EMBO reports

scientific report

Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*

*Vincent Leclerc**, Nadège Pelte**†, Laure El Chamy, Cosimo Martinelli, Petros Ligoxygakis‡, Jules A. Hoffmann & Jean-Marc Reichhart*+

UPR9022 du CNRS, IBMC, Strasbourg, France

The antimicrobial defence of *Drosophila* relies on cellular and humoral processes, of which the inducible synthesis of antimicrobial peptides has attracted interest in recent years. Another potential line of defence is the activation, by a proteolytic cascade, of phenoloxidase, which leads to the production of quinones and melanin. However, in spite of several publications on this subject, the contribution of phenoloxidase activation to resistance to infections has not been established under appropriate *in vivo* conditions. Here, we have isolated the first *Drosophila* mutant for a prophenoloxidase-activating enzyme (PAE1). In contrast to wild-type flies, *PAE1* mutants fail to activate phenoloxidase in the haemolymph following microbial challenge. Surprisingly, we find that these mutants are as resistant to infections as wild-type flies, in the total absence of circulating phenoloxidase activity. This raises the question with regard to the precise function of phenoloxidase activation in defence, if any.

Keywords: *Drosophila*; innate immunity; PAE; prophenoloxidase; serine protease

EMBO reports (2006) **7**, 231–235. doi:10.1038/sj.embor.7400592

INTRODUCTION

Besides the well-documented induction of antimicrobial peptides (Hoffmann, 2003; Brennan & Anderson, 2004; Leclerc & Reichhart, 2004), a second potential defence mechanism in insects (and in most invertebrates) is the activation of phenoloxidase (PO; Cerenius & Soderhall, 2004). In *Drosophila*, the crystal cells, a specific class of haemocytes, synthesize PO as an inactive prophenoloxidase (proPO) precursor. Biochemical data derived from large insects, such as *Manduca sexta*, led to the present model of PO activation, in which the recognition of microorganisms triggers a proPO-activating enzyme (PAE) proteolytic cascade culminating in the liberation of active PO. The activated PO then catalyses the oxidation of tyrosine-derived phenols to quinones. Quinones are believed to be directly toxic to microorganisms and also to polymerize non-enzymatically to form insoluble melanin. Melanin deposition is observed at all infection sites, where it possibly contributes to wound healing and control of microorganism growth.

In spite of several publications on this subject in various invertebrates (for reviews, see Sugumaran, 2002; Cerenius & Soderhall, 2004; Christensen *et al*, 2005), the precise contribution of PO activation to survival to microbial infections has not been genetically investigated. To address this problem, we decided to generate mutant flies that are unable to activate PO, targeting the PAE upstream regulators. In *Drosophila*, except for a proteolytic activity purified from total pupae extracts (Chosa *et al*, 1997), no PAE has been characterized so far. However, we have recently identified a serine protease inhibitor, Serpin27A, involved in the control of PO activation. Loss-of-function mutations in this serpin result in constitutive PO activation and an excessive melanization at the site of injury, indicating that at least one serine protease is involved in PO activation in *Drosophila* (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). Here, we present a new mutation that renders the flies unable to activate circulating PO. Surprisingly, we observed that, in these mutant flies, the survival to infections is not affected.

RESULTS AND DISCUSSION

Detection of proPO cleavage in the haemolymph

The haemolymph of uninfected flies is devoid of any detectable PO activity, and injection of a mix of Gram-negative and Gram-positive bacteria induces a marked level of activity in 3 h, as reported earlier (Ligoxygakis *et al*, 2002b). *Drosophila* and *Anopheles* proPOs share a high sequence conservation, and antibodies raised against *Anopheles* PPO2 protein (Muller *et al*, 1999) have been successfully used to detect *Drosophila* proPO in crystal cells, by immunolocalization (Duvic *et al*, 2002). Assuming that the induction of PO activity results from the cleavage of proPO in the haemolymph of the flies (Chosa *et al*, 1997), we used an anti-*Anopheles* PPO2 serum on western blot. We detected a single band of about 75 kDa in the absence of infection, and a

UPR9022 du CNRS, IBMC, 15 rue Descartes, 67084 Strasbourg, France
†Present address: German Cancer Research Center (DKFZ/B110), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany
‡Present address: Genetics Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK
*First co-authors, alphabetical order
+Corresponding author. Tel: +33 388 417 034; Fax: +33 388 606 922;
E-mail: jm.reichhart@ibmc.u-strasbg.fr

Received 18 May 2005; revised 25 October 2005; accepted 26 October 2005; published online 2 December 2005

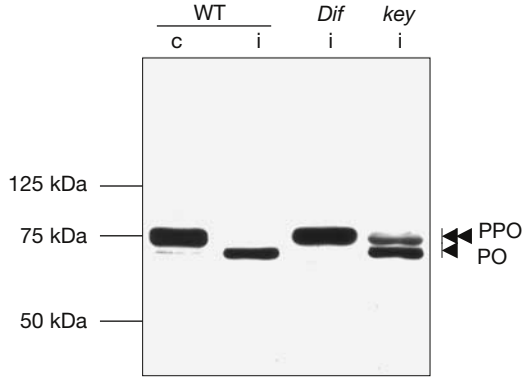

Fig 1 | Anti-*Anopheles gambiae* PPO2 antibodies detect prophenoloxidase (proPO) cleavage in *Drosophila*. Immunoblotting experiments detect a single band of about 75 kDa in control (c) wild-type *Drosophila* (WT) haemolymph and a faster migrating band 4 h after infection (i) with a mixture of Gram-positive (*Micrococcus luteus*) and Gram-negative (*Escherichia coli*) bacteria (the same infection procedure is used in all figures unless otherwise stated). After immune challenge, the low-molecular-weight band is absent in *Dif* but not in *kenny* (*key*) mutants. The high-molecular-weight band corresponds to the proPO (PPO, double arrowhead) and the lower band to phenoloxidase (PO, single arrowhead). The same symbols are used in all figures. Note that, depending on the experiment, some proPO is still present after microbial challenge (see wild type in Fig 2A for example).

second band of about 72 kDa following microbial challenge (Fig 1), which correlates with the calculated molecular weight for *Drosophila* proPO and PO, respectively. As expected, we could not detect any band in the haemolymph of the *Black cell* (*Bc*) mutant, which is devoid of circulating PO activity (Rizki *et al*, 1980; Fig 2A).

We had previously shown that PO activation in the haemolymph was dependent on the Toll pathway but did not require genes of the immune deficiency (IMD) pathway (Ligoxygakis *et al*, 2002b). Using the anti-proPO antibodies, we could confirm that, after microbial challenge, the lowest band is absent from the haemolymph of Toll pathway mutants (*Dif*; Fig 1) but still present in the IMD pathway mutant *kenny* (*key*; Fig 1), although reduced in intensity. Therefore, in all conditions tested, the appearance on western blots of the 72 kDa band correlates with the detection of PO activity (see supplementary Fig 1 online for analysis of antibody specificity). This validates the use of the anti-*Anopheles* PPO2 antibodies as a tool to detect activation of PO in the *Drosophila* haemolymph.

CG3066 encodes a PAE

Biochemical studies on several invertebrates have shown that the PAE serine proteases contain a regulatory clip domain that has to be cleaved for activation of the enzyme (reviewed by Cerenius & Soderhall, 2004). A total of 24 genes encoding clip domain-containing serine proteases are present in the *Drosophila* genome.

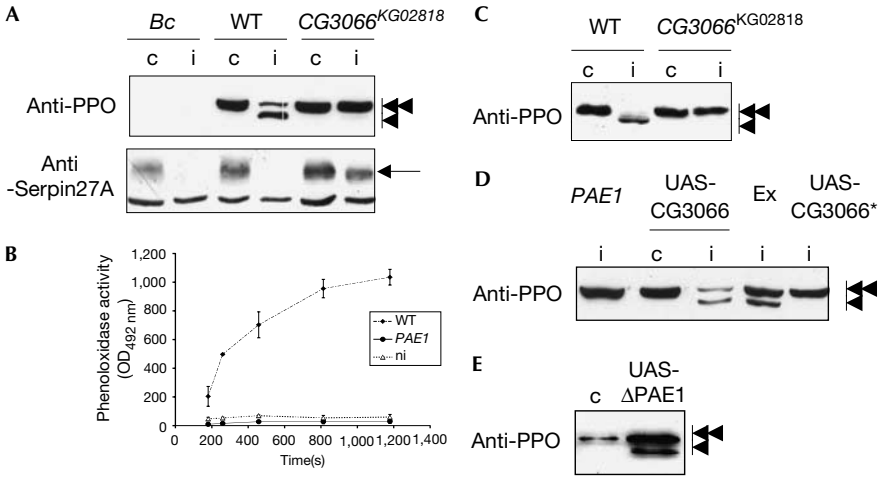

Fig 2 | *CG3066* encodes a prophenoloxidase-activating enzyme. (**A**) Prophenoloxidase (proPO), which is absent from the haemolymph of *Black cell* (*Bc*) flies, is cleaved after immune challenge in wild-type (WT) flies but not in flies homozygous for the $CG3066^{KG02818}$ insertion ($CG3066^{KG02818}$). The blot, incubated with anti-proPO antibodies (anti-PPO), was stripped and re-probed with anti-Serpin27A antibodies (anti-Serpin27A, arrow, the lowest band is unspecific; Ligoxygakis *et al*, 2002b). Serpin27A is depleted after immune challenge in the haemolymph of WT and *Bc* mutant flies, but not in the haemolymph of flies homozygous for $CG3066^{KG02818}$. (**B**) Phenoloxidase activity is detected (as absorbance at an optical density of 492 nm ($OD_{492\,nm}$) after conversion of L-3,4-dihydroxyphenylalanine) in the haemolymph of immune-challenged WT flies, but not in the haemolymph of $CG3066^{KG02818}$ immune-challenged flies (*PAE1*) or WT non-challenged flies (ni). (**C**) proPO fails to be cleaved when $CG3066^{KG02818}$ homozygous flies are infected (i) with the yeast *Candida albicans*, as is the case after bacterial infection. (**D**) Immunoblotting experiments with anti-proPO antibodies on the haemolymph of control (c) or infected (i) flies show that the proPO cleavage, which is absent in $CG3066^{KG02818}$ (*PAE1*) mutant flies, is restored when the KG02818 insertion is excised (Ex) or when a WT copy of the *CG3066* gene (UAS-CG3066), but not a version mutated in the catalytic domain of the protein (UAS-CG3066*), is expressed in the $CG3066^{KG02818}$ background. (**E**) In the absence of any challenge (c), the expression of an activated form of the *CG3066*-encoded protein (UAS-ΔPAE1) leads to the constitutive cleavage of proPO.

Among these, ten have been classified as potential PAEs on the basis of sequence similarity with the PAEs purified from several insect species (Ross *et al*, 2003).

The serine protease Easter is one of these candidate PAEs. Together with Snake and Gastrulation defective, Easter forms the proteolytic cascade that activates, under the control of Serpin27A, the Toll receptor during the establishment of the dorso-ventral axis in the *Drosophila* early embryo (Anderson, 1998; Hashimoto *et al*, 2003; Ligoxygakis *et al*, 2003). However, in *easter*, *snake* or *gastrulation defective* mutant flies, proPO cleavage is similar to that in wild-type flies (supplementary Fig 2 online), indicating that the embryonic proteolytic cascade is not used for proPO activation in adult *Drosophila*.

To identify the *Drosophila* PAEs, we screened the databases for transposon insertions in the vicinity of candidate proteases and found that the P element KG02818 could potentially inactivate *CG3066*, as it is inserted in the second exon of the gene. In flies homozygous for the $CG3066^{KG02818}$ insertion, PO cleavage could not be detected after immune challenge with a mix of Gram-positive and Gram-negative bacteria or with the yeast *Candida albicans* (Fig 2A,C). This is correlated with an absence of PO activity in the haemolymph (Fig 2B). We verified by two different rescue experiments that the phenotype is due to the insertion of the transposon into *CG3066*. In the first experiment, we precisely excised the KG02818 element from the mutant chromosome (Fig 2D, Ex), and, in the second, we expressed the *CG3066* complementary DNA in the homozygous $CG3066^{KG02818}$ mutant background, using the UAS-GAL4 system (Brand & Perrimon, 1993) and a female fat-body-specific yolk promoter driving *Gal4* (Fig 2D, UAS-CG3066). In both cases, proPO cleavage was fully restored after immune challenge. The proteolytic function of the *CG3066*-encoded protein is absolutely required, as the expression of a mutated non-catalytic form of the serine protease, in which the serine of the active site was replaced by a glycine (Fig 2D, UAS-CG3066*), could not restore a normal cleavage of the proPO in the mutant background.

The activation of the melanization cascade is known to be under the control of the Toll pathway (Fig 1; Ligoxygakis *et al*, 2002b). The Toll pathway is activated by a proteolytic cascade, and the observed lack of proPO cleavage in the *CG3066* mutant background could result from a default in the activation of this pathway. We observed that the expression of the Toll pathway target gene *Drosomycin* was affected neither in a $CG3066^{KG02818}$ mutant background nor in flies overexpressing *CG3066*, using the UAS-GAL4 system (data not shown). *CG3066* is thus not involved in the activation of the Toll pathway. The protein meets all the requirements for a bona fide proPO-activating enzyme, and we propose to name this protease PAE1.

proPO is activated by a proteolytic cascade in *Drosophila*

Biochemical studies with large insect species have established a model of PO activation, in which proPO is cleaved after the activation of a cascade of serine proteases. Under normal circumstances, this hypothetic cascade is kept inactive by Serpin27A in *Drosophila*. Serpin27A could potentially inhibit the end protease of the proPO activation cascade in the same way as it inhibits Easter, the end protease of the cascade that controls embryonic dorso-ventral axis formation (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b, 2003; Hashimoto *et al*, 2003).

Serpin27A is degraded in wild-type flies shortly after immune challenge, probably through interaction with its target protease (Fig 2A; Ligoxygakis *et al*, 2002b), but not in *PAE1* mutant flies (Fig 2A, $CG3066^{KG02818}$), which means that the serpin inhibits either PAE1 itself or a downstream protease. Moreover, *Spn27A*; $CG3066^{KG02818}$ double mutants still present spontaneous large melanization spots, with the same frequency as *Spn27A* simple mutants (data not shown), indicating that PAE1 is not required for the activation of proPO in the absence of Serpin27A and is therefore not its target. PAE1 does not directly cleave proPO, but activates a downstream protease ultimately responsible for the cleavage of proPO and regulated by Serpin27A. To our knowledge, this is the first genetic demonstration that PO activation requires a cascade of at least two proteolytic enzymes.

Regulation of proPO activation

As previously shown, the Toll pathway controls PO activation, probably by inducing the expression of a protease resulting in disequilibrium in the respective concentrations of serpin and proteases and the subsequent activation of the cascade (Ligoxygakis *et al*, 2002b). *PAE1* transcription is controlled by the Toll pathway and increases as early as 1.5 h after an infection (De Gregorio *et al*, 2001, 2002b; Irving *et al*, 2001). However, overexpression of PAE1 in the absence of challenge does not induce proPO cleavage (Fig 2D), suggesting that PAE1 is unable to self-activate without immune challenge.

We designed an activated form of PAE1 (ΔPAE1), with the same strategy as that used to construct an activated form of Easter lacking the amino-terminal regulatory Clip domain (Chasan *et al*, 1992; Ligoxygakis *et al*, 2003). When expressed under the control of the fat-body-specific *yolk-Gal4* driver, it resulted in a constitutive proPO cleavage in the haemolymph (Fig 2E, UAS-ΔPAE1). The expression of an activated PAE1 is therefore able to turn on the downstream target protease and override its inhibition by Serpin27A. During an infection, the protease cascade has to be activated, probably by the recognition of microorganisms. Shortly after a challenge, a dark melanized spot is usually observed at the site of the wound. In *Spn27A* mutants, this spot is considerably enlarged, presumably because Serpin27A is not able to restrict anymore proPO activation to the site of injury (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). Flies overexpressing the full-length PAE1 protein present no constitutive proPO cleavage (Fig 2D), but show the same phenotype of excessive melanization around the infection site after pricking (supplementary Fig 3 online). Hence, proPO activation seems to be controlled by both the Toll pathway-driven expression of a protease that is usually present in limited amounts and by the activation signal resulting from the recognition of microorganisms. Peptidoglycan recognition protein-LE is a good candidate for a receptor delivering such a signal, as it activates PO in larval haemolymph when overexpressed (Takehana *et al*, 2002), and is required for PO activation after *Escherichia coli* infection (Takehana *et al*, 2004). When the activated form of PAE1 was driven by *daughterless-Gal4,* no or very few adult flies emerged, probably because of the toxic effect of the ectopic protease activity. This larval lethality associated with large melanization spots is similar to the lethality observed for *Spn27A* mutant larvae. Therefore, we can assume that a strict double control is required to prevent inappropriate activation of PO that is deleterious to the flies.

Absence of susceptibility to infections

As the PAE1 mutant is unable to activate PO in the haemolymph after an infection, it is a good tool to test the potential function of PO activity in defence against infections. We analysed the susceptibility of *PAE1* mutants to infections with different microorganisms: natural infection with the entomopathogenic fungus *Beauveria bassiana* and challenge with a needle contaminated with the yeast *C. albicans*, the Gram-negative bacteria *Agrobacterium tumefaciens* and *E. coli* or the Gram-positive bacteria *Enterococcus faecalis* and *Staphylococcus aureus*. Surprisingly, the *PAE1* mutant flies survived as wild-type flies after all infections that we could test (Fig 3; data not shown). Accordingly, the number of *E. coli* bacteria still alive inside the body cavity 20 h after infection was similar in PAE1 mutant flies and wild-type flies and almost 500 times lesser than in IMD pathway mutant flies (supplementary Fig 4 online). This correlates well with recent results showing that *Drosophila* PO has no major killing effect on bacteria (Bidla *et al*, 2005).

These results clearly indicate that PO activity in the haemolymph of adult flies is dispensable for survival to infections, raising the question of its role, if any, in immune defence in *Drosophila*. A possible role for PO was suggested by the analysis of the *Bc* mutant phenotype. This mutation is characterized by the absence of PO in the haemolymph (Rizki *et al*, 1980; Lanot *et al*, 2001). The gene corresponding to the *Bc* mutation has not yet been isolated, but it probably affects a gene required for secretion of proPO into the haemolymph. *Bc* mutant flies are slightly susceptible to all infections, with a survival rate lower than wild-type flies but significantly higher than that of Toll or IMD pathway mutant flies (Fig 3; De Gregorio *et al*, 2002a). This indicates that the *Bc* mutation affects a gene or function that has a weak but general role in resistance to infections. However, this phenotype is independent of the lack of PO secretion. Our results on the *PAE1* mutant indicate that survival of adult flies to microbial infections does not require circulating PO activity.

METHODS

***Drosophila* strains.** Oregon^R flies were used as wild-type controls. Other stocks have been described previously: *Dif*^{1} (Rutschmann *et al*, 2000b), *key*^{1} (Rutschmann *et al*, 2000a), *Bc* (Rizki *et al*, 1980), *daGAL4* (Ligoxygakis *et al*, 2002a) and *ea*^{1}, *ea*^{2}, *snk*^{073}, snk^{233} and *gd*^{7} (Ligoxygakis *et al*, 2003). The transposon insertion P{SUPor-P}CG3066^{KG02818} stock was obtained from the Bloomington Stock Center. We observed a strong susceptibility to *B. bassiana* infections in the original *CG3066*^{KG02818} insertion that is not rescued by the excision of *KG02818* and that we could easily separate, using meiotic recombination, from the P-element insertion responsible for the defective proPO cleavage. Clone SD07170 from the BDGP EST project matches the *CG3066* gene. For the inactive version of the protease, the catalytic serine (Ser 341) was replaced by a glycine using PCR-directed mutagenesis. To obtain an activated form of the protease, we used the strategy described by Chasan *et al* (1992) for Easter: the PCR-amplified proteolytic domain (starting Val 137) with cysteine 264 replaced by serine was linked to the Easter signal sequence. The resulting constructs were cloned into pUAST (Brand & Perrimon, 1993).

Microbial strains and survival experiments. We used the following microbial organisms: *E. coli* (1106), *Micrococcus luteus* (CIP A270), *E. faecalis* (a kind gift from H. Monteil), *S. aureus, A. tumefaciens, C. albicans* (a pathogenic strain isolated in patient no. 3 by Pr. M. Koenig, CHU Strasbourg-Hautepierre) and *B. bassiana* (80.2 strain).

Survival experiments were carried out as described previously (Rutschmann *et al*, 2000b; Ligoxygakis *et al*, 2002a). Each experiment was repeated at least three times.

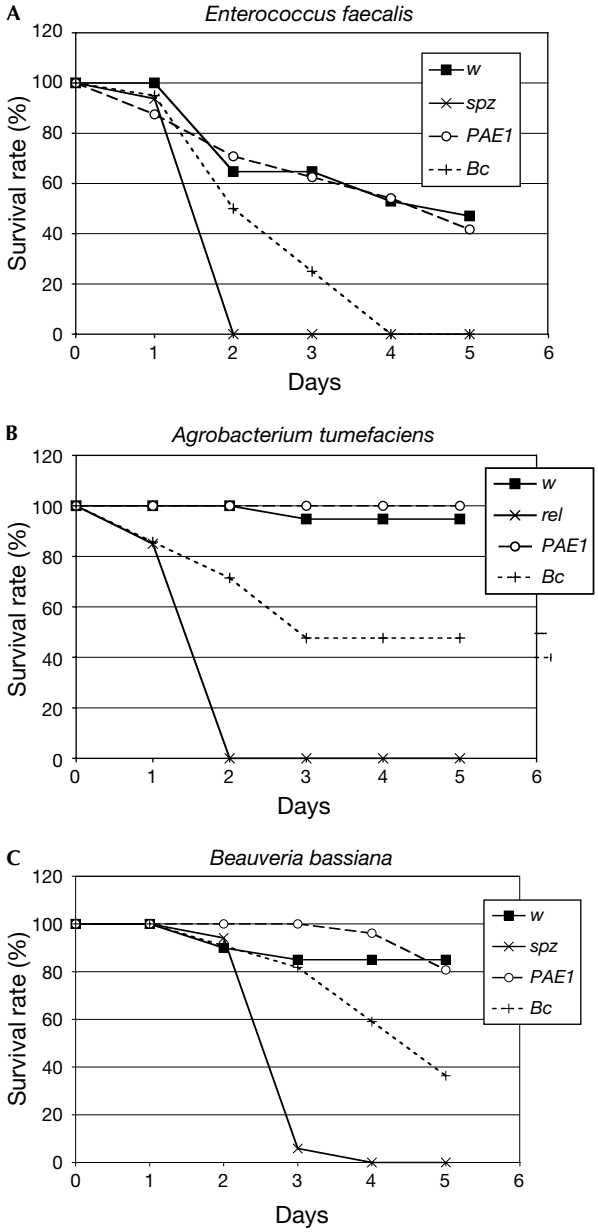

Fig 3 | Prophenoloxidase activation mutants survive similar to wild-type flies to infection. Representative survival curve of *white* (*w*, wild-type control) and *relish* (*rel*, immune deficiency pathway mutant), *spaetzle* (*spz*, Toll pathway mutant), *PAE1* and *Black cell* (*Bc*) mutant flies after infection with Gram-positive bacteria (*Enterococcus faecalis* (**A**)), Gram-negative bacteria (*Agrobacterium tumefaciens* (**B**)) or after natural infection with the fungus *Beauveria bassiana* (**C**).

Sample preparation and analysis. Infections, haemolymph collection, sample preparation, western blot analysis and PO activity assay were as described by Ligoxygakis *et al* (2002b). Western blots were incubated with rabbit anti-glutathione S-transferase–Serpin27A antibodies (Ligoxygakis *et al*, 2002b) and rabbit anti-*Anopheles gambiae* PPO2 antibodies (a generous gift from H.M. Müller) overnight at 4 °C at a dilution of 1/5,000 and 1/10,000, respectively.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We are grateful to A. Meunier, R. Walther and S. Ozkan for technical help. We thank H.-M. Müller for the anti-PPO2 antibodies and D. Ferrandon and M. Meister for a critical reading of the manuscript. This work was supported financially by the Centre National de la Recherche Scientifique and a National Institutes of Health Program Grant PO1 AI44220.

REFERENCES

Anderson KV (1998) Pinning down positional information: dorsal–ventral polarity in the *Drosophila* embryo. *Cell* **95:** 439–442

Bidla G, Lindgren M, Theopold U, Dushay MS (2005) Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. *Dev Comp Immunol* **29:** 669–679

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415

Brennan CA, Anderson KV (2004) *Drosophila*: the genetics of innate immune recognition and response. *Annu Rev Immunol* **22:** 457–483

Cerenius L, Soderhall K (2004) The prophenoloxidase-activating system in invertebrates. *Immunol Rev* **198:** 116–126

Chasan R, Jin Y, Anderson KV (1992) Activation of the easter zymogen is regulated by five other genes to define dorsal–ventral polarity in the *Drosophila* embryo. *Development* **115:** 607–616

Chosa N, Fukumitsu T, Fujimoto K, Ohnishi E (1997) Activation of prophenoloxidase A1 by an activating enzyme in *Drosophila melanogaster*. *Insect Biochem Mol Biol* **27:** 61–68

Christensen BM, Li J, Chen CC, Nappi AJ (2005) Melanization immune responses in mosquito vectors. *Trends Parasitol* **21:** 192–199

De Gregorio E, Spellman PT, Rubin GM, Lemaitre B (2001) Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proc Natl Acad Sci USA* **98:** 12590–12595

De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata S, Lee BL, Iwanaga S, Lemaitre B, Brey PT (2002a) An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Dev Cell* **3:** 581–592

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002b) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* **21:** 2568–2579

Duvic B, Hoffmann JA, Meister M, Royet J (2002) Notch signaling controls lineage specification during *Drosophila* larval hematopoiesis. *Curr Biol* **12:** 1923–1927

Hashimoto C, Kim DR, Weiss LA, Miller JW, Morisato D (2003) Spatial regulation of developmental signaling by a serpin. *Dev Cell* **5:** 945–950

Hoffmann JA (2003) The immune response of *Drosophila*. *Nature* **426:** 33–38

Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Reichhart JM, Hoffmann JA, Hetru C (2001) A genome-wide analysis of immune responses in *Drosophila*. *Proc Natl Acad Sci USA* **98:** 15119–15124

Lanot R, Zachary D, Holder F, Meister M (2001) Postembryonic hematopoiesis in *Drosophila*. *Dev Biol* **230:** 243–257

Leclerc V, Reichhart JM (2004) The immune response of *Drosophila melanogaster*. *Immunol Rev* **198:** 59–71

Ligoxygakis P, Pelte N, Hoffmann JA, Reichhart JM (2002a) Activation of *Drosophila* Toll during fungal infection by a blood serine protease. *Science* **297:** 114–116

Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, Belvin M, Jiang H, Hoffmann JA, Reichhart JM (2002b) A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. *EMBO J* **21:** 6330–6337

Ligoxygakis P, Roth S, Reichhart JM (2003) A serpin regulates dorsal–ventral axis formation in the *Drosophila* embryo. *Curr Biol* **13:** 2097–2102

Muller HM, Dimopoulos G, Blass C, Kafatos FC (1999) A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes. *J Biol Chem* **274:** 11727–11735

Rizki TM, Rizki RM, Grell EH (1980) A mutant affecting crystal cells in *Drosophila melanogaster*. *Roux Arch Dev Biol* **188:** 91–99

Ross J, Jiang H, Kanost MR, Wang Y (2003) Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships. *Gene* **304:** 117–131

Rutschmann S, Jung A, Zhou R, Silverman N, Hoffmann JA, Ferrandon D (2000a) Role of *Drosophila* IKKγ in a Toll-independent antibacterial immune response. *Nat Immunol* **1:** 342–347

Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D (2000b) The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. *Immunity* **12:** 569–580

Sugumaran M (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigment Cell Res* **15:** 2–9

Takehana A, Katsuyama T, Yano T, Oshima Y, Takada H, Aigaki T, Kurata S (2002) Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. *Proc Natl Acad Sci USA* **99:** 13705–13710

Takehana A, Yano T, Mita S, Kotani A, Oshima Y, Kurata S (2004) Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. *EMBO J* **23:** 4690–4700