PVF1/PVR signaling and apoptosis promotes the rotation and dorsal closure of the *Drosophila* male terminalia

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ABSTRACT  The *Drosophila* adult male terminalia originate from the genital disc. During the pupal stages, the external parts of terminalia evert from two ventral stalks; the everted left and right dorsal halves fuse at the dorsal midline. At the same time the male terminalia perform a 360° clockwise rotation. Several mutations are known to affect the rotation of the male terminalia, while none is known to affect dorsal closure. We show here that the *Pvf1* gene, encoding one of the three *Drosophila* homologues of the mammalian VEGF/PDGF growth factors, is required for both processes. Males either mutant for *Pvf1* or bearing a dominant negative form of *Pvr* or *stasis (stai)*, the unique PVF receptor, do not complete either rotation or dorsal closure. *Pvf1* expression in the genital disc is restricted to the A8 cells. However, PVF1/PVR signaling influences A8, A9 and A10 cells, suggesting that the PVF1 protein diffuses from its source. Flies hemizygous for the apoptotic genes *hid*, *reaper* and *grim*, or mutant for *puckered* which encodes a phosphatase that down-regulates the n-Jun-N terminal kinase pathway, lead to the same phenotypes as mutations in PVF1/ PVR. Our results indicate that PVF1/PVR signaling functions not only in apoptotic phenomena but are also required during rotation and dorsal closure of the *Drosophila* male genital disc.

KEY WORDS: PVF1/PVR, VEGF/PDGF, apoptosis, *Drosophila*, male genital disc

Introduction

The adults terminalia of *Drosophila* derive from the genital disc (reviewed in Sánchez and Guerrero, 2001). It is constituted by the fusion of three embryonic abdominal segments: A8, A9 and A10. In the male genital disc the A8 primordium gives rise to a tiny A8 tergite (T8), whereas the A9 forms the male genitalia and A10 the male analia and hindgut. The shaping process of these structures occurs during the pupal stages; among them there is the fusion in the dorsal midline of left and right halves of this bilateral symmetrical disc and a 360° clockwise rotation (Gleichauf, 1936; Adám et al., 2003) (see Fig. 1 A,B) related with the maturation of the internal genitalia (Gleichauf, 1936) (see Fig. 1C). No mutations are known to affect dorsal closure, but several have been reported to affect the rotation of the male terminalia. The latter include mutations at *Abdominal-B (Abd-B)* (Casanova et al., 1986; Sánchez-Herrero and Crosby, 1988), *head involution defective (hid)* (Abbott and Lengyel, 1991; Grether et al., 1995), at the gene encoding transcription factor *TAF250* (Wassarman et al., 2000), *spin* (Adám et al., 2003) and at some unidentified genes located in the region 11A (Fahmy and Fahmy, 1958). Most of those mutants present several degrees of incomplete rotation. The involvement of *hid* is of interest, for it is a pro-apoptotic gene (Grether et al., 1995) and suggests that cell death is required.

In *Drosophila*, the PVF/PVR signaling pathway has been involved in border cell migration in the oocyte (Duchek et al., 2001, McDonald et al., 2003) as well as in migration-survival and proliferation of the hemocytes in embryos and larvae (Heino et al., 2001; Cho et al., 2002, Munier et al., 2002; Brückner et al., 2004).

There are three *Pvfs* genes in *Drosophila*. The mammalian homologues of these ligands could form homo or heterodimers, the type of dimer formed specifies the response of the receptor (Heldin and Westermark, 1999). On the contrary, there is only one receptor for PVFs, *Pvr o stasis (stai)*, with demonstrated binding activity to PVF1 (Duchek et al., 2001).

Abbreviations used in this paper: JNK, n-Jun-N terminal kinase; PDGF, platelet derived growth factor; puc, puckered gene; PVF1, platelet vascular factor I; VEGF, vascular endothelial growth factor; WT, wild-type.

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In this work we show that PVF1/PVR signaling is involved in dorsal closure and rotation of male terminalia, as mutations in genes encoding the ligand or in the receptor alter these processes. We show that Pvfl transcript is only expressed in part of A8 of male genital discs but is required in cells of the three segments, suggesting a diffusion of the product. We also confirm the role of hidn terminalia rotation (Abbott and Lengyel, 1991) and show that blocking cell death by the baculovirus caspase inhibitor p35, or altering the apoptotic n-Jun-N terminal Kinase pathway produce the same phenotype as hid- and the lack of function of PVF1/PVR.

Results

Genetic analysis of the Pvfl gene

To identify genes involved in the development of adult male terminalia we analyzed P-Gal4 lines using the yellow method (Calleja et al., 1996). The line LP23-Gal4 was selected and characterized in detail. Inverse and direct PCR, as well as Southern blot analysis, showed that the insertion is located 250 bp upstream the proposed transcription start site of the Pvfl gene (Fig. 2; Duchek et al., 2001, Cho et al., 2002). The expression pattern driven by LP23-Gal4 in embryos corresponds to a subset of Pvfl expressing cells previously described (Cho et al., 2002; data not shown). LP23-Gal4 males have wild type terminalia. The specific y+ adult expression in the male terminalia suggested a possible function of Pvfl in this region of the body. To characterize its function the LP23 insertion was mobilized to generate deficiencies after imprecise jumps (Gloor et al., 1991). Six putative imprecise excisions were semilethal in males and showed adult male rotated terminalia (Fig. 3A). The Dp (1; Y) W39 (Prado et al., 1999) rescued these phenotype. Since this duplication includes the wild type allele of Pvfl, this result suggested that the phenotype observed in the excised LP23-Gal4 lines were probably due to a defective Pvfl gene. Detailed Southern blot and PCR analyses were performed for two of these mutants, LP23sem1 and LP23sem2 (Fig. 2). It was found that LP23 sem1 is a deletion that removes part of the 5’ region upstream the transcription starts site (Duchek et al., 2001; Cho et al., 2002), the first exon and part of the first intron of the Pvfl gene, as well as most of the P-Gal4 transposon (Fig. 2). LP23sem2 is also a deletion with a breakpoint in the first intron of Pvfl, extending 5’ more distally than LP23 sem1, removing the transposon and deleting the neighboring gene CG7101 (Fig. 2). We also analyzed the mutant Pvfl1624, carrying the transposon EP1624 (Rørth, 1996) inserted in the first intron of the Pvfl gene (Duchek et al., 2001). This allele is homozygous viable (Duchek et al., 2001), albeit there is some pupal lethality and the adults show reduced fertility (not shown). Hemizygous Df (1)LP23sem1 and Pvfl1624 males show a similar rotated terminalia (Fig. 3 A,B). The phenotype is not fully penetrant and the expressivity is variable, but both increase at higher temperatures (Fig. 3 D,E). Occasionally, we noticed a lack of dorsal closure in some individuals (not shown). The variability of the phenotype could be due to a partial redundancy in Pvf1 genes function, as it has been described for other Pvf associated phenotypes (Duchek et al., 2001; Cho et al., 2002).

![Figure 1. Wild type adult male terminalias.](image1)

(A) A wt male with its ventral side up, showing the correct position of external terminalia. In all the figures the position of the genitalia is indicated by G and the analia by A; the degree of rotation reached is expressed in the lower right angle. (B) Schematic representation showing the direction and degree of rotation, ventral corresponds to the upper half. (C) Dissection, showing the turns of the spermiduct around the hindgut (H). The white line indicates the 360° spiral trajectory of the spermiduct; the white star indicates the sperm pump location on the right side of the organism.

![Figure 2. Structure and product of the Drosophila Pvf1 gene.](image2)

The Pvfl locus at cytological position 17E. The open reading frame (ORF) of the transcript is indicated as black boxes. The arrow on P transposon pGawβ, called LP23 indicates the direction of Gal4-induced transcription. The sequences deleted in Pvfl, i.e. Df(1)LP23sem1 and Df(1)LP23sem2 are indicated; dotted extensions indicate the range of uncertainty in deletion endpoints.
The analyses of terminalia phenotypes are easily performed by microscope observation of the spermiduct trajectory and the sperm pump position (Fig. 1C). In wild type males, the spermiduct bends twice around the hindgut and the sperm pump is located in the right side of the abdomen (Fig. 1C). The internal analyses of Pvf1 mutant flies, with various degrees of rotated terminalia, showed that the direction of rotation is not altered in the mutants (Figs. 3C, 1C). The spermiduct normally initiated a clockwise-turn but stops prematurely, while the sperm pump that is on the right side, is on the left in the mutants. All the external structures of the terminalia appear to move coordinately with the internal spermiduct and sperm pump during rotation, because all of them are mis-positioned in an equivalent rotated stage when the movement fail to be completed.

Pvf1 expression in the male genital disc

Pvf1 transcripts are first detected in late third instar larvae, in cells that belong to A8 segment (Fig. 4 A,B). We delimited Pvf1 expression using as a reference the en, dpp and ptc genes (Fig. 4 C,D) genes. ptc expression is closely associated to that of en in the genital disc (Fig. 4D), while dpp is more restricted and co-expresses with en only in few cells (Fig. 4C). In A8, Pvf1 expression may overlap that of en, dpp and ptc domain of A8 cells in the vicinity (Fig. 4F). This observation of the internal spermiduct and sperm pump domain of A8 (Fig. 4A,B). The activity of Pvf1 expression may overlap that of Pvr function was present; sometimes, as observed with Pvf1 mutants, the dorsal closure was not completed.

We tested if there is a more extreme phenotype in males Pvf1+ ; en-Gal4; UAS- PvrDN. All these males have the terminalia rotated between 180° and 270° and show the dorsal split phenotype (not shown). This confirms the specificity of PVF1 for PVR (Duchek et al., 2001). The observation of the internal spermiduct and sperm pump structures in these flies is similar to that observed in the Pvf1 mutants (Fig. 5C).
Taken together these results show that lack of Pvf1 or inactivation of its receptor causes similar phenotypes, so the PVF1/PVR signaling is required. Remarkably, blockage of the signaling mechanism is most sensitive in the en grailed domain (Fig. 5D), where Pvf1 transcript was not detected.

Pvf1 gain of function

The EP11235 line (Rørth, 1998) has a gain of function allele for Pvf1 as the EP element is inserted upstream of the Pvf1 transcript start site (Duchek et al., 2001). With the dpp-Gal4 or ptc-Gal4 drivers EP11235 overexpression has no phenotypic effects, but with en-Gal4 or hh-Gal4 it does (Fig. 5E). Increasing PVF1 levels in males EP11235; en-Gal4:2xUAS-Pvf1 augmented the percent-age of miss-rotations and the split phenotype (Fig. 5F). As the wt expression of Pvf1 does not overlap that of en grailed in the genital disc, this result supports the idea that the diffusion of PVF1 is instructive in terminalia rotation and dorsal closure.

Apoptosis involved in dorsal closure and rotation

hid is a pro-apoptotic gene (Grether et al., 1995). hidd alleles are embryonic lethal and heterozygous individuals are wild-type. Males escapers hid^226 (Grether et al., 1995) over Df(3L)H99 (a deficiency that removes hid and the other pro-apoptotic genes reaper and grim (Grether et al., 1995; Chen et al., 1996; Chao and Nagoshi, 1999) reared at 25ºC have the terminalia rotated between 90º and 270º (Abbott and Lengyel, 1991) (data not shown). Interestingly, 50% of heterozygous Df(3L)H99/+ males have a dominant phenotype of rotated terminalia when grown at 30ºC. To analyze whether Pvf1 and Df(3L)H99 interact in eliciting this phenotype we constructed males of genotypes Df(1)LP23sem; Df(3L)H99/+ and Pvf1^1624; Df(3L)H99^/+ . They showed a notable increase in penetrance, from 20% of Df(1)LP23sem or Pvf1^1624 alone to 80% with the deficiency (Fig. 6 A,B). This increment suggests an interaction between the pro-apoptotic genes included in Df(3L)H99and Pvf1 in the process of terminalia rotation. Since hid is a pro-apoptotic gene (Grether et al., 1995), we hypothesized that the rotated phenotype may be caused by and inhibition or reduction of apoptosis. To test this possibility, the gene encoding the caspase inhibitor p35 (Hay et al., 1994) was expressed in the male genital disc. We find that males of genotype en-Gal4; UAS-p35 and dpp-Gal4; UAS-p35 possess rotated terminalia and a gap in the dorsal midline (Fig. 6C). Driving the expression of p35 to the posterior cells led to a stronger phenotypes than using anterior Gal4 drivers (not shown).

It has been shown that the activity of RAS-MAPK, one of the major signal transduction players of RTK (reviewed in Seger and Krebs, 1995), produces a decreased activity of hid (Kurada and White, 1998; Bergmann et al., 1998). Accordingly, we analyzed the consequence of Ras overexpression in the rotation of the male terminalia. A low percentage (4%) of males carrying the UAS-RasAV12 transgene, a constitutive form of the Ras (Fontini et al., 1992), show at RT, a dominant phenotype of rotated terminalia (not shown). This effect may be based on a basal expression of the construct. However, expression of RasAV12 at 30ºC using the driver LP23-Gal4 led to a much higher percentage (55%) of adult males with rotated terminalia (Fig. 6D). Therefore, overexpressing Ras in the male genital disc causes the same phenotype observed in the eye-antenna disc, as it is reduces hid activity (Kurada and White, 1998; Bergmann et al., 1998).

Role of the JNK signalling pathway

The JNK pathway is involved in apoptosis in the imaginal discs (Moreno et al., 2002 a). Furthermore, JNK, together with Eiger the Drosophila TNF homologous up-regulates hid (Moreno et al., 2002 b). Therefore, we hypothesized that alterations in this pathway could also affect terminalia rotation and dorsal closure.

JNK activity can be down regulated in Drosophila by increasing the expression of the gene puckered (puc), an element of the pathway encoding a dual-specificity phosphatase that generates a negative feedback loop (Martin-Blanco et al., 1998). Targeted expression of UAS-puc in the genital disc using en-Gal4, dpp-Gal4, or ptc-Gal4 causes rotation of the male terminalia and dorsal split, remarkably observed in the ptc-en domains (Fig. 7 B-E). In these
overexpressing Pvf1 in the en domain produce rotated terminalia. (F) Wild type male with its dorsal side up to show the morphology of terminalia in this side, analia (A), anterior genital arch (AGA) (arrow), tergite 8 (T8) (arrowhead). (G) Dorsal view of hh-Gal4:EP11235; 2xUAS-Pvf1, to show the failure in dorsal closure. The tergite 8 (T8), the anterior genital arch and the dorsal anal plates do not fuse in the dorsal midline (arrowhead). (H) Genital disc of hh-Gal4; EP11235; 2x UAS- Pvf1 hybridized with Pvf1 RNA, showing the endogenous and the exogenous expression of Pvf1. (I) hh-Gal4; 3x UAS-Pvf1, the external terminalia was at +270º, the spermiduct is followed by a black line, the duct only does one bend, the sperm pump (SP) is on the left.

flies, the spermiduct and sperm pump are miss-rotated (Fig. 7D), as in Pvf1/Pvr.

To further study the association between the JNK and the PVF1/PVR pathways we examined JNK activity by checking the expression of puc-lacZ transgene in males either puc\textsuperscript{E68} a lethal allele with the puc\textsuperscript{lacZ} insertion, or hh-Gal4; UAS-Pvf1/puc\textsuperscript{E68}. It was observed that the expression domain of puc surrounds that of Pvf1 (Fig. 7F) and extends to A9 cells. Targeted expression of Pvf1 can ectopically activate puc(Fig. 7G). We also analyzed whether increasing the activity of JNK by lowering puc may rescue the dominant phenotype of Df(3L)H99\textsuperscript{+} males. We found that 100% of the males double heterozygous for Df(3L)H99 and puc\textsuperscript{E68} are wild type at 30ºC, whereas 50% of Df(3L)H99\textsuperscript{+} males have a mutant phenotype. This result strongly supports the idea that in the genital disc the JNK pathway is also activating hid as it has already been demonstrated in other genetic context (Moreno et al., 2002 b).

Discussion

The PVF1/PVR pathway and the rotation and dorsal closure of male terminalia

In this work we demonstrate that either mutations at Pvf1 or the expression of a dominant negative form of its unique receptor, Pvr\textsuperscript{DN}, result in various degrees of male rotated terminalia and failure of dorsal closure. These observations indicate that the PVF1/PVR pathway is relevant in these morphogenetic processes. Although the Pvf1 gene is only expressed in a subset of cells from the segment A8, reduction or abolition of PVF1/PVR signaling affects the normal development of all terminalia precursors (A8, A9 and A10). Interestingly, mutations in the Abd-B m function, which affect only the A8 segment (Casanova et al., 1986) have a phenotype of rotated terminalia. Thus, these results highlight the importance of the A8 segment in this process. We propose that A8 cells affect the development of structures originated from A9 and A10 through the activity of the PVF1 protein diffusing from A8. Although our data concern transcript expression, Rosin et al., 2004, demonstrated that PVF1 is capable of extensive lateral diffusion, so it has the properties of a long range signaling molecule.

PVFs could form homo and heterodimers, what opens the possibility of different effects in the binding responses of the receptor. McDonald et al., 2003, observed that homodimers are not equivalent, because PVF1 seems to be the relevant signal for the migration of border cells and Brüncker et al., 2004, describe two function for PVR in the embryonic hemocytes, suggesting a diversity of functions. We have not analyzed other PVFs and although we observed some partial redundancy, it will be necessary to separate PVF individual or associated functions.
We obtained indirect evidence about where the PVR receptor is activated or expressed. First, by recognition of factors that mediate the activity of the PVF1/PVR signaling mechanism (i.e., dpERK), whose expression was located at the periphery of the group of cells expressing Pvf1. Second, blocking PVF1 activity using Pvf1dn and overexpressing Pvf1 the effects are stronger in the engrailed domain where Pvf1 is not expressed. These findings provide additional evidence that there are specific domains for ligand expression and for responsive cells. In the ovary Pvf1 is expressed in the ovule while Pvr is expressed in the follicle cells, the importance of this non-overlapping domains is reflected by the fact that overexpression of a constitutive active form of the receptor (Pvr) produces the same phenotype of its lack of function (Duchek et al., 2001). In the wing disc Rosin et al., 2004 observed that the restrictions in the activity are regulated by a polarized secretion of the ligand in the apical membrane.

**Apoptotic genes and the JNK pathway**

Mutations in the pro-apoptotic gene *hid* have been shown to affect male terminalia rotation (Abbot and Lengay, 1991), although this phenotype was observed in trans heterozygotes for Df(3L)H99, which includes the three pro-apoptotic genes hid, ppr and grim (Grether et al., 1995). Trans heterozygotes for hid mutations are of wildtype phenotype, indicating that the rotated phenotype over deficiency is not only due to hid but to the haploinsufficiency of one or the two other genes. Our result that preventing cell death with p35 leads to miss rotation and split dorsal is also consistent with an involvement of apoptosis in these processes.

Additionally, we show that overexpressing puc results in the same phenotypes as PVF1/PVR and reduction of apoptosis, lowering puc rescues the rotated terminalia defects observed in DfH99/+ males. The level of puc is considered as indicative of the JNK pathway activity (Martin-Blanco et al., 1998; Moreno et al., 2002a-b) so these experiments suggests that JNK promotes apoptosis, probably by up-regulating hid.

**PVF1/PVR, JNK and apoptosis**

The fact that alterations in the PVF1/PVR pathway and in JNK/apoptosis give rise to similar phenotypes suggests a functional link between these two pathways.

The penetrance of the phenotypes of Pvf1 mutations in the terminalia increases when they are additionally heterozygous for Df(3L)H99. This increase is non additive, suggesting PVF1/PVR and the apoptotic machinery affect the same aspect of the process. The overexpression of Pvf1 ectopically activates puc and impedes the normal rotation and closure. This activation would down-regulate the JNK apoptotic pathway (Martin-Blanco et al., 1998), thus reducing apoptosis and giving rise to the terminalia phenotype, but since the JNK pathway is a transcriptional activator of puc (Martin-Blanco et al., 1998), this result opens up the possibility that Pvf1 ectopically activates JNK rather than puc.

Apoptosis is necessary for terminalia rotation and dorsal closure and our results and those of others indicate that it is mediated by JNK activity. PVF1/PVR is also affecting these processes and our data suggest that PVF1/PVR may also affect JNK-mediated apoptosis. It is not clear however, whether all these elements act on the same developmental cascade.

**Materials and Methods**

**Fly stocks**

Mutant Pvf1 alleles used in this study are: Pvf11624 and EP11235, corresponding to EP insertions in the Pvf1 gene (Rarth, 1998; Duchek et al., 2001). Two deficiencies in Pvf1, Df(1)LP23semii and LP23semii, were isolated in this work and are described in the text. Dp(Y)W39 (Prado et al., 1999) is a duplication covering the Pvf1 gene. The Gal4/UAS system (Brand and Perrimon, 1993) was used to drive the expression of several genes constructs: UAS-Pvf1 expressing the long spliced form of the Pvf1 gene (Duchek et al., 2001); UAS-Pvr and UAS-Pvf1 express the native and a dominant negative form of the receptor for the PVF proteins, respectively (Duchek et al., 2001); UAS-RasV12 (Fontini et al., 1992).
phenotype with the targeted expression in the en-ptc domains. (D) Dissected en-Gal4; UAS-puc male. The external terminalia was at 180°, the white line follows the spermduct, the duct does one bend (white star) and the sperm pump is on the left side of the fly. (E,F) Male genital discs. Expression of puc by puc-lacZ transgene revealed by anti-βgal stain. The insertion of P-lacZ in puc generates a recessive lethal mutation called puc^{E69}. In males, puc^{E69} -/+ , wt puc expression is in A8 cells which surround the segment and extend to A9. (F) hh-Gal4; UAS-Pvf1/puc^{E69}, stained with anti-βgal to reveal puc expression, the ectopic expression of Pf1 activates the gene puckered outside its own domain, the arrows point the normal and the ectopic expression.

expresses a constitutive form of the RAS protein, while UAS- puc expresses the wild type allele of the puckered(puc) gene (Martin-Blanco et al., 1998). The driver’s dpp-Gal4, en-Gal4, ptc-Gal4 and hh-Gal4 (Staehling-Hampton, K. et al., 1994; Hinz, U. et al., 1994; Tanimoto, H. et al., 2000), as well as the hid allele hid^{E22} and Df(3L)H99 (Abbott and Lengyel, 1991; Grether et al., 1995), have previously been described. puc^{E69} is a null allele for the gene puc carrying an insertion of a lacZ gene (Martin-Blanco et al., 1998).

Immunostaining, X-Gal staining and in situ hybridization

Immunostaining and X-Gal staining were performed as described (Macias et al., 1990). The anti dpERK used is a monoclonal anti-Map Kinase, Activated antibody (SIGMA #M8159). The detection system used for the immunoreactions was ELITE of Vector Lab. The RNAs probes were transcribed from: Pvf1: EST 30334; CG7101: EST 44815. For in situ hybridization we followed the protocol adapted from Lehner and O’Farrell (1990). Stained embryos or discs were soaked in 87% glycerol, mounted onto cover slips and photographed with a NIKON digital camera Coolpix 950. The images were obtained with a stereomicroscope LEICA MZ 9.5 and a NIKON LABOPHOT light microscope. Given the low amount of products to be analyzed in these studies, incubation and developing times in our protocols were adjusted to optimize signal/background ratios.

PCR and Southern analysis

The LP23-Gal4 insertion was located by inverse PCR (Rehm, J.E; BDGP Resources). The primers used to map the LP23-Gal4 site of insertion are: 5’-CTG AGC GAC TCA CGT CC-3’ and 5’-TAG TCA GCG GAG ACC TTT TGG-3’ for the 5’ end and 5’-TGA CCA TGA TTA CCG CAA GCG-3’ and 5’-CCA AGC ACC AGC AAG TTC CG-3’ for the 3’ end. For Southern analyses, genomic DNA from FM7 (wt control), Pvf1^{E24} and EP11235 (insertions into Pvf1), Df(1)LP23^{sem1} and LP23-Gal4 were digested with the restriction enzymes PstI, HindIII, BglII, XhoI and EcoRI. Blots were hybridized with α-^{32P}-dATP (Amersham) oligolabeled probes (Feinberg and Vogelstein, 1984) prepared from template DNA corresponding to EST 30334, EST 44815, pBluescript KS and the Gal4 gene. EST 30334 is a near full-length cDNA of Pvf1 (Cho et al., 2002).

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