ECOLE POLYTECHNIQUE FEDERALE DE LAUSANNE

LIFE SCIENCES FACULTY

Master Project in Life Sciences and Technologies

Analysis of a specific modular serine protease acting in the *Drosophila* Toll pathway

by

Valentin Sottas

under the supervision of

PROF. Bruno Lemaitre

at the Global Health Institute, EPFL

External expert PROF. Tadeusz Kawecki

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1. Abstract

In *Drosophila*, the Toll pathway plays an important role in the immune defense against Gram-positive bacteria and fungi. Molecular determinants coming from those pathogens are directly detected by pattern recognition receptors (PPRs) circulating in the hemolymph. It has been proposed that several serine protease cascades are activated by PRRs, leading to the activation of a cleaved form of the cytokine-like molecule Spätzle, the ligand of the Toll receptor. The results obtained during my master project demonstrate an essential role for ModSP, a modular serine protease acting in the activation of the Toll pathway upon Gram-positive and fungal infections. We demonstrate that ModSP integrates signals coming from GNBP3 and PGRP-SA recognition molecules and that ModSP sends this signal to Grass, a serine protease already known to activate SPE and thereby Spätzle. Further biochemical experiments show the interaction between ModSP and GNBP1 demonstrating an apical role of this serine protease in the proteolytic cascades leading to Toll pathway activation. We also find that ModSP is expressed in specific vesicles released from the fat bodies into the hemolymph. Also, preliminary studies suggest that ModSP does not participate in the melanization reaction, a secondary but important insect immune mechanism. Biochemical analysis done by some collaborators indicates that ModSP does not cleave Grass and that ModSP exhibits a high level of auto-proteolysis when this molecule is expressed. Our data reveal a conserved role of modular serine protease in the regulation of immune proteolytic cascade in insects.

2. Introduction

Research on the *Drosophila* immune response has constantly increased since the important discovery of the role of the Toll pathway in 1996 by the group of Jules Hoffmann. *Toll* was found to have a role in the regulation of antifungal peptides such as *Drosomycin* in response to fungal infection. Today, we know that the immune response of the fruit fly *Drosophila melanogaster* relies on different mechanisms to fight microbial infection that involve epithelia, specialized haemocytes and the fat body (reviewed in Lemaitre, 2007). Firstly, the epithelia just beneath the cuticle can produce local defenses against pathogens by producing antimicrobial peptides and Reactive Oxygen Species (ROS). Secondly, the haemocytes are more specialized in phagocytosis and encapsulation of invaders entering the body cavity. Last but not least, the fat body is the main organ responsible for the humoral response by producing antimicrobial peptides that are released into the hemolymph.

The *Drosophila* immune response is adapted to the nature of the invading micro-organism (Lemaitre et al, 1997). The analysis of the humoral immune response with the help of a septic injury model led to the distinction of two different intracellular pathways, immune deficiency (IMD) and Toll pathways, that regulate the transcription of many genes by controlling the nuclear uptake of the NF-kB transcription factors (reviewed in Hoffman, 2003). The activation of the Toll pathway is done by Gram-positive bacteria as well as fungi and virulence factors (proteases), whereas the IMD pathway is triggered by Gram-negative bacteria (De Gregorio et al, 2002). The high number of pathogens indicates the existence of several specific mechanisms of microbial recognition.

Unlike mammalian Toll-Like Receptors, whose function is mainly the direct recognition of pathogens, the *Drosophila* Toll receptor is activated by a cleaved form of the secreted cytokine-like molecule *Spätzle* (*Spz*). During the immune response, *Spz* is thought to be processed by secreted serine proteases (SPs) present in the hemolymph which are activated by the recognition of gram-positive bacteria or fungi. The recent identification by an in vivo RNAi approach of five new serine proteases regulating *Spz* activation (Kambris et al, 2006) has revealed the complexity of the cascade acting upstream of Toll. In *Drosophila melanogaster*, the high number of genes encoded serine proteases (211) and their serpins inhibitor (29 compared to the 34 of the human genome) remain a great mystery because a good understanding of their functions is still missing. Nevertheless, it is now clear that serine proteases have an important role in the *Drosophila* immune response (reviewed by Reichhart, 2007).

It is well documented that the immune-induced cleavage of *Spz* is triggered by proteolytic cascades that are similar to vertebrate blood coagulation or to complement activation cascades and that these

proteolytic cascades consist of several serine proteases undergoing zymogen activation. These events are followed by the cleavage of a terminal substrate induced by the downstream protease. It can be seen as a complex mechanism consisting of the amplification of an extra-cellular signals in which lot of reactions of amplification and inhibition happen respectively between the serine proteases and their serpins (serine protease inhibitors). SPE (Spätzle Processing Enzyme) has been identified as the terminal SP that maturates *Spätzle* into its active substrate (Jang et al, 2006).

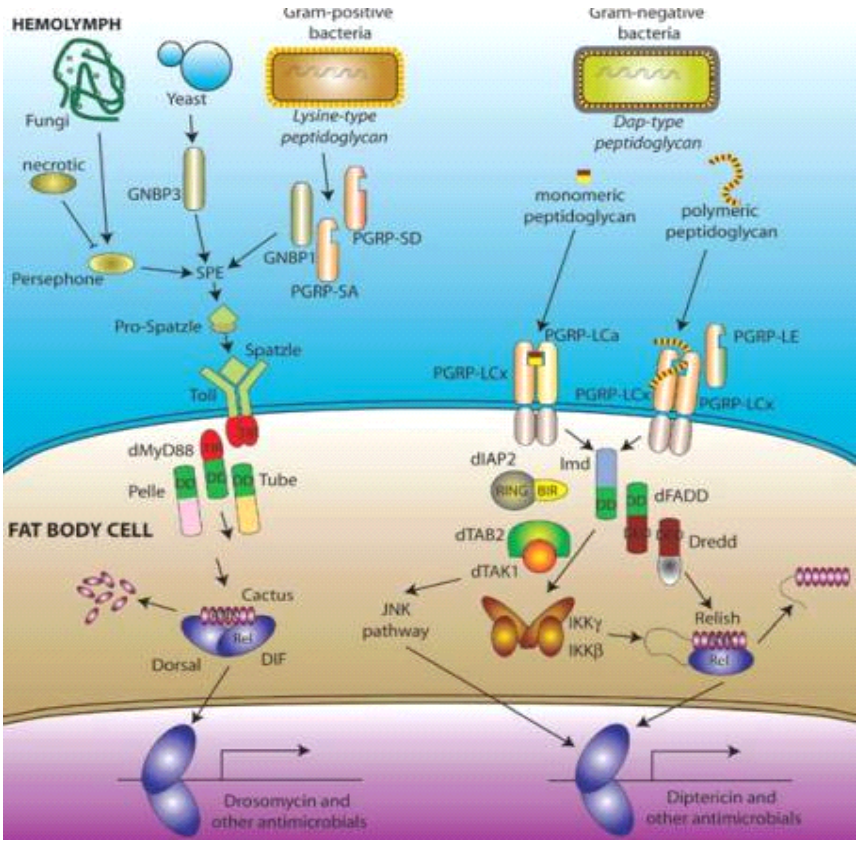

Figure 1: Two pathways, Toll and IMD, are responsible for AMP production. Recognition of pathogens in the Toll pathway is specifically mediated by Psh, GNBP3 and PGRP-SA branches. This induces Spz activation that will in turn provokes the Toll Receptor activation. In the fat body, a complex containing Cactus, Dorsal and DIF is activated upon Toll Receptor activation and thereby induce *Drosomycin* induction. On the other hand, pathogens are directly sensed by PGRP-LC or PGRP-LE in the IMD pathway. The final read-out of the IMD pathway is *Diptericin*.

Three separate pathways leading to the cleavage of *Spz* have been identified:

1. Recognition of Gram-positive bacteria

   Pattern-recognition receptors (PRRs) are thought to be present in the hemolymph where they sense microbial-derived molecules. Members of the peptidoglycan recognition protein

(PGRP) family have been shown to be required for the recognition of Gram-positive bacteria (Michel et al, 2001). PGRP-SA, a secreted PGRP is known to activate the Toll pathway upon detection of lysine-type peptidoglycan, a major component of Gram-positive bacteria. The complex formed with Gram-negative binding protein 1 (GNBP1) and PGRP-SA is sufficient to activate the Toll pathway upon Gram-positive infection (Gobert et al, 2003). The PGRP-SA/GNBP1/Peptidoglycan complex activates a proteolytic cascade that involves Grass (El Chamy et al, 2008) that functions upstream of SPE and *Spz* (figure 1).

2. Recognition of fungi

GNBP3, another member of the Gram-negative binding protein family, is required for the detection of fungal cell components (Gottar et al, 2006) such as ß-glucan (Lee et al, 2006). A null mutation of this gene induces a high rate of death when injection of fungi such as *Candida albicans* was performed in mutant flies. This pathway also converges to Grass (figure 1).

3. Recognition of proteases and virulence factors

The Persephone (Psh) pathway is the third branch responsible for pathogen recognition in the Toll pathway. It has been proposed that proteases such as PR1 produced by entomopathogenic fungi cleave the Psh serine protease. The latter would then activate SPE, the activator of *Spätzle* thereby activating the Toll pathway (Gottar et al, 2006) (Figure 1).

The serine proteases cascades acting on the melanization pathway have already been linked to the serine proteases cascade acting on the Toll pathway (Gottar et al, 2007). The principal goal of the melanization reaction is the rapid synthesis of melanin at the site of injury to fight against a microbial pathogen or to facilitate wound healing. Two SPs, MP1 and MP2, and two serpins, Spn 27A and 28D, have been identified for their role in the *Drosophila* melanization process, however their interconnection and position in the pathway are not well defined yet. It is known that these proteolytic cascades take place in the hemolymph and lead to the cleavage of the phenoloxidase (PO) a key enzyme in melanin biosynthesis.

Although the function of the Toll pathway and the recognition molecules serving for pathogens recognition have been clearly defined, a lot of problems remain unsolved. What are the serine protease cascades acting on the Toll pathway? For instance, the apical SP linking PRR recognition to the cleavage of *Spz* is not known resulting in an important gap in our knowledge of the Toll pathway activation. Are the fungi and the Psh pathway interconnected by a specific serine protease?

Is serine protease cascade regulating Toll pathway activation also responsible for the melanization reaction, thereby connecting the two pathways?

In order to shed some light on these questions, we have studied a specific serine protease. An *in vitro* study in *Tenebrio molitor*, has suggested an important role for a modular serine protease (Tm-MSP) in the activation of *Toll* by PRRs (Kim et al, 2008). In *Tenebrio molitor*, GNBP1 and PGRP-SA complexes induce the activation of the TM-Modular serine protease that will in turn activate another serine protease (Tm-SAE) resulting in *Spz* and melanization activation (Figure 2). This result motivated us to study the *Drosophila* homolog of the Tm-MSP that is encoded by the *CG31217* gene. In this study, we have demonstrated the essential role of CG31217 in the activation of the Toll pathway by PRRs.

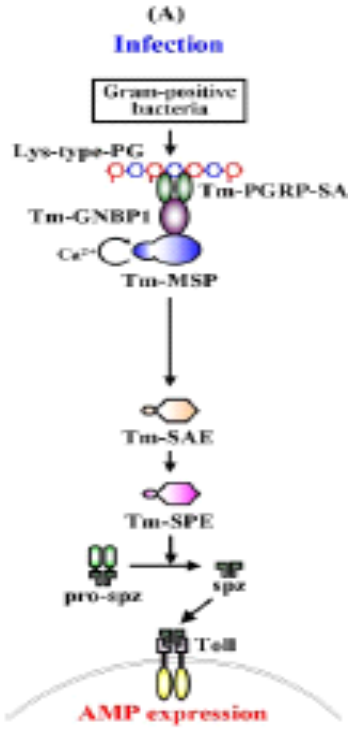

Figure 2: *Spz* activation in *T. Molitor*. Pathogens are directly sensed by *Tm*-PGRP-SA. The complex formed between *Tm*-GNBP1 and *Tm*-PGRP-SA activates *Tm*-ModSP. This induces a proteolytic cascade leading to the activation of *Tm*-SAE. *Tm*-SAE is the serine protease responsible for Spz maturation in this insect.

3. Materials and methods

3.1 Genetic tools

Some powerful genetic tools available in *Drosophila melanogaster* has been used for characterizing and ordering the components in the *Drosophila* Toll pathway.

Firstly, the balancer chromosomes present the advantage to maintain a mutation in a fly stock along generation. They prevent genetic recombination during meiosis between homologous chromosomes. Many balancer chromosomes, for the X, second or third chromosomes exist for genetic studies of *Drosophila melanogaster*. Balancer chromosomes contain both a lethal recessive mutation and a dominant marker (such as genes responsible for the wings or the hairs). Therefore, it is possible using balancers to determine visually which flies in the stock carry a homozygous or a heterozygous mutation.

Secondly, *UAS-Gal4* system in the *Drosophila melanogaster* model, allows us to study the over-expression of some genes and their effect on the immune response of the flies. That system uses two components: a yeast transcription activator protein *Gal4* and a region *UAS* (Upstream Activation Sequence) responsible for the binding of the *Gal4* to enhance gene transcription. In details, the *Gal4* is used with a driver region gene where an over-expression of our proteins is desired. Haemocytes, fat body and the whole organism are the most common drivers for the *Gal4* system in *Drosophila* genetics. On the other hand, the *UAS* portion controls the target gene, that is the gene to over-express in the given driver region.

Finally, crosses between flies are performed to create double mutants lines as well as lines expressing specific proteins in a certain driver using the *UAS-Gal4* system. Recombinant double mutant flies are also obtained with genes located on the same chromosomes. The number of crosses depends on the frequency of recombination and thereby on the distance between the two loci. Another way to use crosses in fly genetics is the use of virgin flies containing four distinct balancer chromosomes to map mutations. It helps us to determine on which chromosomes the mutation is carried.

3.2 Creation of pathogens (bacteria, fungi)

Bacteria and yeasts (*Enteroccocus Faecalis, Candida Albicans, Microccocus Luteus, E. Carotovora, Staphylococcus Aureus, Lysteria Monocytogenes*) were precolonized in Petri dishes. A centrifugation pellet was carried out after leading over night the bacteria/yeasts at different

temperatures according to the type of bacteria/yeasts (37°C for *E. faecalis*, 30°C for the others). All pathogens were enhanced in LB or YPGA before centrifugation.

Beauvaria Bassiana spores were colonized in separated Petri dishes. Sleeping flies were shaken on the *B. Beauvaria* spores in order to fully cover the cuticles of the flies. *Aspergilus Fumigatus* pellet was already done and could be obtained upon request.

3.3 Bacterial and fungal infections for survival experiments

Bacterial and fungi infections were performed by pricking adults in the thorax with a thin needle previously dipped into a concentrated pellet of a microbial culture (OD = 200 for all the pathogens and OD = 20 especially for *E. Faecalis*). A total of approximately 80 flies were infected for each genotype. At different time points, flies were counted to monitor survivals. Injection of Peptidoglycans and proteases (concentration 1:1500) were performed with a Nanoinject apparatus (World Precision Instruments Corporation).

3.4 RNA extraction, RT-PCR, qPCR

For RNA extraction, flies were crushed in tubes containing Trizol and crushing balls. Chloroform was then added to bind to the RNA. After centrifugation, supernatant containing only RNA was collected. After multiple washings with ethanol and isopropanol, the optical density of RNA present in our samples was measured on a nanoject device (Roche Diagnostics) in order to obtain the same concentration for all samples (0.5 ug/ul).

Reverse Transcriptase-PCR (RT-PCR) was done to produce double stranded DNA from our single stranded RNA samples (Eppendorf AG device). It consists of a two-steps process: the RT reaction and PCR amplification. SuperScript III reverse transcriptase (Invitrogen) was used for the RT reaction process. Random primers (Eurogentec) were used according to manufacturers' instructions.

Quantitative PCR was performed using LightCycler system (Roche Diagnostics). Different primers from Eurogentec (ModSP, Drs, Grass, Rp49) were used in a concentration and a temperature given by the manufacturers. Ratio of Rp49_Ct and Target_Ct according also to their efficacies were used to determine mRNA relative levels.

3.5 Microscopy devices

For image viewing, the flies were dissected in PBS and directly mounted in an antifading solution (Citifluor). The samples were observed with an Axioplot (Zeiss) and photos taken using the deconvolution properties of an apotome grid (Zeiss). The staining of lipid vesicles were done with a hemolymph extraction from 20 flies expressing UAS-ModSP-GFP under the control of the *c564-Gal4* driver. The hemolymph was fixed on slides containing PBS and rinsed with a solution of oil red O within 5 minutes.

3.6 Western Blotting

After standard protein extraction from 30 flies in Lysis Buffer containing some protease inhibitors (Complete lysis kit), the samples were placed in a Laemly Buffer containing 20% glycerol for a perfect migration. In case of co-immunoprecipitation, the samples were stored in balls containing HA-beads over night. After some rinsing, the samples were heated at 95°C during 5 minutes to denaturate properly the proteins. Migration of proteins (HA-beads with linked proteins in case of co-immunoprecipitation) was done with a constant voltage of 120 V in Transfert Buffer during approximatively 2 hours. The migration device was a power supply named EPS 2A/200 (Amersham Biosciences). Tris-glycine gels with an agarose concentration of 4 to 20% (Invitrogen) were chosen to migrate the samples. After migration, transfert on a nitrocellulose membrane was done during 15 minutes with the help of a regular iBlot Gel transfer stacks (Invitrogen). The membrane was deposited in milk during 15 minutes before the addition of the first antibody and an over night stay. After the addition of the secondary antibody and some washes with PBT 0.5 %, the membrane was processed during 5 minutes with a SuperSignal West Dura Kit (Thermo scientific). Proteins were then revealed in a black chamber on a specific film (Invitrogen).

4. Results

4.1 Generation of a CG31217 mutation by homologous recombination

The CG31217 protein named ModSP for Modular Serine Protease contains four Low Density Lipoprotein-receptor class A (LDLa) domains and one Complement Control Protein (CCP) module at its N-terminus (Figure 3). In contrast to many SP involved in the immune response, the *modSP* gene is not inducible transcriptionnaly upon infection and encodes an SP devoided of any CLIP domain.

Figure 3: ModSP contains four Low Density Lipoprotein-receptor class A (LDLa) domains and one Complement Control Protein (CCP) module at its N-terminus. The LDL receptor class A domain contains a cluster of negatively charged amino acids that form the LDL Receptor binding sites for LDL and calcium. The CCP module is found in a wide variety of complement like molecules and adhesion proteins.

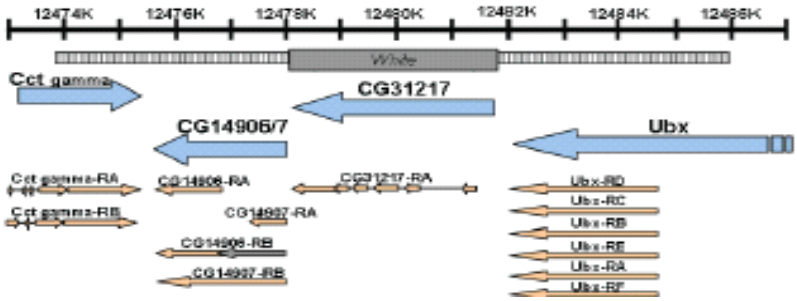

Figure 4: Homologous recombination between *modSP* locus (*CG31217*) and the *white* gene. The gene map was adapted from FlyBase. We controlled that flanking genes were not affected by the recombination

To determine ModSP function, a mutation by homologous recombination was generated, consisting of a replacement of the *modSP* locus by the *white* gene (Figure 4). Two independent mutations were obtained named $modSP^1$ and $modSP^2$. To confirm the absence of the CG31217 transcription, a RT-qPCR was performed on homozygous mutant with primers of the CG31217 locus. Results are shown in Figure 5. The expression of *modSP* in the two *modSP* mutants was 0% in comparison to that of wild-type flies which expressed the normal quantity of this gene (set to 100%). In this figure, the expression of *modSP* in two different RNAi lines targeting the *modSP* gene is also shown. The reduction of *modSP* expression in RNAi flies was significantly lower compared to that of *modSP* mutant. Moreover, we also checked that the recombination event in *modSP* mutants did not affect

the expression of flanking genes by the same methods (RT-qPCR). The two mutants were completely viable with no morphological defects. In the presentation of the results below, we will only describe the results obtained with the *modSP*^{1} flies, because we observed that the two mutant lines responded in the same way.

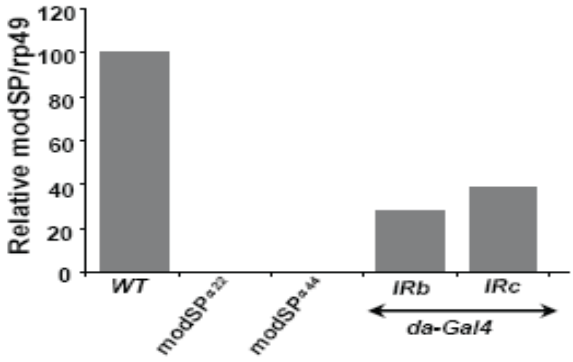

Figure 5: Relative *modSP* expression in wild-type, *modSP*^{1}, *modSP*^{2} and two RNA fly lines. The *modSP* expression is shown as a ratio over the expression of the housekeeping gene *rp49*. The two mutants did not express *modSP* (0%). The two RNAi lines under the control of a *daughterless* driver expressed 30-40 % of *modSP* compared to wild-type flies.

4.2 Role of ModSP in the response to Gram-positive bacteria infection

Injection of Gram-positive bacteria with a needle under a septic injury (SI) model, was performed with a panel of species (*Enteroccocus Faecalis, Staphylococcus Aureus, Lysteria Monocytogenes*).

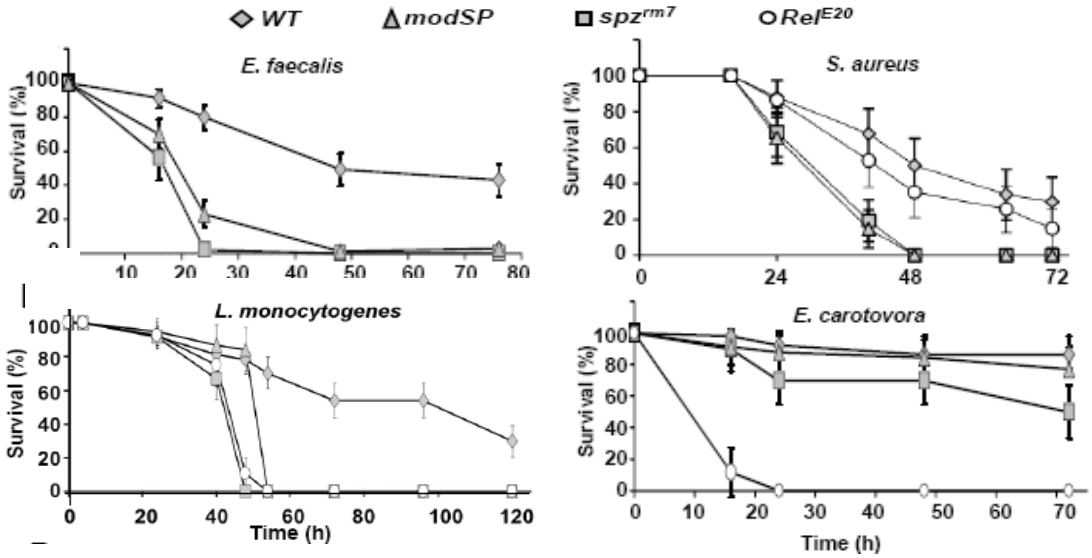

Figure 6: Survival experiments done with Gram-positive bacteria (*E. Faecalis, S. aureus, L. Monocytogenes*) and Gram-negative bacteria (*E. carotovora*). Flies were counted at different times of infection. Infection by septic injury with a needle dipped in a concentrated pellet of each microbe (OD=200, OD=10 for *E. faecalis*) was performed. *modSP*^{1} exhibited almost the same phenotype as *spz*^{rm7}. *modSP*^{1} did not exhibit a general immune deficiency as shown on *E. carotovora* survival curve.

Figure 6 summarizes all survival experiments done with the *modSP* mutant flies (Gram-positive and Gram-negative bacteria). It was evident from the survival curves that *modSP* deficient flies died rapidly after infection with Gram-positive bacteria. Compared to the spz^{rm7} mutant, the phenotype of $modSP^{1}$ was similar albeit weaker. More interestingly, *modSP* mutant flies did not exhibit a general immune deficiency as observed on the *Erwinia carotovora* survival curve. This Gram-negative bacterium was known to activate strongly the IMD pathway but not the Toll pathway. As a consequence, Relish mutant flies (rel^{E20}) rapidly die after infection with this bacterium. Also, since $modSP^{1}$ did not show any susceptibility to this kind of infection, we can deduce that ModSP did not participate in the immune response against Gram-negative bacteria.

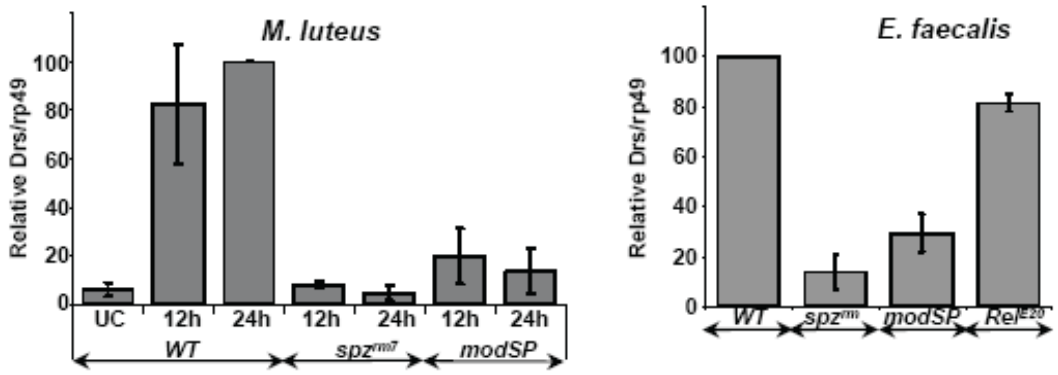

Figure 7: *Drs* gene expression was monitored by RT-qPCR with total RNA extracted from wild-type, spz^{rm7}, rel^{E20} and $modSP^{1}$ adult flies collected at different time points after septic injury with *M. luteus* or *E. faecalis*. *modSP*^{1} blocked *Drs* expression with a ratio of *Drs/rp49* of 20% after *M. luteus* infection and of 30% after *E. faecalis* infection (compared to WT flies whose expression level was set to 100%). rel^{E20} flies served as positive control and did not completely block *Drs* expression.

To test whether this immune susceptibility was due to an improper function of the Toll pathway, we monitored by RT-qPCR the expression of the antifungal peptide gene *Drosomycin* (*Drs*), a target of the Toll pathway. As shown in Figure 7, $modSP^{1}$ exhibited a weak level of *Drs* expression compared to wild-type flies (Oregon) after infection with *Micrococcus Luteus* or *Enteroccocus Faecalis*. It is also visible that spz^{rm7} showed a great susceptibility to this kind of infection (Ratio of *Drs/rp49* was two times less than that observed with $modSP^{1}$). Another experiment was done with peptidoglycan deriving from Gram-positive bacteria. Those lysine-type peptidoglycans are strong inducers of the Toll pathway when injected in the flies through a Nanoinject device. Figure 8 shows a strong reduction of the *Drs* expression in $modSP^{1}$ flies as observed for the spz^{rm7} flies. We conclude that ModSP functions at a place downstream of PRRs induced by peptidoglycan from the Gram-positive bacteria in the activation of the Toll pathway.

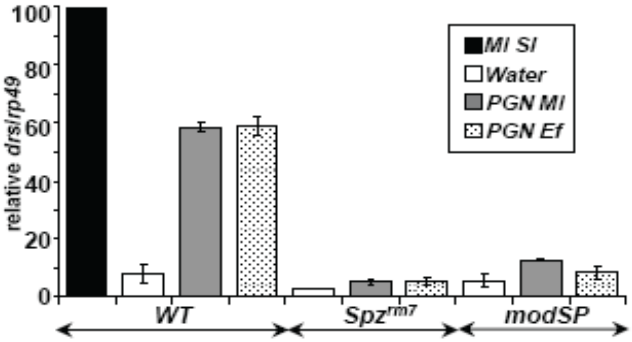

Figure 8: Relative *Drs* expression in wild-type, *modSP*[1] and *spz*^{rm7} flies after injection of peptidoglycan from two different Gram-positive bacteria. Injection of water served as negative controls. *modSP*[1] (approximately 10% of *Drs* induction) exhibited almost the same phenotype as *spz*^{rm7}, whose level of *Drs* expression reached 7% of the level of wild-type infected flies.

The last Gram-positive bacterium that was tested was *L. Monocytogenes*. This bacterium, unlike all other previously described Gram-positive, contains Diaminopimelic (DAP) –type peptidoglycan. *L. Monocytogenes* activates both IMD and Toll immune pathways of the flies . As shown in Figure 9, *modSP*^{1} blocked the expression of *Drs* but did not block the expression of *Diptericin* (*Dpt*), a readout of the IMD pathway. *rel*^{E20} flies were used as negative controls and did not show any *Dpt* expression upon *L. Monocytogenes* infection. Moreover, the expression of *Dpt* was not blocked in *modSP* mutants upon infection with *E. Carotovora,* confirming the absence of role of ModSP in the IMD pathway (Figure 10).

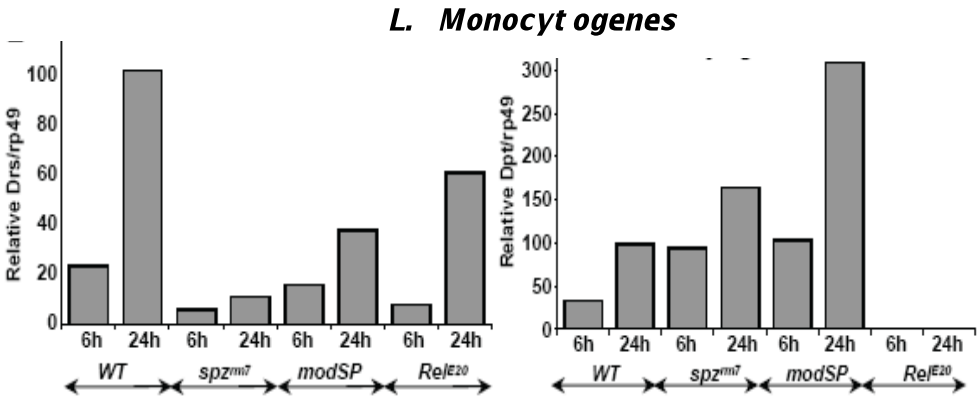

Figure 9: Relative *Drs* and *Dpt* expression in wild-type, *spz*^{rm7}, *modSP*[1] and *rel*^{E20} flies upon *L monocytogenes* infection after 6h and 24h. *modSP*[1] blocked *Drs* expression to a level of 40% but did not block *Dpt* expression. We conclude that ModSP regulates the Toll pathway but not the IMD pathway.

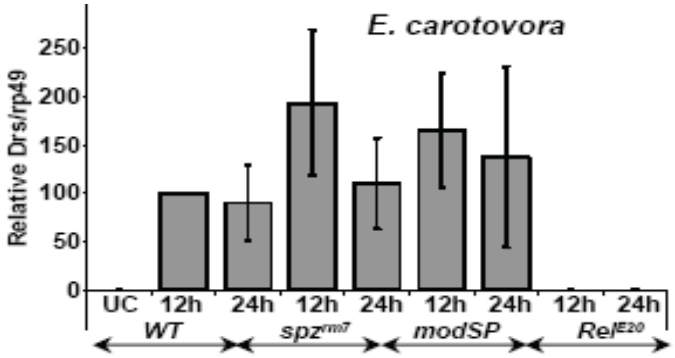

Figure 10: Relative *Drs* expression in wild-type, spz^{rm7}, $modSP^{1}$ and rel^{E20} upon *E. carotovora* infection. $modSP^{1}$ failed the capacity to block the IMD pathway as could do rel^{E20}.

In conclusion, this study demonstrates an essential role of ModSP in the activation of the Toll pathway against Gram-positive bacteria challenge.

4.3 ModSP overexpression is sufficient to activate the Toll pathway

Other experiments were carried out to confirm that *modSP* mutation was indeed responsible for the observed phenotype. First of all, we did the same experiments (survival assay and *Drs* expression) with flies carrying $modSP^{1}$ over a deficiency (*Df(3R)P10, Df(3R)Spf*) removing the *modSP* locus. We observed the same immune deficient phenotype indicating that no added mutation was responsible for the phenotype that we observed before. This also indicated that $modSP^{1}$ behaves as

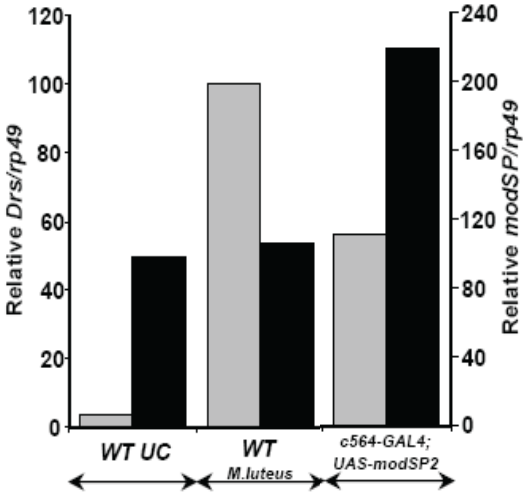

Figure 11: Relative *Drs* expression and *modSP* expression in wild-type flies and flies over-expressing *modSP* under the control of a *c564-Gal4* driver (region = haemocytes+ fat body). Flies containing over-expressing *modSP* were able to induce *Drs* expression to a level of 60% compared to wild-type infected flies (ratio set at 100%). Ratio of *modSP/rp49* was increased threetimes in *modSP* over-expressing flies.

an amorphic mutation.

Then, we generated some fly stocks over-expressing ModSP under the control of an *UAS* element. We over-expressed ModSP in the whole organism using a *daughterless* driver *(da-gal4),* but we saw straight away that it led to larval lethality (Only balanced flies were observed in the vials). Another driver *c564-Gal4* expressing *Gal4* in the fat bodies and haemocytes (genotype: *C564-gal4, UAS-modSP*) was therefore used. Figure 11 shows that *Drs* expression was expressed at a level of 60% compared to that of wild-type flies collected 16 hours after infection with *M. Luteus.* Moreover, expression of the *modSP* gene was clearly seen in over-expressing flies. The ratio of *modSP/rp49* was two to three times greater than that of wild-type unchallenged flies (as reminder: ModSP is not inducible upon infection).

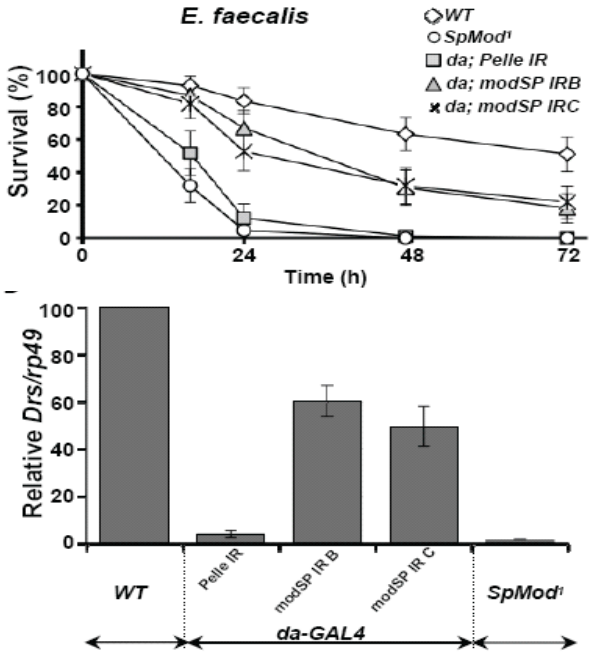

Figure 12: Survival experiments and relative *Drs* expression level of two RNAi lines under the control of a *daughterless* driver. RNAi flies were susceptible to *E. faecalis* infection and had also the capacity to block Drs expression to a level of 50%, but they exhibited a weaker phenotype than *modSP*[1] flies in the two experiments An *UAS-pelle-IR* construct targeting the intracellular component of the Toll pathway Pelle was used as a positive control.

Last of all, we used two *modSP*-RNAi fly lines from the Vienna Drosophila RNAi Center to knockdown the expression of the gene *in vivo*. We built some fly lines expressing *modSP-IR* in the whole organism with the *daughterless* driver and saw, as mentioned earlier, a depletion of the *modSP* level of expression (Figure 5). *modSP* gene expression was 30 to 40 % compared to wild-type flies. Moreover, *modSP*-RNAi fly were more susceptible to *E. faecalis* infection even if

modSP^{1} flies were showing a greater susceptibility (Figure 12). *Pelle*-RNAi flies were also used as a control of the RNAi and showed a high susceptibility to *E. faecalis* infection. A last experiment of RT-qPCR was performed with the RNAi lines. The two *modSP-IR* exhibited a reduced level of *Drs* after *M. Luteus* infection as expected (Figure 12). The results were not as clear as that observed with the *modSP* mutation or the *Pelle-IR*, whose two expression levels came almost to 0%.

All these results demonstrate that ModSP is essential for Toll activation by Gram-positive bacteria and that over-expression of full-length ModSP is sufficient to activate the Toll pathway.

4.4 Epistasis analysis of ModSP

Then, we performed a series of epistasis experiments to analyse where ModSP acts exactly in the cascade of the Toll pathway activation. We already knew that over-expression of both GNBP1 and PGRP-SA activates the Toll pathway, thereby inducing a constitutive amount of *Drs* even without the presence of an immune challenge (Gobert et al, 2003). In figure 13, we saw that *Drs* expression after an over-expression of GNBP1 / PGPSA arrived at a level of 17% compared to WT flies challenged by *M. Luteus*. Moreover, the *modSP*^{1} mutation fully suppressed *Drs* expression in GNBP1/PGRP-SA over-expressing flies. The level of expression was similar to that of unchallenged flies. In the same figure, we can see the results of an experiment showing that *Drs* expression upon over-expression of ModSP was not affected by the presence of a *PGRP-SA* mutation, (*PGRP-SA*^{seml}). Together these experiments indicate that ModSP functions downstream of PGRP-SA.

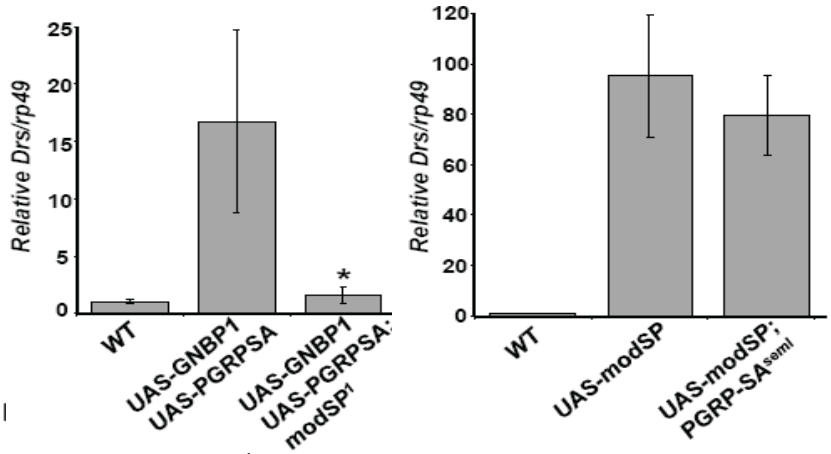

Figure 13: Relative *Drs* expression in two different epistasis experiments *modSP*^{1} blocked *Drs* expression induced under an over-expression of PGRP-SA and GNBP1. On the other hand, over-expression of *modSP* did not block *Drs* expression in a *PGRP-SA* mutant context. Percentage has been calculated in function of wild-type infected flies whose *Drs* expression level was set at 100%.

We then performed a series of experiments shown in figure 14, in which genes encoding different

components of the Toll pathway were over-expressed in a normal context and a *modSP* mutant context. The over-expressed gene were a gain-of-function allele of Toll (*UAS-Toll10b),* a matured and constitutively active form of Spz (*UAS-spz**) and an active form of SPE (*UAS-SPE**). As expected, we could see that *modSP*^{1} did not block under those three different conditions the expression of *Drs*, in agreement with an upper role of ModSP in the cascade.

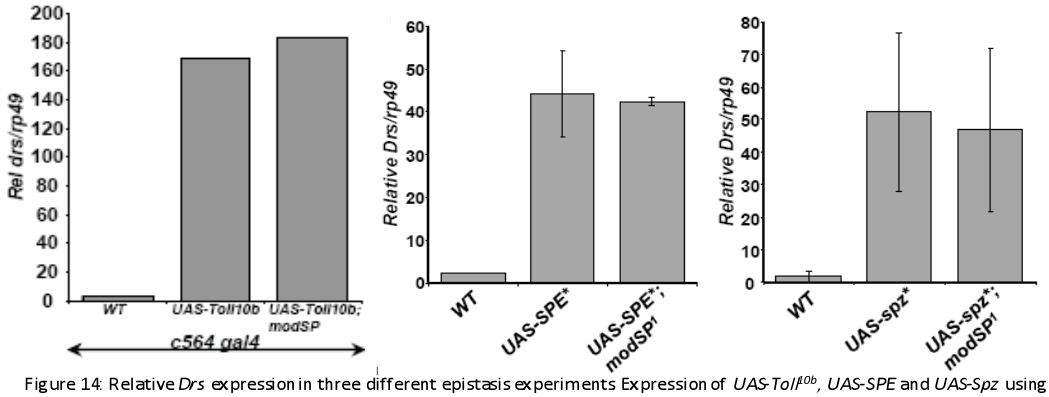

Figure 14: Relative *Drs* expression in three different epistasis experiments Expression of *UAS-Toll*^{10b}, *UAS-SPE* and *UAS-Spz* using the C564-gal4 driver were able to express a high level of *Drs* in wild-type or in a *modSP*^{1} mutant context *Drs* expression was calculated as a ratio of the level obtained with wild-type flies infected *with M. luteus* whose expression level was set to 100%.

Figure 15 shows the results obtained from another important epistasis experiment in which *Drs* expression was monitored in flies for which ModSP was over-expressed in wild-type or *grass* mutation context (*Grass*^{Herrade}). Interestingly, *Drs* expression under ModSP over-expression alone reaches a level of 70% of wild-type flies that have been challenged with *M. Luteus*. Figure 15 shows that the *Grass*^{Herrade} mutation strongly reduced the level of *Drs* expression induced by ModSP. This important result shows that ModSP is active upstream of Grass.

Altogether, this epistatic analysis indicates that ModSP functions downstream of PGRP-SA and GNBP1 and upstream of Grass in the branch that links Gram-positive bacterial recognition to the

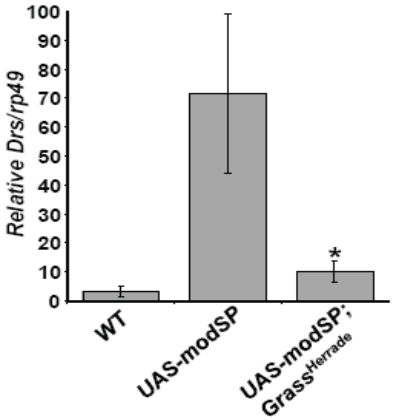

Figure 15: Relative *Drs* expression in a single epistasis experiment Under a Grass^{Herrade} mutant context, ModSP was not able to induce *Drs* expression explaining its upper role in the Toll pathway. *Drs* expression was calculated in function of wild-type infected flies whose expression level was set to 100%.

Toll pathway activation.

4.5 Role of ModSP in the response to fungal infection

As already said, yeasts are recognized by GNBP3 via the recognition of β-glucans. *GNBP3^{hades}*, a mutation in the *GNBP3* gene induces a phenotype of reduced survival when challenged by *C. albicans* (Gottar et al, 2007). It was also shown that in absence of an immune challenge, over-expression of GNBP3 activates by itself the Toll pathway. We next investigated whether ModSP also operates downstream of GNBP3 in the recognition of yeasts.

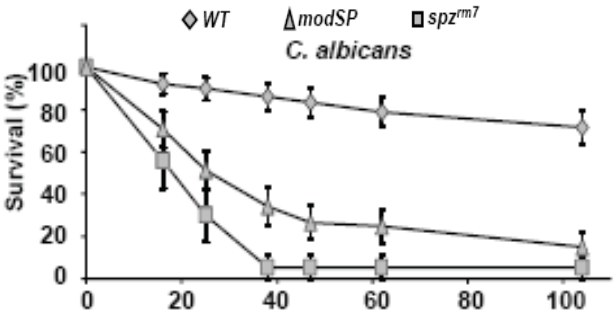

Figure 16: Survival experiments upon *C. albicans* infection. *modSP^1* was susceptible compared to wild-type curve, although the phenotype was weaker than that observed with *spz^{rm7}*.

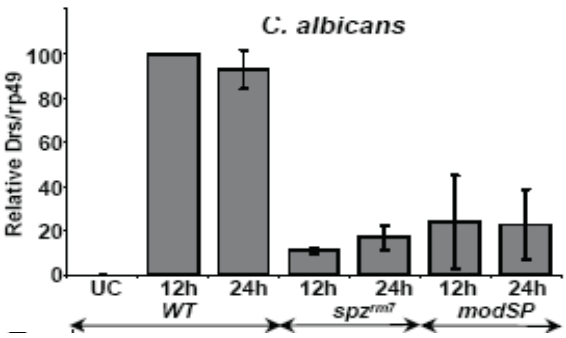

Figure 17: *Drs* expression in wild-type, *spz^{rm7}* and *modSP^1* flies upon *C. albicans* infection collected at 12h and 24h post-infection. *modSP^1* blocked *Drs* expression to a ratio of 20% compared to *Drs* expression in wild-type flies set at 100%.

To test this hypothesis, we first performed a survival experiment with *modSP* mutants to yeast infection. Figure 16 shows that *modSP* deficient flies die rapidly after *C. albicans* infection. The *spz^{rm7}* phenotype appeared to be more significant, but a great amount of *modSP* mutant flies already died after 40 hours with the same kinetics compared to *GNBP3^{hades}* flies. Furthermore, RT-qPCR experiments (Figure 17) shows that *modSP* mutation reduces the amount of induced *Drs* to a threshold of 20% compared to wild-type flies after 12 hours. This phenotype was again a little weaker than the *spz^{rm7}* phenotype whose peak of induction arrived at 12% after the same period of time. Those results suggest that ModSP functions downstream of GNBP3, in parallel to the Psh pathway, explaining the differences between *spz^{rm7}* and *modSP^1* phenotypes. The relevance of GNBP3 to Toll activation is particularly important when flies were injected with dead *C. albicans*, a condition limiting the activation of the Toll pathway through Psh (El chamy et al, 2008). Thus,

another experiment with dead *C. albicans* injection was performed. Figure 18 shows that the same reduction of around 20% of *Drs* expression compared to wild-type is visible in *modSP* mutant flies. The only difference was the amount of *Drs* induced in *spz* deficient flies, with a value around 18% higher than before. This fact already suggests an important role of the Psh pathway for the detection of yeast. The latter is studied in more details in the next section.

Figure 18: Relative *Drs* expression after injection of dead *C albicans* in wild-type, spz^{rm7} and $modSP^{1}$ flies. The level of *Drs* expression in $modSP^{1}$ was similar to that of spz^{rm7} (17%).

The interaction between GNBP3 and ModSP was then investigated with the help of epistasis experiments. Unfortunately, we have not been able to induce *Drs* by over-expression of GNBP3 in the same manner done with GNBP1 and PGRP-SA over-expression. Nevertheless, one experiment has turned out to be successful, in which *Drs* was induced upon GNBP3 over-expression to a level of 10% compared to wild-type flies collected 16 hours after infection with *M. luteus* (Figure 19). In this unique, unreproducible experiment, we observed a reduction of the *Drs* induction by $modSP^{1}$

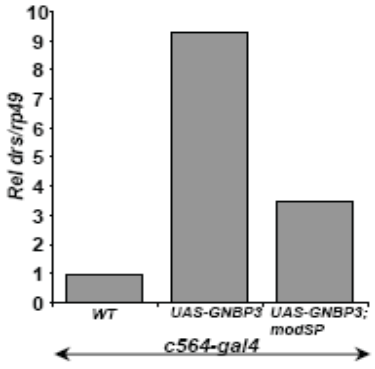

Figure 19: $modSP^{1}$ blocked *Drs* expression induced by the over-expression of GNBP3. Over-expression of GNBP3 without $modSP^{1}$ mutation arrives at 9.5%. Those results were unreproductible

flies that can be compared to the amount of *Drs* produced with unchallenged flies.

These experiments demonstrate that not only a requirement of ModSP in the Gram-positive recognition pathway is necessary to activate the Toll pathway, but also that this protein is also required in the yeast recognition pathway.

4.6 Role of ModSP in the sensing of proteases activity

The Psh pathway is activated by proteases from fungal or bacterial origins. Virulence factors such as the PR1 protease derived from fungi are known to activate this specific branch (El chamy et al, 2008). The absence of a role for ModSP in this branch was suggested by our preliminary results showing that dead *C. albicans* injection does not change the quantity of *Drs* obtained compared to normal *C. albicans* injection. The latter contains virulence factors and is in this way able to activate the Psh branch. If no changes are observed, we can already determine that ModSP does not play any role in the Psh branch. Additional experiments were performed to confirm that ModSP was absent of the Psh branch.

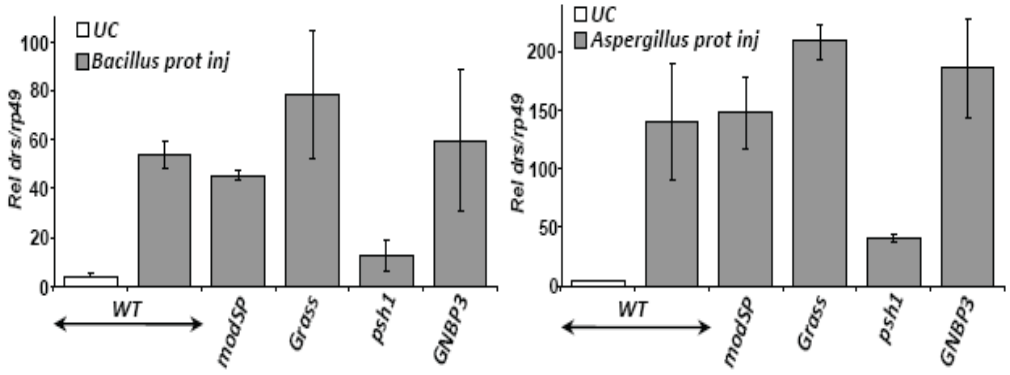

Figure 20: Relative *Drs* expression after injection of bacterial or fungal proteases. *modSP*[1] was unable to block *Drs* expression in the two experiments (compared to *psh*[1] phenotype). Each graph represents the mean of four independent experiments

RT-qPCR analysis was performed to monitor the level of *Drs* after injection of proteases in various mutant flies affected in different branches of the Toll pathway. For this, we use protease extracts derived from *Bacillus subtilis* or *Aspergillus oryzae.* The results of these experiments are represented in figure 20. We observed that expression of *Drs* was different between mutants: the *modSP*[1] mutation did not impair Toll pathway activation by proteases, because the same level of *Drs* was observed in *modSP* flies compared to the wild-type infected flies. Similar results were

obtained with *GNBP3*^{Hades} and *Grass*^{Herrade} flies as previously demonstrated. In contrast, *psh*^{1} mutant flies expressed a low level of *Drs* expression upon protease injection, confirming the important immune role of Psh in the defense against proteases.

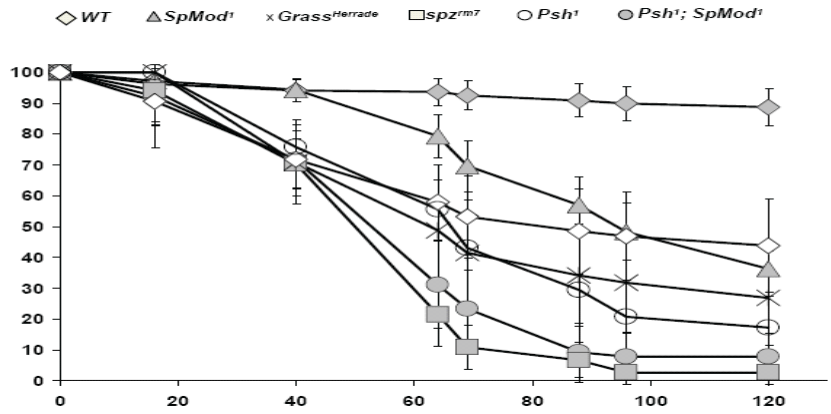

Figure 21: Survival experimentson wild-type, *modSP*^{1} *Grass*^{Herrade}, *spz*^{rm7}, *psh*^{1} and *psh*^{1};*modSP*^{1} flies upon *A. fumigatus* infection. The susceptibility of *psh*^{1};*modSP*^{1} flies was similar to that of *spz*^{rm7} phenotype. *modSP*^{1} alone exhibited a susceptibility almost similar to *psh*^{1}.

Another experiment was done with a new fly stock containing both the *psh*^{1} and *modSP*^{1} mutations. We monitored the resistance of this double mutant against the injection of spores of *A. fumigatus,* a filamentous fungus known to produce proteases. The survival curve from this experiment is shown in figure 21. Both *modSP*^{1} and *psh*^{1} flies were shown to have a moderate susceptibility against this pathogen as compared to the curve of the wild-type flies. More interesting was the double mutant phenotype whose curve was greatly depressed with similar kinetics as found in the *spz*^{rm7} curve. The RT-qPCR experiment shown in figure 22 gave no surprising results. The level of Toll activity in *modSP*^{1} flies and to a lesser extent in *psh*^{1} flies was reduced, although remaining generally higher than the levels observed in *spz*^{rm7} mutants. In double mutant flies, the Toll pathway activation by *A. fumigatus* was reduced to a level comparable to the *spz*^{rm7} mutant.

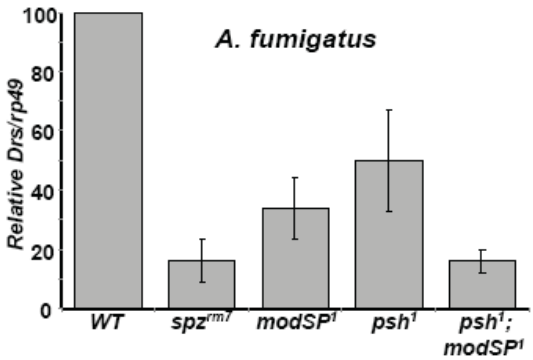

Figure 22: Relative *Drs* expression in *modSP*^{1} and *psh*^{1};*modSP*^{1} upon *A. fumigatus* infection. *psh*^{1};*modSP*^{1} blocked more *Drs* expression than *modSP*^{1} alone explaining a synergistic action of the two branches.

These results suggest a synergistic action of the Psh branch and the GNBP3 branch to fight against *A. fumigatus* spores. ModSP and the Psh branches of the Toll pathway contribute independently to the resistance to this fungus. ModSP, like Grass (El chamy et al, 2008) does not participate in the psh-dependant branch of the Toll pathway.

4.7 Interaction between ModSP and other components of the Toll pathway

We next investigated the interaction between ModSP and other components of the Toll pathway using a biochemical approach. We first obtained some fly lines containing an *UAS-ModSP* with a HA-TAG. After the successful chromosomal mapping of the insertions, we crossed those fly lines with other flies containing a *daugtherless* driver to express the tagged protein everywhere in the flies. Surprisingly, this over-expression did not induce a larval mortality like the *UAS-ModSP* (without the HA-tag) did. The goal of those biochemical experiments was to check by co-imunoprecipitation whether ModSP interacts with other components of the Toll pathway especially GNBP1.

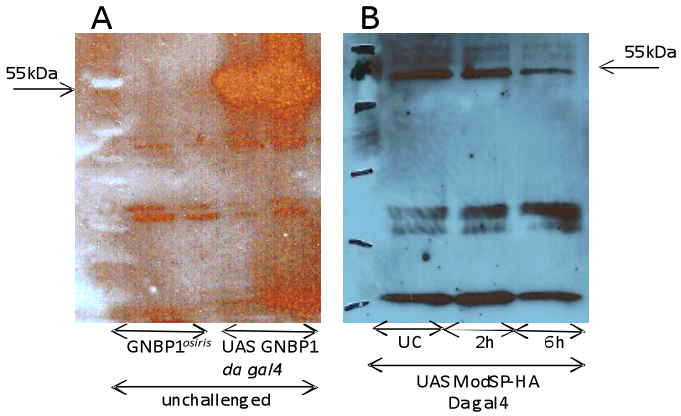

Figure 23: A. An anti-rabbit antibody directed against GNBP1 recognizes a band of 55kDa that was not observed in GNBP1^{osiris}. A strong signal was observed in flies over-expressing GNBP1 (genotype *Da-gal4; UAS-GNBP1, UAS-PGRP-SA*). This confirmed the specificity of the GNBP1 antibody. B. Co-immunoprecipitation of HA-tagged ModSP using the GNBP1 antibody. A signal corresponding to GNBP1 was observed in protein extracts immuno-precipitated with an anti-HA. This figure suggests that the quantity of GNBP1 interaction with ModSP-HA decrease upon infection.

The results obtained from two experiments are shown in figure 23. The use of an antibody directed against GNBP1 revealed that a band for GNBP1 at the expected weight of 55 kDa was detected by Western Blot analysis. In the first picture, it can be observed that this band was not observed with extracts from *GNBP1*^{osiris} mutant flies. Furthermore, an increased amount of GNBP1 was observed

with extracts from flies over-expressing GNBP1 (Genotype: *da-gal4; UAS-GNBP1*). Those results demonstrated that the anti-GNBP1 antibodies were specific of GNBP1 and could be used in co-immunoprecipitation experiments with GNBP1 and the HA-tagged ModSP.

Figure 23 B shows the results obtained from this experiment. Here, flies containing the HA-tagged ModSP were infected at different points with *M. Luteus*. The times of infection were respectively unchallenged (UC), 2 hours and 6 hours. Three bands at the expected weight of 55 kDa can be clearly observed on all samples that were co-immunoprecipiated using the anti-HA antibody. These results suggest that ModSP and GNBP1 are part of a complex, but it was impossible to know if the reaction between those two proteins was direct or the result of other reagents.

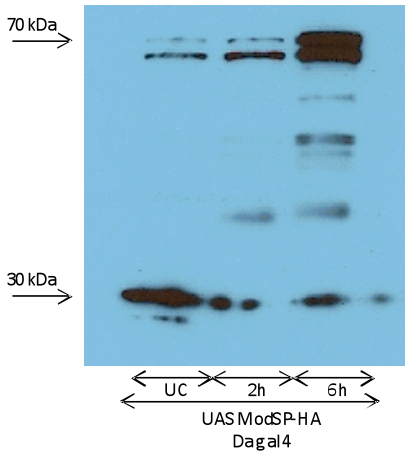

Figure 24: Western blot analysis of the expression of ModSP using an anti-HA antibody. Two bands are clearly visible with intensities that depend on the infection time. The band of 70 kDa corresponds to the full-length form while the band of 30 kDa corresponds to the cleaved form. This experiment suggests that the amount of the full-length form ModSP increases upon infection, while the cleaved form tends to decrease in intensities along the infection.

We next used the UAS-ModSP-HA fly lines to analyse the cleavage of ModSP in response to bacterial infection, we performed a western blot analysis with proteins extracted from total flies over-expressing the HA-tagged ModSP (using the *daughterless* driver). The membrane was incubated in a solution containing first antibodies against HA-tagged ModSP. The results are represented in figure 24 which shows three samples collected at different time point (UC, 2 hours, 6 hours as before). Two bands at 70 kDa and 30 kDa can be distinguished. The cleaved form of ModSP (30kDa) appeared to decrease upon the infection, while the normal form (70 kDa) appeared to increase. Thereby, These results can be explained by an auto-cleavage of this protein without infection. Then we believe that upon infection, GNBP1 interacted with ModSP and impaired its

auto-cleavage function to enable them to react at its full normal form. Additional experiments are nevertheless required to confirm this theory, because those suppositions are only based on western blots showing different intensities in the bands, with no ideas on the quantities of proteins that were present in the samples (despite that the same quantity of flies was placed in vials before crushing them).

Altogether, these results suggest a direct link between GNBP1 and ModSP in agreement with the idea that ModSP was an apical SP reacting with the PRRs during infection with Gram-positive bacteria. Moreover, ModSP performs an auto-cleavage on itself without any pathogen challenge, but when it occurs, normal form of ModSP seems to increase along the infection.

4.8 Analysis of ModSP localization using a ModSP-GFP reporter gene

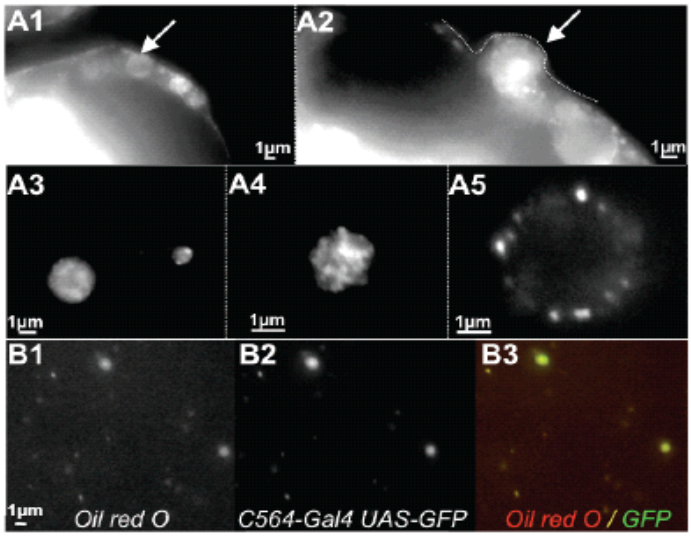

Figure 25: ModSP-GFP is released from the fat body in vesicles. A1-A2: Production of lipid vesicles containing ModSP-GFP from the fat body. A3-A4-A5: Lipid vesicles green-tagged are observed in the hemolymph of flies over-expressing ModSP-GFP. B1: Oil red O signal alone. B2: GFP alone. B3: Oil red O and GFP merge.

We generated some flies over-expressing a ModSP-GFP fusion protein using the fat body *Gal4* driver (*c564-Gal4)* to analyze the localization of the ModSP protein in the hemolymph and the fat bodies (genotype: *c564-Gal4;UAS-ModSP-GFP)*. The figure 25 (A1 and A2) shows that ModSP-GFP protein was produced by the fat bodies in the form of small lipid vesicles. More interesting was the discovery that ModSP-GFP was found in lipid vesicles in the hemolymph. Collectively, this indicated that ModSP was most probably secreted from the fat body into the hemolymph at the

surface of small lipid vesicles of 0.2-5 μm. Such transport was not observed with other SPs, but it could be consistent with the presence of LDLa repeats that could anchor ModSP to lipoproteins specially found in lipid vesicles. Another experiment was performed to show that the fat body of *c564-Gal4; UAS-ModSP-GFP* flies was not apoptotic using a staining against Caspase-3 activity. This indicated that the observed vesicles were not due to cell death linked to the over-expression of ModSP-GFP.

This important result allows us to speculate about the fact that the association of ModSP to vesicles can be important to nucleate the activation of downstream SP in the hemolymph.

4.9 A role for ModSP in the melanization pathway?

We next investigated whether ModSP plays a role in the melanization reaction resulting in the activation of phenoloxidase (PO). A melanin deposition can be observed at the injury site to control wound healing and pathogen growth. A proteolytic cascade containing many SPs is involved in the activation of PO. Two clip-domain SPs, MP1 and MP2 and two serpins Spn27A and Spn28D have already been discovered to regulate the PO cascade, but their localization in the melanization pathway is not well defined. It has also been shown that the melanization cascade is regulated at the transcriptional level by the Toll pathway in adults (Ligoxygakis et al, 2002). From the above results, we know that ModSP plays an important role in the Toll pathway activation and we propose the idea that this SP could also play a role in melanization reaction as observed for *T. Molitor* ModSP (Kim et al, 2008).

We first observed the diminution of the melanin deposition on the injury site of $modSP^{1}$ flies compared to wild-type Oregon flies (Figure 26). Black cells (Bc) flies are mutant flies that can not induce any melanization reaction because of the absence of the PO enzyme. Those flies were used as negative controls. On the other hand, spn^{27A} mutant flies served as positive controls, because the absence of this serpin enhanced the melanization reaction. We can see a reduction of melanin deposition in $modSP^{1}$ flies compared with wild-type flies and spn^{27A} deficient flies. The reduction was less marked than that of Bc flies. A speculate pathway can be visible in figure 27, in which ModSP was localized in both Toll pathway and melanization reaction.

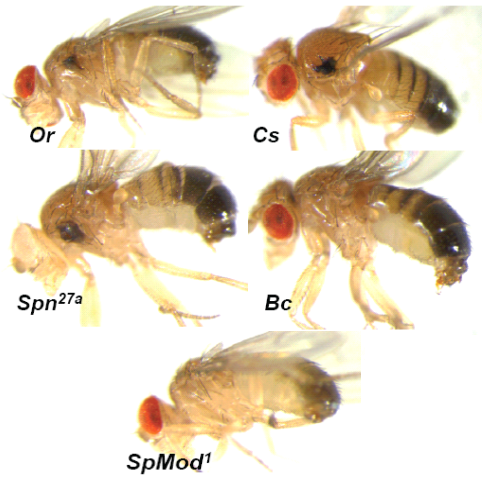

Figure 26: The melanization reaction at the injury site was observed in wild-type flies (Oregon and Canton), *spn*^{27A} flies (excessive melanization reaction), *Bc* flies (no melanization) and *modSP*^{1} flies. We observed almost no melanization in *modSP*^{1} flies.

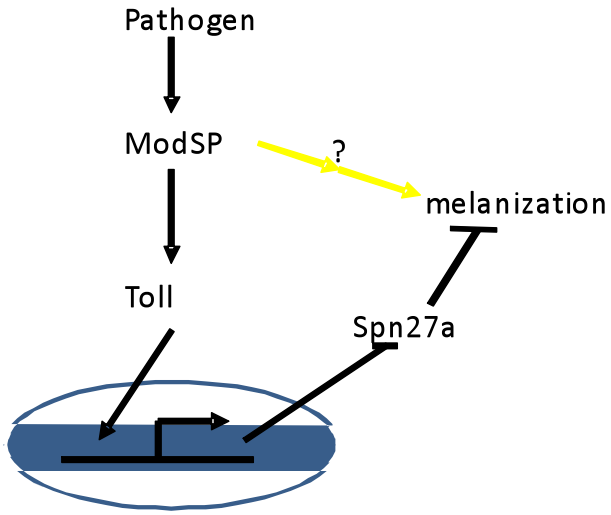

Figure 27: Suggestion of ModSP acting in Toll pathway and also in the melanization reaction. We know already that Toll pathway promotes transcription and maturation of Spn acting in the melanization pathway.

Those speculations prompted us to do a specific experiment to measure PO activity in hemolymph of *modSP*^{1} flies compared to wild-type, *spz*^{rm7} and *Grass*^{Herrade} flies. Figure 28 shows the PO activity of hemolymph extract collected 5 hours after infection with *M. Luteus*. This experiment reveals that *modSP*^{1} flies blocked the melanization pathway to the same extent that the *spz*^{rm7} or the *Grass*^{Herrade} mutation.

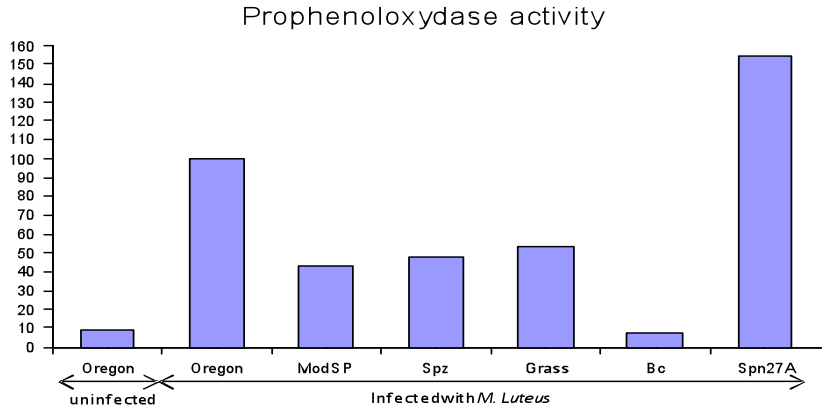

Figure 28: Quantification of the hemolymph phenoloxydase activity in wild-type, *modSP*^{1}, *spz*^{rm7}, *grass*^{Herrade}, *Bc* and *spn*^{27A} flies. The same level of PO activity was observed in *modSP*^{1} and other mutants affecting the Toll pathway. This suggest that the *modSP* mutation reduced the level of phenoloxidase activity indirectly by reducing the synthesis of enzyme participating in the melanization that are under the control of the Toll pathway.

Those results suggest that ModSP does not participate in the melanization pathway, but it indirectly controls the melanization cascade as observed for other components of the Toll pathway.

5. Discussion

In this thesis, we demonstrated that a null mutation in the *modSP* gene blocks the activation of the Toll Pathway. We show that ModSP is a serine protease acting in the proteolytic cascade necessary for the maturation of *Spz* the ligand of the Toll receptor. We also demonstrated its importance to activate the Toll pathway in response to both Gram-positive bacteria and fungi. This notion was supported by both survival and RT-qPCR analysis of flies infected with those pathogens. Epistatic analyses also demonstrated that ModSP acts downstream of PGRP-SA and GNBP3, but upstream of Grass. Moreover, we showed that ModSP did not participate in the Psh-dependant branch of the activation of Toll, as shown with the infection of proteases from bacterial and fungal origins. Also, the analysis of a double mutant $psh^{1};modSP^{1}$ suggested a synergistic action of the ModSP and Psh pathways in the response against filamentous fungi. Those fungi can be directly detected by PRRs from GNBP3 branch or through their virulence factors for the activation of the Psh-dependant branch. Another experiment with *Beauvaria Bassiana* (Figure 29) showed similar results to that obtained with *A. Fumigatus,* although we saw that the Psh-dependant branch plays a more significant role for the defense against this entomopathogenic fungi.

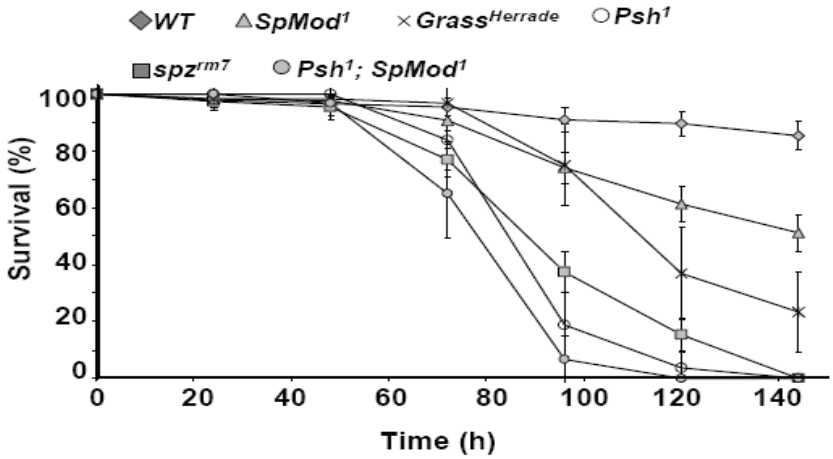

Figure 29: Survival experiment of wild-type and *modSP*[1] flies upon infection with *B. Bassiana*. *modSP*[1] *fliesshows a moderate* susceptibilityto *B. bassiana* while*modSP*[1]*;psh*[1] were highly susceptible to this fungus. This experiment indicates that the Psh-dependantbranch is moreimportantin the defense against*B. Bassiana*.

We also showed that Grass and ModSP function in a common SP cascade and that ModSP is the apical SP of the proteolytic cascade. Those results are supported by biochemical experiments that suggested a direct interaction between ModSP and GNBP1. Moreover, biochemical analyses in *T.*

molitor indicated that *Tm*-MSP directly interacts with the PRR complexes involved in the sensing of peptidoglycan (Kim et al, 2008) like *Dm*-MSP. The participation of ModSP and SPE in an extracellular pathway linking PRRs recognition to *Spz* activation in both *T. molitor* (Coleoptera) and *D. melanogaster* (Diptera), which diverged about 250 million years ago, demonstrated the conservation of this extracellular signalling module in two different insects. Moreover, in the lepidopteran *Manduca sexta,* a modular SP hemolymph protein 14 (*Ms*-HP14) similar to ModSP, regulates the melanization cascade in response to microbial infection (Wang et al, 2006). This indicates the existence of similar mechanism regulating the immune SP cascades in various insects. Interestingly, a similar organization was also observed in the proteolytic cascade that regulated Toll during dorso-ventral patterning of the embryo in which the apical SP is Gastrulation Defective. Thus, we have been able to reveal a similar level of organization for various proteolytic cascades in different insects (Figure 29). Nevertheless, data obtained from our Korean collaborators suggests that ModSP does not cleave Grass (BL Lee, personal communication). This suggests that the cascade regulating Toll pathway activation in *Drosophila* is more complex and may involve more than 3 Sps.

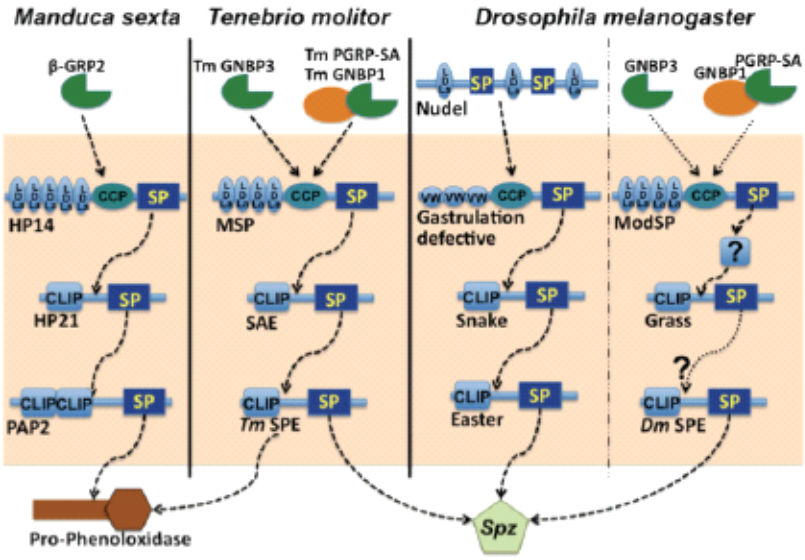

Figure 29: Extracellular cascades of serine proteases in insects (see discussion).

Another important result coming from B-L Lee laboratory was the fact that ModSP exhibited a high level of auto-proteolysis (BL Lee, personal communication). Those results support a model in which recruitment of ModSP by PRRs, when challenged by pathogens, can increase its local

concentration, a situation sufficient for its auto-proteolysis. The fact that ModSP over-expression was sufficient to activate the Toll pathway also support this idea. In fact, we showed that over-expression of a full length version of ModSP was sufficient to reach a high level of Toll activation, in contrast to other SPs that generally required the over-expression of a pre-activated form to fully induce the cascade. This demonstrates again that ModSP can be auto-activated, in case of over-expression of a non-reactive ModSP. Moreover, it had also been proved that a recombinant form of ModSP produced in Baculovirus appeared to be unstable as a zymogen. This was certainly due to a high level of auto-proteolysis. This high level of auto-proteolysis did not permit *in vitro* reconstitution experiments using ModSP, GNBP1 and PGRP-SA, but we had already provided biochemical experiments suggesting that ModSP interacts with GNBP1.

We are far away from a total understanding of the Toll pathway activation. The high number of SPs encoded in the *Drosophila* genome (Ross et al, 2003) complicates the analysis of this question, since a lot of them have not been already linked to a specific function yet. We have nevertheless evidence that an additional SP functions between ModSP and Grass in the proteolytic cascade leading to the Toll pathway activation. Further work has to be done to identify this SP and to fill the gap between the ModSP active form and Grass activation. Furthermore, no serpin responsible for the regulation of this proteolytic cascade has been discovered yet despite the critical role of this family in the negative control of such process. Further experiments combining genetics, biochemistry and cell biology are required to identify additional components of this cascade and to clarify *in vivo* how and where proteolytic cascades downstream of PGRP-SA or GNBP3 are activated in the hemolymph compartment.

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7. References

1. Lemaitre B & Hoffmann J (2007) The host defence of *Drosophila melanogaster*. *Annu Rev Immunol* 25:697-743 .
2. Jang IH, Nam HJ, & Lee WJ (2008) CLIP-domain serine proteases in Drosophila innate immunity. *BMB Rep* 41(2):102-107 .
3. Kambris Z*, et al.* (2006) Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. *Curr Biol* 16(8):808-813.
4. Jang IW*, et al.* (2006) A Spätzle-Processing Enzyme Required for Toll Signaling Activation in Drosophila Innate Immunity. *Dev Cell* 10:45-55.
5. Michel T, Reichhart JM, Hoffmann JA, & Royet J (2001) Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* 414(6865):756-759.
6. Gobert V*, et al.* (2003) Dual activation of the Drosophila toll pathway by two pattern recognition receptors. *Science* 302(5653):2126-2130.
7. Gottar M*, et al.* (2007) Dual detection of fungal infections in Drosophila by recognition of glucans and sensing of virulence factors. *Cell* 127:1425-1437.
8. El Chamy L, Leclerc V, Caldelari I, & Reichhart JM (2008) Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signalling pathways 'upstream' of Toll. *Nat Immunol* 9(10):1165-1170 .
9. Kan H*, et al.* (2008) Molecular control of phenoloxidase-induced melanin synthesis in an insect. *J Biol Chem* 283(37):25316-25323 .
10. Kim CH*, et al.* (2008) A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced toll pathway in an insect. *J Biol Chem* 283(12):7599-7607 .
11. De Gregorio E, Spellman PT, Tzou P, Rubin GM, & Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *Embo J* 21(11):2568-2579.
12. 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.

13. Piao S*, et al.* (2005) Crystal structure of a clip-domain serine protease and functional roles of the clip domains. *Embo J* 24(24):4404-4414.

14. Ligoxygakis P, Pelte N, Hoffmann JA, & Reichhart JM (2002) Activation of Drosophila Toll during fungal infection by a blood serine protease. *Science* 297(5578):114-116.

15. Wang L*, et al.* (2006) Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA. *Embo J* 25(20):5005-5014 .

16. Wang Y & Jiang H (2006) Interaction of beta-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in Manduca sexta. *J Biol Chem* 281(14):9271-9278 .

17. Wang Y & Jiang H (2007) Reconstitution of a branch of the Manduca sexta prophenoloxidase activation cascade in vitro: snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenoloxidase-activating proteinase-2 precursor. *Insect Biochem Mol Biol* 37(10):1015-1025 .

18. Han JH, Lee SH, Tan YQ, LeMosy EK, & Hashimoto C (2000) Gastrulation defective is a serine protease involved in activating the receptor toll to polarize the Drosophila embryo. *Proc Natl Acad Sci U S A* 97(16):9093-9097.