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**Aeromonas salmonicida** secreted protein **AopP** is a potent inducer of apoptosis in a mammalian and a *Drosophila* model

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**Abstract**

Some pathogens are able to establish themselves within the host because they have evolved mechanisms to disrupt host innate immunity. For example, a number of pathogens secrete preformed effector proteins via type III secretion apparati that influence innate immune or apoptotic signaling pathways. One group of effector proteins that usurp innate immune signaling is the YopJ-like family of bacterial effector proteins, which includes AopP from *Aeromonas salmonicida*. *Aeromonas* species are known to cause gastrointestinal disease in humans, and are associated mainly with subcutaneous wound infections and septicemia in other metazoans, particularly fish. AopP has been reported to have inhibitory activity against the NF-κB pathway in cultured cells, although the pathological outcomes of AopP activity have not been examined. Here, we show that AopP has potent pro-apoptotic activity when expressed in cultured mammalian macrophage or epithelial cells, or when ectopically expressed in *Drosophila melanogaster* hemocytes or imaginal disk epithelial cells. Furthermore, apoptosis was significantly elevated upon concurrent AopP expression and TNF-α cellular stimulation. Together, our results demonstrate how the specificity of a YopJ-like protein toward signaling pathways directly governs cellular pathological outcome in disease.

**Keywords**

*Aeromonas*; NF-κB; apoptosis; AopP; bacterial effector protein

**Introduction**

*Aeromonads* are Gram-negative, facultative anaerobic bacteria found in saline and fresh waters. The majority of studies relating to the pathogenicity of *Aeromonas* species are associated with wound infections and septicemia in vertebrates including fish, frogs, snakes and birds. Some aeromonads are also pathogenic to humans, causing severe wound infections and a number of less well described illnesses including (but not limited to) infections of the eye, joints and bones. Other reports have shown evidence that some aeromonads cause gastroenteritis in humans (Albert et al., 1999; Gluskin et al., 1992) although it is unclear if fecal bacteria are the direct agent of diarrheal disease and thus their role in gastroenteritis remains controversial (Abbott et al., 2003; Abbott et al., 1998), and comprehensively reviewed by (Parker and Shaw, 2011).
Disease causing pathogens can establish themselves within host cells because they possess mechanisms to subvert innate immune or apoptotic signaling pathways. Some pathogens secrete preformed proteins into host cells via a “type III secretion apparatus” to facilitate pathogen invasion and/or colonization. Recent studies have shown that *Aeromonas salmonicida*, which has been extensively studied in the context of a fish pathogen, harbors genes encoding a type III secretion system (Burr et al., 2002). Disruption of these genes forming the secretion apparatus substantially reduced *A. salmonicida* virulence (Burr et al., 2005; Burr et al., 2002). Furthermore, certain isolates of *A. salmonicida* contain a plasmid harboring the *aopP* gene (Fehr et al., 2006). The closest known AopP orthologues are members of the YopJ-like family of bacterial effector proteins including YopJ from *Yersinia enterocolitica* (Mukherjee et al., 2006; Orth et al., 1999), AvrA from *Salmonella typhimurium* (Jones et al., 2008; Collier-Hyams et al., 2002; Hardt and Galan, 1997) and VopA from *Vibrio parahaemolyticus* (Trosky et al., 2007; Trosky et al., 2004) - all of which are known to cause gastroenteritis in humans, and are secreted into the host cell via a “type III secretion apparatus” (Staskawicz et al., 2001). These proteins have been shown to exhibit disparate inhibitory activities against MAPKs or NF-κB signaling. Specifically, YopJ inhibits ERK, p38 and JNK MAPK pathways as well as the NF-κB pathway, while also inducing rapid apoptotic death in infected macrophages (Orth et al., 1999; Ruckdeschel et al., 1998) AvrA from *Salmonella typhimurium* has been shown by our research group to be a potent inhibitor of the JNK and NF-κB pathways, thereby inhibiting both cytokine production and apoptosis during invasion and infection (Du and Galan, 2009; Jones et al., 2008; Collier-Hyams et al., 2002). VopA is an inhibitor of ERK, p38 and JNK, but not of NF-κB and is reported to not display any pro-apoptotic activity (Trosky et al., 2004).

A study by Fehr et al. (2006) investigated the prevalence of *aopP* in *A. salmonicida* field isolates. Furthermore, they also showed that AopP inhibited nuclear translocation of p65, but did not inhibit I-κB phosphorylation. However, in their investigations measuring I-κB phosphorylation, *aopP* transfected cells were stimulated with TNFα for only 3 minutes before analysis of lysates. We deemed that the inhibitory effects of AopP warrant further investigation, particularly after extended periods of TNFα stimulation. Additionally, previous studies of AopP did not address to the pathogenic outcome of AopP activity in cultured cells or animal models. The importance of this study is further highlighted by the plasmid based location of the *aopP* gene, compared to YopJ, AvrA and VopA which are chromosomally encoded. Thus *aopP* has a higher potential for horizontal gene transfer within bacterial populations, including those associated with gastroenteritis or skin infection in humans.

To further study the effects of AopP on NF-κB and MAPK signaling pathways in vivo, we used directed expression of AopP in specific *Drosophila melanogaster* tissues, thus allowing controlled examination of the physiological effects of AopP in intact animal tissues through well-defined genetic inducible expression systems. We show that AopP is a potent inhibitor of the *Drosophila* IMD pathway and Toll NF-κB pathways normally activated during bacterial infection. AopP also potently induced apoptosis in *Drosophila* epithelial tissue, and reduced phagocyte numbers. These data indicate that the *A. salmonicida* AopP can modulate host defenses by inhibiting innate immune responses and also potently induce an apoptotic response in the eukaryotic host tissues.

**RESULTS**

**AopP expression in the *Drosophila* fat body suppresses NF-κB pathway activation**

In order to examine AopP function in a whole animal model, we created transgenic *Drosophila* harboring either wild type *A. salmonicida* protein AopP or a catalytically inactive mutant form of AopP (mAopP) (C181A transversion) under the transcriptional
control of the yeast UAS promoter, allowing tissue specific expression by crossing to GAL4 driver lines (Brand, 1994). We expressed AopP and mAopP in the Drosophila fat body using the c564-GAL4 driver fly line. Previous reports showed that AopP expression in cultured mammalian cells inhibited p65 NF-κB translocation. The NF-κB pathways in Drosophila, namely the Toll pathway and the Imd pathways, have been shown to be necessary for the antimicrobial response to bacteria. We thus examined the extent to which expression of AopP could suppress NF-κB responses following bacterial challenge. We expressed AopP and mAopP using the heat shock-GAL4 (hs-GAL4) driver line. Adult Drosophila were subjected to heat shock for 1 hour at 37°C, and survival was monitored. Expression of AopP resulted in complete loss of viability up to 96 hours post heat shock (Fig. 1A). We then assessed the ability of Drosophila expressing AopP to respond to infection. Flies were subjected to heat shock, and then incubated for 24 hours at 25°C before injury with a sterile needle as control, or parenteral infection with either the Gram-negative fly pathogen Erwinia carotovora, or with the Gram-positive Micrococcus luteus. Monitoring of Drosophila survival revealed no significant increase in mortality rate between uninfected AopP expressing Drosophila, and isogenic flies injured with a sterile needle (Fig. 1B). However, AopP significantly dampened the ability of Drosophila to respond to both Gram-negative and Gram-positive infections, compared to expression of the mutant and catalytically inactive mAopP, or hs-GAL4 alone (Fig. 1C and Fig. 1D). Furthermore, bacterially infected AopP expressing flies exhibited significantly increased mortality rates compared to isogenic flies injured with a sterile needle (Fig 1B, Fig. 1C and Fig. 1D). No significant differences were detected between the survival rates of infected mAopP expressing flies compared to infected hs-GAL4 alone in all experiments. AopP also inhibited anti-microbial peptide gene expression in response to both Gram-negative and Gram-positive infections (Fig. 1E and Fig. 1F). Furthermore, AopP inhibited the nuclear translocation of the imd pathway transcription factor Relish (Fig. 1G), and the Toll pathway transcription factor Dif (Fig. 1H), in response to immune challenge whereas expression of mAopP did not. Together, these data show potent AopP-mediated immunosuppression through inhibition of the Drosophila NF-κB Imd or Toll pathways.

AopP expression is directly apoptotic in Drosophila tissue

Developing Drosophila tissue is highly susceptible to induced apoptosis, and thus affords a robust assay for cell death. We examined whether ectopic AopP expression resulted in altered tissue phenotypes characteristic of increased cell death. Expression of aopP in the Drosophila eye using the GMR-GAL4 driver resulted in an altered eye phenotype compared to the transcriptionally silent UAS-aopP parental strain, or a strain expressing the catalytically inactive mAopP (Fig. 2A). To confirm that the altered eye phenotype is due to aberrant apoptosis, we immunostained eye imaginal disks from wandering third instar larvae using an antibody against cleaved Caspase-3. Consistent with the gross morphological eye phenotypes, AopP induced the activation of cleaved Caspase-3 in the developing eye, whereas the aforementioned controls did not (Fig. 2A). Importantly, co-expression of Drosophila Inhibitor of Apoptosis-1 (dIAP-1) in the GMR-GAL4 UAS-aopP genetic background markedly rescued the altered eye phenotype, and inhibited AopP-induced activation of active Caspase-3 (Fig. 2A). We also expressed AopP in the Drosophila wing margin under the c96-GAL4 driver. Adult Drosophila expressing AopP in the wing margin exhibited dose dependant wing margin notching, a phenotype characteristic of elevated apoptosis in this tissue (Fig. 2B). Importantly, wing notching could be completely rescued by the co-expression of dIAP, or by expression of the IAP-like molecule p35 (Fig. 2B). In order to confirm that notching was due to elevated apoptosis, we analyzed AopP-expressing wondering third instar larval imaginal wing disks by immunostain using apoptotic markers. AopP-expressing larval wing disks exhibited increased levels of cleaved caspase-3 in the developing wing margin (Fig. 2C). Again, co-expression of UAS-dIAP1 in

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the c96-GAL4 UAS-aopP background inhibited AopP-induced Caspase-3 cleavage (Fig. 2C). Finally, consistent with the markedly altered morphology in AopP expressing fat bodies (Fig. 1E and Fig. F), examination of fat bodies from c564-GAL4 UAS-aopP revealed considerable numbers of TUNEL positive cells compared to control larvae (Fig. 2D). TUNEL positive cells were also detected in the fat bodies of hs-GAL4 UAS-aopP larvae at 72 hours post heat shock, albeit to markedly reduced penetrance (Fig. 2D). We conclude that AopP-mediated induction of apoptosis, and the dampening of the imd and Toll innate immune pathways, render Drosophila highly susceptible to immune challenge.

AopP does not inhibit MAPK signaling in Drosophila tissue

Members of the YopJ-like family of bacterial effector proteins have been shown to inhibit ERK, p38 and JNK MAPK signaling, (reviewed by) (Jones and Neish, 2011). Fehr et al. (2006) reported that AopP has no inhibitory effects on ERK signaling as detected in transfected cultured cells stimulated with EGF. In order to examine whether AopP can inhibit MAPK signaling in Drosophila, we investigated the effects of AopP on ERK (Rolled in Drosophila) MAPK pathway signaling. The ERK signaling pathway has been shown to be required for normal eye development where loss of function alleles of ERK pathway intermediates result in severe altered eye phenotypes due to ommatidial mis-specification (Kumar et al., 2003; Kumar et al., 1998). We co-expressed the ERK pathway gain of function alleles, including constitutively active UAS-RasV12, UAS-rl(sem) or UAS-pointedP2 in the genetic background of GMR-GAL4 UAS-aop, and observed no discernable differences in eye morphology, indicating that AopP has no inhibitory effects on ERK signaling in Drosophila developing tissue (Figure 3A). This is in contrast to the “rough eye” phenotype induced as a result of expression of the Vibrio VopA protein which is reported to block ERK and JNK MAPK signaling while having no effect on NF-κB signaling. This VopA-dependent rough eye phenotype is completely rescued by constitutive ERK pathway expression downstream of Rolled (Fig. 3A). Additionally, we examined whether AopP has inhibitory effects on the JNK (Basket in Drosophila) signaling pathway. Eiger is the Drosophila ortholog of mammalian TNF and is a potent activator of JNK signaling and apoptosis (Moreno et al., 2002). Constitutive expression of Eiger in the retina resulted in an extreme small eye phenotype, consistent with past reports (Igaki et al., 2002). AopP expression did not rescue the Eiger-induced apoptotic eye phenotype to the same extent as AvrA, a known inhibitor of JNK signaling that completely repaired the rough eye phenotype. Intriguingly, while the size of the eye was marginally larger, black necrotic tissue was clearly visible within eye tissue following eye specific co-expression of Eiger and AopP (Fig. 3B).

AopP decreases Drosophila immune cell number and compromises cellular immune responses following 5 days of infection

Drosophila contain three blood cell types (hemocytes) - plasmatocytes, crystal cells and lamellocytes – with functions similar to the vertebrate myeloid lineage (Lemaitre and Hoffmann, 2007)(Wood and Jacinto, 2007). Plasmatocytes, which constitute 95% of total hemocyte numbers are analogous to mammalian macrophages, and have been shown to function in immune defense via the phagocytosis of invading bacteria pathogens (Lemaitre and Hoffmann, 2007). Consistently, when invasive pathogens of the mammalian gut enter the sub-mucosa, they are rapidly phagocyted by macrophages. Following internalization, certain pathogens secrete pro-apoptotic bacterial effector proteins directly into the macrophage, thus usurping the host’s ability to respond to bacterial threat. We examined the outcome of direct AopP expression under the hml-GAL4 driver, which expresses the GAL4 protein only in Drosophila plasmatocytes. We also concurrently expressed UAS-gfp in these flies to facilitate plasmatocyte detection. Hemolymph was collected from third instar larvae expressing AopP or mAopP and GFP positive cells were counted. Expression of AopP in
Drosophila plasmatocytes resulted in about a 60% reduction in cellular population, whereas expression of mAopP, or the non-proapoptotic AvrA protein did not (Fig. 4A and 4B). A similar reduction in total plasmatocyte numbers was also detected when Eiger was expressed under hml-GAL4. These data indicate that AopP expression reduces the total number of Drosophila plasmatocytes when directly expressed in these cells. We then examined the extent to which AopP-induced apoptosis of Drosophila plasmatocytes affected fly survival following bacterial challenge. Flies expressing AopP, or the Eiger control, in plasmatocytes had significantly reduced survival rate following parenteral infection with M. luteus only between 5 and 8 days post infection, compared to flies expressing mAopP, (Fig. 4C). AopP expressing flies infected with E. carotovora also exhibited noticeably reduced survival between 5 and 8 days post infection, although these differences were not statistically significant. No significant differences were detected between AopP expressing flies and controls when stabbed with a sterile needle. These results indicate that the fat body is the primary immune responder to infection during the first 5 days of infection, and are concordant with previous reports (Charroux and Royet, 2009) (Defaye et al., 2009).

AopP is highly pro-apoptotic in mammalian cultured cells

To determine whether AopP induces apoptosis of mammalian cells, we co-transfected plasmids expressing wild type AopP (pCMVmyc-AopP) or the catalytically inactive mAopP (pCMVmyc-mAopP) together with a plasmid expressing GFP into cultured HeLa cells. Transfected cell populations were then stained with Annexin V and propidium iodide, and analyzed by Fluorescent-activated cell sorting (FACS). GFP-positive HeLa cell populations expressing AopP contained significantly increased numbers of Annexin V positive cells compared to cells expressing with mAopP or with vector alone (Fig. 5A and 5B). Furthermore, HeLa cells transfected with pCMVmyc-AopP and stimulated with TNF-α exhibited further increased numbers of apoptotic cells compared to unstimulated transfected cells (Fig. 5A and 5B). Together, these data show that AopP is pro-apoptotic towards mammalian cultured cells and that the apoptotic population is increased in the presence of TNF-α, a pleiotropic activator of the NF-κB, ERK, p38 and JNK pathways.

AopP attenuates NF-κB pathway activation and dysregulates JNK pathway signaling in response to TNF-α stimulation

Previous studies reported that AopP inhibited NF-κB nuclear translocation, but did not inhibit I-κB phosphorylation at 3 minutes post TNFα stimulation in cultured mammalian cells (Fehr et al., 2006). Our studies confirm the potent AopP-mediated inhibition of the NF-κB pathway in Drosophila. Previous investigations focusing on the AopP homologues YopJ and AvrA, showed YopJ- or AvrA-induced alter I-κB phosphorylation and degradation kinetics up to 1 hour after TNF-α stimulation (Jones et al., 2008; Mittal et al., 2006). Therefore we decided to investigate the mechanism of AopP-mediated NF-κB pathway inhibition in more detail. We transfected plasmids expressing AopP, mAopP in to cultured HEK 293T cells, along with a plasmid expressing GFP to measure transfection efficiency, which was estimated at >75% of the cell population (data not shown). Transfected cells were stimulated with TNFα and cell lysates were analyzed by immunoblot for innate immune related signaling intermediates. We observed that AopP mediated a marked attenuation in the degradation of the phosphorylated I-κB in response to TNF-α stimulation (Fig. 6A). This response is similar to the activity of AvrA reported in Fig. 3C of Jones et al. (2008), and YopJ reported in Fig. 3B of Mittal et al. (2006), which also resulted in prolonged stabilization of phosphorylated I-κB. Interestingly, despite the observation that YopJ does not inhibit TNFα-induced phosphorylation of I-κB, it was also shown that YopJ has acetyltransferase activity against IKKβ (Mittal et al. 2006), indicating that YopJ inhibition of NF-κB signaling occurs at the level of IKKβ. We thus investigated the extent to which AopP can inhibit IKKβ phosphorylation. We co-transfected HEK293T cultured cells with a
plasmid expressing IKKβ (pCMV2-flag-IKKβ), AopP and (pCMV-mycAopP) or mAopP (pCMV-myc-mAopP) respectively. Cells were incubated for 24 hours before stimulation with TNFα for 1 hour. Although expression of IKKβ was reduced when co-expressed with AopP, probably due to AopP-induced apoptosis, it is still evident that AopP markedly inhibits the phosphorylation of IKKβ in response to TNFα (Fig. 6B), consistent with the activity of YopJ.

Finally, to identify the mechanism of AopP-mediated cell death, we assessed the extent to which AopP activity inhibited the expression of NF-κB responsive Inhibitor of Apoptosis (IAP) proteins. AopP, but not mAopP or vector control blocked cIAP-2 expression in response to TNFα stimulation (Fig. 6C), and reduced the levels of IAP-2 in cell lysates of transfected cells (Fig. 6D). Thus, AopP-induced cellular apoptosis plausibly occurs due to inhibition of the expression of IAP proteins. Together, these data indicate that in unstimulated conditions, apoptosis results from AopP-mediated NF-κB pathway inhibition and subsequent inhibition of the expression of anti-apoptotic factors.

**DISCUSSION**

The *Drosophila* animal model has been utilized to investigate several areas of experimental biology due to the ease of working with this species and its “genetic tractability”. Proteins can be expressed in specific tissues without lethal effects to the whole organism. There is also striking structural and functional homology between the *Drosophila* and mammalian innate immune pathways (Lemaitre and Hoffmann, 2007) and as a consequence *Drosophila* has been exploited to study the activity of several prokaryotic or viral effector proteins on innate immune networks (Jones et al., 2008; Guichard et al., 2006; Jia et al., 2006; Leulier et al., 2003). Here, we utilized *Drosophila* to undertake functional analyses of the pathological outcome of AopP enzymatic activity in host cells. Our experiments show that AopP mediates the inhibition of the IMD and Toll NF-κB pathways in *Drosophila*. Transgenic *Drosophila* expressing AopP were highly susceptible to Gram-negative or Gram-positive infection, and were unable to upregulate Gram-negative or Gram-positive specific anti-microbial peptides in response to infection. AopP also inhibited Relish and Dif cytoplasmic-to-nuclear translocation. The effects of the *A. salmonicida* AopP on cellular innate immune signaling has been studied using mammalian cell-based assays (Fehr et al., 2006). These experiments showed that constitutive AopP expression suppressed the canonical NF-κB pathway by inhibiting nuclear translocation of the p50/p65 Rel proteins. These observations are consistent with our findings of AopP-mediated inhibition of Relish and Dif translocation and suppression of Rel-dependent Diptercin activation, and Dif-dependent Drosomycin activation in the fat body of *Drosophila*. These results illustrate the conservation of AopP-mediated effects on this pathway.

The closest orthologs to AopP are secreted by bacteria that are known to cause gastroenteritis. During pathogenesis, these proteins repress the host innate immune response via the blockade of the MAPK or NF-κB signaling pathways. For example, YopJ of *Yersinia* has a wide range of inhibitory activities against ERK, p38, JNK, and NF-κB (Orth et al., 1999) VopA of *Vibrio parahaemalyticus* has been shown to inhibit ERK, p38 and JNK, but not NF-κB (Trosky et al., 2004) and AvrA of *Salmonella typhimurium* has been shown to inhibit the JNK and NF-κB pathway, while having no inhibitory effect on ERK or p38 (Jones et al., 2008). In this study, and as reported by Fehr et al. (2006), we show that AopP activity is specific to NF-κB while having no effect on MAPK pathways. These data indicate that this family of bacterial effector proteins has evolved to differentially inhibit members of the MAPKKs and IKKs, and indicates that bacteria interrupt innate immunity by targeting one or multiple pathways.
The inhibitory profile exhibited by bacterial effector protein enzymatic activity ultimately influences cellular innate immune outcome, be it survival and inflammation or apoptosis. For example, YopJ has been shown to induce apoptosis and dampen cytokine production via broad inhibition of the MAPK and NF-κB pathways. AvrA, on the other hand, dampens cytokine production but is anti-apoptotic due to activity against the JNK pathway, while VopA, which inhibits all three MAPKs but not NF-κB, is reported to have no pro-apoptotic activity (Jones and Neish, 2011). These data indicate that blockade of the NF-κB pathway leads to cellular apoptosis. Our data examining AopP activity, which inhibits NF-κB pathway and not MAPK is concordant with this model. We show that AopP expression is potently pro-apoptotic in both Drosophila and cultured mammalian cells. Fehr et al. 2006 reported that AopP does not inhibit IkBα phosphorylation, and thus conclude that AopP inhibits the NF-κB pathway downstream of I-κB kinase (IKK) activation. In their experiments HeLa cells were transfected with a plasmid harboring AopP and stimulated with TNFα for 3 minutes. In this study, we expand the analysis to include time intervals up to 1 hour after TNFα stimulation, a time period which we have successfully used to characterize the inhibitory profiles of AvrA and YopJ (Jones et al., 2008). Similar to AvrA and YopJ, we found that AopP attenuated IkBα phosphorylation. Furthermore, we show that AopP inhibits IKKβ phosphorylation thus proving that AopP-mediated inhibition of NF-κB occurs upstream of I-κB phosphorylation. However, we and others still detect TNFα-induced I-κB phosphorylation in the presence of AopP, YopJ or AvrA, arising speculation of an alternative mechanism of I-κB phosphorylation, yet one that is insufficient to eventuate in NF-κB nuclear translocation.

Altruistic cell loss as a defense mechanism against pathogen invasion is conserved across metazoans, including mammals and invertebrates (Hoffmann, 2003; Silverman and Maniatis, 2001; Weinrauch and Zychlinsky, 1999). Our data show that AopP influences innate immune activation in disparate in vivo or cultured cell model systems. In fish, Aeromonas spp. induces an innate immune or apoptotic response in host tissue characterised by the formation of subcutaneous wound infections and septicemia. The pro-apoptotic activity of AopP may account for the destructive effects observed in Aeromonas infections. Consistently, the AopP ortholog in Yersinia, YopJ (Mukherjee et al., 2006; Ruckdeschel et al., 1998; Ruckdeschel et al., 1997) due to its enzymatic activity against a range of MAPKK and IKK, rapidly kills phagocytic cells and mediates a highly virulent infection. This is in contrast to the effects of Salmonella protein AvrA which, due to its enzymatic specificity against JNK and IKK, apparently allows the invading bacterium to dampen innate immune signaling but also prevents the apoptotic elimination of cells that have perceived microbial compromise. Together, these data illustrate that this family of bacterial effector proteins mediate the fine tuning of host responses, and govern the pathological outcomes of diseases caused by bacteria from which they are secreted.

**Experimental procedures**

**Plasmids and constructs**—A plasmid encoding the aopP coding sequences were a gift from J. Frey (Universität Bern, Switzerland). We generated a mutant and catalytically inactive version of AopP, named mAopP where the cysteine residue at position 177 is mutated to an alanine (C177A). The aopP and mAopP coding sequences were cloned into pCMV-myc, creating pCMV-myc-aopP and pCMV-myc-maopP, respectively. DNA amplicons myc-aopP or myc-maopP were cloned into pP[UAST] (a gift from Kevin Moses, Emory University) creating pP[UAST]-myc-aopP or pP[UAST]-myc-maopP respectively.

**Drosophila lines**—The vectors pP[UAST]-myc-aopP and pP[UAST]-myc-maopP were micro-injected into W1118 embryos, creating fly lines harboring UAS-myc-aopP and UAS-myc-maopP. Other fly stocks used include, UAS-p35, UAS-dIAP and UAS-eiger. Driver
lines include hs-GAL4 (heat shock), GMR-GAL4 (specific expression of GAL4 in omatidial cells during development), and c96-GAL4 (expression of GAL4 in the wing margin), and hml-GAL4 (expression of GAL4 in plasmatocytes).

**Experimental infection**—A total of 5ml culture of *E. carotovora* (grown at 25°C) or *M. luteus* (grown at 30°C) was grown to approximately $1 \times 10^9$ cfu/ml, and centrifuged at 14000 rpm, resulting in a pellet of approximately $5 \times 10^9$ cfu. For infections, a thin tungsten needle was dipped in a concentrated bacterial pellet, and adult *Drosophila* were infected by stabbing the dorsal side of the thorax. To determine the number of bacteria present on the needle, a single needle dipped into the concentrated pellet was transferred into 1ml of liquid culture media and then the population was enumerationed by plate colony forming unit (cfu) count. We detected $5\times 10^4$ cfu per needle (Std. dev. = $2.4\times 10^4$, n=10) for *E. carotovora*, and $3\times 10^4$ cfu per needle (Std. dev. = $1.9\times 10^4$, n=10) for *M. luteus*. For infections of hs-GAL4 *Drosophila* lines, flies were raised at 18°C until 4 days into adulthood. Flies were then subjected to heat shock for 1 hour at 37°C, incubated at 25°C for 24 hours, and then subjected to infection, or wound infliction with a sterile needle. Flies were then return to 25°C and survival rate was determined by counting survivors at regular intervals.

**Quantitative RT-PCR analysis**—Total RNA was extracted from the abdomens of 10 adult *Drosophila* using TRizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription reactions were performed using the QuantiTect Reverse Transcription kit (Qiagen, Valencia CA) and PCR reactions were performed using QuantiTect SYBR Green PCR kit (Qiagen). Primers used for PCR reactions include rpl32(forward) 5′-cagtcggatcgatatgctaagctg-3′, rpl32(reverse) 5′-taaccgatgtgacatcagatac-3′, Dipt(forward) 5′-ttcaccattgccgtcgccttactt-3′, Dipt(reverse) 5′-ccacgacgccttcctgataac-3′, Dros(forward) 5′-gcgccactgtctcggagaataag-3′ and Dros(reverse) 5’-ttgcatcctgcaacacacactcagt-3′. Dipterecin and Drosomycin gene expression were standardized against rpl32 transcript levels in the same sample, and experimental results recorded as fold increase relative to measurements in uninfected *Drosophila*. PCR reactions were performed in triplicate using two separate RNA preparations for each data point.

**Antibodies and Reagents**—Antibodies against phospho-IκB, phosphor-IKKβ and actin were purchased from Cell Signaling Technology (Danvers, MA) and anti-myc was purchased from Clontech (Mountain View, CA). Active Caspase-3 for larval eye disk and histological staining was purchased from Cell Signaling. Antibodies against *Drosophila* tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Relish N-terminus antibody used for both immuno staining was a gift from Svenja Stöven, Umea University, Sweden. The anti-DIF antibody was a gift from Ylva Engström and Gunnel Björklund, Stockholm University, Sweden. DNA was stained with SYTO24 (Molecular Probes). Secondary antibody incubations were done using goat anti-rabbit Cy5 or goat anti-mouse FITC (Jackson ImmunoReserarch, West Grove PA).

**Immunoblot assays and Immunohistochemistry**—Immunoreactive species were detected using anti-rabbit HRP or anti-mouse HRP followed by visualization with ECL chemiluminescence detection reagent (GE Healthcare Biosciences Piscataway NJ). For immuno-staining procedures, third instar larvae eye or wing imaginal disks were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. The tissues were washed 3 x 10 min in 0.1% Triton X-100, and then placed in blocking solution (1% goat serum in 0.1% Triton X-100) for 30min. The tissues were incubated in primary antibody for 1 h at 37°C with gentle rocking, before being washed 3 x 10 min in 0.1% Triton X-100, and incubated in the secondary antibody at 4°C overnight.
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References


Fig. 1. AopP expression in the Drosophila fat body suppresses innate immunity in response to Gram-negative and Gram-positive infection

Flies harboring hs-GAL4 crossed to UAS-aopP or UAS-maopP were subjected to heat shock for 1 hour at 37°C. All parenteral infections, or infliction of wound with sterile needle were carried out at 24 hours post heat shock. All statistical analysis were computed by Log-rank [Mantel-Cox] test. The P value computed and presented with in a figure is for simultaneous comparison of the curves within the figure. n=100 for each genotype tested.

(A) Survival rates of control hs-GAL4 Drosophila, or of Drosophila expressing AopP (hs-GAL4 UAS-aopP), or of isogenic control Drosophila harboring (hs-GAL4 UAS-maopP). P = <0.0001, n=100.

(B) Survival rates of Drosophila genotypes described in (A) following parenteral wound with a sterile needle. n=100. Comparison of hs-GAL4 UAS-aopP (uninfected) from Fig. 1A vs hs-GAL4 UAS-aopP (sterile needle) from Fig. 1B, P = 1.000, n=100.

(C) Survival rates of Drosophila genotypes described in (A) following parenteral infection with E. carotovora. Mean of 5x10^4 cfu infected per fly with Std. deviation of 2.4x10^4, n=10. Comparison of hs-GAL4 UAS-aopP (sterile) from Fig. 1B vs hs-GAL4 UAS-aopP (E. carotovora) from Fig. 1C P = <0.0001, n=100.

(D) Survival rates of Drosophila genotypes described in (A) following parenteral infection with Micrococcus luteus. Mean of 2.4x10^4 cfu infected per fly with Std. deviation of 1.9x10^4, n=10. Comparison of hs-GAL4 UAS-aopP (sterile) from Fig. 1B vs hs-GAL4 UAS-aopP (M. luteus) from Fig. 1D, P = <0.0001, n=100.

(F) Quantitative RT-PCR analysis of *drosomycin* gene expression in *Drosophila* genotypes hs-GAL4, hs-GAL4 UAS-aopP, or hs-GAL4 UAS-maopP, following 24 hours infection with *M. luteus*. Error bars represent S.E.M. n=3.

(G) Immunostain analysis of *Drosophila* larval fat body tissue from c564-GAL4 UAS-aopP, or from isogenic flies harboring c564-GAL4 UAS-maopP, following parenteral *E. carotovora* (Gram-negative) infection. Fat bodies were analyzed for Relish distribution using an antibody against the N-terminal domain of Relish.

(H) Immunostain analysis of *Drosophila* larval fat bodies from *M. luteus*-infected third – instar larvae expressing AopP or mAopP using an anti-Dif antibody.
Fig 2. AopP expression in *Drosophila* epithelial tissue induces apoptosis

(A) (upper panels) Phenotypes of adult *Drosophila* eyes expressing AopP, or mAopP under the eye specific driver GMR-GAL4 respectively. Suppression of AopP mediated small eye phenotypes by p35 or dIAP confirm the phenotype is a result of apoptotic events. (lower panels) Immunostain analysis of third instar larval eye imaginal disks dissected from phenotypes described in A using antibodies against Cleaved Caspase-3 or myc.

(B) Phenotypes of adult *Drosophila* wings expressing AopP, or mAopP under the wing specific driver c96-GAL4. Suppression of AopP mediated wing notching by p35 or dIAP confirm the phenotype is a result of apoptotic events.

(C) Immunostain analysis of dissected third instar larval wing disks from phenotypes described in (B) using antibodies against Cleaved Caspase-3 or myc.

(D) TUNEL analysis of fat bodies dissected from *Drosophila* larvae expressing AopP or mAopP, under the fat body specific c564-GAL4 driver, or under hs-GAL4 at 72 hours post heat shock.
Fig. 3. AopP does not inhibit MAPK pathway activation in Drosophila

(A) Phenotypes of adult Drosophila eyes constitutively expressing AopP or VopP in combination with activators of ERK (Rolled (rl) in Drosophila) pathway. Note, the AopP induced rough eye phenotype is not rescued by the activation of the ERK signaling pathway, whereas the VopA induced rough eye is completely rescued by the activation of the ERK pathway at the level of Rolled.

(B) Phenotypes of adult Drosophila eyes resulting from constitutive expression of the JNK pathway activator Eiger (Drosophila homologue of mammalian TNF), and from a co-expression of Eiger with the indicated bacterial protein. Note that AopP only results in a slight increase in eye size whereas AvrA, a potent inhibitor of JNK activity, completely rescues the Eiger-induced small eye phenotype. Also, note appearance of black necrotic-like tissue the eye where Eiger and AopP are co-expressed.
Fig 4. Constitutive expression of AopP in *Drosophila* immune cells decreases plasmatocyte number and compromises the innate immune response after 5 days of infection.

(A) *Drosophila* plasmatocytes constitutively co-expressing GFP and AopP, or mAopP, or Eiger, or AvrA were bled from third instar larvae and visualized by confocal microscopy (20X).

(B) Numerical representation of GFP expressing cells from (A). Error bars indicate S.E.M. n=20 20x fields.

(C) Survival rates of adult *Drosophila* constitutively expressing AopP or mAopP in hemocytes following parenteral Gram-negative *E. carotovora* or Gram-positive *Micrococcus luteus* infection. All statistical analysis were done by Log-rank [Mantel-Cox] test. The *P* value computed and presented with in a figure is for simultaneous comparison of the curves within the figure. n=100 for each genotype tested.
Fig 5. AopP is highly pro-apoptotic in cultured mammalian cells
(A) FACS analysis of HeLa cultured cells transfected with vector alone or with plasmids expressing AopP or mAopP for 24 h. Cells were further incubated for an additional 24 h with or without 10 ng/ml TNF-α, and stained with Annexin V and propidium iodide.
(B) Numerical representation of Annexin V positive cells from Fig.5A. Error bars indicate S.E.M. n=5. *p=<0.01.
Fig 6. AopP represses NF-κB activation in mammalian cultured cells at the level of IKK-β

(A) HEK293T cultures cells transfected with vector control or plasmids harboring myc-AopP or myc-mAopP were stimulated with 10 ng/ml TNF-α for up to 1 hour and assayed by immunoblot with the indicated antibodies.

(B) Immunoblot analysis of HEK293T cells transfected with the indicated plasmids and stimulated with 10 ng/ml TNF-α for 1 hour, using antibodies against phosphorylated IKKβ, myc, flag and actin.

(C) Immunoblot analysis for cIAP-2 in HeLa cultured cells transfected with plasmids harboring myc-AopP or myc-mAopP, and stimulated with 10 ng/ml TNF-α for up to 24 hours.

(D) Quantitative RT-PCR analysis of IAP-2 gene expression in HeLa cultured cells transfected with plasmids harboring AopP or mAopP, and stimulated with 10 ng/ml TNF-α for 2 hours. Error bars represent standard errors of mean (S.E.M.). n=3. *=p<0.01.