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A Spätzle-Processing Enzyme Required for Toll Signaling Activation in *Drosophila* Innate Immunity

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Summary

The Toll receptor was originally identified as an indispensable molecule for *Drosophila* embryonic development and subsequently as an essential component of innate immunity from insects to humans. Although in *Drosophila* the Easter protease processes the pro-Spätzle protein to generate the Toll ligand during development, the identification of the protease responsible for pro-Spätzle processing during the immune response has remained elusive for a decade. Here, we report a protease, called Spätzle-processing enzyme (SPE), required for Toll-dependent antimicrobial response. Flies with reduced SPE expression show no noticeable pro-Spätzle processing and become highly susceptible to microbial infection. Furthermore, activated SPE can rescue ventral and lateral development in embryos lacking Easter, showing the functional homology between SPE and Easter. These results imply that a single ligand/receptor-mediated signaling event can be utilized for different biological processes, such as immunity and development, by recruiting similar ligand-processing proteases with distinct activation modes.

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Introduction

Innate immunity is an evolutionary conserved host defense system throughout the animal and plant kingdoms. In response to microbial infection, the host initiates various inflammatory signaling pathways, such as NF-κB, to induce immune effector molecules. In *Drosophila*, there are two genetically identified NF-κB pathways, Toll and *immune deficiency* (IMD) (Boutros et al., 2002; Brennan and Anderson, 2004; Hoffmann and Reichhart, 2002; Hultmark, 2003; Lemaitre, 2004; Silverman and Maniatis, 2001). Toll was originally identified as a type I transmembrane receptor that controls the dorsal-ventral patterning of the *Drosophila* embryo (Anderson et al., 1985a, 1985b; Hashimoto et al., 1988) and subsequently was shown to be involved in host resistance against fungal and Gram-positive (G+) bacterial infections (Lemaitre et al., 1996; Ligoxygakis et al., 2002a; Michel et al., 2001). A family of Toll-like receptors (TLRs) has also been found in humans, where they act as pattern recognition receptors (PRRs) for the activation of immune responses by recognizing pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan (PG), and β-1-3-glucan (βG) (Beutler and Rietschel, 2003; Iwasaki and Medzhitov, 2004). However, in contrast to TLRs, *Drosophila* Toll does not function as a bona fide PRR (Levashina et al., 1999; Ligoxygakis et al., 2002a). Instead, an enzymatically cleaved form of the pro-Spätzle gene product functions as the direct extracellular ligand for Toll (Weber et al., 2003). During early embryonic patterning, Easter, a CLIP domain serine protease, is known to activate Toll by cleaving pro-Spätzle (Chasan and Anderson, 1989). However, as the null mutant of Easter exhibits normal Toll activation after immune challenge, the involvement of other protease(s) with Easter-like activity has been suggested during Toll-dependent immune response (Lemaitre et al., 1996). Exposure and interaction of PAMPs to soluble *Drosophila* PRRs such as PG recognition proteins (PGRPs) and gram-negative bacteria binding proteins (GNBPs) during infection is believed to initiate the sequential activation of extracellular serine protease zymogens, leading to the cleavage of pro-Spätzle, analogous to the cascade involving Easter during embryonic patterning (Gobert et al., 2003; Levashina et al., 1999; Ligoxygakis et al., 2002a; Michel et al., 2001). The *persphone* (*psh*) gene encodes the only known serine protease required to activate Toll in response to fungal infection, but not G+ bacterial infection (Ligoxygakis et al., 2002a). Thus, in the case of G+ bacteria, another protease cascade may be involved in Toll activation (Gobert et al., 2003; Kim et al., 2000b; Michel et al., 2001). As both cascades would presumably lead to the conversion of pro-Spätzle to its active form through limited proteolysis, they must share a "downstream" protease that cleaves pro-Spätzle in response to both G+ bacteria and fungi. However, this key pro-Spätzle-processing protease has remained elusive for a decade.

In this study, we have taken a biochemical and genetic approach using two model insects, the silkworm

Bombyx mori and *Drosophila melanogaster*, to identify a Spätzle-processing enzyme (SPE) involved in immunity. SPE is specifically activated by either fungi or G+ bacteria and is essential for Toll activation and host defense.

Results

Purification and Characterization of a *Bombyx* Serine Protease Specifically Activated after Either PG or βG Treatment

Drosophila is a powerful tool for genetic studies, but *Bombyx mori* is a better model for biochemical studies, in particular of serine proteases involved in host defense, due to its large size and greater volume of extractable hemolymph (Ashida, 1971, 1990). The plasma fraction of *Bombyx* hemolymph contains a serine protease named BAEEase (based on its ability to hydrolyze the synthetic substrate N^{α}-benzoyl-L-arginine ethyl ester, BAEE) that can be activated from a zymogen form by two independent PAMPs (PG and βG) (Katsumi et al., 1995), a property shared with the hypothetical Spätzle-processing enzyme involved in *Drosophila* immunity. To investigate the role of BAEEase, we purified it from silkworm hemolymph (Figures 1A–1C). BAEEase was purified in a zymogen form that was specifically activated through limited proteolysis after either PG or βG treatment in the presence of a biochemical preparation containing all necessary upstream components for prophenoloxidase (pro-PO) activation (Figure 1D). This BAEEase is distinct from the *Bombyx* protease involved in activating pro-PO during melanization of invading pathogens (Ashida and Brey, 1995; Ashida et al., 1983; Kanzaki et al., 2003; Ochiai and Ashida, 1988, 1999, 2000; Yoshida et al., 1996), which is also activated by both PG and βG, suggesting a different role for BAEEase in host defense. Cloning and DNA sequence made possible by the purified protein revealed that BAEEase is initially synthesized as an inactive zymogen containing a NH_2-terminal CLIP domain and a COOH-terminal serine protease catalytic domain (Figure S1; see the Supplemental Data available with this article online). Biochemical analysis showed that, after the signal peptide removal (cleavage between G24 and Q25), the BAEEase zymogen was subjected to two additional limited proteolyses (between R83 and S84, and between R112 and I113) during PG- and βG-induced activation (Figure 1D and Figure S1). The cleavage between R112 and I113 ($DR^{\downarrow}IFGG$) was found to be essential for acquiring BAEEase enzymatic activity (data not shown). These results showed that *Bombyx* BAEEase exists as a zymogen and can be activated by upstream serine protease cascade components in the presence of PG and βG.

Identification of SPE, a *Drosophila* Homolog of *Bombyx* BAEEase that Activates Spätzle and Toll Signaling

The fact that *Bombyx* BAEEase can be activated by both PG and βG suggests that this enzyme is a good candidate to be the hypothetical Spätzle-processing enzyme that is activated by both PAMPs. To test this idea by using genetic analysis, we searched for the *Drosophila* homolog of *Bombyx* BAEEase. Among the 24 CLIP domain serine proteases having trypsin-like specificity, including Easter (Ross et al., 2003), only one (Flybase annotation: CG16705) has the identical cleavage site ($DR^{\downarrow}IFGG$) for zymogen activation between the CLIP domain and the catalytic domain as BAEEase (Figure 2A). This *Drosophila* homolog of *Bombyx* BAEEase was subsequently named SPE for Spätzle-Processing Enzyme based on its ability to process Spätzle in vitro and in vivo, as shown below.

Easter, an essential serine protease for Toll activation during early embryogenesis, has been shown to cleave Spätzle between R143 and V144 to yield a COOH-terminal fragment of 106 amino acids that corresponds to the Toll ligand (DeLotto and DeLotto, 1998; Weber et al., 2003). To test whether SPE can cleave Spätzle, we first generated an activated form of SPE consisting of just the catalytic domain after signal sequence cleavage (see Experimental Procedures). When this activated SPE was expressed with Spätzle in *Drosophila* S2 cells, a processed form of Spätzle was produced that matched the size of the C-terminal fragment produced by Easter cleavage (Figure 2B). Consistent with cleavage at the same site used by Easter, a mutant form of Spätzle having L143 and N144 instead of R143 and V144 was not cleaved when expressed with activated SPE (Figure 2B). Two additional experiments demonstrated that SPE directly cleaves Spätzle, rather than another protease acting between SPE and Spätzle. First, wild-type Spätzle, but not the cleavage mutant, was cleaved when coexpressed with activated SPE in human embryonic kidney cells (Figure S2A). Second, using purified recombinant proteins made in S2 cells, we found that wild-type Spätzle, but not the cleavage mutant, was cleaved when incubated with activated SPE (Figure S2B). In flies, expression of activated SPE caused the production of a processed form of endogenous Spätzle corresponding in size to the C-terminal form of Spätzle generated by cleavage between R143 and V144 (Figure 2C). These results suggest that SPE processes Spätzle identically to Easter in vitro and in vivo.

To test whether SPE can activate Toll signaling, we expressed activated SPE in S2 cells and in flies, and we then assayed the expression of the gene for Drosomycin (Drs), an antifungal peptide known to be induced by Toll signaling in response to microbial infection (Lemaitre et al., 1996). In both cases, Drs expression was significantly induced in the absence of infection, suggesting that activated SPE can trigger Toll signaling by processing endogenous pro-Spätzle in S2 cells and flies (Figures 2D and 2E). These results demonstrate that SPE can process pro-Spätzle to generate the ligand that activates Toll signaling.

Activated SPE Rescues Ventral and Lateral Development in Embryos Lacking Easter

To test further the ability of SPE to activate Spätzle in vivo, we assayed SPE activity by RNA injection into embryos lacking maternally encoded Easter. In the early embryo, Easter processes pro-Spätzle to activate Toll signaling, which is necessary for the development of ventral and lateral cell types and the establishment of dorsoventral polarity (Chasan and Anderson, 1989; DeLotto and DeLotto, 1998). Consequently, in the embryo lacking Easter, Toll signaling fails to be activated,

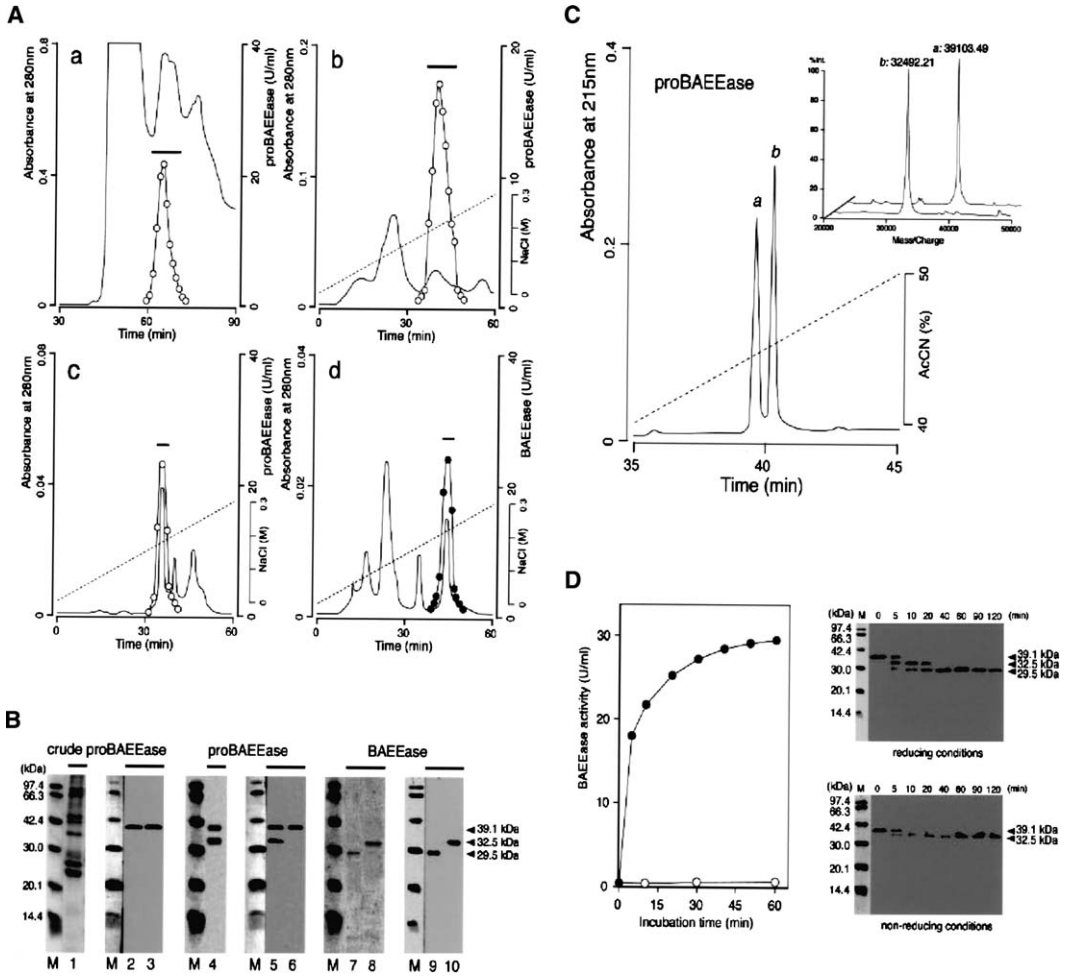

Figure 1. Purification and Characterization of *Bombyx* Pro-BAEEase and BAEEase

(A) *Bombyx* pro-BAEEase and BAEEase were purified by using four sequential chromatography steps involving (Aa) Superdex 75, (Ab and Ac) Q-Sepharose, and (Ad) heparin-Q-Sepharose, and their activities were assayed as described in Supplemental Experimental Procedures. In (Aa)–(Ac), the horizontal bar represents fractions that were pooled for the next purification step, which, in (Ac), also involved a treatment to convert pro-BAEEase into BAEEase, whereas in (Ad) it represents the pooled fractions providing the purified BAEEase preparation. Open circle, pro-BAEEase; closed circle, BAEEase; solid line, absorbance at 280 nm; broken line, NaCl concentration.

(B) SDS-PAGE and immunoblotting of pro-BAEEase and BAEEase. A crude pro-BAEEase fraction (lanes 1–3, 60 μg protein/lane), purified pro-BAEEase (lanes 4–6, 0.2 μg protein/lane), and purified BAEEase (lanes 7–10, 0.1 μg protein/lane) were subjected to SDS-PAGE under reducing conditions, except in the case of lanes 8 and 10, which were run under nonreducing conditions. Lanes 1, 4, 7, and 8 were stained with Coomassie brilliant blue R-250, whereas the remaining numbered lanes were subjected to Western blot analysis with antibody against the catalytic domain (lanes 2, 5, 9, and 10) or the CLIP domain (lanes 3 and 6) of BAEEase. The molecular sizes of marker proteins (M) are indicated at the left.

(C) Reversed-phase octyl column chromatography and MALDI-TOF mass spectroscopy of purified pro-BAEEase. Purified pro-BAEEase (10 μg, see lanes 4–6 of [B]) was applied to the column, and peak fractions a and b were subjected to MALDI-TOF mass spectroscopy. The numbers at the top of each peak in the spectra (inset) are the means of ten observed mass/H^+ values.

(D) Purified pro-BAEEase was activated with PG as described in Supplemental Experimental Procedures. BAEEase activity over time is shown at the left. Closed circles, complete reaction mixture; open circles, control reaction in which PG or purified pro-BAEEase was omitted. An almost identical activation profile was obtained when pro-BAEEase was activated by βG (data not shown). Processing of pro-BAEEase to BAEEase over time is shown at the right, as detected by Western blotting with antibody against the catalytic domain of BAEEase under reducing (upper panel) and nonreducing (lower panel) conditions. The numbers at the left refer to the sizes of marker proteins (M).

and, thus, cells at all dorsoventral positions assume the fate of dorsal cell types in the wild-type embryo (Figures 3A–3C). We found that injection of synthetic RNA encoding activated SPE very efficiently rescued ventral and lateral development in embryos lacking Easter (Figures 3D–3F). RNA encoding full-length SPE had no rescuing activity. Rescue by activated SPE-RNA showed a dose dependence, with higher levels of SPE required to induce the most ventral cell fate than to induce lateral cell types, as was previously observed in similar injection experiments with activated Easter (Chasan et al., 1992; Misra et al., 1998). In addition, ventral or lateral cell types were induced at all dorsoventral positions, indicating that activated SPE, like activated Easter, is diffusible and therefore can be active everywhere in the embryo; however, normally, Easter activity is ventrally restricted. These results provide further evidence that SPE processes pro-Spätzle identically to Easter, thereby generating the ligand that activates Toll signaling.

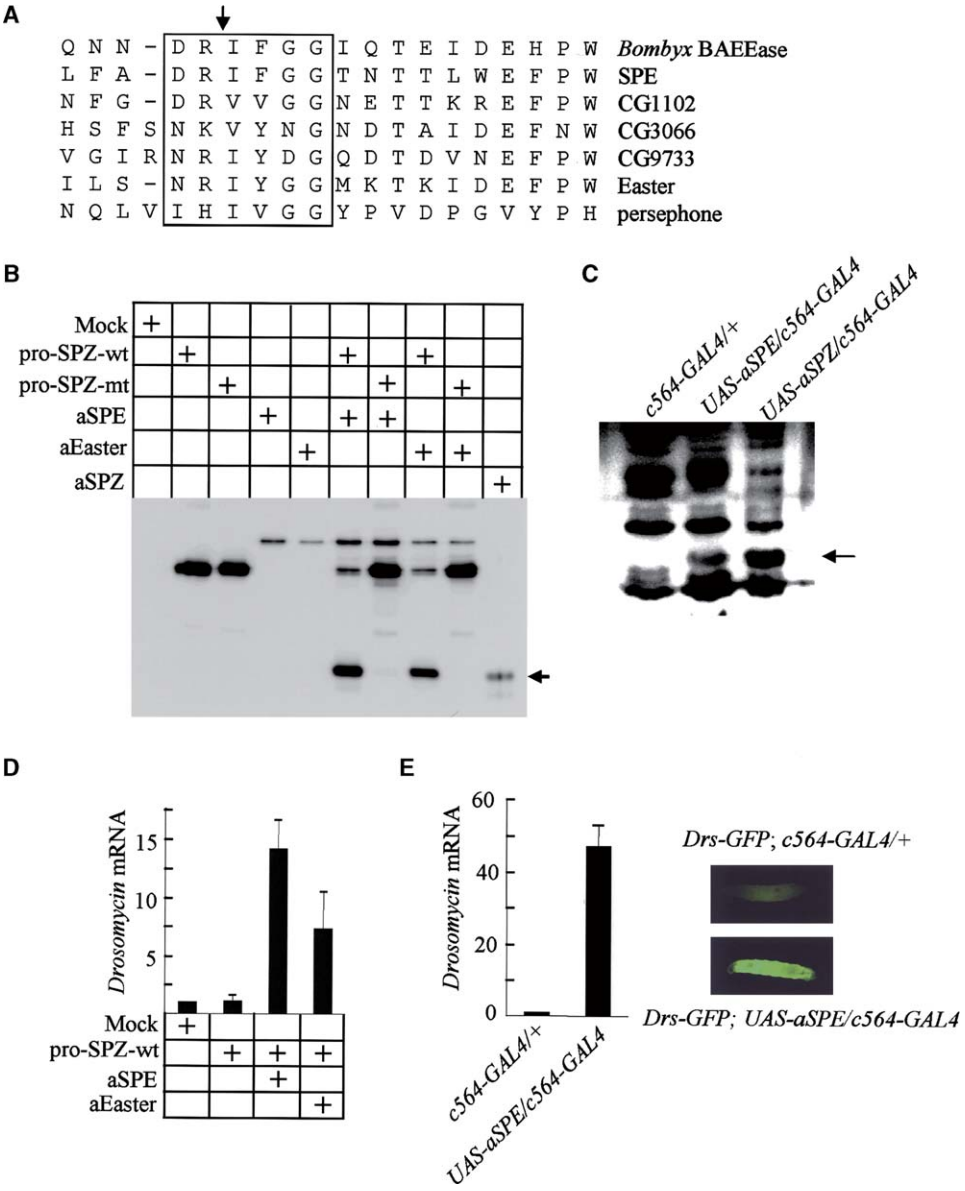

Figure 2. Identification and Characterization of the Spätzle-Processing Enzyme

(A) *Drosophila* Spätzle-processing enzyme (SPE) and *Bombyx* BAEEase have identical cleavage sites for zymogen activation. BAEEase, SPE, and other *Drosophila* CLIP proteases are compared in the cleavage region between CLIP and the catalytic domains (boxed). The cleavage site in pro-BAEEase is indicated by an arrow.

(B) SPE can process Spätzle in vitro. Spätzle (pro-SPZ-wt) was expressed in *Drosophila* S2 cells and was detected by Western blot analysis with an antibody against the C-terminal V5 epitope. Coexpression of either activated SPE (aSPE) or activated Easter (aEaster) resulted in processed Spätzle corresponding in size to the C-terminal fragment of 106 amino acids (aSPZ is indicated by an arrow), the active Toll ligand. Mutant Spätzle (pro-SPZ-mut) having an altered cleavage site was not cleaved.

(C) Activated SPE induces processing of Spätzle in flies. Transgenic expression of activated SPE (*UAS-aSPE/c564-GAL4*) results in the production of processed Spätzle that matches the size of the C-terminal fragment of Spätzle (arrow) expressed from a transgene (*UAS-aSPZ/c564-GAL4*). Western blot analysis was performed with an anti-Spätzle antibody.

(D) Expression of activated SPE (aSPE) or activated Easter (aEaster) induces Drosomycin mRNA expression in S2 cells in the presence of ectopic pro-Spätzle expression as measured by real-time PCR analysis. The amount of Drosomycin expression in the mock-transfected cells was taken arbitrarily to be 1, and the results are presented as relative expression levels. T bars represent the mean and standard deviations of at least three independent experiments.

(E) Transgenic expression of activated SPE (*UAS-aSPE/c564-GAL4*) induces expression of Drosomycin mRNA (left panel) and the Drosomycin reporter Drs-GFP in larvae (right panel). The amount of Drosomycin expression in the *c564-GAL4/+* flies was taken arbitrarily to be 1, and the results are presented as relative expression levels. T bars represent the mean and standard deviations of at least three independent experiments.

SPE Is Essential for Host Resistance to Fungi and Gram-Positive Bacteria

As our data indicated that SPE can process pro-Spätzle to generate the Toll ligand in vitro and in vivo, we investigated whether SPE plays a role in activating Toll signaling during the immune response. Because a SPE mutant was not available, we decided to assess the loss-of-function phenotype for SPE by using RNAi and therefore

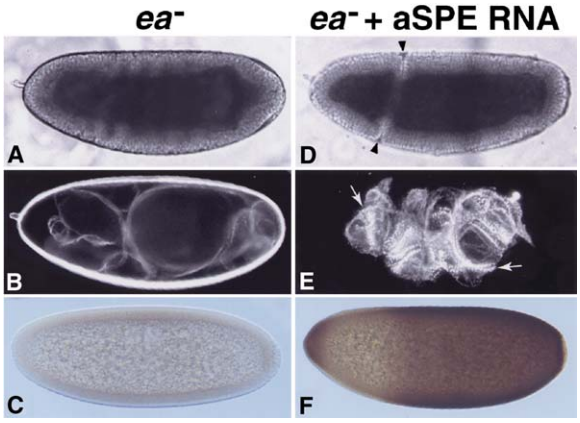

Figure 3. Injection of Activated SPE-RNA Rescues Ventral and Lateral Development in Embryos Lacking Easter

(A–C) All embryos are oriented with the anterior end toward the left and the dorsal side facing up. Embryos lacking the maternal Easter protease develop a dorsalized phenotype resulting from cells at all dorsoventral positions assuming the fate of dorsal cell types in the wild-type embryo. The dorsalized embryo exhibits at gastrulation (A) multiple transverse folds representing extensions of normally dorsal restricted folds, (B) differentiates a cuticle containing only dorsally derived structures (the bright oval structure encircling the cuticle is the vitelline envelope), and (C) fails to be specifically stained with antibodies to the Twist protein, a marker for the most ventral cell fate.

(D and E) Lateral cell types were induced around the dorsoventral circumference of an embryo injected with 1.0 μg/μl activated SPE-RNA, and these cell types were discernible at gastrulation by the extension of a (D) normally lateral head fold to both ventral and dorsal sides of embryo (arrowheads) and in the (E) cuticle by the presence of denticle bands normally made by lateral cells (arrows).

(F) The most ventral cell fate was induced in an embryo injected with 1.3 μg/μl activated SPE-RNA, as evident by the expression of Twist at all dorsoventral positions along two-thirds of its length from the site of injection at the posterior pole. Lateral cell types appeared to be induced in the anterior one-third of this embryo, as suggested by the patch of Twist staining at the anterior pole, which is typically seen in lateralized embryos (Misra et al., 1998). Rescue by activated SPE-RNA was very efficient; the lateralized gastrulation pattern was seen in 60/60 embryos injected with 0.05 μg/μl RNA, while Twist expression was detected in 28/28 embryos injected with 1.3 μg/μl RNA.

generated transgenic flies carrying a construct targeting SPE for RNAi-mediated gene knockdown under UAS control. Flies in which this construct was activated ubiquitously were completely viable, despite having SPE-RNA at only ~10% of the wild-type level (Figure S3). However, they were susceptible to fungal and G+ bacterial infections (Figure 4). Their survival rate was not affected by G− bacterial infection (Figure 4). These results demonstrate that SPE is essential for host resistance against fungal and G+ bacterial infection.

SPE Is Required for Toll-Dependent Immune Gene Expression

To confirm that the immune susceptibility of flies in which SPE-RNAi was activated is due to the impairment of Toll-dependent immune gene expression, we examined the expression of antimicrobial peptides in these flies. We found that the induction of the antifungal peptide Drs by both fungi and G+ bacteria was severely impaired (Figures 5A and 5B). However, expression of the antibacterial peptide Diptericin (Dipt), which is solely controlled by the IMD pathway, was not affected after G− bacterial infection (Figure 5B). When we examined these flies for endogenous pro-Spätzle processing in response to septic injury, pro-Spätzle processing was found to be completely blocked (Figure 5C). These results demonstrate that SPE is essential for infection-dependent pro-Spätzle processing and Toll-dependent immune gene expression.

Interestingly, in addition to being required to activate Toll, SPE appears to be induced by Toll signaling. We found that SPE gene expression in flies infected by fungi and G+ bacteria was reduced when Toll signaling, but not the IMD signaling pathway, was blocked (Figure 5D). This observation, which is consistent with previous microarray analysis (De Gregorio et al., 2002b), suggests that a positive feedback loop involving Toll signaling regulates the level of SPE.

SPE Is Downstream of the *psh* and PGRP-SA Pathways

Toll signaling in response to fungal or G+ bacterial infection requires *psh*, which encodes a CLIP serine protease (Ligoxygakis et al., 2002a), or the PRRs GNBP1 and PGRP-SA (Gobert et al., 2003; Michel et al., 2001), respectively. To determine their epistatic relationship with SPE, we overexpressed *psh* or both GNBP1 and PGRP-SA while simultaneously activating SPE-RNAi. We found that induction of Drs by overexpression of *psh* or GNBP1 and PGRP-SA was completely blocked when SPE-RNAi was activated (Figure 6A). This result indicates that *psh* and the PRRs GNBP1 and PGRP-SA require SPE to activate Toll signaling during fungal and G+ bacterial infections, respectively.

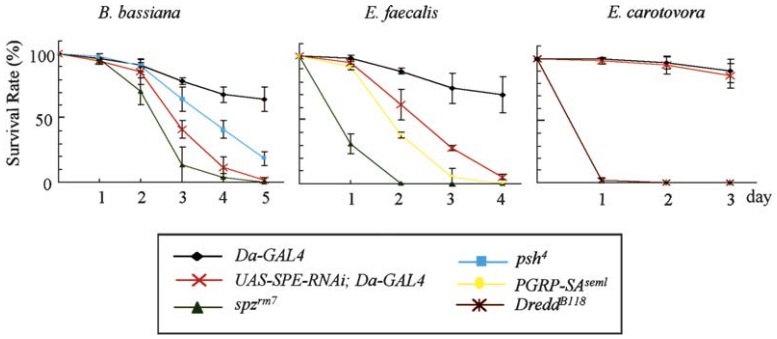

Figure 4. SPE Is Essential for Host Resistance against Fungal and G+ Bacterial Infection, but Not G− Bacterial Infection

Flies in which the SPE-RNAi construct was induced (*UAS-SPE-RNAi; Da-GAL4*) were infected with the fungus *B. bassiana*, the G+ bacterium *E. faecalis*, or the G− bacterium *E. carotovora carotovora-15*, and their survival rates (%) were measured. The *Da-GAL4*, spz^{rm7}, $PGRP\text{-}SA^{seml}$, psh^{4}, and $Dredd^{B118}$ flies were used as controls. Results are expressed as the mean and standard deviations of at least three independent experiments.

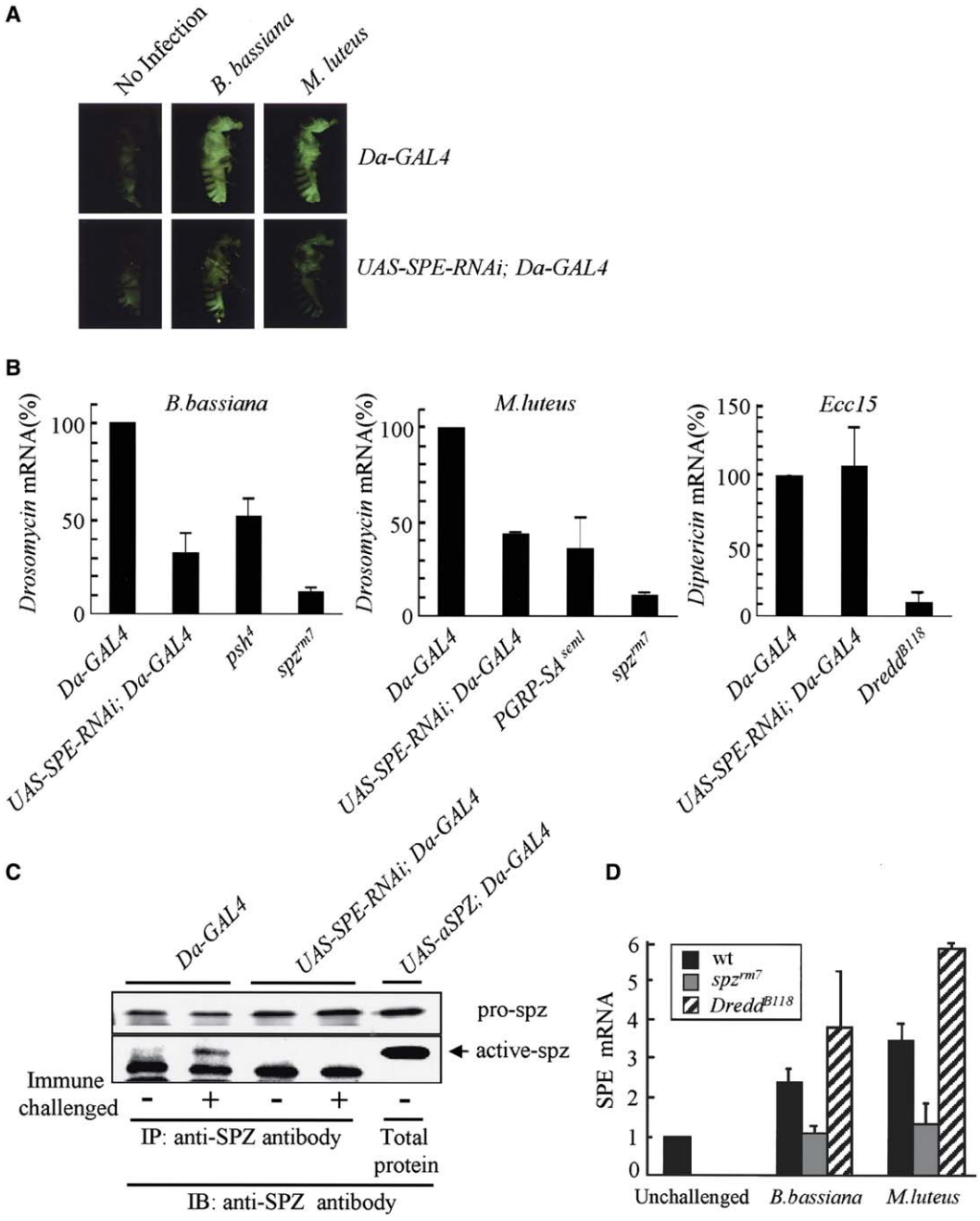

Figure 5. SPE Is Essential for Infection-Induced Spätzle Processing and Toll-Dependent Immune Gene Expression

(A) Expression of Drs-GFP after fungal (*B. bassiana*) or G+ bacterial (*M. luteus*) infection as seen in control flies (*Da-GAL4*) was blocked by SPE-RNAi (*UAS-SPE-RNAi; Da-GAL4*).

(B) Flies in which SPE-RNAi was activated (*UAS-SPE-RNAi; Da-GAL4*) had reduced levels of Drosomycin mRNA in response to infection with either *B. bassiana* or *M. luteus*, which are known to activate the Toll signaling pathway. They showed normal levels of Diptericin expression in response to infection with the G− bacteria *E. caratovora carotovora-15* (Ecc-15), which activates the IMD pathway. Mutations that block the Toll (*psh*^{4}, *spz*^{rm7}, and *PGRP-SA*^{seml}) and IMD (*Dredd*^{B118}) pathways were used as controls. Drosomycin or Diptericin expression is shown relative to the 100% level in infected control flies (*Da-GAL4*). T bars represent the standard deviation of at least three independent experiments.

(C) Spätzle processing in flies seen after 1.5 hr of infection with the mixture of *B. bassiana* and *M. luteus* (*Da-GAL4*, challenged) was blocked by SPE-RNAi (*UAS-SPE-RNAi; Da-GAL4*, challenged). Spätzle (spz) was detected by Western blot analysis of immunoprecipitates from fly extracts by using an anti-Spätzle antibody in both steps. The entire extract of flies (*UAS-aSPZ/Da-GAL4*) expressing the processed active form of Spätzle was used as a control size marker.

(D) SPE can be rapidly upregulated in response to infection via the Toll pathway, but not the IMD pathway. Wild-type (wt) flies or flies mutant for the Toll (*spz*^{rm7}) or IMD (*Dredd*^{B118}) pathway were infected with *B. bassiana* or *M. luteus* as previously described (Lemaitre et al., 1996). The amount of SPE expression was measured at 6 hr postinfection by real-time PCR, and the results are shown relative to the arbitrary level of 1 in unchallenged flies. T bars represent the standard deviation of at least three independent experiments.

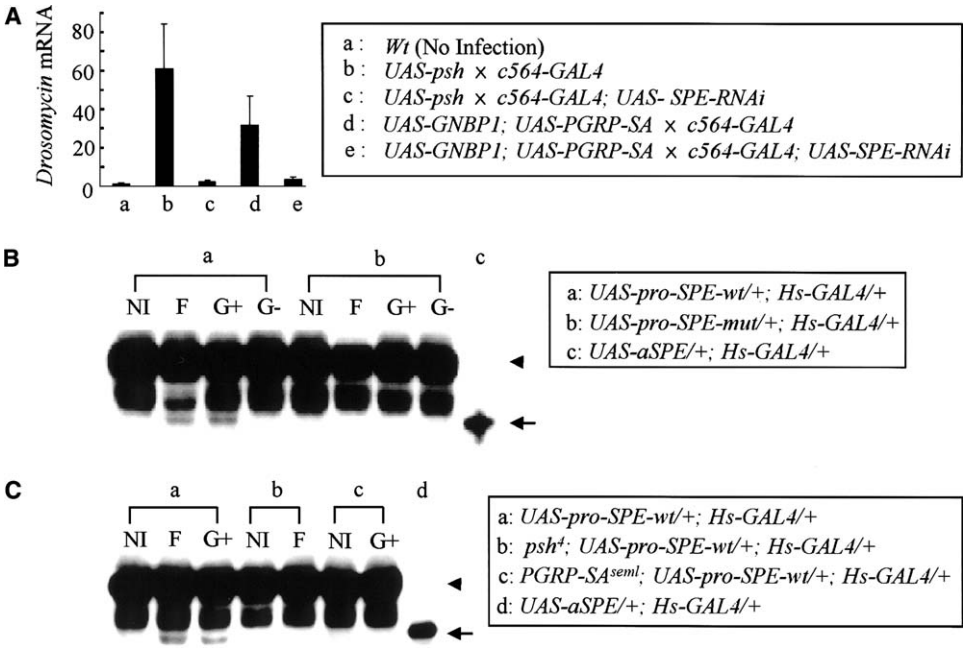

Figure 6. SPE Is Activated by Both *psh*- and PGRP-SA/GNBP1-Dependent Pathways

(A) Induction of Drosomycin in flies by overexpression of (b) psh or (d) GNBP1 and PGRP-SA is blocked by (c or e) SPE-RNAi. The amount of Drosomycin expression in the wild-type flies in the absence of infection was taken arbitrarily to be 1, and the results are presented as relative expression levels. T bars represent the mean and standard deviations of at least three independent experiments.

(B) The processed form of SPE corresponding to activated SPE (aSPE, arrow) from the zymogen form of SPE (arrowhead) is detected in transgenic flies (a: *UAS-pro-SPE-wt/+; Hs-GAL4/+*) after 1 hr of fungal (F) or G+ bacterial infection, but not after 1 hr of G− bacterial infection. It is also not detected in transgenic flies (b: *UAS-pro-SPE-mut/+; Hs-GAL4/+*) expressing SPE with a mutant zymogen activation site (DA-IFGG rather than DR-IFGG). NI represents the uninfected control. Fly extracts (40 μg total protein) were analyzed by Western blot with anti-V5 antibody to detect epitope-tagged SPE.

(C) Processing of pro-SPE (arrowhead) to the activated form (aSPE, arrow) after fungal (F) or G+ bacterial infection was blocked in flies mutant for (b) psh (*psh^{4}; UAS-SPE-wt/+; Hs-GAL4/+*) or (c) PGRP-SA (*PGRP-SA^{seml}; UAS-SPE-wt/+; Hs-GAL4/+*). The (a) *UAS-SPE-wt/+; Hs-GAL4/+* flies were used as a positive control. The entire extract of flies (*UAS-aSPE/+; Hs-GAL4/+*) expressing the processed active form of SPE was used as a control size marker. Western blot analysis was performed as described in (B) above.

We next investigated the possibility that *psh* and PGRP-SA function upstream to promote processing of SPE to an active protease. In flies expressing an epitope-tagged version of the SPE zymogen, infection by either fungi or G+ bacteria, but not by G− bacteria, resulted in the appearance of a polypeptide corresponding in predicted size (~37 kDa) to the C-terminal catalytic domain of SPE (Figure 6B). This polypeptide was not detected under the same conditions in flies expressing a mutant form of SPE in which the cleavage site for zymogen activation was mutated from DR$^{\downarrow}$IFGG to DA$^{\downarrow}$IFGG (Figure 6B). Furthermore, production of this polypeptide in response to fungal or G+ bacterial infection was completely blocked in flies mutant for *psh* (Ligoxygakis et al., 2002a) or PGRP-SA (Michel et al., 2001), respectively (Figure 6C). These results demonstrate that *psh* and PGRP-SA function to promote processing of the SPE zymogen to an active protease in response to fungal and G+ bacterial infection. Thus, SPE is the terminal protease activated by two distinct pathways, as defined by *psh* and PGRP-SA, leading to activation of Toll signaling during the immune response.

Discussion

We have identified a serine protease named SPE that processes the Spätzle protein to generate the ligand that activates Toll signaling and presented evidence that this function of SPE is essential for the immune response to fungal and G+ bacterial infection in *Drosophila*. SPE therefore functions in the immune response as the counterpart of the Easter protease that activates Spätzle and Toll signaling to establish dorsoventral polarity of the *Drosophila* embryo.

An important question is how SPE itself is activated during the immune response. SPE does not appear to be activated by Snake, the protease that activates Easter, as SPE must be preactivated to rescue ventral and lateral development in embryos lacking Easter (Figure 3 and Results); moreover, the SPE zymogen is not cleaved by Snake in vitro (Figure S4). One candidate to be a direct activator of SPE is the protease encoded by *psh*, which is required to activate Toll signaling in response to fungal infection (Ligoxygakis et al., 2002a) and, as we have shown, is also required to process SPE into an active protease (Figure 6). However, the SPE zymogen is not cleaved by the Psh protease when coexpressed in human embryonic kidney cells (data not shown), suggesting that another protease directly activates SPE. While the direct activator of SPE still needs to be identified, our work has nonetheless defined a new, to our knowledge, protease cascade involving Psh and SPE with a role in immunity. As SPE is also activated during the immune response to G+ bacterial infection, which does

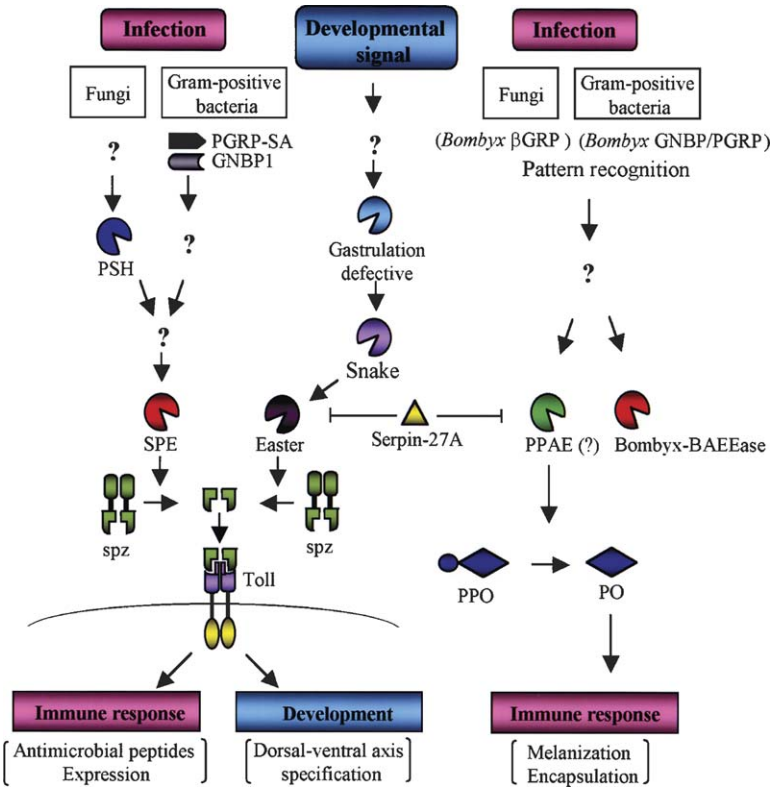

Figure 7. Protease Cascades Involved in Immune and Developmental Signaling

Depicted are several protease cascades in immunity and development, including the one involving SPE described in this study. See Discussion for details.

not involve *psh*, there may exist another protease cascade in which SPE is activated (Figures 6 and 7). The possible existence of two protease cascades that converge on SPE as a terminal protease provides for a versatile immune system in which the same signaling pathway that activates a potent immune response can be used for defense against distinct pathogens. Fungal and G+ bacterial infection also appears to trigger the activation of another cascade in which the terminal protease, pro-PO activating enzyme (PPAE), activates a key enzyme in the melanization reaction that encapsulates pathogens (Ashida and Brey, 1995, 1998; Ashida and Yamazaki, 1990; De Gregorio et al., 2002a; Ochiai and Ashida, 2004; Satoh et al., 1999) (Figure 7). The activation of two different immune responses involving SPE and PPAE by a common trigger would be an advantageous mechanism for enhancing host survival after microbial infection.

Protease cascades have diverse biological roles in vertebrates and invertebrates, ranging from digestive processes to fertilization, immunity, development, and tissue remodeling (Krem and Cera, 2002). Our work highlights the functional relationships between protease cascades involved in distinct processes such as embryonic development and innate immunity. SPE and Easter are the terminal proteases of two different protease cascades involved in development and immunity, yet both process the Spätzle protein to activate the Toll signaling pathway. The essential difference between SPE and Easter appears to be that they require distinct mechanisms for activation, which allows the Toll signaling pathway to be activated in response to different triggers and thus used in very different physiological processes. Another link between development and immunity is provided by Spn27A, a serine protease inhibitor that regulates both Easter and the pro-PO cascade (De Gregorio et al., 2002a; Hashimoto et al., 2003; Ligoxygakis et al., 2002b, 2003) (Figure 7). Interestingly, Spn27A does not appear to regulate SPE, as evidenced by the fact that Toll signaling is not constitutively activated in flies mutant for Spn27A (De Gregorio et al., 2002a). Thus, although SPE and Easter possess common substrate specificity and similar enzymatic activity, the striking regulatory differences between SPE and Easter in terms of their activation/inhibition mode may confer dual physiological functions on the Spätzle-Toll signaling cassette. These structural and functional relationships between the protease cascades involved in *Drosophila* development and immunity support the idea that an ancestral protease cascade gave rise to those with diverse functions in present day organisms (Krem and Cera, 2002).

Experimental Procedures

Constructs and Cell Transfection

The pMT/V5-His vector (Invitrogen) was used for protein expression in transfected *Drosophila* S2 cells, the pUAST vector was used for protein expression in transgenic flies, and the pSP64T vector was used for synthesis of RNA for embryo injection. Pro-SPE-wt is full-length SPE (amino acids 1–400), activated SPE consists of the Easter signal peptide fused directly to the SPE catalytic domain (amino acids 135–400), pro-SPE-mut has a mutant zymogen activation site (DA-IFGG) created by site-directed mutagenesis, activated EA was made as described previously (Chasan et al., 1992), pro-Spätzle is full-length Spätzle (amino acids 1–227), the active form of Spätzle was made by fusing signal peptide directly to the C-terminal 106 amino acids (122–227), and pro-Spätzle-mut has a mutant processing site (L121–N122) created by site-directed mutagenesis. In both pro-SPE-wt and pro-SPE-mut, the catalytic Ser at position 346 was mutated to Ala to stabilize the active form after zymogen cleavage as described (LeMosy et al., 2001).

To make the SPE-RNAi construct, a DNA fragment encoding amino acids 15–193 of SPE was amplified by PCR with a cDNA template. In order to eliminate potential problems with nonspecificity, we verified that the double-stranded RNA made from this DNA does not have a perfect match of 19–21 nucleotides to other sequences in the fly genome by BLAST analysis. The amplified DNA fragment was subcloned in an inverted orientation, with an intronic spacer in the middle (Reichhart et al., 2002), to make pUAST-SPE-RNAi.

Drosophila S2 cells (ATCC CRL-1963) were maintained exactly as described previously (Han et al., 1998). Transfection of these cells was performed according to a standard protocol with $CaPO_4$ (Di Nocera and Dawid, 1983), and protein expression was induced in cells by the addition of $CuSO_4$ to the culture medium at a final concentration of 500 μM. Cells were induced for 48 hr before harvesting (Kim et al., 2000a).

Fly Strains

UAS constructs (pUAST-SPE-RNAi, pUAST-activated-SPE, pUAST-pro-SPE-wt, and pUAST-pro-SPE-mt) were injected into w^{1118} embryos to generate transgenic animals by P element-mediated transformation (Rubin and Spradling, 1982). The fly stocks used in this study have been described previously: spz^{rm7} (Lemaitre et al., 1996); psh^{4} (Ligoxygakis et al., 2002a); *PGRP-SA*^{seml} (Michel et al., 2001); *Dredd*^{B118} (Leulier et al., 2000); ea^{4} and $ea^{5022rx1}$ (Chasan and Anderson, 1989); *Da-GAL4*, *Hs-GAL4*, and *c564-GAL4* (Ligoxygakis et al., 2002a; Takehana et al., 2004); *UAS-psh* (Ligoxygakis et al., 2002a), *UAS-activated-Easter* (Ligoxygakis et al., 2002a), *UAS-GNBP1* (Gobert et al., 2003), *UAS-PGRP-SA* (Gobert et al., 2003), and *UAS-active-Spätzle* (Pili-Floury et al., 2004); and *Drs-GFP* (Ferrandon et al., 1998).

Antibodies

Thioredoxin fusion proteins containing the BAEEase catalytic domain (S84–Q369), the BAEEase CLIP domain (Q25–R83), or V144–G243 of *Drosophila* Spätzle (Morisato and Anderson, 1995) were synthesized by using the *E. coli* expression system pET Trx fusion System 32 (Novagen) according to the manufacturer's instructions. The recombinant proteins were purified to homogeneity by chromatography on Superdex 75 pg and reversed-phase octyl (wide pore from YMC, AP-203) columns. The purified recombinant proteins (0.30–0.35 mg) in physiological saline were emulsified with Freund's incomplete adjuvant and injected subcutaneously into rabbits. Blood from the rabbits was collected after two additional booster injections administered at 14-day intervals. IgG fractions from the immunized rabbit sera were purified by Protein-A Sepharose column chromatography. Anti-V5 antibody was purchased from Invitrogen.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from cells and flies with RNAzol reagent. The cDNA was synthesized by using a first cDNA synthesis kit (Roche) according to the manufacturer's instructions. Fluorescence real-time PCR was performed with double-stranded DNA dye SYBR green (Perkin-Elmer) to quantify the amount of gene expression. Primer pairs for Drs (sense, 5′-GCAGATCAAGTACTTGTTCGC CC-3′; antisense, 5′-CTTCGCACCAGCACTTCAGACTGG-3′), Dipt (sense, 5′-GGCTTATCCGATGCCCGACG-3′; antisense, 5′-TCTGTA GGTGTAGGTGCTTCC-3′), SPE (sense, 5′-GGCTGGGGACTTACC GAGAAC-3′; antisense, 5′-ACCGCATGTATCCACGCCCAACTG-3′), and the control Rp49 (sense, 5′-AGATCGTGAAGAAGCGCAC CAAG-3′; antisense, 5′-CACCAGGAACTTCTTGAATCCGG-3′) were used to detect the target gene transcripts. All samples were analyzed in triplicate, and the levels of mRNAs detected were normalized to the control Rp49 values as previously described (Leulier et al., 2003).

Embryo Injections

RNA for injection into embryos was made by SP6 transcription by using the mMessage mMachine kit (Ambion). Embryos with no detectable Easter were obtained 0–1 hr after egg deposition from $ea^{4}/ea^{5022rx1}$ females and were injected according to a standard procedure (Anderson and Nusslein-Volhard, 1984). Injected embryos were observed during gastrulation, and their cuticles were prepared as previously described (Wieschaus and Nüsslein-Volhard, 1986). Immunostaining of injected embryos was performed by using anti-Twist antibodies provided by Siegfried Roth (Universität zu Köln) and diaminobenzidine as the histochemical reagent essentially as described earlier (Patel et al., 1994; Stein and Stevens, 1991).

Supplemental Data

Supplemental Data including Supplemental Experimental Procedures, figures, and references are available at http://www.developmentalcell.com/cgi/content/full/10/1/45/DC1/.

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Accession Numbers

The nucleotide sequence of *Bombyx* pro-BAEEase has been deposited in the DDBJ/EMBL/GenBank with accession code AB035418.