

# Migration of the *Drosophila* primordial midgut cells requires coordination of diverse PS integrin functions

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Accepted 1 September; published on WWW 21 October 1999

## SUMMARY

Cell migration during embryogenesis involves two populations of cells: the migrating cells and the underlying cells that provide the substratum for migration. The formation of the *Drosophila* larval midgut involves the migration of the primordial midgut cells along a visceral mesoderm substratum. We show that integrin adhesion receptors are required in both populations of cells for normal rates of migration. In the absence of the PS integrins, the visceral mesoderm is disorganised, the primordial midgut cells do not display their normal motile appearance and their migration is delayed by 2 hours. Removing PS integrin function from the visceral mesoderm alone results in visceral mesoderm disorganization, but only causes a modest delay in migration and does not affect the appearance of the migrating cells. Removing PS integrin function from the migrating cells causes as severe a delay in migration as the complete loss of PS integrin

function. The functions of PS1 and PS2 are specific in the two tissues, endoderm and mesoderm, since they cannot substitute for each other. In addition there is a partial redundancy in the function of the two PS integrins expressed in the endoderm, PS1 ( $\alpha_{PS1}\beta_{PS}$ ) and PS3 ( $\alpha_{PS3}\beta_{PS}$ ), since loss of just one  $\alpha$  subunit in the midgut results in either a modest delay ( $\alpha_{PS1}$ ) or no effect ( $\alpha_{PS3}$ ). We have also examined the roles of small GTPases in promoting migration of the primordial midgut cells. We find that dominant negative (N17) versions of Rac and Cdc42 cause a very similar defect in migration as loss of integrins, while those of Rho and Ras have no effect. Thus integrins are involved in mediating migration by creating an optimal substratum for adhesion, adhering to that substratum and possibly by activating Rac and Cdc42.

Key words: Migration, *Drosophila*, Integrin, Signaling, GTPase

## INTRODUCTION

Cell migration is a vital component of embryonic development. During embryogenesis, cellular migration takes place in a variety of morphogenetic processes, from gastrulation to the formation of the nervous system. Many cell types travel substantial distances to reach their final destination, where they aggregate to form tissues. In the adult organism, migration remains prominent, for example, leukocytes migrate into areas of inflammation and fibroblasts migrate into wounds. Cell migration is a multistep process in which cells extend processes that adhere to the substratum through specific receptors (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996; Sheetz et al., 1998). The stabilization of these adhesion sites is required before the cell can exert force on the substratum and therefore pull the cell forward. Thus the process of assembling and stabilizing adhesion sites is a crucial controlling step of migration. Among the adhesion receptors that have been found to be involved in migration are the integrins, which are a family of  $\alpha\beta$  transmembrane receptors. By linking the cytoskeleton of the cell to the extracellular matrix, integrins transmit forces and signals necessary for migration.

A variety of experiments have shown that integrins

containing the  $\beta_1$  subunit are important for cell migration during animal development. Each integrin contains two type I transmembrane subunits, and the widely expressed  $\beta_1$  subunit forms heterodimers with a large number of  $\alpha$  subunits (Hynes, 1992). Blocking  $\beta_1$  integrins with diverse approaches can perturb quail neural crest cell migration, mesodermal cell migration on fibronectin during gastrulation in *Pleurodeles* and gastrulation in *Xenopus* (for references see Fassler and Meyer, 1995). However, analysis of the phenotype of chimeric mouse embryos containing a mixture of wild-type and  $\beta_1$  integrin-deficient cells has shown that the mutant cells are able to migrate into most tissues (Fassler and Meyer, 1995), suggesting that migration does not require  $\beta_1$ -containing integrins. These conflicting results might indicate that migration can be achieved by receptors other than  $\beta_1$ , which could be other integrins or non-integrin receptors or, alternatively, mutant cells could passively migrate by interaction with normal cells (Fassler and Meyer, 1995).

Regulation of the formation and dissolution of adhesive complexes is crucial to the control of cell migration (Lauffenburger and Horwitz, 1996). Integrin ligand binding and subsequent clustering leads to the generation of intracellular adhesion complexes, which contain cytoskeletal proteins that may provide the necessary anchor for migration.

In addition, these adhesion complexes contain signaling molecules that may play a role in the regulation of the assembly of the complexes and their link with cytoskeletal filaments (Yamada and Geiger, 1997). One family of signaling molecules associated with integrin complexes that is particularly important during migration is the Rho family of small GTPases, including Rho, Rac and Cdc42. The results obtained with dominant negative mutant forms of these molecules suggest that they are important components of integrin-mediated signaling pathways to regulate cytoskeletal organization (Clark and Brugge, 1995). However, it has proven difficult to exactly predict the roles of individual members of this family in a particular morphogenetic event, since their roles appear to vary depending on the type of cell examined or morphogenetic assay used. In Swiss 3T3 cells, Rho but not Rac was found to be involved in cell motility (Takaishi et al., 1993) whereas, in Rat1 cells, Rac rather than Rho promotes motility in an invasion assay (Michiels et al., 1995; Qiu et al., 1995). Diverse requirements for specific GTPase subfamily proteins have also been seen during animal development. In *Drosophila*, Rac and Cdc42 dominant negative proteins interfere with neuronal differentiation, myoblast fusion and organization of epithelial cells in the wing imaginal disc, however their requirements are qualitatively different (Luo et al., 1994; Eaton et al., 1995). Rac is also required for the process of dorsal closure and for the migration of the border cells during oogenesis, while Cdc42 is not (Harden et al., 1995; Murphy and Montell, 1996).

In the *Drosophila* embryo, the development of the midgut provides a particularly attractive system for the study of the role of integrins and associated signaling molecules in migration. The *Drosophila* midgut is composed of two germ layers, the visceral mesoderm and the endoderm. The visceral mesoderm arises from a small block of cells in each segment, which involute and join together to produce continuous bands of cells on each side of the embryo (Dunin Borkowski et al., 1995). The midgut endoderm arises from two spatially separated primordia: the anterior midgut primordium, which is situated at the anterior-ventral limit of the embryo and the posterior midgut primordium at the posterior pole of the embryo. The formation of the midgut endoderm is initiated by these two primordia undergoing an epithelial-to-mesenchymal transition, followed by migration of the resultant cells over the two bands of visceral mesoderm (Skaer, 1993). The visceral mesoderm is a substratum for endodermal cell migration since mutations in genes required for visceral mesoderm specification lead to a failure in midgut migration (Reuter et al., 1993; Tepass and Hartenstein, 1994). Once the migrating primordia meet in the middle they undergo a conversion back from mesenchymal to epithelial, forming two bands of cells. Together with the visceral mesoderm, these bands stretch ventrally and dorsally to enclose the yolk and form a continuous tube.

The *Drosophila* position-specific (PS) integrins are most similar to the  $\beta_1$  family of vertebrate integrins. All three position-specific PS integrins, PS1 ( $\alpha_{PS1}\beta_{PS}$ ), PS2 ( $\alpha_{PS2}\beta_{PS}$ ) and PS3 ( $\alpha_{PS3}\beta_{PS}$ ), are expressed in the developing midgut, with PS1 and PS3 expressed in the midgut endoderm and PS2 in the visceral mesoderm (Brown, 1993; Stark et al., 1997). The PS integrins have been shown to be involved in midgut migration since embryos that lack them exhibit delays in

endodermal cell migration (Roote and Zusman, 1995). There are a variety of possible ways that the PS integrins could be important for midgut migration. They could be required in the visceral mesoderm to assemble a substratum for the endodermal cells to migrate upon, which could consist of extracellular matrix or transmembrane proteins. They could be required in the migrating endodermal cells to bind to the substratum and provide anchor points to resist the force of traction. Finally, they could signal in the migrating cells to promote the reorganization of the actin cytoskeleton that is required for migration.

In this work, we have analysed the specific roles of each PS integrin in the cell migration of the midgut of the *Drosophila* embryo. We show that they are required in both tissues, the visceral mesoderm substratum and the migrating endodermal cells. The PS2 integrin is required in the visceral mesoderm to make an optimal substratum for migration. The PS1 and PS3 integrins are required in a partially redundant way for normal migration of the endodermal cells. Loss of the PS1 integrin results in a modest slowing of the rate of migration, loss of PS3 has no effect on migration, while loss of both PS1 and PS3 results in a dramatic reduction in the rate of the migration. In the absence of all three PS integrins, the endodermal cells no longer display a migratory morphology, lacking the prominent projections observed in the wild-type embryo. These results show that midgut migration requires coordinated functions of all three PS integrins in both tissues the visceral mesoderm and the endoderm. We find that Rac and Cdc42, but not Rho or Ras, are required in the endodermal cells for their migration, suggesting that an essential role of the PS1 and PS3 integrins is to promote migration by signaling through Rac and Cdc42.

## MATERIALS AND METHODS

### *Drosophila* strains

The integrin mutant alleles used in this study are the null allele *ifB4* (Brown, 1994), the null allele *mew<sup>mb6</sup>* (Brower et al., 1995), the null allele *mys<sup>XG43</sup>* (Bunch et al., 1992) and the allele *scb<sup>JG</sup>* (Stark et al., 1997). The GAL4 enhancer trap lines used are *twist-GAL4* and *24B*, expressed in the mesoderm (Brand and Perrimon, 1993; Greig and Akam, 1993), and *48Y*, expressed in the endoderm from stage 12 onwards (Martin-Bermudo et al., 1997). The *UAS-tau-lacZ* gene is described in Hidalgo et al. (1995), the *UAS-CD2* gene in Dunin-Borkowski and Brown (1995), and the *UAS-mys* ( $\beta_{PS}$ ) in Martin-Bermudo and Brown (1996). The midgut enhancer trap line 258 (Martin-Bermudo and Brown, 1999) was used to visualize the endodermal midgut. To assess the role of members of the Rho family of GTPases, we crossed the *48Y* GAL4 line with the following UAS constructs: *UAS-Rho<sup>N19</sup>* (Strutt et al., 1997), *UAS-Cdc42<sup>N17</sup>*, *UAS-Cdc42<sup>V12</sup>*, *UAS-Rac<sup>N17</sup>*, *UAS-Rac<sup>V12</sup>* (Luo et al., 1994) and *UAS-Ras<sup>N17</sup>* (Lee et al., 1996).

### Generation of germline clones

To examine the role of PS integrins during midgut migration, we expressed *tau-lacZ* in embryos lacking both maternal and zygotic *mys* function by generating germline clones for *mys<sup>XG43</sup>*. This was achieved using the FLP-recombinase system and the dominant female-sterile mutation *ovo<sup>D1</sup>* (Chou and Perrimon, 1992). Virgin females of the genotype *y w mys<sup>XG43</sup> FRT<sup>101</sup>; UAS-tau-lacZ* were crossed with males that were *w ovo<sup>D1</sup> FRT<sup>101</sup>; FLP<sup>F38</sup>*. Larvae of this cross were heat shocked two times at 37°C for 2 hours. The larvae were allowed to recover for 2 hours at room temperature between the heat shocks. The female progeny *y w mys<sup>XG43</sup> FRT<sup>101</sup>/w ovo<sup>D1</sup>*

*FRT<sup>101</sup>; UAS-tau-lacZ/FLP<sup>F38</sup>* were then crossed to *48Y* to drive expression of *tau-lacZ* in the endoderm. By staining with anti- $\beta$ -galactosidase antibody, we visualized the morphology of endodermal cells as they migrated.

To create embryos that only express  $\beta$ ps in either the visceral mesoderm or the endoderm, we first eliminated both maternal and zygotic  $\beta$ ps function using the same strategy. In this case, virgin females of the genotype *y w mys<sup>XG43</sup> FRT<sup>101</sup>; UAS-mys* were used to generate the germline clones. The female progeny *y w mys<sup>XG43</sup> FRT<sup>101/w</sup> ovo<sup>D1</sup> FRT<sup>101</sup>; UAS-mys/FLP<sup>F38</sup>* were then crossed to either *twist-GAL4; 24B* or *48Y UAS-CD2* to drive expression of  $\beta$ ps in either the visceral mesoderm or the endoderm, respectively. To visualize the endodermal cells as they migrated, we used anti-Hindsight or anti-CD2 antibodies.

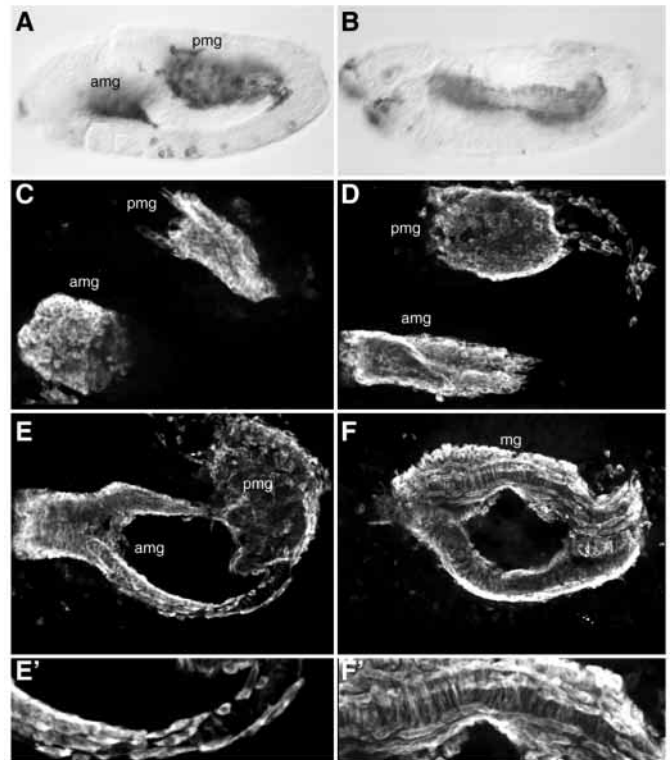
### Antibody staining

Whole-mount staining of embryos was performed using standard procedures. The primary antibodies used were rabbit anti- $\beta$ -galactosidase (Cappel Laboratories, Malvern, PA), and the following mouse monoclonal antibodies: 1G9 anti-Hindsight (DSHB, University of Iowa, IA 52242), OX-34 anti-CD2 (Dunin-Borkowski and Brown, 1995), and DA1B5 anti-Fasciclin III (Brower et al., 1980). We used a biotin-labelled secondary antibody, followed by the Vectastain Elite ABC Kit (Vectorlabs) enhancement to stain the embryos, or streptavidin-rhodamine for immunofluorescence. Stained embryos were photographed with either a Zeiss Axiophot microscope and the images scanned using a Nikon Coolscan, or a Spot digital camera on a Leica DMR microscope, or directly from the MRC1024 Confocal microscope. The digital images were assembled with Adobe Photoshop 5.0, and labelled in Freehand 8.0 on a Power Macintosh.

## RESULTS

### The PS integrins are required for active migration of the endodermal midgut cells

Ultrastructural analysis has shown that cells of the endoderm go through changes in morphology during midgut development (Tepass and Hartenstein, 1994). The two primordia start as parts of epithelial layers within the stomodeal and proctodeal invaginations. These cells then undergo an epithelial-to-mesenchymal transition and start migrating. Electron microscopic analysis has shown that the surfaces of these migrating cells are characterized by multiple slender processes (Tepass and Hartenstein, 1994). We have found that the morphology of these cells can also be visualized by expressing a microtubule marker, tau- $\beta$ -gal (Hidalgo et al., 1995) in these endodermal cells using the GAL4 line *48Y* (Martin-Bermudo et al., 1997) (Fig. 1). By stage 10 (4.20-5.20 hours after egg laying, staging according to Campos-Ortega and Hartenstein, 1985) the anterior midgut (AMG) and the posterior midgut (PMG) primordium have undergone an epithelial-to-mesenchymal transition and are visible as two separate groups of small round cells positioned at both ends of the embryo (Fig. 1C). During the end of stage 10 and beginning of stage 11 (5.20-7.20 hours) both rudiments, AMG and PMG, begin to migrate towards the posterior and the anterior, respectively, along the two bands of visceral mesoderm, which leads to each of them splitting into two arms (Fig. 1D,E). At this point, the migrating cells extend processes that can be detected with an anti- $\beta$ -gal antibody that reveals the expression of the tau- $\beta$ -gal marker in these cells (Fig. 1E'). In the figure, it can also be seen that the posterior midgut cells move as a group due to the

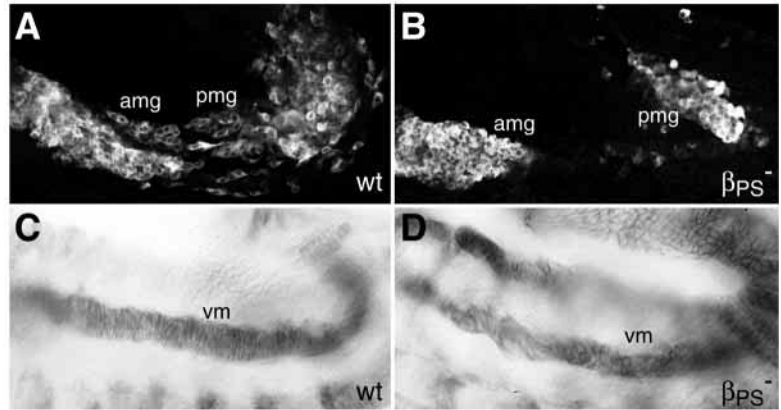


**Fig. 1.** Morphological analysis of wild-type midgut migration. The expression of *UAS-tau-lacZ* driven by the GAL4 line *48Y* and detected with an anti- $\beta$ -gal antibody allows visualization of changes in the morphology of the endodermal cells as they migrate (A,B; anterior to the left, dorsal top). (C-F) Higher magnification views of the embryos of the same genotype using a fluorescence secondary antibody for confocal microscopy. (C) At the end of stage 10, the anterior (amg) and posterior (pmg) midgut primordia can be identified as two groups of rounded mesenchymal cells, which are localized at the anterior and posterior end of the embryo respectively. (D) During stage 11, cells from both primordia send projections towards the visceral mesoderm and start migrating as two arms. Included within the cells migrating from the posterior end are caudal visceral mesoderm cells. (E) By stage 12, cells of both primordia span the length of the germband and their leading tips have met on either side of the yolk. Cells send lots of projections at this stage, as it can be seen in a higher magnification in E'. (F) By stage 13, when germband retraction is completed, cells of the midgut have become arranged as an epithelial layer of columnar cells. (F') Higher magnification of (F).

retraction of the germband. Shortly before germband retraction is completed (end of stage 12, 7.20-9.20 hours), the leading tips of AMG and PMG meet (Fig. 1E). During stage 13 (9.20-10.20 hours), the midgut mesenchyme transforms back into epithelial columns of cells (Fig. 1F,F'; Reuter et al., 1993; Tepass and Hartenstein, 1994). The Gal4 driver *48Y* also directs expression in a second population of cells that migrates anteriorly from the posterior, the caudal visceral mesodermal cells, which give rise to longitudinal visceral muscles (Georgias et al., 1997). Some of the observations that follow may therefore also apply to these cells.

We next used this method of visualizing the migration of the midgut to examine the role of the PS integrins in this process. To completely eliminate PS integrin function, we

**Fig. 2.** Midgut cells of embryos lacking the  $\beta_{PS}$  subunit do not form projections and are delayed in their migration. Examination of the morphological problems associated with the defects in midgut migration in embryos lacking the  $\beta_{PS}$  subunit, by expressing the *UAS-tau-lacZ* in the gut of  $\beta_{PS}^-$  embryos. In the absence of the  $\beta_{PS}$  subunit, the midgut primordia has failed to migrate.  $\beta_{PS}^-$  cells remain rounded and cell projections are not detected (B), as compared with wild-type cells of a similar stage (A). The  $\beta_{PS}^-$  mutant embryos (stage 12) show a disorganized visceral mesoderm (vm) (D), visualized with anti-FasIII antibody, as compared to wild-type embryos (C).



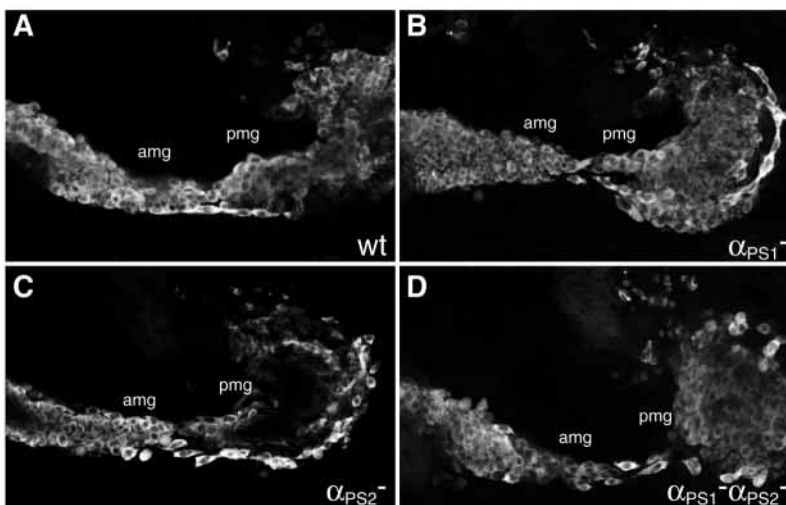
made germline clones of a  $\beta_{PS}$  null mutation to produce embryos lacking both maternal and zygotic  $\beta_{PS}$  contributions (see Materials and Methods). We observed that in the absence of the PS integrins the cells remain rounded, do not have projections and do not migrate at the normal rate (Fig. 2A versus B). In these mutant embryos, the AMG and PMG do eventually meet, but not until stage 15 (11.20-13 hours), and therefore migration is delayed by approximately 2 hours. This demonstrates that, while integrin-mediated migration is not absolutely essential for the endodermal cells to reach each other and reform an epithelium, it is required for it to occur at the normal rate.

There are several possible explanations for this delay in migration.  $\beta_{PS}$  mutant embryos show interruptions and irregularities in the visceral mesoderm (Fig. 2D), the substratum for the endoderm migration. Therefore, the defects in migration could be due to defects in the visceral mesoderm. Alternatively, the PS integrins might be required in the endodermal cells for their migration or they could be required in both tissues. Two different  $\alpha_{PS}$  integrin subunits have been shown to be required for the normal formation of the midgut:  $\alpha_{PS2}$ , which is expressed in the visceral mesoderm, and  $\alpha_{PS1}$ , which is expressed in the endoderm (Brown, 1994; Martin-Bermudo and Brown, 1999). Both are required to convert the initial short fat midgut into a long slender tube. By examining the phenotype of embryos lacking one or other  $\alpha$  subunit, we can analyse integrin function in midgut migration independently in the visceral mesoderm and the endoderm.

### Integrin function is required in each of the two tissues involved in midgut migration

We have expressed the tau- $\beta$ -gal marker in embryos lacking either  $\alpha_{PS1}$  or  $\alpha_{PS2}$  and we have found that migration occurs almost normally (Fig. 3). These  $\alpha$  subunit genes do not have any detectable maternal contribution (Brown, 1994; Brower et al., 1995). In each case, the migration of midgut endodermal cells is modestly delayed, so that anterior and posterior mutant cells do not contact each other until stage 13 when wild-type cells have already fused (Fig. 3A-C), a delay of approximately 30 minutes. This is contrary to a previous analysis that indicated that embryos lacking  $\alpha_{PS1}$  or  $\alpha_{PS2}$  show equivalent irregularities in the migration of the primordial midgut as those lacking  $\beta_{PS}$  (Roote and Zusman, 1995). Furthermore, we have seen that, in contrast to the lack of the  $\beta_{PS}$  subunit, in embryos lacking  $\alpha_{PS1}$  or  $\alpha_{PS2}$  the endodermal cells still send projections towards the visceral mesoderm (Fig. 3A-C). Therefore, we find that loss of either  $\alpha_{PS1}$  or  $\alpha_{PS2}$  separately does not produce the same migration phenotype as loss of  $\beta_{PS}$ . However, loss of the PS2 integrin does produce the same defects in the organisation of the visceral mesoderm as loss of all PS integrins (data not shown; Roote and Zusman, 1995).

There are at least two possible explanations for this result: (1) either  $\alpha_{PS}$  subunit is sufficient to mediate migration, or (2) there is another  $\alpha$  subunit that can partially compensate for the loss of  $\alpha_{PS1}$  or  $\alpha_{PS2}$ . In order to distinguish between these possibilities, we examined embryos mutant for both  $\alpha_{PS1}$  and  $\alpha_{PS2}$ . If the first explanation were correct then we would expect the double mutant phenotype to be as strong as the loss of  $\beta_{PS}$ . Endodermal cells from  $\alpha_{PS1}\alpha_{PS2}^-$  embryos show a greater delay in cell migration than that observed in the single  $\alpha$  subunit mutants. This can be seen when only one or two cells get in contact in the middle in the double mutant embryos at a time when several cells have



**Fig. 3.** The absence of the PS1 and PS2 integrins does not mimic the phenotype of the lack of all PS integrins. Endodermal midgut cells of embryos lacking the  $\alpha_{PS1}$  (B) or  $\alpha_{PS2}$  (C) subunits (stage 12) show a delay in midgut migration (compare to wild type A), but they still have stable projections directed towards the visceral mesoderm. This phenotype is enhanced by the removal of both subunits (D) but it is still less severe than that observed in  $\beta_{PS}^-$  cells.

**Table 1. Summary of the specific roles of PS integrins in both the endoderm and the visceral mesoderm in midgut cell migration**

	Visceral mesoderm palisade	Delay in migration	Cell projections
$\beta_{PS}^-$	mutant	***	-
$\alpha_{PS1}^-$	wt	*	+
$\alpha_{PS2}^-$	mutant	*	+
$\alpha_{PS1}^- \alpha_{PS2}^-$	mutant	**	+
$\alpha_{PS1}^- \alpha_{PS3}^-$	wt	***	nt
$\beta_{PS}^- + \beta_{PS}$ endoderm	mutant	*	+
$\beta_{PS}^- + \beta_{PS}$ v.m.	wt	***	nt

First column: Phenotype of the arrangement of visceral mesodermal cells into the normal palisade structure.

Second column: Extent of the delay in endodermal cell migration: \*\*\* strong delay (1.5-2 hours); \*\* moderate delay (1 hour); \* mild delay (20-40').

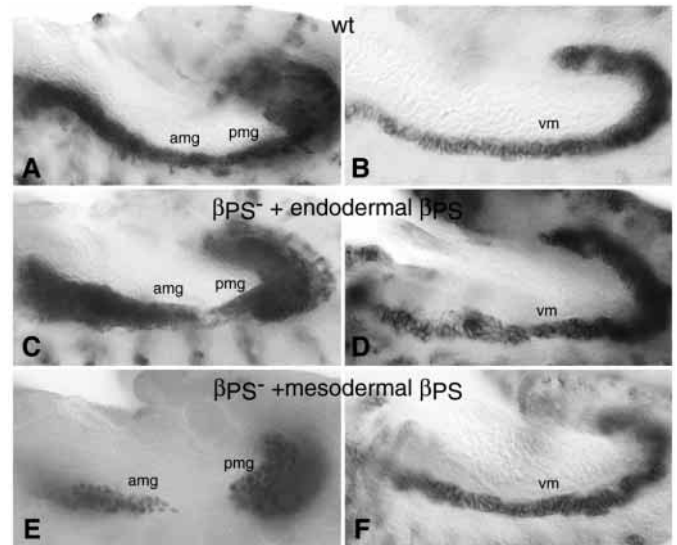
Third column: Presence + or absence - of endodermal cell projections. nt, not tested.

met in the single mutant embryos (Fig. 3B-D). Nevertheless, in this double mutant combination, the endodermal cells send projections and the delay in cell migration (1 hour) is still less severe than that observed in  $\beta_{PS}^-$  embryos (compare Fig. 2B to Fig. 3D and see Table 1 for summary). This situation is different from the role of the integrins in muscle attachment, where loss of PS1 and PS2 integrins is equivalent to the loss of all PS integrins (Prokop et al., 1998).

Therefore, these results suggest that additional  $\alpha$  subunits exist that can compensate for the loss of  $\alpha_{PS1}$  and  $\alpha_{PS2}$ . These  $\alpha$  subunits could be required in the endoderm, in the visceral mesoderm or in both tissues. Since loss of PS2 appears to affect the formation of the visceral mesoderm substratum as strongly as loss of the  $\beta_{PS}$  subunit, and this does not substantially delay migration, we favour a model in which other  $\alpha$  subunits are required in the endoderm. As  $\alpha_{PS3}$  is also expressed in the endoderm (Stark et al., 1997), it is an obvious candidate for a second  $\alpha$  required in the endoderm for cell migration. Therefore, as the next step in analyzing the role of integrins in midgut migration, we first investigated the specific requirements of the  $\beta_{PS}$  subunit in the two different tissues, and second tested the role of  $\alpha_{PS3}$ .

### Distinct and independent functions of $\beta_{PS}$ in the endoderm and in the visceral mesoderm during midgut migration

In order to analyze the specific requirements of the  $\beta_{PS}$  subunit in each of the two tissues involved, visceral mesoderm and endoderm, we have generated embryos that express  $\beta_{PS}$  in just one tissue. This was achieved using the GAL4 system to express the  $\beta_{PS}$  subunit in the endoderm (using *48Y*), or in the visceral mesoderm (using a combination of two GAL4 lines, *twist-GAL4* and *24B*) in embryos that lack both maternal and zygotic  $\beta_{PS}$  function (see Materials and Methods). To be able to visualize the projections, the endodermal cells were marked by also expressing UAS-CD2, a cell surface marker (Dunin-Borkowski and Brown, 1995). When we express the  $\beta_{PS}$  subunit in the endoderm of embryos that completely lack the  $\beta_{PS}$  subunit, the ability of endodermal cells to send projections and to migrate is rescued (Fig. 4C). There is a small delay in the migration, a phenotype that closely resembles that of  $\alpha_{PS2}$

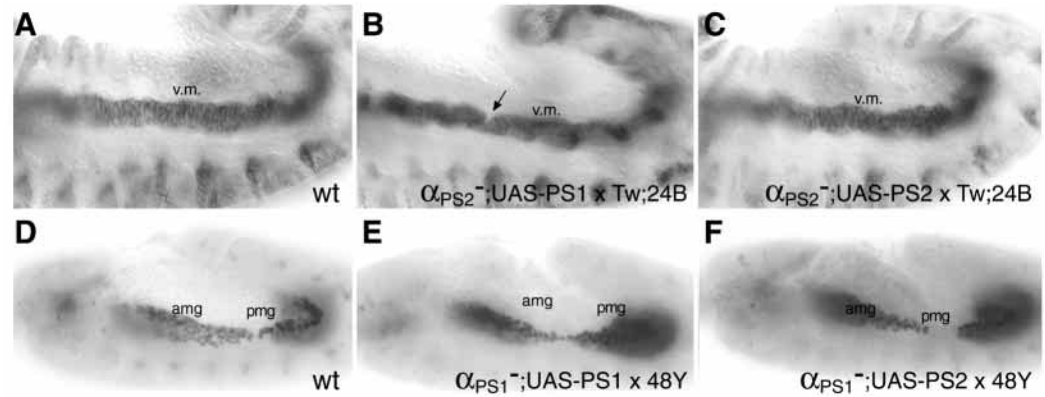


**Fig. 4.** The migration phenotype of  $\beta_{PS}$  mutant embryos can be rescued by putting back  $\beta_{PS}$  in the endoderm only. Endodermal cells were visualized by coexpressing the transmembrane protein CD2 with  $\beta_{PS}$ , and staining with an anti-CD2 antibody (A,C), and by staining with anti-hindsight (E). The visceral mesodermal cells were visualized with anti-FasIII (B,D,F). Using the GAL4 system,  $\beta_{PS}$  expression in the endoderm only almost completely rescues the phenotype of migration (compare C with wild-type embryo in A) while the visceral mesoderm remains affected (D). Expression of  $\beta_{PS}$  only in the mesoderm rescues the visceral mesoderm phenotype (compare F with B, wild type), but cells of the endoderm do not recover their ability to migrate (E).

mutant embryos. As expected, these embryos exhibit the disorganised visceral mesoderm phenotype due to the lack of  $\beta_{PS}$  in the visceral mesoderm (compare Fig. 4D to Fig. 2D). In addition, the defects in the retraction of the germband associated with the loss of  $\beta_{PS}$  are not rescued, indicating that the cell movements of germband retraction do not provide any forces that are essential for midgut migration. In contrast, mutant embryos that express the  $\beta_{PS}$  subunit in the visceral mesoderm fail to rescue the migration phenotype (Fig. 4E), even though the visceral mesoderm has been rescued and shows a wild-type shape and organization (Fig. 4F). These results demonstrate that the  $\beta_{PS}$  subunit is specifically required in the endodermal cells for their migration and that this function is independent of the role of the  $\beta_{PS}$  subunit in organizing the visceral mesoderm (Table 1).

We have shown that PS1 and PS2 cannot substitute for each other to mediate adhesion at the muscle attachment site, indicating that PS1 and PS2 have distinct functions at these sites (Martin-Bermudo et al., 1997). However, PS2 is able to substitute for PS1 to regulate gene expression in the gut, showing that the  $\alpha$  subunits do not provide specificity to this signaling event (Martin-Bermudo and Brown, 1999). In order to test whether PS1 and PS2 have specific functions in midgut migration, we examined whether the  $\alpha$  subunits are able to substitute for each other. We expressed  $\alpha_{PS1}$  in the mesoderm of embryos mutant for  $\alpha_{PS2}$  subunit and found that it is unable to completely rescue the visceral mesoderm phenotype (Fig. 5B versus A). Similarly, when we express  $\alpha_{PS2}$  in the endoderm of embryos that lack  $\alpha_{PS1}$ , the migration phenotype is not

**Fig. 5.** PS1 and PS2 function are specific in mediating midgut migration. The visceral mesoderm was visualized using an anti-FasIII antibody and the endodermal cells using the midgut enhancer trap line 258 followed by staining with an anti- $\beta$ -gal antibody. All panels show embryos at stage 12, and A and D are wild-type embryos. Expression of  $\alpha_{PS1}$  in the mesoderm fails to rescue the visceral mesoderm phenotype of embryos lacking the  $\alpha_{PS2}$  subunit (B; arrow shows an irregularity in the visceral mesoderm), while expression of  $\alpha_{PS2}$  can (C). Conversely, endodermal cell migration of embryos lacking the  $\alpha_{PS1}$  subunit is rescued by expressing  $\alpha_{PS1}$  in the endoderm (compare E with D) but it is not when expressing  $\alpha_{PS2}$  (F).



rescued (Fig. 5F versus D). These results demonstrate that PS1 and PS2 integrins each perform a specific function.

#### $\alpha_{PS1}$ and $\alpha_{PS3}$ cooperate to mediate endodermal cell migration

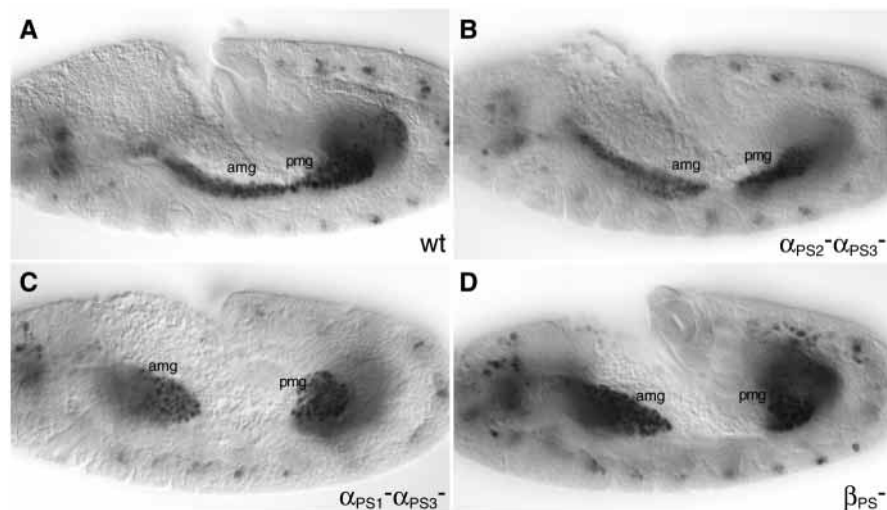
Our analysis of embryos mutant for  $\alpha_{PS3}$  (data not shown) supports previous evidence indicating that there is no defect in midgut migration (Stark et al., 1997), showing that the PS3 integrin is not essential for midgut migration. However, it is possible that there is some redundancy between the two  $\alpha$  subunits expressed in the endoderm, so that  $\alpha_{PS1}$  compensates for the lack of  $\alpha_{PS3}$  and vice versa. To test this, we generated embryos lacking both PS1 and PS3 integrins and examined midgut migration. As  $\alpha_{PS1}$  and  $\alpha_{PS3}$  genes are on different chromosomes, it was difficult to use the GAL4 system to express any marker, so we used an enhancer trap line, 258, which is expressed in the endodermal cells as they migrate. This is a nuclear marker that allows us to identify the position of the endodermal cells and therefore to monitor their migration although it does not reveal the morphology of the cells. We found that, in the absence of both  $\alpha_{PS1}$  and  $\alpha_{PS3}$  subunits, the defect in the migration of the midgut is as strong as in the absence of the  $\beta_{PS}$  subunit (compare Fig. 6C and D), consisting of a delay of approximately 2 hours. We also

examined embryos doubly mutant for  $\alpha_{PS3}$  and  $\alpha_{PS2}$  subunits, and found no difference in midgut development compared to the loss of  $\alpha_{PS2}$ , showing that there is no overlap in function of the PS2 and PS3 integrins (Fig. 6B and Table 1). In conclusion, the main requirement for PS integrins during migration is provided by the combination of PS1 and PS3 integrins.

The PS1 integrin is also required in the endoderm to regulate gene expression (Martin-Bermudo and Brown, 1999). To investigate whether PS3 is also required to regulate gene expression in the midgut, we have analysed the pattern of expression of the two integrin target genes that we identified in embryos that lack the  $\alpha_{PS3}$  subunit. We found that loss of PS3 function does not cause any changes in their expression (data not shown). Thus, we have found that PS1 function in migration can also be partially performed by the PS3 integrin (there is a small delay in the absence of PS1), while PS1 function in the regulating gene expression or adhesion to the visceral mesoderm (as judged by the later defect in midgut elongation) cannot be performed by PS3.

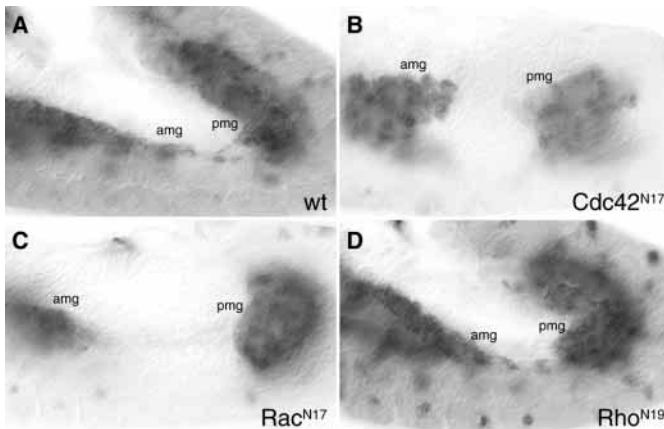
#### Is signaling through Rho family members required for midgut migration?

To test the roles of members of the Rho family in midgut migration, we expressed dominant-negative forms of Rac, Rho and Cdc42 in the midgut endoderm using the GAL4 driver *48Y*. These dominant negative forms are point mutations (Asn-17 for Thr-17)



**Fig. 6.** PS1 and PS3 collaborate in the endoderm to mediate midgut migration. Panels show migration of wild-type (A) and mutant (B-D) endodermal cells at stage 12, visualized using the midgut enhancer trap line 258 and staining the embryos with an anti- $\beta$ -gal antibody. Cells of embryo that lack both  $\alpha_{PS2}$  and  $\alpha_{PS3}$  subunits (B) show a mild delay in migration, which is similar to the one observed in  $\alpha_{PS2}^-$ . However, when  $\alpha_{PS1}$  and  $\alpha_{PS3}$  (C) are absent, we observe a strong phenotype in migration, which is very similar to the one observed in embryos that lack the  $\beta_{PS}$  subunit (D).





**Fig. 7.** Migration defects produced by mutant small GTPase proteins. All embryos are stage 12. The GAL4 line 48Y has been used to express dominant negative forms of Rho family member proteins in combination with the CD2 cell surface marker, and the migrating cells visualized by staining with an anti-CD2 antibody. Expression of a dominant negative form of Cdc42 (B) or Rac (C) causes a strong delay in midgut migration which is very similar to the one observed in  $\beta_{PS}$ - embryos. In contrast, expression of the dominant negative form of the RhoA protein does not seem to cause any effect (D).

which stabilize the GDP-bound stage. They are thought to inactivate the endogenous proteins by sequestering the rate-limiting guanine nucleotide exchange factor (Farnsworth and Feig, 1991). Expression of Cdc42<sup>N17</sup> or Rac<sup>N17</sup> in the developing midgut causes a delay in endodermal migration (Fig. 7B,C). This phenotype closely resembles that observed in embryos lacking both zygotic and maternal  $\beta_{PS}$  contribution. These cells were marked by also expressing UAS-CD2, which showed that Rac and Cdc42 are required for the formation of the endodermal cell projections. No midgut migration defect was observed with Rho<sup>N17</sup> (Fig. 7D), or a dominant-negative version of the related GTPase Ras (data not shown). Activated Cdc42 and Rac1 have been shown to promote a motile phenotype, as detected by a three- to five-fold increase in motility on collagen (Keely et al., 1997). This increased cell motility appears to be mediated by integrins since it is inhibited by antibodies against the  $\beta_1$  or  $\alpha_2$  subunits. To test whether Rac and Cdc42 activity are limiting factors in regulating midgut migration, the activated forms, Rac<sup>V12</sup> and Cdc42<sup>V12</sup>, were expressed in the endodermal cells, but no sign of accelerated migration was observed (data not shown). These results are consistent with results showing that the activated form of Rac cannot accelerate migration of border cells, even though a dominant negative form inhibits their migration (Murphy and Montell, 1996).

## DISCUSSION

In this work, we have used a variety of tools to dissect the role of integrins in a model migratory process that occurs during *Drosophila* embryogenesis. We have found that the migration of primordial midgut cells along a substratum provided by the visceral mesoderm requires the coordinated function of all three PS integrins. The PS2 integrin is required in the visceral

mesoderm for the formation of an optimal substratum for migration. The PS1 and PS3 integrins are required in the endodermal cells for their migration over the visceral mesoderm. These two integrins are partially redundant in the midgut, but PS3 cannot fully replace PS1 integrin function. We have characterised one important step that is likely to be downstream of integrins, the activation of certain small GTPases and the pathways that they initiate.

Our use of tau- $\beta$ -gal to visualise the morphology of the migrating endodermal cells has revealed that, contrary to the conclusions of previous work (Roote and Zusman, 1995), loss of single integrins does not produce equivalent migratory defects as the loss of all PS integrins. Only in the absence of all  $\beta_{PS}$  function do the endodermal cells fail to have projections that are labelled by tau- $\beta$ -gal. The protein tau is a microtubule-associated protein, and so this marker labels tubulin-containing projections. The PS integrins could have a structural role in the formation of these projections, by attaching the cellular extensions to the substratum and stabilising them. Integrins, which normally diffuse freely in the membrane, upon ligand binding attach to the cytoskeleton in newly extended lamellipodial regions of fibroblasts (Felsenfeld et al., 1996). Integrins could also activate signaling pathways that regulate the organization of the cytoskeleton and therefore the formation of cellular extensions required for migration. Integrin engagement and clustering has been shown to cluster and/or activate signaling molecules such as focal adhesion kinase (FAK), c-Src, Abl and Syk (Miyamoto et al., 1995; Hanks and Polte, 1997). A potential connection between this integrin signaling and reorganization of the cytoskeleton might come from the fact that the Rac/Cdc42 GTPase-activating protein, Graf, is associated with FAK and localizes to focal contact sites (Hildebrand et al., 1996).

We have found that the abnormal migration of  $\beta_{PS}$  mutant midgut is rescued when  $\beta_{PS}$  function is restored only in the endoderm but not if it is restored in the visceral mesoderm. Therefore, the migration of the midgut primordia is mediated largely by  $\beta_{PS}$  function in the endoderm. These results are similar to our previous finding that  $\beta_{PS}$  function is more important in the somatic muscles for muscle attachment than in the epidermis (Martin-Bermudo and Brown, 1996), but contrast with the wing where the two integrins are equally required for the adhesion between the two surfaces (Brabant and Brower, 1993; Brower et al., 1995). We have found that the PS1 and PS3 integrins work in a partially redundant way to mediate midgut migration. While a role for PS3 integrin in cell migration had been suggested by the defects that occur in the absence of the  $\alpha_{PS3}$  subunit in some tissues, such as the trachea and the pericardial cells, there is no requirement of PS3 for midgut migration (Stark et al., 1997). Our results have shown that, in the absence of the PS1 integrin, PS3 is required for migration of the midgut. Collaborative interactions between different integrins in mediating migration have already been shown to occur in other systems. For example, experiments *in vitro* have shown that expression of the  $\alpha_5\beta_1$  fibronectin receptor in CHO cells is required for directed cell motility on vitronectin, suggesting that the fibronectin receptor plays a cooperative role with vitronectin receptors (e.g.  $\alpha_v\beta_3$ ) in cell motility (Bauer et al., 1993). Our experiments have shown that, while PS1 can fully substitute for PS3, the reciprocal is not true, suggesting that PS1 and PS3 are not equally functional

for midgut migration. One way to explain this result relates to the recent evidence that the PS1 integrin can regulate gene expression in the *Drosophila* gut and, as shown here, PS3 cannot (Martin-Bermudo and Brown, 1999). While the nature of these signals and their relation to cell migration is not yet clear, it seems possible that PS1 integrin signaling is important for cell migration. This signaling event appears to be distinct from that leading to changes in gene expression, since PS2 is not able to substitute for PS1 in migration.

While the migratory appearance of the midgut cells and close to normal rates of migration only require PS integrin function in the migrating cells, we do see a modest role for the PS2 integrin in this process. As shown here, the normal appearance of the visceral mesoderm requires  $\beta_{PS}$  function in these cells, consistent with the observation that PS2 mutant embryos, and not PS1 or PS1 + PS3 mutant embryos, show irregularities in the visceral mesoderm (our own unpublished observations and Roote and Zusman, 1995). Therefore, the delay in migration observed in PS2 mutant embryos can be explained as a consequence of irregularities in the substratum. Experiments *in vitro* have shown that changes in the substratum, like ligand levels, can regulate cell migration (Huttenlocher et al., 1995; Palecek et al., 1997). Integrins are also required for assembly of the extracellular matrix *in vivo*. Intracellular injection of antibodies against the cytoplasmic domain of the  $\beta_1$  subunit prevent fibronectin matrix assembly in amphibians (Darriber et al., 1990). It is also tempting to suggest that lack of PS2 integrin in the visceral mesoderm affects the assembly of the extracellular matrix so that ligands for the PS1 and PS3 integrins are not assembled properly or are reduced in amount, leading to the observed irregularities in cell migration. If this is true, one might expect the reciprocal to occur in some cases, such that the PS1 integrin is required to assemble a matrix for the PS2 integrin to bind to. There is some evidence to suggest that this is the case. Lack of PS1 function in the endodermal cell layer results in the detachment of the visceral muscles in the larval gut, a defect also observed in the absence of PS2 function (Martin-Bermudo and Brown, 1999). Another possible explanation for the role of the PS2 integrin in producing normal rate migration of the endodermal cells, which cannot be ruled out yet, is that PS2 signaling induces the secretion of diffusible molecules from the visceral mesoderm that promote endodermal migration.

The cells that lack PS integrin function exhibit reduced motility but they still manage to migrate, suggesting other pathways can also trigger migration. External signals like growth factors have also been shown to be important for cell migration (Huttenlocher et al., 1995). It appears to be the combination of adhesion-initiated events and growth factor signaling that regulates cell migration. Furthermore, recent experiments have suggested that integrins and growth factors use different signaling molecules to promote migration. These experiments have shown that, while signaling events initiated by integrins require the Src family kinases (SFK) to regulate migration, those initiated by the platelet-derived growth factor may be SFK independent (Klinghoffer et al., 1999). Therefore, growth-factor-mediated signaling may account for the residual migration observed in the absence of integrins.

Finally, our results have shown that Cdc42 and Rac are required to mediate migration but Rho is not. Distinct functions

for Rho, Rac and Cdc42 in cell migration have also been characterized in *Drosophila* oogenesis, where Rac activity was specifically required for the migration of the border cells. In that case, the expression of a dominant negative form of Cdc42, or a novel Rho family member, RhoL, did not affect migration of the border cells (Murphy and Montell, 1996). Another Rho family member, encoded by the *mig2* gene in *C. elegans*, has been shown to be required for cell migration and axon guidance (Zipkin et al., 1997). The sequence of Mig2 shows that this new member is most closely related to Rac and Cdc42. These results and ours are consistent with the fact that Cdc42 has been shown to be required for the extension of filopodia (Nobes and Hall, 1995). Therefore, in the developing embryo different migratory events appear to require different members of the Rho family. This could be explained by a high degree of cell type specificity within this family of GTPases, functional redundancy or diverse mechanisms of cell migration.

The interactions between the pathways mediated by integrin adhesion and signaling and those triggered by the Rho family members have yet to be defined in detail. It has previously been shown that integrin-ligand interaction and aggregation of integrins in itself is sufficient to trigger the formation of focal adhesion complexes (Miyamoto et al., 1995). In contrast, studies with Swiss 3T3 cells have suggested that integrin engagement of the matrix alone is not sufficient to induce the assembly of vinculin-containing focal complexes; Rho must also be activated by a soluble factor. These results led the authors to propose that rho GTPases could direct clustering of integrins, by modifying their cytoplasmic tails, allowing other proteins of the complex to assemble (Hotchin and Hall, 1995). Studies of the effect of expressing dominant-inhibitory mutants of Cdc42, Rac and Rho in Rat1 cells have defined morphological changes and integrin-mediated signaling pathways that are regulated by the different members of the Rho family of GTPases (Clark et al., 1998). A Rho-dependent pathway regulates the full activation of FAK, phosphorylation of paxillin and assembly of focal adhesions. A Cdc42-dependent pathway activates Akt, and both Cdc42 and Rac regulate actin organization required for membrane ruffling. Therefore, several separable signaling pathways regulated by different members of the Rho family seem to control integrin signaling events that regulate changes in cell morphology and migration. Our results implicate both the integrins and the Rho subfamily proteins Rac and Cdc42 in the migration of endodermal cells. This makes the *Drosophila* midgut an ideal system for investigating how the Rho and integrin signal transduction pathways are connected to regulate cell migration in developing tissues.

We are grateful to A. Brand, L. Kockel, L. Luo, M. Mlodzik and R. Murakami for providing fly stocks. We thank A. Gonzalez-Reyes and I. Palacios for helpful comments on the manuscript. This work was supported by a Royal Society University Research Fellowship and Wellcome Trust project grant 054613 to M. D. M. B. and a Wellcome Trust Senior Fellowship to N. H. B.

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