Psidin Is Required in Drosophila Blood Cells for Both Phagocytic Degradation and Immune Activation of the Fat Body

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Summary
Phagocytic blood cells are critical to innate immune defense: They internalize and destroy microbial invaders and produce signals that trigger other immune responses [1, 2]. Despite this central role, the in vivo contributions of phagocytosis to systemic immune activation are not well understood. Drosophila has proven a fruitful model for the investigation of evolutionarily conserved innate immune mechanisms, including NF-κB-dependent transcriptional induction, RNAi in antiviral responses, and phagocytosis [3–5]. The phagocytes of Drosophila encounter bacterial invaders early in infection and contribute to survival of infection [6–9]. Phagocytosis in flies and mammals is highly homologous: Both rely on scavenger receptors, opsonins, and actin rearrangements for engulfment; have phagosomal cysteine proteases active at low pH; and can be subverted by similar intracellular pathogens [9–13]. Although the role of Drosophila phagocytes in the activation of other immune tissues has not been clear, we show that induction of the antibacterial-peptide gene Defensin in the fat body during infection requires blood-cell contributions. We identify a gene, psidin, that encodes a lysosomal protein required in the blood cells for both degradation of engulfed bacteria and activation of fat-body Defensin. These data establish a role for the phagocytic blood cells of Drosophila in detection of infection and activation of the humoral immune response.

Results and Discussion

psidin Mutants Do Not Induce Defensin after Septic Injury
Genetic screens in Drosophila to uncover mechanisms controlling the activation of antimicrobial peptide (AMP) genes such as Diptericin and Drosomycin in the fat body have led to the elucidation of the Imd and Toll pathways, both of which culminate in the activation of NF-κB-like transcription factors in the fat body [3]. Similarly, the first allele of psidin, ird16, was recovered in a screen for mutant larvae unable to induce Diptericin in response to injected E. coli [14]. The Diptericin induction failure in ird16 mutants was somewhat variable, whereas we found a consistent, strong impairment in the induction of Defensin, which encodes another AMP (Figure 1A). This specific defect differed from that of Imd-pathway mutants, such as ird5 (ikkj), which fail to induce a wide spectrum of antibacterial peptides (Figure 1A). ird16 mutants eventually accumulated melanotic masses in the hemolymph and died after a prolonged third larval instar. A second ethyl methanesulfonate (EMS)-induced allele showed the same spectrum of mutant phenotypes.

Based on meiotic recombination mapping and sequencing (Supplemental Experimental Procedures in the Supplemental Data available online), both ird16 mutations were associated with stop codons in the CG4845 gene, which we renamed psidin (phagocyte signaling impaired) (Figure 1B). Because neither allele showed a stronger phenotype when in trans to a deficiency, we inferred that both were null alleles. The psidin open reading frame has single homologs in yeast, mammals, and C. elegans and shares 22% identity and 45% similarity with predicted human protein FLJ13089. Psidin is 7% identical and 22% similar to Mdm20, the accessory subunit of the NatB N-acetyltransferase (NAT) of Saccharomyces cerevisiae; the homology is distributed across the length of the protein. NatB acylates the N-terminal methionine of a small number of polypeptides as they are synthesized; however, neither the general role of N-terminal acetylation nor the biochemical function of the Mdm20 subunit is known [15–17]. One substrate of NatB is tropomyosin, whose binding of actin is strengthened by acetylation; other substrates are less well characterized [16–18].

Among the Drosophila AMPs, Defensin has the strongest activity against Gram-positive bacteria [19]. To test whether the Defensin-specific impairment correlated with a reduced ability to mount a response to Gram-positive infection, we challenged larvae with different types of bacteria. However, we found that psidin mutants were equally deficient in the induction of Defensin whether the infection was with a Gram-negative or Gram-positive micro-organism (Figure 1C).

psidin Function Is Not Required in the Fat Body for the Induction of Defensin
Ubiquitous expression of a UAS-CG4845 transgene by using the hsp70- or daughterless-GAL4 driver (Figure 2A) rescued all the psidin phenotypes, including lethality. However, expression of psidin in the fat body by using the c564-GAL4 driver [20] rescued neither Defensin induction nor lethality (Figure 2A). The fat body was confirmed as the predominant site of Defensin expression

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after immune challenge by comparing the levels of Defensin transcripts in whole larvae and dissected fat bodies [Figure 2B]. Defensin was more than 20-fold enriched in fat-body RNA compared with whole larvae, which is consistent with the fat-body expression of a Defensin:GFP reporter construct [21]. Our results, therefore, suggest that psidin acts nonautonomously to control the induction of Defensin in the fat body.

psidin Function Is Required in the Blood Cells for the Induction of Defensin

We next tested whether psidin was required in the blood cells for the induction of Defensin during infection. Previous efforts to determine whether the blood cells play a role in the activation of AMP production in the fat body have relied on domino mutant larvae, in which proliferative tissues are disrupted and blood-cell numbers greatly reduced [7]. The activation of most AMPs, including Diptericin, in response to injected E. coli is normal in domino mutants, which suggested that lmd- and Toll-pathway activation are independent of blood-cell function [7]. However, domino mutants fail to induce Diptericin during a Gram-negative gut infection [22], suggesting that the blood cells could relay a signal from the gut to activate lmd signaling in the fat body. Because the original analysis of the domino phenotype did not include Defensin [7], we re-examined the requirement for domino in the induction of AMP genes. We found that domino mutants, like psidin mutants, were specifically impaired in the induction of Defensin in response to septic injury [Figure 2C]. These findings raised the possibility that psidin might act in the blood cells to promote Defensin induction in the fat body. To test this hypothesis, we used two GAL4 drivers that drive expression in both the fat body and the blood cells [23], and peroxidasin-GAL4 drives expression in the blood cells alone [24] (Figure S1A). Expression of UAS-psidin under the control of either CgIV-GAL4 or pxn-GAL4 was sufficient to rescue the inducibility of Defensin (Figure 2D). Although the c564-GAL4 driver that failed to rescue the Defensin defect was originally reported to drive expression in both the fat body and the blood cells [20], we found that it does not drive detectable expression of GFP in the blood cells (data not shown). None of the tissue-specific promoters tested rescued the lethality of psidin, suggesting that expression in other tissues is necessary for successful entry into metamorphosis. These experiments demonstrate that the blood cells are required to activate systemic antimicrobial responses in the fat body in response to disseminated infection, and they identify a specific gene required for this function.

Defensin Induction Is Not Sensitive to Spätzle or Unpaired 3 Signaling

Mammalian phagocytes produce cytokines and other immunostimulatory signals upon exposure to microbes [1, 2]. Drosophila blood cells produce two cytokine-like proteins: Upd3, which activates JAK-STAT signaling, and Spätzle, a ligand for Toll [25, 26]. spätzle mutant larvae showed robust induction of Defensin (Figure 2E), and total induction, which is dependent on Upd3 signaling [25], was normal in psidin mutants (Figure 2F). We therefore infer that neither Spätzle nor Upd3 is the blood-cell signal, and that an unknown signal produced by the blood cells in response to infection modulates the fat-body immune response. It will be interesting to determine whether this signal is a Drosophila cytokine or, alternatively, a bacterial product generated by phagocytic degradation.

Because Diptericin induction is variably impaired in psidin mutants (Figures 1A, 2A, and 2C), we infer that the blood-cell signal is not dedicated to Defensin induction. Studies of AMP regulation found that the Defensin promoter is unusual, and that both aberrant Rel-GATA binding-site spacing and low-affinity Rel binding sites contribute to low levels of expression of a reporter [27]. We propose that Defensin’s low level of expression relative to other AMPs renders it particularly sensitive to perturbations of immune signaling, including a blood-cell-dependent pathway that also activates other AMPs.

psidin Mutant Blood Cells Cannot Degrade Ingested Bacteria

We examined psidin mutant blood cells to learn how the gene might contribute to blood-cell signal production. Unlike domino mutants, which lack most blood cells [7],
psidin mutants had normal blood-cell counts (data not shown). Ten to twenty percent of psidin blood cells were enlarged, but most were normal sized (Figure S1B). psidin mutants died after a prolonged third larval instar and developed melanotic masses two days after the normal time of pupariation. At this late time point, defects in the blood cells were detected, including necrotic and multinucleate cells. However, none of these defects was apparent at the early wandering stage when Defensin induction was assayed. Unlike domino mutants, psidin mutants melanized wound sites normally, suggesting that at least some blood-cell functions are intact [6] (Figure S1C).

Because phagocytosis is an important function of Drosophila blood cells, we tested the ability of psidin mutant cells to engulf fluorescein-conjugated, heat-killed E. coli and protect them from trypan blue, which quenches extracellular fluorescence. psidin blood cells were able to engulf these particles in a manner indistinguishable from that of the wild-type (WT) (Figure 3A). However, when we examined the fate of ingested GFP-expressing E. coli, we found that psidin mutant cells failed to digest them. In the first hour after injection of live E. coli into larvae, intracellular bacteria were seen in both WT and mutant blood cells. However, by 4 hr, whereas very few fluorescent bacteria remained in WT cells, psidin blood cells still contained many bacteria. At 6 hr, almost no bacteria were visible in WT cells, whereas bacteria in psidin mutant cells continued to persist, and even increase in number. In such cases, the bacteria remained clustered, suggesting they may have replicated within phagosomes (Figure 3B). We counted the number of green bacteria in WT and mutant blood cells 4 hr after infection. In 50 WT cells, only a single bacterium was visible. In contrast, 12 of 50 psidin mutant cells contained a single bacterium, and four had two or more intracellular E. coli (Figure 3C). Counts of colony-forming units (CFUs) showed that WT animals cleared an infection of 12,000 CFU within 5 hr, whereas psidin mutant animals had indistinguishable numbers of bacteria at 0 and 5 hr after infection (Figure 3D). The maintenance of stable numbers of bacteria in psidin mutants suggests that the bacterial replication observed within some blood cells may be balanced by some bacterial killing.

These blood-cell phenotypes raised the intriguing possibility that phagocytic degradation of microbes is required for the production of an immunostimulatory...
signal from the blood cells to the fat body to activate Defensin. To further test this hypothesis, we examined the ability of Eater mutants to induce Defensin. Eater encodes a transmembrane blood-cell receptor that binds bacteria and contributes to bacterial internalization [9]. Eater mutants showed normal induction of Dipterocin, but induced Defensin to only 45% of WT levels (Figure 3E). This defect is not as pronounced as in psidin mutants, but is consistent with the finding that Eater mutants retain the ability to phagocytose Gram-negative bacteria at about 50% of WT levels [9]. The finding that two stages of phagocytosis, internalization and degradation, are both required for the blood cells to stimulate fat body Defensin induction indicates that phagocytosis plays a central role in activation of systemic immune responses in Drosophila.

Psidin Localizes to Blood-Cell Lysosomes

Immunostaining with antisera raised against an N-terminal fragment of Psidin showed a punctate distribution throughout the cytoplasm with an additional perinuclear localization in both larval blood cells and S2 cells, a macrophage-like cell line (Figure 4A). Specificity of the antibody was shown by increased staining when the psidin gene was overexpressed (Figure 4B) and reduced staining after RNAi gene silencing in S2 cells (Figure 4C). The perinuclear distribution is predicted for a homolog of Mdm20, which participates in the acetylation of proteins as they emerge from the ribosome [15–17]. The cytoplasmic Psidin-positive structures also were labeled with dArl8, a marker for lysosomes (Figure 4D) [28].

The lysosomal localization for a putative NAT subunit was unexpected and could indicate that Psidin has NatB-independent functions, or that N-terminal acetylation in metazoans is not exclusively cotranslational. In support of this latter possibility, San, the Drosophila homolog of another NAT subunit, also has a cytoplasmic distribution [29]. The lysosomal localization of Psidin does correlate with the defect in phagosomal degradation of ingested bacteria in psidin mutants. N-terminal acetylation of Drosophila Arl8 by the NatC complex is required for its lysosomal localization [28], and it is possible that Psidin acts in a NatB complex to localize other proteins essential for lysosome function.

In summary, we have shown that the psidin gene acts in blood cells, where it plays two roles in the larval immune response: It is required both for the phagocytic degradation of internalized bacteria and for the induction of Defensin in the fat body. Our inability to detect other defects in psidin mutant cells, coupled with the lysosomal localization of the Psidin protein as well as the impairment of Defensin induction in the phagocytic-internalization mutant Eater, leads us to favor the hypothesis that the phagocytic-degradation defect of psidin underlies the inability to activate the fat body. A model whereby phagosome maturation is required for activation of immunostimulatory signaling in Drosophila blood cells is consistent with the close relationship in mammals between endosome/phagosome function and immune activation. However, whereas the mechanisms underlying the processing and presentation of endosomal peptides and lipid antigens in adaptive immunity

Figure 3. Defensin Induction Defects Are Associated with Phagocytosis Deficiencies

(A) Differential interference contrast (DIC) and fluorescence images of live blood cells 2 hr after injection of larvae with FITC-labeled E. coli Bioparticles. Blood cells suspended in trypan blue to quench extracellular fluorescence.

(B) DIC and fluorescence images of live blood cells 6 hr after injection of larvae with live, GFP-expressing E. coli.

(C) Quantitative comparison of the ability of wild-type and psidin/Df blood cells to degrade GFP-expressing E. coli. Number of blood cells containing 0, 1, or 2 or more bacteria 4 hr after infection are indicated.

(D) CFUs in WT versus psidin at 0 and 5 hr after infection with E. coli. Each dot represents a single animal.

(E) Northern-blot analysis of AMP induction in psidin (psidin/Df) and Eater [Df(3R)D605/Df(3R)TI-l] mutant larvae.
Figure 4. Psidin Is Expressed in Blood Cells in a Ring around the Nucleus and in Lysosomes
(A) Immunolocalization of Psidin protein in larval blood cells. Arrowhead indicates perinuclear staining.
(B) Immunostaining of Psidin protein in larval blood cells showing increased staining when psidin is overexpressed under the control of the peroxidasin GAL4 driver.
(C) Immunostaining of Psidin protein in S2 cells showing reduction of protein expression when psidin expression levels are knocked down by RNAi.
(D) Immunolocalization of Psidin and dArl8 proteins.

have been studied extensively, the events linking phagosome maturation with initiation of innate immunity are still unclear. Future genetic studies on Defensin induction in Drosophila could be a useful system for the in vivo analysis of phagocytosis, including postengulfment steps, and its coupling to immunostimulatory mechanisms.

Supplemental Data
Supplemental Data include Experimental Procedures and one figure and are available with this article online at: http://www.current-biology.com/cgi/content/full/17/1/67/DC1/.

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References


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**Supplemental Experimental Procedures**

**Infection Protocols**

Larvae were infected with *E. coli* by injecting 50 nl of an overnight culture into the body cavity. Infected larvae were grown for 3 hr at 25° before RNA extraction.

**Isolating an Additional Allele of psidin**

Five hundred males of genotype ru h th st cu sr e ca were fed 25 mM EMS and mated to virgins carrying the TM6C balancer. F1 males were mated singly to psidin?/TM6 Tb virgins. Nine hundred and fifty lines were scored for melanotic masses and late larval lethality (absence of long pupae), and one allele was recovered.

**Mapping and Cloning the psidin Mutation**

The melanotic-mass phenotype of psidin1/Df (absence of long pupae), and one allele was recovered. The genotypes are psidin1/Df (absence of long pupae), and one allele was recovered. The proximal limit was the distal breakpoint of Df(3R)K9 (psidin*) (near hs65f), and the distal limit was the distal breakpoint of Df(3R)12 (psidin*) (near CG10887). Twelve candidate genes in the interval were sequenced for both alleles, and only one sequence change was identified for each allele.

**Molecular Biology**

A UAS-psidin rescue construct was generated as follows: A 3460 bp cDNA from EST GM28696 was excised from pOT2 in a partial XhoI digest and cloned into pUAST. Transgenic flies were obtained by using standard methods.

**Phenotypic analysis**

Northern blot analysis was performed as follows: Total RNA was photoexcised from larvae. Three to ten micrograms were separated in 1.5% agarose MOPS [3-(N-morpholino)-propanesulfonic acid] formaldehyde gels and then transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech). 32P-labeled probes were made by random priming from DNA templates generated by PCR with the following primers: att, forward 5'-TGGGGGCTGAT GCTCGTTT-3' and reverse 5'-TCCACTTGGCCTCCTTG-3'; cec, forward 5'-CTCTCTTTGGGCCTACACC-3' and reverse 5'-GGTC AACCTGCGGCAATGCTG-3'; def, forward 5'-TATGGCATCTCAGTCGGTG-3' and reverse 5'-TCTCAGCTTGGAGTG-3'; dipt, forward 5'-TCTTGGAGGGTTTCTGG-3' and reverse 5'-TGTTTAAAGAC TCGAGCTG-3'; totA, forward 5'-CTAGGTGCTTTGCTCTGC-3' and reverse 5'-GCCCTTCACACTGCGAGA-3'; and rp49, forward 5'-CAGGCCCAAGGATGTTGAA-3' and reverse 5'-CAAATGTTGATTCCCG-3'. Hybridization was conducted at 68° in RapidHyb solution (Amer sham Biosciences). Blots were stripped in boiling 0.1% SDS and then reprobed with rp49 as a loading control. Blots were exposed to film, and signals were quantified by measuring band densities with Scion Image analysis software. Northern-blot results shown are representative of multiple experiments.

**Psidin antibodies**

An N-terminal fusion protein of 6xHis and amino acids 1–147 of the psidin open reading frame was expressed in *E. coli* by using the pET-28a vector (Novagen). This was used as an antigen to generate rat antisera (Pocono Farms). For immunostaining, antisera were used at a 1:1,000 dilution, with 2° goat anti-rat Alexa 594 (1:500) (Molecular Probes).

**RNAi Experiments**

A 460 bp PCR product amplified from the large 5° exon of psidin, and bearing the T7 promoter at each end, served as the template for dsRNA transcription and was generated with the primers 5'-TAAATAGCATCCTCCTTGGCAGACCA-3'; metch, forward 5'-GAGATGAGTCACGCTACTGCTG-3'; drom, forward 5'-GCTGTTGGCATCAGATTG-3'; and reverse 5'-ATTTAGCATCCTTCGAGACTCCA-3'; cec, forward 5'-TCTTGGAGGGTTTCTGG-3' and reverse 5'-TGTTTAAAGAC TCGAGCTG-3'; totA, forward 5'-CTAGGTGCTTTGCTCCTTG-3' and reverse 5'-GCCCTTCACACTGCGAGA-3'; and rp49, forward 5'-CAGGCCCAAGGATGTTGAA-3' and reverse 5'-CAAATGTTGATTCC CGACC-3'. Hybridization was conducted at 68° in RapidHyb solution (Amer sham Biosciences). Blots were stripped in boiling 0.1% SDS and then reprobed with rp49 as a loading control. Blots were exposed to film, and signals were quantified by measuring band densities with Scion Image analysis software. Northern-blot results shown are representative of multiple experiments.

**Phagocytosis Experiments**

For internalization assays, fluorescein-conjugated, heat-killed *E. coli* (Biparticles, Molecular Probes E-2861) were washed and resuspended in a 2× volume of water. Fifty nanoliters was injected. After 2 hr, larvae were bled into trypsin blue, which quenches the fluorescence of extracellular particles. For phagocytic-degradation assays, an overnight culture of GFP-expressing *E. coli* was diluted 5× in PBS. One hundred nanoliters (10,000–15,000 cfu) was injected. Following indicated incubation times, larvae were bled into Schneider’s media, and blood cells were examined by light and fluorescence microscopy.

**Figure S1. Blood Cells in psidin-GAL4 Larvae**

(A) Brightfield and fluorescence microscopy images of blood cells from larva of genotype psidin-GAL4/UAS-GFP. GFP is expressed in the blood cells and no other tissues. (B) Live blood cells. Right-hand panel shows an oversized psidin (psid?/Df) blood cell. Mutant cells up to twice the normal diameter were found. The scale bar represents 5 μm. (C) Posterior of third-instar larvae 30 min after septic injury. Arrows indicate melanization sites. The genotypes are domino (dom?) and psidin (psid?/Df).