Spatial and temporal distribution of Patched-related protein in the Drosophila embryo

Carmen Bolatto a, Cristina Parada a, Fiorella Revello a, Alejandro Zuñiga b, Pablo Cabrera b, Verónica Cambiazo a, *,

a Laboratorio de Biología del Desarrollo, Departamento de Histología y Embriología, Facultad de Medicina-Universidad de la República, Montevideo, Uruguay
b Laboratorio de Bioinformática y Expresión Génica, INTA-Universidad de Chile and Fondap Center for Genome Regulation (CGR), Santiago, Chile

Abstract

Patched-related (Ptr) encodes a protein with 12 potential transmembrane domains and a sterol-sensing domain that is closely related in predicted topology and domain organization to Patched, the canonical receptor of the Hedgehog pathway. Here we describe the production of an antibody specific for Drosophila Ptr and analyse its spatial and temporal distribution in the embryo. We find that at early developmental stages Ptr is predominantly localized at cell periphery but later on it becomes strongly and almost exclusively expressed in hemocytes. Interestingly Ptr null mutant embryos died without hatching. Our findings suggest that Ptr plays an essential function in Drosophila development, perhaps as a new receptor of embryonic hemocytes.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Seven classes of multipass transmembrane proteins carry a conserved domain known as sterol-sensing domain (SSD), which consists of about 180 amino acids that form 5 consecutive membrane spanning segments. This motif was initially identified in HMG CoA reductase (HMGCR) and SREBP cleavage activating protein (SCAP), two proteins involved in sterol synthesis and sterol-dependent transcription, respectively (Hampton, 2002). Subsequently additional proteins such as 7-dehydrocholesterol reductase (Witsch-Baumgartner et al., 2001), Niemann–Pick disease type C1 (NPC1) (Carstea et al., 1997), Patched (Ptc) (Hooper and Scott, 1989) and Dispatched (Disp) (Burke et al., 1999) with roles in cholesterol biosynthesis, lipid transport and transport of cholesterol-modified morphogen Hedgehog (Hh) have been shown to carry the SSD motif. More recently a group of membrane proteins named as Patched-related (Ptr), which is closely related in predicted topology to Ptc and Disp, were characterized in C. elegans (Kuwabara and Labouesse, 2002). The Ptr proteins seem to function in multiple aspects of development, including cell growth, patterning, and molting, a process that depends on the availability of sterols (Zugasti et al., 2005).

A Drosophila Ptr gene (CG11212) was originally isolated in a subtractive hybridization screening designed to identify genes that...
are differentially expressed at the beginning of gastrulation (Zuniga et al., 2009). We cloned the gene and deduced that the product is a 1061 amino acids protein closely related in predicted topology and domain organization to the protein encoded by the Drosophila segment polarity gene ptc. In addition, our biochemical analysis revealed that Ptr is associated with embryo membranes and immunohistochemistry analyses allowed us to localize this protein to the growing plasma membranes in Drosophila blastoderm and in vesicles-like structures of S2 cells overexpressing a Ptr-V5 fusion protein (Zuniga et al., 2009; Pastenes et al., 2008).

Here we describe the production of an antibody specific for Drosophila Ptr and analyse its temporal and spatial distribution in the embryo. In addition, we present an initial characterization of a null mutation in the Ptr gene, created by imprecise P-element excision. Ptr null mutant embryos died without hatching, indicating that Ptr plays an essential function in Drosophila development.

2. Results

2.1. Generation of antibody specific for Ptr protein

The spatial and temporal pattern of Patched-related expression has been only briefly reported (Pastenes et al., 2008) with antibodies no longer available, so a new polyclonal antiserum against a 275 amino acids peptide from the carboxyl termini of Ptr (aa 855 to 1129) was generated. The quality of the signal displayed by the new anti-Ptr antibody, was first tested by Western blot assays using a purified recombinant Ptr protein consisting of the 275 amino acids peptide fused to a poly-His tag (Fig. 1, lane A). Then, Western blot assays were performed using lysates of cl-8 cells that overexpress a Ptr-V5 construct under the control of an inducible metallothionein promoter (see Sections 4.2–4.4). To confirm the specificity of the signal, these experiments included as control the use of a monoclonal anti-V5 antibody to recognize the protein tag. The results showed that both the polyclonal anti-Ptr and the monoclonal anti-V5 antibodies recognized a single band at 95 kDa (Fig. 1 left, lanes B and C). The apparent molecular weight of Ptr was different to that predicted by its sequence (120 kDa). This biochemical behaviour, termed “gel shifting”, was reported previously in other studies and appears to be frequent for membrane proteins (Rath et al., 2009; Nybo, 2012). The origin of gel shift behaviour might derive from altered binding caused by the detergent. In our experiment, the need to avoid boiling the sample to prevent protein aggregation conspired against the uniform binding of SDS to the sample proteins. Nevertheless, the correspondence of the 95 kDa signal to Ptr was confirmed by the co-staining with anti-V5 antibody. Uninduced cells were used as control to confirm the absence of reactivity with anti-Ptr antibody (Fig. 1 left, lane D). In addition, transfected cl-8 were processed for immunofluorescence using the anti-Ptr and anti-V5 antibodies (green and red signal, respectively). Ptr and V5 signals colocalized in vesicle-like structures (Fig. 1, right). The specificity of the anti-Ptr antiserum was further demonstrated by the absence of immunostaining in a Ptr null mutant embryos (see Section 2.3). Thus, we concluded that the antiserum generated specifically recognized the Ptr protein.

2.2. Expression pattern of Ptr during Drosophila embryogenesis

The structural similarities of Ptr and Ptc along with the association of Ptr with embryo membranes (Zuniga et al., 2009; Pastenes et al., 2008) prompted us to compare the expression pattern of Ptr and Ptc at early and late stages of embryogenesis. To do this, we performed double immunofluorescence analyses by staining embryos with anti-Ptr and anti-Ptc antibodies. The spatial distribution of Ptc protein observed here was consistent with that reported previously (Capdevila et al., 1994; Taylor et al., 1993) allowing us to compare it with that of Ptr.

In early embryos (cellular blastoderm and gastrula stages) the immunofluorescence signals of Ptr and Ptc showed a uniform distribution in almost all cells (Fig. 2 A–L). As previously showed (Pastenes et al., 2008), Ptr is distributed around the periphery of the cell and accumulates in cytoplasmic dots (Fig. 2 D–F).
subcellular arrangement is reminiscent to that described for Ptc. Ultrastructural localization of anti-Ptr using electron microscopy and a pre-embedding immunogold method demonstrated that Ptr is associated to vesicular and plasma membranes (Fig. 2C–I). At stage 12 of embryogenesis when germ band shortening takes place, Ptc is expressed in 15 alternating stripes over the trunk region (Capdevila et al., 1994), whereas the anti-Ptr antibody stained conspicuous zones with high concentration of the protein (Fig. 2 M–U) that were not coincident with the Ptc-positive stripes. During germ band shortening, distribution of Ptr positive cells was reminiscent of the spatial pattern of migrating hemocytes. Thus, Ptr positive cells were concentrated at both ends of the embryo (Fig. 2N, arrows) from where they migrated towards the middle of the embryo. Migration took place along different paths that were highly coincident with those previously described as the stereotyped paths of hemocyte migration: ventral surface of ventral nerve cord (vs), dorsal surface of ventral nerve cord (ds) and dorsal epidermis (de) (Fig. 2Q and T). There is a fourth path followed by hemocytes during migration, but it is inside the embryo, surrounding the gut and it was nor clearly visible in our immunofluorescence stainings (Evans et al., 2010; Wood and Jacinto, 2007; Tepass et al., 1994). At a later stage of development Ptr positive cells were observed around the brain (Fig. 2W, arrowheads) and bilaterally around the amnioserosa (Fig. 2V, as).

In order to verify whether Ptr staining was enriched in the hemocyte population of cells, (Evans et al., 2010; Olofsson and Page, 2005) embryos expressing UAS-lacZ under the control of hemocyte-specific GAL4 driver (crr-GAL4) were immunostained using anti-Ptr and anti-beta galactosidase (beta-gal) antibodies (Fig. 3). We found that the patterns of immunostaining for beta galactosidase and Ptc were highly similar, indicating that Ptr is enriched in the hemocytes at late stages of embryonic development. Although expression pattern of lacZ driven by crr-GAL4 was according to previous descriptions (i.e. starts at germ band retraction stage), the overlap between anti-Ptr and anti-beta-gal signals was incomplete and denoted a potential subset of Ptr positive embryonic macrophages. The specificity of the Ptr staining in blood cells was confirmed by the absence of immunofluorescence in Ptc null mutant embryos (Fig. 4).

2.3. Generation of a Ptr mutant and antibody validation

We searched the Flybase for P-element insertion lines near Ptr gene and identified the line PSUPor-PKG01682 in which the PSUPor-P element (Bellen et al., 2004) was adjacent to the predicted 5′-UTR of Ptc. The line was homozgyous viable lacking an obvious mutant phenotype. To identify the complete 5′-UTR of Ptc gene, we performed a 5′ Race using embryos at stage 5. Sequencing of the 5′ Race PCR products revealed two 5′-UTR sequences, 5′-UTR 1 (357 bp) and 5′-UTR 2 (614 bp) (data not shown). Based on EST (expressed sequence tag) sequence data, the 5′-UTR 1 appears to be predominantly transcribed (Flybase, http://flybase.bio.indiana.edu). This result allowed us to map the insertion site of the P-element 335 bp 5′ of the 5′-UTR 1. To obtain mutants for Ptr we mobilized the P element in a standard cross with the mobilized P element 335 bp 5′ edu). This result allowed us to map the insertion site of the P-element in a standard cross with the mobilized P element 335 bp 5′.

The embryo-to-embryo variability of the Ptc staining in blood cells was confirmed by the absence of immunofluorescence in Ptc null mutant embryos (Fig. 4).

We obtained one excision line (23C) that was homozgyous lethal, the extent of the genomic deletion is shown in Fig. 4A. Line 23C has a deletion of 3852 bp removing the transcription start site and 1212 bp encoding the 5′-UTR and part of the first intron of the gene. In the 23C line the neighbouring gene CG30432 is completely deleted. CG30432 is reported as a gene moderately expressed in imaginal discs and testis, with very low or no expression during embryonic development (Graveley et al., 2011), suggesting that the observed lethality could be caused by the mutation in Ptc.

3. Discussion

Previous work indicate that Ptc displays a similar structure and topology with Ptc, a negative regulator of the Hh pathway (Tajpale et al., 2002; Chen and Struhl, 1996; Ingham et al., 1991). Here, we showed that Ptc and Ptc display distinctive expression patterns during embryogenesis. At early stages, Ptc and Ptc immunoreactivity seem to be predominantly associated to cell membranes. At later stages, when Ptc-positive stripes are formed, Ptc becomes highly enriched in the migrating hemocytes. According to the temporal transcriptome of Drosophila published by the modENCODE project (Graveley et al., 2011) the transcription profile of Ptc shows a sharp increment from mid-embryogenesis, co-temporal with hemocyte differentiation and migration as phagocytic cells (Tepass et al., 1994). This is consistent with our findings of strong Ptc immunoreactivity in hemocytes at this stage. In this regard, Ptc immunostaining in embryos expressing UAS-lacZ under the control of the crr-GAL4 driver showed that a subset of migrating hemocytes expressing beta-galactosidase coexpressed Ptc, suggesting the existence of a subset of Ptc-positive cells. To our knowledge, distinct subsets of cells have not been characterized among migrating hemocytes, although the expression of a Tn antigen, with putative roles in immune response, has been detected in a subgroup of embryonic hemocytes (Yoshida et al., 2008).

Hemocytes are circulating cells responsible for the phagocytosis of apoptotic cells in Drosophila and other invertebrates. During embryogenesis, hemocytes carry out essential functions within the embryo, in addition, they are important for embryonic tissue formation as well as organ remodelling during metamorphosis (Tepass et al., 1994; Franc et al., 1996; Abrams et al., 1993; Franc et al., 1995). The ability of the hemocytes to recognize, migrate towards and engulf dying cells is controlled by transmembrane proteins acting as receptors. For example Croquemort (Crrq), a member of the CD36 family of proteins, is a Drosophila macrophage receptor with roles in the binding and clearance of apoptotic cells in Drosophila embryos (Franc et al., 1996). Another receptor that is involved in apoptotic engulfment is Draper that functions in phagocytosis of apoptotic cells by migrating hemocytes (Manaka et al., 2004). On the other hand, the PDGF/VEGF receptor (Pvr) is part of the mechanism of hemocyte migration, directing hemocyte precursors along stereotyped migratory routes (Heino et al., 2001;
Fig. 2. Expression pattern of Ptr during Drosophila embryogenesis. Confocal microscopy images of whole-mount wild-type embryos double stained for Ptc (green signal: A, D, J, M, P, S, V) andPtr (red signal: B, E, K, Q, T, W). Yellow indicates signal overlap in the merged images (C, F, I, O, U, Y). (A–C) Stage 5 embryo (blastoderm cellularization), (D–F) Higher magnification images of blastoderm stage to show similar distribution of the two proteins. (G–I) Pre-embedding immunogold using anti-Ptr to show the association of the protein with plasmatic (arrow) and vesicular (arrowhead) membranes at blastoderm stage. Magnified area in H and I is outlined in G by a black rectangle. (J–L) Stage 7 embryo (gastrulation), (M–U) Stage 12 embryo (germ band shortening), (V–Y) Stage 13 embryo (completion of germ band shortening). (A–B and J–R) Lateral and (S–Y) ventral views. At late stages of embryogenesis the antibody detected conspicuous zones with high concentration of Ptr. The distribution of these zones is coincident with some of the stereotyped paths of hemocyte migration: ventral surface of ventral nerve cord (vs), dorsal surface of ventral nerve cord (ds), dorsal epidermis (de), around the brain and around the amnioserosa (as). Scale Bars: (C) 50 µm, (F) 10 µm and (H–I) 500 nm.
In summary, our results help to determine the expression pattern of the lacZ gene product under control of the macrophage driver crg-GAL4 (red signal: A, D) indicated that Ptr (green signal: B, E) accumulates in hemocytes. (C and F). Merged images indicate signal co-localization (yellow) in the entire embryo (C, F) or in a region close to its dorsal surface (G) to show hemocytes placed in the ventral surface of ventral nerve cord. Magnified area (G) is outlined in C by a white rectangle. Scale bars: (F) 50 μm and (G) 20 μm.

Fig. 3. Ptr is expressed in embryonic macrophages. Confocal images of Drosophila embryos at stage 13/14, viewed (A–C) laterally or (D–F) dorsally with the anterior end to the left of the panel. The expression pattern of the lacZ gene product under control of the macrophage driver crg-GAL4 (red signal: A, D) indicated that Ptr (green signal: B, E) accumulates in hemocytes. (C and F). Merged images indicate signal co-localization (yellow) in the entire embryo (C, F) or in a region close to its dorsal surface (G) to show hemocytes placed in the ventral surface of ventral nerve cord. Magnified area (G) is outlined in C by a white rectangle. Scale bars: (F) 50 μm and (G) 20 μm.

Cho et al., 2002; Wood et al., 2006). In addition, it is relevant to promote the survival of blood cells in the embryo. Thus, Pvr mutant embryos have large numbers of apoptotic hemocytes and drastically reduced total hemocyte number. Interestingly, the absence of Pvr also seems to affect the number of the migrating hemocytes at least at late stages of embryogenesis, suggesting that the lack of Pvr alters cell proliferation or induces hemocyte apoptosis. Further studies will be necessary to determine the role of Pvr on the development and differentiation of embryonic hemocytes.

Although it has been detected a close correlation between the pattern of apoptosis and the migratory routes travelled by hemocytes in the embryo (Tepass et al., 1994; Abrams et al., 1993), it is not clear how morphogenetic processes are able to coordinate developmental migrations with hemocyte homing toward dying cells along stereotyped migration pathways and how hemocytes are able to sense apoptotic cells at a distance (Wood and Jacinto, 2007; Cho et al., 2002). Thus, hemocytes have to respond to a diversity of signals arriving from extracellular matrix (ECM) or from other cells to perform vital functions: the clearance of apoptotic cells and the deposition of ECM. Because receptors and intracellular pathways activated by such signals are still partially known, it is attractive for us to trace the presence of Pvr, a transmembrane protein, to the migrating hemocytes.

Hemocyte malfunction has been related with alterations in the intestine and nervous system (Brown, 1994; Yarnitzky and Volk, 1995; Schmid et al., 2002; Sears et al., 2003). Future studies will clarify the existence of such malformations in Pvr null embryos. In this sense, the observation that these mutant embryos fail to hatch from the vitelline membrane encouraged us to de

In summary, our results help to determine the expression pattern of Pvr, a SSD-containing protein whose role in the development has not yet been established. It was demonstrated that at later stages of embryogenesis, Pvr protein was highly expressed in a hemocyte-specific pattern. In addition, lack of Pvr seems to affect the number and distribution of hemocytes. These observations were unexpected and encourages us to delineate more sophisticated functional investigations to elucidate the role of Pvr in these cells.

4. Experimental procedures

4.1. Production of Pvr anti-serum

To generate the Pvr-GST fusion protein, an 825 bp fragment of Pvr, encompassing residues 855 to 1129, was cloned into a pGEX-6P-1 vector (Amersham Biosciences). The construct was then used to transform BL21 competent cells. An overnight culture of bacteria carrying the fusion protein construct was inoculated (1:100 dilution) into 200 ml of LB plus ampicillin. Expression of the fusion protein was induced by the addition of IPTG to a final concentration of 1 mM and culture was grown 1 h at 37 °C. The bacteria were harvested by cold centrifugation (4 °C) at 8000× g for 10 min. The pellet was resuspended in 10 ml of phosphate buffered saline (PBS) with protease inhibitors and sonicated on ice (ten times for 10 s each, with 1 min rest between sonications). Then, TritonX-100 was added to a final concentration of 1%, the mix was incubated for 20 min and centrifuged at 12,000× g (4 °C) for 20 min. Examination of Coomassie blue stained gels revealed that after induction of bacteria transformed with pGEX constructs, the 275 amino acids Pvr peptide was expressed as part of a GST fusion protein. The GST fusion protein was purified using Glutathione Superflow resin (Qiagen) according to the manufacturer’s instructions. The release of the recombinant polypeptide from its GST tag was performed by on-column procedure using PreScission protease (GE Healthcare). After the cleavage, the product was dialysed using Amicon (molecular weight cutoff 30 kDa, EMD Millipore) and employed to immunize a rabbit. Freund’s complete and incomplete adjuvants were used for the primary immunization and for subsequent boosts, respectively. After several boosts, the presence of antibodies specific for Pvr was detected in the serum of this animal by Western blot (data not shown). Finally, the serum was affinity purified against Pvr fusion protein without GST. In western blot analysis the antibody was used at a dilution 1:1000 whereas in immunofluorescence the same antibody was used at a dilution 1:100.

The same 825 bp fragment of Pvr was PCR amplified and cloned into pTrchIs2Topo vector (Invitrogen). The resulting histidine-tagged protein was purified using Ni-NTA agarose (Invitrogen) according to the manufacturer’s instructions and employed as positive control on western blot studies.
4.2. Cell cultures and transfection

Cl-8 cells (obtained from the Drosophila Genomics Resource) were cultured in Shields and Sang M3 insect medium (Sigma) supplemented with 2% heat-inactivated fetal bovine serum, 2.5% fly extract, 0.5 mg/ml insulin and antibiotics. To overexpress Ptr, cells were transfected with 2 mg of pMT/V5-His-Topo (Invitrogen) vector containing the coding sequence of gene Ptr (residues 1 to 1129) using Calcium phosphate transfection kit (Invitrogen) according to the manufacturer's instructions. Expression of the constructs was induced 48 h post-transfection by adding CuSO₄ to the cell medium (final concentration 0.5 mM). After 24 h, induced and uninduced (control) cells were harvested, transferred onto coverslips, and fixed with 4% paraformaldehyde.

4.3. Cell lysate and western blotting

Cell extract was obtained by lysing with 600 µl cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and protease inhibitor cocktail) three 60 mm culture dishes of cl-8 cells induced or uninduced with CuSO₄ to express Ptr-V5. The lysate was centrifuged for 10 min at 16,000 x g at 4 °C and the supernatant was stored at -80 °C. Protein determination was performed on aliquots using Bradford reagent (Fermentas). To detect the presence of Ptr-V5, 16 µg of the cell lysate was separated by SDS-PAGE and transferred to PVDF membrane for 2 h at 100 V. Before loading the gel, the samples were incubated with Laemmli sample buffer 30 min at 37 °C (not boiled) to avoid the formation of protein aggregates. The membrane was blocked (5% low-fat milk in Tris-buffered saline pH 7.4) for 1 h at room temperature and...
incubated for 16 h at 4 °C with rabbit anti-Ptr or mouse anti-V5 (Santa Cruz Biotechnology) antibodies diluted 1:1000 and 1:100. To detect the primary antibodies, anti-rabbit or anti-mouse secondary antibodies conjugated to peroxidase were used (Thermo Scientific).

4.4. Immunostaining of cells and embryos

Cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100 for 15 min, and then incubated in blocking buffer (3% BSA, 0.1% Triton X-100 in PBS) for 45 min prior to incubation with primary antibodies: monoclonal anti-V5 (1:500, Sigma) and polyclonal anti-Ptr (1:100). Cells were washed three times in PBS plus 0.1% Triton and incubated with the secondary antibodies and probes: anti-mouse Alexa 488 (Molecular Probes, 1:800), anti-rabbit Alexa Fluor 546 (Molecular Probes, 1:800) and DAPI (Molecular Probes, 1:5000).

Embryos of all stages were collected from grape juice agar plates and dechorionated (50% commercial bleach solution in PBS, 0.05% Triton X-100). Embryos were staged, fixed in 1:1 (v/v) mixture of n-heptane and 4% formaldehyde for 30 min, and their devitellinization was performed by the hand-peeling procedure. Embryos were then postfixed in 4% paraformaldehyde (Sigma—Aldrich) for 20 min, incubated at 4 °C for 30 min in blocking buffer (3% BSA, 0.1% Triton X-100, 50 mM glycine in PBS), and then incubated with Ptr affinity purified antibody (diluted 1:100) or anti-Ptc (Apa1, Developmental Studies Hybridoma Bank-DSHB, diluted 1:100) or anti-fascin (5n C, DSHB, diluted 1/20) or anti-β-galactosidase (Hybridoma Bank, diluted 1:10 or ebioscience, diluted 1:400). After overnight incubation at 4 °C the embryos were washed four times (15 min each) with blocking buffer and incubated for 1 h with anti-mouse Alexa Fluor 488 (1:800, Molecular Probes) or anti-rabbit Cy3 antibodies (1:1000 Jackson ImmunoResearch). DAPI (1:5000, Molecular Probes) was added together with the secondary antibodies to stain the nuclei. Digital images were captured with laser confocal microscopy using either a Leica TCS-SP5-DMI6000 or a Nikon C2 Plus-SiR microscope.

4.5. Electron microscopy

Embryos were collected and fixed for 1 h in a 1:1 mixture of heptane and freshly prepared 4% paraformaldehyde (Fluka) in PBS (pH 7.3) at room temperature. Then, embryos were manually devitellinized in PBS, transferred again to fixative for 30 min, washed in PBS and incubated in 3% BSA, 0.03% saponin and 1% goat serum in PBS for 40 min (two changes). The embryos were incubated with anti-Ptr antibody diluted 1/20 in the same buffer and incubation was performed for 16 h at 4 °C. Then, embryos were washed and incubated with secondary antibodies conjugated to 15 nm colloidal gold particles (anti-rabbit, EMS) in the same buffer for 1 h at room temperature. The embryos were then washed in PBS plus 0.03% saponin and incubated for 60 min, post-fixed in 2.5% glutaraldehyde (Fluka) in PBS for 10 min and washed again using PBS and stained overnight at 4 °C with 2% uranyl acetate (Merck). Then, the samples were dehydrated through an acetone series and embedded in Araldite for sectioning. Thin sections were obtained with a RMC MT–X ultramicrotome, stained with lead citrate, observed under a JEOL JEM-1010 transmission electron microscope and photographed using Hamamatsu Digital Camera CCD 4742–95.

4.6. Drosophila strains and genetic manipulations

All Drosophila stocks were maintained and crossed at 25 °C according to standard procedures. ThePtr mutant lines were generated by imprecise excision using the following stocks: w^{67C3}+, P[w^{+}mDm{y}^{2}, w^{BR}REBR = SUPor-P]KG01682 (P element line), w^{+}; w^{SP}^{0}\!/CyO; ry^{506}, Dr^{1}, P[ry^{1/2}] = Delta2-3)J99B/TM6 (transposase line), w^{SP}^{0}\!/CyO, P[^{ry}^{1/2}] = fzs/lacB]E3 (CyO balancer line). The primer sequences used to characterize the deletion are described in Table 1. For lethal phase determination we balanced Ptr alleles over CyO, kr–GFP balancer gently donated by R. Canteras. Each mutant line was crossed with wild-type (Canton-S strain) flies. Males and females heterozygous for the mutation (Ptr23C+/+) were subsequently crossed to each other. Embryonic hemocytes were detected using embryos from a cross between w^{+}w^{*}; P[w^{1}+mC] = crq–GAL4) 2 (crq–GAL4) and w^{11B}w^{*}; P[US-lacZ-B]BG4) 2-4b (UAS-lacZ). All the stocks were obtained from the Bloomington Stock Center.

4.7. Rapid Amplification of cDNA Ends (RACE)

The 5′-end of the Ptr transcript was amplified using the RLM-RACE GeneRacer™ kit, according to the manufacturer’s instructions (Invitrogen Carlsbad, CA). Briefly, 5 μg of total RNA from Drosophila melanogaster stage 5–7 embryos was used as starting material. The adapter-ligated cDNA was PCR amplified using Platinum®Taq DNA Polymerase (Invitrogen Carlsbad, CA), GeneRacer™ 5′ Primer and 10 μM of gene-specific primer RACE11212-a2 under the following conditions: 2 min at 94 °C, 5 cycles of 30 s at 94 °C and 40 s at 72 °C, 5 cycles of 30 s at 94 °C and 40 s at 70 °C and 25 cycles of 30 s at 94 °C, 30 s at 65 °C and 40 s at 68 °C. The resulting PCR products were used in a Nested PCR reaction with GeneRacer™ 5′ Nested Primer and the gene-specific primer RACE 11212-a1 (see primer sequences in Table 1). The Nested PCR program comprises 2 min at 94 °C and 25 cycles of 30 s at 94 °C, 30 s at 65 °C and 2 min at 68 °C. The PCR products were cloned into pGEM-T Easy vector and sequenced.

4.8. Embryo genotyping

The method for genotyping was described by Ghanim and White (Ghanim and White, 2006). Briefly, heterozygous fly lines carrying the Ptr mutation were allowed to lay eggs on grape agar plates for 1 h. The collected embryos were dechorionated in 50% bleach and staged under light microscope. To perform RNA extraction of 10–15 homozygous embryos, one hundred embryos in stage 5 of development were selected and individually transferred into PCR tube containing 14.5 μl of extraction buffer (100 mM Tris–HCl ph8.2, 1 mM EDTA and 25 mM NaCl). After homogenization with a pipette tip, 11 μl from each single embryo extract were individually stored in a new tube containing 30 μl of RNAseW2 reagent from Ambion (Austin, TX, USA) and frozen (-20 °C) to preserve RNA integrity. The remaining extract (3.5 μl) was incubated at...
28 °C for 30 min with 200 μg/ml of proteinase K (Sigma–Aldrich, St. Louis, MO, USA), followed by incubation at 95 °C for 2 min. After the incubation with proteinase K the extract was used in PCR. In our case, absence of lacZ or GFP specific band together with the presence of a control band (gene CG0650) were indicators of homozygous lethal embryos.

4.9. RNA extraction

Extracts of homozygous mutant staged embryos preserved in RNAWIZ reagent (N = 10 to 15) were pooled adding 300 μl of the reagent. To perform adequate tissue disaggregation, the samples were carefully homogenized in a 1.5 ml Eppendorf tube on ice with the aid of RNase-free polypropylene pellet pestle. After complete the volume of each sample up 1 ml with RNAWIZ, RNA extraction was performed using standard protocols. To increase the recovery of RNA, precipitation was made with the addition of glycogen (Ambion, Austin, TX, USA) to the isopropanol step. After 15 min of centrifugation at 13,000 × g, RNA quality and quantity was evaluated by spectrophotometry (OD260/280) and by electrophoresis (1.2% formaldehyde–agarose gel). Samples were treated with TURBO DNA-free DNase (Ambion, Austin, TX, USA) to remove contaminating DNA.

Acknowledgements

We would like to thank Soledad Astra, Lucia Guggeri, Lucia Pastro and Fiorella Scandroglio for their technical assistance; Beatriz Garat and Rafael Cantera for their advice and critical comments during the work; and the Bloomington Drosophila Stock Center for providing stocks used in this study. The Apa1 monoclonal anti-Ptc developed by Isabel Guerrero, the 40-1a monoclonal anti-Galactosidase developed by Joshua Sanes and sn 7C monoclonal anti-fascin developed by Lynn Cooley were obtained from the Developmental Studies Hybridoma Bank (DSHB) under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Some materials were received through the Drosophila Genomics Resource Center. This work was supported by CSIC 1-D 2010 to CB and Fondecy N° 1120254 to VC. CP and Fr were supported by CSIC 1-D 2010. AZ was supported by Postdoctoral Fondecyt N° 3110147. CB was also supported by fellowships from: CSIC (Programa de Recursos Humanos), PEDECIBA, AMSUD-Pasteur (Regional Training Fellowship Program) and Proyecto 720 (Concurrentia Convenios-UdeLaR).

References

Abrams, J.M, White, K., Fessler, L.L, Steller, H., 1993. Programmed cell death during Drosophila embryogenesis. Development 117 (1), 29–43. Belen, H.J, Lewis, R.W, Liao, G., He, Y., Carlson, J.W., Tsang, G., Evans-Holm, M., Hiesinger, P.R., Schulze, K.L., Rubin, G.M., et al., 2004. The BDGP gene disruption materials were received through the Drosophila Genomics Resource Center. This work was supported by CSIC 1-D 2010 to CB and Fondecy N° 1120254 to VC. CP and Fr were supported by CSIC 1-D 2010. AZ was supported by Postdoctoral Fondecyt N° 3110147. CB was also supported by fellowships from: CSIC (Programa de Recursos Humanos), PEDECIBA, AMSUD-Pasteur (Regional Training Fellowship Program) and Proyecto 720 (Concurrentia Convenios-UdeLaR).