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PVF2, a PDGF/VEGF-like growth factor, induces hemocyte proliferation in *Drosophila* larvae

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Blood cells play a crucial role in both morphogenetic and immunological processes in *Drosophila*, yet the factors regulating their proliferation remain largely unknown. In order to address this question, we raised antibodies against a tumorous blood cell line and identified an antigenic determinant that marks the surface of prohemocytes and also circulating plasmatocytes in larvae. This antigen was identified as a *Drosophila* homolog of the mammalian receptor for platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF). The *Drosophila* receptor controls cell proliferation *in vitro*. By overexpressing *in vivo* one of its putative ligands, PVF2, we induced a dramatic increase in circulating hemocytes. These results identify the PDGF/VEGF receptor homolog and one of its ligands as important players in *Drosophila* hematopoiesis.

INTRODUCTION

Blood cells (hemocytes) play important roles in tissue remodeling during embryogenesis and metamorphosis and in immune defenses of insects. *Drosophila* contains three distinct hemocyte types: plasmatocytes, lamellocytes and crystal cells (reviewed in Lanot *et al.*, 2001). Plasmatocytes represent the major hemocyte type (~95%). They exhibit potent phagocytic capabilities. During embryogenesis and metamorphosis, plasmatocytes are involved in the clearance of apoptotic cells and histolytic debris (Tepass *et al.*, 1994; Franc *et al.*, 1996). During immune defenses, they engulf microbial particles, synthesize and secrete some of the antimicrobial peptides and possibly concur to activate the production of antimicrobial peptides by the fat body (reviewed in Hoffmann and Reichhart, 2002; Ramet *et al.*, 2002). Lamellocytes are very large and flattened cells that appear in the blood in response to parasitic infections and encapsulate large-sized invaders (e.g. wasp eggs). Crystal cells are observed only during late embryogenesis and in larvae (Lebestky *et al.*, 2000) and are involved in melanization and melanotic tumor formation.

Significant progress has recently been made in understanding the genetic control of blood cell differentiation during development (Lebestky *et al.*, 2000). However, our information on the molecular facets of the recognition of microorganisms by blood cells is still fragmentary. In addition, the mechanisms by which blood cell proliferation and differentiation are triggered during an immune response have remained elusive so far. To identify hemocyte membrane receptors implicated in defense and proliferation, we decided to raise monoclonal antibodies directed against the surface proteins of hemocytes. A similar approach had led to the characterization of numerous clusters of differentiation (CDs) on the surface of mammalian blood cells. These CDs serve to follow cell lineages, identify activation states of blood cells and isolate distinct functional subpopulations.

Using this strategy, we have identified an antibody that inhibits proliferation of hemocyte-like cells *in vitro*. We identified the antigenic determinant as the *Drosophila* homolog of the mammalian receptor for the cytokines platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). *Drosophila* contains three genes encoding a putative ligand for this receptor, and we show that one of these triggers a dramatic proliferation of hemocytes when overexpressed *in vivo*.

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RESULTS

Production of antibodies and characterization of 18G as a proliferation-inhibiting antibody

As *Drosophila* blood cells are difficult to obtain in sufficient numbers for immunization of mice, we raised antibodies against the cells of the tumorous blood cell line, mbn-2 (Gateff *et al.*, 1980), which are functionally close to plasmatocytes. These cells phagocytose microorganisms and also synthesize and secrete antimicrobial peptides when exposed to bacteria or bacterial cell components (Dimarcq *et al.*, 1997). We injected live mbn-2 cells into BALB/c mice and selected hybridomas for their capacity to produce antibodies that recognize cell surface antigens on live mbn-2 cells. This recognition was monitored by flow cytometric analysis (Figure 1A and B), and selected antibodies were tested for their abilities to affect the mitotic rate of mbn-2 cells. In these series, one antibody, 18G, strongly inhibited the proliferation of mbn-2 cells when added to the culture medium (number 6 in Figure 1C). The antiproliferative capacity of 18G was further ascertained by measuring its dose-dependent effect (Figure 1D). Western blots performed with the 18G antibody on extracts of mbn-2 cells (under non-reducing conditions) revealed several protein bands of high molecular weight (>75 kDa; Figure 1E). Stained high-molecular-weight proteins were also detected in extracts of larval blood cells, as well as in *Drosophila* Schneider 2 (S2) cells.

The 18G determinant recognizes prohemocytes and circulating plasmatocytes

We next examined the distribution of the 18G determinant on the different *Drosophila* hemocyte types by immunocytochemistry. Wild-type and $hop^{Tum\text{-}l}$ mutants were used for this purpose. $hop^{Tum\text{-}l}$ is a Janus kinase (JAK) gain-of-function mutation resulting in an overproliferation of circulating blood cells, of which a large number are lamellocytes (Hanratty and Dearolf, 1993; Luo *et al.*, 1995). As shown in Figure 2A and C, plasmatocytes were stained and lamellocytes were not. In $hop^{Tum\text{-}l}$ mutants, the most strongly reacting cells were small rounded cells that correspond to circulating progenitor blood cells (prohemocytes). In wild-type larvae, the presence of prohemocytes is mostly restricted to lymph glands. We observed strong staining on the prohemocytes of wild-type lymph glands (Figure 2E). In *hop*-$^{Tum\text{-}l}$ lymph glands, the small rounded prohemocytes were also strongly stained, but other cells that have been described as lamellocytes by Lanot *et al.* (2001) did not react with the antibody (Figure 2G). Stained circulating crystal cells could not be observed due to their fragility. We never observed any staining on larval tissues other than hemocytes, namely on fat body, muscles, imaginal discs, epidermal cells, brain or trachea (data not shown).

The 18G antibody recognizes a *Drosophila* homolog of the mammalian receptor for VEGF/PDGF

Three protein bands positive for 18G staining in mbn-2 cell extracts were purified by immunoaffinity chromatography and subjected to Edman degradation. The N-terminal sequences obtained with each of the three protein bands were identical: VPLQQFSPDP. The *Drosophila* genome contains a single match to this sequence, namely in a gene encoding a homolog of

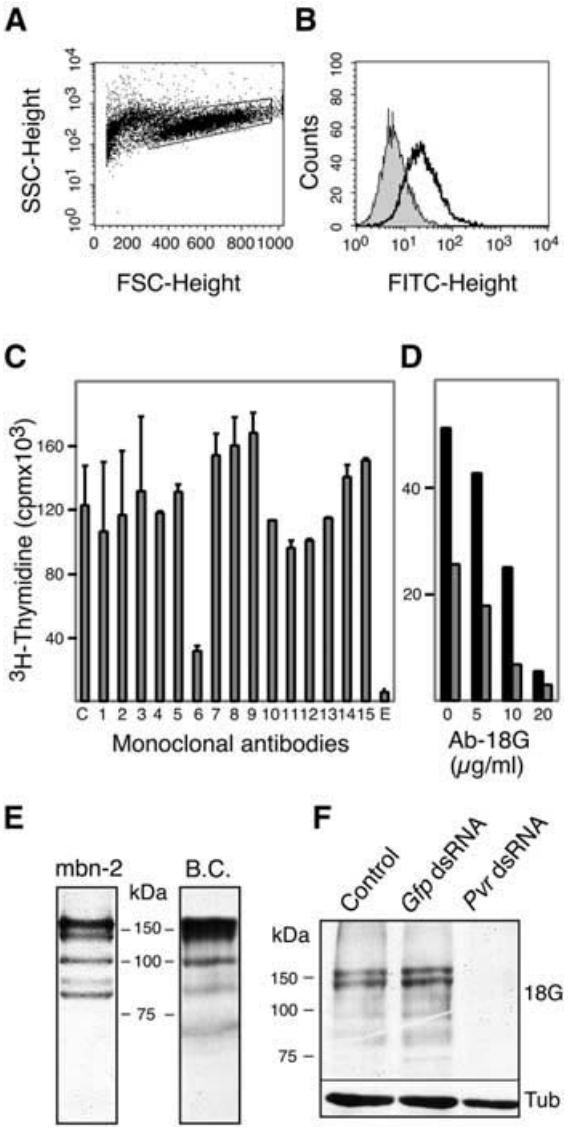

Fig. 1. Generation of monoclonal antibodies against cell surface antigens. (**A**) Flow cytometric analysis: live mbn-2 cells are gated according to their forward and side scatter profiles (FSC- and SSC-Height). (**B**) Flow cytometric detection of mbn-2 cells labeled with antibody. A secondary FITC-labeled antibody allows the detection of stained cells (black curve). Cells incubated only with the secondary FITC antibodies served as control to measure fluorescence background (plain gray curve). (**C**) Mitotic rate of mbn-2 cells measured by [^{3}H]thymidine incorporation incubated with different antibodies (1–15; 18G is number 6). C, control: cells without antibody. E, cells incubated with 20-hydroxyecdysone; this ecdysteroid was used as a control because it has been shown to impair proliferation of mbn-2 cells (Gateff *et al.*, 1980; Dimarcq *et al.*, 1997). (**D**) The effect of 18G on cell proliferation increases in a dose-dependent manner. Black bars, 1×10^5 cells/well; gray bars, 5×10^4 cells/well. (**E**) Western blot analysis (under non-reducing conditions) of mbn-2 cell and larval blood cells (B.C.) extracts using 18G antibody. (**F**) Western blot analysis (under non-reducing conditions) using 18G antibody of S2 cell extracts after mock transfection (control) or transfection with *Gfp* dsRNA and *Pvr* dsRNA (experiments were performed as in Duchek *et al.*, 2001). Molecular weight markers are indicated (in kDa).

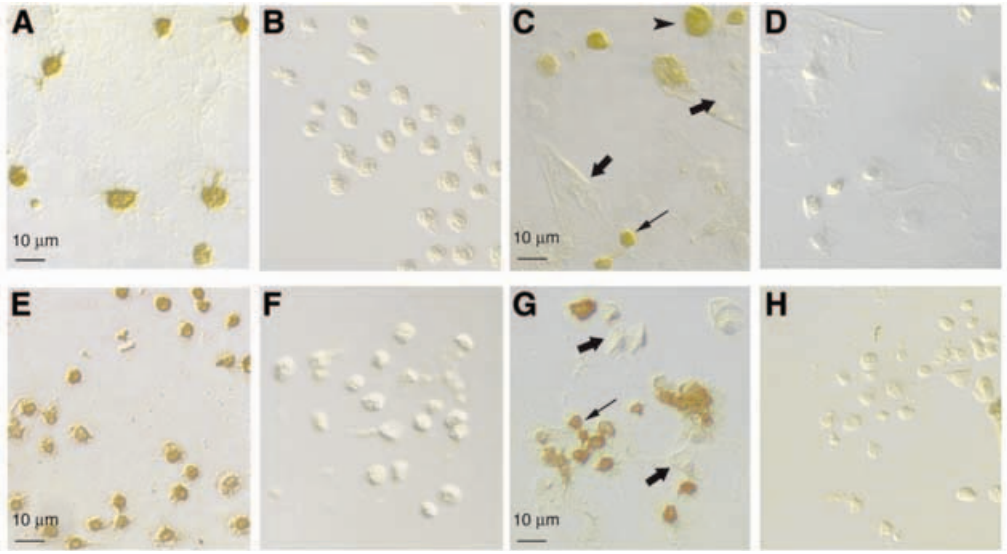

Fig. 2. The 18G antibody stains prohemocytes and circulating plasmatocytes. Blood smears from Oregon third instar larvae (**A** and **B**) or from *hop*$^{Tum\text{-}l}$ third instar larvae (**C** and **D**). Dissected and dilacerated lymph glands of Oregon third instar larvae (**E** and **F**) or from *hop*$^{Tum\text{-}l}$ third instar larvae (**G** and **H**). Staining was performed using 18G antibody followed by a peroxidase-conjugated goat anti-mouse IgG (A, C, E and G) or for controls an irrelevant IgG (human CD45) mouse antibody followed by a peroxidase-conjugated goat anti-mouse IgG (B, D, F and H). The arrowhead points to plasmatocytes, the thin arrows to prohemocytes and the thick arrows to lamellocytes.

mammalian receptors for PDGF and VEGF. Independent studies have recently identified expression of this gene in ovarian border cells and embryonic hemocytes, and the receptor is now referred to as PVR (PDGF/VEGF receptor; Duchek *et al.*, 2001; Heino *et al.*, 2001; Cho *et al.*, 2002). *Pvr* RNA interference experiments in S2 cells confirmed that 18G recognizes this receptor (Figure 1F).

Functional analysis of the 18G/PVR protein and its putative ligand

We next attempted to study the role of PVR on *Drosophila* blood cell proliferation *in vivo*, using mutants or transgenes of *Pvr*. A transposon insertion (line PBc2195) in the 11th intron of the *Pvr* gene generates a null mutant that leads to embryonic lethality (Cho *et al.*, 2002). Larvae heterozygous for this mutation (*Pvr*^{c2195}/*CyO*) did not show defects in hemocyte counts (data not shown). Overexpression of the *Pvr* cDNA by using a *UAS-Pvr* transgenic line crossed with either a *daughterless-GAL4* line (for ubiquitous expression) or *e33c-GAL4* line (for preferential lymph gland expression) was lethal at the embryonic stage (data not shown). As an alternative, to establish the role of this receptor in hemocyte proliferation in larvae, we analyzed the effect of ectopic expression of two of its putative ligands: *Pvf1* (CG7103) and *Pvf2* (CG13780) (Duchek *et al.*, 2001; Heino *et al.*, 2001; Cho *et al.*, 2002).

We generated *UAS-Pvf1* and *UAS-Pvf2* transgenic fly lines and directed PVF1 and PVF2 expression using *daughterless-GAL4* and *e33C-GAL4* drivers as described above and examined larval hemocytes. We observed that PVF2 expression in both cases resulted in a dramatic increase (up to 300-fold; $4 \times 10^5 \pm 1 \times 10^5$/μl of blood, compared with $1.5 \times 10^3 \pm 0.5 \times 10^3$ in *UAS-Pvf2/+*) in the number of blood cells in third instar larvae (Figure 3A and B) and led to pupal lethality. Apart from hemocytes and lymph glands, no proliferation was observed in other tissues (data not shown). In contrast, overexpression of PVF1, using the same *GAL4* drivers, resulted in a mild and variable effect on blood cell counts that did not exceed a 2-fold increase compared with controls (Figure 3C and D). Overexpression of PVF1 also resulted in pupal lethality.

On average, the hemocytes in PVF2-overexpressing flies were noticeably smaller than in the wild type (~6 μm in diameter, compared with 10 μm for wild-type plasmatocytes). All cells were reactive to the 18G antibody, indicating that they are prohemocytes or plasmatocytes (data not shown). However, only a small percentage (5%, compared with 80% in controls) was able to phagocytose injected India ink, thus qualifying as fully mature plasmatocytes (Figure 4A and B). Staining with anti-phosphohistone H3 antibody showed that a large number of circulating hemocytes were in the process of division (Figure 4C and D), indicating that PVF2 stimulates proliferation rather than promotes cell survival. The vast majority of the PVF2-induced hemocytes are therefore to be considered as prohemocytes. Crystal cells were noticeably absent in *w; UAS-Pvf2/+; e33C-GAL4/+* larvae (Figure 4E and F).

We counted blood cells in two lines with a transposon inserted in the *Pvf2* gene, XPd2444 and PBc6947 (Cho *et al.*, 2002). These lines are homozygous viable and showed no obvious defects in blood cell counts (XPd2444, $0.2–0.8 \times 10^3$ cells/μl; and PBc6947, $0.6–0.7 \times 10^3$ cells/μl). A *Pvf1* loss-of-function mutant, *Pvf1*^{1624} (Duchek *et al.*, 2001), similarly showed no defect in larval blood cell counts. However, in this line, pupal lethality was observed (40–60% lethality).

DISCUSSION

By using an antibody screening approach against a *Drosophila* blood cell line, we have identified the receptor tyrosine kinase

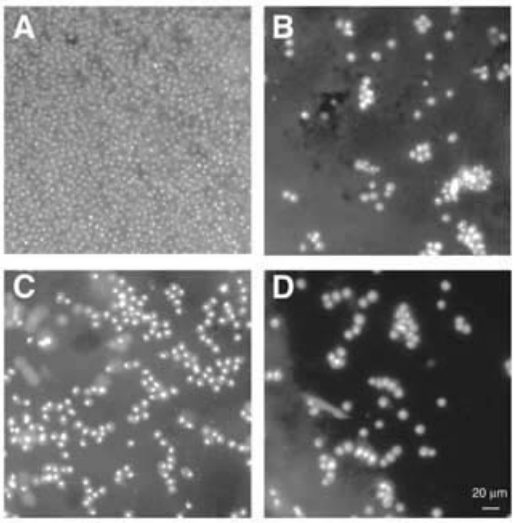

Fig. 3. Ectopic expression of PVF2 promotes the proliferation of hemocytes. (**A**) Blood drop of a third instar larva overexpressing PVF2 (*w; UAS-Pvf2/+; e33C-GAL4/+*). (**B**) Blood drop of control larva (*w; UAS-Pvf2/+; TM6B/+*). (**C**) Blood drop of a third instar larva overexpressing PVF1 (*w; UAS-Pvf1/+; e33C-GAL4/+*). (**D**) Blood drop of a control larva (*w; UAS-Pvf1/+; TM6B/+*). Nuclei of blood cells are stained with DAPI.

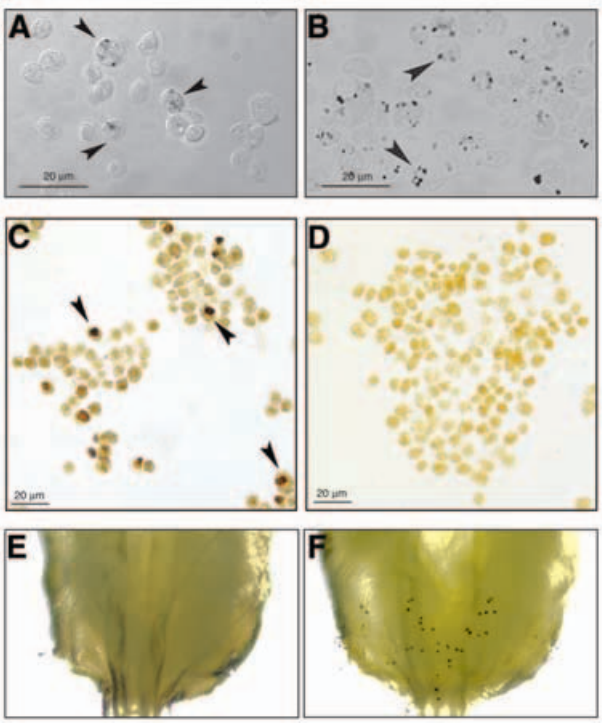

Fig. 4. Blood cell types present in larvae overexpressing PVF2. (**A** and **B**) Plasmatocytes are evidenced by their phagocytotic activity revealed by the presence of India ink (arrowheads; Lanot *et al.*, 2001). (A) Blood smears of larvae overexpressing PVF2 (*w; UAS-Pvf2/+; e33c-GAL4/+*). (B) Blood smears of control larva (*w; UAS-Pvf2/+; TM6B/+*). (**C** and **D**) Dividing cells, prohemocytes, are evidenced by immunostaining with an anti-phosphohistone H3 antibody. (C) Blood smears of larvae overexpressing PVF2 (*w; UAS-Pvf2/+; e33c-GAL4/+*). (D) Blood smears of control larva (*w; UAS-Pvf2/+; TM6B/+*). (**E** and **F**) The presence of crystal cells is revealed by a 10 min heating at 60°C; with this treatment, they turn black (Rizki *et al.*, 1980). (E) No crystal cells are observed in larvae overexpressing PVF2 (*w; UAS-Pvf2/+; e33c-GAL4/+*), as opposed to (F) control larvae (*w; UAS-Pvf2/+; TM6B/+*).

(RTK) PVR as a marker of larval *Drosophila* hemocytes. This receptor is found on lymph gland prohemocytes and on the surface of mature circulating plasmatocytes/macrophages. The anti-PVR antibody inhibits thymidine incorporation in blood cell lines, whereas overexpression of one of its ligands, PVF2, induces the proliferation of hemocytes *in vivo*. These results strongly suggest that the PVF2/PVR couple is involved in hemocyte proliferation. PVR belongs to the PDGF/VEGF subfamily of RTKs. These receptors are characterized by an extracellular sequence composed of five (in: c-Kit, Flt-3, c-Fms, PDGFR α and β) or seven (in VEGFRS: Flt1, KDR, Flt4) Ig domains and a cytoplasmic split tyrosine kinase domain. In vertebrates, receptors of this RTK family function in both cell proliferation and cell migration.

PVR has recently been identified as a chemotactic receptor guiding cells to a source of PVF ligand, both in the context of ovarian border cell migration and in embryonic macrophage migration. The three PVFs (PVF1, PVF2 and PVF3) encoded by the *Drosophila* genome are thought to function redundantly during migration. The misexpression of a PVR ligand can disrupt the normal migration of border cells (i.e. PVF1 misexpression) or embryonic hemocytes (i.e. PVF2), but the removal of a single ligand is insufficient to block the migration process (Duchek *et al.*, 2001; Cho *et al.*, 2002). A similar misexpression strategy was used here to study larval hemocyte proliferation, with markedly different outcomes depending on the misexpressed ligand. Our study clearly establishes that PVF2, and not PVF1, promotes hemocyte proliferation. More complex to explain is the complete absence of crystal cells in larvae overexpressing PVF2. This phenotype could result from the persistence of PVR-positive prohemocytes in a constant mitotic state by PVF2 stimulation, preventing their differentiation into crystal cells (Lebestky *et al.*, 2000). Finally, the absence of any abnormal blood cell phenotype in transposon insertion lines of the *Pvf2* gene implies that PVF2 is sufficient, but not absolutely required, in hemocyte proliferation.

The overexpression of PVF1 or PVF2 resulted in lethality during pupal development. In the case of PVF2, we attribute this phenotype to the enormous amount of blood cells that could disturb overall physiology of the larvae instead of metamorphosis *per se*. On the other hand, PVF1 misexpression reveals a more complex role for the PVR pathway in metamorphosis, possibly by disrupting the homing of hemocytes to, and/or engulfment of, larval apoptotic tissue. PVF1 seems important in metamorphosis, because a loss-of-function allele in the gene ($Pvf1^{1624}$) results in 40–60% pupal lethality but has wild-type hemocyte numbers (data not shown).

In vertebrates, hematopoietic stem cells are defined by their ability to self-renew and contribute to all lineages of mature blood cells. This self-renewal and differentiation are driven by numerous receptors that co-exist on the surface membrane of hematopoietic cells, among which are RTKs of the PDGFR family (c-Kit, Flt-3, c-Fms, PDGFR; Scheijen and Griffin, 2002). These factors act in combination with intracellular signal transducers. For example, it has been shown that JAK2 activation triggers extensive self-renewal of stem cells only if it is

complemented by a second signal from c-Kit or Flt-3. Each of these proteins, JAK2, Flt-3 or c-Kit, alone is unable to sustain this activity (Zhao *et al.*, 2002).

In *Drosophila*, it has been known for some time that gain-of-function alleles in the JAK Hopscotch (e.g. $hop^{Tum\text{-}l}$) cause overproliferation of hemocytes (Hanratty and Dearolf, 1993; Luo *et al.*, 1995). Our report raises the obvious question as to what kind of interconnection exists between pathway(s) activated by PVR and the JAK/STAT pathway itself. Some cross-talk could occur, such as phosphorylation of STAT by PVF2-induced PVR activation. In mammals, for instance, PDGFR can directly activate some STATs (Vignais and Gilman, 1999). Conversely, evidence exists that JAK can activate the D-raf/D-MEK/MAP kinase pathway, one that is frequently activated by RTKs (Porter and Vaillancourt, 1998; Luo *et al.*, 2002). As is the case for PVF2, however, neither JAK nor STAT seem absolutely required for blood cell proliferation. Indeed, in loss-of-function mutants of *hop* or *stat* that permit larval development, blood cell counts are normal (Remillieux-Leschelle *et al.*, 2002). This leaves open the possibility that upstream components of the JAK/STAT pathway, e.g. the receptor Domeless (DOME; Brown *et al.*, 2001) and its ligand Unpaired (Upd; Harrison *et al.*, 1998), could act in synergy with the PVF2/PVR pathway. Both DOME and Upd are implicated in embryonic pair-rule gene expression, but their role in hematopoeisis awaits investigation.

In summary, our data indicate that PVR integrates two functions shared by mammalian receptors of the same subfamily. Like its mammalian VEGFR homologs (Flt1, KDR and Flt4), it regulates cell migration; and like c-Kit, Flt-3, c-Fms and most PDGFRs, it is implicated in the control of blood cell proliferation. In the light of the importance of hemocytes in development and in the innate immune response, it would be highly relevant to investigate further the interaction between PVFs, PVR, the JAK/STAT pathway and the downstream mitogenic factors that they induce.

METHODS

Production of monoclonal antibodies, flow cytometric analysis, cell proliferation assays and interference with dsRNA. See Supplementary data available at *EMBO reports* Online.

Immunocytochemistry and western blot analysis. Hemolymph was collected from third instar larvae spread out on polylysine-treated slides. Hemocytes were fixed for 10 min in cold 1% formaldehyde 0.1 M phosphate buffer, rinsed in PBS. Blocking (3% BSA), antibodies incubation and washing steps were performed in PBS Tween 0.1%. Antibody 18G was used at 1:200 dilution (or human-CD45 mouse antibody as a control, same dilution; Jackson ImmunoResearch). We used a peroxidase-conjugated AffiniPure goat anti-mouse IgG (Fc, Jackson ImmunoResearch) as secondary antibody. For anti-phosphohistone H3 staining, a rabbit anti-phosphohistone H3 antibody (Upstate Biotechnology) was used at 5 µg/ml. Secondary antibody was a peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L, Jackson ImmunoResearch). For *in situ* staining, wandering third instar larvae were opened, fixed in cold 1% formaldehyde 0.1 M phosphate buffer, rinsed in PBS and stained as described for blood smears. Samples were subsequently rinsed in water, dehydrated with ethanol and mounted in Eukitt.

Purification of the 18G antigenic determinant. The 18G determinant was purified using affinity chromatography followed by gel electrophoresis and transferred to PVDF membrane. Proteins were sequenced directly from the PVDF membrane (see Supplementary data; Mozdzanowski *et al.*, 1992; Coligan *et al.*, 1999).

Fly strains and crosses. Oregon was used as the standard wild-type strain. The $hop^{Tum\text{-}l}$ mutant strains were as described previously (Hanratty and Ryerse, 1981). The *daughterless-GAL4* (Giebel and Campos-Ortega, 1997) and EP1624 ($Pvf1^{1624}$) lines were obtained from the *Drosophila* stock center (Bloomington, IN). The *e33C-GAL4* line (Harrison *et al.*, 1995) was a gift from C. Dearolf (Boston, MA). *Pvf1*, *Pvf2* and *Pvr* cDNAs and PBc2195, XPd2444 and PBc6947 strains were from Dr Felix Karim (Genetics Department, Exelixis). The cDNAs were subcloned into pUAST between *Xho*I–*Eco*RI sites (for *Pvf1*) and *Not*I–*Kpn*I sites (for *Pvf2* and *Pvr*), and transgenic lines were generated by injection of w^{1118} flies.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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