subcloned into the EcoRI-NotI sites of pCite 4A* using the EcoRI-NotI capu fragment from the Capu-pEG202 expression plasmid described by Manseau et al. (1996). The Cta and Capu ORFs were expressed from the T7 promoter using the Promega TnT in vitro expression kit ([35 S]methionine labeled). The Rho1 full-length cDNA was subcloned into the BamHI-EcoRI sites of pGEX-3X (AMRAD Corp.). The GST-Rho1 fusion protein was expressed at 30°C to improve protein solubility. The GST-Rho1 protein was incubated with GDF or GTP\(\gamma\)S and the binding assays were performed as described by Lu and Settleman (1999), then analyzed by SDS-PAGE. The in vitro translated full-length Hairy protein and GST-dCtBP fusion protein used as a negative control were described previously (Poortinga et al., 1998).

RESULTS

Molecular characterization of the Rho1 gene
We have identified two P-element insertions within the Drosophila homologue of the mammalian RhoA gene from a collection of lethal P-element insertion lines (allele designations: Rho1\(^{(2)k07236}\) and Rho1\(^{(2)k02107}\); Török et al., 1993; Hariharan et al., 1995; Sasamura et al., 1997). Plasmid rescue and sequence analysis of the genomic DNA flanking the P elements indicated that the Rho1\(^{(2)k07236}\) element is inserted in an intron 33 bp upstream of the ATG, whereas the Rho1\(^{(2)k02107}\) element is inserted in an intron within the coding sequences (see Fig. 1A). These P elements localize to cytological region 52E (data not shown; Hariharan et al., 1995; Strutt et al., 1997).

Genomic libraries were screened using probes from the sequences flanking the P element and a restriction map of the genomic region was generated. This map was confirmed by sequencing roughly 17 kb of genomic DNA surrounding the Rho1 locus and is shown in Fig. 1 (see Materials and Methods). The same fragment was then used to screen embryonic and ovarian cDNA libraries (see Materials and Methods), and cDNAs ranging in size from 1.2 to 2.1 kb were recovered. Sequence analysis of these cDNAs revealed that they encompass at least six different classes of transcripts, all of which share an identical ORF encoding a putative protein of 192 amino acids. The variation in sizes among the cDNAs results from alternative splicing at both the 5′ and 3′ untranslated ends of the message (Fig. 1A and data not shown).

Using a cDNA fragment encompassing the entire Rho1-coding region as a probe on a developmental northern blot, four transcripts were identified. Two major transcript classes, corresponding to 1.3 and 2.1 kb, are expressed at all stages of development, although relative levels of each vary between stages (Fig. 1B). In addition, there are two transcripts of 3.1 and 3.7 kb, which are first expressed at pupariation (day 6) and persist until adulthood in females (but are not detected in males). The sizes of the cDNAs identified suggest that each of the major transcript bands on the northern represent multiple transcript forms. In the future, it will be important to determine the dynamic temporal or spatial changes associated with each Rho1 splice form and the relative contribution/developmental consequences associated with each form.

Disruption of zygotic Rho1 function is associated with defects in morphogenetic processes
Rho1 is essential for zygotic function as progeny that are homozygous for either the Rho1\(^{(2)k07236}\) or Rho1\(^{(2)k02107}\) mutations die as embryos. Rho1\(^{(2)k07236}\) fails to complement Rho1\(^{(2)k02107}\), and their maternal and zygotic phenotypes are identical. Both Rho1\(^{(2)k07236}\) and Rho1\(^{(2)k02107}\) are strong alleles as imprecise excisions of the P element do not differ greatly in their phenotypic severity (data not shown). All subsequent analyses were done using an imprecise excision allele derived from the Rho1\(^{(2)k07236}\) allele (Rho1\(^{rev220}\)), from this point on referred to as Rho1; (see Materials and Methods).

Heterozygous Rho1 embryos are viable and have no embryonic cuticle defects, while homozygous Rho1 embryos die with holes in the dorsal anterior region of the cuticle and a disruption of the dorsal surface that stretches the ventral surface.
and causes the cuticles to bow slightly (Fig. 2B). To better identify the processes leading to such disruptions, scanning electron microscopy (SEM) was used to visualize embryonic morphology throughout gastrulation. The most striking defects occur late in gastrulation when the embryos fail to undergo head involution, a process whereby the anterior structures of the embryo are internalized through dramatic cell shape changes and movements. The procephalon, which cannot secrete cuticle, remains on the exterior, leading to a characteristic dorsal anterior hole in the larval cuticle (B) is a result of failed internalization of head structures on the Rho1 embryo (D), resulting in the brain remaining on the anterior dorsal surface with the subsequent failure of this region to secrete cuticle. Anterior is left and dorsal is up in all images.

Reduced maternal Rho1 function disrupts actin cytoskeletal structure in ovaries

Previous studies utilizing ectopic expression of dominant-negative and constitutively active transgenes of UAS-DRhoA or UAS-Dcdc42 expressed with Hs-GAL4. They observed segmentally reiterated spaying of cells and loss of myosin staining and phosphotyrosine nodes in leading edge cells flanking the segment border. While we observe aberrant cell shapes and inappropriate constriction of cells along the leading edge, we do not observe the segmental reiteration of these losses in the Rho1 loss-of-function mutation. In addition, nodes of phosphotyrosine expression are still visible in splayed out cells.

To look further at cell shape along the leading edge, we looked at the expression of fasciclin III (fas III). While fas III is present in the leading edge cells of wild-type embryos, it is not present in the dorsalmost edge of these cells (Fig. 4G,I) until they are touching the cells of the opposing leading edge epithelia (Fig. 4H). When cells along the leading edge in Rho1 mutations constrict inappropriately, they appear to recognize their lateral neighbors as those on the opposing leading epithelial edge and elicit downstream events prematurely. Consistent with this possibility, we sometimes observe the appearance of secondary ‘midlines’ perpendicular to the major midline (Fig. 4F).

During dorsal closure in wild-type embryos, actin and myosin localize along the leading edge of the dorsal lateral epidermis as they extend dorsally and have been proposed to act as part of the driving force of the cell shape changes occurring at this time (Young et al., 1993; Edwards et al., 1997). We examined dorsal closure in wild-type and Rho1 mutants using phalloidin to visualize actin structures at successive developmental time points. Actin is enriched in the cortices of individual cells, allowing the cellular morphology during dorsal closure to be documented. As the two leading epithelial edges meet in wild-type embryos, they fuse from either end to form a straight, seamless dorsal midline (Fig. 3A,E,I,M). Embryos homozygous for the Rho1 mutation undergo dorsal closure, in that the lateral epithelia do come together at the dorsal surface of the embryo (Fig. 3B,F,J,N); however, this process is disorganized compared to wild type. At higher magnification, cells along the dorsal midline in wild-type embryos are well ordered and columnar in shape (Figs 3C,G,K,O, 4A). In Rho1 mutant embryos, cells along the dorsal midline are inappropriately shaped, pinched together in some regions and stretched out in others (Figs 3D,H,L,P, 4B). This is especially clear in the later stages of dorsal closure, after the epithelia have come together (Figs 3N,P, 4B). We do not observe any disruptions to the microtubule network as visualized by β-tubulin staining (Fig. 4C,D), suggesting that Rho1 primarily affects actin cytoskeletal components.

Harden et al. (1999) have reported disruptions to the leading edge cytoskeletal components using dominant negative and constitutively active transgenes of UAS-DRhoA or UAS-Dcdc42 expressed with Hs-GAL4. They observed segmentally reiterated spaying of cells and loss of myosin staining and phosphotyrosine nodes in leading edge cells flanking the segment border. While we observe aberrant cell shapes and inappropriate constriction of cells along the leading edge, we do not observe the segmental reiteration of these losses in the Rho1 loss-of-function mutation. (Fig. 4). In addition, nodes of phosphotyrosine expression are still visible in splayed out cells (Fig. 4F).
of actin resulting from incomplete cytokinesis that allow for the exchange of cytoplasmic material between nurse cells and the developing oocyte. Egg chambers from females with reduced Rho1 function show a general disruption of the actin cytoskeleton, particularly in the outer ring canals and oocyte cortex (Table 1; Fig. 5E-H). The inner ring canals appear relatively normal and oogenesis is able to proceed in these females leading to inviable embryos with patterning defects (see below).

Reduced maternal Rho1 function also disrupts segmentation

The maternal effect of the Rho1 mutation on embryos is distinct from its zygotic effects: embryos derived from mothers with reduced Rho1 function show a general disruption of the actin cytoskeleton, particularly in the outer ring canals and oocyte cortex (Table 1; Fig. 5E-H). The inner ring canals appear relatively normal and oogenesis is able to proceed in these females leading to inviable embryos with patterning defects (see below).

Maternal Rho1 activity does not contribute to the Rho1 zygotic phenotype

The Rho1 loss-of-function mutation does not exhibit all the phenotypes expected if it is the primary target of genes such as DRhoGEF2: whereas DRhoGEF2 mutations and ectopic expression of dominant negative Rho1 block gastrulation (Barrett et al., 1997; Häcker and Perrimon, 1998), we do not observe this phenotype with the Rho1 loss-of-function zygotic mutation (Fig. 2). To determine if maternal Rho1 activity was masking this phenotype, we looked at the Rho1 zygotic phenotypes when maternal Rho1 activity was reduced. Homozygous Rho1 mutant embryos derived from mothers with reduced maternal Rho1 activity exhibit both the maternal segmentation phenotype and the zygotic morphogenetic phenotypes (Fig. 6K-M). New phenotypes are not uncovered and the zygotic Rho1 phenotype is not enhanced in this background (compare to Fig. 2D).
Engrailed and Wingless expression is disrupted in maternal Rho1 mutants

The segmentation phenotype in embryos with reduced maternal Rho1 activity indicates a maternal role for Rho1 in patterning events that establish the embryonic body plan. To identify the developmental stage at which Rho1 is necessary, we stained embryos derived from mothers with reduced maternal Rho1 activity with a collection of antibodies recognizing segmentation gene products, including Bcd (maternal), Hb and Kr (gap), Eve and Ftz (pair rule), and En (segment polarity). All of the antibodies tested show that segmentation products are set up properly in Rho1 maternal embryos (data not shown). However, while the En segment polarity protein is initially expressed properly (Fig. 7D), it fails to maintain its proper expression and shows severe aberration in pattern by stage 9 (Fig. 7E,F). Since Wingless (Wg) signaling is necessary for the maintenance of En expression (DiNardo et al., 1988), we next examined Wg expression in these embryos. Like En, Wg expression is initiated correctly (not shown), but fails to be maintained properly (Fig. 7H).

Rho1 does not interact with members of the JNK pathway

Because Rho GTPases have been linked to a number of pathways in cellular systems, we looked at Rho1 in the background of other mutations to see whether we could genetically link Rho1 to similar pathways in Drosophila (cf. Noselli, 1998) and thereby correlate the numerous Rho pathways to specific roles in organismal development. We tested Rho1 with putative interacting mutations (pim) using trans-heterozygous (Rho1+/+;pim+/+), dose sensitive (Rho1/Rho1;pim/+ or Rho1+/+;pim/pim) and double mutant (Rho1/Rho1;pim/pim) combinations. No genetic interactions were detected with hemipterous and basket (members of the JNK signaling pathway), chickadee (cytoskeletal protein), puckered (dorsal closure mutant), wingless and armadillo (Wingless signaling), anterior open (ras signaling), or EGFR (EGF receptor) (Baker, 1988; Peifer et al., 1991; Cooley et al., 1992; Lai and Rubin, 1992; Glise et al., 1995; Rogge et al., 1995; Riesgo-Escovar et al., 1996; Glise and Noselli, 1997; Martin-Blanco et al., 1998). We could not detect a genetic interaction with DDrhoge42 (Barrett et al., 1997); however, we only examined trans-heterozygous interaction since DDrhoge42 and Rho1 map next to each other (<1 map unit) and we have not yet recovered a recombinant double mutant chromosome.

The lack of genetic interactions of Rho1 with Wg and JNK signaling components during oogenesis and early embryonic development was unexpected in light of Rho1’s requirement for dpp’s expression in the leading edge cells (cf. Glise and Noselli, 1997; Hou et al., 1997). Since inability to detect genetic interactions does not rule out a role for Rho1 in JNK signaling, we also examined dpp and puc expression in embryos
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homozygous mutant for Rho1 (Fig. 8). Consistent with the lack of genetic interactions, dpp and puc are expressed in Rho1 homozygous mutant embryos. Egg chambers from mothers with reduced Rho1 maternal activity (E-H) or trans-heterozygous for Rho1 and either dpp or puc mutations (M-P) display defects in actin cytoskeleton structures, including defects at the nurse cell boundaries, along the cortex of the developing oocyte, and in the outer ring canals, cellular bridges formed by incomplete cytokinesis of the germline cells. Egg chambers from homozygous mothers show similar, albeit weaker, disruptions to the actin cytoskeleton. In addition, aberrant accumulation or disruption of actin is visible by punctate phallolidin staining within the follicle cells.

**Table 1. Ring canal morphology is disrupted in egg chambers with reduced Rho1 maternal activity**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild type (%)</th>
<th>Mutant (%)</th>
<th>No. scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>wimp/+</td>
<td>67</td>
<td>33</td>
<td>359</td>
</tr>
<tr>
<td>Rho1/+</td>
<td>62</td>
<td>38</td>
<td>194</td>
</tr>
<tr>
<td>Rho1+/wimp/+</td>
<td>39</td>
<td>61</td>
<td>615</td>
</tr>
<tr>
<td>Rho1 capu/capu(1D2) +</td>
<td>25</td>
<td>75</td>
<td>203</td>
</tr>
<tr>
<td>Rho1 capu/capu(1D2) +</td>
<td>33</td>
<td>67</td>
<td>166</td>
</tr>
<tr>
<td>Rho1 capu/capu(1D2) +</td>
<td>26</td>
<td>74</td>
<td>197</td>
</tr>
<tr>
<td>Rho1 capu/capu(1D2) +</td>
<td>76</td>
<td>24</td>
<td>123</td>
</tr>
<tr>
<td>hep73+/Rho1+</td>
<td>81</td>
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<td>155</td>
</tr>
<tr>
<td>arm+/Rho1+</td>
<td>76</td>
<td>24</td>
<td>168</td>
</tr>
</tbody>
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cappuccino (capu; formin homologue; Emmons et al., 1995) and concertina (cta; Gα protein; Parks and Wieschaus, 1991). Egg chambers from mothers heterozygous for Rho1 (Rho1+/+) exhibit normal actin morphology (Table 1). Egg chambers from mothers trans-heterozygous for Rho1 and capu (Rho1 capu+/+) or cta (Rho1 cta+/+) exhibit disruptions of the actin cytoskeleton (Table 1; Fig. 5I-P), similar to that in females with reduced maternal Rho1 activity (Table 1; Fig. 5E-H). While capu has been shown to affect actin integrity during oogenesis, similar studies have not been reported for cta. We examined phallolidin staining in egg chambers from homozygous cta mothers and find similar, albeit weaker, disruptions to the actin cytoskeleton. In addition, aberrant accumulation or disruption of actin is visible by punctate phallolidin staining within the follicle cells. Egg chambers from homozygous cta mothers show similar, albeit weaker, disruptions to the actin cytoskeleton.

We also used an in vitro binding assay to examine the interaction specificity between the Rho1 and Cta or Capu proteins. Rho1 fused to glutathione S-transferase (GST-Rho1) was expressed in bacteria and immobilized on glutathione-Sepharose beads. Rho1 was then tested for its ability to bind [35S]-labeled full-length Cta or Capu proteins. Consistent with the observed genetic interactions, Rho1 specifically pulls down full-length Cta or Capu (Fig. 9). Interestingly, Capu preferentially
interacts with GTP-bound Rho1, whereas Cta interacts equally with GTP- or GDP-bound Rho1.

**DISCUSSION**

Rho GTPases play a central role in diverse biological processes such as actin cytoskeleton organization, gene transcription, cell cycle progression and adhesion. It is not yet clear, however, if the same or different biochemical mechanisms are used by Rho family proteins to regulate these activities. Rho GTPases are required during early *Drosophila* development where morphogenetic movements of individual cells and groups of cells are important for the proper formation of the embryonic body plan. Our characterization of the *Rho1* maternal and zygotic phenotypes suggests that, while *Rho1* is likewise mediating diverse biological pathways during embryonic development, the underlying mechanism used for each process may be similar.

**Rho1’s zygotic phenotypes appear to result from direct effects on the cytoskeleton**

Dorsal closure involves the migration of the lateral epithelial sheets to cover the dorsal region of the embryo, a process requiring the proper localization of actin and myosin along the leading edge. Mutants that fail to undergo this process appropriately cannot secrete cuticle on the dorsal surface and exhibit a large dorsal hole or ‘dorsal open’ cuticular phenotype. Dorsal closure mutants can be divided into two main classes: those that affect cellular architecture such as the cytoskeleton (e.g., *capu*, *zipper*) or cell junctions (e.g., *coracle*, *canoe*), and those that are involved in signaling pathways (e.g., *basker* and *hemipterous*), which encode components of the MAPK-like JNK pathway (reviewed in Noselli, 1998).

Studies of dominant negative and constitutively active Rho1, dRac and dCdc42 suggest they play roles in dorsal closure: the resulting embryos display large dorsal holes and puckering of the dorsal surface (Hariharan et al., 1995; Harden et al., 1995, 1999; Barrett et al., 1997; Strutt et al., 1997). More recently, activated forms of dRac and dCdc42 have been linked genetically with components of the p38 MAPK and JNK signaling pathways during dorsal closure (Glise et al., 1995; Harden et al., 1995, 1996, 1999; Riesgo-Escovar et al., 1996; Glise and Noselli, 1997; Hou et al., 1997; Noselli, 1998), suggesting a signal transduction mechanism leading to proper control of cell morphology.
Fig. 7. Disruption of Engrailed and Wingless expression in maternal Rho1 mutants. (A-C) Wild-type embryos at progressively older stages stained with anti-En antibodies. En stripes are initiated and maintained in these embryos. (D-F) Embryos from mothers with reduced Rho1 activity stained with anti-En antibodies. While En expression is initiated properly in these mutant embryos (D), some stripes are not apparent in late stage embryos, indicative of a failure in En maintenance. (G) Wild-type embryo stained with anti-Wg antibody showing the normal pattern of Wg expression in stripes 2, 3 and 4 of late stage embryos. (H) A similarly staged embryo with reduced maternal Rho1 activity demonstrating abnormal Wg expression in these same stripes, suggestive of a failure in Wg autocrine signaling. Anterior is left in all images.

The dorsal closure phenotype that the Rho1 loss-of-function mutations display is distinct from that observed with ectopic expression of its dominant negative or constitutively active forms and from that of other dorsal closure mutants as well. While cells in Rho1 loss-of-function mutations are inappropriately shaped and do not form a straight seamless dorsal midline, they complete dorsal closure. However, Rho1 loss-of-function mutations do exhibit severe defects in head involution. Head invagination is a complex and well-ordered rearrangement of cells that results in the internalization of anterior head structures. The anterior dorsal hole observed in Rho1 homozygous mutant larval cuticle preparations is a consequence of failed head involution, rather than aberrant dorsal closure (see Fig. 2). In addition, we find that Rho1 does not activate downstream genes (dpp or puc) or interact genetically with basket or hemipterous, suggesting that Rho is not utilizing the same JNK signaling pathways as its relatives dRac and dCdc42 to effect changes in cell morphology at the dorsal midline.

Work on fibroblasts suggests that Rho proteins act hierarchically (Cdc42>Rac>Rho; Nobes and Hall, 1995). Cdc42 can induce Rac (filopodia are usually present with lamellipodia), and Rac, in turn, can induce Rho, albeit weakly. In neuronal cells, however, the existence of a linked pathway is less clear. In these cells, the consequences of activated Rac or Cdc42 expression appears to act opposite to that of Rho: Rac or Cdc42 expression results in formation of lamellipodia and filopodia along neurite extensions, whereas Rho expression induces neurite retraction and cell rounding (Kozma et al., 1997; Hall, 1998). Recent work on Drosophila PKN and ectopically expressed dominant negative and constitutively active Rho1 suggests these proteins are likely not functioning in a hierarchy and mediate a pathway that is independent of JNK signaling (Harden et al., 1999; Lu and Settlemen, 1999). Our results are consistent with Rho1 acting in a different fashion than that reported for dRac and dCdc42: Rho1 appears to have a direct effect on regulation of cytoskeletal components necessary for proper coordination of cell shape changes, rather than as part of a signaling pathway, during early Drosophila development.

Phenotypes resulting from reduced maternal Rho1 activity may result from direct effects on cytoskeletal components

Ectopic expression studies using dCdc42(dn) and dRhoL(dn) indicate that these Rho1 relatives affect actin structure in the ovary: the expression of these proteins results in nurse cell collapse and subcortical actin breakdown (Murphy and Montell, 1996). Reducing Rho1 function maternally also affects the integrity of the actin cytoskeleton. Egg chambers derived from females with reduced Rho1 activity exhibit generally disorganized actin cytostructure, as well as disruptions to more specialized cytoskeletal structures such as the ring canal morphology. While reduced Rho1 maternal activity affects ring canal morphology, especially that of the outer ring canal, it does not affect their generation or growth. Some of the molecules involved in ring canal morphogenesis have been identified (Cooley and Theurkauf, 1994; Robinson and Cooley, 1997). Phosphorylation events seem to play an important role, and recent studies have implicated two tyrosine kinases, Src64 and Tec29, in normal ring canal development (Cooley, 1998; Dodson et al., 1998; Roulier et al., 1998). Mutations in these genes show defects in ring canal growth that we do not observe in Rho1 mutants. Hu-li tai shao (Hts) encodes a protein localized to ring canals following the increase in phosphotyrosine levels that may direct the addition of actin filaments to the developing ring canal. Kellc encodes another protein localized to ring canals after Hts, which may bundle actin filaments (Robinson et al., 1994). Most of these mutations lead to a ‘dumpless’ phenotype: ring canals are blocked by nuclei not properly held in place by the disordered actin filaments, thus preventing the transport of cytoplasm to the oocyte from the adjoining nurse cells. We do not observe a dumpless phenotype in egg chambers from mothers with reduced Rho1 activity. Despite the actin disorganization observed, oogenesis proceeds in these ovaries.

Consistent with Rho interacting directly with cytoskeletal proteins, we observe a strong genetic interaction between Rho1 and capu, suggesting that capu is a downstream effector of Rho1’s organization of the actin cytoskeleton of the ovary. capu encodes a member of the formin homology (FH) class of proteins, which includes diaphanous in Drosophila, cyk-1 in C.
elegans, BNII and BNR1 in budding yeast, cdc12 in fission yeast and the founding member of the family, the limb deformity locus in mice (Mass et al., 1990; Castrillon and Wasserman, 1994; Emmons et al., 1995; Watanabe et al., 1997; Petersen et al., 1998; Swan et al., 1998). These genes have been implicated in processes involving cytoskeletal regulation such as cytokinesis and the establishment of cell polarity. The yeast formin, BNI1p, has been shown to interact with a yeast Rho gene, Rho1p (Imamura et al., 1997; Watanabe et al., 1997). Similarly, we find that Rho1 and Capu interact physically in an in vitro binding assay.

Embryos obtained from mothers with reduced Rho1 activity are able to proceed through embryogenesis, however, they do not hatch and show severe patterning defects, suggestive of effects on transcriptional regulation. We have shown that the early steps of the transcriptional cascade controlling segmentation are normal in embryos with reduced Rho1 maternal activity. The observed segmentation defects are first manifested by the failure to maintain Engrailed (En) expression. En maintenance has been shown to be dependent on the presence of the Wingless (Wg) protein (reviewed in: Martinez-Arias, 1993). Wg is also required for its own maintenance (Dinardo et al., 1998). Wg is made in a cell adjacent to the cell expressing En. It is then secreted and taken up by the En-expressing cell (paracrine signaling) and by the secreting cell itself (autocrine signaling). The segmentation phenotype associated with reduced maternal Rho1 activity could result from different biochemical mechanisms. The segmentation phenotype could be due to direct effects of the actin cytoskeleton: secretion or endocytosis could be disrupted whereby the Wg- and/or En-expressing cell fail to take up Wg protein. Consistent with this possibility, a role for Rho in receptor-mediated endocytosis has been described (Lamaze et al., 1996). Alternatively, Rho1 could be a component of the Wg signaling pathway that affects transcription of segmentation genes in the En-expressing cell. While further experiments will be required to distinguish between these possibilities, this segmentation phenotype either provides an in vivo system to look at Rho1’s effects on transcriptional regulation or complements Rho1’s other phenotypes providing an additional process in which to investigate Rho’s effects on cytoskeletal components.

Rho GTPase regulatory pathways
The numerous seemingly distinct biological responses of the Rho GTPase suggests that its activation must be both temporally and spatially regulated. Part of this regulation is likely to come from interaction of Rho with different GEFs. The mechanisms that lead to activation of Rho family proteins by extracellular signals are thought to be similar to that of Ras: mediated by GEFs linked to heterotrimeric G protein coupled membrane receptors (reviewed in: Machesky and Hall, 1996; Van Aelst and D’Souza-Schorey, 1997; Zohn et al., 1998). A large family of RhoGEFs have been identified in mammalian systems, some of which are specific for a particular family member (e.g., Lbc for Rho, Tiam1 for Rac), while other GEFs act on all members (Michiels et al., 1995; Zheng et al., 1995; Olson et al., 1996). Three RhoGEFs have been
identified in *Drosophila*, but little is known about their specificity. No mutations corresponding to DRhoGEF1 have been reported (Werner and Manseau, 1997). DRhoGEF2 (*shar pei*) has been shown to affect many of the morphogenetic movements associated with gastrulation and suppress genetic phenotypes associated with overexpression of wild type or constitutively active Rho1 (Barrett et al., 1997; Häcker and Perrimon, 1998). However, while both Rho1 and DRhoGEF2 loss-of-function mutations affect gastrulation, their phenotypes are very different and we do not detect trans-heterozygous genetic interactions between DRhoGEF2 and loss-of-function Rho1 mutations. The third *Drosophila* RhoGEF, Pebble, does interact genetically with Rho1 loss-of-function mutations (Prokopenko et al., 1999). Since the identified RhoGEFs do not have completely overlapping phenotypes with Rho1 loss-of-function mutations, it is likely that additional RhoGEFs exist. Similar, since phenotypes associated with loss-of-function mutations in dRac, dCdc42 and dRhoL have not yet been reported, the specificity of the existing RhoGEFs is not yet known.

Work in fibroblasts suggests a role for subunits of the heterotrimeric Gα proteins (G12 and G13) in Rho-mediated signaling. While the exact link between the G proteins and Rho family proteins has not been described, a physical interaction between specific RhoGEFs and Gα proteins has not been described, a physical interaction to appropriately organize cell shape changes during *Drosophila* gastrulation (Parks and Wieschaus, 1991; Morizé et al., 1998). Ectopic expression studies utilizing dominant-negative Rho1 led to its implication in the cell shape changes leading to proper ventral furrow formation, consistent with studies showing disruption of *Drosophila* celularization after microinjection of the botulinum C3 exoenzyme Rho-specific inhibitor (Barrett et al., 1997; Crawford et al., 1998). While Rho1 loss-of-function mutations do not show the same severe cellularization or gastrulation phenotypes of DRhoGEF2, Rho1 does interact both genetically and physically with cta, suggesting that Rho1 is likely to be a downstream effector of the Cta Gα protein in the ovary. Interestingly, Cta interacts equally with the GTP- and GDP-bound forms of Rho1 and may form a complex including GEFs.

Proper oogenesis and morphogenesis in *Drosophila* are dependent on Rho1 activity. Because these are complicated developmental processes involving multiple cellular events, it is expected that a large number of genes are involved in regulating and executing them. To understand the biochemical mechanisms through which Rho family proteins regulate the organization of the actin cytoskeleton, gene transcription, and their other associated activities, identification of regulatory factors and cellular targets is essential. *Drosophila* offers a genetically amenable system in which to systematically identify components of the Rho pathway required for the proper execution of these events. Future genetic screens with loss-of-function Rho1 mutations should also help in identification of regulators and effectors, an important step in describing the pathways through which Rho acts in the organism.

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