The *Drosophila* homolog of the putative phosphatidylinerse receptor functions to inhibit apoptosis

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Exposure of phosphatidylinerse is a conserved feature of apoptotic cells and is thought to act as a signal for engulfment of the cell corpse. A putative receptor for phosphatidylinerse (PSR) was previously identified in mammalian systems. This receptor is proposed to function in engulfment of apoptotic cells, although gene ablation of PSR has resulted in a variety of phenotypes. We examined the role of the predicted *Drosophila* homolog of PSR (dPSR) in apoptotic cell engulfment and found no obvious role for dPSR in apoptotic cell engulfment by phagocytes in the embryo. In addition, dPSR is localized to the nucleus, inconsistent with a role in apoptotic cell recognition. However, we were surprised to find that overexpression of dPSR protects from apoptosis, while loss of dPSR enhances apoptosis in the developing eye. The increased apoptosis is mediated by the *head involution defective* (*Wrinkled*) gene product. In addition, our data suggest that dPSR acts through the c-Jun-NH₂ terminal kinase pathway to alter the sensitivity to cell death.

KEY WORDS: Phosphatidylinerse, Engulfment, Apoptosis, *Drosophila*

INTRODUCTION

The terminal event of apoptosis is often engulfment of the apoptotic corpse by a phagocytic neighbor or professional phagocyte. Molecular events in the apoptotic cell are important for this process, including the removal of ‘don’t eat me’ signals, such as CD31 (Brown et al., 2002), and the exposure of ‘eat me’ signals, such as phosphatidylinerse (PS) (Verhoven et al., 1995). PS is normally confined to the inner leaflet of the plasma membrane in living cells, but moves to the outer surface in apoptotic cells (Schlegel and Williamson, 2001). This surface alteration is conserved from *Drosophila* through mammals (van den Eijnde et al., 1998). The exposure of PS provides a recognition cue for phagocytes and is thought to drive engulfment of the apoptotic cell (Fadok et al., 1998).

A number of proteins are known to bind to PS on the surface of apoptotic cells, and some have been shown to bridge apoptotic cells to molecules on the surface of phagocytes. These bridging molecules include annexin V, annexin I, milk fat globule EGF-like protein 8 (MFGE8), Developmental endothelial locus-1 (DEL-1) and growth arrest-specific 6 (GAS6) (Vermes et al., 1995; Scott et al., 2001; Hanayama et al., 2002; Arur et al., 2003; Hanayama et al., 2004).

In contrast to bridging molecules, a phagocyte receptor that directly binds PS on apoptotic cells has been described (Fadok et al., 2000). In this work, a monoclonal antibody that blocked apoptotic cell uptake in a PS-dependent manner was identified. The epitope recognized by the antibody was isolated by phage display and the gene encoding this epitope was identified as the PS receptor (PSR). PSR was predicted to be a type II membrane spanning protein. Expression of PSR in cells that normally do not engulf apoptotic cells increased their efficiency of engulfment (Fadok et al., 2000). In addition, PSR was found to have a role in the inhibition of inflammatory cytokine release after apoptotic cell engulfment (Hoffmann et al., 2001; Huynh et al., 2002). These studies suggested that PSR might be a key regulator of the response of phagocytes to apoptotic cells.

Gene knockout models of the PSR gene in mouse (*Jmjδ6* – Mouse Genome Informatics), *Caenorhabditis elegans* (psr-1 – Wormbase) and zebrafish (*jmjd6* – ZFIN) have been reported, but these studies do not unequivocally implicate PSR in engulfment. Conflicting phenotypes have been reported from three PSR knockouts in mice (Li et al., 2003; Bose et al., 2004; Kunisaki et al., 2004). Perinatal lethality was observed in all three knockouts; however, different effects on the proliferation and differentiation of certain tissues, as well as on apoptotic cell engulfment, were observed. Defects in apoptotic cell engulfment were found in two studies, but not in a third (Li et al., 2003; Bose et al., 2004; Kunisaki et al., 2004). Disruption of the PSR gene in *C. elegans* showed a very slight inhibition of apoptotic cell engulfment (Wang et al., 2003). Unengulfed apoptotic cells were also observed in zebrafish during development (Hong et al., 2004). More recently, no engulfment defect or altered response to apoptotic cells was observed in a fibroblast line established from PSR-deficient mice (Mitchell et al., 2006).

An additional issue in understanding PSR function has been disagreement regarding the localization of the PSR protein. Original reports indicated a potential transmembrane domain in the protein (Fadok et al., 2000). PSR also contains a region of homology to a jumonji C (JmjC) domain that has been implicated in modifying nuclear proteins (Ayoub et al., 2003). A fusion protein of mammalian PSR to GFP was shown to localize to the nucleus of nuclear proteins (Ayoub et al., 2003). A fusion protein of mammalian PSR to GFP was shown to localize to the nucleus. This result is consistent with the localization of PSR in mammals and raises the question of whether PSR functions to inhibit apoptosis in a cell surface receptor or a nuclear receptor.

Given these conflicting results, we investigated the function of the highly conserved PSR homolog (dPSR) in *Drosophila*, to determine if we could find a role for dPSR in engulfment or in other developmental events. In the fly embryo, the majority of apoptotic cells are engulfed and degraded by circulating phagocytic...
hemocytes, referred to as macrophages (Tepass et al., 1994). Membrane proteins involved in the uptake of apoptotic cells have been identified in *Drosophila. croquemort*, a homolog of the mammalian scavenger receptor CD36, was identified as a hemocyte protein important for engulfment of apoptotic cells (Franco et al., 1999), *draper*, a homolog of the *ced-1* gene in *C. elegans*, has also been identified as a phagocyte protein important for apoptotic cell engulfment (Freeman et al., 2003; Manaka et al., 2004).

Through analysis of a mutant for *dPSR*, we determined that *dPSR* is not required for the engulfment of apoptotic cells by macrophages in the developing embryo. However, we have detected a role for *dPSR* in protection from apoptosis. We found that increased *dPSR* inhibited apoptosis, while flies that lack *dPSR* showed inappropriate apoptosis during eye development. This protection from apoptosis appears to involve suppression of the apoptosis regulator Head involution defective (Hid; also known as Wrinkled – FlyBase). In addition, activation of the c-Jun-NH$_2$ terminal kinase (JNK) pathway suppresses phenotypes caused by *dPSR* overexpression, suggesting that *dPSR* may act to suppress the JNK pathway. These results provide an alternative explanation for phenotypes observed in PSR knockouts in other model organisms.

**MATERIALS AND METHODS**

**Fly stocks and cDNAs**

*P(EP)561* was obtained from the Szeged Stock Center; *Srp-hemo-GAL4, UAS-src:EGFP (srp-hemo GFP)* flies were kindly provided by Katja Bruchner and Norbert Perrimon (Bruchner et al., 2004); *dronc$^{224}$ FRT 2A/TM1 and *dronc$^{220}$ FRT2A/TM3* were kindly provided by Andreas Bergmann (Xu et al., 2005). Other flies used in these studies were *SM1, GMR-hid/Sco, CyO 2X GMR-rrp/Sco* (Kurada and White, 1998), *GMR-grim* (Chen et al., 1996), *GMR-GAL4* (Freeman, 1996), *UAS-grim* (Wing et al., 1998), *UAS-hid* (Zhou et al., 1997), *hid$^{P50}$TM6B, GMR-hid* (Grether et al., 1995), *Df(3L)H99/TM3* (White et al., 1994) and *Puc-lacZ$^{69}$* (Martin-Blanco et al., 1998). All experiments were conducted at 25°C.

**Generation of UAS-PSR flies**

The cDNA for *dPSR* (CG5383) was excised from cDNA clone LD25827 (Invitrogen) using *Bgl* II and *Xho* I, and ligated into the pPUBST vector. Transgenic flies were generated by the Transgenic Fly Core Facility of the Cutaneous Biology Research Center at Massachusetts General Hospital.

**Generation of dPSR$^{FM1}$ flies**

The *P(EP)561* element was mobilized with Δ2-3, PCR mapping was performed to determine loss of *dPSR* using various primers. DNA was isolated from *dPSR$^{FM1}$* homozygous flies for inverse PCR using the method described by E. J. Rehm on the Berkley Drosophila Genome Project website (http://www.fruitfly.org/about/methods/inverse.pcr.html). Briefly, DNA was isolated, digested with *Sal*III, and recircularized. The DNA was then amplified via PCR with primers *PSR1: ATGGTGTCGTCCATGTTGAG*, *Psrl-H11032* and *PSRR (5'-TCCAGATCT-ATCCACATTGATCCACTGGG-3')* and *PSR2* (5'-GGCTAGCCATGATGGGAGG-AAGCGGATGAGCCCGCGGACTGAA* and primers cloned and ligated into the *Bgl* II and *XhoI* sites in the pCEPF-N1 vector (Clontech) to make a *PSR-ECFP* fusion. The *PSR-ECFP* was excised with *Bgl* II and *NotI* and cloned into the *BamH* I and *NolI* sites of the pIE-1 vector (Novagen). S2 cells were transfected using 5 μg DNA and Cellfectin reagent (Invitrogen) in 1 ml serum-free Schneider's S2 media (Gibco). After 3 days in complete media, the cells were stained with 5 μg/ml Hoechst 33342 (Molecular Probes) and visualized using fluorescence microscopy.

**Apoptosis and engulfment assays**

Acridine Orange staining was done according to White et al. (White et al., 1994). To assess engulfment, embryos were fixed and apoptotic cells were visualized by staining with 7-aminoactinomycin D (Franco et al., 1999), or TUNEL (described below). The embryos were examined by confocal microscopy to visualize srp-hemo GFP-labeled hemocytes and the punctate 7-AAD- or TUNEL-labeled apoptotic nuclei. At least five single hemocytes from a minimum of eight embryos of each genotype were scored for engulfment from similar regions of age-matched *dPSR* homozygous embryos. The phagocytic index was calculated as the mean number of corpses per hemocyte for each genotype.

**Immunohistochemistry**

To assess pupal eye morphology, eye discs were taken from pupae at the indicated time after puparium formation at 25°C, dissected in 0.1 M sodium phosphate buffer (PB), and stained with a 1:20 dilution of mouse anti-DisC large antibody 4F3 (Developmental Studies Hybridoma Bank) overnight in PB with 0.1% Triton-X-100 (PBT) and 5% goat serum. The discs were washed three times in PBT for 10 minutes each, and then incubated with a 1:200 dilution of goat anti-mouse FITC conjugate (Jackson Immunochemicals) in PBT and 5% goat serum overnight at 4°C. The discs were washed three times for 10 minutes each and mounted using Fluormount-G (Southern Biotechnologies Associates). The discs were visualized by confocal microscopy.

Differentiation and proliferation in third instar eye discs were assayed by staining with *dPSR* FM1, or with *GMR-GAL4/UAS-dPSR;+* third instar eye discs with anti-phospho-histone antibody (1:200, Upstate), and rat anti-ElA antibody (1:200, Developmental Studies Hybridoma Bank), followed by secondary antibodies, goat anti-mouse FITC conjugate (1:200, Molecular Probes) and goat anti-rat Alexa 568 conjugate (1:200, Molecular Probes). The discs were visualized by confocal microscopy.

**In situ hybridization**

Fixed embryos were subjected to in situ hybridization using an antisense riboprobe specific to *dPSR*, and compared to a sense probe as described by Grether et al. (Grether et al., 1995).

**Reverse transcription PCR**

Total RNA was isolated from 100 third instar larval eye discs of the indicated genotype. Equal amounts of total RNA were reverse transcribed using an oligo dT primer and MMLV reverse transcriptase (Ambion). The cDNA encoding *dPSR* was then amplified using Platinum Taq (Invitrogen) with primers *PSRF (5'-ATCCACATTGATCCACTGGG-3')* and *PSRR (5'-AGCTGAAATTTGGACTG-3').

**TUNEL labeling**

Cell death in embryos was assayed using the In Situ Cell Death Detection Kit, TMR red (Roche). Embryos were dechorionated and fixed at the interface of heptane and 4% formaldehyde for 20 minutes, and then dehydrillized with methanol for 2 minutes. Embryos were washed once with methanol, twice with ethanol (2 minutes each), and incubated in 70% ethanol overnight at –20°C. The embryos were washed with 30% ethanol for 10 minutes and twice with PBS for 10 minutes each, and then permeabilized with 0.3% Triton X-100 for 20 minutes and incubated in PBS + 0.1% Triton X-100 (PBS) with 1:1000 anti-GFP antibody (Invitrogen) overnight at 4°C. The embryos were washed three times in PBSB for 10 minutes each, and incubated with secondary antibody (Alexa 488-conjugated donkey anti-rabbit antibody, Molecular Probes), in 50 μl TUNEL labeling mix/enzyme solution, overnight at 4°C. The embryos were washed three times in PBSB for 10 minutes, mounted in Fluormount, and visualized with confocal microscopy. To assure that TUNEL signal was specific to apoptotic cells, H99 homozygous embryos that lack cell death were analyzed and found to lack TUNEL staining.
Pupal eye discs were dissected at 49 hours after pupation, fixed in 4% formaldehyde in PBS for 20 minutes, and stained as described for anti-Discs large. The TUNEL reaction was carried out for 2 hours at 37°C. The Alexa 488-conjugated goat anti-mouse secondary antibody was added directly to the TUNEL labeling solution. The discs were then washed three times in PBST, mounted with Fluormount and visualized under confocal microscopy.

RESULTS
To identify genes involved in apoptotic cell engulfment we stained embryos with Acridine Orange (AO). Apoptotic cells can be visualized by AO, and can be seen to cluster in embryos, because of engulfment by macrophages (Abrahm et al., 1993). In wild-type embryos, clustering of AO-positive cells can be observed in the anterior and posterior, and along the nervous system (Fig. 1A). We screened a collection of genomic deletions for a lack of apoptotic cell clustering (N. Franc, C. Marion, B. Zhai, R.J.K. and K.W., unpublished). Decreased clustering could indicate a defect in phagocytic clearance, or could be due to problems in macrophage development or migration.

Our screens identified several genomic regions with possible defects in engulfment. Embryos homozygous for Df(3R)Sc1 contained numerous unclustered AO-positive cells (Fig. 1B). Interestingly, this deletion includes the gene encoding the Drosophila homolog of the putative phosphatidylserine receptor, dPSR (Fadok et al., 2000). The predicted Drosophila gene encodes a 408 amino acid protein that is 70% identical and 82% similar to the human gene, with conservation extending throughout the gene (data not shown). This prompted us to further examine the role of this gene in engulfment. We generated dPSR mutants by P-element excision of EP(S61), a P-element insertion 101 bp 5’ of dPSR (Fig. 1D). Five imprecise excision mutants of dPSR were generated. The strain dPSRFM1 was found to be a deletion that removed sequence from the P-element insertion into the gene, deleting the coding sequence for the first 210 amino acids of dPSR, while a portion of the P element and the upstream gene remained (Fig. 1D).

Homozygous dPSRFM1 flies were viable and fertile with no obvious morphological defects and were established as a homozygous line for further analysis. We found that dPSR is ubiquitously expressed in wild-type embryos, but not expressed in dPSRFM1 embryos (see Fig. S1A,B in the supplementary material), indicating that dPSRFM1 is likely to be a null mutation. In addition, no expression of dPSR could be detected by RT-PCR in eye discs from third instar dPSRFM1 larvae, whereas dPSR was expressed in control eye discs (see Fig. S1C in the supplementary material).

PSR is not important for apoptotic cell engulfment
To determine the requirement for dPSR in apoptotic cell engulfment, we examined dPSRFM1 embryos by AO staining and found that the numbers and pattern of clustered apoptotic cells was similar to those seen in wild-type flies (Fig. 1C). This indicates that the AO-positive cell clustering phenotype detected in the Df(3R)Sc1 embryos is not due to deletion of the dPSR gene alone.

To quantify apoptotic cell engulfment in wild-type and mutant embryos, we analyzed the number of apoptotic cells per macrophage using srp hemo-GAL4, UAS-src-EGFP (Srphemo GFP). In these animals, GFP is expressed specifically in macrophages (Bruckner et al., 2004). The number of GFP-labeled macrophages and apoptotic cells was similar in wild-type and dPSRFM1 embryos (see Fig. S2 in the supplementary material). We counted the number of engulfed cell corpses, marked by 7-aminoactinomycin D or TUNEL staining, in macrophages of similarly staged embryos. Importantly, we did not detect a decrease in apoptotic cell engulfment by macrophages in dPSRFM1 embryos (Fig. 2A,B). These data indicate that dPSR is not required for apoptotic cell clearance by macrophages in the developing embryo.

Drosophila PSR is a nuclear protein
If Drosophila PSR functions in macrophages to recognize dying cells, one would expect this protein to be localized to the cell surface. However, analysis of the sequence of dPSR using PSORT II predicts that dPSR is a nuclear protein (Nakai and Horton, 1999). This was consistent with reports that mammalian and Hydra PSR contain nuclear localization signals and significant homology to nuclear proteins (Cikala et al., 2004; Cui et al., 2004; Mitchell et al., 2006). To determine whether Drosophila PSR is localized to the nucleus, the sequence was fused to the N-terminus of ECFP and expressed in S2 cells under a constitutive promoter. ECFP alone was found throughout the cell (Fig. 3A), while PSR:ECFP was found to co-localize with the DNA stain Hoechst 33342 (Fig. 3B-D). These data indicated that dPSR is likely to be localized to the nucleus of Drosophila cells, and is unlikely to act as a cell surface receptor for apoptotic cells.

A role for PSR in protection from cell death
In addition to its role in apoptotic cell recognition and engulfment, work on PSR in mammalian systems suggest that PSR is important for other processes such as proper development and TGF-β release (Hoffmann et al., 2001; Bose et al., 2004). To explore the signaling pathways activated by PSR, we generated transgenic flies that
expressed dPSR under control of the GAL4-UAS system (Brand and Perrimon, 1993). Expression of dPSR under two different ubiquitous promoters, Act5c-GAL4 and Tub-P-GAL4, resulted in a rotated male genital defect similar to that seen in mutants of the hid gene (Fig. 4A-C) (Abbott and Lengyel, 1991). hid is one of a cluster of genes important for regulating developmental apoptosis in the fly. Loss of hid results in semi-lethality, with adult escapers showing a rotated male genital phenotype and a lack of wing blade fusion resulting from lack of cell death (White et al., 1994). A genital rotation phenotype has also been observed in flies that express the baculovirus pan-caspase inhibitor p35, confirming a role for apoptosis in the correct orientation of the developing male genital disc (Macias et al., 2004). We also observed the rotation defect in flies that lack dronc (Nc – FlyBase), an initiator caspase (Fig. 4D) (Xu et al., 2005). This further indicates a role for cell death in this phenotype.

dPSR expression driven by the apterous driver resulted in a phenotype in the wing, ranging from a wavy wing, to a puffy wing that lacked wing blade fusion. This is another phenotype shared by hid and dronc mutants (Fig. 4F-I) (Abbott and Lengyel, 1991; Xu et al., 2005). These results suggest that dPSR overexpression inhibits Hid-dependent apoptosis in multiple Drosophila tissues.

To further test the hypothesis that dPSR can protect from apoptosis, we examined the effects of increasing or decreasing dPSR function on cell death induced by the overexpression of pro-apoptotic genes in the eye. If dPSR promotes cell survival in the eye, then increased dPSR should suppress apoptosis and loss of dPSR should enhance apoptosis. We co-expressed dPSR in the eye with the pro-apoptotic proteins Hid or Grim. Co-expression of dPSR suppressed the small rough eye induced by expression of Hid, the pro-apoptotic genes in the eye. If dPSR promotes cell survival in the eye, then increased dPSR should suppress apoptosis and loss of dPSR should enhance apoptosis. We co-expressed dPSR in the eye with the pro-apoptotic proteins Hid or Grim. Co-expression of dPSR suppressed the small rough eye induced by expression of Hid, resulting in an average increase in eye size of 19% (P = 0.0002). The rough eye induced by Grim was also suppressed when dPSR was co-expressed (Fig. 5A-D).

To determine whether loss of dPSR enhances death, we examined the effect of removing dPSR on Rpr-, Hid- or Grim-induced cell killing in the developing eye. We found that deletion of one copy of dPSR enhanced the rough eye of GMR-Rpr, GMR-Hid or GMR-Hid or Grim.
Grim. Loss of both copies of dPSR further enhanced this death (Fig. 6A-I). Taken together, these results suggest that the normal function of dPSR in the eye is to suppress cell death.

To determine whether dPSR was required to block apoptosis in the absence of exogenous stimuli, we examined the eye for altered apoptosis at various stages of development. There was no detectable alteration in apoptosis, as detected by TUNEL staining in third instar larval eye discs from dPSRFM1 mutants or from larvae expressing dPSR under control of a GMR promoter (data not shown). Proliferation and differentiation were also normal in these discs, as assessed by anti-phosphohistone and anti-Elav antibody staining, respectively (data not shown) (Robinow and White, 1991).

During pupal life, the characteristic hexagonal lattice structure of the adult eye is formed. The cells that make up each ommatidial unit can be observed using anti-Discs large, an antibody to a septate junction protein that labels cell membranes (Parnas et al., 2001). During the formation of the hexagonal lattice, many extra interommatidial cells undergo apoptosis, leaving 12 lattice cells per ommatidium (Brachmann and Cagan, 2003). The apoptosis of these extra cells and formation of the lattice is normally complete by 30 hours after puparium formation (APF) (Brachmann and Cagan, 2003). In wild-type pupal eye discs, a characteristic hexagonal lattice pattern of the secondary and tertiary pigment cells of the ommatidial clusters is observed (Fig. 7A). At 40 hours APF, pupal eye development was normal in the mutants and a characteristic hexagonal lattice of interommatidial cells was seen (Fig. 7D). By 50 hours APF we detected loss of interommatidial cells, which in some cases resulted in ommatidial fusions (Fig. 7B,C). The loss of cells was not uniform, as some parts of the eye discs appeared normal at this time. To determine if the loss of the lattice cells was due to apoptotic death of these cells, we performed TUNEL staining of eye discs at 49 hours APF. We observed TUNEL-stained nuclei below the plane of the lattice in mutant discs (Fig. 7I,J). In contrast to the loss of cells seen in the absence of dPSR, overexpression of dPSR in the eye led to the appearance of extra lattice cells (Fig. 7E). These data indicate that dPSR functions to regulate apoptosis during the pupal phase of eye development.

dPSR inhibits apoptosis upstream of Hid

To test directly whether the ectopic death seen in dPSR mutants was due to Hid-dependent apoptosis, we examined the effect of loss of hid on the dPSRFM1 pupal eye phenotype. We found that the absence of both hid and dPSR resulted in extra interommatidial cells, similar to the phenotype caused by expressing cell death inducers in the eye. Removing one copy of dPSR (B,E,H) enhances the small or rough phenotype caused by expressing cell death inducers in the eye. Removing both copies of dPSR (C,F,I) further enhances the rough eye phenotype.
to that seen in the absence of hid alone (Kurada and White, 1998) (Fig. 7F,G). These results indicate that the increased apoptosis in dPSR-null eyes is due to Hid-dependent apoptosis.

dPSR and hid have opposite effects on apoptosis. To determine if dPSR acted genetically downstream of hid, we tested whether loss of dPSR could modify the hid genital phenotype. Flies that lack both hid and dPSR displayed a penetrance of the rotated genital phenotype similar to flies that lack hid alone (Fig. 4E). These data indicate that dPSR is unlikely to act downstream of hid to regulate cell death. Taken together, our epistasis analysis in both the eye and genitals suggest that dPSR functions to suppress Hid activity and thus promote cell survival.

dPSR inhibits apoptosis upstream of JNK activity

The JNK pathway has previously been shown to regulate Hid activity and apoptosis during development (Moreno et al., 2002). The JNK pathway has also been shown to be important for the proper rotation of the developing male genitalia, a process that requires apoptosis (Macias et al., 2004). Increasing JNK activity by removing one copy of its negative regulator, Puckered (Puc), suppresses a genital rotation defect observed in males heterozygous for the H99 deletion, which deletes hid, grim and rpr (Macias et al., 2004). Inhibition of JNK by overexpression of Puc can also induce rotated genitalia (Macias et al., 2004). These data support the model that decreased JNK activity suppresses Hid activity, resulting in similar rotation defects to those seen in hid mutants.

To determine whether the JNK pathway has a role in the rotated genital phenotype induced by dPSR expression, we examined the effect of loss of one copy of puc on the dPSR overexpression phenotype. Loss of one copy of puc almost completely suppressed the rotated genital phenotype resulting from increased dPSR. The mis-rotation phenotype was seen in 52% (336/642) of dPSR-overexpressing flies, but in only 1.6% (8/496) of flies that overexpress dPSR but have one copy of puc disrupted (Fig. 8A). Thus, the dPSR anti-apoptotic effects are counteracted by increasing JNK pathway activity, suggesting that dPSR may suppress Hid activity by suppressing the JNK pathway (Fig. 8B).

DISCUSSION

We have examined the function of the Drosophila homolog of the putative phosphatidylserine receptor. Our data indicate that this protein is not required for apoptotic cell engulfment by macrophages in the Drosophila embryo, but rather functions to suppress apoptosis. We have considered how these findings can be interpreted in light of the diverse data on the function of PSR in other organisms.
PSR may not be involved in engulfment

In addition to the original cell-based studies supporting a role for PSR in engulfment, gene ablation experiments in a variety of models also provide some evidence for a role for PSR in this process. PSR gene ablation in C. elegans resulted in a delay in the engulfment of apoptotic cells, as evidenced by the presence of more cell corpses in the mutant animals (Wang et al., 2003). In two of the three reported murine PSR gene-knockout models, defects in apoptotic cell engulfment were reported (Li et al., 2003; Kunisaki et al., 2004).

We found no evidence for a role of dPSR in engulfment in Drosophila. Evidence against a role for PSR in engulfment also comes from two other knockout models and from data on the localization of the protein. One of the reported mouse knockouts showed no difference in engulfment of apoptotic cells by macrophages in the mutant, although PSR−/− macrophages were generally inhibited in their release of pro- and anti-inflammatory cytokines (Bose et al., 2004). In addition, fibroblast lines established from PSR−/− embryos showed no defect in apoptotic cell engulfment or in their response to apoptotic cells (Mitchell et al., 2006). Zebrafish lacking PSR accumulated dead cells, but were not definitively shown to have defects in apoptotic cell engulfment (Hong et al., 2004). Finally, localization data from our work and from a number of labs strongly supports a nuclear localization for the protein (Ayoub et al., 2003; Cui et al., 2004; Mitchell et al., 2006). This is not consistent with a role for PSR as a surface receptor for the recognition for apoptotic cells, although PS could theoretically modulate the activity of this protein within the cell.

PSR could be required for cell differentiation and survival

Our observations support a role for dPSR in cell survival. In zebrafish, reduction of PSR resulted in an increase in the number of apoptotic cells present during development (Hong et al., 2004). In particular, the brains of these fish were shrunken and had an increase in apoptotic cells. In two of the mouse knockout models an increase in apoptotic cells was detected (Li et al., 2003; Kunisaki et al., 2004). However, all three knockouts resulted in perinatal lethality, with defects in differentiation in a variety of tissues (Li et al., 2003; Bose et al., 2004; Kunisaki et al., 2004). We speculate that defects in engulfment detected in some of the gene ablation models could reflect a role for PSR in the proper differentiation of macrophages. Increased apoptosis seen in our studies and by others might also be due to defects in proper differentiation in the absence of PSR.

What insight can be gained from our studies into the function of dPSR in differentiation and cell survival? We have shown that increased dPSR results in a cell survival phenotype that is suppressed by activation of the JNK pathway, while loss of dPSR results in apoptosis, activated by the cell death regulator Hid, a known target of JNK activation in apoptosis (McEwen and Peifer, 2005). Taken together, these data suggest that dPSR may normally act to suppress JNK activation of Hid-induced apoptosis.

JNK activation is important for many processes in cells, including cell death, proliferation and differentiation. A role for JNK in apoptosis was found in many mammalian cell types (Yang et al., 1997; Dong et al., 1998; Tournier et al., 2000). Data from mouse knockouts of JNK also suggest a role for JNK in proliferation and differentiation (Dong et al., 1998; Tournier et al., 2000). In addition, JNK activation in dying cells is required for proliferative signals originating from apoptotic cells in Drosophila (Ryoo et al., 2004). Interestingly, defects in proliferation and differentiation of many tissues were observed in mice that lack PSR (Li et al., 2003; Bose et al., 2004; Kunisaki et al., 2004). Taken together with our observations of increased cell death in dPSR mutant flies, these observations suggest that some of the phenotypes seen in mouse and fish models of PSR gene ablation might be due to inappropriate activation of the JNK pathway.

Role of PSR in Drosophila development

Based on our genetic assays, we propose that one function of dPSR is to suppress Hid activation. Flies that lack dPSR show increased apoptosis in the developing pupal eye, which is suppressed in the absence of hid, while overexpression of dPSR results in ectopic cell survival. Hid function is required for the death of the interommatidial cells in the pupal retina (Kurada and White, 1998). Our results also showed that expression of dPSR can inhibit death induced by the expression of Hid or Grim in the eye, and that loss of dPSR enhances Rpr-, Hid- or Grim-induced death in the eye. Interestingly, loss of one copy of hid can also suppress cell death induced by Rpr or Hid expression in the eye (Bergmann et al., 1998; Kurada and White, 1998). Therefore alterations of dPSR levels in the eye may be altering Hid activity to modify the Grim- and Rpr-induced eye phenotypes.

JNK activation has been shown to increase hid activity (McEwen and Peifer, 2005). However, Hid activity is also modulated by activation of the Ras/Erk pathway (Bergmann et al., 1998; Kurada and White, 1998). Ras activation results in the survival of ectopic interommatidial cells, through the downregulation of Hid activity (Miller and Cagan, 1998). Ectopic Ras activation also results in genital rotation defects, similar to those seen with dPSR overexpression (Macias et al., 2004). This suggests that PSR overexpression might activate the Ras/Erk pathway. Based on our data, it is not clear whether dPSR might activate Ras and thus suppress JNK activity, whether dPSR could suppress JNK and thus activate Ras, or whether dPSR might act independently in an opposing manner on the JNK and Ras pathways.

By examining the function of dPSR in the Drosophila system, we have provided new insight into the controversy regarding this protein. Although we do not find evidence that this protein plays a role in engulfment, we have found that it is important in cell survival. This is consistent with phenotypes seen in gene ablation models in other organisms. Furthermore, we have shown that dPSR affects the JNK pathway, and this may provide a clue as to its diverse functions in mammals.

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