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Two Proteases Defining a Melanization Cascade in the Immune System of *Drosophila*[*]

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The melanization reaction is used as an immune mechanism in arthropods to encapsulate and kill microbial pathogens. In *Drosophila*, the serpin Spn27A regulates melanization apparently by inhibiting the protease that activates phenoloxidase, the key enzyme in melanin synthesis. Here, we have described the genetic characterization of two immune inducible serine proteases, MP1 and MP2, which act in a melanization cascade regulated by Spn27A. MP1 is required to activate melanization in response to both bacterial and fungal infection, whereas MP2 is mainly involved during fungal infection. Pathogenic bacteria and fungi may therefore trigger two different melanization cascades that use MP1 as a common downstream protease to activate phenoloxidase. We have also shown that the melanization reaction activated by MP1 and MP2 plays an important role in augmenting the effectiveness of other immune reactions, thereby promoting resistance of *Drosophila* to microbial infection.

Drosophila utilizes multiple defense mechanisms against microbial pathogens (1). A major mechanism is the synthesis of antimicrobial peptides that kill pathogens in the hemolymph. Genetic studies have revealed that the Toll and Imd signaling pathways regulate antimicrobial peptide expression. The Toll pathway is important for the defense against Gram-positive bacteria and fungi (2). Upon infection, secreted pattern recognition receptors recognize microbial cell wall components and then trigger cleavage of the Spätzle protein to generate the ligand that activates Toll signaling in the fat body, an immune-responsive tissue. GNBP1 and PGRP-SA are two pattern recognition receptors that act upstream of Toll and recognize Gram-positive bacteria (3–5). Toll activation by fungal infection is independent of GNBP1 and PGRP-SA but involves the serine protease Persephone (6) and regulation by the serpin-type protease inhibitor Necrotic (7). Antimicrobial peptides against Gram-negative bacteria are induced under the control of the Imd pathway (8). The Toll and Imd pathways are homologous to the mammalian Toll-like receptor and tumor necrosis factor pathways, respectively, reflecting the evolutionary conservation of the innate immune systems between insects and mammals (1).

The melanization reaction is the most immediate immune response against invading pathogens in *Drosophila* (9). Although melanization is apparently not used as a defense mechanism in mammals, it serves as a major immune response against the malaria parasite in the mosquito (10). Melanization is visible by the blackening of a wound site or the surface of pathogens, which results from the synthesis and deposition of melanin. In addition to being important for wound healing, melanin can encapsulate and sequester pathogens, and the reaction intermediates appear to be directly toxic to microbes as well (11). During melanization, the enzyme phenoloxidase (PO)[4] catalyzes the oxidation of phenols to quinones, which then polymerize non-enzymatically to form melanin. In *Drosophila* and other arthropods, PO is produced and released into the hemolymph as an inactive zymogen called prophenoloxidase (PPO). Biochemical studies in larger insect species have identified proteases involved in melanization, including PPO-activating enzyme (PPAE) that directly cleaves and activates PPO at the end of a cascade upon microbial infection (12–14). These studies have also identified serpins as being involved in regulating the melanization cascade.

Recent genetic studies in *Drosophila* have provided new insights into the mechanisms that regulate the melanization reaction. The pattern recognition receptor called PGRP-LE is involved in inducing melanization in response to Gram-negative bacteria (15). In addition, the serpin Spn27A inhibits activation of the melanization reaction (16, 17). The target of Spn27A is thought to be PPAE, the protease that cleaves PPO, as recombinant Spn27A inhibits the beetle PPAE *in vitro* (16). However, the endogenous target of Spn27A in *Drosophila* has remained unknown.

Here, we have described the identification of two proteases named MP1 and MP2 (melanization protease) that define a melanization cascade regulated by Spn27A, which is activated by microbial infection in *Drosophila*. Our data suggest the existence of two melanization cascades, one involving MP2 activated primarily by fungal infection and the other involving an analogous protease activated by bacterial infection, which

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[4] The abbreviations used are: PO, phenoloxidase; PPO, prophenoloxidase; PPAE, PPO-activating enzyme; MP, melanization protease; RNAi, RNA interference.

converge on MP1 as a common activator of phenoloxidase. Our data also suggest that the melanization reaction, although not essential for survival following microbial infection, plays a critical role in enhancing the effectiveness of other immune reactions in *Drosophila*.

MATERIALS AND METHODS

Genetics and Molecular Biology—Oregon R was used as the wild-type strain unless otherwise indicated. The spz^{rm7} (2), rel^{E20} (18), and $spn27A^{1}$ (16) mutations, as well as the UAS-Spn27A (17), UAS-dFadd RNAi (19), and UAS-Dif RNAi (20) lines have been described earlier. The Bloomington Stock Center was the source of all other stocks.

In our hands, ~6% of $spn27A^{1}$ homozygotes were viable as adults, and of these, ~80% showed constitutive melanization (see Fig. 1*A*). The two deficiencies that suppressed *spn27A* melanization >5-fold were Df(3R)ME15 and Df(3R)dsx11, which respectively uncovered the genes *CG1102* and *CG3066*, encoding clip proteases named MP1 and MP2.

The cDNAs GH02921 and LP12377 for MP1 and MP2, respectively, were obtained from the *Drosophila* Genomics Resource Center. To make constructs for overexpression of MP1 and MP2, DNA fragments encoding full-length MP1 and MP2 protein or just the catalytic domain of these proteases were generated by PCR using cDNA templates and cloned into the pUASP vector (21). The Easter signal sequence was attached to the catalytic domains of MP1 and MP2 to make the preactivated forms of these proteases (22). To make the MP1 and MP2 RNAi constructs, PCR fragments of ~600 bp of the appropriate cDNAs were cloned as inverted repeats into the pWIZ vector (23). Results were obtained with at least two independently generated transgenic lines bearing the MP1 and MP2 RNAi constructs. The Spn27A RNAi construct was made using the pUAST-R57 vector essentially as described earlier (20). Quantitative real-time PCR was performed essentially as described previously (5).

Microbial Infection and Survival Measurement—For septic injury, adult flies were pierced in the lateral side of the thorax with a tungsten needle of 0.1 mm diameter previously dipped into a concentrated microbial culture (24). The flies were photographed ~24 h after infection. For *Beauveria bassiana* infection, anesthetized flies were placed onto a plate containing a carpet of the fungus, agitated until covered by spores, and incubated at 29 °C for further analysis (24). To measure survival after microbial infection, the flies were transferred to fresh vials every 2–3 days after infection, and the number of live insects was recorded (24).

PO Activity Assay—PO activity was assayed 4 h after septic injury or 24 h after natural fungal infection, essentially as described previously (25). The hemolymph of 30 adult flies was collected with a microcapillary and then squeezed into 20 μl of phosphate-buffered saline containing protease inhibitors on ice. Protein concentration was measured using the Bio-Rad Protein Assay. Five μg of total hemolymph proteins in 40 μl of phosphate-buffered saline containing protease inhibitors was mixed with 120 μl of phosphate-buffered saline saturated with L-3,4-dihydroxiphenylalanine. After incubation at room temperature for 30 min, the absorbance at 490 nm of the samples was measured. Each experiment was repeated at least three times.

RESULTS

Identification of Proteases MP1 and MP2 in a Screen for Suppression of the spn27A Melanization Phenotype—The serpin Spn27A regulates the melanization reaction in *Drosophila*, as loss of *spn27A* function leads to constitutive melanization (Fig. 1*A*) and semilethality (16, 17). In the melanization reaction, Spn27A is presumed to inhibit PPAE, the terminal protease that activates PO (Fig. 1*B*). Spn27A is also maternally required for embryonic dorsoventral patterning (26, 27), where its target is Easter, the terminal protease in a cascade leading to activation of Toll signaling (Fig. 1*B*). Loss of maternal Spn27A results in a ventralized embryo phenotype, which can be partially rescued by the reduction in gene dosage of Easter (26). By analogy, we thought that the suppression of the constitutive melanization phenotype caused by the loss of *spn27A* function could be used as a genetic strategy to uncover the target of Spn27A and other proteases in the melanization cascade. Using standard crosses, we generated flies homozygous for the null mutation $spn27A^{1}$ and heterozygous for one of 12 chromosomal deficiencies that uncovered 13 different serine proteases of the clip family. We focused on clip proteases, which contained an N-terminal clip domain in addition to a C-terminal catalytic domain (Fig. 1*C*), because Easter is a clip protease and all known PPAEs identified biochemically in arthropods are also clip proteases (9). Of the 12 deficiencies screened, two showed significant (5–10-fold) suppression of the *spn27A* melanization phenotype (see "Materials and Methods"). These two deficiencies uncovered the genes CG1102 and CG3066, which encoded two clip proteases, hereafter called MP1 and MP2, respectively. In the catalytic domain, MP1 and MP2 were 56 and 44% identical to Easter, respectively, and similar to Easter, had sequence features (such as codon usage for the catalytic serine) shared by serine proteases that apparently define the most evolutionarily ancient class and occupy the most downstream positions within cascades (Fig. 1*C*) (28). Interestingly, MP1 and MP2 were shown by microarray analysis to be up-regulated upon bacterial and fungal infection in a Toll-dependent manner (29, 30).

To test that suppression of the *spn27A* melanization phenotype was due to functional reduction of MP1 or MP2 instead of another gene within the two deficiencies that uncover these proteases, we wanted to specifically lower the expression level of MP1 and MP2. We decided to use RNAi to knock down their expression and therefore generate transgenic flies that could express double-stranded RNA targeting either MP1 or MP2 for RNAi under the control of the UAS sequence element and the Gal4 transcriptional regulator (31). The act-Gal4 or da-Gal4 "driver," in which Gal4 protein is broadly expressed under the actin or *daughterless* gene promoter, induces the expression of UAS-coupled genes throughout development in virtually all tissues. To drive such ubiquitous activation of MP1 or MP2 RNAi, we generated flies carrying the act-Gal4 driver and the UAS construct encoding double-stranded RNA targeting MP1 or MP2. These MP1 and MP2 RNAi flies were viable to the adult stage, except for ~50% of the insects that died at the late pupal

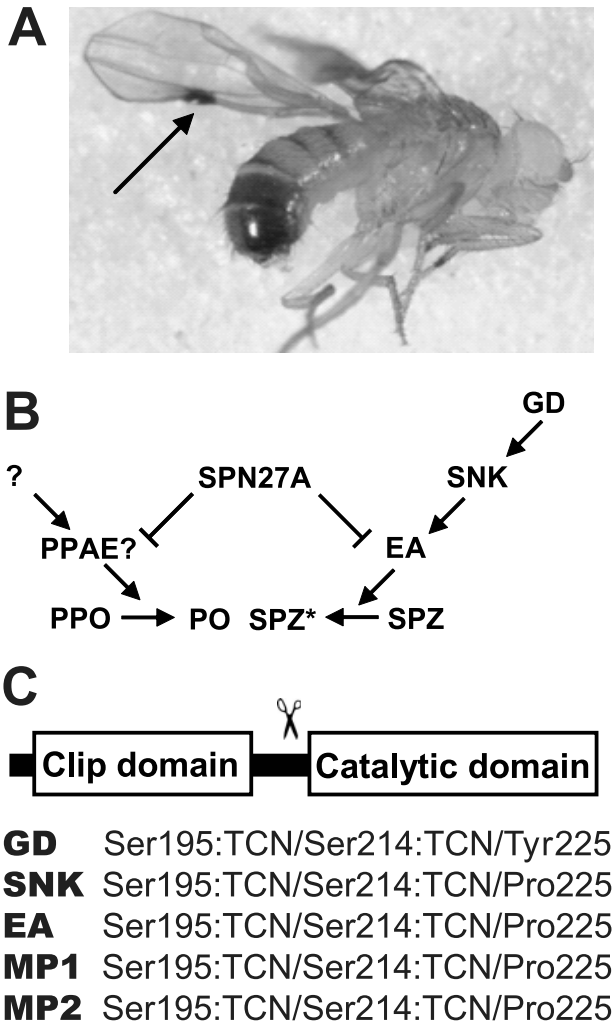

FIGURE 1. **Screening for suppressors of the *spn27A* melanization phenotype identifies two serine proteases, MP1 and MP2.** *A*, the null mutation *spn27A*^{1} for the serpin Spn27A results in constitutive melanization, as seen here on the wing (*arrow*), and in semilethality. *B*, in melanization, Spn27A is presumed to inhibit the protease PPAE that cleaves PPO to generate PO, a key enzyme in melanin biosynthesis. Spn27A also regulates embryonic dorsoventral patterning, where its target serine protease is Easter (*EA*). *GD* and *SNK* are serine proteases that act sequentially in a cascade to activate EA, which cleaves the Spätzle protein (*SPZ*) to generate the Toll ligand (*SPZ**). *C*, MP1 and MP2 proteases contain a clip domain in the N-terminal region and a C-terminal catalytic domain. Cleavage (indicated by *scissors*) is required to activate clip protease. Chymotrypsin-like serine proteases can be phylogenetically classified based on sequence patterns that serve as evolutionary markers (28). The marker for the most primordial class of serine proteases is Ser^{195}:TCN/Ser^{214}:TCN/Pro^{225}, where the catalytic serine (Ser^{195}) and the highly conserved serine adjacent to the active site (Ser^{214}) are encoded by a TCN (N = any base) codon and the residue 225 (chymotrypsinogen numbering) is Pro. MP1, MP2, EA, and SNK belong to this class, whereas GD belongs to a modern class one evolutionary transition removed from the primordial class, with residue 225 being Tyr that, unlike Pro, enhances catalysis caused by Na^{+} binding. The GenBank™ accession numbers for the protein sequences of GD, SNK, EA, MP1, and MP2 are AAF48122, AAF54897, AAF55170, AAF52151, and AAF54143, respectively.

stage or during eclosion. Reverse transcription-PCR analysis indicated that MP1 and MP2 expression was specifically knocked down in each case ~10-fold from the wild-type level (data not shown). In addition, the lethality and constitutive melanization caused by loss of normal Spn27A function was completely suppressed in flies in which either MP1 or MP2 RNAi was simultaneously induced with Spn27A RNAi using the da-Gal4 driver (data not shown). These results suggest that MP1 and MP2 are involved in the melanization reaction that is negatively regulated by Spn27A. While we were preparing this work for publication, MP2 was reported to be involved in activating melanization (32, 33).

MP1 and MP2 Are Required to Activate Melanization and PO Activity upon Microbial Infection—To test directly a requirement for MP1 and MP2 in the melanization reaction, we examined the ability of MP1 and MP2 RNAi flies to activate melanization upon microbial infection. Flies were pierced with a tungsten needle previously dipped into a concentrated solution of the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Micrococcus luteus*. A few hours after piercing, significant melanization around the wound site was seen in control flies (Fig. 2*A*) but not in flies in which MP1 or MP2 RNAi was induced with act-Gal4 (Fig. 2, *B* and *C*).

To examine whether MP1 and MP2 are required to activate PO, we assayed PO enzymatic activity in hemolymph extracted from adult flies (Fig. 2*D*). In uninfected wild-type flies, only a low level of PO activity was detected; however, 4 h after septic injury with *E. coli* and *M. luteus*, a dramatically higher level of PO activity was seen. Overexpression of Spn27A blocked the induction of PO activity by bacterial infection. When MP1 RNAi flies were similarly infected, no significant PO activity was detected in the hemolymph 4 h after infection. In MP2 RNAi flies after bacterial infection, PO activity was significantly lowered but not completely blocked. Similar results were obtained by infecting MP2 RNAi flies with *E. coli* and *M. luteus* separately (data not shown).

We also checked hemolymph PO activity after natural infection by the fungus *B. bassiana* (Fig. 2*E*). In wild-type flies, significant PO activity was induced 24 h after *B. bassiana* infection, although the level was lower than that after bacterial septic injury. MP1 RNAi flies again showed only a background level of PO activity after fungal infection. Interestingly, no significant PO activity was seen in MP2 RNAi flies after fungal infection, in contrast to the lowered activity seen after bacterial infection.

Altogether, these data indicate that MP1 and MP2 are required to activate melanization and PO after microbial infection. They also suggest that MP1 is involved in the response to bacteria and fungi, whereas MP2 is more specifically involved in the response to fungi.

MP1 or MP2 Activity Is Sufficient to Induce Constitutive Melanization and PO Activity—To check whether MP1 or MP2 activity is sufficient to induce constitutive melanization and PO activity, we generated transgenic flies in which the UAS/Gal4 system could be used to overexpress a preactivated form of MP1 or MP2 consisting of just the catalytic domain after signal sequence cleavage. Larvae in which preactivated MP1 was induced with act-Gal4 developed more slowly than wild-type larvae. Approximately 85% of these larvae showed constitutive melanization and eventually died with melanization visible over the whole body (Fig. 3*B*). The remaining 15% developed to the pupal and adult stages with no apparent defect. When we used the c564-Gal4 driver expressed in the fat body and hemocytes to induce the overexpression of preactivated MP1, most of the insects were able to survive to or beyond the pupal stage; however, 10% of the pupae and 30% of the adults showed constitu-

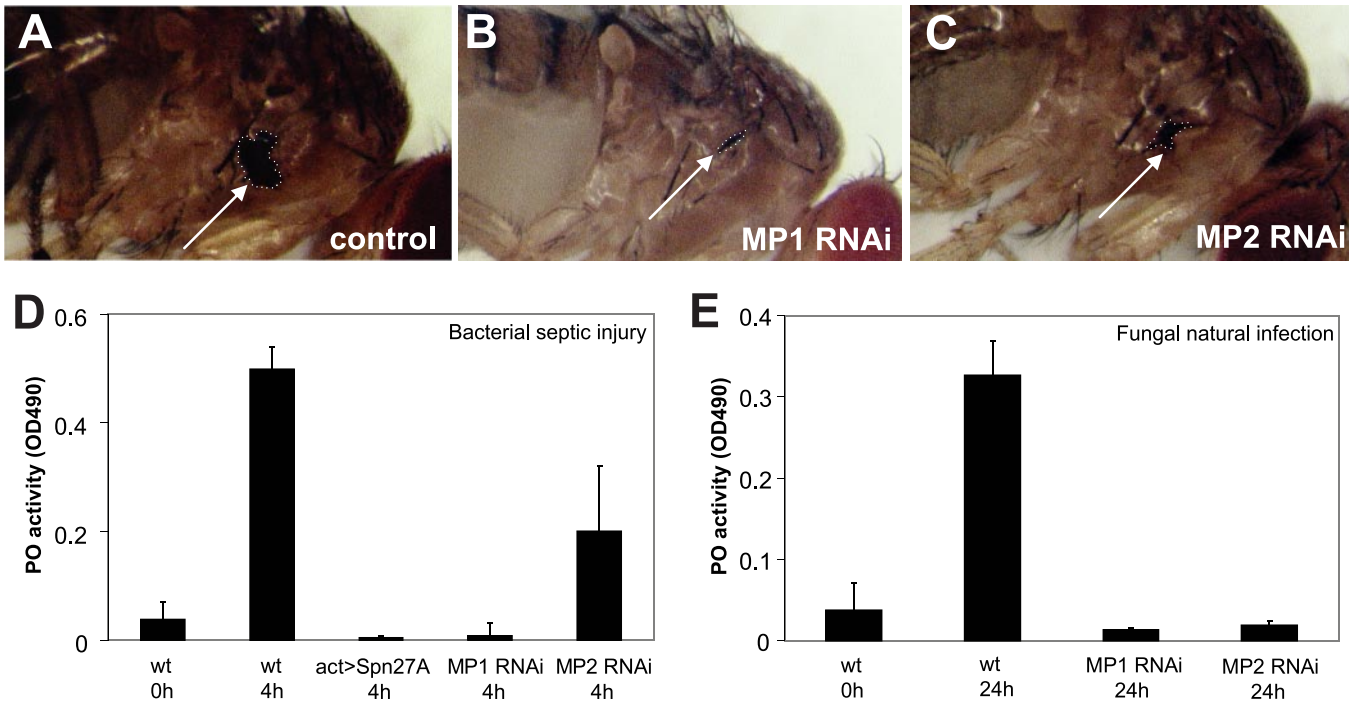

FIGURE 2. **MP1 or MP2 RNAi compromises activation of melanization and phenoloxidase activity after microbial infection.** Flies were pierced with a tungsten needle harboring *E. coli* and *M. luteus*. At the injury site (*arrow*), control flies (*A*) showed pronounced melanization, whereas MP1 RNAi flies (*B*) and MP2 RNAi flies (*C*) showed significantly reduced melanization. The extent of melanization is *outlined*. *D*, PO activity in adult hemolymph was assayed 4 h after bacterial septic injury. In wild-type (wt) flies, PO activity was significantly induced by infection (0 and 4 h). As a control, when Spn27A was overexpressed (*act>Spn27A*), PO activity was comparable with the un-induced level. In MP1 RNAi flies, PO activity was also at the un-induced level, whereas in MP2 RNAi flies, PO activity was significantly reduced but with greater variability. *E*, hemolymph PO activity was assayed 24 h after natural infection by the fungus *B. bassiana*. In wild-type flies, PO activity was significantly induced, although it was not as high as seen after bacterial septic injury (*wt*, 0 and 24 h). In either MP1 or MP2 RNAi flies, PO activity after natural fungus infection was comparable with the un-induced level. RNAi was induced with act-Gal4. *Bars* indicate S.D. of at least three independent experiments.

tive melanization. The ubiquitous overexpression of preactivated MP2 seemed to have a weaker effect. Approximately 10% of the larvae showed constitutive melanization (Fig. 3*C*), and most larvae developed into pupae, of which ~40% showed constitutive melanization (Fig. 3*E*). Most of these melanized pupae died. Among ~50% of the insects that survived to adulthood, ~5% showed a constitutive melanization phenotype (Fig. 3*G*). Most of the melanization appeared to be under the epithelia and in the hemolymph.

We also checked PO activity in the hemolymph of flies overexpressing preactivated MP1 or MP2 (Fig. 3*H*). Consistent with the melanization phenotype described above, a high level of hemolymph PO activity was detected in these flies even without any microbial infection. This level was nearly comparable with the levels seen in wild-type flies 4 h after microbial infection or in *spn27A*^{1} flies. These results indicate that preactivated MP1 or MP2 is sufficient to induce the activation of melanization and PO activity.

Flies ubiquitously overexpressing the full-length form of either MP1 or MP2 were completely viable. Only a few (2–3%) displayed some melanization spots, and none produced significant constitutive PO activity (data not shown). These results indicate that MP1 and MP2 must be activated by zymogen cleavage to induce melanization and PO activity.

The induction of melanization and PO activity by overexpression of preactivated MP1 or MP2 was blocked by simultaneous overexpression of Spn27A (Fig. 3*H*). This result indicates that MP1 and MP2 cannot act downstream of the direct target of Spn27A, which is presumed to be the terminal protease in the melanization cascade that activates PO.

MP1 and MP2 Function Sequentially in Activating Melanization and PO Activity—Lethality and melanization caused by the loss of Spn27A function are completely suppressed by RNAi knockdown of either MP1 or MP2. Moreover, MP1 and MP2 are both required, whereas preactivated MP1 or MP2 is each sufficient to induce melanization and PO activity. These observations suggest that the two proteases act in a common pathway rather than in independent pathways. To test whether MP1 and MP2 act sequentially, as in a zymogen activation cascade, we examined whether RNAi of one protease could block the induction of melanization and PO activity caused by overexpression of the preactivated form of the other protease. We found that melanization and PO activity induced by overexpression of preactivated MP2 was completely blocked by MP1 RNAi (Fig. 3*H*). In the reciprocal experiment, MP2 RNAi did not block the melanization of larvae induced by overexpression of preactivated MP1 (as seen in Fig. 3*B*). PO activity in adult hemolymph could not be assayed in this case, as simultaneous induction of MP2 RNAi and preactivated MP1 overexpression led to complete lethality, compared with 85% lethality from preactivated MP1 overexpression alone (see above) before the adult stage. These results are consistent with MP2 functioning genetically upstream of MP1 to activate melanization and PO.

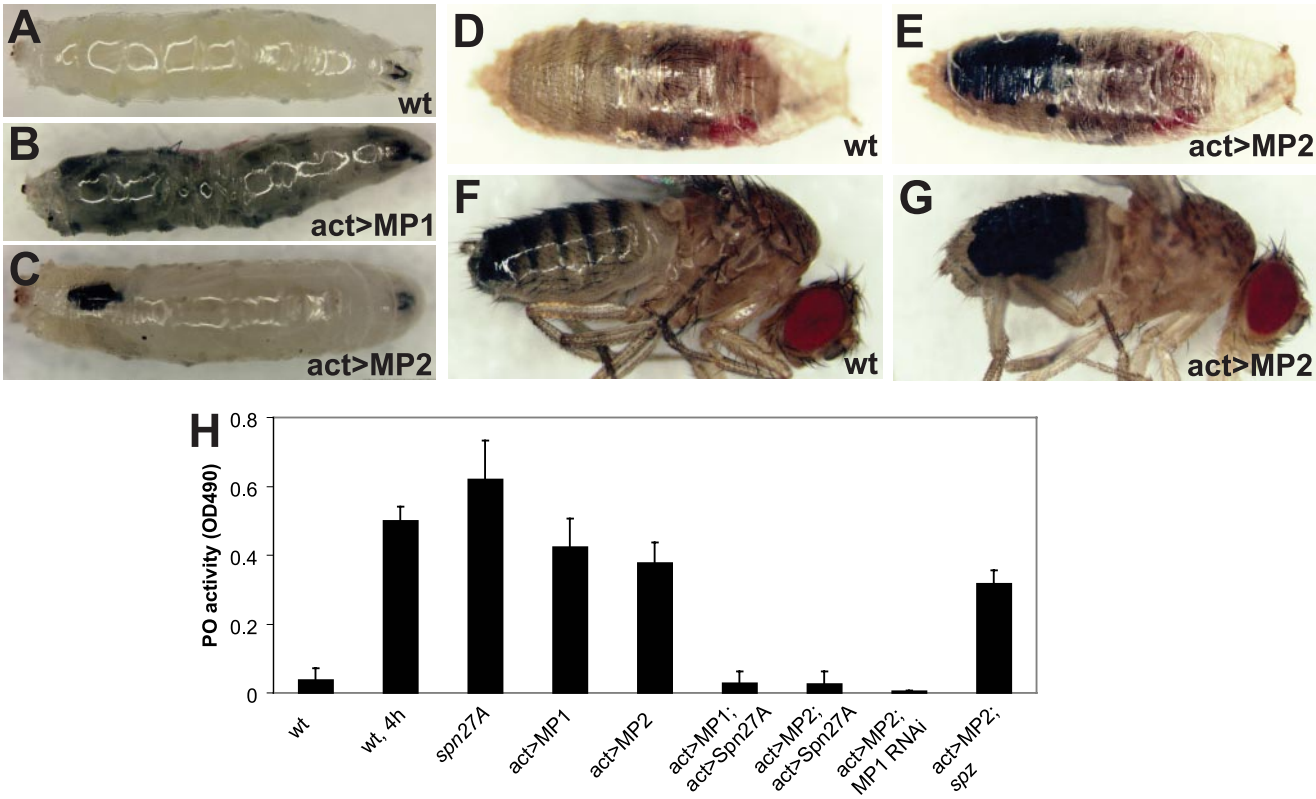

FIGURE 3. **Overexpression of preactivated MP1 and MP2 induces constitutive melanization and PO activity.** Larva, pupa, and adult were examined for melanization in the absence of microbial infection. Wild-type larva (*A*), pupa (*D*), and adult (*F*) typically show no evidence of melanization. Overexpression of preactivated MP1 (*act>MP1*) resulted in most insects dying at the larval stage, with melanization spots visible over the whole body (*B*). Overexpression of preactivated MP2 (*act>MP2*) results in ~10% of larvae (*C*), 40% of pupae (*E*), and 5% of adults (*G*) displaying constitutive melanization. *H*, PO activity in adult hemolymph. Uninfected wild-type (*wt*) flies have only a low level of PO activity. Control *spn27A*^{1} homozygotes (*spn27A*) have a high level of PO activity, as in wild-type flies, 4 h after infection (*wt*, 4 h). Flies that overexpress preactivated MP1 (*act>MP1*) or preactivated MP2 (*act>MP2*) have a similarly high level of PO activity. This constitutive PO activity was blocked by overexpression of Spn27A (*act>MP1*; *act>Spn27A* and *act>MP2*; *act>Spn27A*). MP1 RNAi (but not the *spz*^{rm7} mutation) was able to block the high PO activity induced by overexpression of preactivated MP2, consistent with MP1 acting downstream of MP2 and with MP2 acting independently of Toll pathway, respectively (*act>MP2; MP1 RNAi* and *act>MP2; spz*).

MP1 and MP2 Do Not Require Toll Signaling to Activate Melanization—An earlier study suggests that melanization requires activation of the Toll signaling pathway to deplete Spn27A (17). To investigate whether MP1 and MP2 activate melanization through Toll, we overexpressed preactivated MP1 or MP2 in flies homozygous for *spz*^{rm7}, a loss-of-function mutation in the *spätzle* gene encoding the Toll ligand. We found that *spz*^{rm7} did not block constitutive melanization (as seen in Fig. 3*B*) and larval lethality caused by overexpression of preactivated MP1. Indeed, *spz*^{rm7} enhanced the partial lethality caused by preactivated MP1 overexpression alone (see above), causing complete lethality before the adult stage that precluded the assay of PO activity in adult hemolymph. However, we determined that *spz*^{rm7} did not block the high PO activity produced by overexpression of preactivated MP2 (Fig. 3*H*). Thus, MP1 and MP2 appear to regulate melanization and PO activation, in large part, independently of the Toll signaling pathway.

Melanization Is Important for Enhancing the Effectiveness of Other Immune Reactions—As no significant PO activity is detectable in MP1 RNAi flies after bacterial or fungal infection (Fig. 2), we used these flies to address whether the melanization reaction is important for resistance to microbial infection. Flies were challenged with a Gram-negative bacterium (*Erwinia carotovora*), a Gram-positive bacterium (*Enterococcus faecalis*) or the fungus *Candida albicans*, or naturally infected with the entomopathogenic fungus *B. bassiana*. The survival rate of MP1 RNAi flies was not significantly different from wild-type flies after all types of infection (Fig. 4, *A–C*, and data not shown). Similar results were obtained for MP2 RNAi flies after infection with bacteria (Fig. 4, *A* and *B*) or the fungus *C. albicans* (data not shown). These results suggest that the melanization reaction is largely dispensable for resistance to microbial infection. A similar conclusion was reached from a recent independent study of MP2 (33). Interestingly, however, we found that the melanization reaction was important for resistance to microbial infection in flies impaired in the Toll or the Imd pathway controlling antimicrobial peptide synthesis. For example, after infection with the Gram-negative bacterium *E. carotovora*, the survival rate was moderately lower for flies in which MP1 (or MP2) and dFadd in the Imd pathway were both knocked down by RNAi compared with flies in which just dFadd was knocked down (Fig. 4*D*). A similar decrease in the survival rate of flies from RNAi knockdown of MP1 (or MP2) and Dif in the Toll pathway, compared with knockdown of just Dif, was observed after infection with the fungus *C. albicans* (Fig. 4*F*). Such a distinct synergistic effect was less discernible after infection with the Gram-positive bac-

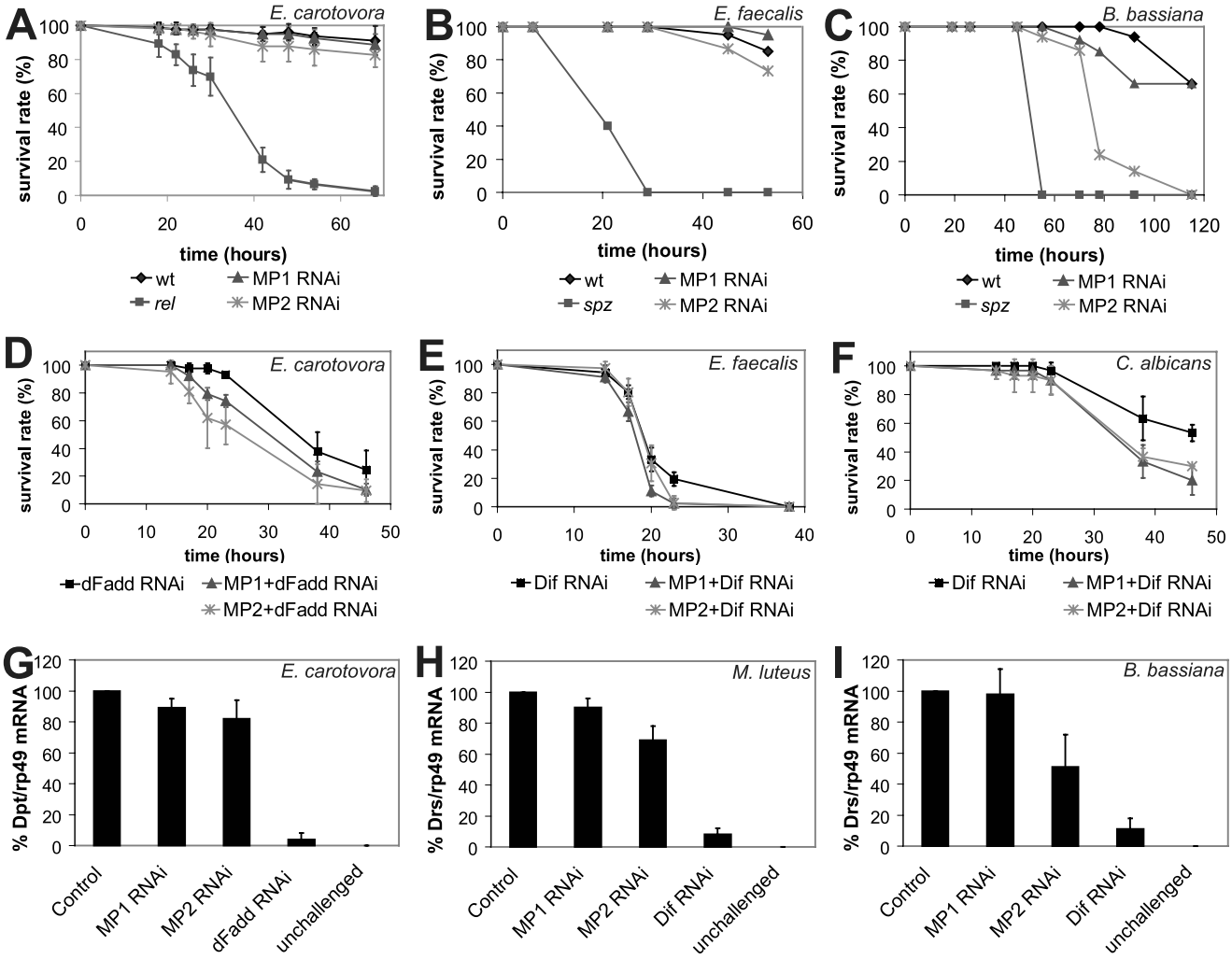

FIGURE 4. **Role of melanization in resistance to microbial infection.** MP1 and MP2 RNAi flies were examined for survival (*A–F*) and antimicrobial peptide expression (*G–I*) after bacterial and fungal infection. Knockdown of MP1 or MP2 did not compromise survival (*A*) nor affect *Diptericin* induction (*G*) after infection with the Gram-negative bacterium *E. carotovora*, unlike in the case of *rel* mutant or dFadd RNAi flies that lack a functional Imd pathway. MP1 RNAi flies resisted infection by the Gram-positive bacterium *E. faecalis* (*B*) and natural infection by the fungus *B. bassiana* (*C*) in a similar manner to control flies and showed a wild-type level of *Drosomycin* induction after bacterial or fungal infection (*H* and *I*). MP2 RNAi flies showed increased susceptibility to natural fungal infection (*C*) that correlated with decreased *Drosomycin* induction (*I*), although the effect was much less than seen with the *spz* mutation or Dif RNAi impairing the Toll pathway. The survival rate of flies after Gram-negative bacterial infection (*D*) was lowered when both melanization and the Imd pathway were impaired (*MP1* + or *MP2* + *dFadd RNAi*) compared with when just the Imd pathway was disrupted (*dFadd RNAi*). A similar synergistic relationship was seen between melanization and the Toll pathway (*MP1* + or *MP2* + *Dif RNAi versus Dif RNAi*) after infection with the fungus *C. albicans* (*F*). MP1 and MP2 RNAi flies showed no appreciable susceptibility to *C. albicans* infection (data not shown). *A–F* show a representative of 2–3 independent experiments, whereas the data in *A* and *D–F* represent the mean with S.D. of triplicate samples. Bacteria and *C. albicans* were used at a concentration equivalent to a liquid culture at absorbance (600 nm) of 60 (*A*), 30 (B), 50 (*D*), 10 (*E*), and 75 (*F*). Note the lower concentration of the same bacterium used for *D versus A* and *E versus B*. Antimicrobial peptide expression in *G–I* was monitored by reverse transcription-PCR using total RNA extracted from flies 6 h after septic injury with *E. carotovora*, 24 h after septic injury with *M. luteus*, and 48 h after natural infection with *B. bassiana* spores. Each column represents the mean of 4–5 independent experiments with S.D. Survival and antimicrobial peptide gene expression were monitored using flies carrying one copy each of the da-Gal4 driver and, except in the wild-type control, of the UAS-RNAi construct.

terium *E. faecalis*, perhaps because of the rapid death of the flies (Fig. 4*E*). We note that the differences in relative survival rates described here, although modest, were reproducible despite the variability in absolute survival rates between experiments. These observations suggest that the melanization reaction, although not essential for resistance to microbial infection, can play an important role in enhancing the effectiveness of other immune reactions.

Interestingly, compared with MP1 RNAi flies, MP2 RNAi flies were less viable after natural infection with the fungus *B. bassiana* (Fig. 4*C*). The pronounced difference in susceptibility to fungal infection made us wonder whether MP2 might have a role in activating the Toll antifungal pathway in addition to its role in activating melanization. Consequently, we monitored in MP1 RNAi and MP2 RNAi flies the expression of *Drosomycin*, an antifungal peptide gene regulated mainly by the Toll pathway, and of *Diptericin*, an antibacterial peptide gene regulated by the Imd pathway. As measured by quantitative real-time reverse transcription-PCR (Fig. 4, *G–I*) and reporter gene expression (data not shown), the knockdown of MP1 or MP2 had no significant effect on the induction of *Diptericin* after infection by *E. carotovora*, indicating that MP1 and MP2 do not regulate the Imd pathway and that the fat body, the site of antimicrobial peptide

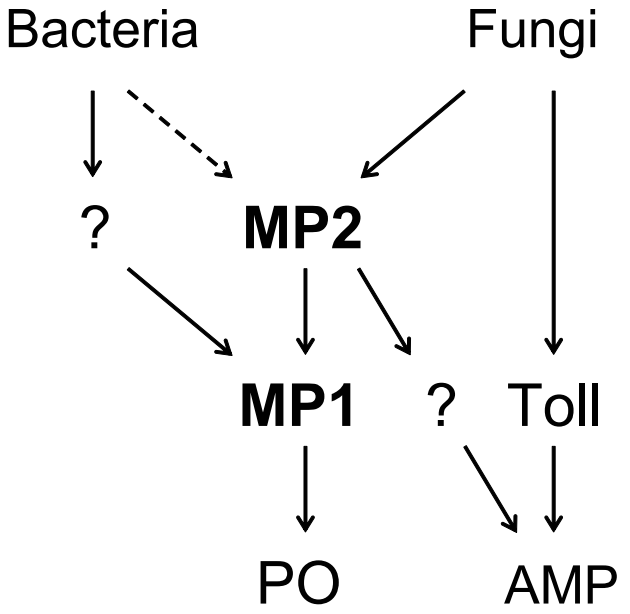

FIGURE 5. **Model depicting MP1 and MP2 proteases in multiple immune pathways.** MP1 and MP2 act in the melanization cascade to activate PO, the key enzyme in melanin synthesis. This cascade is triggered primarily by fungi and to a lesser degree by bacteria. Another melanization cascade involving an unidentified protease and MP1 may be more specifically induced during bacterial infection. MP2 is also involved in inducing antimicrobial peptide expression by an unknown mechanism potentially involving cross-talk with the Toll pathway. *Arrows* represent genetic relationships and not necessarily direct biochemical interactions.

expression, is still functional (Fig. 4*G*). However, MP2 RNAi (but not MP1 RNAi) adults showed reduced *Drosomycin* expression, ~50% of the wild-type level, after infection with the fungus *B. bassiana* (Fig. 4*I*). MP2 RNAi did not similarly reduce *Drosomycin* expression after challenge by the Gram-positive bacterium *M. luteus* (Fig. 4*H*). These results suggest that MP2 has a role in activating *Drosomycin* expression in response to natural fungal infection.

DISCUSSION

Melanization is a conserved host defense reaction in insects and other arthropods, such as in the mosquito, where it is a critical determinant of resistance to the malarial parasite. A recent key finding revealed that Spn27A, a serpin-type protease inhibitor, is a negative regulator of melanization in *Drosophila*. In this study, we have used suppression of the *spn27A* melanization phenotype as a genetic strategy to identify two *Drosophila* proteases, MP1 and MP2, having essential roles in activating melanization in response to microbial infection.

Our data are consistent with MP2 acting genetically upstream of MP1 and thus with MP2 activating MP1 in a protease cascade leading to melanization (Fig. 5). Interestingly, the application of evolutionary markers suggests that MP1 and MP2 belong to the primordial class of serine proteases that tend to function most downstream in a protease cascade, as in the case of Easter and its direct activator involved in activating Toll during development (Fig. 1*C*). However, we have not been able to demonstrate that MP2 directly activates MP1, as MP2 failed to cleave the zymogen form of MP1 when co-expressed in transfected *Drosophila* S2 cells, thereby suggesting that another protease acts in between MP2 and MP1. As the protease downstream of MP2 that acts either in parallel to or upstream of Spn27A, MP1 is a candidate to be PPAE, the terminal protease in the melanization cascade that cleaves PPO as well as the putative direct target of Spn27A. We have not detected cleavage of PPO by MP1 when assayed by co-expression in transfected S2 cells.[5] However, biochemical studies in the beetle and tobacco hornworm have identified a non-enzymatic cofactor required for PPO cleavage by PPAE to generate PO activity *in vitro* (13, 34), which may indicate that such a cofactor is required to demonstrate PPO cleavage by MP1.

Our data suggest that another melanization cascade exists in *Drosophila* besides the one that we have defined involving MP1 and MP2. In activating melanization and PO activity, MP1 is essential during both bacterial and fungal infection, whereas MP2 is essential during fungal infection and partially required during bacterial infection (Fig. 2*D* and *E*). Thus, another protease may function analogously to MP2 in activating MP1 during bacterial infection (Fig. 5). The convergence of two different melanization cascades on MP1 is consistent with the idea that MP1 is the shared terminal protease of both cascades that activates phenoloxidase.

We presume that MP1 and MP2 activate melanization in the hemolymph. As both MP1 and MP2 have an N-terminal signal sequence for secretion, they may be secreted by the fat body and/or blood cells into the hemolymph. This possibility is consistent with the detection of MP1 and MP2 in extracted hemolymph when epitope-tagged versions of the full-length proteases are overexpressed with act-Gal4 (data not shown) and with constitutive melanization induced by overexpression of preactivated MP1 using the c564-Gal4 driver, which is expressed in the fat body and hemocytes (see "Results").

MP1 and MP2 define a protease cascade distinct from the one that may activate the Toll pathway, as they activate melanization independently of this pathway (Fig. 3). However, we found that MP2 (but not MP1) is important for the induction of *Drosomycin* expression and for viability of *Drosophila* following natural fungal infection (Fig. 4). One explanation is that MP2 activates two distinct pathways, one leading to melanization and the other leading to the induction of *Drosomycin* expression (Fig. 5), and that this dual role is important for resistance to natural fungal infection. Preliminary experiments indicate that overexpression of preactivated MP2 does not induce *Drosomycin* expression in non-infected adult flies, unlike in the case of the Persephone protease (6), thereby suggesting that MP2 does not induce *Drosomycin* expression by directly activating the Toll pathway. Nonetheless, there may exist cross-talk between melanization and the Toll pathway mediated by MP2 involving an as yet undefined mechanism.

Surprisingly, the melanization reaction does not appear to be critical for survival of *Drosophila* after bacterial or fungal infection (Fig. 4; 33). However, we observed that the inability to activate melanization is detrimental when flies are also defective in the Toll or the Imd pathway controlling antimicrobial peptide synthesis (Fig. 4). Melanization is an immediate immune response that temporally precedes the induction of

[5] H. Tang and C. Hashimoto, unpublished data.

antimicrobial peptide synthesis, which requires gene transcription. Consequently, melanization may play a crucial role in weakening a microbial infection, thereby enhancing the effectiveness of subsequent immune reactions. Having a single switch, such as MP2, to activate a temporal sequence of immune reactions would therefore seem to be an advantageous mechanism for ensuring a potent defense against a microbial pathogen.

In conclusion, we have identified two serine proteases among the large set of serine proteases encoded in the *Drosophila* genome as being essential components of a melanization cascade activated by microbial infection. A major goal in future studies will be to delineate the entire cascade from the pattern recognition receptor that triggers the cascade to the putative cofactor required for phenoloxidase activation.

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